Journal of Chromatography B, 879 (2011) 1226–1240

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Review

# Derivatization of carbohydrates for GC and GC–MS analyses☆

A.I. Ruiz-Matute[a], O. Hernández-Hernández[b], S. Rodríguez-Sánchez[b], M.L. Sanz[b], I. Martínez-Castro[b,*]

[a] Instituto de Fermentaciones Industriales-CIAL (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain
[b] Instituto de Química Orgánica General (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

ARTICLE INFO

*Article history:*
Received 15 July 2010
Accepted 8 November 2010
Available online 8 December 2010

*Keywords:*
Derivatization
Carbohydrates
GC
GC–MS

ABSTRACT

GC and GC–MS are excellent techniques for the analysis of carbohydrates; nevertheless the preparation of adequate derivatives is necessary. The different functional groups that can be found and the diversity of samples require specific methods. This review aims to collect the most important methodologies currently used, either published as new procedures or as new applications, for the analysis of carbohydrates. A high diversity of compounds with diverse functionalities has been selected: neutral carbohydrates (saccharides and polyalcohols), sugar acids, amino and iminosugars, polysaccharides, glycosides, glycoconjugates, anhydrosugars, difructose anhydrides and products resulting of Maillard reaction (osuloses, Amadori compounds). Chiral analysis has also been considered, describing the use of diastereomers and derivatives to be eluted on chiral stationary phases.



**Contents**



*Abbreviations:* 3-DG, 3-deoxy-glucosone, 3-deoxy-D-*erythro*-hexos-2-ulose; $Ac_2O$, acetic anhydride; AGEs, advanced glycation end products; BSA, *N,O-bis*(trimethylsilyl)acetamide; BSTFA, *N,O-bis*(trimethylsilyl)trifluoroacetamide; CMP, κ-casein macropeptide; DFA, difructose dianhydride; DNJ, deoxynojirimycin; DMSO, dimethylsulfoxide; HFB, heptaflourobutyrate; HFBAA, heptafluorobutyric anhydride; HMDS, hexamethyldisilazane; KDN, 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid; KDO, 3-deoxy-D-*manno*-2-octulosonic acid; LPS, lipopolysaccharides; MAAS, methyl alditol acetates; MR, Maillard reaction; MSA, *N*-Methyl-*N*-trimethylsilylacetamide; MSTFA, *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide; Neu5Ac, sialic acid, 5-N-acetyl neuraminic acid; NSP, non-starch polysaccharides; *O*-PFP, *O*-pentafluropropionyl; PAANs, partially methylated aldononitrile acetates; PMAAs, partially methylated alditol acetates; PFBOA, *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; THM, thermally assisted hydrolysis and methylation; TMCS, trimethylchlorosilane; TMSDEA, *N*-trimethylsilyldiethylamine; TMSDMA, *N*-trimethylsilyldimethylamine; TMSI, *N*-trimethylsilylimidazole.

☆ This paper is part of the special issue "Enhancement of Analysis by Analytical Derivatization", Jack Rosenfeld (Guest Editor).

* Corresponding author. Tel.: +34 91 2587559; fax: +34 91 5644853.
*E-mail address:* iqomc16@iqog.csic.es (I. Martínez-Castro).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.11.013

## 1. Introduction

Carbohydrates, present in both vegetable and animal kingdoms (including terrestrial and marine organisms), represent the major form of photosynthetically assimilated carbon in the biosphere [1]. Besides the large isomer diversity, carbohydrates in nature display a great variety of functional groups as suited to the different biological roles that they play in the living organisms. Apart from their nutritional role, they also serve as a structural material, component of membranes and participate in cellular recognition. Carbohydrates occur as simple or complex mixtures which makes chromatographic techniques necessary for their determination. GC presents several characteristics such as high resolution, high sensitivity and easy coupling to different detectors, including mass spectrometers, which make it suitable for the analysis of complex mixtures. In turn, mass spectrometry is a technique which affords rather complete structural information.

The preparation of appropriate derivatives is required for the application of GC or GC–MS to the analysis of carbohydrates. This subject has been frequently covered in reviews [2–5] and books [6–8] but often only the most common sugars (aldoses, ketoses, polyalcohols and acids) are considered. However, preparation of carbohydrate derivatives for GC and GC–MS present several difficulties. Firstly, there is a high number of functional groups in the molecule (about one in each carbon atom, most of them being hydroxyls). Secondly, the presence of different tautomeric forms in solution gives rise to complex chromatograms. Finally, the lability of some molecules and in certain cases, the steric hindrance must be considered. Regarding MS behaviour, it is worth noting that fragmentation of close isomers is also very similar and often retention time becomes a decisive datum for their identification.

The present review aims to collate the most important methodologies currently used for GC and GC–MS analyses of carbohydrates, taking special note of the different isomers and functionality. Most classes of carbohydrates have been taken into account including neutral carbohydrates (saccharides and polyalcohols), acid sugars, amino and iminosugars, polysaccharides, glycosides, glycoconjugates, anhydrosugars, difructose anhydrides, products resulting of Maillard reaction, and carbohydrate enantiomers.

## 2. Common sugars (neutral saccharides and polyalcohols)

Due to the relatively low volatility of carbohydrates, GC analysis is limited to derivatized sugars of low molecular weight, mainly mono-, di- and trisaccharides. However, it has been reported that using suitable derivatives and appropriate GC conditions, carbohydrates up to 12 monosaccharide units can be analysed [9]. GC and GC–MS has also been applied for structural determination of polysaccharides, and this will be commented on detail in Section 9.

In general, one-step reactions are preferred, but sometimes, two-step derivatizations are a good choice when the obtained derivatives show better chromatographic behaviour or more adequate mass fragmentation.

Classical derivatization methods consist in the substitution of the polar groups of carbohydrates in order to increase their volatility. Table 1 summarizes the most common methods used for sugar analysis. Due to the high number of applications that can be found in literature, only selected articles will be commented as representative examples of the current state of the art.

### 2.1. Ethers and esters

Methyl ethers, acetates, trifluoroacetates and trimethylsilyl ethers are the most common derivatives used for carbohydrate determination [6]. The good volatility and stability characteristics of the derivatives formed make trimethylsilyl (TMS) ethers the most popular derivatives applied to GC analysis of saccharides and polyalcohols. Due to the simplicity of the method described by Sweeley et al. [10], it is still applied (cited 366 times in Scopus since 1996), sometimes with slight modifications [11–14]. Silylation generally consists of the use of hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) as derivatizating agents and pyridine as a solvent. Other reagents such as *N*-trimethylsilylimidazole (TMSI), *N*-methyl-*N*-trimethylsilylacetamide (MSA), *N*-trimethylsilyldiethylamine (TMSDEA), *N*-trimethylsilyldimethylamine (TMSDMA), *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), *N*,*O*-*bis*(trimethylsilyl)acetamide (BSA) and *N*,*O*-*bis*(trimethylsilyl) trifluoroacetamide (BSTFA) have also been used for silylation, at different temperatures for different reaction times [6]. Although several aprotic solvents can be used, the good solubility of the carbohydrates and their derivatives in pyridine makes it the most commonly used for sugar analysis. In many cases, the need for a solvent is eliminated with silylating reagents which act as a solvent themselves. The addition of hexane and water is recommended in some cases in order to eliminate the excess of reagents or interferences of the sample.

The above methods are adequate for silylation of polyalcohols as well as non-reducing saccharides. When dissolved, sugars with free carbonyl groups can be present as different tautomers. A different

**Table 1**
Main types of derivatives used for analysis of neutral carbohydrates.

| Derivatives | Anomeric centre | Derivatization reaction | Advantages | Drawbacks |
|---|---|---|---|---|
| Methyl ethers | Non-modified (Multiple peaks) | $ROH + CH_3–X \rightarrow R–O–CH_3 + HX$ | - Suitable for analysis of low molecular weight carbohydrates and for structural analysis of polysaccharides | - Complex chromatograms due to the different tautomeric forms<br>- Time-consuming preparation<br>- Thermal degradation at high temperatures<br>- Lack of good resolution in GC |
| Acetates | Non-modified (Multiple peaks) | $ROH + Ac–X \rightarrow R–O–Ac + HX$ | - Suitable for analysis of low molecular weight carbohydrates<br>- Chemical and thermical stability | - Complex chromatograms due to the different tautomeric forms<br>- Low volatility comparing to trimethylsilyl ethers |
| Trifluoroacetates | Non-modified (Multiple peaks) | $ROH + CF_3CO–X \rightarrow R–O–COCF_3 + HX$ | - More volatile than either acetates or trimethylsilyl ethers<br>- Applicable to carbohydrates of a wide range of molecular weights<br>- Allows the use of lower analysis temperatures | - Complex chromatograms due to the different tautomeric forms<br>- Difficulty in achieving satisfactory quantitation |
| Trimethylsilyl ethers | Non-modified (Multiple peaks) | $ROH + (CH_3)_3Si–X \rightarrow R–O–Si(CH_3)_3 + HX$ | - More volatile, less polar and more thermally stable than methyl ethers and acetates<br>- Rapid derivatization method which proceeds under mild conditions<br>- Large number of silylating reagents available<br>- Useful for quantifying equilibrium forms of carbohydrates in solution<br>- Derivatives most widely utilized | - Complex chromatograms due to the different tautomeric forms.<br>- Samples must be completely dried<br>- Silylation reagents are moisture sensitive |
| Trimethylsilyl oximes | Modified (Two peaks) | (1) $R–CHOH–CHO + NH_2OH \rightarrow R–CHOH–C{=}NOH + H_2O$<br>(2) $R–CHOH–C{=}NOH + (CH_3)_3Si–X \rightarrow R–CHOSi(CH_3)_3–C{=}NO–Si(CH_3)_3 + HX$ | - Decreases the number of chromatographic peaks of reducing carbohydrates to *anti* (*E*) and *syn* (*Z*) isomers<br>- Applicable to both aldoses and ketoses | - Samples must be totally dry<br>- Derivatives are stable for a long time |
| Trimethylsilyl alkyl oximes | Modified (Two peaks) | (1) $R–CHOH–CHO + NH_2OR' \rightarrow R–CHOH–C{=}NOR' + H_2O$<br>(2) $R–CHOH–C{=}NOR' + (CH_3)_3Si–X \rightarrow R–CHOSi(CH_3)_3–C{=}NO–R' + HX$ | - Decreases the number of chromatographic peaks of reducing carbohydrates to *anti* (*E*) and *syn* (*Z*) isomers<br>- Applicable to both aldoses and ketoses | - Samples must be totally dry |
| Alditol acetates | Modified (One peak) | (1) $R–CHOH–CHO + NaBH_4 \rightarrow R–CHOH–CH_2OH$<br>(2) $R–CHOH–CH_2OH + Ac–X \rightarrow R–CHOAc–CH_2O–Ac + HX$ | - Elimination of the anomeric centre giving one chromatographic peak for each aldose<br>- Very stable compounds<br>- Used for sugar composition analysis of macromolecules<br>- Allows separation of complex mixtures | - Different sugars can lead to the same alditol<br>- Each ketose yields a mixture of two alditols<br>- Frequently, tedious and time-consuming derivatization procedure<br>- Reaction products (acid by-products) often need to be removed before analysis<br>- Acylation reagents are moisture sensitive |
| Aldononitrile acetates | Modified (One peak) | (1) $R–CHOH–CHO + NH_2OH \rightarrow R–CHOH–C{=}NOH + H_2O$<br>(2) $R–CHOH–C{=}NOH + Ac–X \rightarrow R–CHOAc–C{\equiv}N + HX$ | - Give only one product for each aldose<br>- Water does not interfere with the reaction | - Formation of non-volatile products for ketoses<br>- Non-appropriate for GC analyses of real samples where aldoses and ketoses exist simultaneously |
| Dialkyl dithioacetals | Modified (One peak) | (1) $R–CHOH–CHO + R'–SH \rightarrow R–CHOH–CH(S–R')_2 + H_2O$<br>(2) $R–CHOH–CH(S–R')_2 + (CH_3)_3Si–X \rightarrow R–CHOSi(CH_3)_3–CH(S–R')_2$ | - Form a single peak for each aldose<br>- Stable products | - Difficulties in the preparation procedure basically related to the use of a thiol as regent of derivatization<br>- Variable yields are obtained<br>- In some cases, formation of byproducts contaminating the chromatographic system |

derivative is obtained for each tautomeric form of the sugar and GC analysis gives, therefore, a different peak, producing complicated chromatograms. This may interfere in qualitative identification and in quantitative measurement of complex sugar mixtures. Different strategies of derivatization have therefore been conducted to reduce this effect.

### 2.2. Oximes

Trimethylsilyl oximes (TMSO) can be obtained by a two step derivatization procedure (oximation and silylation) producing two peaks corresponding to the *syn* (*E*) and *anti* (*Z*) forms per reducing sugar. A single peak is obtained for any non-reducing carbohydrate present (which do not form oximes). These derivatives are applicable to both aldoses and ketoses and have been widely used for carbohydrate determinations of complex mixtures, as they present good GC properties and provide simple chromatograms [15–22]. The samples are generally dissolved in pyridine containing 2.5% hydroxylamine hydrochloride and the solution is heated for 30 min at 75 °C. Then, for the silylation step, HMDS and TFA are added and the mixture is allowed to react for further 30 min at 45 °C [23,24]. In some cases, the application of different derivatives can be used in order to obtain complementary information of the sample under study. Soria et al. [12] identified for the first time *scyllo*-inositol and sedoheptulose (D-*altro*-2-heptulose) in carrots, using both TMS and TMSO derivatives for complete identification of the latter.

