Chapter 11
Current Analytical Methods for Qualitative and Quantitative Measurement of D-Xylitol

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Abstract D-xylitol is a naturally-occurring five-carbon sugar alcohol. It can also be derived from the chemical reduction of D-xylose. It is widely used in recent years and will continue to be used as a food additive and sweetening agent in the food industry. The qualitative detection and quantification of D-xylitol in the presence of other sugars and sugar alcohols in fruits, vegetables and other natural sources is essential for industry production. A number of analytical methods have been developed over the years for qualitative detection and quantitative measurement of D-xylitol. Since most samples to be analyzed contain a mixture of compounds, highly efficient and sensitive analytical methods for D-xylitol in the mixture are required. Current analytical methods are usually comprised of two components: (1) an efficient separation unit, and (2) a structure identification unit. In this chapter, we provide an overview on these analytical methods used for the qualitative and quantitative determination of D-xylitol in samples from various sources. Chromatography-based techniques including GC, HPLC and CE methods with different detection options, such as UV, RI, ELS, etc., have been widely used. More advanced analytical instruments derived from hyphenation of

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chromatography with structure determination tools such as MS and NMR are becoming more and more accessible. The GC–MS, LC–MS and LC–MS/MS have now become routine methods for D-xylitol measurement. The coupling of spectroscopic methods such as NMR and MS to the chromatography methods can also provide structural information of the compounds being analyzed. Other methods such as the immunoassay and enzymatic assay methods are also discussed.

Keywords: D-xylitol · Analytical method · Detection method · Chromatographic separation · Spectroscopy · Biosensor

Abbreviations

H NMR	Proton nuclear magnetic resonance
BSA	Bovine serum albumin
CE	Capillary electrophoresis
CZE	Capillary zone electrophoresis
ELISA	Enzyme-linked immunosorbent assay
ELS	Evaporative light-scattering
ESI-MS	Electrospray ionization mass spectrometry
FIA	Flow injection analyisis
FID	Flame Ionization Detector
FT-ICR	Fourier transform ion cyclotron resonance
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
HPAEC	High-pH anion exchange chromatography
HPLC	High-performance liquid chromatography
IC-ELISA	Indirect competitive enzyme-linked immunosorbant assay
IDC	1-isopropyl-3-(3-dimethylaminopropyl) carbodiimide perchlorate
Ig E	Immunoglobulin E
Ig G	Immunoglobulin G
ISTD	Internal standard
ITP	Capillary isotachophoresis
LC-MS	Liquid chromatography-mass spectrometry
LC-NMR	Liquid chromatography-nuclear magnetic resonance
MS	Mass spectrometry
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
OPLC	Optimum performance laminar chromatography
PAD	Pulsed amperometric detector
RI	Refractive index
RSD	Relative standard deviation
SIM	Selective-ion-monitoring
TLC	Thin layer chromatography

TMS	Trimethylsilylation
XDH	D-xylitol dehydrogenase
XYO	D-xylitol oxidase

11.1 Introduction

D-xylitol is a naturally-occurring five-carbon sugar alcohol derived from the reduction of D-xylose. It is also known as 1, 2, 3, 4, 5-pentahydroxypentane, and its IUPAC name is designated as (*2R, 3r, 4S*)-pentane-1, 2, 3, 4, 5-pentol. It has been used as a food additive and sweetening agent in the food industry since the 1960s (Mickenautsch and Yengopal 2012). D-xylitol was first discovered and reported in 1891 by the German Nobel Prize winning chemist Emil Fischer (Granström et al. 2007). It is present in a wide variety of fruits and vegetables, as well as in corn husks and mushrooms (Jaffe 1978; Mitchell 2006). Currently D-xylitol is produced commercially by the chemical reduction of D-xylose which is derived mainly from birch bark, corn husks and stone fruit (James 2009). It has attracted huge interest worldwide because of its usefulness in treating a number of health-related conditions. D-xylitol has almost the same sweetness as sucrose but has lower energy value than sucrose (2.4 cal/g vs. 4.0 cal/g). It has been shown to resist fermentation by microorganisms into acids (Russo 1976), thus it has been used as a sugar substitute in dietary foods, especially for insulin-deficiency patients. In addition, D-xylitol is believed to have 'active' anticariogenic properties and has been widely used in the odontological industry. Furthermore, studies have shown that D-xylitol can prevent acute ear infection (otitis media) in small children (Mäkinen 1992; Uhari et al. 1996).

D-xylitol is currently sold at the price of $4–5/kg and its global market is estimated to be $340 million per year, which will definitely increase in the future (Chen et al. 2010). It is un-economical to extract large amounts of D-xylitol from vegetables and fruits due to its low content. In industrial scale production, D-xylitol is manufactured through catalytic chemical reduction of pure D-xylose which is obtained from hemicellulose (Chen et al. 2010).

The importance of simple, fast, low cost, but sensitive and accurate methods of separating and measuring D-xylitol in various sources cannot be over-emphasized. The qualitative detection and quantification of D-xylitol in the presence of other sugars and sugar alcohols in fruits, vegetables and other natural sources are essential because such information reveals very important characteristics of these natural foods, such as the flavor, maturity, quality, authenticity, and storage conditions (Martínez Montero et al. 2004). The product analysis and quality control of processed food, beverage, and health care products also require sensitive determination methods of D-xylitol. In more recent years, increasing efforts are

directed toward the discovery and development of new bioprocesses for the production of D-xylitol as a natural occurring sugar substitute. Such investigations also depend on efficient detection methods for D-xylitol.

