Quality control of sweet medicines based on gas chromatography-mass spectrometry

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Summary

Sweet medicines are a relatively untapped source of new drugs. Their biological activities are closely correlated to their chemical characteristics. However, accurately defining the chemical characteristics of glycans is a challenge due to their chemical heterogeneity and diversity. Gas chromatography-mass spectrometry (GC-MS) is an excellent technique for the analysis of glycans even though the preparation of adequate derivatives is necessary. We reviewed and discussed the most important methodologies currently used for glycan analysis in sweet medicines based on GC-MS, including the derivatization for monosaccharide analysis, hydrolysis methods for polysaccharide analysis, glycosidic linkage analysis based on methylation, and pyrolysis gas chromatography in carbohydrate analysis. Finally a strategy for quality control of sweet medicines based on quantification analysis is proposed.

Keywords: Carbohydrate, gas chromatography, derivatization, methylation, hydrolysis, pyrolysis gas chromatography

1. Introduction

Sugars occur in a variety of forms and locations in nature. Besides their roles in metabolism and as structural building blocks, sugars are fundamental constituents of every cell surface, which play critical roles in many cellular functions and disease. Sugar-based drugs are a relatively untapped source of new drugs and therefore offer an exciting new generation of drug therapies (1,2). Depending on their degree of polymerization (DP), simple sugars are often referred to as monosaccharides such as glucose and disaccharides (DP 1-2). Oligosaccharide typically refers to a bit longer chains (DP 3-9), whereas much larger molecules are defined as polysaccharides (DP > 9). Those attached with proteins or lipids are known as glycoconjugates or, more specifically, glycoproteins and glycolipids (3,4). Although studies of those activities lag behind research into genes and proteins, several carbohydrate-based molecules are known for their wide range of pharmacological activities and have been clinically used to treat different ailments (5,6).

Naturally occurring sugars are abundant, and can be derived from plants, fungi, bacteria, algae and animals (1,7). Low-molecular-weight heparin, derived from animal tissue, is the prominent example that successfully developed as clinical medicine for anticoagulants (8). Carbohydrates have also established themselves as the most clinically relevant antigens of those tested and subsequently developed for vaccines against infectious diseases, which initially isolated from bacteria (9). So far carbohydrate vaccines are widely derived from bacteria, protozoa, helminths, viruses, fungi and especially from cancer cells for immunotherapy on cancer (10). Lentinin, isolated from the fruit body of *Lentinula edodes*, is one of the host-mediated anti-cancer drugs and has been shown to affect host defense immune systems (11). Structure-activity relationship studies showed that (1→3)-β-D-glucan with (1→6)-glucosyl side groups and triple-
helical types play the decisive roles in its anti-cancer activity (12).

The structure of naturally occurring carbohydrates is often complex. The biological activities of them are closely correlated to their physico-chemical properties, such as molecular size, types and ratios of constituent monosaccharides, and features of glycosidic linkages (e.g., configuration and position of glycosidic linkages, and sequence of monosaccharides) (13). Characterization of carbohydrates is therefore necessary to ensure their efficacy and safety (14). Gas chromatography – mass spectrometry (GC-MS) is an excellent technique for analysis of carbohydrates for its high resolution and high sensitivity. It is irreplaceable for both qualitative and quantitative analysis of structurally similar monosaccharides (14). However, the preparation of volatile derivatives is required for different functional groups in carbohydrates. This review aims to collect the most important methodologies currently used for the carbohydrates analysis of sweet medicines based on GC-MS. The aspects include the derivatization for monosaccharide analysis, hydrolysis methods during polysaccharide analysis, glycosidic linkages analysis based on methylation, and pyrolysis gas chromatography in carbohydrate analysis. Finally a strategy for quality control (QC) of sweet medicines based on quantification analysis is proposed.

2. Qualitative analysis of monosaccharides

Monosaccharides are the simplest carbohydrates, which are the basic unit to compose disaccharides, oligosaccharides and polysaccharides. Monosaccharides can be found naturally as free carbohydrates or are produced by the hydrolysis of polymeric carbohydrates including oligosaccharides, polysaccharides and glycoconjugates (15). Generally analysis of polymeric carbohydrates in sweet medicines based on GC-MS would convert to monosaccharides analysis after various derivatization and hydrolysis (Figure 1). The strategy for carbohydrates analysis of sweet medicines based on GC-MS is shown in Figure 1. The targets include

**Figure 1. Quality control of sweet medicines based on GC-MS.** P-N: polysaccharides composed of neutral monosaccharides, P-A: polysaccharides contained sugar acids, P-B: polysaccharides contained amino sugars or iminosugars, M-N: neutral monosaccharides, M-AL: alditols, M-A: sugar acids, M-B: amino sugars or iminosugars.
neutral carbohydrates (aldoses and ketoses), alditols, sugar acids, amino sugars and iminosugars in both of free and polymeric carbohydrates in sweet medicines. Derivatization of carbohydrates for gas chromatography (GC) and GC-MS analyses was reviewed recently (14). Herein we discuss the aspects related to QC of sweet medicines and updated applications based on GC-MS.

