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A method for the analysis of sugars in biological systems using reductive amination in combination with hydrophilic interaction chromatography and high resolution mass spectrometry

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ABSTRACT

Separation of sugar isomers extracted from biological samples is challenging because of their natural occurrence as alpha and beta anomers and, in the case of hexoses, in their pyranose and furanose forms. A reductive amination method was developed for the tagging of sugars with the aim of it becoming part of a metabolomics work flow. The best separation of the common hexoses (glucose, fructose, mannose and galactose) was achieved when $^{2}H_{5}$-aniline was used as the tagging reagent in combination with separation on a ZICHILIC column. The method was used to tag a range of sugars including pentoses and uronic acids. The method was simple to perform and was able to improve both the separation of sugars and their response to electrospray ionisation. The method was applied to the profiling of sugars in urine where a number of hexose and pentose isomers could be observed. It was also applied to the quantification of sugars in post-mortem brain samples from three control samples and three samples from individuals who had suffered from bipolar disorder.

1. Introduction

Reducing sugars can exist as alpha and beta anomers and also in pyranose and furanose ring forms. The pyranose form of glucose is a 6-membered hemiacetal while the furanose form is a 5-membered hemiacetal. Neutral sugars are some of the most difficult analytes to characterise during metabolomics analyses by LC-MS and this is for two reasons, firstly, they do not ionise strongly in negative ion mode given their lack of ionisable groups and secondly as discussed above, they tend to give jagged peaks which are broad because they can exist in four different ring forms which are in equilibrium with each other. Raising the pH of the mobile phase can partly overcome these problems since this speeds up the equilibration rate between the four forms so that it becomes more rapid than the chromatographic mass transfer processes [1,2]. However, additives such as trimethylamine or ammonia which have been used to promote mutarotation are not mass spectrometry friendly and in addition many chromatography columns are not stable to the high pH values required to increase the rate of mutarotation. Complete chromatographic separations of the common monosaccharides using liquid chromatography are rare[1]. In our previous work a calcium ligand exchange column was partly able to resolve the problem of sugar separation and was able to separate glucose and fructose but was not mass spectrometry compatible since the sugars tended to form a complex mixture of calcium adducts in the mass spectrometer making spectra hard to interpret [3]. The separation of the sugar isomers by using gas chromatography is achievable although even in this case the elution times of the isomers are close particularly when oximation is used which produces two peaks for each sugar [4–6]. In GC-MS analysis analytes are subjected to electron impact ionisation and this results in uninformative spectra where there is no molecular ion and the fragments ions are not definitive of a sugar structure. In the case of high resolution mass spectrometry under electrospray conditions all the ion current is carried by the molecular ion thus giving high sensitivity and precise identification since the exact molecular weights the sugar isomers are predictable. This allows data from biological samples to be mined for unknown or unexpected sugars. Derivatising agents can be used to introduce ionisable functional groups into the sugars thereby both improving separation and the detection of the poorly ionising sugars by electrospray mass spectrometry [7–19]. A frequently adopted strategy for sugar deriva-

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tisation prior separation by liquid chromatography is to carry out reductive amination of the sugars at low pH [7–10]. The aldehyde group of the sugar reacts with the amine to form a Schiff's base and then, since a Schiff's base can exist in syn- and anti-forms thus potentially producing two chromatographic peaks and it is also not particularly stable, the reaction is pushed to completion by reducing the Schiff's base to produce a single amine product which is stable against hydrolysis. There have been several reviews of the methods used for derivatising sugars [13–15] and three basic rules have been established.