Other alternatives to TMSO are trifluoroacetylated oximes, trimethylsilyl *O*-methyloximes, and *O*-methyloxime acetates, all of which also give the two isomeric forms (*E* and *Z*). Funcke and von Sonntag [25] reported that the major *O*-methyl oximes formed from aldohexoses are the *syn* forms (which can be an aid in the identification), while for ketohexoses almost equal proportions of *syn* and *anti* are formed. Trifluoroacetylated oximes can be prepared at low temperatures and requires low column temperatures for GC but their use is less common [2]. Some drawbacks have also been reported for TMS methyloxime application such as the insufficient efficiency of the derivatization steps and the low stability of the products obtained in high moisture environment [26].

Derivatization of aldose and ketose monosaccharides to their respective *O*-methyloxime acetates for GC–MS has also been demonstrated to be an easy method for determination of serum glucose and fructose, having a lower detection limit than other methods [26]. Glucose and fructose methyloxime peracetates showed characteristic mass fragment ions which were used in the quantitative determination of glucose and fructose in clinical samples.

### 2.3. Alditol acetates

The reduction of the carbonyl group of aldoses and the subsequent formation of alditol acetates simplifies chromatograms by producing a single peak for each derivatized sugar. Once formed, alditol acetates are stable allowing post-derivatization cleanup and storage of treated samples for extended periods [6] and has been widely used for monomer analysis of macromolecules [27]. One of the main drawbacks of these derivatives is that the reduction of different aldoses and ketoses can yield the same alditol upon reduction (i.e., fructose produces mannitol and glucitol and the latter can also arise from glucose) producing a significant loss of information of the original sample. However, some studies are being developed to overcome this problem. Brunton et al. [28] succeeded in the quantitation of reducing sugars of potato tubers (including fructose) with an acceptable reproducibility by converting fructose into glucitol and mannitol hexa-acetates in a fixed proportion, which allowed the estimation of the carbohydrates of the samples. Derivatives were formed by adding 1 mL of a sodium borohydride solution (0.5 M in dimethyl sulfoxide) and incubating at 40 °C for 90 min. After leaving overnight at 4 °C, the samples were acetylated with g. acetic acid:1-methylimidazole:acetic anhydride (1:2:10 v:v:v; 40 °C for 10 min).

Another difficulty with alditol acetates is the large number of manual processing steps needed, which makes the procedure time consuming and tedious to perform. Although significant improvements have recently been made simplifying the derivatization procedure, some common versions of this method still require tedious evaporations to remove the excess of borate before acetylation.

### 2.4. Aldononitriles

An alternative approach to eliminate the anomeric centre of sugars is by dehydration of aldose oximes to their aldononitrile acetates. These give a unique peak for every sugar, and derivatization procedure is relatively rapid. However, the validity of quantitative analysis using these derivatives has been called into question [28], and they cannot be applied for ketoses [26]. Zhang et al. [29] developed a method for the simultaneously quantitation of ribose, rhamnose, arabinose, xylose, fucose, mannose, glucose, and galactose in soil by GC. After hydrolysis with TFA the sugars were converted into aldononitrile acetate derivatives. In comparison with TMS derivatives, the aldononitrile acetates were more stable, gave better recovery, and the reproducibility of ribose analysis improved significantly [29].

Ye et al. [30] developed a quantitative method for the analysis of aldoses and ketoses by GC–MS using two different derivatizations. Firstly, sugar oximes were obtained by the addition of hydroxylamine and pyridine (90 °C for 30 min). As a second step, acetylation (acetic anhydride, 90 °C, 30 min) was performed for aldoses while trimethylsilyl derivatives (HMDS:TMCS, 80 °C, 1 h) were employed for ketoses. Both aldononitrile acetates and TMS oximes showed good results in the qualitative and quantitative analysis of eight monosaccharides present in a *Lyceum barbarum* L. extract [30].

### 2.5. Dithioacetals

Trimethylsilylated diethyl dithioacetals proposed by Honda et al. [31] are derivatives that also give a single peak for each sugar. Although this procedure is simpler than the alditol acetate method, the resolution is such that only simple mixtures of sugars can be analysed and therefore the technique has not gained general acceptance. A simplified method was developed by Englmaier [32] for obtaining dithioacetal–trimethylsilyl ethers and dithioacetal–trifluoroacetates. However, several difficulties have been noted in the use of thiols for derivatization. Variable yields are obtained, and in some cases, the formation of byproducts makes the derivatization reaction inefficient, leading to the contamination of the chromatographic system [33]. Lluveras et al. [33] developed a method to analyse polysaccharides and other components present in binders and varnishes from works of art. It consisted of a microwave-assisted acidic hydrolysis, a cleanup step to eliminate inorganic material, and derivatization using mercaptalation followed by silylation. Important modifications were introduced in the cleanup and derivatization steps to avoid the above-mentioned problems. Samples were submitted to a three step derivatization procedure: (i) ethanethiol/trifluoroacetic acid were added to dried samples (room temperature for 10 min). (ii) A first silylation step was performed by adding BSTFA to the mercaptalation mixture (15 min at 60 °C). (iii) a second derivatization step (necessary to ensure the complete silylation of the mercaptal derivatives) using BSTFA with 1% TMCS in pyridine. This procedure gave a single peak for all pentoses, hexoses, and methyl hexoses analysed. However, multiple peaks were detected for ketoses submitted to the three-

step derivatization procedure indicating that these compounds undergo decomposition reactions during mercaptalation [33].

Price [34] developed dialkyldithioacetal acetates for monosaccharide GC–MS determination, and the use of various thiolating agents (1,2-dithioethane, 1,2-dithiopropane and 1,3-dithiopropane) was evaluated. These derivatives were suitable for measuring isotopic enrichment into the characteristic anomeric carbon of a large number of aldose sugars. In the same work, different derivatives (peracetates, deuterioalditol and aldononitrile acetates) were evaluated for obtaining positional and quantitative isotopic information by GC–MS. Peracetates resulted to be more useful for isotopomer analysis of ketoses than aldoses due to the presence of cyclic forms which complicated the MS analysis and isotope localization. Alditol acetates provided good chromatographic separation but were less useful for localizing carbon isotopes as they were symmetrical to the mass spectrometric analysis. The introduction of a deuterium in the anomeric aldehyde with sodium borodeuteride provided asymmetry, but the mass difference compared to the metabolically incorporated $^{13}C$ was too small to allow accurate distinction. GC–MS analysis of mass isomers of different monosaccharides established the usefulness of aldononitriles for direct metabolic flux analysis [34]. However, these derivatives are limited by lack of cleavage of the aldose C1–C2 bond that permits an estimation of $^{13}C$ enrichment into the anomeric carbons. The use of both aldononitrile and dialkyldithioacetal acetates (which presents fragment ions arising from C1–C2 bond cleavage) allows comprehensive estimation of isotope distribution and quantitation in the majority of cellular aldose sugars [34].

## 3. Amino and iminosugars

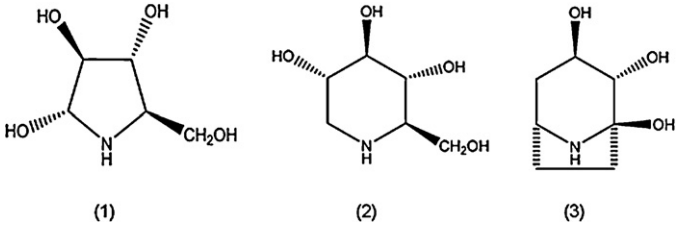
### 3.1. Aminosugars

Aminosugars are found both free or as part of glycoproteins, glycolipids or polysaccharides, therefore, a previous hydrolysis step before their analysis is commonly necessary. Samples are usually hydrolyzed with 6 M HCl although more details about hydrolysis procedures will be shown in Sections 9 and 10. Classical procedures of derivatization for carbohydrate analysis, such as alditol acetates, TMS ethers and aldononitrile acetates have been assayed [35,36].

The alditol acetate method has been used by many authors [37–39]. Aminosugars are previously submitted to a reduction step similarly to neutral carbohydrates (Section 2) with sodium borodeuteride or borohydride at 4 °C and further acetylated with acetic anhydride for 15 h at 100 °C. However, as previously noted in Section 2, the preparation of alditol acetates is time consuming and retention times in GC analyses were high.

TMS ethers have been commonly used for aminosugar derivatization using MSTFA, BSTFA [40], BSA with TMSI and TMCS [41] or HMDS/TMCS (1:0.5, v:v) with or without BSA [42] as silylation reagents. MSTFA is the most volatile, and its use is recommended for amines and amino acid derivatization for GC analysis [43].

Mawhinney et al. [35] proposed an alternative derivatization procedure, in which *O*-methyloximes were prepared using *O*-methylhydroxylamine hydrochloride in dry methanol, pyridine and 1-dimethylamino-2-propanol (70 °C, 20 min). These derivatives can be then converted into either TMS ethers or acetates for their further GC analysis. The acetylation was achieved with pyridine–acetic anhydride solution (1:3, v:v, 70 °C for 25 min). For the conversion into TMS derivatives, *O*-methyloximes were dissolved in anhydrous pyridine and reacted with TMSI:BSTFA:TMCS. The *O*-acetylated-*O*-methyloximes were the most suitable derivatives for aminosugars [35]. Gullberg et al. [43] optimized both oximation and silylation for the anlaysis of aminosugar, sugars, amino acids, fatty acids, etc. Methoximation using methoxyamine in pyridine (1 h, 60 °C) followed by 16 h at room temperature and silylation with MSTFA (1 h, room temperature) was the best combination. This demonstrated that alkoxyoxime and TMS derivatization is more suitable for the identification of all the compounds by GC–MS analysis compared with oxime and TMS derivatization. Moreover, the silylation step developed was simple and quite easily automated. This procedure has been recently used by other authors [44].




(1) (2) (3)

**Fig. 1.** Structure of some iminosugars: 5-amino-5-deoxy-D-glucopyranose, nojirimycin (1); 1-deoxy-nojirimycin, DNJ (2); calystegine $A_3$ (3).

Aldononitrile acetates are easy to prepare, stable and well separated by GC, and also give good mass spectra. However, the lack of reproducibility for some aminosugars such as glucosamine, galactosamine and mannosamine has been reported [35]. Guerrant and Moss [36] described that by increasing the derivatization time for oximation from 5 to 25 min a single peak was obtained for each hexosamine sugar, indicating that the oximation process was complete. Several authors [45–49] have followed the method proposed by Zhang and Amelung [50], where aldononitrile acetate derivatives are prepared using a mixture of hydroxylamine hydrochloride and 4-(dimethylamino) pyridine in pyridine–methanol (4:1, v:v). Samples were heated for 35 min to 75–80 °C. In a further acetylation step, acetic anhydride is added, and the resulting solution is again heated for 25 min. After cooling, the aminosugar derivatives were recovered with dichloromethane and HCl. When necessary, the excess of derivatization reagents is removed by successive washes with deionized water. Finally, the organic phase is dried at 45 °C and resuspended in ethyl-acetate hexane (1:1, v:v), ready for analysis by GC.

### 3.2. Iminosugars

Iminosugars or azasugars such as fagomine, deoxynojirimycin (DNJ) or calystegines (Fig. 1) are monosaccharides where the O atom in the cycle has been replaced by a N atom. They can be either synthetically produced or found in natural sources. Iminosugars are considered potent glycosidase inhibitors due to the resemblance in structure to common sugars and potential uses against different diseases such as cancer or viral infections has been described for some of them [51]. Recently, Molyneux et al. [4] have extensively covered the chromatographic methods existing for the analysis of these compounds and polyhydroxyalkaloids, including GC analysis and the required derivatization procedures. Trimethylsilylation confers the necessary stability and volatility for GC analysis to the hydroxyl groups of iminosugars. Different derivatization reagents (TMCS:HMDS (1:3); MSTFA; BSTFA, etc.) and conditions have been used [4]. MSTFA was found the most suitable reagent for the silylation of iminosugars, the reaction being completed in 1 h at 60 °C [52].

However, partial derivatization of iminosugars is the major drawback of this procedure. While the hydroxyl groups are readily silylated, the reaction for the imino group depends on the reagent and on the reaction conditions, resulting in one or two peaks for each iminosugar, according to the extent of silylation. Different silylation reagents have been compared for derivatization of DNJ. MSTFA gave rise to two silyl derivatives differing by the substitution

on the imino group, whereas BSTFA, HMDS + TFA and TMSI + TMCS gave only a single peak, due to the non-derivatized imino group [53].

Reaction conditions with TMCS–HMDS–pyridine have also been studied [4]. Dräger [54] reviewed the extraction and GC analysis of calystegines. Before analysis, these compounds were treated with MSTFA in pyridine for 12 h at 60 °C. Both hydroxyls and the imine group were derivatized, although these aggressive conditions led to some decomposition of calystegines. However, the use of HMDS:TMCS led to complete derivatization of the hydroxyl groups without imino group substitution, and without calystegine degradation.