2.1. Derivatization

The common sugars existed in nature in free and polymeric forms mainly include neutral carbohydrates (aldoses and ketoses), alditols, acid sugars, amino and iminosugars. Derivatization is crucial for non-volatile carbohydrates converted to volatile derivatives amenable to GC analysis. Due to the relatively low volatility of carbohydrates, GC analysis is limited to derivatized sugars of low molecular weight, mainly mono-, di- and trisaccharides (14). Generally, the diversity of naturally occurring carbohydrates makes the derivatization difficult to cover all kinds of sugars. Therefore adopting a suitable choice based on the individual samples is very important. The most used derivatization method available for different kind of sugars in sweet medicines especially the chromatographic behaviors and thier applications were summarized in Table 1 and discussed as follows.

2.1.1. Neutral carbohydrates (aldoses, ketoses) and alditols

Neutral carbohydrates are the most common sugars existed in sweet medicines, such as aldoses including arabinose, xylose, ribose, fucose, rhamnose, mannose, glucose, galactose and ketoses such as fructose. Alditols such as erythritol, rhamnitol, mannotol, sorbitol, xylitol, etc. However the different chemical properties of aldoses, ketoses and alditols, which are induced by a high number of functional groups in the molecule and tautomeric forms in solution, lead to different derivatives and chromatographic behavior.

A variety of derivatives, including acetates, trimethylsilyl (TMS) derivatives, alditol acetates, aldononitrile acetates and oxime derived compounds, have been widely used for the analysis of carbohydrates in sweet medicines. One-step reaction, including acetylation and silylation, focuses on the increase of volatility by substituting the polar groups in carbohydrates. It is preferred because of its simple and time-saving derivatization procedure.

Acetates are prepared directly by reaction of the sugar with acetic anhydride or together with a basic solvent such as pyridine. It is rapid and applicable for aldoses, ketoses and alditols (14,16). Especially when catalyst such as 1-methylimidazole is used, the reaction will be sped up (17,18). Multiple peaks formed corresponding to one sugar limit the application of this method. Researchers focused on this problem recently developed a methyl sulfoxide (Me2SO)/1-methylimidazole system to esterification reactions, and 23 free saccharides (80% MeOH extracts) including aldoses, ketoses, alditols, amino sugars as well as trehalose and sucrose were acetylated. Only one peak was formed of each analyte for quantification analysis Figure 2a (17). Besides microscale sampling and derivatization is environmentally friendly and speed up the total sample preparation procedure for GC-MS analysis, therefore promising for the future carbohydrates analysis in sweet medicines (18).

TMS ethers have better volatility and stability than acetates and are more popularly applied for GC analysis of carbohydrates in sweet medicines Table 1. TMS ethers also prepared directly with derivatization regents or together with aprotic solvents. Generally, pyridine is the most commonly used solvent among several aprotic solvents for good solubility of the carbohydrates. Pyridine and silylation reagents are volatile and can be easily evaporated before the sample is analyzed. Sometimes complete reaction mixture can be injected directly into the gas chromatograph, thus avoiding any cleanup stages. There are many silylation reagents that have been applied for the analysis of carbohydrates in sweet medicines at different temperatures for different reaction times (19-22). Hexamethyldisilazane (HMDS), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) are the most commonly used reagents. Alkylsilyl derivatives for gas chromatography are summarized in a previous review (23). Sometimes, a mixture of different silylation regents is also used. HMDS and chlorotrimethylsilane (TMSCI)

### Table 1. Derivatization methods available for neutral carbohydrates and alditols in sweet medicines (data from 243 journal articles collected in Web of Science mainly dated 2010-2014)

