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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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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.

Keywords: HILIC-MS/MS; artificial sweeteners; steviol glycosides; HILIC method development;

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 flavouring 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 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 4ºC, 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 minutes. 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 five minutes 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 pseudotransition in the negative mode was chosen (317.30 → 317.40).
<insert Table S1. Supplementary material>

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 x 2 mm, 1.8 µm (Knauer), Ascentis Express Si 150 mm x 2.1 mm, 3 µm (Supelco) and Acclaim™ Trinity™ P2 100 mm x 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.
<insert 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. Three columns were chosen to develop a method based on the HILIC approach: BlueOrchid PAL-HILIC, Ascentis Express Si and Acclaim™ Trinity™ P2. The Ascentis Express Si column is packed with high purity bare silica (core-shell technology, 0.5 µm thick porous shell with 1.7µm solid impenetrable core). In the BlueOrchid PAL-HILIC a high purity, fully porous silica modified with polymer amine ligands was used. This column was designed for the separations in anion exchange and in

HILIC modes. Third column – Acclaim™ Trinity™ P2 is based on nanopolymer-silica hybrid technology. The sorbent is 3 μm silica coated with charged particles of a nanopolymer: the inner pores of silica particles are modified with a covalently bonded hydrophilic layer, while the outer surface is modified with anion-exchange nanopolymer particles. This approach offers cation exchange retention in the innerpore region and anion-exchange retention on the outer surface. Moreover, the hydrophilic surface can be used in HILIC chromatography.

In HILIC, partitioning mainly occurs between the water-rich surface and water-deficient bulk mobile phase. In addition to this mechanism other phenomena are present: adsorption of molecule's polar functional groups on the stationary phase, ion exchange, and partial reversed-phase retention on the hydrophobic parts of bonded ligands [31].

ACN modified with acetic acid (up to 0.05% v/v) was chosen as the main organic component in the optimization of chromatographic runs. Two buffer solutions: ammonium formate and ammonium acetate, were tested as the aqueous parts of the mobile phase. However, better results in term of peak shapes and efficiency were obtained with NH_4Ac buffer on all columns. The initial composition of mobile phase was identical for all three tested columns. The mobile phase was composed of ACN (0.01% AA v/v) and 10 mM of NH_4Ac. The initial content of the aqueous component of the mobile phase was kept at 2-3% to enhance the separation of early eluting compounds. Further on, the gradient conditions were optimized to separate later eluting compounds. Additionally, other combinations of aqueous/organic parts of mobile phases were tested: the amount of NH_4Ac buffer was increased (25, 40, 50 up to 75mM), together with the amount of AA (up to 0.05% v/v) in the organic component of the mobile phase. The last step was to choose the temperature of separation in the range from 30-50˚C. Example chromatograms obtained with BlueOrchid PAL-HILIIC and Ascentis Express Si columns under optimized conditions are presented in Figure 1.

<insert Figure 1>

Regardless of the mobile phase composition (buffer amount, AA amount in ACN, initial organic component content) the separation of the acesulfame-K, saccharin and steviol on Ascentis Express Si column was not sufficient. These analytes eluted close to the void time of the system and were poorly separated from each other, which is typical in the case of the bare silica columns, and was previously reported [26]. The increased content of buffer (above 10 mM) resulted in small changes in peak shapes and in suppressing the signal. The increased content of AA in ACN (>0.05% v/v) had similar effect. Moreover, the peaks of neotame,

aspartame and alitame showed severe tailing. The peaks of steviol glycosides were well separated and the order of elution was correct for HILIC chromatography – from the lowest to the highest molecular mass. In the case of separation done on BlueOrchid PAL-HILIC the peak shapes for tailing compounds (aspartame, alitame) were improved at the cost of the peak width of saccharin and acesulfame-K. The optimum buffer concentration in case of this column was 25 mM. Lower concentrations (5 and 10 mM) yielded poor peak shapes for aspartame and alitame, while higher concentrations (40, 50 and 75 mM) resulted in overall lower sensitivity. In all experiments the detection sensitivity was comparable up to 40 mM of NH_4Ac buffer, despite the different gradient programmes. Above 40 mM the detection sensitivity of all transitions was suppressed, probably due to the increased amount of ions present in the eluate sprayed into the ionization chamber. In case of separation of steviol glycosides on BlueOrchid PAL-HILIC, the retention was not characteristic for the HILIC-type chromatography (reverse retention of rebaudioside C and A), hence different mechanisms of separation were involved. Most likely the exposed steviol moiety of rebaudiosides A and C was attracted to the hydrophobic ligand chain.

The Acclaim™ Trinity™ P2 column was originally designed for ion chromatography to determine the counter ions of some pharmaceuticals. This specific sorbent offers HILIC separation mechanisms as well. A higher buffer content (40 mM) than suggested by the producer (10 mM as minimal to force HILIC mechanism) was applied. The buffer content was increased in order to saturate the stationary phase sufficiently. Two example chromatograms obtained with the Acclaim™ Trinity™ P2 column with two different buffer concentrations (10 and 40 mM) as aqueous mobile phase are presented in Figure 2. The concentration of buffer smaller than 40 mM resulted in irregular peak shapes for ionic compounds (saccharin and acesulfame-K) and substantially longer retention times for all analytes (see Figure 2). To reduce retention times and improve peak shapes the buffer concentration was increased to 40 mM. Another factor to favour buffer at 40 mM was the high content of the organic component at the initial conditions (96%) necessary to separate steviol and shift its peak away from the dead time. However, buffer concentrations higher than 40 mM of NH_4Ac did not improve neither separation nor peak shapes, while the detection sensitivity started to decrease, similarly to the previously described cases. It is suspected that the high concentration of ions derived from ammonium acetate in the mobile phase (salting out effect) can limit the formation of hydrogen bonds between molecules of the analytes and stationary phase. Further analysis of the mechanism of separation is difficult because of

commercial confidentiality concerning the structure of silica gel with nanoparticles. The order of elution of steviol glycosides with the use of the Acclaim™ Trinity™ P2 column is classic for HILIC (from the smallest mass - steviolbioside to the largest mass - rebaudioside A). Detailed information about chromatographic parameters is presented in section 3.2. For further analysis and for comparison with the previously developed RPLC method [9] the HILIC method employing the Acclaim™ Trinity™ P2 column was chosen due to the best peak shapes and separation factors.

<insert Figure 2>

3.2 Comparison of chromatographic parameters

Two developed methods based on the HILIC and RPLC modes were compared in terms of basic chromatographic parameters: reduced retention time (t'_R), width at baseline (w), width at 50% of height ($w_{50\%}$), tailing factor at 10% of height ($TF_{10\%}$), efficiency (N) and plate height (H). The compared results are presented in Table 2.

