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Identification of disaccharides by gas chromatography–Fourier transform infrared spectroscopy

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Abstract

The use of GC–Fourier transform (FT) IR of methylsilyl ethers of disaccharides to positively identify disaccharides is reported for the first time. The conditions required to separate these high-molecular-mass compounds by GC, in the quantities required for FT-IR detection are reported. Trimethylsilyl ethers can be used successfully. The use of dimethylsilyl ethers is not recommended as their instability, under the required operating conditions, resulted in fragmentation and rearrangements.

Keywords: Derivatization, GC; Disaccharides; Carbohydrates

1. Introduction

The ability to analyse carbohydrates is of considerable importance in the biological sciences. The wide range of possible structures and function of carbohydrates contributes to the problems encountered when trying to analyse these materials.

A number of techniques exist for carbohydrate analysis, but none of them will solve every analytical problem. Techniques such as nuclear magnetic resonance and mass spectroscopy can yield a positive identification in many cases, but require a pure sample to work with. Whilst GC, HPLC, RPLC etc. can separate mixtures of saccharides, their identification can only be tentative based on retention times of standards run under identical conditions. For these reasons the normal procedure is to use one of the separation technologies coupled to one of the methods for positive identification. Probably the most widely used procedure is GC–MS of either trimethylsilyl (TMS) ethers or alditol acetates.

Alditol acetates of stereoisomers have mass spectra with only minor differences in relative intensities rather than in fragmentation pattern [1]. In addition both the α- and β-anomers give identical alditol acetates. The TMS ethers also give mass spectra of stereoisomers that only differ in their relative intensities [2].

Mass spectroscopy is extremely good at differentiating on the basis of major structural differences or differing molecular mass but less so at differentiating on the basis of stereochemical changes. Infrared (IR) spectroscopy is effective at differentiating stereochemical changes but is less able to differentiate on the basis of molecular size as in homologous series [3].

Since many disaccharides have only minor stereochemical differences between them then IR should more readily distinguish them than MS. We have already demonstrated that this was true for the TMS ethers of the monosaccharides [4]. This previous work showed that unambiguous identification of monosaccharides could be readily achieved. Here we report on the extension of this technique to cover

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disaccharides. The molecular mass of the TMS ether of the disaccharide maltose is 916. Successful GC, using a flame ionization or MS detector, of such high-molecular-mass compounds would normally require a narrow bore column with a thin phase film (high phase ratio β), operated at high temperatures. Whilst the higher molecular mass of oligosaccharides makes their separation by GC increasingly difficult, methods have been reported for the separation of oligosaccharides, by GC, with a degree of polymerisation of 6–7 [5]. The phase ratio (β) is defined as V_G/V_S where V_G and V_S are the volumes of the mobile and stationary phases respectively [6]. For GC–Fourier transform (FT) IR these conditions cannot be adhered to as thick film columns (low β) are required to handle the sample size required by the less sensitive FT-IR detector. In addition temperature is limited by the maximum operating temperature of the light pipe.

To increase β of a capillary column and at the same time retain the sample handling capacity requires that the bore of the column be increased allowing the same total volume of phase to be deposited as a thinner film.

Previous workers had proposed the use of dimethylsilyl (DMS) rather than TMS ethers for GC of saccharides and claimed a reduction in retention times of 50% [7]. For maltose the molecular mass of the DMS ether would be 804, a reduction of ≈12% which should result in a reduction of the retention time.

2. Experimental

TMS ethers were prepared by the methods previously reported [4,8]. DMS ethers were prepared by adding 500 μl of anhydrous pyridine to ≈10 mg of the disaccharide followed by 500 μl of tetramethyldisilazane and 250 μl of dimethylchlorosilane. After allowing the reaction to proceed for one h at room temperature the reaction mixture was evaporated to dryness under a stream of nitrogen gas and redissolved in 50–200 μl of *n*-hexane. Tetramethyldisilazane and dimethylchlorosilane were obtained from Fluka, Gillingham, UK. All other reagents and disaccharide standards were obtained from Sigma, Poole, UK.

Between 1 and 3 μl was injected in splitless mode into an 8600 gas chromatograph coupled to a 1720X FT-IR spectrometer by means of a 1700 GC–IR interface, all equipment from Perkin–Elmer, Beaconsfield, UK. The GC system was fitted with a sample splitter at the outlet of the column allowing 10% of the sample to be directed to the flame ionisation detection (FID) system to give the normal GC trace whilst the remainder was fed to the FT-IR system. The FT-IR system was fitted with a mercury cadmium telluride (MCT) detector cooled with liquid nitrogen.