<table>
<thead>
<tr>
<th>Derivatization Methods</th>
<th>Reaction steps</th>
<th>Neutral carbohydrates</th>
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<tbody>
<tr>
<td>Acetates</td>
<td>One</td>
<td>M&quot;/S°</td>
<td>M/S</td>
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<tr>
<td>TMS derivatives</td>
<td>One</td>
<td>M</td>
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<tr>
<td>Alditol acetates</td>
<td>Two</td>
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<td>D°</td>
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<tr>
<td>Oximes and derived compounds</td>
<td>Two</td>
<td>D</td>
<td>D</td>
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<td>Aldononitrile acetates</td>
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<td>Others</td>
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*M: multiple peaks; †: single peak; °: double peaks; -: not applicable.
were together used for the derivatization of aldoses, ketoses, and uronic acid simultaneously to characterize the polysaccharides from Kadsura marmorata fruits, which is a commonly used traditional Chinese medicines (TCMs) Figure 2b (22). Different silylation regents including HMDS, MSTFA and BSTFA were compared for determination of carbohydrates in medicinal plants. Mono-, di- and tri-saccharides (glucose, sucrose, and raffinose) were all taken into the consideration. The results showed that BSTFA delivered both satisfactory chromatographic behavior (two signals of glucose and one signal for sucrose and raffinose) and signal intensity. MSTFA delivered comparable results with BSTFA, however with lower signal intensity. HMDS has drawn negative attention because of multiple peaks and one signal for sucrose and raffinose) were all taken into the consideration. The results showed that BSTFA delivered both satisfactory chromatographic behavior (two signals of glucose and one signal for sucrose and raffinose) and signal intensity. MSTFA delivered comparable results with BSTFA, however with lower signal intensity. HMDS has drawn negative attention because of multiple peaks and very low signal intensity (24). Furthermore, trimethylsilyl cyanide (TMSCN) was developed for evaluating the carbohydrates simultaneously with amino acids, small organic acids, phenolic acids, flavonoids and triterpenoids in plant extracts. The results indicated that TMSCN achieved 8.8 times higher intensities than MSTFA (25). However, TMSCN should be employed with special care since it hydrolyzes to give hypertoxic hydrogen cyanide, which limits its applications. For TMS derivatizations, it should be noted that the silylation reagents are moisture sensitive, the entire derivatization process needs to avoid the introduction of water.

Both acetates and TMS ethers are achieved by one-step derivatization and could be applied for the analysis of aldoses, ketoses and alditols. However, the anomeric centre leads to isomers peaks make the chromatography complicated and embarrass the accurate identification. For more authentic identification and accurate quantification, two-step derivatization is appreciated: one step to modify the anomeric centre, another to improve the volatility. The commonly used modification methods about the anomeric centre of carbohydrates are 1) reduction or 2) oximation.

Reduction of the carbonyl group in aldoses and subsequent acetylation to form alditol acetates could simplify chromatograms by producing a single peak for each aldose. The reduction commonly achieved by NaBH4/NaBD4 or NaBH4/NaBD4 in NH4OH (usually cost several hours) and acetylation with pyridine-acetic anhydride or 1-methylimidazole-acetic anhydride. The aldoses, sugar acids and amino sugars were successfully derivatized as single peaks corresponding to every sugar by this method (except N-acetyl-neuraminic acid (NAcNeu)) (Figure 2c) (26). One of the main drawbacks of these derivatives, which make it lose the original information of complex samples, is that ketoses produce two alditol acetates. What’s more, aldoses and ketoses could yield the same alditol acetates. Take fructose as an example, it produces mannitol and glucitol after reduction, while glucose also produces glucitol after the reduction. In fact, fructose could be reduced into glucitol and mannitol in a fixed proportion and samples containing glucose and fructose simultaneously could be quantified with acceptable reproducibility (27). Another difficulty in preparation of alditol acetates is that the step of reduction is time-consuming; furthermore, removing the excess NaBH4 makes the procedure tedious to perform. Nevertheless, alditol acetate is still the most commonly used approach for carbohydrate analysis in sweet medicines (Table 1), because of good chromatographic behavior for identification and quantification. Additionally, the achieved derivatives are stable.