Natural iminosugars are commonly found in the presence of complex sugar mixtures, thus derivatization procedures should be carefully chosen to avoid overlapping peaks in the GC analysis. Rodríguez-Sánchez et al. [53] have recently suggested the use of TMS oximes which give only two peaks for each reducing sugar, making it easier to resolve them from the imino sugars present.

Acetylation of iminosugars using acetic anhydride in pyridine (20 °C, 24 h) was described by Magalhães et al. [55]. This allowed the identification of five polyhydroxyalkaloid acetates from their corresponding mass spectra, and a fragmentation pathway for these derivatives was suggested.

An oximation step before acetylation has been also suggested for the analysis of iminosugars in the presence of interfering monosaccharides [53]. Reducing sugars give rise to per-acetylated aldononitriles (PAAN) for aldoses and acetylated oximes for ketoses. Although no co-elution was observed between iminosugars and interfering carbohydrates, this derivatization gave inaccurate results for ketoses due to incomplete derivatization [53].

## 4. Sugar acids

Sugar acids (polyhydroxy carboxylic acids) are sugars with one or more carboxylic acid function. Aldonic acids are obtained by oxidation of the aldehyde group of an aldose to form a carboxylic acid group (for example, glucose gives rise to gluconic acid). When the terminal hydroxyl group of the chain is oxidized instead of the terminal aldehyde, the compound formed is an uronic acid (e.g., glucuronic acid), while oxidation of both terminal ends yields an aldaric acid (e.g., glucaric acid).

Aldonic acids commonly appear in many biological systems, and they are generally in equilibrium with their corresponding lactones (1–4 and 1–5 lactones). Uronic acids are more commonly found in nature than aldonic or aldaric acids, being present as part of structural and/or extracellular polysaccharides of plant and bacteria [56]. Polysaccharides only composed of uronic acids can also be found, such as alginates, which are linear copolymers of (1–4)-linked β-D-mannuronic and α-L-guluronic acids. The analysis of these compounds requires a hydrolysis or a methanolysis before derivatization as will be discussed in Section 9.

Neuraminic acid (5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid or 5-amino-4,6,7,8,9-pentahydroxy-2-oxo-nonanoic acid) is a monosaccharide with nine carbon atoms. *N*- or *O*-substituted derivatives of neuraminic acid (commonly known as sialic acids) are found in nature as part of animal tissues, fungi and bacteria, especially in glycoproteins and gangliosides. Acetyl, glycolyl, methyl, sulfate, and phosphate groups can be present as hydroxyl substituents, *N*-acetylneuraminic acid being the most common in mammalian cells. Other sugar acids such as 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid (KDN) or 3-deoxy-D-*manno*-2-octulosonic acid (KDO) are also found in biological systems [57].

Other naturally occurring sugar acids include muramic acid, which is formed from lactic acid and glucosamine. It generally occurs as *N*-acetylmuramic acid as part of biopolymers in bacteria cell walls. Derivatization of this sugar acid is also treated here, although it can be considered as an aminosugar (Section 3).

Derivatization procedures of sugar acids are typically based on silylation or acetylation. Drozd [7], Knapp [6] and Churms [2] have reviewed different methods for the derivatization of these carbohydrates. Most are currently used, although some modifications and new procedures are emerging.

### 4.1. Previous treatments: reduction and/or hydrolysis

Sugar acids, and more specifically uronic acids, can appear in several different structural forms (one acyclic, two anomeric for each pyranose and furanose and at least one lactone; [56]) and therefore, in their GC analysis can yield multiple peaks. Reduction of the sugar carbonyl groups to hydroxyls (alditols) is one of the possibilities for decreasing this high number of peaks. However, some sugar acids C2 epimers can yield the same alditol.

GC characterization of polysaccharides that contains sugar acids requires a hydrolysis step. However, quantitative recovery is not easily achieved, because glycosidic linkages between uronic acids and other monosaccharides are acid resistant during acid treatments. Several methods using a reduction step prior to the hydrolysis have been tested, and will be detailed in Section 9.1

### 4.2. Trimethylsilyl derivatives

Different silylation procedures for sugar acids commonly use either HMDS/TMCS [6], BSTFA/TMCS [6], BSTFA [58], BSTFA/TMSI [59], or MSTFA [60], with dimethylsulfoxide (DMSO) or pyridine as solvents. Methoxime or oxime TMS derivatives can also be formed using methoxylamine HCl or hydroxylamine HCl in pyridine before silylation [60]. As reported by Tisza et al. [61], the TMS oxime derivatives of sugar acids (glucaric, gluconic and glucuronic acids), neutral sugars and other organic acids from different apple varieties could be simultaneously analysed by GC–MS. As it will be discussed in Section 5, a recent work [62] identified aldonic and saccharinic acids in Maillard model systems produced through a postpyrolytic *in situ* derivatization procedure using TMSDEA. Their origin was verified through $^{13}$C-labeling studies.

BSTFA, HMDS/TMCS and TMSI/TMCS have also been used for the derivatization of sialic acids using anhydrous pyridine as solvent. Silylation should be carried out under inert conditions to avoid the degradation of neuraminic acid [63]. Different applications, such as the analysis of both phosphorylated and dephosphorylated KDO from lipolysaccharides (LPS) previously converted to their TMS derivatives [64], or the determination of muramic acid in organic dust [65] have been carried out.

Bonaduce et al. [66] compared different silylation procedures (BSTFA or HMDS and pyridine, with or without TMCS) for aldoses and uronic acids liberated form plant gums and previously converted into their diethyl-dithioacetals. Although both BSTFA and HDMS gave good yields, best results were obtained using BSTFA. TMCS caused the formation of ammonium chloride which interfered with the drying of the sample, and adversely affected yields. Standard deviation of the sugar peaks areas was smaller when pyridine was used [66].

### 4.3. Acylation

Sassaki et al. [67] determined that acetates of uronic acids are unsuitable for GC since their C6 carboxylic group does not allow elution from the column. As shown in Fig. 2B these authors developed a modified acetylation of acidic monosaccharides and compared it with conventional processes (Fig. 2A). De-lactonization and reduction were undertaken to determine the best reagents for these

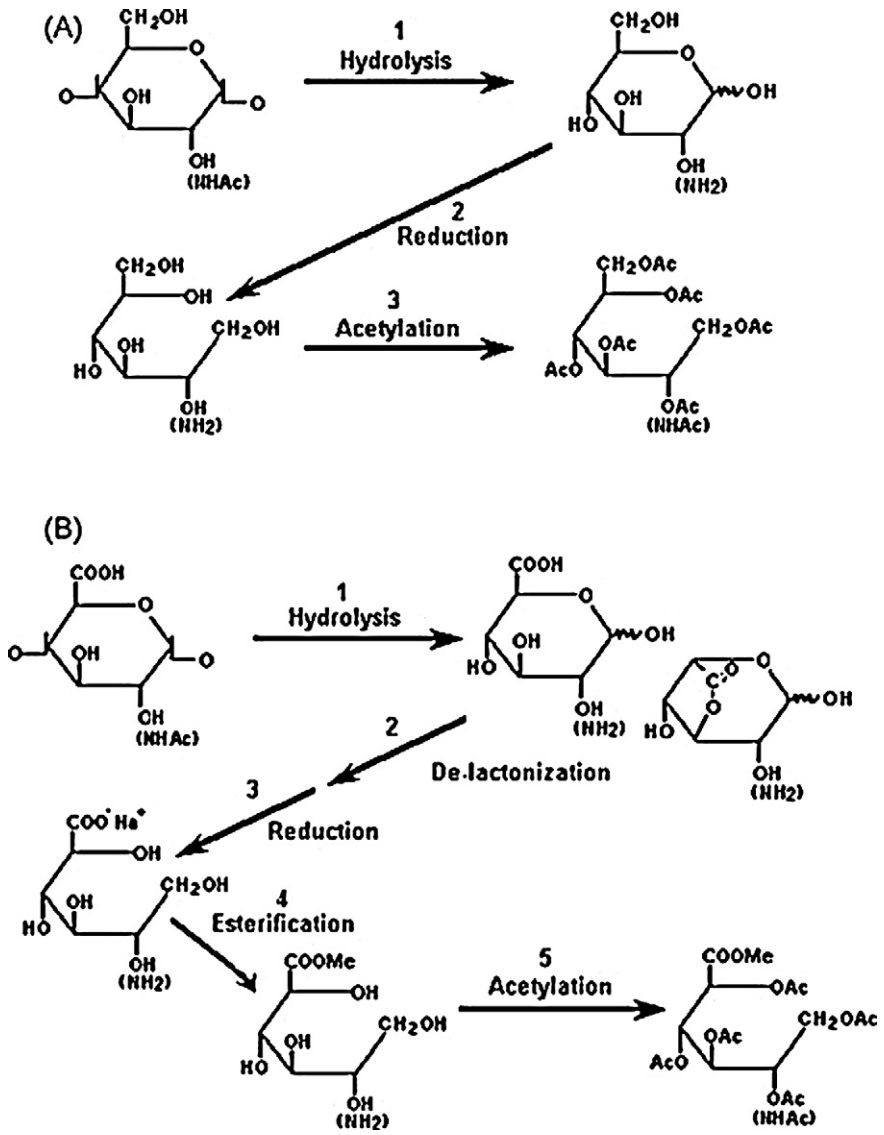



**Fig. 2.** (A) Conventional preparation of alditol acetates. (B) Preparation of methyl-alditol-acetates (MAAs) according to Sassaki et al. [67]. Reprinted with permission of Elsevier.

purposes. GC–MS noise was lower for samples de-lactonizated with $NH_4OH$ at room temperature than with $Na_2CO_3$ at 100 °C. Under the former conditions, treatment with $NaBH_4$ gave rise to a rapid reduction of the carbohydrates. Aldonic acids were methyl esterified using methanolic HCl (15 min, 100 °C), evaporating the solvent under nitrogen and acetylated using acetic anhydride in pyridine [6]. The methyl ester alditol acetates (MAAs) were stable for several months. Moreover, a single peak was obtained after GC–MS analyses for each sugar acid.

Acetylation conditions for sialic acids and related compounds previously reduced with $NaBH_4$ have been evaluated. As expected, acetylation under alkaline conditions (acetic anhydride-pyridine) gave less by-products at room temperature than at 100 °C for 30 min. Derivatization under alkaline conditions gave higher yield by GC-FID than under acidic conditions (trifluoroacetic anhydride-acetic acid at room temperature for 15 min) [68].

Lehrfeld [69] proposed the conversion of lactones into *N*-(1-alkyl)-aldonamides by treatment with a primary amine in pyridine for the analysis of aldonic acids followed by acetylation. The *N*-(1-alkyl) reagents used were: 1-propyl, 1-butyl, 1-pentyl and pyrrolidinyl. Normally, the tendency of aldonic acids to form lactones introduces difficulties into their analysis, taking into account that the ratio lactone:free acid varies with pH, temperature, time and solvent. The aldonamides are heat-stable and give only one peak in GC analyses. Another advantage is that the *N*-(1-alkyl)-aldonamides preserve the symmetry of the molecule which would be lost by other procedures. Moreover, it has been seen that aldonolactones may form dehydrated products when treated with pyridine and acetic anhydride. However, this does not occur with the *N*-(1-alkyl)-aldonamides.

This derivatization procedure has been found useful for the analysis of complex mixtures containing both aldonic acids and aldoses. Aldonic acids are converted to their *N*-(1-alkyl)-aldonamides whereas aldoses are reduced to their corresponding alditols (Fig. 3). Alduronic acids are also derivatized by this method by reduction of alduronates which are obtained from lactones using a weak base solution [56,69]. This has also been used for the analysis of sugar acids in pectins from sugar beet and pumpkin [70] and to determine galactonic acid in human urine [71].

Trifluoroacetyl or alditol acetate derivatives have been used to analyse sugar acids such as KDO [72] or muramic acid [73]. Manual and automatic preparations of alditol acetates of sugars and sugar acids from bacterial cellular polysaccharides have been reviewed by Fox [38]. However, these methods have been limited because of

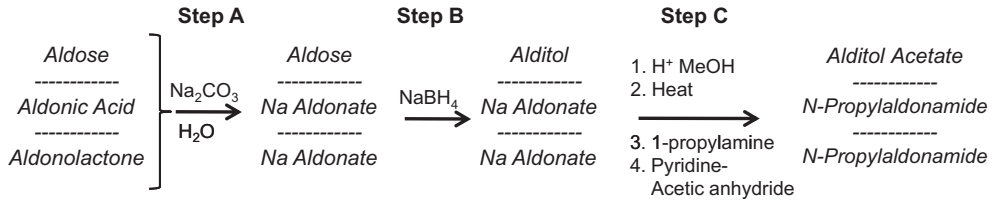



**Fig. 3.** Derivatization procedure for the simultaneous analysis of aldoses and aldonic acids. Redrawn from [69] with permission of the American Chemical Society.

the difficulty of preparing volatile and stable derivatives and the inadequate resolution of some compounds [74].