A good number of analytical methods have been developed for the qualitative and quantitative measurement of D-xylitol. Since most samples to be analyzed contain a mixture of compounds, efficient analytical methods for D-xylitol are usually comprised of two components: (1) an efficient separation unit, and (2) a structure identification unit. Earlier methods mainly rely on the retention time of analytes in chromatography for the identification of D-xylitol, while more recently developed methods incorporate advanced spectroscopic methods such as mass spectroscopy (MS) and nuclear magnetic resonance (NMR) spectroscopy for structure determination of D-xylitol. In the following sections, we provide an overview on current analytical methods for the qualitative and quantitative determination of D-xylitol-containing samples from various sources.

11.2 Detection Methods of D-Xylitol

Based on their separation principles, the current methods for D-xylitol detection may be classified as gas chromatography (GC)-based methods, high performance liquid chromatography (HPLC)-based methods, and capillary electrophoresis (CE)-based methods. The relatively newly developed biotechnology-based methods can analyze compound mixtures without prior separation because D-xylitol is recognized specifically by biomolecules through non-covalent molecular interactions.

11.3 GC-Based Methods

11.3.1 Uncoupled GC

GC is a very useful technique for the analysis of volatile compounds. For the determination of sugars and sugar alcohols, the gas chromatographic method has been widely used due to its good selectivity and small amount of sample needed. It is also rapid and very sensitive. However, the main drawback is that sugars and sugar alcohols including D-xylitol are not volatile and must therefore be converted to volatile derivatives prior to measurement. The common derivatization methods include trimethylsilylation (TMS) (Sweeley et al. 1963; Namgung et al. 2010) and acetylation of all hydroxyl groups present in a molecule (Mäkinen and Söderllng 1980; Lee and Chung 2006). The flame ionization detector (FID) is a type of gas detector used in GC for detecting organic compounds such as proteins, nucleotides, and pharmaceuticals. FID is best for detecting hydrocarbons and other easily flammable components. They are very sensitive to FID and their response tends to

be linear across a wide range of concentrations (Namgung et al. 2010). GC with FID is one of the early methods developed for the detection of D-xylitol (Mäkinen and Söderllng 1980), and it is still the method of choice for the determination of D-xylitol in a complex matrix (Sreenath and Venkatesh 2010; Namgung et al. 2010).

In evaluating the quality of Doenjang, a traditional fermented food widely consumed in Korea, which varies considerably by its basic ingredients, species of microflora, and the fermentation process, the classification of metabolites present in Doenjang samples was performed by using GC-FID. A significant amount of D-xylitol was detected among other metabolites such as amino acids, organic acids, sugars and sugar derivatives, and fatty acids (Namgung et al. 2010).

11.3.1.1 GC–MS

Gas chromatography-mass spectrometry (GC–MS) is one of the so-called hyphenated analytical method in which the gas chromatography separates the components of the mixture and the mass spectroscopy characterizes each of the components. The coupling of GC with MS has greatly enhanced the capacity of this analytical method. GC–MS has high reproducibility, high resolution and few matrix effects (Kopka 2006). It has become one commonly used method for D-xylitol detection (Namgung et al. 2010; Lee and Chung 2006; Clayton et al. 2008). Figure 11.1 shows a typical ion chromatogram and mass spectra of polyols, which were separated as peracetylated derivatives and detected in selective-ion-monitoring (SIM) mode (Lee and Chung 2006). The method was successfully used to simultaneously measure urinary polyols and quantify as little as 0.5–1.0 ng/μL of these polyols.

GC–MS has been used extensively in the medical, pharmacological, environmental, and law enforcement fields. Roboz et al. (1984) used gas chromatography-chemical ionization mass spectrometry with selected ion monitoring of the M-59 ions to determine polyols (as their peracetyl derivatives) in serum. They used 2-deoxygalactitol as an internal standard. A rapid capillary gas chromatographic method was also described by Haga and Nakajima (1989) for the profile analysis of urinary polyols as their trifluoroacetyl derivatives. Ten urinary polyols including D-xylitol and myo-inositol were measured for the first time and verified by GC–MS method. Polyol species in cerebrospinal fluid and plasma-ribitol, arabitol, D-xylitol, 1, 5-anhydrosorbitol, myo-inositol, mannitol, sorbitol, and galactitol were quantified by a capillary gas chromatography/ion trap mass spectrometric method. Microliter volumes of cerebrospinal fluid or plasma were mixed with internal standard (deuterium labeled myo-inositol), deproteinized, and evaporated to dryness. The samples were converted to acetylated derivatives resolved on a capillary column bonded with 50 % phenyl-50 % methyl polysiloxane. Chemical ionization mass spectra for the acetate derivatives of polyols were generated in an ion trap using acetonitrile as reagent gas (Shetty et al. 1995).

