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Abstract

Non-nutritive or low calorie sweeteners are commonly used worldwide in the food industry, often in combination in order to limit undesirable tastes. The list of allowable sweeteners varies among countries and it is important for regulatory agencies and food safety laboratories to monitor these highly consumed products to ensure compliance with worldwide regulations. Current analytical methods for confirmation and quantification of sweeteners must allow for confirmation of analyte identity in order to be compatible with today's standards. Various methods for the determination of non-nutritive sweeteners have been reported in the literature. The most common multi-sweetener methods involve high performance liquid chromatography (HPLC) with different types of detection. The modern technique of HPLC-MS/MS is the current method of choice for the determination and confirmation of sweeteners in foods. In addition to multi-sweetener analyses there is also a need for single sweetener analytical methods in certain circumstances.

***Keywords*:** Non-nutritive sweeteners, foods, LC-MS/MS

2.1 Introduction

Non-nutritive sweeteners are commonly used in foods as alternatives to sugar to provide a sweet taste with little or no calories [1]. They are an important class of food additives which are added to foods to cause

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a technical effect such as sweetening [2]. Sweeteners are grouped into two main categories, bulk and intense sweeteners. Bulk sweeteners, such as sugar alcohols, provide texture and preservative effects to low calorie foods, with equivalent or less sweetening strength relative to sucrose. Sugar alcohols have been given *quantum satis,* meaning that they are harmless enough to have no specific quantity restriction [3].

Intense sweeteners have sweetening capacities greater than sucrose with varying potencies. These compounds can be synthetic, semi-synthetic or natural. The majority are synthetic compounds, including aspartame (ASP), sucralose (SCL), saccharin (SAC), cyclamate (CYC), acesulfame-potassium (ACS-K), alitame (ALI), neotame (NEO) and dulcin (DUL). Neohesperidine dihydrochalcone (NHDC) is a semi-synthetic sweetener, while stevioside (STV) and rebaudioside (REB) A are natural sweeteners [4]. The list of allowable sweeteners varies among nations worldwide [5]. For example, CYC and NHDC are not approved for use as food additives by the US Food and Drug Administration (FDA) but are authorized in the European Union (EU) [6].

The oldest sweetener on the market, SAC is approved for use in nearly 90 countries. It has a sweetening strength about 450 times that of sucrose and exhibits high water solubility and storage stability [7]. In the 1980s, its consumption was linked with bladder cancer in rats and as such was prohibited in Canada [8]. Despite its bitter metallic aftertaste it is approved for use in many foods and beverages [9]. Unlike SAC, DUL does not have a bitter aftertaste and has a sweetening capacity about 250 times that of sucrose. However, DUL has not gained widespread use due to concerns over its toxicity [7]. It is not approved for use in the USA.

Discovered in 1967, ACS-K exhibits good storage stability [9]. It is 200 times sweeter than sucrose and its use is associated with a slight bitter aftertaste at high concentrations [8]. ACS-K is widely used and approved in 90 countries with few health problems linked with its use [9]. It has very good water solubility and is stable at high cooking and baking temperatures [7].

In contrast, ASP is the most controversial artificial sweetener regarding its health effects. There have been reports about adverse neurological effects and cancer in rats. It is 180 times sweeter than sucrose and thus only small quantities are added to foods to achieve the desired sweetness. Since ASP is not heat-stable it degrades in liquids during prolonged storage [8]. Therefore, it cannot be used in baking or cooking and beverage products with ASP have expiry dates for acceptable consumption [9]. It has been approved for use by the US FDA and the EU. Phenylalanine is a metabolite of ASP, which cannot be metabolized by people with phenylketonuria, a

rare genetic disorder. Excessive intake of phenylalanine has been linked to brain damage [7]. As a result, all products containing ASP must be labeled to indicate the presence of a phenylalanine source [8].

A derivative of ASP, NEO is an odorless, white crystalline powder. It is safer for consumption by people with phenylketonuria because the 3,3-dimethyl group in its structure blocks the breakdown to phenylalanine [10]. NEO is 7000–13000 times sweeter than sucrose, with a taste very similar to sucrose. Its use is not associated with any bitter aftertaste and it has extensive shelf-life stability in dry conditions. It is also very stable in aqueous solutions in the neutral and acidic pH ranges [7]. In addition, NEO is heat stable and thus can be used in cooking and baking. It is approved for use in the USA, Australia, New Zealand and the EU.

The dipeptide sweetener ALI has a sweetening capacity 2000 times greater than sucrose. Due to the presence of an amide moiety in its structure, ALI is relatively heat stable [7]. It has no aftertaste and is characterized by a clean, sweet flavor. It is approved for use as a sweetener in Australia and Mexico but not in the USA or EU [7].