Firstly the reaction should be conducted at low pH so that the acyclic form of the sugar is favoured over the cyclic form, since it is the aldehyde form that reacts to form the Schiff base. Thus it is better to carry out the reaction at a fairly low pH (e.g. in dilute acetic acid). Having to work at low pH determines the second rule. Since bases are protonated at a low pH, and it is the unprotonated form of a base that reacts with the aldehyde, then to get a successful reaction it is necessary to use a weak base that is not fully protonated at low pH. A good example of such a base is aniline which has been used for many years to detect sugars following their separation by thin layer chromatography. The pKa value of aniline is about 4.5 so even working at around pH 3.5 there is still enough of the unprotonated form of the base to react with the sugar. A base such as aminobenzamide has an even lower pKa value (*ca* 2.0) and may be even better than aniline for the derivatisation of sugars [17]. The presence of the amide group at the para-position to the amino group increased the electron withdrawing effect away from the nitrogen of the basic group, which weakens the latter. Finally the third rule is that the reducing agent used to reduce the Schiff's base should be stable under acidic conditions. Sodium borohydride is often used in reductive amination reactions but it is of no use in the current case since it is unstable under acidic conditions. Thus it is necessary to use a suitable acid-stable reducing agent such as 2-methylpyridine-borane complex which is stable at low pH [10]. The aim of the current study was to develop a reductive amination method which would allow separation of the common hexoses fructose glucose, galactose and mannose in the identification of unknown sugars detected in biological samples.

2. Materials and methods

2.1. Chemicals and materials

HPLC grade acetonitrile, formic acid, acetic acid, aniline. HCl, $^{2}H_{5}$-aniline, picoline borane complex, glucose, fructose, mannose, galactose, ribose, xylose, arabinose, glucuronic acid, galacturonic acid, *N*-acetylglucosamine and *N*-acetylneuraminic acid were obtained from Sigma Aldrich, Dorset, UK. Synthesis of 4-amino-*N,N,N*-trimethylbenzenaminium was carried out as described in the supplementary material.

2.2. Biological samples

Urine samples were available from a previous study [20] and were collected in this study with permission from the University ethics committee. Post-mortem brain samples were also available from a previous study [21] and were provided by from the Sudden Death Bank collection held in the MRC Edinburgh Brain and Tissue Banks. Full ethics permission has been granted to the Banks for collection of samples and distribution to approved researchers, the University of Strathclyde Ethics Committee also approved the local study of this material.

2.3. Sugar derivatisation

Solutions of the sugar standards were prepared at 1 mg/ml in methanol and were then derivatised with either aniline, deuterated aniline, 4-amino-*N,N,N*-trimethylbenzenaminium as follows. The tagging agents were prepared at 10 mg/ml in methanol/water containing 10% v/v acetic acid (50:50) (solution A) and a solution containing 10 mg/ml of picoline-borane in methanol/water (1:1) (solution B) was also prepared. An aliquot of each sugar (50 µl) solution was mixed with 40 µl of solution A. The mixture was then heated in a heating block at 40 °C for 30 min. Then 20 µl of solution B was added and the resulting mixture again heated at 30 °C for 45 min. The samples were then blown to dryness in a stream of nitrogen gas and re-dissolved in 0.2 ml of water containing 0.1% formic acid, before adding 1 ml of acetonitrile.

2.4. Profiling of biological samples

Urine samples were derivatised as follows: Urine (0.2 ml) was mixed with 0.2 ml of methanol/water containing 10% v/v acetic acid (1:1) and centrifuged at 3000*g*. This sample was then treated as described above using deuterated aniline as the reagent.

Brain tissue (200 mg) was extracted by homogenising with 1 ml of acetonitrile/water containing 1 µg/ml of $^{13}C_{6}$-glucose. The sample was centrifuged at 3000*g* and then the supernatant was blown to dryness under a stream of nitrogen and the residue was dissolved the 200 µl of methanol and then derivatised as described above. A calibration curve was prepared by diluting stock solutions of sugar standards and carrying out derivatisation as described above to give solutions containing 1 µg of $^{13}C_{6}$-glucose and 0, 0.1, 0.2, 0.4, 0.8, and 1.6 µg of fructose, glucose, galactose and mannose.