*O*-Methyl oxime acetates have also been used for the derivatization of different sugar acids such as muramic acid [75] or KDO [76]. In example, acetylated-*O*-methyloximes were proposed for the analysis of KDO [76] in LPS of different bacteria, using *O*-methoxyamine hydrochloride plus 4-(dimethylamino)pyridine and acetic anhydride as reagents. The KDO was liberated from LPS using HCl hydrolysis prior to the formation of *O*-methyl-oxime acetate derivatives. This provided stable derivatives and a single symmetric peak for KDO in the GC analysis.

Cooper et al. [77] used three different derivatives: (1) ethyl ester/*O*-trifluoroacetates, (2) isopropyl ester/*O*-trifluoroacetates and (3) isopropyl ester/*O*-pentafluoropropionates for the analysis of C3 to C6 straight-chained aldonic acids. Lactone rings were opened by heating samples to 100 °C for 5 min in a slight molar excess of $NH_4OH$ followed by drying. Thus, in a first step aldonic acids were converted either into their isopropyl or ethyl esters by heating the acids at 60 °C for 30 min in excess isopropanol/acetyl chloride or ethanol/acetyl chloride mixtures. Secondly, hydroxyl groups were derivatized to their *O*-trifluoroacetyl (*O*-TFA) or to their *O*-pentafluoropropionyl (*O*–PFP) derivatives. *O*-TFA were obtained by heating samples in mixtures of TFAA:THF for 5 min at 50–60 °C and allowing them to sit at room temperature from 30 to 60 min. *O*-PFP derivatives were prepared in PFPA:ethyl acetate with no heating. C3 to C5 acids were readily converted into their isopropyl or ethyl esters, but C6 acids are less soluble and esterification was only acceptably achieved using acidic ethanol/acetyl chloride mixtures. Similar solubility problems occur in the formation of TFA derivatives which could only be done using fresh anhydrous tetrahydrofuran (in excess) combined with the anhydride [77].

### 4.4. Heptafluorobutyrate (HFB) derivatives

Heptafluorobutyrate (HFB) derivatives are produced by heating the sugar acids in heptafluorobutyric anhydride (HFBAA) and acetonitrile. The analysis of the muramic acid HFB derivative has also been reported [78]. Pons et al. [79] also determined the sialic acid, monosaccharide and amino acid composition of glycoproteins by GC–MS analysis of their HFB derivatives. This was also used by Zanetta et al. [80] using HFBAA for the determination of sialic acids. These were first liberated by mild acid hydrolysis, then esterified using diazomethane in methanol.

The simultaneous GC–MS analysis of pentoses, hexoses, deoxyhexoses, hexosamines, heptoses, KDO, KDN, uronic, muramic and neuraminic acids from bacteria has been described. The sugars were liberated through methanolysis prior to their conversion into their HFB derivatives, without requiring any purification step [81]. The advantages of HFB derivatives reported by these authors were based on the (i) simplicity of handling; (ii) quantitative simultaneous derivatization of alcohol (phenol) and amino groups; (iii) stability with time of the derivatives in the reagent; (iv) the possibility to form stable derivatives on impure material; and (v) easy removal of reagents and by-products of the acylation under a stream of nitrogen without any loss of the most volatile compounds.

## 5. Sugars characteristic of the Maillard reaction

The Maillard reaction (MR) or non-enzymatic browning is a complex group of reactions between reducing sugars and free amino groups of amino acids, peptides and proteins. This involves the formation of Amadori compounds, followed by multiple reactions and a high number of by-products [82,83]. Different beneficial biological properties of MR products have been reported [84–87] as well as some negative biological effects [88,89]. MR is commonly divided into three stages: the early, intermediate and final reaction steps [90]. Final products are polymers (melanoidins) which are not amenable to GC analysis, but this technique has been successfully applied to the early and intermediate MR products.

### 5.1. Derivatives of Amadori compounds

Amadori compounds are considered the main early products of MR. Few papers have been devoted to GC analysis of these compounds. However, D-glucosylamines and their Amadori rearrangement products, 1-deoxy-1-(*N*-substituted) amino-D-fructoses, formed in a model system Maillard reaction were derivatized with different reagents before GC and MS analyses. A mixture of BSA, TMSI and TMCS (5:5:1; 1 h; room temperature) was selected as optimum conditions [91]. Wittmann and Eichner [92] reported the separation of different Amadori compounds which were converted to TMS oximes using hydroxylamine chloride in pyridine, *N*,*O*-*bis*-trimethylsilyl acetamide and TMCS. A slightly different method has been recently reported [93] to detect an Amadori compound ($N^{\alpha}$-(1-deoxy-D-fructos-1-yl)-L-histidine) in tomato powder: oximation was carried out with a mixture of hydroxylamine-HCl, methanol, pyridine and 1-dimethylamino-2-propanol, and TMSI and *N*,*O*-*bis*(trimethylsilyl)-trifluoroacetamide were used in the silylation step (60 °C, 10 min).

In spite of the results presented by the above-cited authors, these methods have been scarcely cited and perhaps deserve more attention.

### 5.2. Derivatives of intermediate compounds

Deoxysugars, α-dicarbonyl sugars and sugar acids are found as intermediate products produced during Maillard reaction [94]. Among deoxysugars, 3-deoxypentulose was firstly identified by GC in heated milk as its TMS ether, which was prepared with TMSI:TMCS (2:1) in pyridine at room temperature [95]. α-Dicarbonyl sugars have been extensively studied. Among them, 3-deoxy-D-*erythro*-hexos-2-ulose (3-deoxyglucosone, 3-DG) has biological relevance since it is a precursor of advanced glycation end products (AGEs) responsible for cellular toxicity and development of diabetes and uremic complications. For these reasons the accurate quantification of 3-DG in biological samples is especially relevant [96].

α-Dicarbonyl sugars have been frequently analysed as quinoxalin derivatives, which are formed by condensing with ortho-diamines, such as 1,2-diaminobenzene or 2,3-diaminonaphtalene (Fig. 4). These aromatic derivatives are UV absorbant and are

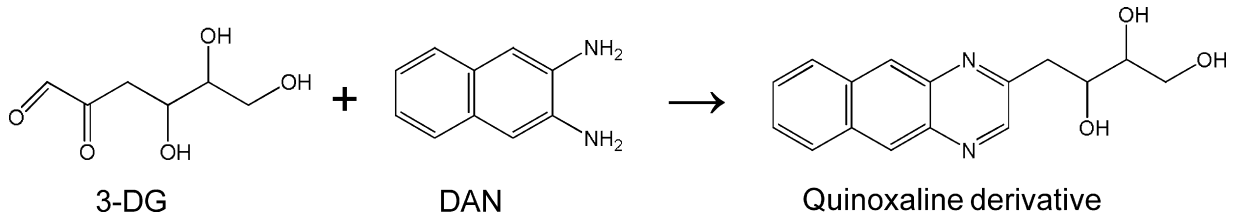



**Fig. 4.** Formation of quinoxalines (2-(2,3,4-trihydroxy butyl)-benzo[g]quinoxaline) from α-dicarbonyl sugars (3-DG) by treatment with 2,3-diaminonaphthalene (DAN).

therefore also useful for HPLC analysis. When the OH groups of the sugar moiety are converted to acetates or TMS ethers, the resulting substituted quinoxalines are volatile enough for analysis by GC and GC–MS [97,98]. For example, 1,2-diaminobenzene (*o*-phenylenediamine) was used to derivatize α-dicarbonyls [97], and the adducts were then acetylated (acetic anhydride, pyridine, 18 h, 60 °C). In this way, 3-deoxypentosulose has been identified as a predominant compound in MR of oligosaccharides in aqueous solution [97]. A similar procedure has been used to identify 3-DG and D-*arabino*-hexos-2-ulose (D-glucosone) in model systems [98].

Data regarding GC–MS analysis of 3-DG can be found in the review of Niwa [96]. Methodology for 3-DG analysis in biological samples has been controversial, and some quantitative discrepancy has been reported [96]. Different derivatives have been assayed however, it seems that the main cause of discrepancy relies on deproteinization and not on derivatization [99].

Madson and Feather [100] quantified 3-DG and D-*arabino*-hexos-2-ulose using per-*O*-trimethylsilyl-1,2-dioximes, prepared with hydroxylamine hydrochloride in pyridine plus TMSI and BSTFA. The derivatives were satisfactory for GC–MS analysis. TMS methoximes of 3-DG were obtained by Tsukushi et al. [99], by adding methoxylamine hydrochloride (70 °C, 30 min) in the first step of derivatization process and BSTFA + 1% TMCS (60 °C, 20 min) in the second step. With this method two isomers of 3-DG were obtained [99]. Although four isomers can be formed due to the presence of two vicinal carbonyl groups (each one in forms *syn* and *anti*), Niwa [96] reported that the methoxime-trimethylsilyl derivatives of 3-DG showed only two peaks by GC–MS. These arise as consequence of the restricted rotation around the methoxime double bond [96].

3-DG present in human plasma was determined after reaction with 2,3-diaminonaphthalene, and the adducts were silylated with 20% BSA (70 °C, 10 min) [101], or with MSTFA (30 min, 65 °C) [102]. 3-DG has been determined along with 3-deoxyfructose in urine and plasma by GC–MS after reduction with $NaBH_4$ (overnight at 4 °C). The corresponding alditols were acetylated with acetic anhydride in pyridine (30 min, 110 °C) [103].

A novel derivatization procedure was reported by Wu et al. [104] to enable the simultaneous analysis of 3-DG and other dicarbonyls in human plasma, using two derivatization steps. Firstly with *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBOA; 2 h, 50 °C) and secondly with MSTFA (90 °C, 30 min) (Fig. 5). They found that the most suitable ion for the quantification of 3-DG was the *m*/*z* 563. The chromatogram obtained using this derivatization method showed an excellent resolution between 3-DG and other dicarbonyls.

Voigt and Glomb [105] studied the reactivity of the 1-deoxy-D-*erythro*-hexo-2,3-diulose (1-deoxyglucosone) under Maillard reaction conditions, using two derivatives: TMS ethers (BSA plus 5% TMCS in pyridine, 3 h, room temperature) and trimethylsilyl *O*-benzyloximes (prepared with *O*-benzyl-hydroxylamine in water, 3 h, 37 °C). After extraction with diethylether and drying, the samples were silylated as above. The use of these methods allowed the determination of 1-deoxyglucosone, erythrulose, threosone, 1-deoxythreosone, 3-deoxythreosone and other related compounds.

Other products arising from the cleavage of the α-dicarbonyl compounds are the carboxylic and aldonic acids, and their corresponding lactones. Haffenden and Yaylayan [62] used pyrolysis–GC–MS, in glucose/glycine model systems, with post-pyrolytic derivatization. TMSDEA was directly injected with the sample to determine aldonic and saccharinic acids and the respective lactones, and correctly identifying 6 different lactones. Other sugar acids and deoxysugars, such as D-ribono-1,4-lactone and 2-deoxy-D-arabino-hexono-1,4-lactone, respectively, were identified by Novotný et al. [59], by converting the compounds into their respective trimethylsilyl derivatives, employing equivalent volumes of TMSI and BSTFA.

## 6. Anhydrosugars and carbohydrates formed as heating products

### 6.1. Anhydrosugars

Anhydrosugars appear in nature from two different sources: they can be formed by heating carbohydrates in dry conditions or in low-moisture products, which cause intramolecular loss of water, as the first step of thermal degradation. They also appear in living organisms as important constituents of polysaccharides such as agars and carrageenans.

Having only three OH groups, free anhydrosugars can be analysed by GC without derivatization. In fact, several studies based on pyrolysis GC–MS directly introduce pyrolyzates into GC [106]. Nevertheless, as anhydrosugars usually appear along with their parent sugars and other poly-hydroxylated compounds, derivatization is

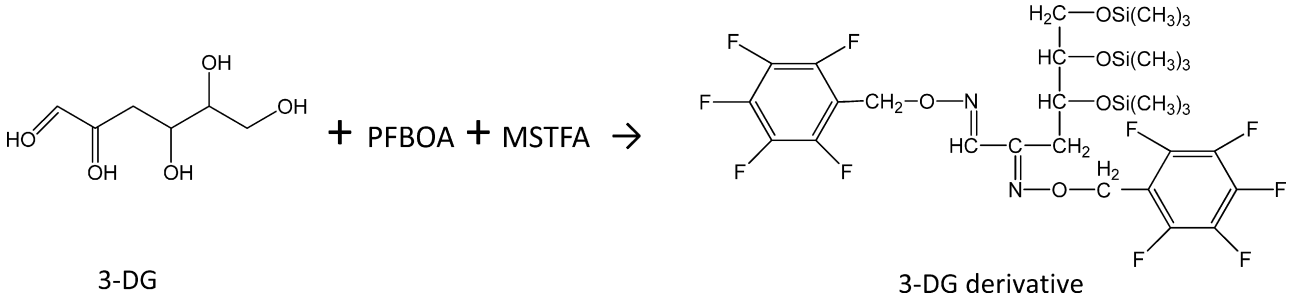



**Fig. 5.** Formation of *bis*(pentafluorobenzyl)-*tris*(trimethylsilyl) derivative of α-dicarbonyl sugars (3-DG) by benzoyloximation and silylation.