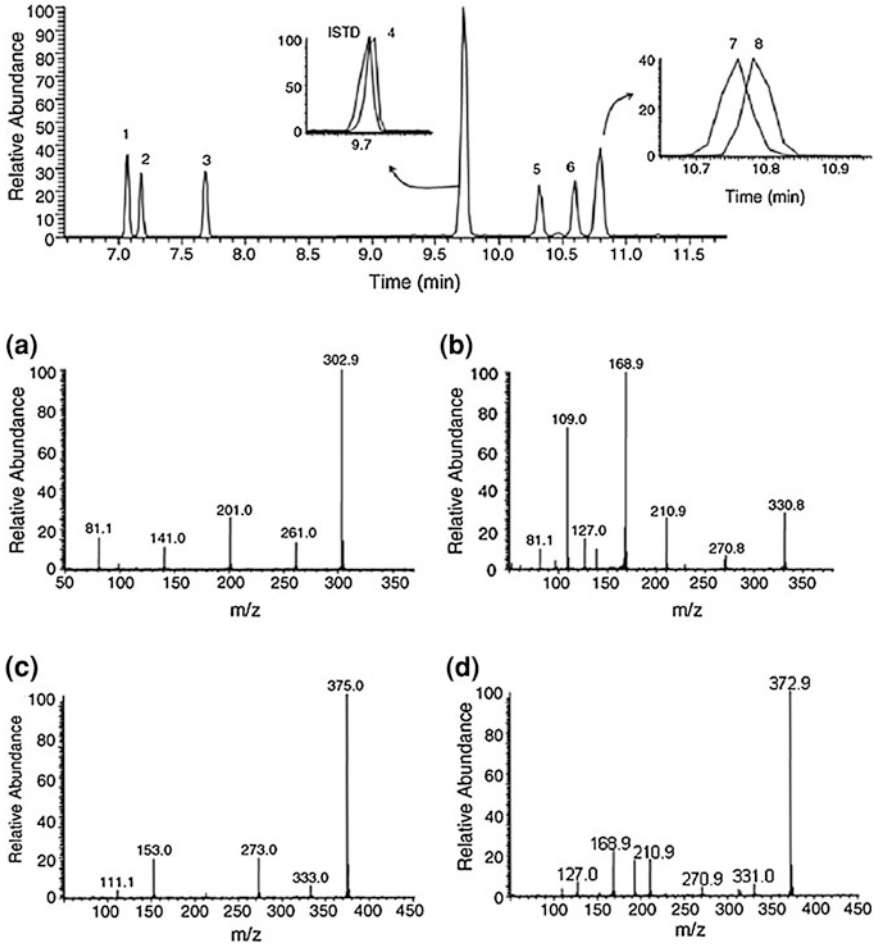

Fig. 11.1 GC-MS analysis of standard polyols as acetylated derivatives separated on an SPB-1701 column (15 m × 0.25 mm i.d., 0.25-μm film thickness). Representative total ion chromatogram (upper layer) and mass spectra (**a**–**d**) obtained in selective-ion-monitoring (SIM) mode. The magnified figures were ion chromatogram at *m/z* 169 and 171 for peak 4 and the internal standard (ISTD), at *m/z* 375 for peak 7, and at *m/z* 373 for peak 8. Peak 1: adonitol; peak 2: arabitol; peak 3: xylitol; peak 4: glucose; peak 5: mannitol; peak 6: dulcitol; peak 7: sorbitol; peak 8: myo-inositol; ISTD: 6, 6-D_2-glucose. Mass spectrum: (**a**) adonitol, arabitol and xylitol; (**b**) glucose; (**c**) mannitol, dulcitol and sorbitol; (**d**) myo-inositol. Adapted from reference Lee and Chung (2006), copyright 2006 Elsevier

The coupling of MS to GC has also increased the accuracy of the GC method in terms of structure determination. Li et al. (2011) reported a GC–MS method for profiling tobacco leaves rich in D-xylitol. From water–methanol-acetonitrile (v/v/v, 3:1:1) extract, nine saccharides, nine alcohols, nine amino acids, 16 organic acids, and phosphoric acid were identified based on standard compounds. Rainey

et al. (2011) also described the chemical characterization of D-xylitol from ground tobacco product. The tobacco product was first derivatized with trimethylsilylation and then analyzed by GC–MS. D-xylitol was detected in mellow Camel sticks.

11.3.2 HPLC-Based Methods

HPLC is a method of separating, identifying, and quantifying compounds in a mixture. Using HPLC does not require the derivatization of the compounds to form volatile derivatives. As early as the 1980s, there were reports on the determination of D-xylitol by HPLC (Galensa 1983; Kertesz et al. 1983; Lohmander 1986). Development in recent years has improved the separation efficiency, sensitivity, and detection accuracy. The HPLC method is simple, fast, and accurate, and is currently the main method for qualitative and quantitative determination of D-xylitol.

11.3.2.1 Uncoupled HPLC

HPLC columns and mobile phases

The separation of D-xylitol from sugars and other sugar alcohols can be quite challenging due to their structural similarity. Different types of columns have been used for that purpose with good separation result. These include amino-based carbohydrate column (Wan and Yu 2006; Bhandari et al. 2008), HPX-87H organic acid column (Park et al. 2005), TSK amide 80 column (Katayama et al. 2006), phenyl column (Wan and Yu 2007), ODS column (Nojiri et al. 1999), and ion-exclusion column (Cheng et al. 2010; Ohsawa et al. 1986). For optimum analytical results, sample pretreatment is usually required prior to applying onto the column. While simple dilution and filtration suffice for liquid samples, solid samples may undergo solid phase extraction (SPE) with different solvents to eliminate a great variety of interfering substances present in the sample matrix (Martínez Montero et al. 2004). The mobile phases most frequently utilized in HPLC separations are mixtures of water/acetonitrile, solutions of NaOH, pure water, sulphuric acid solutions or gradient elution systems (Martínez Montero et al. 2004). The presence of Ba^{2+} ions in an alkaline mobile phase improves selectivity and the reproducibility and reduces the analysis time, because Ba^{2+} ions allow the precipitation of carbonate which would otherwise cause interference (Cataldi et al. 1999).