2.5. LC-MS analysis

LC-MS analysis was carried out using a Surveyor HPLC pump interfaced to an LTQ Orbitrap mass spectrometer operated in positive ion mode with a needle voltage of 4.5 kV, sheath and drying gases flows set a 20 and 50 arbitrary units and a capillary temperature of 250 °C. Separation was carried out on a ZICHILIC column (150×4.6 mm, 3.5 µm particle size, HiChrom Reading, UK) in isocratic mode with an acetonitrile/water mobile phase containing 0.01% formic acid (90:10) at a flow rate of 0.6 ml/min.

3. Results

3.1. Underivatised sugars

Fig. 1 shows extracted ion chromatograms obtained for underivatised glucose and galactose on a ZICpHILIC column with our standard metabolomics screening conditions [21] which uses hydrophilic interaction chromatography at pH 9.2 with ESI-MS with positive

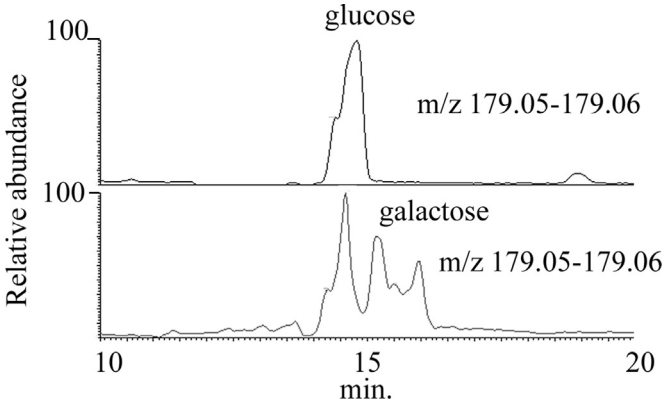

Fig. 1. Extracted ion chromatograms for glucose and galactose in negative ion mode on a ZICpHILIC column (150×4.6 mm, 5 µm) with a gradient between acetonitrile and ammonium carbonate pH 9.2.

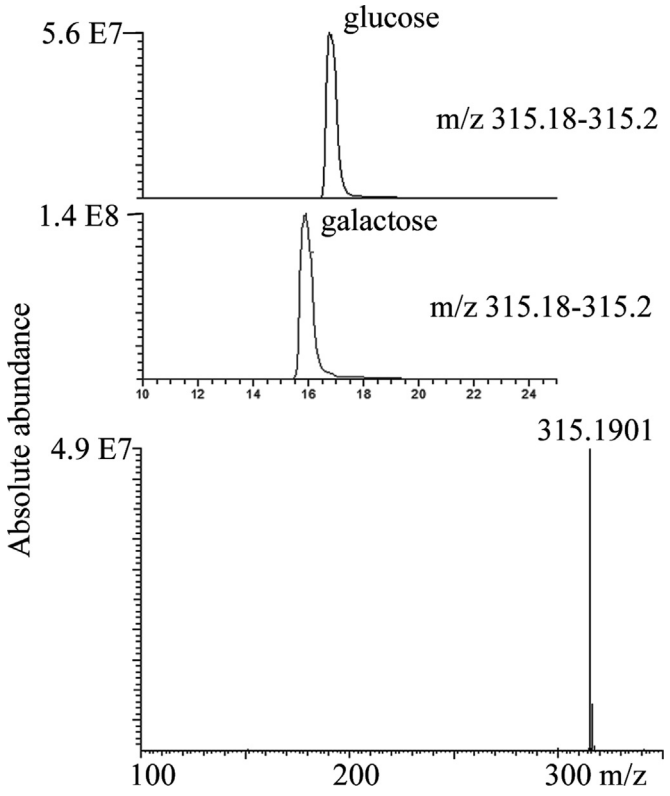

Fig. 2. TMBA derivatives of glucose and galactose analysed on a ZICHILIC column with mobile phase A 0.1% formic acid in water and mobile phase B 0.1% formic acid in acetonitrile.