**Table 2**
Some silylation procedures used to obtain TMS ethers of anhydrosugars.

| Sample | Reagent | Temp./Time | Derivatization | Ref. |
|---|---|---|---|---|
| Monosaccharide anhydrides | (a) TMSI<br>(b) MSTFA + 1% TMCS | 70 °C, 60 min in pyridine | (a) Multiple peaks<br>(b) Single peaks | [112] |
| Polysaccharides | (a) HMDS<br>(b) TMSDEA<br>(c) BSTFA<br>(d) TMSI | Pyrolysis | (a) 75% (areas)[a]<br>(b) 65% (areas)[a]<br>(c) Complete<br>(d) Complete | [111] |
| Monosaccharide anhydrides | BSTFA + 1% TMCS | 3 h, 70 °C pyridine | Single peaks | [1] |
| Polysaccharides in aerosols | BSTFA + 1% TMCS + 0.2% dithioerythritol | 70 °C, 30 min | Single peaks | [113] |

[a] Expressed as ratio of persilylated levoglucosan (LG) peak to summed peak areas of persilylated and disilylated LG peaks.

the best option. The second ring including the glycosidic carbon atom avoids mutarotation generating a single peak for these compounds. Hence, the same derivatives used for common saccharides can be used for free anhydrosugars. Methyl ethers are obtained in pyrolytic methylation of polysaccharides with tetramethylammonium hydroxide [107]. Acetates are typically prepared using acetic anhydride in pyridine (24 h, room temperature) plus a catalytic amount of dimethylaminopyridine [108].

TMS-oximes have been used to analyse sugars released from heated lactose [109], or toasted wood casks [110]. However, several recent papers indicate that partial derivatization is frequent. A mass fragment at $m/z$ 116 has been found to be characteristic of partially silylated anhydrosugars and since it is absent in the persilylated compounds it is a good marker for controlling derivatization [111]. The difficult silylation has been attributed to the high steric hindrance of anhydride sugars. In the case of polysaccharide pyrolysis, the depolymerization reaction probably also interferes with the silylation. Different derivatization procedures have been used to achieve a complete silylation [1,111–113] and are summarized in Table 2.

A different approach is used when the structure of polysaccharides containing anhydrosugars is going to be studied. In this case it is necessary to hydrolyze the polymer preserving the structure of each monomer as it will be commented in Section 9.

### 6.2. Carbohydrate products formed in basic media

Hexoses are first isomerizated and then degradated in basic media to give saccharinic acids, pentoses, osuloses, deoxy-sugars and many low-molecular by-products. Disaccharides are easily hydrolyzated at the start of the reaction, and polysaccharides are isomerized and degradated starting from their reducing end. The formed products can be analysed by GC and the derivative has to be chosen depending on its structure. TMS ethers [114] and ethyl oximes [59] have been used for six-carbon sugars whereas α-dicarbonyls have been analysed as quinoxalines [59]. Several compounds formed by alkaline degradation are also formed in the Maillard reaction. Their derivatization procedures have been considered in Section 5.

### 6.3. Carbohydrate products formed in acid conditions

A group of important products arising from the acidic heating of monosaccharides, specially ketoses, are dihexulose dianhydrides. These are pseudodisaccharides, intermolecular cyclic acetals formed by condensation of two hexuloses with loss of two water molecules [115,116]. They were firstly identified in acid-treated fructose solutions, but they also appear in biological sources and foods. Their GC analysis requires derivatization, and TMS oximes have been successfully used for analysis of difructose dianhydrides in caramels [117], coffee [118] and syrups [119].

Other carbohydrates resulting from acid degradation are similar to those formed in neutral or basic media (acids, dicarbonyls, deoxy compounds) and consequently they are derivatized in a similar way.

## 7. Glycosides

Glycosides are molecules in which a sugar is covalently bound through a glycosidic linkage to a non-carbohydrate moiety (aglycone). The sugar moiety in most natural glycosides is a low molecular weight saccharide or a sugar acid. They have several important roles in living organisms and depending on the aglycone moiety can be classified as alcoholic, anthraquinone, saponins, cyanogenic, flavonoid, phenolic and thio-glycosides, among others.

Glycosides can be directly submitted to derivatization procedures as stated in Section 2 for common sugars, or hydrolyzed into the glycosidic and the aglycone constituents which can be analysed separately. When several sugars are present in the same molecule, classical methylation analysis is required to determine their glycosidic linkages and structures.

Neutral glycosides which contain only hydroxyl groups in the sugar and aglycone moieties are easy to derivatize. Silylation and trifluoroacetylation are the most popular methods, and this can be carried out by using conventional reagents as described in previous sections. As literature on this subject is large, some applications of different types of glycosides have been selected.

Alcoholic glycosides appear in plants and fermented beverages. Ethyl glucoside in sake was converted to a per-trifluoroacetate [120], whereas glyceryl-galactosides [121] and glyceryl-glucosides [122] have been analysed as TMS-ethers.

Terpenic glycosides are common components in plants. Several monoterpene glucosides have been analysed in wines both as trifluoroacetates and as TMS ethers using classical methods [123,124].

Cyanoglycosides such as amygdalin, prunasin, sambunigrin and others have been analysed by GC after conversion to their TFA derivatives. MS fragments allowed a satisfactory characterization [125].

Phenolic glycosides occur in plants, and some have therapeutic properties. Ten glucosides (salicin, picein, arbutin, salidroside, triandrin, tremuloidin, salicortin, tremulacin and 2′-O-acetylsalicortin) in 18 *Salix* species were silylated with TMSI in pyridine [126]. Glucosides with higher molecular weight such as betuloside and platyphylloside in birch were analysed by GC–MS also after derivatization with TMSI in pyridine (10 h, 4 °C) [127].

Anthraquinone glycosides have been derivatized with several reagents, HMDS + TFA anhydride, BSTFA, MTBSTFA, the first one (1 h, 100 °C) was selected as the optimum [128]. Lignanes in plants have been analysed using a two-step silylation. First extracts were treated with hydroxylamine hydrochloride containing pyridine (70 °C, 30 min), and thereafter silylation was performed with HMDS + TFA (100 °C, 60 min). After dilution with HMDS, the samples were analysed by GC–MS [129].

GC analysis of flavonoid glycosides has been recently reviewed [130]. The literature on this subject is scarce because of their high molecular weight. A TMS derivative of hesperidin (M.W. 1186) was

prepared by treating with BSTFA (100 °C, 72 h) [131]. Oleszeck [132] also reports limited applications of GC–MS for the analysis of intact saponins.

Steryl glycosides are usually constituted by glucosides of sterols although acyl esteryl glycosides can be also found. Campesteryl-, stigmasteryl- and β-sytosteryl-3-*O*-glucosides and the corresponding 6-*O*-palmitoyl steryl glucosides from *Eucalyptus* were silylated with BSTFA in pyridine (90 min, 80 °C) for GC–MS analysis [133].

## 8. Sugar phosphates

Sugars substituted with phosphate groups are often used in biological systems to store or transfer energy. Some of the most common sugar phosphates are dihydroxyacetone-phosphate, glucose-6-phosphate and fructose-6-phosphate, phytic acid, teichoic acid, etc. Due to their polarity GC analysis of most of these compounds is not common, however, some applications are described here as examples.

Phytic acid is a constituent of the human diet and important in several beneficial functions on human health. Its analysis by GC has been used as an indirect measure of the *myo*-inositol produced after separation from free inositols present in samples. Hydrolysis is done using either phytases [134] or 2 M HCl at 120 °C [135]. Derivatization of the resulting *myo*-inositol was carried out using HMDS + TMCS [134].

A simultaneous analysis of metabolites of potato tuber including glucose-6-phosphate and fructose-6-phosphate has been carried out by GC–MS using a prior derivatization with methoxylamine hydrochloride in pyridine and MSTFA as silylating agent [136]. More recently, Koek et al. [137] optimized and validated a GC–MS method for the analysis of microbial metabolites, ribose 5-phosphate, glucose 6-phophate and fructose 6-phosphate .The best results were obtained with pyridine, ethoxyamine and MSTFA (40 °C, 90 min for oximation and 50 min for silylation). This derivatization was also used for metabolomic analyses by GC × GC [138].

## 9. Polysaccharides

### 9.1. Hydrolysis and methanolysis

Polysaccharides are commonly composed by ketoses, aldoses, anhydrosugars, aminosugars and sugar acids. GC analyses of these carbohydrates are based on hydrolysis with a subsequent derivatization of the released monosaccharide residues, as previously described in Sections 2, 3, 4 and 6. Therefore, only procedures for the liberation of monosaccharide units will be mentioned here. A recent review [5] summarized the different procedures for hydrolysis of polysaccharides for GC.

Hydrolysis using acids of different nature and concentration and methanolysis have been used for these purposes. Methanolysis is usually performed with HCl in anhydrous methanol. Conditions may vary depending on the polysaccharide composition or the procedure to follow. For example, Ayestarán et al. [139] and Pati et al. [140] used 0.5 M HCl in methanol (80 °C, 16–18 h) for grape and wine polysaccharides, respectively. Kim et al. [141] used 3 N HCl in methanol (2 h, 121 °C) for the analysis of Pneumococcal serotype polysaccharides. Under this treatment monosaccharides are liberated as methyl-glycosides and the carboxyl groups are esterified. Regarding acid hydrolysis, $H_2SO_4$, TFA, HCl and hydrofluoric acid, among others, are the most commonly used hydrolytic agents.

Although it is not very usual, enzymatic hydrolysis can be also carried out for the liberation of monosaccharide residues from a polysaccharide [142–144]. Virkki et al. [145] used a mixture of commercially available enzymes to hydrolyze water soluble arabinoxylans from wheat flour and dough. Advantages of this method were the mild conditions used (low temperature and near-neutral pH) which do not produce unwanted sugar degradation. In this case, enzymatic hydrolysis was almost as efficient as the acid hydrolysis and slightly better than methanolysis [145].

Microwave assisted hydrolysis has been developed for the liberation of monosaccharide residues of polysaccharides before derivatization [5]. The advantages over conventional hydrolysis are shorten reaction times, good yields and reduced decomposition. Bonaduce et al. [66] used the microwave assisted hydrolysis (500 W, 120 °C, 20 min) for the characterization of plant gums. Released aldoses and uronic acids were converted into their diethyl-dithioacetals and their diethyl-dithioacetal lactones, respectively, and further silylated. Using this method single peaks were obtained for each carbohydrate. Sonication has been also assessed for polysaccharide hydrolysis, because this effect makes the large molecules more sensitive towards chemical attacks [146].

#### 9.1.1. Polysaccharides containing sugar acids

De Ruiter et al. [147] compared several procedures for the liberation of all monosaccharides from water-soluble uronic acid-containing polysaccharides from fungi, plants, and animals. Among them, both $H_2SO_4$ and TFA were unable to completely hydrolyze the polysaccharides. The combination of methanolysis and TFA hydrolysis resulted to be the best procedure for the liberation of monosaccharides [147].

Reinders and Thier [148] used HCl, TFA and $H_2SO_4$ and methanolysis for the liberation of the monosaccharide from non-starch polysaccharides (NSP) of tomatoes. However, none of these procedures were entirely effective. Therefore, the use of concentrated methanesulfonic acid (20 min, 20 °C) before methanolysis (anhydrous methanol HCl, 100 °C, 4 h) was suggested for pectin and cellulose fractions, and formic acid (2 h, 100 °C) for hemicellulose fractions. The methyl glycoside derivatives obtained were trimethylsilylated [145].

As noted in Section 4, reduction of carboxylic groups of uronic acids into their corresponding hexoses is carried out prior to hydrolysis to allow the complete liberation of monosaccharides. However, the quantitative recovery of uronic acids and monosaccharides linked to them is generally not complete [149].

An efficient method based on the methyl esterification of uronic acids using DMSO and methanol with diazomethane, was proposed by Fontaine et al. [150]. Reduction of methylester polysaccharides was carried out in imidazole/HCl buffer with potassium borohydride. Comparison of GC profiles of hydrolyzed and derivatized polysaccharide before and after reduction determined the number of glucose residues coming from the native polymer and those from the glucuronic acid residue converted into glucose. This procedure allowed the complete liberation of monosaccharides [150].

Similarly to uronic acids, several acid treatments have been proposed for the release of neuraminic acid from glycoproteins. However, to determine neuraminic acid content the hydrolytic procedure should be carefully chosen, due to the instability of this sugar acid under strong acid conditions. Moreno et al. [63] determined the neuraminic acid content of κ-casein macropeptide (CMP) present in bovine, ovine and caprine milks. Degradation of this acid was observed using TFA, however Neu5Ac was recovered after milder treatments using dry HCl–methanol (0.625 N) and methyl acetate (70 °C, 16 h). *t*-Butyl alcohol was used to remove the excess of acid. However, these authors used a re-acetylation procedure after methanolysis which also resulted in the conversion of *N*-glycolyl-neuraminic acid into the *N*-acetyl-neuraminic acid. In a different work [151], the same authors reported a methanolysis procedure using milder conditions (0.1 N HCl–methanol, 80 °C, 1 h) which minimizes *N*-deacylation. In this work both *N*-glycolyl-neuraminic acid and Neu5Ac were independently quantified.