In case of the RPLC method the smallest t'_Rs values are obtained for the most polar substances, and the highest for the steviol glycosides. The only part of the molecule of steviol glycosides that undergoes interactions with the stationary phase in the RPLC mode (Ascentis Express C18) is the exposed hydrophobic steviol moiety. In the method using the HILIC mode (Acclaim™ Trinity™ P2) interactions between the stationary phase and the steviol glycosides mainly involves the carbohydrates attached to the steviol moiety.

The shape and symmetry of the peaks were evaluated on the basis of w, $w_{50\%}$ and $TF_{10\%}$. The peaks of synthetic sweeteners are narrower in the case of the RPLC mode than in the case of the HILIC mode. In contrast, minor differences in the shape of peaks are observed for steviol glycosides – regardless of the method used. For both chromatographic methods the $TF_{10\%}$ values are within the range of typical values, that is, optimal conditions of migration of analytes through the sorbent were achieved. However, in the case of the RPLC method the obtained peaks have a tendency for tailing ($TF_{10\%}$ above 1), while some peaks in the case of the HILIC method have the fronting tendency ($TF_{10\%}$ below 1). This is probably related to separation conditions (solute-solute interactions) of the substances of highly ionic character: acesulfame-K, saccharin and cyclamate. In order to obtain retention of steviol (low affinity to the stationary phase of the Acclaim™ Trinity™ P2 column) the chromatographic run has to start with the low elution-strength mobile phase (4% of component A).

The RPLC method was developed with the column based on core shell technology,

hence the analytes have a shorter and more uniform diffusional path in comparison to the column with fully porous particles, as in the HILIC method. In general, the core shell particles are more efficient than fully porous particles, due to the reduction of the resistance of mass transfer in the mobile and stationary phases, as well as the reduction of the eddy diffusion.

The number of theoretical plates for the individual peaks is greater for the method based on the HILIC technique, and this is particularly apparent for the steviol glycosides and some synthetic sweeteners. Only in the case of steviol, neotame and alitame the plate number is significantly lower, due to weak interactions with the stationary phase (steviol) and the relatively high peak width (alitame and neotame).

The resolutions for two adjacent peaks were calculated for chromatograms obtained with the developed RPLC and HILIC methods, and the results are presented in Table S2 (supplementary material). For the RPLC mode of separation all peaks are well separated (R_s above 1.5), except rebaudioside A and stevioside (R_s 1.2). As mentioned before, only the steviol part of the molecule is involved in the separation mechanism on the Ascentis Express C18 column, and this might be the reason for the lower R_s. In case of the HILIC mode of separation, three pairs (neotame - acesulfame-K, neohesperidin DC – aspartame and steviolbioside - dulcoside A) are not completely resolved (R_s 1.1-1.2). This is probably connected with the limitations of the column stationary phase and gradient changes.
<insert Table 2>
<insert Table S2. Supplementary material>

3.3 Within laboratory validation

3.3.1 Calibration

For both methods a seven-point calibration curve was made by plotting the ratio of the peak area of the analyte to the peak area of IS versus concentration. Two concentration ranges were used for synthetic and natural sweeteners (see section 2.3). The obtained calibration curves were linear in the tested concentration ranges. Weighting factor *1/x* was applied to all calibration curves in order to increase accuracy at the lower concentration range. Limit of detection (LOD) values were estimated using the following formula; $LOD=3.3S_b/a$, where S_b is the standard deviation of intercept and a is the slope of the calibration curve. To estimate limit of quantitation (LOQ) values the LOD values were multiplied by three. The figures of merit for the RPLC and HILIC methods are presented in Table 3. In general the

LOD values obtained for a given analyte are comparable regardless of the method used (RPLC or HILIC). However, some exceptions can be noticed for example acesulfame-K and sucralose. The high content of ACN in the mobile phase in the HILIC method results in increased detection sensitivity, which partially compensates for the wider peaks of aspartame and alitame. Chromatogram corresponding to the lowest calibration point (5 ng/mL, HILIC mode) is shown in Figure S1.

<insert Table 3>

<insert Figure S1. Supplementary material >

3.3.2 Trueness and repeatability

The trueness and repeatability of the results of the two methods were estimated on the basis of the recovery of analytes from spiked samples. The procedure for the preparation of spiked samples was presented before [9]. Six independent analyses were done for each of the three levels of concentration for both methods. The recoveries of individual analytes are summarized in Table 4. Recoveries for the RPLC method vary from 97.0 to 105.7 %, with relative standard deviations (RSDs) in the range of 0.4 to 4.1%, while recoveries for the HILIC method vary from 99.3 to 106.2%, with RSDs from 0.8 to 4.2%. From these results it can be concluded that both methods are comparable in terms of trueness and precision. No matrix effects were observed for either of the methods, mainly because of the high dilution of the sample, sufficient separation, and use of IS.

Six analyses were performed on three consecutive days for a spiked sample of the desired concentration. The results were used to check the next parameter validation – repeatability. Detailed data about recovery for repeatability estimation are presented in electronic supplementary material (Table S3). Again, no significant differences were observed, and recoveries were in the range 97.6 – 105.5%, with RSDs range 1.1 – 4.5% for the RPLC method, and recoveries from 98.5 to 105.0% with RSDs 1.1 – 4.4% for the HILIC method.

<insert Table 4>

<insert Table S3. Supplementary material>

3.3.3 Comparison of precision and accuracy by the F-Snedecor and t-Student tests.

In order to compare the precision of both developed methods the F-Snedecor (α= 0.05) test was applied. The obtained values were compared with the $F_{critical}$ (5.05) value. The

results are presented in electronic supplementary material (Table S4). For most of the compounds there is no statistically significant difference in precision. The obtained values do not exceed $F_{critical}$ and these two methods do not differ in term of precision in a statistically significant manner. However, some values are close to $F_{critical}$. In order to verify the accuracy of both methods the Student t-test was applied for all the results. The obtained values were compared with the $t_{critical}$ value (2.23) for the Student t-test. For the four compounds (cyclamate, neohesperidin DC, rebaudioside A, dulcoside A) it can be observed that both methods differ statistically in terms of accuracy for two tested concentrations ($t_{critical}$ is exceeded). The values for acesulfame-K, saccharin and steviolbioside are exceeded for one tested concentration.

It should be noted that different integrating peak algorithms in LabSolution software were used for chromatograms obtained with the RPLC and HILIC methods. The integration of wider and tailing peaks is problematic, hence the differences in accuracy. Results from validation clearly indicate the usefulness of the developed methods for identifying and quantifying artificial and natural sweeteners in beverage samples, despite the differences between accuracy and precision. No significant or observable matrix effects were found during analysis.