Two different fused silica GC columns were used, the first being a 25 m×0.53 mm I.D. with 3 μm of BP1 phase, SGE (UK), Milton Keynes, UK. The second column was a 30 m×0.75 mm I.D. with 0.75 μm of SPB35 phase, Supelco, Poole, UK. A wide range of operating conditions were tried on both columns to determine the optimum conditions. The final conditions chosen were: (a) for the 0.53 mm column, carrier gas was helium at 103 kPa, GC was programmed from 175°C, ramp 2.5°C min^{-1} to 260°C hold 50 min; (b) for the 0.75 mm column, carrier gas helium at 207 kPa and the GC was programmed from 225°C hold 2 min, ramp 1°C min^{-1} to 245°C hold 12 min.

For both columns, injector and FID temperatures were 270°C and 320°C, respectively. Make up gas for FT-IR was not used but the instrument was continuously purged with nitrogen at a flow-rate of 20 ml min^{-1}. The spectrometer was set to make four scans at a resolution of 2 cm^{-1} with a slice width of 0.24 min and an interval of 0.25 min. All spectra were recorded on-the-fly and none of the techniques of spectral enhancement were used. Reference spectra were obtained by chromatographing single disaccharides but experiments were also carried out to ensure that mixtures of disaccharides could be resolved on GC and identified by FT-IR.

3. Results and discussion.

3.1. Chromatography

As was expected the two columns performed differently. The 0.53 mm column with the lower β value gave the longest retention times, which varied from 42–77 min for TMS ethers. The 0.75 mm I.D.

column gave appreciably shorter retention times of 13–30 min for the same range of disaccharides. The DMS ethers gave retention times some 15–20% less than the corresponding TMS ethers, but these were not strictly comparable due to the rearrangements that occurred with the DMS ethers. This reduction was significantly less than that claimed by the original workers and was more in line with expectations considering the reduction in molecular mass.

Despite the wide range of conditions tried the more polar SPB35 column tended to result in peaks that were more skewed than those obtained using the BP1 column. This latter column tended to be more tolerant of overloading whilst slight overloading of the SPB35 resulted in pronounced skew. At moderate column loadings however the skew was minimal and the advantage of the shorter retention times was a considerable advantage. The necessity to use smaller sample sizes on the SPB35 column resulted in spectra with a lower signal-to-noise ratio, although they were still distinguishable. We believe that the optimum column would be a 0.75 mm I.D. with 1 μm BP1 phase giving an increased sample capacity over the 0.75 μm SPB35, short retention times and less tendency to give skewed peaks.

The SPB35 column was used to obtain the majority of the spectra because of its shorter retention times. Under the conditions given it gave adequate resolution of both mixtures of disaccharides and of equal importance it resolved the anomers when single sugars were run. A typical result from a mixture of disaccharides chromatographed as their TMS ethers is given in Fig. 1. Because the average peak width was in the order of 0.85 min and the spectra were obtained in 0.24 min it was possible to obtain spectra from just the heart of the peaks and thus the fact that the peaks were not always completely separated was not significant. In the eventuality that peaks did occur with insufficient separation between them then it would be possible to adjust the GC programme, reduce initial temperature or ramp rate so that they become more widely separated. This would however result in increased retention times.