Oxime derivatives are obtained by oximation, which commonly uses hydroxylamine hydrochloride in pyridine (other regents including methoxamine hydrochloride, O-ethylhydroxylamine hydrochloride and O-benzylhydroxylamine hydrochloride also could be used (28)) and subsequent silylation to form trimethylsilyl oximes (TMSO). In some cases, trifluoroacetylation (28) and acetylation (29) also used for...
subsequent derivatization. All of these oximes derivatives produce two peaks corresponding to the syn (E) and anti (Z) forms per reducing sugar and applicable to aldoses, ketoses and alditols (except aldononitrile acetates). As relative simple chromatograms are achieved, these derivatives have also been adopted for carbohydrate analysis in complex mixtures (30-33). The comparison of single-step derivatization trimethylsilylation and two-step approaches including ethoximation-trimethylsilylation (EO-TMS), ethoximation-trifluoroacetylation (EO-TFA), benzoximation-trimethylsilylation (BO-TMS) and benzoximation-trifluoroacetylation (BO-TFA) have been comprehensively studied for derivatization of aldoses and ketoses with regard to chromatographic characteristics. Results showed that two-step EO-TMS was superior to other approaches due to the low number of peaks obtained per carbohydrate, abundant structural information of mass spectra, low limits of detection and quantitation (28). Derivatization of aldose and ketose to their respective O-methyloxime acetates (oximation by methoxiamine hydrochloride and acetylation by acetic anhydride) for GC-MS analysis is also a facile method for the determination when aldoses and ketoses simultaneously existed, and more stable and sensitive than TMSO. Moreover, O-methyloxime acetates derivatives of glucose and fructose showed characteristic fragments both in chemical ionization (CI) and electronic ionization (EI) mode of mass spectrometry (31).

Especially, when aldose oximes are subsequently acetylated with acetic anhydride and dehydrated to aldononitrile acetates, a unique peak is achieved for every aldose. The derivatization procedure is relatively rapid (oximation with hydroxylamine hydrochloride-pyridine at 90°C for 30 min and acetylation by acetic anhydride at 90°C for 30 min). The produced aldononitrile acetates are more stable than TMS derivatives and have better sensitivity, accuracy and reproducibility in the qualitative and quantitative analysis of carbohydrates in complex matrix (34-37). The derivatization of aldose and alditols to aldononitrile acetates was successfully archived single peaks and quantification analysis of carbohydrates in Ganoderma (38). However, the validity of quantitative analysis using these derivatives also has the drawback that they cannot be applied for analysis of ketoses (14,31). Actually, when aldoses and ketoses exist simultaneously in the matrix, samples can go through oximation first and subsequent derivatization by acetylation and silylation, respectively, and finally converted to aldononitrile acetates and TMSO respectively. The developed method showed good chromatographic behavior and quantitative results (39).

2.1.2. Sugar acids

Sugar acids are monosaccharides with one or more carboxyl group and also known as polyhydroxy carboxylic acids. Generally, sugar acids include following classes: i) aldonic acids, in which the aldehyde functional group of an aldose is oxidized; ii) uronic acids, in which the terminal hydroxyl group of an aldose or ketose is oxidized; iii) aldaric acids, in which both ends of an aldose are oxidized (40). Among them uronic acids such as glucuronic acid (GlcA) and galacturonic acid (GalA) are most commonly found in nature and present as parts of structural and/or extracellular polysaccharides or glycoconjugates. Analysis of these compounds requires hydrolysis or methanolysis before derivatization, which will discuss in section 2.2. Conventionally, colorimetric methods using different chromogens including carbozole, 3-phenylphenol and 3,5-dimethylphenol (DMP) are most commonly used methods explored for uronic acid estimation in polysaccharides but these methods counter numerous difficulties when neutral sugars are present in substantial amount (41). GC analysis despite time consuming for derivatization procedure remains the finest method for precise estimation of uronic acids (41,42). The derivatives including acetates (43), TMS derivatives (22,44), oximes derivatives (45), and alditol acetates (26) have been used for the analysis of sugar acids. However, because hydroxyl and carboxyl groups simultaneously exist in one molecule, different lactones will be formed, and furthermore, coupled with anomic centre, complex chromatograms will be generated when uronic acids presented in the samples. The methyl ester alditol acetate is the alternative solutions for decreasing the multiple peaks. Guillerme L. Sassaki proposed methyl ester alditol acetate for simultaneously determined neutral, uronic acids and amino sugars. The mixture was firstly de-lactonized with NH4OH at room temperature, subsequently reduced by NaBH4 to form alditols, and then methyl esters were formed by 0.5 M HCl in MeOH. Finally acetylation of the Me-alditols was performed in pyridine-acetic anhydride (Ac2O) (1:1, v/v) and uronic acids gave characteristic ions at m/z 143, 156 and 173 (26).