<insert Table S4. Supplementary material>

4. Analysis of real samples

Twenty-four samples from different producers of soft and alcoholic drinks, including three instant drinks, were analyzed with the described HILIC method and previously published [9] RPLC method. Majority of the tested samples contained high-potency sweeteners. Three samples were labelled as free of sweeteners. All samples were prepared with the procedure described in section 2.4. The equations of calibration curves for each analyte were used to determine the content of substances with high sweetening power in samples of soft and alcoholic drinks. The results of HILIC analyses are summarized in electronic supplementary material (Table S5). Example chromatograms obtained during the analysis of the samples by the HILIC method are shown in Figure 3.

<insert Figure 3>

<insert Table S5. Supplementary material>

5. Conclusions

The developed methods allow the separation and quantification of substances of various natures exhibiting high sweetening power. In the case of a HILIC-type separation it is possible to completely resolve the steviol glycosides with better efficiency than in the case of RPLC-type separation. For some of the compounds, such as acesulfame-K, neohesperidin DC, neotame and steviol, RPLC separation is superior to HILIC. However, this is closely related to the physicochemical properties of the molecules and their affinity to the stationary phase. Acesulfame-K as an ionic compound may require specific chromatographic conditions (pH below 3). In the case of HILIC-type separation the pH is above 6, and the amino group of acesulfame-K is protonated, and this might be the reason for the wider peak of this compound, hence lower efficiency. For neohesperidin DC the significant difference in efficiency may result from the presence of other hydrophilic parts of its molecule, except moieties of simple sugars. The hydroxyl groups at the phenyl rings may be involved in the separation mechanism. The neotame has two hydrophobic parts: the aliphatic chain and the phenyl ring. These two parts may partially cover the hydrophilic parts (carboxylic groups), hence the interactions with the stationary phase might be disturbed. The steviol molecule is the key element in all steviol glycosides, although as a molecule it is hydrophobic and difficult to separate in the HILIC mode. The steviol molecule might be a marker of the freshness of products, since steviol glycosides may decompose in time (losing moieties of simple sugars) with the formation of steviol.

The two developed methods (HILIC and RPLC) may be used independently or interchangeably, depending on needs, in order to control the content of each sweetener in beverages. The methods provide short analysis time, high repeatability, and simple sample preparation, which is reduced to a minimum and involves only dilution and centrifuging. Using both methods, it is possible to achieve low LODs, high recovery values and good repeatability of results, and make these methods suitable for food quality and safety control. The method using the Acclaim™ Trinity™ P2 column is the first HILIC application where separation of acesulfame-K, saccharin and sucralose is achieved, and the results are comparable to those obtained in RPLC modes.

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Tables

Table 1. Chosen separation conditions of the chromatographic system for the RPLC and HILIC methods

Table 2. Comparison of selected chromatographic parameters for two developed methods based on the RPLC [12] and HILIC modes of separation

Table 3. Quantification and validation data for artificial sweeteners and steviol glycosides for the RPLC and HILIC methods

Table 4. Recoveries of analytes at three independent concentrations.

Figures

Figure 1. Example chromatograms of a mixture of standards of sweeteners (50 ng/mL) under optimized conditions: A) separation on Ascentis Express Si, mobile phase: A – 10 mM NH4Ac, B – ACN 0.05% AA v/v, gradient programme: 0-5 min 3% A, 5-15 min 3-20% A, 15-20min 20% A, temperature of column compartment 30˚C, injection volume 2 µL; B) separation on BlueOrchid PAL-HILIC, mobile phase: A – 25 mM NH4Ac, B – ACN 0.05% AA v/v, gradient programme: 0-3 min 5% A, 3-15 min 5-45% A, 15-20 min 45% A, temperature of column compartment 35˚C, injection volume 2 µL.

2. Figure 2. Example of chromatograms of a mixture of standards of sweeteners (50 ng/mL) separated on Acclaim™ Trinity™ P2 column: A) buffer content 10 mM in aqueous part of mobile phase, B) buffer content 40 mM in aqueous component of mobile phase.

Figure 3. Examples of chromatograms obtained for real samples with the HILIC method. From the top: CNA1, NCNA6 and CA4.

Supplementary material

Table S1. Monitored ion transitions, their parameters and optimal MS/MS operational parameters

Table S2. Resolution factors between adjacent peaks obtained from chromatograms for two developed methods - HILIC and RPLC

Table S3. Recovery values used for estimation of repeatability.

Table S4. Comparison of the precision and accuracy of results obtained with the RPLC and HILIC methods based on the values obtained by the F-Snedecor test and Student t-test.

Table S5. Concentrations of all found sweeteners in soft and alcoholic drinks: analysis of real samples. Only detected compounds are shown

Figure S1. HILIC mode chromatogram obtained after analysis of the most diluted calibration solution (5 ng/mL of each sweetener).

Table 1. Chosen separation conditions of the chromatographic system for the RPLC and HILIC methods

	RPLC method [9]	HILIC method
Column	Ascentis® Express C18 (100 mm × 4.6 mm, 2.7 µm)	Acclaim™ Trinity™ P2 (100 mm × 2.1 mm, 3 µm)
Flow rate	0.8 mL/min	0.6 mL/min
Temperature of thermostated column compartment	40°C	30°C
Injection volume	2 µL	2 µL
Analysis time	16 min	18 min
Mobile chase components	**A**: MeOH+H_2O+ACTN (20+75+5) 0.1% v/v AA **B**: ACN+ACTN (95+5) 0.1% v/v AA	**A**: 40mM NH_4Ac pH=6,8 **B**: ACN 0.01% v/v AA
Gradient elution	0→10 min 0-30%B 10→15 min 30-70%B 15→16 min 70%B	0→2 min 96%B 2→11 min 96-87%B 11→14 min 87%B 14→18 min 87-50%B

MeOH – methanol, ACTN – acetone, ACN – acetonitrile, NH_4Ac – ammonium formate, AA – acetic acid

Table 2. Comparison of selected chromatographic parameters for two developed methods based on RPLC and HILIC mode of separation