Discovered in the 1960s, NHDC has a sweetening strength ~1500 times greater than sucrose. Industrially, it is produced by hydrogenation of a flavonoid (neohesperidin) found in citrus fruits. NHDC is known to have menthol-licorice-like aftertastes and antioxidant properties [8]. It exhibits good stability in aqueous solutions [7].

Sucralose is thermally stable and contains three chlorine atoms in its structure, making it an organochloride. It is about 600 times sweeter than sucrose and can be used during cooking and baking [9]. It is approved for use by the US FDA in a variety of foods and beverages. There is some concern about its safety due to the fact that other organochlorides such as dioxins and pesticides are linked with toxic and carcinogenic effects [8]. However, human and animal studies have shown SCL to be safe for human consumption [9].

Steviol glycosides are natural components in the extract of *Stevia rebaudiana* Bertoni, a plant native to Paraguay [11]. Stevia has been used for years in Japan, Korea, China, Brazil, and Paraguay as a food additive or as a household sweetener [12]. Steviol glycosides under certain conditions are considered Generally Recognized as Safe (GRAS) by the FDA and are approved in the EU. Stevia produces several diterpene glycosides, the most abundant being STV and REB A [13]. Five other steviol glycosides have been identified as minor components of the stevia leaf, including Reb C, D, F, dulcoside A, and rubusoside. The steviol glycosides have similar structures: a steviol aglycone is connected at C-4 and C-13 to mono, di, or trisaccharides consisting of glucose and/or rhamnose residues [14, 15].

Steviolbioside and Reb B are thought to be hydrolysis products of STV and Reb A formed during the extraction process of the glycosides from the plant [16]. The distribution of steviol glycosides in plant extracts can vary greatly depending on the extraction and purification process [17]. One issue preventing the wide use of stevia as an artificial sweetener is the presence of a bitter aftertaste in some extracts. REB A has been reported to have the least bitterness of the major steviol glycosides [18]. The sweetening power of the steviol glycosides also differ, with REB A being 400 times sweeter than sucrose while STV is about 300 times sweeter [16, 19].

Sweeteners are often used in combination to enhance sweetness and limit undesirable aftertastes [7]. A classic example is the blend of SAC-CYC formulated in a 1:10 ratio. The bitter aftertaste of SAC is masked by CYC and due to an additive effect the sweetening power of the mixture is greater. Food products containing sweeteners are heavily promoted as beneficial for the treatment of obesity and management of diabetes [7]. Sweeteners can be found in a large number of food products including the following: tabletop sweeteners, carbonated and non-carbonated beverages, baked goods, preserves and confectionery (icings, frostings, and syrups), alcoholic drinks, candies and dairy products such as yogurt and ice cream [20].

There is considerable controversy surrounding the adverse health effects of non-nutritive sweeteners. Consumers worldwide have reported side effects linked to sweetener consumption, including mood and behavioral changes, skin irritations, headaches, allergies, respiratory difficulties, and cancer [7]. As such, it is important to monitor and control the concentration of sweeteners in foods to ensure compliance with different country-specific regulations. The EU limits the amount of sweeteners added to food and sets a maximum usable dose (MUD) for specific food commodities [20]. In order to ensure that products are in compliance with regulations, it is necessary to have reliable, robust and quantitative methods for the simultaneous determination of several commonly used sweeteners in a single analysis.

In addition to multi-sweetener analyses, there is also a need for single sweetener analytical methods such as in the case of CYC. The non-nutritive sweetener CYC was discovered in the 1930s [21]. It is 30–40 times sweeter than sucrose with its efficacy increasing when used in combination with other sweeteners [22]. It is widely used as a sweetening agent in a variety of low-calorie foods and beverages in many countries [21]. However, CYC is banned for commercial use as a food additive by the US FDA (Code of Federal Regulations 21, §189.135) because of research findings that linked its consumption with bladder cancer in rats [23]. Under the ban, CYC cannot be added to or be detectable in food. Since there is an increasing

number of foods sold in the USA that are imported from other countries, where CYC is approved for use as a food additive, it is important to have analytical methodology for the detection and confirmation of CYC in foods [22].

2.2 Sample Preparation

Sample preparation/cleanup is the process of isolating target analytes from interferences in food matrices prior to instrumental analysis. This is often the most time-consuming part of the analytical method and is essential to analyte determination. In order to be able to determine whether or not a sample contains sweeteners and authenticate the presence and concentrations of these analytes in various foods, simple to extensive sample cleanup is necessary. Sweeteners are widely used in drinks, candies and yogurts, which are commonly consumed products [24]. Foods are complex matrices due to the considerable differences in their composition, which includes the presence of macromolecules, color additives and preservatives. Furthermore, sweeteners are present in food products at levels that require prepared samples to often be significantly diluted in order to bring the analyte concentrations within the linear range of the method. There are many components in food matrices that have similar polarities to sweeteners, most of which are water soluble, with the exception of DUL and NHDC. Therefore, it is very difficult to isolate sweeteners from food matrix.