Li et al. [152] combined a colorimetric method to determine uronic acids and a methanolysis-acetylation-GC method for the quantification of monosaccharide units of xylan.

*9.1.2. Polysaccharides containing anhydrosugars*

Anhydrosugars are acid-labile and require protection during hydrolysis from polysaccharides. Reductive hydrolysis has been carried out by Usov [153], by adding borane-4-methyl-morpholine to the hydrolysis mixture (2 M TFA, 100 °C, 8 h). This yielded galactosan acetate from 3,6-anhydrogalactose methyl glycoside. Hydrolysis of galactans at lower temperature and acid concentration (0.5 M TFA, 65 °C, 8 h) gave rise to reduced disaccharides having terminal 3,6-anhydrohexitol moieties. Mercaptolysis has also been assessed for quantitative determination of anhydrogalactose in algal galactans [154]. These authors assayed different concentrations of the acid reagent and temperatures of hydrolysis, the optimum ones being 0.5 N HCl/ethanethiol/methanol (60 °C, 6 h). The monosaccharide diethyl mercaptals released were evaporated and silylated with HMDS plus TMCS in pyridine (room temperature, 30 min).

*9.2. Pyrolysis*

Depolymerization of polysaccharides using pyrolysis can be also used prior to their characterization by GC. Derivatization is usually recommended to avoid the retention of hydroxyl groups of pyrolizated products which give rise to peak broadening and loss of resolution [155]. Silylation is the most appropriate procedure for the on-line derivatization, considering the thermal stability and volatility of the TMS derivatives.

The solid sample is added with the silylation reagent and submitted to temperatures commonly around 600–700 °C for 10 s for pyrolysis. Special care should be taken with the temperature of the pyrolysis–GC interface to avoid fast volatilization of the silylation reagent (i.e., Chiantore et al. [155] used 280 °C for BSTA and 150 °C for HMDS). Resulting products of pyrolyzation and silylation of polysaccharides are, in general, propanones, furanones, anhydrosugars, acids, etc. Fabbri and Chiavari [156] used this procedure for the analysis of cellulose, laminaran and agar, whereas Kuroda et al. [157] characterized different lignins.

Different silylation reagents (HDMS, BSTFA, HMDS/TMCS, BSTFA/TMCS) for derivatization of plant gums submitted to pyrolysis have been assayed. The best results were obtained with HMDS/TMCS [155].

Advantages of this method are mainly the shorter times of treatment and the smaller consumption of both reagents and samples. These analyses are useful for obtaining fingerprints for a rapid characterization of polysaccharides.

*9.3. Structural analysis of polysaccharides*

The establishment of the position of glycosidic linkages between sugar residues of polysaccharides is classically determined by GC–MS by permethylation/hydrolysis/acetylation procedures commonly called “methylation analysis”. Jay [158] has reviewed the different methods used for methylation reaction in carbohydrate analysis and solvents, reagents and conditions have been described in detail in this review. Moreover, a table with recommended procedures for the analysis of polysaccharides depending on their chemical structure is included.

To achieve a complete permethylation is critical for a correct analysis, and should be carried out under strictly controlled conditions (absence of water, base catalyst, appropriate reagents, etc.). These permethylation reactions have been recently critically reviewed by Ciucanu [159]. In a more recent study [160], two classical methylation reactions (Ciucanu and Kerek, [161] and Hakomori [162] methods) have been compared. While Ciucanu and Kerek [161] method uses NaOH as base catalyst and DMSO as solvent, Hakomori procedure uses sodium hydride which reacts with DMSO giving rise to its methyl sulfinyl anion, also called dimsyl ion. This last reagent was found to be effective for the permethylation of β-cyclodextrin. However, the NaOH-catalyzed reactions produced an uncompleted permethylation of 3-hydroxy groups as shown by GC–MS analyses after hydrolysis and acetylation. Nevertheless, preservation of dimsyl ion under oil and the difficulty in handling (it should be prepared without air and water presence) are some drawbacks of this reagent. Therefore, one or other procedure can be applied depending on polysaccharide linkages and the completeness of the reaction should be evaluated before the next steps in an analysis.

Hydrolysis of polysaccharides is usually carried out using TFA (2 N, 120 °C, 2 h) although conditions can vary as commented before for unmethylated polysaccharides. Special care should be taken when ketoses are involved (i.e., fructans). Milder conditions (0.15 N, 100 °C, 1 h) have been used in these cases [163]. Free methylated monosaccharide residues are converted into alditols before acetylation by reduction with $NaBH_4$ or $NaBD_4$. Acetylation of the remaining hydroxyl groups is achieved with $Ac_2O$/pyridine or $Ac_2O$/sodium acetate. The partially methylated alditol acetates (PMAAs) obtained in this assay can be directly analysed by GC–MS. An oximation step to give partially methylated aldononitrile acetates (PAANs) can also be used.

Thermally assisted hydrolysis and methylation (THM) procedures to determine the chemical structures of polysaccharides are also used [164]. The formation of methylated saccharinic acids has been reported.

## 10. Glycoconjugates

Many biological functions have been attributed to glycoconjugates, mainly to their glycosidic chain. To determine the structure/function relationship, analytical techniques such as GC–MS using a previous hydrolysis and derivatization are required. Similar hydrolytic procedures to those described in Section 9 have been applied, therefore only some recent examples are mentioned here. Derivatization methods are common to those previously described in Sections 2–4.

In order to release monosaccharide components from glycoconjugates [67] different acids were tested: hot TFA, formic acid, acetic acid, aqueous HCl and MeOH–HCl, at different times and temperatures. Best results were obtained by the combination of MeOH and HCl [67] as indicated in Section 4.

Methylation analysis to determine the carbohydrate structure of glycoprotein glycans has been widely carried out [165–167]. Constituents of glycolipids liberated after acid-catalyzed methanolysis have been determined by GC–MS as heptafluorobutyrate derivatives. Similar procedures have been followed for the analysis of glycoprotein and proteoglycan constituents [81]. Hydrolysis with 4 M TFA (100 °C, 4 h) liberated monosaccharide residues from glycoconjugates which were converted onto their alditol acetates before to their GC and GC–MS analysis [168].

## 11. Derivatives for enantiomer analysis

Carbohydrates are chiral molecules and it is often necessary to determine its absolute configuration. Two main approaches have been used for enantiomer GC analysis of sugars. The first consist of the reaction of sugars with a chiral reagent giving diastereomers which could be separated with a conventional GC column. The second consist of the preparation of conventional derivatives, which

**Table 3**
Procedures for preparation of diastereomeric derivatives of sugars.

| Sample | First step: chiral reagent | Conditions | Second step | Ref. |
|---|---|---|---|---|
| Monosaccharides and lipopolysaccharide | *R*-(−)-2-butanol or (±)2-butanol, 1M HCl | 80 °C 8 h, neutralize with $Ag_2CO_3$ | Silylation (HMDS/TMCS/Pyridine) | [169] |
| Monosaccharides and polysaccharides | (+)-2-Octanol + TFA | 130 °C overnight | Acetylation acetic anhydride) | [170] |
| Plant polysaccharides | TFA + (+)-1-phenyletanethiol | 30 min at room temperature | Silylation (BSTFA/TMCS) | [171] |
| Polysaccharides | *O*-(−)-bornyl hydroxylammonium chloride + sodium acetate | 80 °C, 1 h | Trifluoroacetylation (TFA) | [172] |
| Polysaccharides | (*S*)-1-amino-2-propanol + AcOH + $NaBH_3CN$ in MeOH | 65 °C, 1–2 h | Acetylation (acetic anhydride) | [173] |
| Neutral sugars and uronic acids | TFA + (+)-1-phenyletanethiol or (−)-1-phenyl ethanethiol | 30 min at room temperature | Acetylation (acetic anhydride) or silylation (HMDS/TMCS/Pyridine) | [174] |

are then chromatographed on a capillary GC column with a chiral stationary phase.

### 11.1. Formation of diastereomers

Different chiral reagents proposed to form diastereomers of sugars are summarized in Table 3. The most popular methods are the formation of 2-butyl glycosides by reaction with 2-butanol [169] which has been cited in about 600 papers, and 2-octyl glycosides formed by reaction with 2-octanol [170] cited in about 300 papers, being often complementary of the first. Among the numerous applications which have been published, the following are mentioned as examples: polysaccharide from microrganisms [175–178], algae [179,180], marine microorganisms [181], medicinal fungus [182], cuttlefish [183], wheat germ [184], edible vegetables [185] and lactic bacteria [186]. A method based in reductive amination [173] was developed for the study of algal galactans and is used by groups working in this field [187–189].

It is noteworthy a study about antigenic polysaccharide from *Escherichia coli* [190] which required the use of four of the above described methods [169–171,174].

### 11.2. Derivatives for analysis on chiral stationary phases

The second approach is based on the different interaction energies of both members of an enantiomeric pair when they are eluted onto a chiral stationary phase. Such energies are very similar; hence the separation requires high resolution columns and working at low temperatures. The first reported separation of sugars on a chiral phase appeared in 1981 [191]. Eight monosaccharides were converted to trifluoroacetyl derivatives and separated on a silicone-substituted with L-valine-(*S*)-α-phenylethylamide column. A large number of peaks was obtained for each sugar, since the usual four tautomeric form could be found in D or L configuration. Trifluoroacetylated methyl glycosides also gave satisfactory separations, and this method was applied to the determination of L-galactose in snail polysaccharides, with a previous step of hydrolysis [192].

A similar commercially available column (Chirasil-Val) was used to determine the chiral configuration of *myo*-inositol-1-phosphate in plants. The product was first persilylated with (*N,O-bis*-trimethylsilyl trifluoroacetamide + 10% TMCS). Redissolving in 10% methanol in diethylether removed TMS groups from phosphate, which was then methylated with diazomethane [193].

Konig et al. [194] introduced pentylated α-cyclodextrin as a chiral stationary phase for GC separation of enantiomeric carbohydrates. Numerous monosaccharides, anhydroalditols, polyols and other compounds were trifluoroacetylated and readily separated on this column. Other cyclodextrin phases have also been evaluated [195,196]. The use of new derivatives was also tested: D and L permethylated monosaccharides and 1,5-anhydroalditols obtained from polysaccharide hydrolysis could be separated on β- and γ-cyclodextrins [197]. Lindqvist and Jansson [198] also assayed trifluoroacetylated diethyl dithioacetals, although only some enantiomeric pairs could be resolved on γ-cyclodextrin. Enantiomeric aldonic acids from meteorites have been separated on a permethylated β-cyclodextrin (Chirasil-Dex CB) using ethyl ester/*O*-trifluoroacetyl, isopropyl ester/*O*-trifluoroacetyl and isopropyl ester/*O*-pentafluropropionyl derivatives. The carboxylic groups were firstly derivatized by heating at 60 °C with the corresponding alcohol and acyl chloride, and the hydroxyl groups were then heated with TFA in THF (5 min, 50–60 °C). *O*-PFP derivatives were prepared at room temperature for 45 min [199].

The use of cyclodextrins (and probably also other macrocycles) as stationary phases requires a special condition. The derivatized sugar molecule has to accommodate into the chiral cavity of the stationary phase; at least in the case of cyclodextrins the substituents size on the sugar molecule has to be small. Trifluoroacetyl and methyl derivatives are the best substituents available. TMS and acetyl groups are not adequate because their molecular size and volatility, respectively. At present, separation of enantiomeric sugars on chiral phases in GC are less used than diastereomer formation and separation on conventional phases.

## 12. Conclusions

Much work has been devoted to the preparation of derivatives for different families of carbohydrates before their GC–MS analysis. These have been focussed on two main aims: (i) to simplify the derivatization process; (ii) to reduce the number of peaks in the chromatogram.

At present, there are a number of derivatization methods with one or two steps which can be satisfactorily carried out in the laboratory. The large number of chromatographic peaks per sugar is still mentioned as a problem and different methods have been developed to reduce this effect. However, in certain cases multiple peaks may be an aid for carbohydrate identification.

In the last few years the number of emerging derivatization methods has been limited. These new applications are resolved with optimization and experiment designs in order to find specific conditions for each sample which afford the best yield and reproducibility. In certain cases, the use of two different derivatives provides important structural information, either through MS fragmentation or through retention data.

In conclusion, derivatization along with the coupling of GC with MS allows invaluable information about composition and structure of real samples.

## Acknowledgements

This work has been supported by projects AGL2009-11909 (from Ministerio de Ciencia e Innovación) and ANALISYC-II S2009/AGR-1464 (from Comunidad de Madrid). O. Hernández thanks the CSIC for a JAE-PreDoc PhD-grant.