Analyte	RPLC	HILIC	RPLC	HILIC	RPLC	HILIC	RPLC	HILIC	RPLC	HILIC	RPLC	HILIC
	t'_R [min]		w [min]		$w_{50\%}$ [min]		$TF_{10\%}$		N [th.]		H [µm]	
acesulfame-K	1.75	7.48	0.157	0.278	0.072	0.134	1.24	0.93	4.1	19.7	24.38	5.06
saccharin	2.12	8.72	0.166	0.298	0.079	0.140	1.23	0.92	4.8	24.0	20.93	4.16
aspartame	2.93	12.84	0.149	0.469	0.064	0.213	1.22	1.17	13.1	21.7	7.63	4.60
sucralose	3.39	7.83	0.130	0.219	0.057	0.100	1.21	0.96	22.0	38.2	4.55	2.62
cyclamate	3.65	9.06	0.173	0.280	0.073	0.129	1.19	0.93	15.3	30.4	6.55	3.29
alitame	4.32	13.77	0.141	0.523	0.059	0.252	1.23	1.21	32.9	17.8	3.04	5.61
IS	5.85	8.22	0.241	0.292	0.101	0.146	1.08	0.94	19.7	19.9	5.06	5.04
neohesperidin DC	7.30	12.53	0.128	0.286	0.056	0.127	1.15	0.96	98.2	58.0	1.02	1.72
neotame	9.78	7.21	0.130	0.343	0.060	0.158	1.13	1.11	153.3	13.2	0.65	7.55
rebaudioside A	10.34	16.87	0.122	0.136	0.056	0.061	1.11	1.15	196.3	444.3	0.51	0.23
stevioside	10.42	16.59	0.123	0.116	0.056	0.052	1.12	1.17	196.9	590.8	0.51	0.17
rebaudioside C	11.09	16.38	0.123	0.135	0.056	0.056	1.13	1.16	222.5	503.4	0.45	0.20
dulcoside A	11.38	15.91	0.123	0.128	0.057	0.055	1.11	1.15	231.4	493.2	0.43	0.20
steviolbioside	12.35	15.80	0.103	0.141	0.047	0.059	1.14	1.04	395.0	427.7	0.25	0.23
steviol	15.07	1.36	0.108	0.143	0.049	0.062	1.10	1.15	530.8	2.7	0.19	37.11

t'_R – reduced retention time, w - width at baseline, $w_{50\%}$ - width at 50% of peak height, $TF_{10\%}$ - tailing factor at 10% of height, N – efficiency, H - plate height.

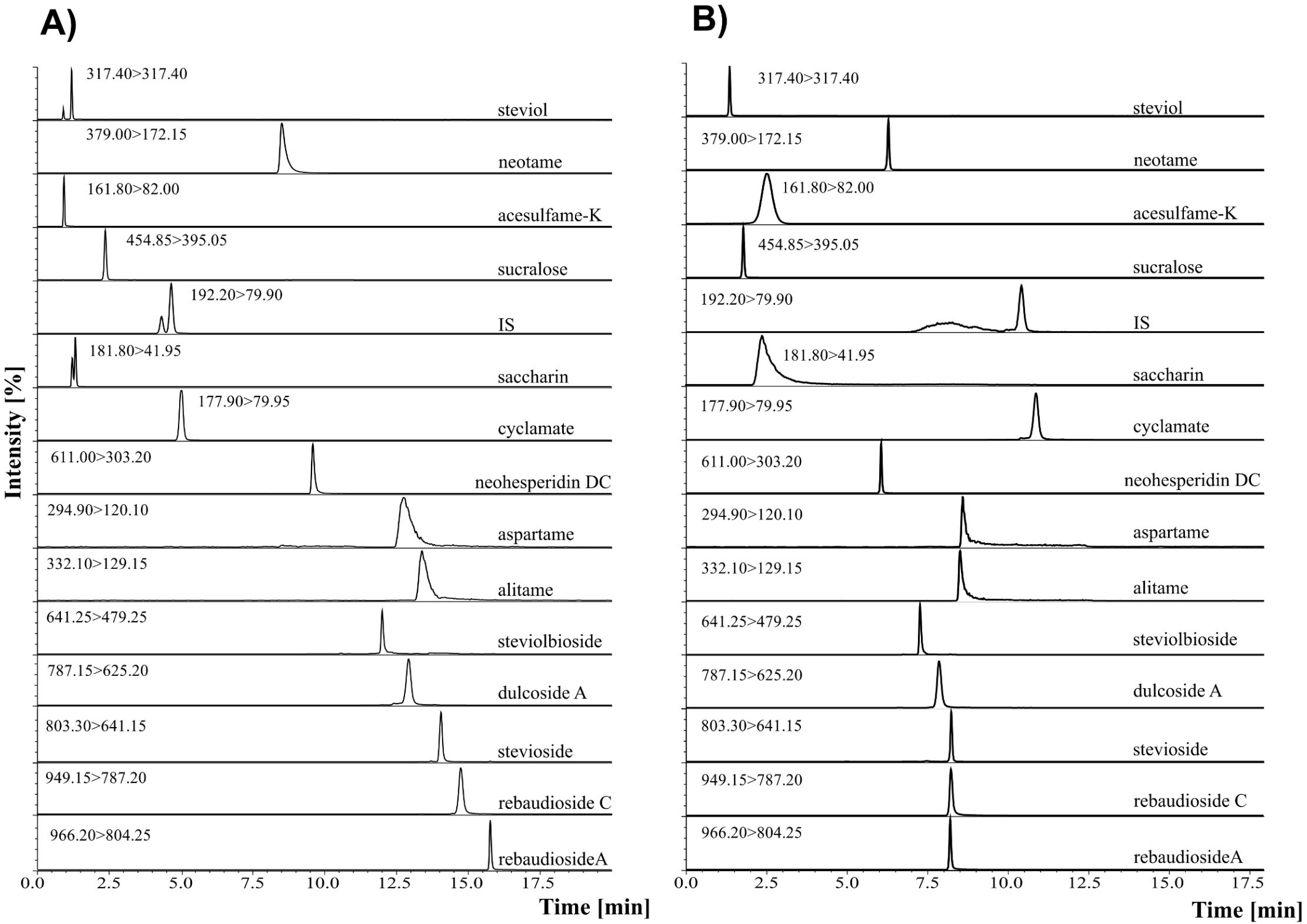
Table 3. Quantification and validation data for artificial sweeteners and steviol glycosides for RPLC and HILIC method