There are considerable differences in the concentrations of sweeteners in drinks, possibly due to beverage manufacturing processes that may contribute to these variations. Differences are most likely due to the varying sweetening strengths of these compounds relative to sucrose. Therefore, differing amounts of sweeteners are added to produce the desired sweetening effect [3]. Furthermore, there are significant differences in chemical properties among sweeteners such as solubility and thermal stability [3]. As such, some sweeteners function better in certain food types while others are best suited for use in drinks.

Generally, hard candies, drinks and tabletop sweeteners require minimal sample preparation prior to instrumental analysis. Normally, hard candies and tabletop sweeteners are weighed and dissolved in H_2O by the process of shaking and/or vortexing. The samples are then diluted to obtain an analyte concentration within the linear range of the method. This procedure should produce complete dissolution of the candy or tabletop samples, resulting in transparent solutions with no visual insoluble

material remaining after shaking. Drink samples are simply diluted with H_2O or mobile phase and filtered with sonication of carbonated beverages to remove dissolved gases [25]. Replicate analysis should be performed on all samples and if products are packaged in individual servings (candy, tabletop sweeteners), separate packages should be analyzed.

Liquid-liquid extraction (LLE) is sometimes used as a simple, low-cost method to prepare samples prior to instrumental analyses [5]. LLE involves addition of an organic solvent to the food in liquid form. Sweeteners are then extracted from the liquid aqueous phase into the organic phase [6]. Solid-liquid extraction (SLE) is the process of partitioning target analytes from a solid state into a solvent prior to dilution and filtration. Solid samples can be homogenized, vortexed and centrifuged to separate the supernatant [5, 22, 26].

Yang and Chen [5] used LLE and SLE to extract sweeteners from a water/methanol solution (50:50, v/v). Beverages were degassed when necessary and solid samples were homogenized and extracted. The method was applied to the determination of eight non-nutritive sweeteners in foods. Lim *et al.* modified the LLE and SLE procedures developed by Yang and Chen to analyze nine artificial sweeteners in Korean foods. Samples analyzed included candies, beverages and yogurts. Sheridan and King [22] applied SLE with homogenization to the analysis of CYC in a wide range of foods, including dried prunes and beans, jarred mangos and peaches, grape tomatoes and strawberry cake. Since CYC is water-soluble the aqueous extract could be centrifuged, filtered and significantly diluted, which limits matrix interferences and MS signal suppression [22]. Scotter *et al.* also used LLE and SLE for the analysis of CYC in carbonated beverages, fruit juices, milk-based desserts, jams and spreads. Additionally, Carrez I and II solutions (reagents used to precipitate proteins and fats) were prepared and added to the foods under slightly heated conditions for sample clarification [7, 26]. This is followed by centrifugation to separate proteins and fatty material from the water-soluble supernatant in complex matrices such as ice-cream, chocolate syrup and coffee creamers [27]. The supernatant can then be filtered and diluted in preparation for instrumental analysis. Centrifugation without protein separation may be needed to separate solid particles present in some fruit juices [28]. Solvents that are commonly used for extraction are methanol (MeOH), acetonitrile (ACN) and water [28].

Another technique to prepare solid samples, such as dried fruits, uses a cryogenic grinder. Dried fruits are cut into small pieces and placed into a cryogenic blender. Liquid nitrogen is then poured over the pieces until they are immersed. Once the liquid nitrogen completely evaporates and

the pieces are frozen they are blended into a fine powder using an analytical mill. Solvent is then added to a weighed amount of the powder with subsequent vortex mixing, centrifugation, dilution and filtration [28]. This procedure results in a more homogeneous and uniform sample mixture than achieved with normal homogenization because the solid is broken down into very fine particles.

One of the biggest challenges in food analysis is the effect of matrix composition on the performance of the analytical method. In order to determine method accuracy and selectivity, a representative from each food commodity containing no target analytes is fortified with known amounts of sweeteners. The sweeteners chosen for spiking experiments should encompass the range of polarities, including most polar, intermediate and nonpolar compounds. Food products are fortified in triplicate at three different concentrations in accordance with agency guidelines and analyzed alongside an unfortified sample.