2.1.3. Amino sugars and iminosugars

Amino sugars are the hydroxyl group of monosaccharides replaced by the amino group, and sometimes by the N-acetyl-amino group. As with the deoxy sugars, theoretically any hydroxyl group can be replaced. The most commonly occurring amino sugars are D-glucosamine (2-amino-2-deoxy-D-glucose, GlcN), D-galactosamine (2-amino-2-deoxy-D-galactose, GalN), N-acetylglucosamine (N-acetyl-D-glucosamine, GlcNAc) and N-acetylgalactosamine (N-acetyl-D-galactosamine, GalNAc) (40). Iminosugars are found both free or as part of glycoproteins, glycolipids or polysaccharides, therefore, a previous hydrolysis step before their analysis is commonly necessary. Iminosugars are monosaccharides where the O atom in the cycle has been replaced by N atom such as fagomine and deoxyojoirimycin (DJN). The derivatives
including acetates (29), TMS derivatives (29,46,47), oximes derivatives (29) and alditol acetates (47,48) have been used for the analysis of amino sugars and iminosugars in sweet medicines. Derivatization methods of aminoglycosides have been reviewed before (49). Alditol acetates have been widely used for determination of neutral and basic monosaccharides simultaneously (30-52) however failed in uronic acids detection without forming methyl esters (52). The derivatization procedure including silylation, acetylation, oximation + acetylation and oximation + silylation have been compared for the analysis of iminosugars (DNJ and fagomine) and other low molecular weight carbohydrates. Results indicated that two-step derivatization including oximation + acetylation and oximation + silylation allowed the separation of target compounds, whereas TMS and acetylated derivatives showed several co-elutions. Oximation + acetylation were discarded for giving inaccurate results for ketoses. TMSO formed by oximation + silylation was successfully applied for simultaneous determination of iminosugars and other carbohydrates including mono-, di-saccharides and alditols (29).

Currently, there is considerable interest in developing the simple and quick method for derivatization and separation of carbohydrates in complex matrices. Microwave-assisted derivatization has been successfully applied for carbohydrates analysis in complex matrix (53-56). Taking the advantage of high efficiency of microwave, the derivatization procedure could be significantly shortened. Silylation was finished within 4 min with HMDS, BSTFA or MSTFA as derivative reagents (24). Microwave-assisted derivatization combined with comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (2D GC-TOF-MS) has been successfully applied for carbohydrate analysis in complex extracts (57).

Although some of the existing procedures for preparing GC derivatives are quite satisfactory, and some of them have even been improved, one of the goals of these methods to achieve only one chromatographic peak for each individual sugar seem to need further work. Generally, when the analytes composed of neutral carbohydrates (ketose and aldose), alditols and amino sugars, novel developed methyl sulfoxide (Me2SO)/1-methylimidazole system to acetylation is recommended (Figure 2a). Aldononitrile acetate is also a good choice for quantitative analysis of aldose (Figure 1, Table 1). Oximes and TMS derivitives are the alternative methods when ketose and aldose simultaneously existed (Figure 2b, Figure 1). When aldose, alditols, sugar acids, amino sugar as well as iminosugars taken into the considerations alditol acetates should be an ideal choice (Figure 2c and Figure 1).

2.2. Hydrolysis

Hydrolysis is a necessary and crucial step both in the compositional monosaccharide analysis and linkage analysis of polysaccharides. The hydrolysis conditions are varying depending on the nature of samples and their compositional sugars. The compositional sugars in sweet medicines are diverse, and additionally their chemical properties are varying. Therefore, different hydrolysis methods are developed for the accurate identification and quantification of sugars in sweet medicines.

2.2.1. Acidic hydrolysis

Acidic hydrolysis is the most commonly used methods for releasing monosaccharides. Two of the most common reagents for hydrolysis are trifluoroacetic acid (TFA) and sulfuric acid. TFA is most commonly used for soluble polysaccharides such as isolated polysaccharides and secreted polysaccharides. It is volatile therefore easily be removed. It accounts 73% of hydrolysis in characterization of polymeric carbohydrates in sweet medicines shown in Figure 3, which is based on the data from 224 journal articles in web of science. While sulfuric acid commonly used for insoluble samples such as plant cell walls or samples difficult to complete hydrolysis (14). What's more the hydrolysis based on sulfuric acid need further cleanup to remove excessive and involatile sulfuric acid, which make the hydrolysis procedure more complex and time-consuming. However this problem partly solved when microscale analysis applied, samples hydrolyzed by sulfuric acid were neutralized with N,N-diocytlyamine (DOM) in chloroform, followed by successive washes with the same solution. This procedure effectively removed the sulfuric acid and allowed derivatization of monosaccharides in one tube. However the chromatography achieved by this procedure is not as clean as the TFA hydrolysis, and unknown peaks will appear in the chromatogram (58). It should be noted that some acid-sensitive sugars would decompose during the acidic hydrolysis. Therefore, identification and quantification of these sugars by hydrolysis should be performed carefully. Fructose is easily decomposed under acidic conditions in both acid hydrolysis and methanolysis conditions (59,60). Some alternative methods have been developed to solve the problem such as enzymatic hydrolysis (61,62) or