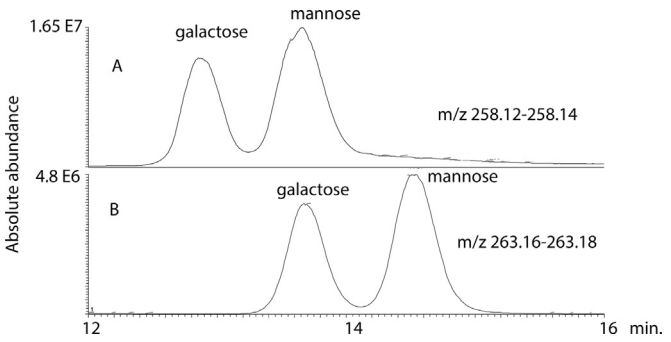

Fig. 3. Comparison of the separation of aniline (A) $^{2}H_5$ –aniline (B) derivatives galactose and mannose run on a ZICHILIC column in acetonitrile/water containing 0.01% formic acid at 0.6 ml/min.

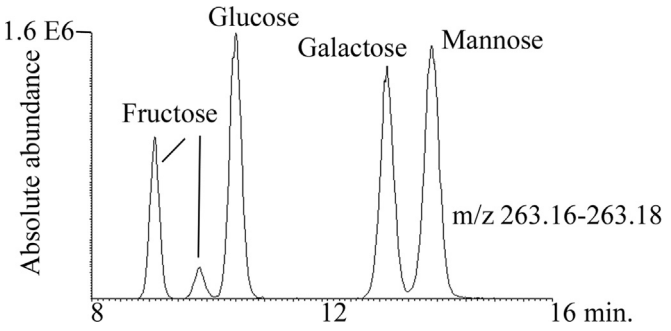

Fig. 4. $^{2}H_5$ –aniline of 1 μg amounts of hexose standards run on a ZICHILIC column in acetonitrile/water containing 0.01% formic acid at 0.6 ml/min.

or negative ion detection. There is little chance of getting a good separation as long as all the four forms are of the sugars are present since the peaks are broad and misshapen even under the conditions used to produce the chromatograms shown in Fig. 1 which were obtained at a pH of 9.2.

3.2. Derivatives formed with 4-amino-N,N,N-trimethylbenzenaminium (TMBA)

In order to improve the sensitivity of the method and remove the problem of anomeric forms a TMBA was synthesised. TMBA is a weak base due to the electron withdrawing effect of the quaternary ammonium group and it was found that it gave efficient tagging of the sugars producing derivatives with narrow peaks and high response factors in positive ion mode (Fig. 2). However, there was not sufficient resolution to separate the four common hexose sugars even when the % of aqueous phase was lowered to 5% with an isocratic run. The positive charge on the tag appears to dominate the separation and the stereochemistry of the sugar attached has little impact on the separation.

3.3. Tagging with aniline and deuteroaniline

Aniline has been used for many years a tagging agent for sugars and it was found that when used to tag the sugars it produced good separation between glucose and galactose and reasonable separation between galactose and mannose (Fig. 3A). The original intention had been to use $^{2}H_5$ -aniline as a stable isotope tagging agent so that for instance, control and disease samples could be analysed in the same run in the manner pioneered by Guo et al. [22]. However, it was found when $^{2}H_5$ –aniline was used as a tagging agent there was slightly better separation between mannose and galactose and better peak shape (Fig. 3B). Fig. 4 shows the separation of fructose, glucose, galactose and mannose as their $^{2}H_5$ –aniline derivatives on a ZICHILIC column under isocratic conditions using acetonitrile/water containing 0.01% formic acid (90:10). Mannose and galactose are almost baseline resolved and are well separated from glucose. Fructose produces two peaks since reduction of the Schiff's base introduces an additional stereocentre and thus forms a pair of diastereomers. These derivatives offer both resolution and sensitivity and also the fractional mass increment conferred by the tag is quite distinctive because of the presence of five deuterium atoms giving high confidence in identification of unknown tagged components extracted from a biological matrix. The derivatisation process can be conducted easily and the formation of the derivatives increases the response for the sugars under ESI conditions. The effect of incorporation deuterium atoms in both reversed phase liquid chromatography and gas chromatography is well known [23]. In these chromatographic modes deuterated isotopomers run earlier than their non-deuterated counterparts indicating that deuterated isotopomers are less lipophilic. The reason for this has not been fully explained but may be related to the lower vibrational energy of the carbon deuterium bond [23] in comparison with the carbon hydrogen bond. Conversely in the case of the HILIC method used in the current study the sugars tagged with the $^{2}H_5$-aniline are more hydrophilic than those tagged with aniline and run *ca* one minute later. This increased retention is sufficient to produce slightly improved resolution between galactose and mannose in comparison with the aniline derivative. Lowering the water content further did not greatly improve the separation of the aniline tagged derivatives because of increased band broadening with retention time. It would seem that the larger the contribution of the tag to the retention properties the less the sugar stereo chemistry of the sugar contributes to retention and, in preliminary work, the use of aminobenzamide as a tag gave very little separation of the hexose isomers. It was found that the best separation of the $^{2}H_5$ –aniline derivatives was produced by using a low concentration of acid modifier and a relatively high flow rate for a mass spectrometric method of 0.6 ml/min. The tagging method was applied to a selection of sugars of biological interest (Table 1).