An improved chromatographic technique known as high-pH anion exchange chromatography (HPAEC) was also developed to separate carbohydrates. It takes advantage of the weakly acidic nature of carbohydrates to give highly selective separations at high pH. Coupled with a pulsed amperometric detector (PAD), it allows direct quantification of non-derivatized carbohydrates at low picomole levels with minimal sample preparation and clean-up. This has allowed the detection of carbohydrates in a variety of complex matrices, for instance, foods,

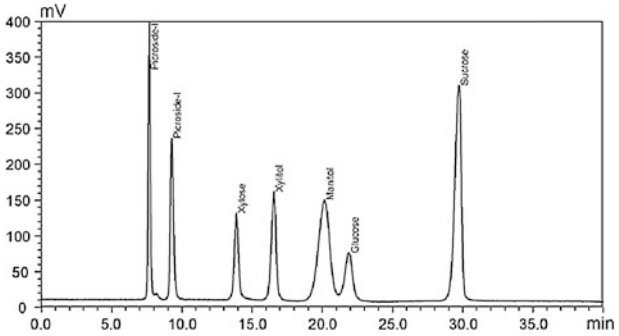

Fig. 11.2 HPLC-ELSD chromatogram of sugars and sugar derivatives: picroside-II, picroside-I, xylose, xylitol, mannitol, glucose and sucrose. Adapted from reference Bhandari et al. (2008), copyright 2008 Elsevier

beverages, dairy products, etc. Both HPAEC and PAD need high pH mobile phases to allow the chromatographic separation of the anionic sugars through ion exchange mechanisms (Martínez Montero et al. 2004).

HPLC detection methods

A variety of detection methods, including UV detection, electrochemical detection, infrared detection, refractive index (RI) detection, and evaporative light-scattering (ELS) detection are available for HPLC methods. The ideal detector must have an adequate sensitivity, good stability and reproducibility, linear response and must be non-destructive. Since D-xylitol lacks chromophoric and fluorophoric moieties, less sensitive detection methods such as RI (Park et al. 2005; Cheng et al. 2010; Ling et al. 2011; Salgado et al. 2010; Altamirano et al. 2000) and ELS (Bhandari et al. 2008) are more commonly used for its determination. Detection limits and sensitivity depend on the type of detector hyphenated to the HPLC. The RI and ELS detection methods typically provide detection limits in the range of 0.05–1.2 μg/injection for D-xylitol (Wan and Yu 2006). Pulsed amperometric detection has also be used for the detection of D-xylitol when coupled with ion chromatography, e.g., using Dionex column and a strongly basic mobile phase (pH > 12).

Bhandari et al. (2008) established a sensitive, selective and reliable HPLC method based on ultrasonic extraction and ELS detection for the simultaneous determination of sugars and sugar alcohols including D-xylitol (Fig. 11.2). The method showed good reproducibility for the quantification of seven analytes in *Picrorhiza* species. The method is simple, accurate and specific and can be used in laboratories that lack sophisticated analytical instruments, such as LC–MS or GC–MS.

Derivatization for more sensitive HPLC detection

Since RI and ELS detection methods are of relatively low sensitivity, pre-column derivatization of D-xylitol for a more sensitive HPLC detection has been

investigated. Derivatization enhances selectivity and sensitivity, but has some disadvantages as well, e.g., it is time consuming and the presence of reagents in analytical samples can interfere with the analysis. Several derivatization methods have been reported in the literature suitable for more sensitive detections based on UV absorbance, fluorescence, and specific optical rotation (Galensa 1983; Katayama et al. 2006; Yamamoto et al. 1998). A simple and sensitive derivatization method with benzoic acid was reported by Katayama et al. (2006) for the determination of diabetic markers in serum, including D-glucose, 1, 5-anhydro-*D*-glucitol, D-xylitol, and other related sugar alcohols. The samples were first treated with benzoic acid in the presence of 1-isopropyl-3-(3-dimethylaminopropyl) carbodiimide perchlorate (IDC) and 4-piperidinopyridine at 80 °C for 60 min. The benzoylated derivatives were separated on a TSK amide 80 column and detected with a fluorescence detector at λ_{ex} 275 and λ_{em} 315 nm. D-xylitol was detected as its mono-benzoyl ester with the detection limit of 10 ng/mL.

Similarly, nitrobenzoylation has been reported as a highly sensitive method for the determination of D-xylitol among other sugar alcohols using UV detection at 260 nm (Wan and Yu 2007; Martínez Montero et al. 2004; Nojiri et al. 1999 and 2000). P-nitrobenzoyl chloride is a rapid and quantitative derivatizing agent for amines and hydroxylated compounds, providing strong UV-absorbing amine and sugar alcohol derivatives allowing for more sensitive detection (Martínez Montero et al. 2004). Nojiri et al. (2000) determined D-xylitol and five other sugar alcohols after derivatization with p-nitrobenzoyl chloride and analyzed on a phenyl column with UV detection. The detection limit of this method was 0.1 % for the above sugar alcohols contained in the samples. The sensitivity of the p-nitrobenzoylation method was 10–1000 times higher than that of HPLC with RI detection or GC with flame ionization detection (Nojiri et al. 1999).