Analite	Calibration curve equation (7 points, n=3)	S_a	S_b	r	LOD [ng/mL]	LOQ [ng/mL]
RPLC [9]						
acesulfame-K	y=0.04286x+0.099	0.00079	0.059	0.9987	4.52	13.56
saccharin	y=0.004583+0.0025	0.000025	0.0018	0.9997	1.32	3.95
aspartame	y=0.04189-0.070	0.00028	0.021	0.9996	1.63	4.90
sucralose	y=0.010964-0.0158	0.000062	0.0046	0.9997	1.38	4.14
cyclamate	y=0.02994-0.0454	0.00013	0.0098	0.9998	1.08	3.23
alitame	y=0.02816-0.024	0.00021	0.015	0.9994	1.78	5.35
neohesperidin DC	y=0.011370-0.0362	0.000095	0.0070	0.9993	2.04	6.12
neotame	y=0.04840-0.124	0.00045	0.033	0.9991	2.28	6.84
rebaudioside A	y=0.004872-0.0012	0.000020	0.0029	0.9998	1.98	5.95
stevioside	y=0.004625-0.0005	0.000019	0.0029	0.9998	2.04	6.11
rebaudioside C	y=0.016005-0.011	0.000071	0.010	0.9998	2.16	6.48
dulcoside A	y=0.007403-0.0168	0.000035	0.0052	0.9997	2.33	7.00
steviolbioside	y=0.002522+0.0022	0.000014	0.0021	0.9997	2.69	8.08
steviol	y=0.05032+0.131	0.00028	0.042	0.9997	2.74	8.23
HILIC						
acesulfame-K	y=0.02930x+0.047	0.00026	0.019	0.9993	2.14	6.42
saccharin	y=0.0019827x-0.00231	0.0000066	0.00048	0.9999	0.81	2.42
aspartame	y=0.003794x-0.0193	0.000018	0.0013	0.9998	1.11	3.33
sucralose	y=0.004268x+0.0029	0.000039	0.0029	0.9992	2.24	6.73
cyclamate	y=0.018984x-0.0094	0.000057	0.0042	0.9999	0.74	2.21
alitame	y=0.003217x-0.0175	0.000017	0.0012	0.9998	1.20	3.60
neohesperidin DC	y=0.0005855x-0.00218	0.0000062	0.00046	0.9989	2.59	7.78
neotame	y=0.04902x-0.145	0.00032	0.023	0.9996	1.57	4.70
rebaudioside A	y=0.00013818x-0.002533	0.00000047	0.000089	0.9999	2.12	6.37
stevioside	y=0.00010864x-0.00194	0.00000051	0.00010	0.9998	3.13	9.40
rebaudioside C	y=0.0003665x-0.00769	0.0000017	0.00031	0.9998	2.83	8.50
dulcoside A	y=0.0002334x-0.00413	0.0000010	0.00020	0.9998	2.88	8.65
steviolbioside	y=0.00006585x-0.001302	0.00000025	0.000047	0.9998	2.36	7.08
steviol	y=0.06131x-0.806	0.00032	0.061	0.9997	3.30	9.89

S_a standard deviation of the slope, S_b standard deviation of the intercept, r correlation coefficient, LOD limit of detection, LOQ limit of quantitation, n number of measurements

Table 4. Recoveries of analytes for three independent concentrations.