Solid-phase extraction (SPE) is a reproducible technique that can be used to isolate sweeteners based on their affinity to a stationary phase. The SPE sorbents are silica- or polymer-based beds that are modified with polar or nonpolar functional groups [29]. There are many types of commercially available SPE cartridges that are packed with C8, C18 and ion-exchange sorbent beds [29]. For the isolation of sweeteners from foods, the most successful SPE cartridges have been those with reversed-phase (RP) sorbents such as C8 or C18 [30].

Zygler *et al.* developed a method for the determination of nine non-nutritive sweeteners using Strata-X polymeric RP 3 mL cartridges packed with 200 mg sorbent bed for the cleanup of beverages, yogurts, and fish products [20]. These SPE cartridges were chosen because extensive testing of different SPE columns, including Chromabond C18ec, Strata-X RP, and Bakerbond Octadecyl, revealed optimal recoveries for all sweeteners were achieved [29].

Scheurer *et al.* [8] tested several different SPE cartridges and determined that Bakerbond Isolute SDB-1 achieved best recoveries for the extraction of ACS-K, SAC, ASP, CYC, NEO, SCL and NHDC in waste and surface waters. Yogurts represent a much more complex mixture of ingredients than beverages or hard candies, thus requiring a thorough sample cleanup prior to chromatographic analyses [31]. This ensures better long-term performance of the instrument and minimizes ion suppression effects when using mass spectrometric detection.

Shah *et al.* [32] modified and optimized a previous SPE method for the analysis of yogurts using Macherey-Nagel Chromabond® C18ec 3 mL cartridges packed with 500 mg sorbent bed [29]. Several SPE parameters were

tested, including sorbent phase type, cartridge size, sample load volume, and extraction buffer. As previously seen, the most critical factor affecting analyte recoveries was the composition of the extraction buffer [29]. The use of formic acid and N,N- diisopropylethylamine (DIPEA) at pH 4.5 yielded the best recoveries for the sweeteners from yogurts. Compared to triethylamine (TEA), the ion pairing agent DIPEA allows for improved recoveries as it enables a stronger hydrophobic interaction between the sorbent bed and sweeteners [29]. As a result, this enables better retention of the sweeteners on the SPE cartridge, especially ACS-K and CYC. The authors reported that it is imperative to prevent the cartridge from drying out during the course of this SPE procedure.

Yang and Chen [33] developed a SPE method using a Waters Oasis HLB cartridge for the isolation of NEO from beverages, preserved fruits and cake. Dairy and fruit juice beverages were pretreated with MeOH, mixed, centrifuged and loaded on the SPE cartridge. Preserved fruits and cake were homogenized, vortexed, sonicated, centrifuged, and then loaded onto the SPE cartridge. The cartridge was conditioned prior to sample loading and then washed with water followed by MeOH to remove impurities. NEO was eluted with MeOH and concentrated to dryness by vacuum and reconstituted with MeOH prior to filtration into HPLC vials [33].

A dispersive SPE procedure was developed by Chen *et al.* for the determination of ACS-K, SAC, CYC, ASP, STV and NEO in red wine. The method allows for the quick magnetic separation of target analytes from matrix interferents using ethylenediamine-functionalized magnetic polymers (IEDA-MP) as the adsorbent. This technique allows for the easy clean-up of red wine using magnetic iron oxide particles to remove pigments, organic acids and sugars under a magnetic field. Recoveries ranged from 78.5% to 99.2% [34].

If available, a standard reference material containing certified values of sweeteners fortified in a food matrix can be obtained from an institution, such as the National Institute of Standards and Technology (NIST) or the International Union of Pure and Applied Chemistry (IUPAC), and analyzed. This material is analyzed to confirm that the method is valid and accurate for its designed purpose.

2.2.1 Internal Standards

Generally, it is important to have internal standards for quantitation to account for possible ion suppression from matrix interferences in the complex composition of foods [5]. Although it is ideal to have isotopically labeled standards for MS detection methods for each compound

being analyzed, these are sometimes unavailable and cost prohibitive. Therefore, similar chemical and physical properties to the target analytes are the criteria used for internal standard choice. Shah *et al.* used saccharin-d4, sodium cyclamate-d11 and D-Sorbitol-1-13C as the three internal standards for the analysis of fourteen sweeteners in foods [32]. Cycloheptylamine was used as the internal standard for the determination of CYC in foods by RP HPLC-UV [26]. Huang *et al.* used tiopronin as the internal standard for the determination of CYC in foods using ion-pair HPLC coupled to ESI-MS [21]. Sodium warfarin has been used as an internal standard in previous multi-sweetener methods for determination of several target analytes [5, 6]. Sucralose-d6 was used as the internal standard for the determination of SCL by ESI-LC/MS-MS in waste and surface waters [8].