Figure 3. Hydrolysis methods used for releasing monosaccharides (data from 224 journal articles collected in Web of Science mainly dated 2010-2014).
determined by phenol-acetone-boric acid reagent (PABR) which introduced by Boratynski (60,63). Besides, anhydrosugars that are common in red/brown algal cell walls such as 3,6-anhydrogalactose need to be analyzed by reductive hydrolysis (64). Mild methanolysis is required for the detection of 3-deoxy-D-manno-oct-2ulosonic acid (KDO), which is used by bacteria in the synthesis of lipopolysaccharides (65-67). GlcNAc also easily destroyed during hydrolysis therefore mild acid condition or methanolysis is needed (68-71). On the contrary, liberation of all monosaccharides from polysaccharides is also not easily achieved, especially uronic acid-containing polysaccharides because glycosidic linkages between uronic acids and other monosaccharides are acid resistant during acid treatments. Therefore the reduction of carboxylic groups of uronic acids into their corresponding hexoses or methanolysis is recommended to carry out to allow the complete liberation of monosaccharides (14,58). The strategy for releasing neutral, amino sugars and acidic sugars was proposed. Firstly for releasing the neutral and amino sugars from lipopolysaccharides, samples were hydrolyzed with 2 M TFA (120°C, 2 h). While to release acidic sugar components, lipopolysaccharides were subjected to methanolysis (1 M HCl in methanol, 85°C, 16 h), finally the hydrolysis products were converted to alditol acetates for GC-MS analysis (66).

Microwave-assisted hydrolysis of polymeric carbohydrates showed its power in complete glycosidic cleavage and conversion of polysaccharides into monosaccharides (72). The optimization of microwave-assisted hydrolysis and derivatization of hydroxyethylstarch showed that hydrolysis could be finished in 2 min at 1,200 W, 100°C and derivatization could be achieved within 5 min at 1,020 W, 100°C. The sample preparation time is greatly shortened by this procedure, compared with traditional hydrolysis and derivatization (73). It should be noted that optimization procedure should be carefully carried out to avoid the degradation of monosaccharides during the hydrolysis.

2.2.2. Enzymatic hydrolysis

Although it is not commonly used as acidic hydrolysis (only accounting for 1% of hydrolysis in Figure 3), enzymatic hydrolysis plays an irreplaceable role for its mild hydrolysis condition and avoiding sugar degradation. Enzymatic hydrolysis is commonly used for the analysis of fructose-rich carbohydrates (61,74) such as inulin and fructans. The amount of inulin in the samples was quantified for the QC as the amounts of hydrolyzed sugars (fructose, glucose and sucrose) after inulinase treatment minus the amounts of free sugars (the existing sugars in the original sample) (61). Enzymatic hydrolysis has also been used for releasing carbohydrates from plant-derived arabinoxylans and uronic acid-containing polysaccharides (62,75-77). The applications of enzymatic hydrolysis in the utilization and analysis of carbohydrates have been summarized in previous reviews (78-81).

2.2.3. Others

Methanolysis is another commonly used approach for releasing monosaccharides (accounting for 14% of hydrolysis in Figure 3) and is usually performed with HCl in anhydrous methanol. Monosaccharides are liberated as methyl glycosides and the carboxyl groups are esterified. Polysaccharides with the inclusion of uronic acid residues can be determined by methanolysis (14). Prebiotic oligosaccharides from Corylus avellana L., composed mainly of GaA and GalNAc, have been successfully quantified by methanolysis (1 M MeOH-HCl at 80°C for 24 h) combined with TMS derivatives (82).