Another derivatization method for UV detection employs phenylisocyanate as a reagent forming phenylurethanes (Indyk and Woollard 1994). Isocyanates have high reactivity with compounds possessing active hydrogen atom(s) and provide highly stable derivatives with carbohydrates and sugar alcohols, allowing detection down to nanogram levels. Interestingly, Yamamoto et al. (1998) reported a method of forming metal complex for the detection of sugars and sugar alcohols. The multi-hydroxylated alditols readily form anionic complexes with molybdate which have remarkably large specific optical rotations (in contrast to the initial alditols showing almost no optical rotation), thus providing a highly selective detection system for these sugar alcohols.

Column-switching HPLC

Recent development in column-switching techniques for chromatography allows the coupling of different separation modes to resolve a wide range of compounds in complex samples (Cheng et al. 2010; Fukushima et al. 2007). Cheng et al. (2010) reported a column-switching HPLC technique by coupling H^+ and Pb^{2+} ion-exclusion columns to study the enzyme hydrolysis of waste cellulosic biomass. The column-switching HPLC with RI detection was connected on-line to an immobilized enzyme reactor for successive on-line desalting and simultaneous

analysis of carbohydrates and D-xylitol in a hydrolysate of waste paper and waste tree branch by incorporating the heart-cut and the elution-time-difference techniques.

11.3.2.2 LC–MS and LC–MS/MS

Electrospray ionization mass spectrometry (ESI–MS) is one of the most important advancements in MS technology. ESI is a desorption ionization method that can be performed on solid or liquid samples, thus allowing the analysis of nonvolatile or thermally unstable samples such as carbohydrates, peptides, proteins, and some inorganic molecules. The ions observed by ESI–MS are usually quasimolecular ions created by the addition of a proton ($\rightarrow [M + H]^+$) or another cation such as sodium ion ($\rightarrow [M + Na]^+$) under positive mode, or the removal of a proton ($\rightarrow [M - H]^-$) under negative mode. Multiply charged ions such as $[M + H + Na]^{2+}$ and $[M - 2H]^{2-}$ are often observed as the result of adding/removing multiple protons and/or cations under positive and negative ionization mode, respectively. The method has high sensitivity and can be used for accurate quantitative and qualitative measurements.

Although the advent of ESI–MS has significantly increased the speed of MS analysis of complex mixtures, mass spectrometry alone is seldom used to analyze D-xylitol-containing samples. Watkins et al. (2005) reported an ESI–MS method equipped with Fourier transform ion cyclotron resonance (FT-ICR) in order to gain detailed structure information of polyols and polyol mixtures including D-xylitol. The analytes were first ionized by positive mode ESI and then allowed to react with the neutral reagent diethylmethoxyborane. Consecutive ion–molecule reactions between the hydroxyl groups of polyols and the borane reagent resulted in products which were separated by 68 mass units, along with 30 mass shifts as a result of intra-molecular derivatization of the primarily formed products. The data provided structural information about the number of hydroxyl groups and their relative positions in the polyols.

In general, MS analysis provides better results for clean samples than mixtures or dirty samples. Thus, the incorporation of a purification/separation component such as HPLC greatly enhances the analytical capacity of MS. Liquid chromatography hyphenated to mass spectrometry (LC–MS) has emerged as a popular and powerful tool for analyzing samples containing multiple compounds. Compared to GC–MS, sample pretreatment is usually simplified with LC–MS because sample derivatization is usually not required.

Wan and Yu (2006) reported the determination of D-xylitol in atmospheric aerosols using LC–MS with positive ESI. Samples were extracted from aerosol filters in methanol and after the evaporation of the solvent reconstituted with 5 mM ammonium acetate in water prior to the LC–MS analysis. D-xylitol was efficiently separated on polymer-based amino columns from eight other sugars and sugar alcohols (glycerol, erythritol, D-mannitol, D-xylose, D-glucose, levoglucosan, sucrose and a trisaccharide melezitose). Isocratic elution was applied with a

mobile phase consisting of 20 % of 10 mM NH_4Ac aqueous solution, 8 % of methanol, and 72 % of water. The addition of an ammonium ion resulted in $[M + NH_4]^+$ which was found to be abundant and used for monitoring and quantification of D-xylitol. The analytes were measured at the levels of 100, 500 and 1000 μg/L and their recovery rates were in the range of 78–102, 94–112, and 92–110 %, respectively. The limit of detection for D-xylitol was 4.7 pmol/injection.

In a similar study by Wan and Yu (2007), LC–MS with negative ESI was used to analyze sugars and sugar alcohols in atmospheric aerosols. Sugars and sugar alcohols do not deprotonate easily to form $[M - H]^-$ ions because they lack highly acidic groups, therefore their ionization is not effective under negative mode ESI without derivatization. However, the post-column addition of chloroform into acetonitrile was found to greatly enhance the ionization of these compounds by forming chloride adduct ion under negative mode ESI. The detection limit of D-xylitol was 0.016 μM based on the quantification of $[M + {}^{35}Cl]^-$ ion.

Tandem mass spectrometry, also known as MS/MS or MS^2, involves multiple steps of mass spectrometry with some form of fragmentation occurring in between the stages. Specific precursor ion(s) generated in the first mass analyzer may be selected for fragmentation, and the product ions are scanned in the second mass analyzer. The first step MS may be considered as a separation unit to provide individual molecular ions (and their daughter ions) of the compounds present in the sample being analyzed. As such, MS/MS not only provides more detailed structural information of the analyzed molecule, but also allows the analyis of samples containing a mixture of compounds. Combined with liquid chromatogrphy, LC–MS/MS has become a routine technique for analyzing samples of complex mixtures.