	RPLC [9]			HILIC		
	Recovery (RSD) [%] of analyte (n=6)					
Analyte	10×10^3 ng/mL	25×10^3 ng/mL	60×10^3 ng/mL	10×10^3 ng/mL	25×10^3 ng/mL	60×10^3 ng/mL
acesulfame-K	100.4 (2.5)	103.5 (2.3)	102.2 (0.6)	99.4 (3.2)	105.3 (1.0)	106.2 (0.9)
saccharin	100.2 (1.9)	97.0 (1.9)	99.0 (1.8)	102.7 (2.4)	100.5 (1.7)	99.8 (1.5)
aspartame	103.3 (2.7)	100.3 (1.1)	102.0 (1.4)	103.0 (3.1)	102.4 (2.4)	100.6 (1.9)
sucralose	104.4 (2.1)	103.0 (1.9)	100.5 (2.4)	103.2 (4.1)	102.7 (1.1)	99.6 (1.7)
cyclamate	101.4 (1.7)	98.0 (2.0)	98.9 (1.1)	105.7 (1.1)	102.8 (1.4)	98.6 (0.8)
alitame	103.8 (2.4)	101.3 (1.4)	101.7 (2.9)	102.9 (0.7)	100.7 (2.1)	101.4 (1.0)
neohesperidin DC	101.9 (3.3)	100.3 (2.3)	98.4 (1.3)	100.1 (2.8)	103.2 (1.1)	100.4 (1.5)
neotame	102.4 (1.8)	99.5 (1.6)	102.1 (1.9)	101.4 (2.3)	101.3 (2.5)	100.7 (2.8)
rebaudioside A	98.4 (2.1)	101.4 (2.9)	103.1 (1.4)	103.3 (2.6)	100.0 (1.5)	99.9 (1.6)
stevioside	102.9 (1.3)	102.4 (3.1)	101.5 (1.7)	101.9 (1.4)	103.9 (1.9)	100.7 (1.2)
rebaudioside C	103.1 (3.4)	102.0 (3.1)	101.9 (2.2)	102.1 (4.1)	99.5 (1.5)	102.0 (1.5)
dulcoside A	105.7 (3.6)	102.6 (2.4)	102.9 (1.4)	99.3 (3.5)	99.9 (0.8)	101.0 (1.4)
steviolbioside	98.0 (4.1)	100.3 (1.8)	102.7 (3.4)	101.8 (2.4)	104.0 (1.0)	99.2 (2.8)
steviol	103.6 (2.8)	100.7 (1.7)	102.1 (2.5)	103.4 (3.7)	100.6 (1.5)	100.7 (2.5)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IS
181.80>41.95
saccharin
177.90>79.95
cyclamate
611.00>303.20
neohesperidin DC
294.90>120.10
aspartame
332.10>129.15
alitame
641.25>479.25
steviolbioside
787.15>625.20
dulcoside A
803.30>641.15
stevioside
949.15>787.20
rebaudioside C
966.20>804.25
rebaudiosideA
Intensity [%]
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2.5
5.0
7.5
10.0
12.5
15.0
17.5
Time [min]
B)
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steviol
379.00>172.15
neotame
161.80>82.00
acesulfame-K
454.85>395.05
sucralose
192.20>79.90
IS
181.80>41.95
saccharin
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cyclamate
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neohesperidin DC
294.90>120.10
aspartame
332.10>129.15
alitame
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steviolbioside
787.15>625.20
dulcoside A
803.30>641.15
stevioside
949.15>787.20
rebaudioside C
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17.5
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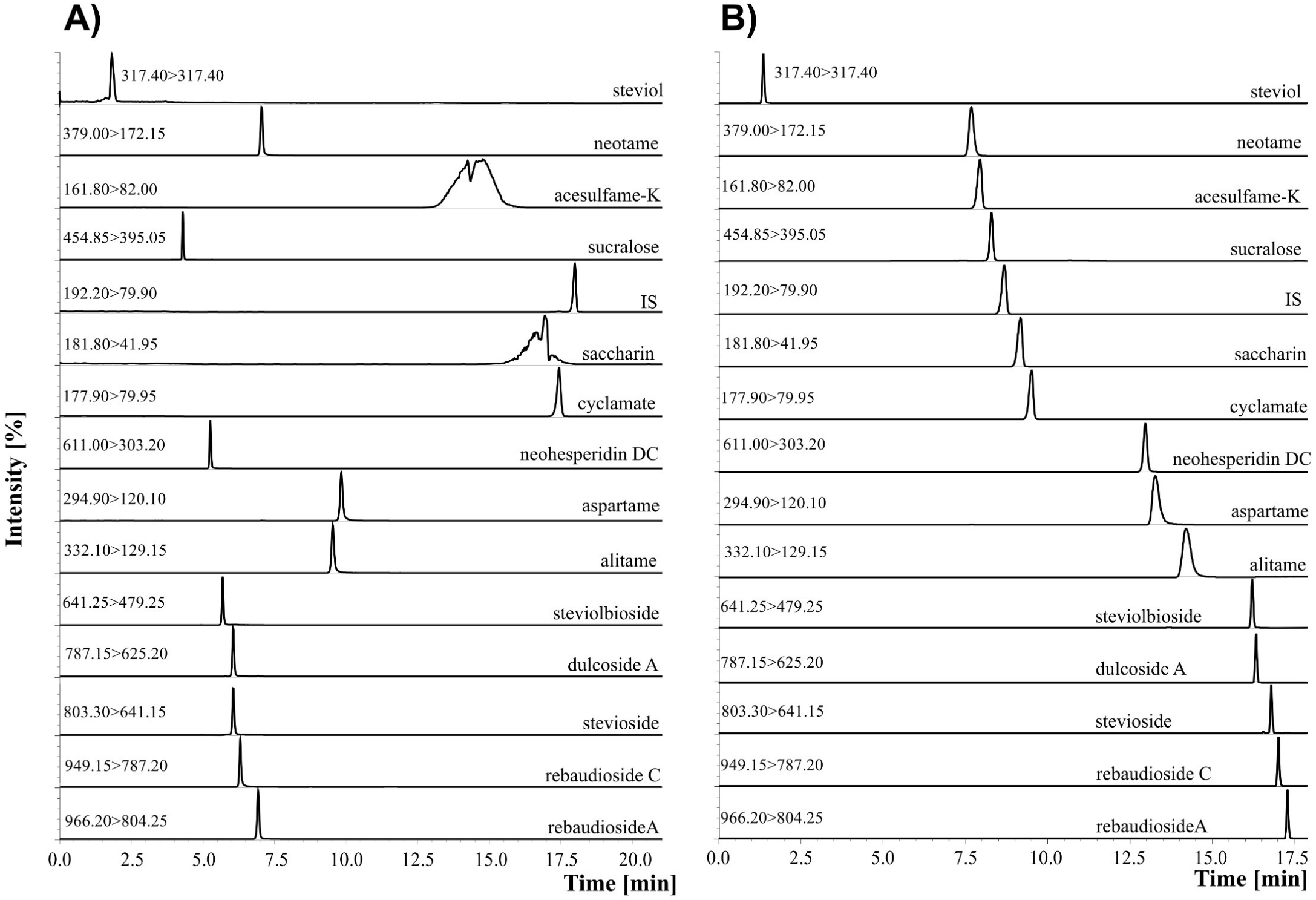
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B)
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neotame
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sucralose
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saccharin
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alitame
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steviolbioside
787.15>625.20
dulcoside A
803.30>641.15
stevioside
949.15>787.20
rebaudioside C
966.20>804.25
rebaudiosideA
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5.0
7.5
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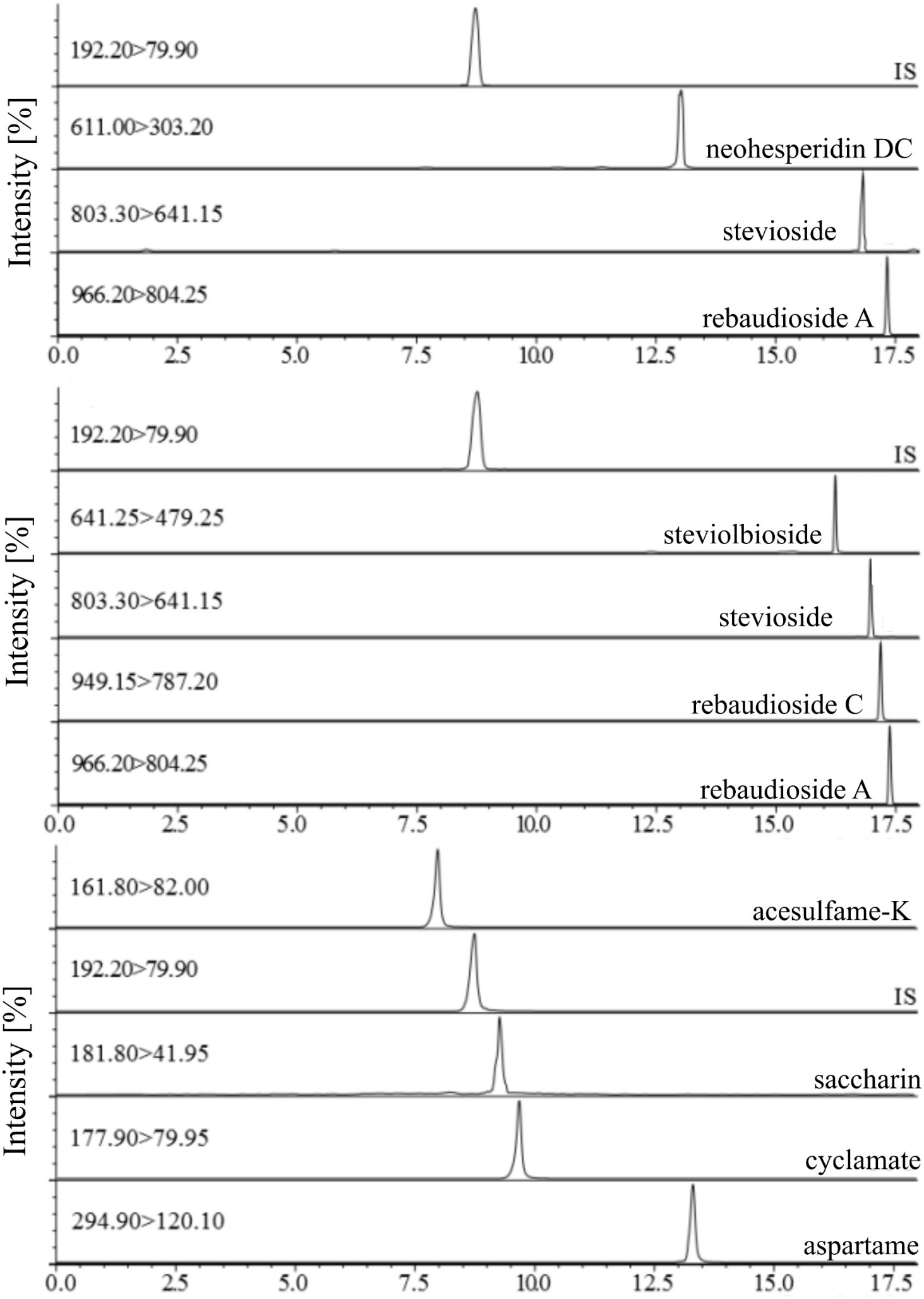
17.5
20.0
Time [min]