2.3 Analytical Methods

2.3.1 Instrumental Analyses

2.3.1.1 HPLC-UV-VIS/DAD/ELSD Detection

Non-nutritive sweeteners are a class of compounds that have significantly different physical and chemical properties. This makes it very challenging to develop a single method for their separation and isolation from matrix interferences. In the past, the most common technique for screening sweeteners was thin-layer chromatography (TLC). The FDA has used the AOAC Official Method #969.27, TLC method for the determination of some non-nutritive sweeteners in food samples [35]. This method lacks specificity and is limited to the qualitative determination of a select few sweeteners for routine regulatory analyses. In addition, this method lacks confirmation criteria compatible with today's standards.

More recently, high performance liquid chromatography (HPLC) with reversed-phase (RP) ion-pair, ion and hydrophilic interaction chromatography (HILIC) have all been applied to the analysis of sweeteners. Gas chromatography is seldom used today for the analysis of sweeteners due to their low volatility and difficulty to form volatile derivatives. Therefore, GC will not be further discussed here. The FDA has used ion chromatography (IC) coupled to suppressed conductivity detection for the determination of ASP, CYC, ACK-S and SAC [36]. However, IC has proved to lack selectivity in certain matrices such as those that contain citric acid. The authors report significant interference from a very large citric acid peak in this anion-exchange separation which can adversely impact target analyte

determinations [36]. Furthermore, the scope of the method is narrow and does not incorporate all sweeteners of regulatory interest [37, 36].

Most sweeteners have poor chromophoric properties and determination by HPLC with an ultraviolet (UV) detector requires derivatization prior to analyses. Furthermore, HPLC-UV lacks specificity especially in food matrices. Additionally, sweeteners encompass a wide range of polarities and molecular size with very different pKa values that makes chromatographic separation difficult. For example, ERY is a very small highly polar compound compared to REB A, which is considerably larger and relatively more hydrophobic (Figure 2.1). Although several analytical methods for the determination of artificial sweeteners have been published, many are not appropriate for routine regulatory analyses.

Various detection techniques for the determination of non-nutritive sweeteners have been reported in the literature. The most common multi-sweetener methods involve HPLC with different types of detection [20].

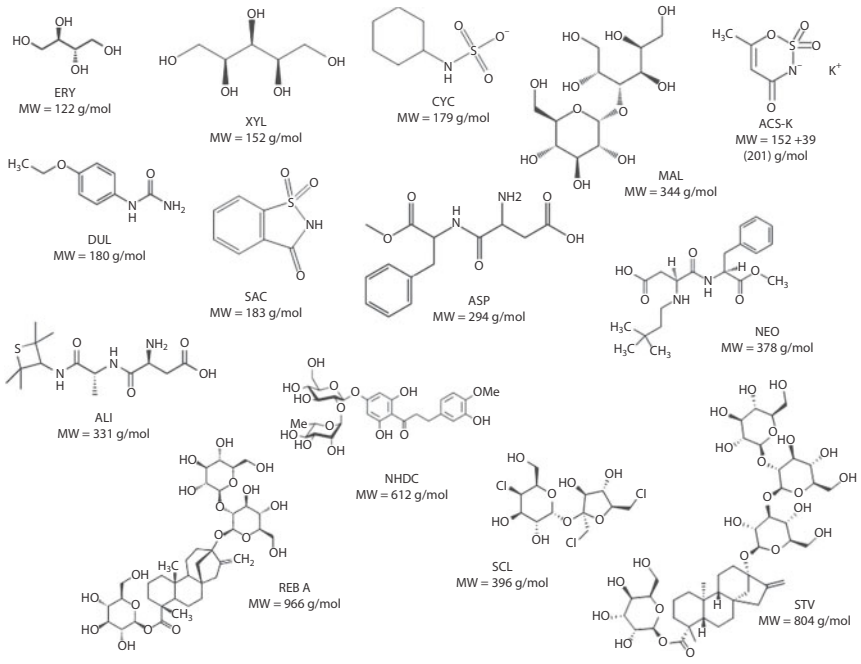

Figure 2.1 Chemical structures of the non-nutritive sweeteners of varying molecular sizes and polarities: ERY, erythritol; XYL, xylitol; CYC, cyclamate; DUL, dulcin; SAC, saccharin; ACS-K, acesulfame potassium; ASP, aspartame; ALI, alitame; MAL, maltitol; NEO, neotame; SCL, sucralose; NHDC, neohesperidine dihydrochalcone; STV, stevioside; REB A, rebaudioside A; and MW, molecular weight.

An HPLC-UV method is reported for the determination of CYC, SAC and ASP using a simple RP separation and detection at 196 nm. The method does not require derivatization of CYC or sample preparation prior to HPLC-UV. However, in order to achieve baseline resolution of CYC and SAC, the pH of the phosphate buffer mobile phase needs to be maintained at 2.5, which could severely compromise the integrity of a RP column [38]. Furthermore, many foods and beverages contain UV-active species which could interfere with the analysis if chromatographic separation was not achieved. This method was applied to the analysis of CYC, SAC, and ASP in beverages [38].