Wamelink et al. (2005) reported an LC–MS/MS method used to analyze the content of sugar alcohols in urine samples. The urine samples were supplemented with isotope-labeled internal standards ($[^{13}C_4]$ erythritol, $[^{13}C_2]$ arabitol and $[^{2}H_3]$ sorbitol) and desalted by a mixed-bed ion-exchange resin. Separation was achieved by using an Aminex HPX-87C column. Multiple reactions monitoring polyol detection were achieved by tandem mass spectrometry with an ESI source operating in the negative mode. Age-related reference ranges of D-xylitol and other polyols (erythritol, treitol, arabitol, ribitol, galactitol, mannitol, sorbitol, sedoheptitol and perseitol) in urine were established. The method was also applied to the quantification of the abnormal polyol concentrations observed in patients with transaldolase deficiency, ribose-5-phosphate isomerase deficiency and classical galactosaemia.

11.3.2.3 LC–NMR

Proton nuclear magnetic resonance (^1H NMR) spectroscopy is a routine tool for fast and comprehensive characterization of organic compounds. However, compound mixtures often result in extensive signal overlapping and spectral

complexity which can be a serious hindrance in analyzing mixtures. The development of the LC–NMR technique, which couples an HPLC step immediately prior to NMR measurement, allows compound separation and analysis to be carried out quickly (Griffiths 1995).

Graça et al. (2008) reported an LC-NMR method for the metabolic profiling of human amniotic fluid and identified more than 60 compounds including D-xylitol in the fluid. Both reversed-phase and ion-exchange liquid chromatography were carried out in order to separate a range of metabolites including amino acids, sugars, xanthenes, organic acids and their derivatives. The NMR spectrometer was equipped with a 3-mm probe head (60 μL active volume) and coupled to an ION300 ion exchange column with a mobile phase composed of 2.5 mM H_2SO_4 in 100 % D_2O. Subsequent NMR and MS analysis enabled the rapid identification of over 60 compounds, five of which were detected for the first time from the human amniotic fluid. Since NMR spectroscopy provides structural information about the atom connectivity and the stereochemistry of molecules, the LC-NMR method has a higher degree of accuracy in the determination of D-xylitol compared to other methods.

11.3.2.4 Optimum Performance Laminar Chromatography

Thin layer chromatography (TLC) is a simple chromatography technique used to separate mixtures for both analytical and preparative purposes. Due to its simplicity and low cost, it is widely used for rapid sample/product profiling. Altamirano et al. (2000) used TLC to detect D-xylitol while trying to rapidly identify the best D-xylitol producing yeast strains isolated from different natural sources. Silica gel TLC analysis was carried out with ethyl acetate: 2-propanol: water (130:57:23) as the developing solvent and bromocresol green-boric acid as the staining agent. The visualization of a yellow spot on a blue background of the TLC plate indicated the presence of D-xylitol, and the relative intensity of the yellow spot correlated with the amount of D-xylitol present in the analyzed samples.

OPLC, also known as over-pressured layered chromatography or forced-flow TLC, is a pumped-flow chromatography system that combines the user friendly interface of HPLC with the capacity of flash chromatography and the inexpensive and multidimensional aspects of TLC (Tyihak et al. 2001). OPLC may be considered as a TLC technique of which the mobile phase is moved under pressure, or alternatively, an HPLC technique that uses a square and planar two-dimensional column format. OPLC allows side-by-side analysis of several samples in a single run, provides added loading capacity for a single sample, and improves separation efficiency. Several types of detection methods have been used in conjunction with OPLC including radiation detection, fluorescence detection, UV detection, and Raman spectroscopy detection (Poole and Poole 1995).

Tamburini et al. (2006) reported an OPLC method for the separation and quantitative determination of three alditols (D-xylitol, L-arabitol, and D-glucitol)

and four aldoses (D-xylose, L-arabinose, D-glucose, and L-rhamnose). The four sugars were present in hemicellulose hydrolyzates used as substrates for the production of D-xylitol from D-xylose by yeast. All four aldoses and three alditols were detected in the final fermented broth. The separation was performed on aluminum foil-backed silica gel OPLC–HPTLC plates with acetonitrile–acetic acid–water (63:33:5, v/v/v) as a mobile phase. The developed plates were derivatized with lead (IV) acetate dichlorofluorescein reagent (Jork et al. 1990) and the fluorescence intensity measured at $\lambda = 313$ nm. The upper limits of linearity were determined to be in the range of 140–600 ng and the detection limits were 15–50 ng per spot.

11.3.2.5 Capillary Electrophoresis

Analytical separation of aldoses and alditols in complex mixtures has been achieved by several other methods including capillary electrophoresis (CE) (Corradini et al. 1998; Guttman 1997; Martínez Montero et al. 2004). CE is a powerful separation technique for the separation of a variety of analytes in relatively complex matrices. The application of CE for the analysis of sugar polyols has been reviewed previously (Martínez Montero et al. 2004). Sugar polyols lack both a charge and a strong UV chromophore, and thus their detection by UV absorbance can be carried out after derivatization (Guttman 1997). Alternatively, underivatized sugar polyol may be detected by indirect UV detection by using high-alkaline pH medium to ionize the polyol in a buffer solution.