Intensity [%]
192.20>79.90
IS
611.00>303.20
neohesperidin DC
803.30>641.15
stevioside
966.20>804.25
rebaudioside A
0.0
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17.5
Intensity [%]
192.20>79.90
IS
641.25>479.25
steviolbioside
803.30>641.15
stevioside
949.15>787.20
rebaudioside C
966.20>804.25
rebaudioside A
0.0
2.5
5.0
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Intensity [%]
161.80>82.00
acesulfame-K
192.20>79.90
IS
181.80>41.95
saccharin
177.90>79.95
cyclamate
294.90>120.10
aspartame
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5

Table S1. Monitored ion transitions, their parameters and optimal MS/MS operational parameters

Compound	Polarity	Ion transition	Q1 Prebias [V]	Collision energy [V]	Q3 Prebias [V]
acesulfame-K	-	161.80→82.00	17	15	30
saccharin	-	181.80→41.95	18	27	14
aspartame	+	294.90→120.10	-30	-14	-19
sucralose	-	454.85→395.05*	22	11	17
cyclamate	-	177.90→79.95	18	26	30
alitame	+	332.10→129.15	16	15	20
IS	-	192.20→79.90	14	28	30
neohesperidin DC	-	611.00→303.20	26	37	30
neotame	+	379.00→172.15	-18	-21	-19
rebaudioside A	-	966.20→804.25	38	26	40
stevioside	-	803.30→641.15	30	29	30
rebaudioside C	-	949.15→787.20	38	33	38
dulcoside A	-	787.15→625.20	32	22	30
steviolbioside	-	641.25→479.25	24	42	22
steviol	-	317.40→317.40**	24	15	30

*adduct with acetic acid

**compound does not undergo fragmentation

MS/MS operation parameters					
Nebulising Gas Flow [L/min]	Heating Gas Flow [L/min]	Interface Temperature [˚C]	DL Temperature [˚C]	Heat Block Temperature [˚C]	Drying Gas Flow [L/min]
3	10	350	250	500	10

DL – Desolvation line

Table S2. Resolution factors between adjacent peaks obtained for two developed methods - HILIC and RPLC [9].

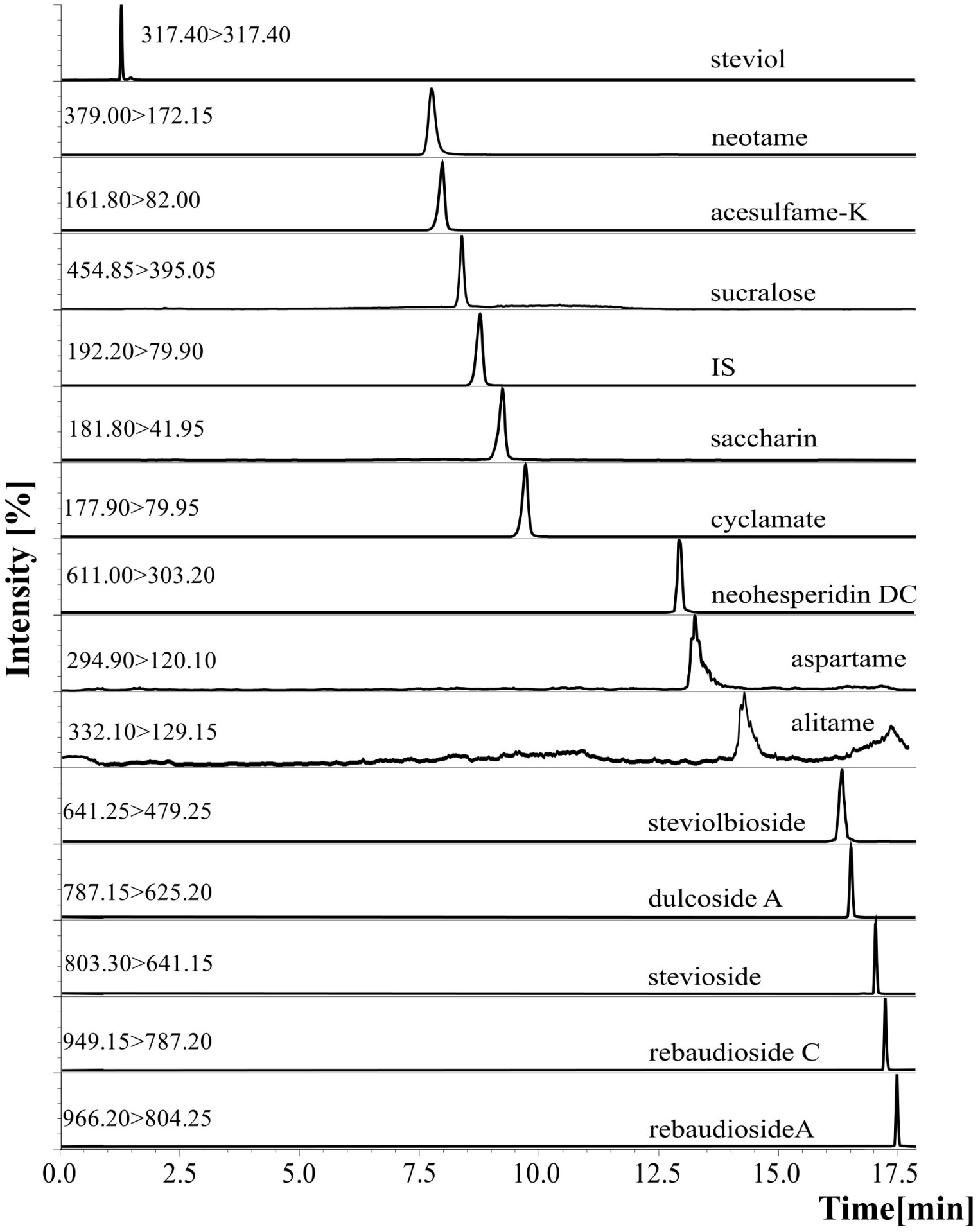
RPLC		**HILIC**	
	Rs [-]		**Rs [-]**
$Rs_{(acesulfame\text{-}K,\ saccharin)}$	2.9	$Rs_{(steviol,\ neotame)}$	34.2
$Rs_{(saccharin,\ aspartame)}$	6.7	$Rs_{(neotame,\ acesulfame\text{-}K)}$	1.1
$Rs_{(aspartame,\ sucralose)}$	4.5	$Rs_{(acesulfame\text{-}K,\ sucralose)}$	1.8
$Rs_{(sucralose,\ cyclamate)}$	2.4	$Rs_{(sucralose,\ IS)}$	1.9
$Rs_{(cyclamate,\ alitame)}$	6.0	$Rs_{(IS,\ saccharin)}$	2.1
$Rs_{(alitame,\ IS)}$	11.3	$Rs_{(saccharin,\ cyclamate)}$	1.6
$Rs_{(IS,\ neoheseperidin\ DC)}$	10.9	$Rs_{(cyclamate,\ neohesperidin\ DC)}$	15.9
$Rs_{(neohesperidin\ DC,\ neotame)}$	25.1	$Rs_{(neohesperidin\ DC,\ aspartame)}$	1.1
$Rs_{(neotame,\ rebaudioside\ A)}$	5.7	$Rs_{(aspartame,\ alitame)}$	2.4
$Rs_{(rebaudioside\ A,\ stevioside)}$	1.2	$Rs_{(alitame,\ steviolbioside)}$	7.7
$Rs_{(stevioside,\ rebaudioside\ C)}$	7.0	$Rs_{(steviolbioside,\ dulcoside\ A)}$	1.1
$Rs_{(rebaudioside\ C,\ dulcoside\ A)}$	3.0	$Rs_{(dulcoside\ A,\ rebaudioside\ C)}$	5.0
$Rs_{(dulcoside\ A,\ steviolbioside)}$	11.0	$Rs_{(rebaudioside\ C,\ stevioside)}$	2.3
$Rs_{(steviolbioside,\ steviol)}$	33.3	$Rs_{(stevioside,\ rebaudioside\ A)}$	2.9