## References

[1] P.M. Medeiros, B.R.T. Simoneit, J. Chromatogr. A. 1141 (2007) 271.
[2] S.C. Churms, J. Chromatogr. 500 (1990) 555.
[3] I. Molnár-Perl, J. Chromatogr. A. 891 (2000) 1.
[4] R.J. Molyneux, D.R. Gardner, L.F. Jamesn, S.M. Colegate, J. Chromatogr. A. 967 (2002) 57.
[5] M.L. Sanz, I. Martínez-Castro, J. Chromatogr. A. 1153 (2007) 74.
[6] D.R. Knapp, Handbook of Analytical Derivatization Reactions, 1st ed., Wiley Interscience, New York, 1979.
[7] J. Drozd, Chemical Derivatization in Gas Chromatography, 1st ed., Elsevier, Amsterdam, 1981.
[8] C.J. Biermann, G.D. McGinnis, Analysis of Carbohydrates by GC and MS, CRC Press, Boca Raton, 1989.
[9] N.G. Carlsson, H. Karlsson, A.S. Sandberg, J. Agric. Food Chem. 40 (1992) 2404.
[10] C.C. Sweeley, R. Bentley, M. Makita, W.W. Wells, J. Am. Chem. Soc. 85 (1963) 2497.
[11] V.A. Isidorov, L. Szczepaniak, J. Chromatogr. A 1216 (2009) 8998.
[12] A.C. Soria, M.L. Sanz, M. Villamiel, Food Chem. 114 (2009) 758.
[13] A. Caseiro, I.L. Marr, M. Claeys, A. Kasper-Giebl, H. Puxbaum, C.A. Pio, J. Chromatogr. A 1171 (2007) 37.
[14] D. Carlavilla, M. Villamiel, I. Martíinez-Castro, M.V. Moreno-Arribas, Am. J. Enol. Viticult. 57 (2006) 468.
[15] A. Farhadi, A. Keshavarzian, J.Z. Fields, M. Sheikh, A. Banan, J. Chromatogr. B. 836 (2006) 63.
[16] A. Farhadi, A. Keshavarzian, E.W. Holmes, J. Fields, L. Zhang, A. Banan, J. Chromatogr. B. 784 (2003) 145.
[17] A. Montilla, J. Van de Lagemaat, A. Olano, M.D. del Castillo, Chromatographia 63 (2006) 453.
[18] M.L. Sanz, J. Sanz, I. Martinez-Castro, J. Chromatogr. A. 1059 (2004) 143.
[19] Z. Füzfai, Z.F. Katona, E. Kovács, I. Molnár-Perl, J. Agric. Food Chem. 52 (2004) 7444.
[20] Z. Füzfai, I. Boldizsár, I. Molnár-Perl, J. Chromatogr. A 1177 (2008) 183.
[21] M.E. Alañón, A.I. Ruiz-Matute, I. Martínez-Castro, M.C. Díaz-Maroto, M.S. Pérez-Coello, J. Sci. Food Agric. 89 (2009) 2558.
[22] A.I. Ruiz-Matute, M. Brokl, A.C. Soria, M.L. Sanz, I. Martínez-Castro, Food Chem. 120 (2010) 637.
[23] K.M. Brobst, C.E. Lott, Cereal Chem. 43 (1966) 35.
[24] B.W. Li, P.J. Schumann, J. Food Sci. 46 (1981) 425.
[25] W. Funcke, C. von Sonntag, Carbohydr. Res. 69 (1979) 247.
[26] P.N. Wahjudi, M.E. Patterson, S. Lim, J.K. Yee, C.S. Mao, W.-N.P. Lee, Clin. Biochem. 43 (2010) 198.
[27] R.H. Furneaux, Carbohydr. Res. 113 (1983) 241.
[28] N.P. Brunton, T.R. Gormley, B. Murray, Food Chem. 104 (2007) 398.
[29] W. Zhang, H. He, X. Zhang, Soil Biol. Biochem. 39 (2007) 2665.
[30] F. Ye, X. Yan, J. Xu, H. Chen, Phytochem. Anal. 17 (2006) 379.
[31] S. Honda, N. Yamauchi, K. Kakehi, J. Chromatogr. 169 (1979) 287.
[32] P. Englmaier, J. Sep. Sci. 13 (1990) 121.
[33] A. Lluveras, I. Bonaduce, A. Andreotti, M.P. Colombini, Anal. Chem. 82 (2010) 376.
[34] N.P.J. Price, Anal. Chem. 76 (2004) 6566.
[35] T.P. Mawhinney, M.S. Feather, G.J. Barbero, J.R. Martinez, Anal. Biochem. 101 (1980) 112.
[36] G.O. Guerrant, C.W. Moss, Anal. Chem. 56 (1984) 633.
[37] K.F. Fox, D.S. Wunschel, A. Fox, G.C. Stewart, J. Microbiol. Meth. 33 (1998) 1.
[38] A. Fox, J. Chromatogr. A. 843 (1999) 287.
[39] M.M. Corsaro, S. Lazzaroni, Anal. Biochem. 282 (2000) 256.
[40] E. Maes, G. Strecker, P. Timmerman, Y. Leroy, J.P. Zanetta, Anal. Biochem. 267 (1999) 300.
[41] D.A. Stead, J. Chromatogr. B. 747 (2000) 69.
[42] J. Kärkkäinen, R. Vihko, Carbohydr. Res. 10 (1969) 113.
[43] J. Gullberg, P. Jonsson, A. Nordström, M. Sjöström, T. Moritz, Anal. Chem. 331 (2004) 283.
[44] T. Vandenbrouk, O.A.H. Jones, N. Dom, J.L. Griffin, W. De Coen, Environ. Int. 36 (2010) 254.
[45] C. Liang, J.A. Pedersen, T.C. Balser, J. Chromatogr. A 1216 (2009) 5296.
[46] H. He, H. Xie, X. Zhang, Y. Wang, Y. Wu, Rapid Commun. Mass Spectrom. 19 (2005) 1993.
[47] H. He, H. Xie, X. Zhang, Soil Biol. Biochem. 38 (2006) 1083.
[48] C. Liang, T.C. Balser, Soil Biol. Biochem. 42 (2010) 904.
[49] X. Ding, X. Zhang, H. He, H. Xie, J. Soils Sediments 10 (2010) 758.
[50] X. Zhang, W. Amelung, Soil Biol. Biochem. 28 (1996) 1201.
[51] P. Compain, O.R. Martin, Iminosugars: From Synthesis to the Therapeutic Applications, 1st ed., John Wiley & Sons, New York, 2007.
[52] I. Pastuszak, R.J. Molyneux, L.F. James, A.D. Elbein, Biochemistry 29 (1990) 1886.
[53] S. Rodríguez-Sánchez, O. Hernández, A.I. Ruiz-Matute, M.L. Sanz, Food Chem. 126 (2011) 353.
[54] B. Dräger, J. Chromatogr. A. 978 (2002) 1.
[55] A.F. Magalhães, C.C. Santos, E.G. Magalhães, M.A. Noriega, Phytochem. Anal. 13 (2002) 215.
[56] J.S. Walters, J.I. Hedges, Anal. Chem. 60 (1988) 988.
[57] S. Inoue, K. Kitajima, Glycoconj. J. 23 (2006) 277.
[58] S. Lewis, C.N. Kenyon, J. Melli, A.L. Burlingame, Anal. Chem. 51 (1979) 1275.
[59] O. Novotný, K. Cejpek, J. Velíšek, Czech J. Food Sci. 26 (2008) 117.
[60] D.L. Callahan, U. Roessner, V. Dumontet, N. Perrier, A.G. Wedd, R.A.J. O'Hair, A.J.M. Baker, S.D. Kolev, Phytochemistry 69 (2008) 240.
[61] S. Tisza, P. Sass, I. Molnarl-Perl, J. Chromatogr. A 676 (1994) 461.
[62] L.J. Haffenden, V.A. Yaylayan, J. Agric. Food Chem. 56 (2008) 1638.
[63] F.J. Moreno, M. Villamiel, R. Lopez-Fandino, A. Olano, Chromatographia 53 (2001) 525.
[64] S.E. Zamze, M.A.J. Fergusson, E.R. Moxon, R.A. Dwek, T.W. Rademacher, Biochem. J. 245 (1987) 583.
[65] Z. Mielniczuk, E. Mielniczuk, L. Larsson, J. Chromatogr. B 670 (1995) 167.
[66] I. Bonaduce, H. Brecoulaki, M.P. Colombini, A. Lluveras, V. Restivo, E. Ribechini, J. Chromatogr. A 1175 (2007) 275.
[67] G.L. Sassaki, L.M. Souza, R.V. Serrato, T.R. Cipriani, P.A.J. Gorin, M. Iacomini, J. Chromatogr. A. 1208 (2008) 215.
[68] Y. Nygren, A.A. Fredriksson, B.J. Nilsson, Mass Spectrom. 31 (1996) 267.
[69] J. Lehrfeld, Anal. Chem. 57 (1985) 346.
[70] D.A. Zhemerichkin, N.M. Ptitchkinal, Food Hydrocolloids 9 (1995) 147.
[71] P. Schadewaldt, H.W. Hammen, S. Stolpmann, L. Kamalanathan, U. Wendel, J. Chromatogr. B 801 (2004) 249.
[72] R.I. Hollingsworth, D.A. Lillelghanian, J. Biol. Chem. 264 (1989) 14039.
[73] A. Fox, R.M.T. Rosario, Indoor Air: Int. J. Indoor Air Qual. Climate 4 (1994) 239.
[74] G. Guerrant, C.W. Moss, J. Clin. Microbiol. 23 (1986) 425.
[75] R.E. Hicks, S.I. Newell, Anal. Biochem. 128 (1983) 438.
[76] G.O. Guerrant, K.H. Wong, C.W. Moss, FEMS Microbiol. 42 (1987) 1.
[77] G. Cooper, M. Sant, C. Asiyo, J. Chromatogr. A 1216 (2009) 6838.
[78] A. Tunlid, G. Odham, J. Microbiol. Meth. 1 (1983) 63.
[79] A. Pons, C. Richet, C. Robbe, A. Herrmann, P. Timmerman, G. Huet, Y. Leroy, I. Carlstedt, C. Capon, J.P. Zanetta, Biochemistry 42 (2003) 8342.
[80] J.P. Zanetta, A. Pons, M. Iwersen, C. Mariller, Y. Leroy, P. Timmerman, R. Schauer, Glycobiology 11 (2001) 663.
[81] A. Bohin, F. Bouchart, C. Richet, K. Osarath, Y. Leroy, P. Timmerman, G. Huet, J.P. Bohin, J.P. Zanetta, Anal. Biochem. 340 (2005) 231.
[82] Z. Lin, J. Zheng, Appl. Microbiol. Biotechnol. 86 (2010) 1613.
[83] S.I.F.S. Martins, M.A.J.S. Van Boekel, Food Chem. 90 (2005) 257.
[84] F. Chevalier, J.M. Chobert, C. Genot, T. Haertlé, J. Agric. Food Chem. 49 (2001) 5031.
[85] J.A. Rufián-Henares, S. De la Cueva, J. Agric. Food Chem. 59 (2009) 432.
[86] H. Jing, D.D. Kitts, Food Res. Int. 33 (2000) 509.
[87] F. Lin Gu, J.M. Kim, S. Abbas, X.M. Zhang, S.Q. Xia, Z.X. Chen, Food Chem. 120 (2010) 505.
[88] R. Nishigaki, T. Watanabe, T. Kajimoto, A. Tada, T. Takamura-Enya, S. Enomoto, H. Nukaya, Y. Terao, A. Muroyama, M. Ozeki, M. Node, T. Hasei, Y. Totsuka, K. Wakabayashi, Chem. Res. Toxicol. 22 (2009) 1588.
[89] S. Sarwar Gilani, E. Sepehr, J. Nutr. 133 (2003) 220.
[90] J.M. Silván, J. van de Lagemaat, A. Olano, M.D. del Castillo, J. Pharm. Biomed. Anal. 41 (2006) 1543.
[91] M.L. Wolfrom, N. Kashimura, D. Horton, J. Agric. Food Chem. 22 (1974) 791.
[92] R. Wittmann, K. Eichner, Z. Lebens, Unters. Forsch. 188 (1989) 212.
[93] V.V. Mossine, T.P. Mawhinney, J. Agric. Food Chem. 55 (2007) 10373.
[94] J. Gobert, M.A. Glomb, J. Agric. Food Chem. 57 (2009) 8591.
[95] E. Troyano, A. Olano, M.L. Jimeno, J. Sanz, I. Martínez-Castro, J. Dairy Res. 59 (1992) 507.
[96] T. Niwa, J. Chromatogr. B 731 (1999) 23.
[97] A. Hollnagel, L.W. Kroh, J. Agric. Food Chem. 50 (2002) 1659.
[98] T. Fiedler, L.W. Kroh, Eur. Food Res. Technol. 225 (2007) 473.
[99] S. Tsukushi, K. Shimokata, T. Niwa, J. Chromatogr. A. 731 (1999) 37.
[100] M.A. Madson, M.S. Feather, Carbohydr. Res. 94 (1981) 183.
[101] S. Lal, F. Kappler, M. Walker, T.J. Orchard, P.J. Beisswenger, B.S. Szwergold, T.R. Brown, Arch. Biochem. Biophys. 342 (1997) 254.
[102] W.H. Hoffman, F. Kappler, G.G. Passmore, R. Mehta, Clin. Biochem. 36 (2003) 269.
[103] K.J. Knecht, M.S. Feather, J.W. Baynes, Arch. Biochem. Biophys. 294 (1992) 130.
[104] M.Y. Wu, B.G. Chan, C.D. Chang, M.H. Huang, T.G. Wu, D.M. Chang, Y.J. Lee, H.C. Wang, C.I. Lee, C.L. Chern, R.H. Liu, J. Chromatogr. A. 1204 (2008) 81.
[105] M. Voigt, M.A. Glomb, J. Agric. Food Chem. 57 (2009) 4765.
[106] Y. Ma, M.D. Hays, C.D. Geron, J.T. Walker, M.J.G. Gichuru, Atmos. Chem. Phys. 10 (2010) 4331.
[107] M. Blazso, S. Janitsek, A. Gelencser, P. Artaxo, B. Graham, M.O. Andreae, J. Anal. Appl. Pyrol. 351 (2003) 68.
[108] I. Simkovic, I. Surina, M. Vrican, J. Anal. Appl. Pyrol. 70 (2003) 493.
[109] H. Hohno, K. Suyama, S. Adachi, J. Dairy Sci. 66 (1983) 11.
[110] M.E. Alañon, H. Rubio, M.C. Diaz-Maroto, M.S. Perez-Coello, Food Chem. 119 (2010) 505.
[111] D. Fabbri, G. Chiavari, S. Prati, I. Vassura, M. Vangelista, Rapid Commun. Mass Spectrom. 16 (2002) 2349.
[112] Z. Zdrahal, J. Oliveira, R. Vermeylen, M. Claeys, W. Maenhaut, Environ. Sci. Technol. 36 (2002) 747.
[113] C.L. Hsu, C.Y. Cheng, C.T. Lee, W.H. Ding, Talanta 72 (2007) 199.