Table 1
Retention and molecular ion data for some sugar standards tagged with of $^{2}H_5$- aniline and run on a ZICHILIC column in acetonitrile/water containing 0.01% formic acid at 0.6 ml/min.

Sugar	m/z of $^{2}H_5$- aniline derivative	Retention time min.
Fructose	263.1646	9.1, 9.9
Glucose	263.1646	10.5
Galactose	263.1646	13.1
Mannose	263.1646	13.9
Ribose	233.1356	8.0
Xylose	233.1256	7.1
Glucuronic acid	277.1436	15.3
Galacturonic acid	277.1436	18.5
N-acetyl glucosamine	304.1905	9.4
N-acetyl neuraminic acid	392.2063	27.7

Table 1 summarises the retention times and the molecular ions obtained for $^{2}H_5$- aniline derivatives of some sugars.

3.4. Profiling of sugar isomers in urine

In order to illustrate the potential of this method for uncovering unusual patterns the derivatisation procedure was applied to the analysis of a sample of human urine and the extracted ion trace for the sugar derivatives is shown in Fig. 5. The most abundant hexose in urine is glucose with small amounts of mannose and galactose being present. In addition there is an unknown abundant hexose running between glucose and mannose and galactose. Mannose is derived from microbial breakdown of complex polysaccharides in the diet and it is likely that the other hexoses, pentoses and deoxy sugars observed in the urinary profile are similarly derived. Unusual sugars do occur in urine and for instance the hexose talose has been observed to be a marker of renal injury [24].

3.5. Quantification and profiling of sugars in post-mortem human brain

In our earlier paper we showed that post-mortem brains from subjects who had schizophrenia, depression and bipolar disorder had elevated levels of polyols in comparison to controls [21] the details of the samples are given in the earlier paper [21]. Fig. 6 shows extracted ion traces for hexoses and pentoses extracted from post-mortem human brain. The major hexoses which could be observed in the brain samples were glucose, galactose and mannose. Table 2 shows the calibration data for the three hexose derivatives obtained by plotting the ratio of the peak area for each hexose against the $^{13}C_6$-glucose internal standard in the range 0.1–1.6 μg.

Table 3 shows the concentrations of hexoses in bipolar and control brains. The level of glucose in the three bipolar brains in all cases is higher than in the control brains with one of the bipolar brains containing a huge amount which was far above the range of calibration curve. The glucose levels in the bipolar samples fluctuate widely and a larger sample number would be required to achieve a statistically significant comparison. In addition, mannose and galactose levels are much more consistent in the control samples in comparison with the bipolar samples. In addition, it was possible to observe several other sugars in the brain samples including N-acetylneuraminic acid and glucuronic acid, which matched the retention times of their derivatised standards (Table 1), three isomers of N-acetylglucosamine one of which matched the standard for this compound, an abundant deoxy hexose and two isomers corresponding to reductive amination products probably derived from the ketone sedoheptulose (Fig. 7).