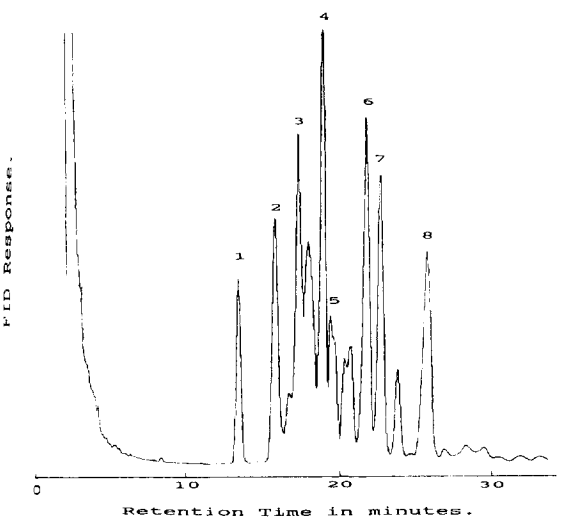

Fig. 1. GC trace obtained from a mixture of disaccharides chromatographed as their TMS ethers. GC conditions: column 30 m×0.75 mm fused silica 0.75 μm SPB35 phase; carrier gas helium at 207 kPa; temperature programme 225°C, hold 2 min, ramp 1°C min^{-1} to 245°C, hold 12 min. Peaks identified: 1. lactose; 2. sucrose; 3. α-maltose; 4. β-maltose; 5. α-cellobiose; 6. α-turanose; 7. β-turanose; 8. β-cellobiose.

3.2. Dimethylsilyl ethers

The spectrum obtained from maltose DMS with an injector and detector temperatures of 270°C is shown in Fig. 2. The same five prominent "groups" of peaks previously reported for the monosaccharides were seen [4]. An extra peak was observed at 2134 cm^{-1} which was absent from the TMS ethers. This peak could be assigned to Si–H stretching [3] and confirmed that the product was a DMS ether. Group one with a major absorbance at 2961 cm^{-1} was not the doublet due to CH_3 stretch that was expected but

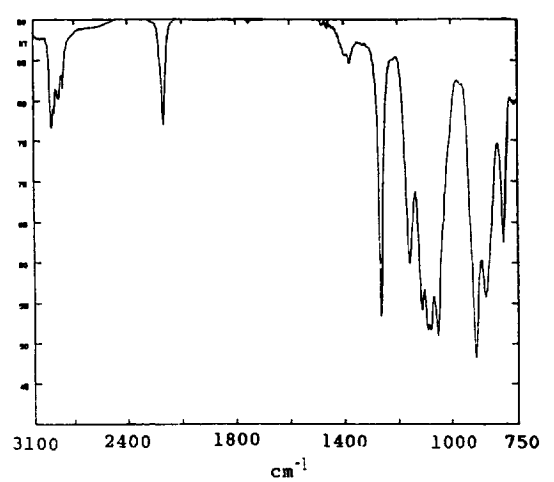

Fig. 2. Vapour phase FT-IR spectrum of DMS ether of maltose.

consisted of four peaks. This pattern was seen in the monosaccharides only when the sugar ring contained a CH_2 group either in a deoxy sugar or from a pyranose form of a pentose sugar such as arabinose or lyxose. The GC trace showed unexpected peaks with short retention times in addition to the major peak corresponding to the product. When the injector and detector temperature were 320°C the GC trace showed multiple peaks which were not reproducible. These results suggested that the DMS ethers were thermally unstable. When the temperature was 320°C extensive fragmentation occurred and no major peak was observed. At the lower temperature of 270°C fragmentation was less (as judged by the appearance of peaks with short retention times) and the DMS ether of a deoxy version of maltose was formed. It is known that the hydrogen atom on the dimethylsilyl is highly reactive [9]. One possible mechanism by which this deoxy compound could have been formed was by the elimination of Me_2SiO. This compound would require a trigonal planar structure and since these are not found in silicon chemistry it is probable that they would have formed cyclic polymers. These would then account for the short retention time peaks observed in the GC trace.

A second example of the instability of DMS ethers is given by the case of melibiose. Fig. 3 shows the GC trace of this preparation with injector and detector temperatures of 270°C and Fig. 4 the FT-IR spectrum of the major product. The GC trace showed

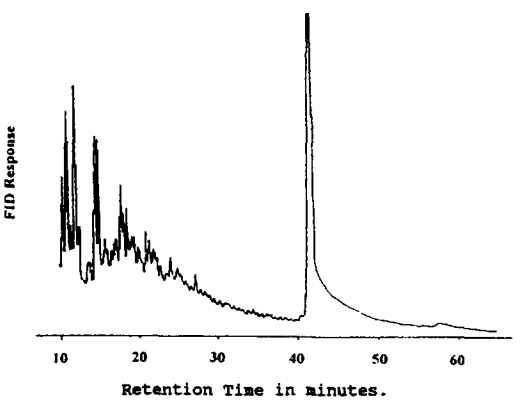

Fig. 3. Melibiose DMS GC trace. GC conditions: column 25 m×0.53 mm I.D. with 3 μm BP1 phase; carrier gas helium at 103 kPa; temperature program 175°C ramp 2.5°C min^{-1} to 260°C, hold 50 min.