[114] A. Olano, I. Martinez-Castro, Michwissenschaft 36 (1981) 533.
[115] M. Manley-Harris, G.F. Richards, Adv. Carbohydr. Chem. Biochem. 52 (1997) 207.
[116] M.I. García-Moreno, J.M. Benito, C. Ortiz-Mellet, J.M. García-Fernández, Molecules 13 (2008) 1640.
[117] V. Ratsimba, J.M. García-Fernández, J. Defaye, H. Nigay, A. Voilley, J. Chromatogr. A. 844 (1999) 283.
[118] A. Montilla, A.I. Ruiz-Matute, M.L. Sanz, I. Martinez-Castro, M.D. del Castillo, Food Res. Int. 39 (2006) 801.
[119] A. Ruiz-Matute, A.C. Soria, I. Martinez-Castro, M.L. Sanz, J. Agric. Food Chem. 55 (2007) 7264.
[120] T. Imanari, Z. Tamura, Agric. Biol. Chem. 35 (1971) 321.
[121] A. Cardelle-Cobas, C. Martínez-Villaluenga, M.L. Sanz, A. Montilla, Food Chem. 114 (2009) 1099.
[122] A.I. Ruiz-Matute, M.L. Sanz, M.V. Moreno-Arribas, I. Martínez-Castro, J. Chromatogr. A. 1216 (2009) 7296.
[123] S.G. Voirin, R.L. Baumes, Z.Y. Gunata, S.M. Bitteur, C.L. Bayonove, J. Chromatogr. 590 (1992) 313.
[124] A. Nasi, P. Ferranti, S. Amato, Chin. Food Chem. 110 (2008) 762.
[125] D. Chassagne, J.C. Crouzet, C.L. Bayonove, R.L. Baumes, J. Agric. Food Chem. 44 (1996) 3817.
[126] R. Julkunen-Tiitto, J. Chromatogr. 442 (1988) 175.
[127] P. Vainiotalo, R. Julkunen-Tiitto, M.R. Juntheikki, P. Reichardt, S. Auriola, J. Chromatogr. 547 (1991) 367.
[128] I. Boldizsar, Z. Szucs, Z. Fuzfai, I. Molnár-Perl, J. Chromatogr. A 1133 (2006) 259.
[129] I. Boldizsár, Z. Füzfai, F. Tóth, É. Sedlák, L. Borsodi, I. Molnár-Perl, J. Chromatogr. A 1217 (2010) 1674.
[130] E. de Rijke, P. Out, W.M.A. Niessen, F. Ariese, C. Gooijer, U.A.Th. Brinkman, J. Chromatogr. A 1112 (2006) 31.
[131] A.S. Pereira, M. Costa Padilha, F. Radler, N. de Aquino, Microchem. J. 77 (2004) 141.
[132] W.A. Oleszeck, J. Chromatogr. A. 967 (2002) 147.
[133] A. Gutierrez, J.C. del Río, Rapid Commun. Mass Spectrom. 15 (2001) 2515.
[134] J.G. March, B.M. Simonet, F. Grases, J. Chromatogr. B 757 (2001) 247.
[135] A.J. Koning, Analyst 119 (1994) 1319.
[136] U. Roessner, C. Wagner, J. Kopka, R.N. Trethewey, L. Willmitzer, Plant J. 23 (2000) 131.
[137] M.M. Koek, B. Muilwijk, M.J. van der Werf, T. Hankemeier, Anal. Chem. 78 (2006) 1272.
[138] M.M. Koek, B. Muilwijk, L.L.P. van Stee, T. Hankemeier, J. Chromatogr. A 1186 (2008) 420.
[139] B. Ayestarán, Z. Guadalupe, D. León, Anal. Chim. Acta 513 (2004) 29.
[140] S. Pati, M.T. Liberatore, C. Lamacchia, E. La Notte, Carbohydr. Polym. 80 (2010) 332.
[141] J.S. Kim, E.R. Laskowich, F. Michon, R.E. Kaiser, R.G. Arumughama, Anal. Biochem. 358 (2006) 136.
[142] L. Johannson, P. Tuomainen, M. Ylinen, P. Ekholm, L. Virkki, Carbohydr. Polym. 58 (2004) 267.
[143] B.V. McCleary, A. Murphy, D.C. Mugford, AOAC Int. 83 (2000) 356.
[144] O. Dahlman, A. Jacobs, A. Liljenberg, A.I. Olsson, J. Chromatogr. A 891 (2000) 157.
[145] L. Virkki, H.N. Maina, L. Johansson, M. Tenkanen, Carbohydr. Res. 343 (2008) 521.
[146] N. Kardos, J.L. Luche, Carbohydr. Res. 332 (2001) 115.
[147] G.A. De Ruiter, H.A. Schols, A.G.J. Voragen, F.M. Rombouts, Anal. Biochem. 207 (1992) 176.
[148] G. Reinders, H.P. Thier, Eur. Food Res. Technol. 209 (1999) 43.
[149] E. Quintero, K. Ishida, G. Gordon, G. Geesey, J. Microbiol. Meth. 9 (1989) 309.
[150] T. Fontaine, B. Fournet, Y. Karamanos, J. Microbiol. Meth. 20 (1994) 149.
[151] F.J. Moreno, A. Olano, M. Villamiel, Milchwissenschaft 56 (2001) 669.
[152] J. Li, K. Kisara, S. Danielsson, M.E. Lindstrom, G. Gellerstedt, Carbohydr. Res. 342 (2007) 1442.
[153] A.I Usov, Hydrobiologia 260–261 (1993) 641.
[154] Y. Hama, H. Nakagawa, M. Kurosawa, T. Sumi,.X. Xia, K. Yamaguchi, Anal. Biochem. 265 (1998) 42.
[155] O. Chiantore, C. Riedo, D. Scalarone, Int. J. Mass Spectrom. 284 (2009) 35.
[156] D. Fabbri, G. Chiavari, Anal. Chim. Acta 449 (2001) 271.
[157] K. Kuroda, J. Anal. Appl. Pyrol. 53 (2000) 123.
[158] A. Jay, J. Carbohydr. Chem. 15 (1996) 897.
[159] I. Ciucanu, Anal. Chim. Acta 576 (2006) 147.
[160] N. Price, Appl. Biochem. Biotechnol. 148 (2008) 271.
[161] I. Ciucanu, F. Kerek, Carbohydr. Res. 131 (1984) 209.
[162] S. Hakomori, J. Biochem. 33 (1964) 205.
[163] M. Politi, J. Alvaro-Blanco, P. Groves, A. Prieto, J.A. Leal, F.J. Cañada, J. Jiménez-Barbero, Eur. J. Org. Chem. (2006) 2067.
[164] J.M. Challinor, J. Anal. Appl. Pyrol. 61 (2001) 3.
[165] B. Fournet, G. Strecker, Y. Leroy, J. Montreuil, Anal. Biochem. 116 (1981) 489.
[166] R. Geyer, H. Geyer, S. Kühnhardt, W. Mink, S. Stirm, Anal. Biochem. 133 (1983) 197.
[167] K.R. Anumula, P.B. Taylor, Anal. Biochem. 203 (1992) 101.
[168] T. Lehr, H. Geyer, K. Maaß, M.J. Doenhoff, U. Geyer, Glycobiology 17 (2006) 82.
[169] G.J. Gerwig, J.P. Kamerling, J.F.G. Vliegentart, Carbohydr. Res. 62 (1978) 349.
[170] K. Leonstein, B. Lindberg, J. Lonngen, Carbohydr. Res. 62 (1978) 359.
[171] M.R. Little, Carbohydr. Res. 105 (1982) 1.
[172] H. Schweer, J. Chromatogr. 259 (1983) 164.
[173] M.R. Cases, A.S. Cerezo, C.A. Stortz, Carbohydr. Res. 269 (1995) 333.
[174] L. Lindqvist, P.E. Jansson, J. Chromatogr. A 769 (1997) 253.
[175] E. Vinogradov, J. Li, I. Sadovskaya, S. Jabbouri, I.M. Helander, Carbohydr. Res. 339 (2004) 1637.
[176] M.E. Soria-Diaz, M.A. Rodriguez-Carvajal, P. Tejero-Mateo, J.L. Espartero, B. Moron, C. Sousa, M. Megias, J. Thomas-Oates, A.M. Gil-Serrano, FEMS Microbiol. Lett. 255 (2006) 164.
[177] N.A. Paramonov, J. Aduse-Opoku, A. Ashim, M. Rangarajan, M.A. Curtis, J. Bacteriol. 191 (2009) 5272.
[178] E. Fragolino, G. Fugazza, E. Galano, V. Gargiulo, P. Landini, R. Lanzetta, B. Lindner, L. Pagani, M. Parrilli, O. Holst, C. de Castro, Eur. J. Org. Chem. 7 (2010) 13450.
[179] T. Rezanka, K. Sigler, Folia Microbiol. 52 (2007) 246.
[180] E.R. Suarez, S.M. Bugden, F.B. Kai, J.A. Kralovec, M.D. Noseda, C.J. Barrow, T.B. Grindley, Carbohydr. Res. 343 (2008) 2623.
[181] K. Mazumder, B.P. Choudhury, G.B. Nair, A.K. Sen, Glycoconj. J. 25 (2008) 345.
[182] Y. Wu, N. Wu, Y. Pan, L. Zhou, X Zhou, Carbohydr. Res. 342 (2007) 870.
[183] C. Liu, X. Li, Y. L.I, Y. Feng, S. Zhou, F. Wang, Food Chem. 110 (2008) 807.
[184] S.S. Zhokhov, J.A. Jastrebova, L. Kenne, A. Broberg, J. Nat. Prod. 72 (2009) 656.
[185] D. Das, S. Mondal, S.K. Roy, D. Maiti, B. Bhunia, T.K. Maiti, S.S. Islam, Carbohydr. Res. 344 (2009) 2581.
[186] S. Gorska, W. Jachymek, J. Rybka, M. Strus, P.B. Heczko, A. Gamian, Carbohydr. Res. 345 (2010) 108.
[187] M.I. Errea, A.A. Kolender, M.C. Matulewicz, Bot. Mar. 44 (2001) 1332.
[188] D.A. Navarro, C.A. Stortz, Carbohydr. Res. 338 (2003) 2111.
[189] J.M. Estevez, M. Ciancia, A.S. Cerezo, Carbohydr. Res. 339 (2004) 2575.
[190] F. Urbina, E.L. Nordmark, Z. Yang, A. Weintraub, F. Scheutz, C. Widmalm, Carbohydr. Res. 340 (2005) 645.
[191] W.A. Konig, I. Benecke, S. Sievers, J. Chromatogr. 217 (1981) 71.
[192] H. Bretting, G. Jacobs, I. Benecke, W.A. Konig, J. Thiem, Carbohydr. Res. 139 (1985) 225.
[193] M.W. Loewus, K. Sasaki, A.L. Leavitt, L. Munsell, W.R. Sherman, F.A. Loewus, Plant Physiol. 70 (1982) 1661.
[194] W. Konig, I. Benecke, H. Bretting, Angew. Chem. Int. 20 (1988) 693.
[195] A. Berthod, W.Y. Li, D.W. Armstrong, Carbohydr. Res. 201 (1990) 175.
[196] J. Roboz, E. Nieves, J.F. Holland, J. Chromatogr. 500 (1990) 413.
[197] J. Heinrich, W.A. Konig, H. Bretting, P. Mischnik, Carbohydr. Res. 299 (1997) 1.
[198] L. Lindqvist, P.E. Jansson, J. Chromatogr. A 767 (1997) 325.
[199] G. Cooper, M. Sant, C. Asiyo, J. Chromatogr. A 1218 (2009) 6838.