A CE method with indirect UV detection was reported for the simultaneous analysis of underivatized acidic, neutral and amino sugars, and sugar alcohols including D-xylitol (Soga and Heiger 1998). Separations were carried out on fused silica capillaries and a combination of 2, 6-pyridinedicarboxylic acid (20 mM) and cetyltrimethylammonium bromide (0.5 mM) was used as the electrolyte. Optimum separation of carbohydrates and sugar alcohols was achieved at pH 12.1 and the minimum detection level for carbohydrates was in the range of 23–71 μM. Under these conditions, D-xylitol was measured along with 18 monosaccharides and other sugar polyols. The applicability of this method was demonstrated in the determination of the monosaccharide composition in the acid hydrolysate of a model glycoprotein fetuin (Soga and Heiger 1998). The advantages of the CE method include direct analysis of sugar and sugar alcohols without derivatization and its high separation capacity for acidic, neutral, and amino sugars and sugar alcohols under a single electrophoretic condition.

Rovio et al. (2007) reported a capillary zone electrophoresis (CZE) method for the separation of D-xylitol, D-mannitol, and ten neutral carbohydrates (sucrose, D-fucose, cellobiose, D-galactose, D-glucose, L-rhamnose, D-mannose, D-arabinose, D-xylose, and D-ribose). The alkaline electrolyte solution was prepared with 130 mM sodium hydroxide and 36 mM disodium hydrogen phosphate and the analytes were detected with direct UV detection at the wavelength 270 nm. The proposed UV-absorbing species at the wavelength 270 nm were enediolate ions

and conjugated enol carbonyl structures that were generated as carbohydrate degradation intermediates under the strongly alkaline condition. The source of UV absorbance in sugar polyols such as D-xylitol under such conditions was not presented, but one may speculate that oxidation reaction followed by some kind of β-elimination reaction in polyols could generate α, β-unsaturated carbonyl system with UV absorbance at around 270 nm. The calibration curves were linear in the range of 0.05–3.0 mM and the detection limits for the analytes were 20–50 μM (Rovio et al. 2007). This method was demonstrated in the determination of monosaccharides in lemon, pineapple, and orange juices and in a cognac sample.

In situ derivatization of polyols with boric acid was described for CE analysis of polyols under less alkaline condition (Pospisilova et al. 2007). Boric acid [B (OH) $_3$] reacts readily with a diol forming a borate diester complex $(RO)_2$ BOH, which is readily ionizable and migrates electrophoretically. Thus, capillary zone electrophoresis (CZE) employing on-column complexation with boric acid and indirect UV detection at 215 nm was used to determine D-mannitol, D-sorbitol and D-xylitol (Pospisilova et al. 2007). The separation of the anionic borate-polyol complexes was carried out in a fused silica capillary at 25 kV with optimized background electrolyte of 200 mM borate buffer (pH 9.3) containing 10 mM 3-nitrobenzoate as the chromogenic co-ion. The rectilinear calibration range was 0.2–2 mg/mL for D-mannitol and D-sorbitol while 1 mg/mL of D-xylitol was used as the internal standard. The limit of detection was approximately 30 μg/mL for both D-mannitol and D-sorbitol.

Similarly, the strategy of forming polyol-borate complexes has been utilized in capillary isotachophoresis (ITP) for the determination of D-xylitol in multicomponent pharmaceutical formulations and foods (Herrmannova et al. 2006). The separation was carried out in a capillary tube of 0.8 mm ID and 90 mm effective length made of fluorinated ethylene-propylene copolymer filled with an electrolyte system consisting of 10 mM HCl + 14 mM Tris (pH 7.7, leading electrolyte) and 5 mM L-histidine + 5 mM Tris (pH 8.3, terminating electrolyte). The analysis was performed at a driving current of 200 μA and detected at a current of 100 μA. The aqueous sample solution was treated with boric acid and the substances of polyhydroxyl nature were converted to borate complexes allowing them to migrate isotachophoretically. Using conductivity detection, the calibration curves were linear in the range up to 2.5 mM for all the components analyzed (acesulfame K, saccharine, aspartame, cyclamate, D-sorbitol, D-mannitol, D-lactitol, and D-xylitol). The detection limits were in the range of 24–81 μM. The concentration of D-xylitol was 52 μM. The good precision of this ITP method was evidenced by the favorable relative standard deviation (RSD) values ranging from 0.8 to 2.8 % obtained at the analyte concentration of 1.0 mM ($n = 6$). Simplicity, accuracy, and low cost of analyses made the ITP an alternative procedure to the current methods for the determination of D-xylitol and other ionizable sweeteners.

11.3.3 Biotechnology-Based Methods

11.3.3.1 Indirect Competitive Immunoassay

Recently, Sreenath and Venkatesh (2010) reported an indirect competitive enzyme-linked immunosorbant assay (IC-ELISA) for the detection and quantification of D-xylitol in food. Heptan-specific anti-D-xylitol antibodies (IgG and IgE) were raised against reductively aminated D-xylitol-albumin conjugate as the immunogen and purified through affinity column. Flat bottom polystyrene microtiter wells were coated with high hapten density, D-xylitol-BSA conjugate (32 haptens/mol) at 100 ng per well, followed by the addition of affinity-purified anti-D-xylitol antibodies (4 ng in 50 μL) and different dilutions of food extracts. Indirect competition was created between the D-xylitoyl epitopes of D-xylitol-BSA conjugate coated on the wells of microtiter plate and free D-xylitol present in the food extracts as they both bind to D-xylitol-specific antibodies. With this immunoassay, the limit of detection was 1 ng for D-xylitol and the linear range for D-xylitol quantification was 5–400 ng. The results were in good agreement with the reported values by HPLC and GC methods. This indirect competitive ELISA may serve as a sensitive analytical method to detect and quantify nanogram amounts of D-xylitol in various biological samples and natural/processed foods (Sreenath and Venkatesh 2010).