Generally, methanolysis and prederivation are highly recommended for uronic acids containing polysaccharides. Acidic hydrolysis is effective in most cases, when neutral and amino sugars are the compositional monosaccharides (Figure 1). Actually, the combination of different hydrolysis and derivatization methods certainly makes the results more reliable (47,83). The methanolysis (1 M MeOH-HCl at 80°C for 16 h) coupled with TMS derivatives and acid hydrolysis (4 N HCl at 100°C for 6 h) coupled with alditol acetate derivatives were successfully applied for the identification of rare monosaccharides in O-antigen capsular polysaccharide from Francisella tularensis. GC-MS analyses of TMS derivative, confirmed the presence of 2-acetamido-2,6-dideoxy-<sub>O</sub>-D-glucose (QuiNAc) in the sample. While GC-MS analyses of alditol acetates showed the presence of QuiNAc and 4,6-dideoxy-4-formamido-D-glucose (Qui4NFm). Besides, two ionization modes were used in the identification, which CI could get fragments related to molecular weight whereas EI could get more fragment ion information (47). A combination of reductive acid hydrolysis and anhydrous mercaptolysis (0.5 M HCl in EtSH:MeOH (2/1, v/v) at 60°C, 6 h) was applied for selective hydrolysis of the 3,6-anhydrogalactosidic linkage in red algal galactan (83). Acid hydrolysis, methanolysis, and enzymatic hydrolysis were compared for depolymerization of different plant materials containing uronic acids. Besides GC (using both HP-1 and HP-5 capillary columns and FID and MS detectors), HPAECPAD and HPAEC-Borate techniques also were compared for subsequent analysis of the released monosaccharides. It was shown that methanolysis combined with GC analysis is a convenient method for obtaining the sugar unit composition from uronic acids containing polysaccharides (76).

2.3. Methylation analysis

Since permethylation reaction was developed for the
linkage analysis between sugar residues in the 1960s, it is a crucial analytical approach for the structural analysis of carbohydrates, called "methylation analysis" (84-86). Methylation analysis traditionally including permethylation-hydrolysis-reduction-acetylation procedures (Figure 4a) and complete permethylation is critical for the correct analysis (16). There are two most commonly used permethylation methods for carbohydrates analysis. One is the method introduced by Hakomori in 1964 (86), and the other is Ciucanu and Kerek introduced in 1984 (87). In Hakomori’s method carbohydrates in dimethylsulfoxide (DMSO) is reacted with methyl iodide catalysed by the methylsulfinyl carbanion, which is prepared from sodium hydride (86). Sometimes with modification for the use of methylsulfinyl carbanion made with bases such as potassium hydride or butyl-lithium (16,21,88). Several years later, Ciucanu and Kerek developed a simple, rapid and quantitative procedure used finely powdered sodium hydroxide as base catalyst and DMSO as solvent (87). These two methods have been compared for the analysis of β-cyclodextrin (β-CD) and Hakomori method showed superior base catalyst than NaOH-DMSO suspension. Under the latter condition, premethylation of β-CD occurs selectively at 3-hydroxy groups, which may because the 3-hydroxy groups are buried within the relatively hydrophobic torus of β-CD where they are excluded from deprotonation by the NaOH base (89). In the same study, however, consistent with this, maltose, which is a linear form of β-CD, is permethylated equally well using either two methods. Even several mannose oligosaccharides are more completely permethylated using NaOH-DMSO (89).

The conclusion is that permethylation conditions are not universally applicable to all carbohydrate types, and it is therefore recommended that the completeness of permethylation of carbohydrate samples should be checked before the acid hydrolysis step such as using infrared spectroscopy to monitor the hydroxyl residues. Besides many researches cited the methods described by Needs and Selvendran in 1993 (90). It is a modified sodium hydroxide-catalysed procedure, in which methylation with sodium hydroxide and methyl iodide is sequentially rather than simultaneously added into samples. The results showed that it was not prone to the oxidative deficiencies of the original and that, given its reduced tendency towards polysaccharide undermethylation. The preparation of permethylated carbohydrates for GC and LC analysis has been the subject of several reviews (91-93).

For the subsequent derivatization, hydrolysis is also necessary in methylation analysis. TFA is still the most frequently used hydrolysis reagents. And what should be noted is that acid-sensitive sugars such as fructose also need hydrolysis in mild conditions after permethylation (94). Then the hydrolyzed free methylated monosaccharide residues are commonly reduced by NaBH₄/NaBD₄ or NaBH₄/NaBD₄ in NH₄OH and converted into alditols. Then the remaining hydroxyl groups acetylated with Ac₂O/pyridine or Ac₂O/1-methylimidazole. The final product partially methylated alditol acetates, known as PMAAs are subject to the GC-MS analysis (Figure 4b). The glycosidic linkage is concluded based on the retention time and mass spectrometry data (Figure 4c). Some databases have been built up to assist the analysis of these data. Such as Spectral Database for PMAA's which is initiated by Complex Carbohydrate Research Center is available online (95). Recently, researchers attempt to synthesize partially O-methylated alditol acetate standards of galactofuranose. These PMAAs could be used as GC-MS standards for simultaneous identification of galactofuranose units with diverse linkages in complex carbohydrates (96).