Scotter *et al.* developed a HPLC-UV method for the determination of CYC using peroxide oxidation of CYC to cyclohexylamine followed by derivatization with trinitrobenzene sulfonic acid. Analytes were separated by RP using a Spherisorb ODS2 C18 column (250 × 4.6 mm, 5 μm). The limit of detection (LOD) values ranged from 1–20 mg/kg in a variety of foods. Recoveries from spiking studies were in the range of 82% to 123%. The method was single-laboratory validated for the analysis of CYC in beverages, fruit preserves, spreads and dairy desserts [26].

Serdar and Knezevic [39] reported two RP methods using diode array detection (DAD) for the determination of ASP, ACS-K, SAC, and CYC in beverages and nutritional products. The first method used a C18 column for the isocratic separation of ASP, ACS-K, and SAC with a mobile phase of phosphate buffer and ACN. The second method used a C18 column for the isocratic separation of CYC with a mobile phase of MeOH and water [39]. However, derivatization of CYC to cyclohexylsulfamic acid was required prior to instrumental analysis, which is unfavorable for routine laboratory use.

A novel technique was reported by Cheng and Wu [40] for the determination of ASP and its hydrolysis products in Coca-Cola Zero. The authors described a two-dimensional HPLC-UV method using a C8 RP column for the first dimension and determination of ASP. The second dimension used a ligand-exchange column with online post-column derivation fluorescence detection for analysis of the hydrolysis products, L- and D-enantiomers of aspartic acid and phenylalanine. Electric or microwave heating was used to induce the formation of the hydrolysis products. The LOD and limit of quantitation (LOQ) for ASP were 1.3 and 4.3 μg/mL, respectively, with a linear range spanning 0–50 μg/mL. The LODs and LOQs for L- and D-aspartic acid were 0.16 and 0.17 μg/mL and for L- and D-phenylalanine were 0.52 and 0.55 μg/mL, respectively [40].

Determination of nine sweeteners by HPLC with evaporative light scattering detection (ELSD) has been published [2]. The method involves SPE

cleanup of samples prior to HPLC-ELSD. Analyte recoveries ranged from 93–109% for spiked concentrations at MUD levels or LOQ concentrations. LOD and LOQ values were < 15 μg/g and < 30 μg/g, respectively, in three matrices, including an energy drink, canned fruit and yogurt. Precision was tested by fortification of sweeteners in three matrices at three different concentration levels on three different days. Intermediate precision was < 8% for all sweeteners with the exception of ASP in canned fruits, due to its degradation as a result of improper storage conditions [2]. This method is suitable for rapid screening of samples for sweeteners but may lack selectivity for target analytes, especially among interferences in the matrix.

A rapid and sensitive method for the determination of steviol glycosides in *Stevia rebaudiana* and stevia products has been developed using ultra-high performance liquid chromatography (UHPLC) with UV and ELSD [41]. Five steviol glycosides are baseline separated on a Waters Acquity UPLC HSS T3 analytical column (100 × 2.1 mm, 1.8 μm) within 12 minutes. The LOD and LOQ values for the steviol glycosides were <10 and <30 μg/mL, respectively. Tested matrices included ground leaves and powder of the stevia plant as well as liquid/solid extracts. Intra- and inter-day precision analyses yielded % RSD values <2.5. The method enabled the quantitation of five steviol glycosides in a single analytical procedure. The use of UV and ELSD enabled confirmation of the characterization of steviol glycosides in *S. rebaudiana* and related products [41]. The method can be applied to the determination of all steviol glycosides of regulatory interest in order to determine total content.

2.3.1.2 HPLC-MS/Tandem MS Detection

HPLC-tandem mass spectrometry (MS/MS) is a technique that affords high specificity and sensitivity and has become widely used for food analysis [21]. MS allows for the direct detection of sweeteners without the need for analyte derivatization prior to instrumental analyses. Electrospray ionization (ESI) is most commonly coupled to HPLC-MS and tandem MS systems for the analyses of sweeteners.

A method for the determination of CYC in foods was developed using ion-pair HPLC ESI-MS in selected ion monitoring (SIM) mode [21]. The separation of CYC was achieved on a Spherigel C8 5 μm column using 5 mM tris(hydroxymethyl)aminomethane as the ion-pair reagent in the mobile phase (pH 4.5) operated under isocratic conditions. Ion-pair chromatography is very sensitive to slight changes in pH that can result in poor chromatographic reproducibility. The linear range spanned 50–5000 ng/mL

with LOD and LOQ values reported as 5 ng/mL and 20 ng/mL, respectively. The method was applied to the analysis of canned orange and mango, dried fruits, ice cream and beverages [21].