11.3.3.2 Enzymatic Assays

Biosensor technology is now widely used in the detection, control and measurement of specific compounds in fermentation broths. Takamizawa et al. (2000) reported a D-xylitol biosensor composed of a partially purified D-xylitol dehydrogenase (XDH) from *C. tropicalis* IFO 0618 and a dissolved oxygen meter (electrode). A flow-injection system using the immobilized XDH was evaluated for on-line D-xylitol monitoring for automatic control of D-xylitol production. The partially purified XDH was most active at pH 8.0 and 50 °C but the optimum operation temperature for the biosensor in the flow-injection system was 30 °C. The biosensor favored the use of NAD^+ over $NADP^+$ in the oxidation reaction of D-xylitol to D-xylulose. Correlation studies indicated that the biosensor was applicable for measurement of D-xylitol in the narrow concentration range of 1–3 mM. The biosensor remained active for two days at 5 °C and was re-used three times with reproducible results. The activity later dropped to 30 % of its original value. The biosensor was completely inactive towards arabitol, xylose, glucose, glycerol and ethanol and non-selective toward sorbitol and ribitol, which were also oxidized at 65 % and 58 % of the D-xylitol rate, respectively.

Vermeir et al. (2007) reported the use of a miniaturized and automated enzymatic assays for fast sugar and acid quantification in apples and tomatoes. The detection of D-xylitol was based on the enzyme sorbitol dehydrogenase which

oxidized D-xylitol to D-xylulose, and at the same time NAD^+ was reduced to NADH. The assay was based on an increase/decrease in absorbance at λ340 nm caused by a change in NADH concentration before and after the addition of the substrate, with the analytical sample containing D-xylitol. The change was stoichiometrically related to the concentration of D-xylitol present in the sample being analyzed. The enzyme sorbitol dehydrogenase reacted with both D-sorbitol and D-xylitol, and this made the method non specific for D-xylitol. The key features of this method were its high throughput (due to automation) and micro-scale. The assays were miniaturized from the standard 3 mL assays in cuvettes into assays of 200 μL or lower in 96 or 384 well micro-plates. Limit of detection, linearity of calibration curve, and repeatability of the assays with standard solutions were proven to be satisfactory. The automated and miniaturized assays were validated with HPLC analyses for the quantification of sugars and acids in tomato and apple extracts. This enzymatic assay may serve as a fast, reliable, and inexpensive alternative to HPLC as the standard analysis technique for the detection of D-xylitol in fruits, vegetables and processed food.

Rhee et al. (2002) developed a flow injection analysis (FIA) system with an immobilized D-xylitol oxidase (XYO) cartridge for on-line monitoring of D-xylitol concentrations during a D-xylitol production process. The enzyme D-xylitol oxidase that oxidized D-xylitol to D-xylose was produced in a recombinant *E. coli*, and the isolated enzyme immobilized on a VA-Epoxy Biosynth E3-support. During the oxidation reaction, oxygen was consumed and hydrogen peroxide produced. The concentration of D-xylitol correlated with the amount of oxygen consumed or the peroxide produced. High activity of the immobilized XYO was attained by using potassium phosphate solution (1 M) with 0.5 g/L Triton X-100 adjusted to pH 8.5 as the carrier solution. High concentrations of certain components including arabinose at 20 g/L, D-xylose at 30 g/L and sodium chloride at 30 g/L in the sample had significant inhibitory effects on the response of the XYO-FIA system.

11.4 Conclusion and Future Recommendations

Primary interest in D-xylitol is due to its properties and its potential use as a sugar substitute. It has a sweetness equivalent to sucrose but its slower metabolism makes it useful as a low caloric sweetener. The cool and fresh taste of D-xylitol makes it ideal for candy and medicinal coatings. Incorporating D-xylitol into confectionery products, beverages and other foodstuffs could reduce the incidence of dental carries.

Currently, there are many analytical methods available for the qualitative and quantitative measurement of D-xylitol. Based on chromatography techniques, these are GC, HPLC, and CE methods with different detection options such as UV, RI, ELS, etc. More sophisticated analytical instruments derived from hyphenation of chromatography with structure determination tools such as MS and NMR have

become more accessible in the past decade. Therefore, GC–MS, LC–MS and LC–MS/MS have become routine methods for D-xylitol detection. The coupling of spectroscopic methods such as NMR, and MS to chromatography provides structural information about elemental connectivity and stereochemistry of unknown compounds. Furthermore, immunoassay and enzymatic assay methods are advantageous over other methods of D-xylitol detection in that they are potentially highly specific for D-xylitol and the cost is low.

Development of novel, specific, sensitive and inexpensive biotechnology-based D-xylitol detection methods will go a long way in improving the qualitative and quantitative determination of D-xylitol in fermentation broth, fruits, vegetable sources and cereals. This is a challenge for future research.

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