Traditional methylation analysis commonly costs several days, of which the permethylation process takes most (97). Microwave-promoted methylation significantly shortens and simplifies this procedure. It was demonstrated that permethylation of plant seed gum with NaOH-dimethyl sulfate was completed in 4 min after exposure to microwave power. And subsequently hydrolysis with 70% aqueous formic acid and 0.5 N H₂SO₄ was finished in 3.32 min (97). Therefore microwave assistant methylation analysis is probably a wise choice to improve the methylation...
analysis efficiency. However, still, there are time-consuming steps. By-products and excessive methylation agents should be separated with partially methylated carbohydrates before hydrolysis. And dialysis or extraction with organic solvents such as dichloromethane and chloroform are performed. Extraction partially methylated carbohydrates by organic solvents is simple and time-saving. After three or more times extraction with organic solvents and wash with water, the organic layer could be separated and obtained the purified partially methylated carbohydrates. However, solvent extraction is not suitable for the high molecular weight polysaccharides because of poor solubility (58). Dialysis is commonly adopted for these polysaccharides. However, dialysis is usually performed "over night". So far, it has still been the rate-limited step in methylation analysis, which needs further improvements.

Even though methylation analysis could provide abundant information about the characterization of carbohydrates, it still could not give the definite linkage of polymeric carbohydrates for the mass spectra of some PMAAs are highly similar. Accurate identification of structures must combine with other approaches such as MALDI-TOF-MS (16), characteristic enzymatic digestion (13) and NMR (65).

2.4. Pyrolysis-gas chromatography (Py-GC)

Pyrolysis-gas chromatography (Py-GC) has been well established as a simple, quick and reliable analytical technique for a range of applications including the analysis of polymeric materials (98,99). The most important application of Py-GC in carbohydrates analysis is characterization of cellulose, hemicellulose and plant gum (100-103). Derivatizations including methylation and silylation are also necessary of Py-GC for improving the behavior of analytes during separation in the column, modifying the thermal degradation pathway or enhancing detectability (98). The derivative reagent including TMCS (103), BSTFA (103,104) and HMDS (105,106) are most commonly used. Recently, on-line analysis of thermally assisted hydrolysis and methylation (THM) gas chromatography commonly used tetramethylammonium hydroxide (TMAH) (107-109) as base reagents, made the analysis simpler and faster (99). Pyrolysis GC-MS as a novel analysis technique to determine the biochemical composition including carbohydrate has been applied for microalgae. The results showed that a linear trend was observed and the method could give a quick estimation of carbohydrate contents (110). The medicinal plant Ginkgo biloba was also successfully identified by THM-GC (111). The greatest advantage of pyrolysis is that, in most cases, only minimal sample preparation is required. Therefore, Py-GC is a promising method for quick identification of sweet medicines and is useful in the QC of sweet medicines (Figure 1).

3. Quantification analysis

Quantitation is crucial for QC of sweet medicines. Compared with free carbohydrates, polymeric carbohydrates such as oligosaccharides and polysaccharides are more difficult to quantify due to their large molecular weights, complex structures and rare of chemical standards (4). However, separation and quantification are possible for free carbohydrates (including mono-, di- and trisaccharides) by GC-MS (24,112). Therefore, suitable hydrolysis of polymeric carbohydrates (discussed in Section 2.2) combined with efficient derivatization methods (discussed in Section 2.1) is an alternative method to quantify the carbohydrates in sweet medicines for QC.

Monosaccharide profile has been successfully applied for QC of Dendrobii Officinalis Caulis, which is a rare medicinal plant (113). Chinese Pharmacopoeia (2010 Edition) documented that the ratio of mannose and glucose in Dendrobii Officinalis Caulis should be 2.4-8.0 (114). The monosaccharide profiles released from polysaccharides have also been used to discriminate different sweet medicines and identify their origins (115-118). The results of those studies indicate that free sugars or sugar profiles obtained after acidic or enzymatic hydrolysis (i.e., amounts and composition of monosaccharides) are crucial for QC of polysaccharides. On the other hand, the characteristic chromatography of PMAAs achieved by GC-MS which reflects the glycosidic linkages is also could be applied for the discrimination of original for sweet medicines, however many works should be carry on to make the procedures involved in methylation analysis more efficient, automated and high-throughput.

4. Conclusion

Biological activities of sweet medicines are highly correlated with their chemical characteristics. The qualitative and quantitative analyses of both free and polymeric active carbohydrates are necessary for QC of sweet medicines. GC coupled with MS, which provides abundant structure and quantitative information, is very helpful in improving QC of sweet medicines.

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