An HPLC ESI-MS method was developed by Yang and Chen [5] for the analysis of seven sweeteners in foods. The MS acquisition was performed in the negative ion mode by applying SIM. The LOD values were <0.1 μg/mL with LOQs <0.3 μg/mL for all analytes [5]. Results from accuracy studies showed recoveries between 95 and 104%. The method was applied to the analysis of beverages, candied fruit, and cake [5].

A multi-sweetener method using RP HPLC with ESI-MS detection in the negative mode has been reported by Zygler *et al.* [20]. Analytes were separated by gradient elution on a Nucleodur C18 Pyramid column (250 × 3 mm, 5 μm). The LOD values for nine non-nutritive sweeteners were < 0.25 μg/mL with LOQs < 2.5 μg/mL. Recovery results from spiking studies were in the range of 84–107%, as determined in a cola beverage, yogurt and fish marinade. There were some disadvantages to this method [20]. The DUL could not be directly detected and was determined as a formic acid adduct. In addition, three commonly used sweeteners ACS-K, SAC and SCL gave nonlinear responses in the tested calibration range [20].

Nine sweeteners were determined in foods using HPLC ESI-MS/MS in multiple reaction monitoring mode (MRM) with improved MS confirmation data [6]. Analytes were separated on a Thermo Hypersil BPS C18 column (250 × 3 mm, 5 μm). However, recovery studies were performed at spiking concentrations significantly below what would be expected in real samples, limiting the value of the accuracy data.

A fast and reliable LC-MS/MS method for the determination of CYC in a variety of food matrices was developed and validated [28]. This method provides both quantitation and the qualitative mass spectral determination important for the analysis of regulatory samples. The method requires minimal sample preparation followed by a RP HPLC separation. Utilization of a CYC-d11 internal standard corrects for potential matrix interferences during sample processing and allows minimal sample preparation. Detection and quantitation were achieved using HPLC ESI-MS/MS with MRM confirmation. Seventeen commercially available food products were fortified at 250 μg/mL and tested as part of the method validation. Recoveries ranged from 72–110%, with relative standard deviations (RSD) ranging from 3–15%. The linear range spanned 0.010–1.00 μg/mL. The LOD values were 0.1 and 0.6 ng/mL, as determined in pomegranate juice and dried fig, respectively. The LOQ values were 0.3 and 1.6 ng/mL, which were significantly lower than needed to measure CYC when it is used as a food additive. This method was validated for the analysis of a variety of

commonly adulterated products, including drinks, dried fruits, jams and hard candies. It was proved suitable for analysis in a regulatory setting as it requires minimal sample preparation due to the selectivity afforded by MS/MS. This method allows the US FDA's ability for accurate quantitation and confirmation of CYC and provides industry and regulatory laboratories a rapid and selective method to monitor imported and domestic food products for the presence of CYC.

In the case of the steviol glycosides, due to the structural similarity between STV and REB A, baseline separation using RP HPLC columns can be difficult. However, baseline separation is necessary for accurate quantitation, even when using MS, because the analytes have a common mass spectral fragment ion (803 m/z). Baseline separation of nine steviol glycosides in plant extracts was achieved using a two-dimensional LC–S/MS system but this method was not applied to food matrices [18]. A direct, versatile method for the determination of steviol and nine steviol glycosides in food products was developed using HPLC ESI-MS in the negative ion mode. Ten stevia compounds were readily separated on an amino column using a gradient separation and HILIC retention mechanism. As previously seen, the use of HILIC with an amino column enabled baseline separation of STV and REB A [16]. Data for analyte quantitation was collected in SIM mode, giving LODs in the range of 0.01–0.34 μg/g and repeatability at the LOQ of 2–15% relative standard deviation (RSD). Thirty-four commercially available food products were tested using the optimized method and in these products REB A and STV comprised 52 to 100% of the total steviol glycoside content. Data was collected in MRM mode for analyte confirmation. This method allows for the characterization of steviol glycosides in foods with minimal sample clean-up and provides accurate identification and quantification of 10 stevia compounds in a single analytical procedure. The information provided by this method is useful for industry and governing authorities to ensure compliance with international regulations [42].

An analytical method was developed by Ordonez *et al.* for the determination of six sweeteners in environmental waters using HPLC/MS-MS. Analytes were separated by RP-HPLC using a Phenomenex Luna C18 column (100 mm × 2.0 mm, 3 μm). The authors reported that RP chromatography was more successful for the separation of sweeteners than HILIC [30]. This method allows quantitation and MRM confirmation of sweeteners using the two most intense transitions for each analyte.