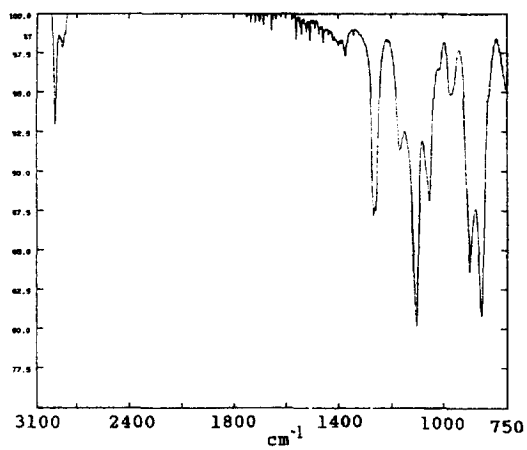

Fig. 4. Vapour phase FT-IR spectrum of DMS ether of melibiose.

multiple peaks at low retention times indicating extensive fragmentation. The FT-IR spectrum was clearly not a DMS ether since the Si–H stretching peak at 2134 cm^{-1} was absent. It is therefore probable that the DMS ether had been converted to a TMS ether with the methylation being carried out by one of the fragments produced. The TMS ether that formed the major peak was not however that of melibiose. The TMS ether of melibiose shown in Fig. 5 was visibly different from that in Fig. 4. The spectrum that was formed was virtually identical to that obtained from the TMS ether of lactose, Fig. 6. This suggests that not only was the DMS ether converted to a TMS ether but the melibiose

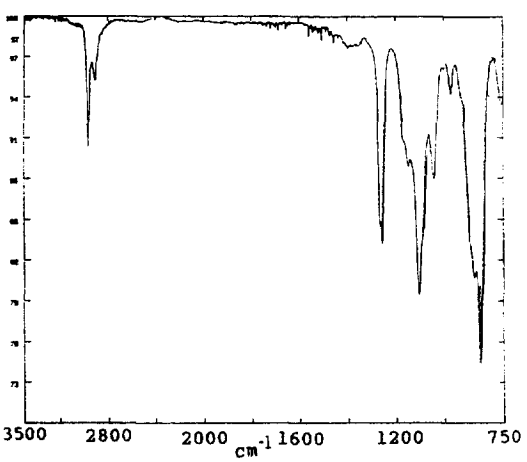

Fig. 5. Vapour phase FT-IR spectrum of TMS ether of melibiose.

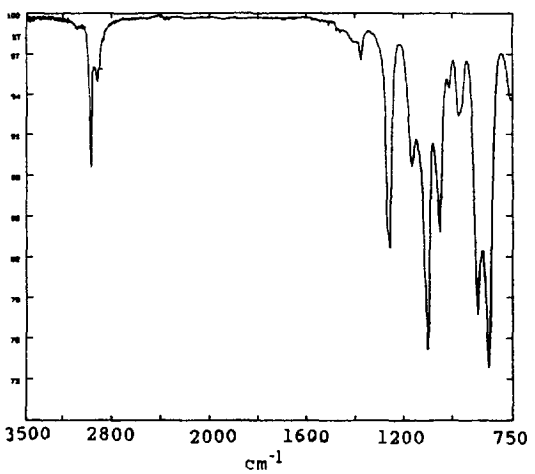

Fig. 6. Vapour phase FT-IR spectrum of TMS ether of lactose.

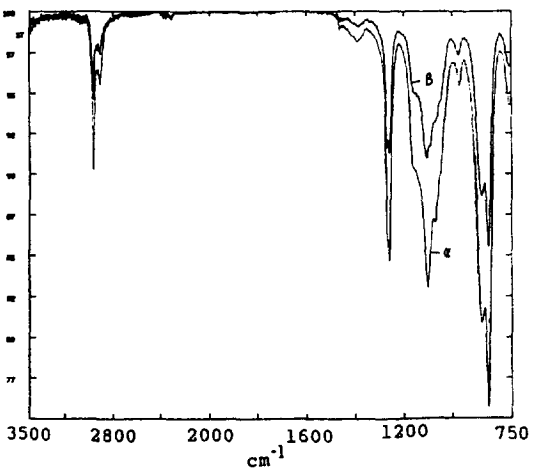

Fig. 7. Vapour phase FT-IR spectrum of TMS ethers of α- and β-turanose.