Table S3. Recovery values used for estimation of repeatability.

	RPLC [9]			HILIC		
	Recovery (RSD) [%] of the analyte (n=6) $60*10^3$ ng/mL					
Analyte	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
acesulfame-K	103.9 (1.5)	103.5 (1.9)	104.0 (2.2)	101.5 (1.9)	104.5 (3.1)	103.7 (1.2)
saccharin	103.0 (2.9)	103.9 (3.5)	105.2 (2.4)	100.5 (2.1)	101.6 (2.5)	102.1 (3.2)
aspartame	98.4 (1.8)	102.2 (1.2)	101.8 (2.1)	103.8 (2.3)	103.7 (3.4)	102.8 (2.6)
sucralose	103.6 (1.9)	99.6 (2.5)	98.5 (2.6)	102.3 (2.9)	102.4 (2.7)	104.3 (2.6)
cyclamate	97.6 (1.3)	98.9 (1.2)	100.0 (1.5)	99.6 (1.3)	99.5 (3.3)	102.6 (2.4)
alitame	97.8 (2.5)	98.7 (2.1)	97.9 (1.5)	98.9 (2.8)	101.0 (1.8)	103.2 (1.5)
neohesperidin DC	98.8 (1.1)	100.0 (2.3)	101.6 (2.9)	102.6 (1.6)	103.4 (3.2)	102.1 (2.8)
neotame	97.6 (1.2)	97.0 (1.5)	98.1 (1.5)	104.9 (3.7)	103.9 (2.2)	105.0 (3.1)
rebaudioside A	103.3 (2.3)	103.0 (1.8)	101.6 (2.3)	101.7 (4.4)	102.6 (3.1)	101.3 (4.0)
stevioside	102.1 (2.5)	98.3 (1.3)	99.4 (2.7)	103.3 (1.8)	101.9 (4.3)	104.2 (2.2)
rebaudioside C	103.6 (3.0)	102.4 (1.3)	102.1 (2.1)	98.9 (2.4)	99.2 (1.6)	98.7 (2.3)
dulcoside A	105.5 (4.5)	103.2 (1.4)	104.0 (1.8)	103.7 (3.5)	101.7 (2.6)	102.3 (3.7)
steviolbioside	101.9 (2.5)	98.9 (3.6)	98.2 (4.1)	102.5 (2.4)	98.5 (2.9)	101.5 (1.4)
steviol	104.6 (2.9)	98.6 (1.3)	100.8 (2.9)	100.9 (2.2)	102.4 (1.1)	101.2 (1.8)

Table S4. Comparison of precision and accuracy of results obtained with RPLC and HILIC methods based on the values obtained by F-Snedecor test and Student t-test

	F-Snedecor test ($F_{critical}$=5.05)			Student t-test ($t_{critical}$=2.23)		
Analyte	F_1 (10×10^3 ng/mL)	F_2 (25×10^3 ng/mL)	F_3 (60×10^3 ng/mL)	t_1 (10×10^3 ng/mL)	t_2 (25×10^3 ng/mL)	t_3 (60×10^3 ng/mL)
acesulfame-K	1.61	5.01	2.43	0.60	1.69	**8.63**
saccharin	1.68	1.16	1.42	1.97	**3.41**	0.84
aspartame	1.31	4.96	1.79	0.17	1.91	1.44
sucralose	3.72	3.00	2.03	0.62	0.33	0.75
cyclamate	2.20	1.85	1.90	**5.07**	**4.84**	0.55
alitame	4.93	2.22	4.78	0.36	0.58	0.26
neohesperidin DC	1.44	4.13	1.39	1.01	**2.76**	**2.48**
neotame	1.60	2.53	2.11	0.82	1.47	1.00
rebaudioside A	1.69	3.84	1.23	**3.54**	1.04	**3.64**
stevioside	1.14	2.59	2.04	1.25	0.98	0.93
rebaudioside C	1.43	4.49	2.15	0.45	1.75	0.09
dulcoside A	1.20	3.71	1.04	**3.04**	**3.81**	2.31
steviolbioside	2.70	3.01	1.58	1.98	**4.35**	1.92
steviol	1.74	1.29	1.03	0.10	0.11	0.96


317.40>317.40
steviol
379.00>172.15
neotame
161.80>82.00
acesulfame-K
454.85>395.05
sucralose
192.20>79.90
IS
181.80>41.95
saccharin
177.90>79.95
cyclamate
611.00>303.20
neohesperidin DC
294.90>120.10
aspartame
332.10>129.15
alitame
641.25>479.25
steviolbioside
787.15>625.20
dulcoside A
803.30>641.15
stevioside
949.15>787.20
rebaudioside C
966.20>804.25
rebaudiosideA
Intensity [%]
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
Time[min]