A method for the determination of three common sweeteners, ASP, SAC and SCL, was developed using HPLC with time-of-flight (TOF) MS [43].

Sweeteners were confirmed by accurate MS measurements of protonated molecules, sodium adducts and main fragment ions [43]. The studied fragmentation pathways of these sweeteners can be used to model their degradation [43]. The method was applied to the analyses of beverages, liquid syrups and environmental water samples, enabling the identification of these sweeteners using high resolution MS.

An improved, efficient, sensitive method for the determination of fourteen non-nutritive sweeteners in food products has been developed using ESI with UHPLC MS/MS in the negative ion mode [32]. Fourteen sweeteners and three internal standards are separated on a RP UHPLC column using a simple gradient program. Chromatographic separations were performed on a Waters (Milford, MA) Acquity UPLC BEH C18 analytical column (100 × 2.1 mm, 1.7 μm). Target analytes include CYC, SAC, SCL, DUL, ASP, NHDC, ACS-K, ALI, NEO, REB A, STV, and the three sugar alcohols, erythritol (ERY), xylitol (XYL) and maltitol (MAL). This method allows quantitation and MRM confirmation of all sweeteners using three isotopically labeled internal standards. An Applied Biosystems Sciex (Foster City, CA) 4000 Q-trap LC/MS/MS system interfaced with an Agilent (Agilent Technologies, Santa Clara, CA) 1290 series UHPLC was used in all experiments. Two structurally significant MS product ions for each sweetener and all three internal standards allow for more selectivity and confirmation of target analytes, while providing an important advantage over previously reported methods [5, 20]. Ion ratios of the two MS transitions were used for identity confirmation of sweeteners in standards and samples. Tested matrices included carbonated and non-carbonated beverages, hard candies, and yogurt samples. Yogurts were processed using an SPE method but minimal sample cleanup was required to analyze beverages and hard candies. The method enabled the quantitation and confirmation of fourteen sweeteners in a single analytical procedure. It will be useful for industry and regulatory authorities in order to monitor sweetener concentrations in commercial products to ensure compliance with country-specific regulations.

Ordonez *et al.* reported a LC-MS/MS method to determine nine high-intensity sweeteners using water and a small amount of ethanol as the mobile phase for separation in 23 minutes. A high temperature gradient with ethanol as the organic modifier allowed for separation of sweeteners using a Shodex ET-RP1 column (150 × 3.0 mm, 4 μm). The LOD values were in the range of 0.05–10 μg/mL with recoveries from spiking studies between 86–110%. The method was applied to the analyses of 25 beverage products [44]. The potential for using simply water and a small

amount of ethanol (green solvent) for elution is promising, however, compounds eluting prior to 10 minutes exhibited peak tailing effects.

2.3.1.3 Capillary Electrophoresis

Capillary electrophoresis (CE) is an alternative to HPLC with a comparable resolving power and low solvent consumption [45]. It is a quick and simple procedure using photometric detection in the UV region or conductivity detection. For compounds not amenable to UV detection due to their poor chromophoric properties, such as CYC, conductivity detection is a good alternative. Capacitively coupled contactless conductivity detection is a fairly new approach for detection with CE [45]. A method was developed for the determination of ASP, CYC, SAC and ACS-K, chromatographically separated in < 6 minutes using a background electrolyte [45]. This method was applied to the analyses of beverages and tabletop sweeteners [45].

A method using CE was developed for the simultaneous analysis of ASP, CYC, SAC and ACS-K by Fernandes *et al.* [46]. Optimum separation conditions were achieved for simultaneous direct (ASP, ACS-K and SAC) and indirect (CYC) UV detection at 215 nm. The method was applied to the analyses of lemon tea sachet samples containing ASP, CYC, SAC, and ACS-K [46].

2.4 Future Trends

Due to its high sensitivity and robustness the current method of choice for the determination of sweeteners in different foods is HPLC-MS/MS. This technique allows for both single- and multi-analyte sweetener determination while providing quantitative and confirmatory analyses. Although CE is an interesting technique with comparable resolving power available at lower cost with less solvent consumption, it is less popular due to limited robustness. As the availability of UHPLC-MS/MS systems rises in laboratories, there will be a growing need for more methods using this procedure. This will allow high-throughput analyses of foods and beverages for sweeteners using rapid chromatographic separations. As discussed in this chapter, multi-sweetener methods are beneficial for a variety of reasons; however, sometimes there is value and a need for single-sweetener methods. In the future, as new analytical methods are developed using the latest instrumentation and sample preparation options, there will be a need for these procedures to be validated and applied to the routine analyses of sweeteners.

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