[galactose(α1-6)glucose] was converted to lactose [galactose(β1-4)glucose]! These changes in structure meant it was not possible to accurately compare the retention times of DMS and TMS ethers.

Attempts were made to make the DMS ethers of lactose, cellobiose, and turanose, all of which showed extensive fragmentation even at the lower injector temperature.

It was thus concluded that the DMS ethers were too unstable and their use was discontinued.

3.3. Trimethylsilyl ethers

TMS ethers prepared from melibiose, cellobiose, lactulose, sucrose, turanose, maltose, lactose, trehalose and lactobionic acid were successfully chromatographed as individual compounds and as mixtures. The spectra of α- and β-turanose are given in Fig. 7. All TMS derivatives gave unique spectra and the GC traces did not show the degradation as did the DMS ethers, even when the injector temperature was 320°C.

As was expected TMS lactobionic acid showed an additional peak at 1738 cm^{-1} due to the presence of C=O.

With these ten sugars even subtle changes in conformation gave rise to distinctly different spectra. For example the anomers of turanose, and all other sugars, only differ by having the O-TMS group on C1 change from its axial to its equatorial position, yet the spectra are significantly different and readily distinguished. Additionally in the case of cellobiose [glc(β1-4)glc] and lactose [gal(β1-4)glc] the only difference between them was that in the galactose portion of lactose the O-TMS on C4 was axial, whilst in the first glucose unit of cellobiose the O-TMS on C4 was in its equatorial position. Also spectra of cellobiose and maltose could be readily differentiated yet the former was [glc(β1-4)glc] whilst the latter was glc(α1-4)glc.

This evidence coupled with the extensive set of data (>100 unique spectra) we obtained previously for the monosaccharides give a clear indication that all other disaccharides when tested will be expected to give unique spectra also. Thus we believe that this technique can be extended to unambiguously identify disaccharides.

Whilst the technique is unlikely to match the sensitivity of GC–MS the quantities required, 100–500 ng on column, are low enough to make the technique useful in a large number of biological investigations. If instead of a light pipe GC–FT-IR a more modern instrument was used wherein each peak of the sample is deposited as a spot on a cooled disc sensitivity would be likely to be 1–2 orders of magnitude greater, thus extending further its range of applications. The major advantages over GC–MS are that it can easily distinguish subtle stereochemical changes that are difficult or impossible to detect in mass spectra. Whilst LC methods of separation do

not require derivatisation and can be coupled to FT-IR the resultant spectra of the underivatised sugars are much more complex and cannot so readily be interpreted [10]. We believe that no other technique can so readily distinguish between disaccharides made from different monosaccharides, between different anomeric linkages and between linkages joining different carbon atoms in the sugar rings.

References

[1] D.C. Dejong, in W. Pigman and D. Horton (Editors), The Carbohydrates, Vol. 1B. Academic Press, London, 2nd ed., 1980, Ch. 37, p. 1333.
[2] M.F. Chaplin, in M.F. Chaplin and J.F. Kennedy (Editors), Carbohydrate Analysis: A Practical Approach, IRL Press, Oxford, 1987, Ch.2 p.35.
[3] L.J. Bellamy, The Infrared Spectra of Complex Molecules, Methuen, London, 2nd ed., 1962.
[4] R.G. Veness and C.S. Evans, J. Chromatogr. A, 721 (1996) 165–172.
[5] D.J. Foulkes and A. Brooks, in S.Z. Dziedzic and M.W. Kearsley (Editors), Glucose Syrups; Science and Technology, Elsevier Applied Science, London, 1984, p.197.
[6] K.D. Bartle, in P.J. Baugh (Editor), Gas Chromatography; A Practical Approach, IRL Press, Oxford, 1993, Ch.1, p.4.
[7] W.W.Wells, C.C. Sweeley and R. Bentley, in H.A. Szymanski (Editor), Biomedical Applications of Gas Chromatography, Plenum Press, New York, 1964, pp. 169–224.
[8] C.C. Sweeley, R. Bentley, M. Makita and W.W. Wells, J. Am. Chem Soc., 85 (1963) 2497.
[9] C.F. Bell and K.A.K. Lott, Modern Approach to Inorganic Chemistry, Methuen, London, 1962, Ch. 9, p.250.
[10] R.G. Veness, unpublished results.