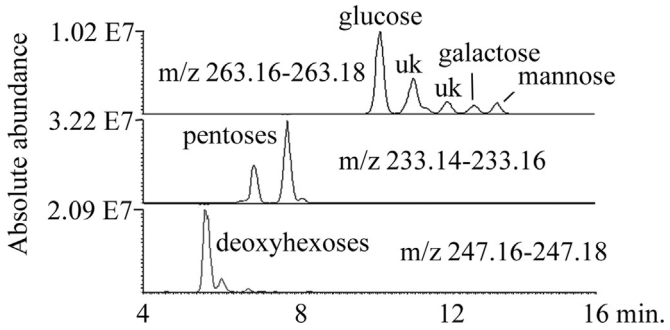

Fig. 5. $^{2}H_5$ –aniline derivatives of hexoses, pentoses and deoxyhexoses in human urine.

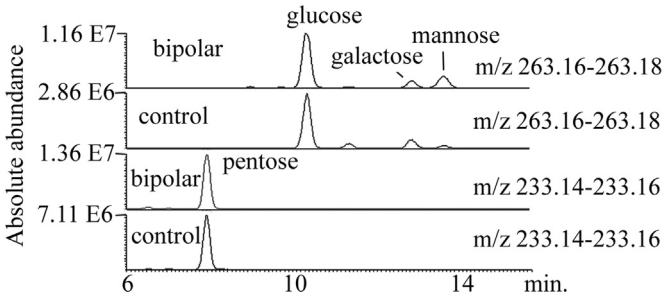

Fig. 6. Hexoses and pentoses extracted from brain tissue obtained from an individual with bipolar disorder and a control sample.

Table 2
Calibration data based on ratio of response of derivatised sugar to response for derivatised $^{13}C_6$ glucose over the range 0.1–1.6 μg.

Sugar	Equation of the line	R^2
Fructose	Y=0.457x–0.0257	0.994
Glucose	Y=0.585–0.0046	0.994
Galactose	Y=0.606–0.001	0.989
Mannose	Y=0.707–0.0152	0.985

Table 3
Concentrations of glucose, mannose and galactose in three bipolar and three control brains.

Brain	Glucose μg/g	Galactose μg/g	Mannose μg/g
Bipolar 1	285.9	4.66	18.31
Bipolar 2	44.4	5.62	9.18
Bipolar 3	18.0	0.91	0.67
Control 1	0.3	2.30	0.62
Control 2	10.3	1.75	0.65
Control 3	4.9	1.82	0.58

3.6. Validation

The precision of the method for the four common hexoses was

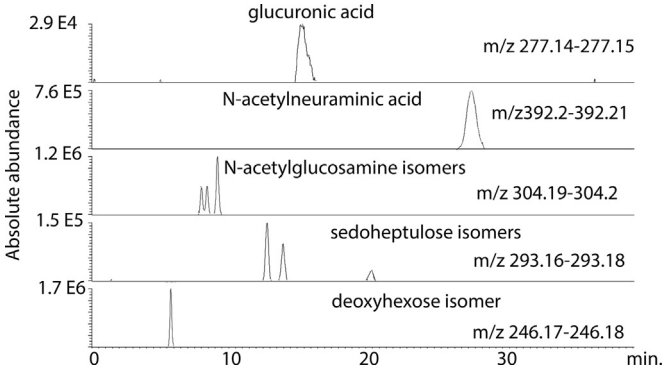

Fig. 7. Additional sugar isomers extracted from brain tissue obtained from an individual with bipolar disorder.

determined by carrying out the analysis of five aliquots taken from the same urine sample. The following concentrations were obtained: glucose 5.79 μg/ml ± 3.6%, galactose 0.307 μg/ml ± 14.8%, mannose 0.127 μg/ml ± 12.2%, fructose 0.664 μg/ml ± 22.7%. As expected the best precision was obtained for glucose where the concentrations were higher and the internal standard closely matched the properties of the analyte. The other sugars were at lower levels and gave poorer precisions which were acceptable in the case of mannose and galactose being < ± 15% as specified by FDA guidelines for bioanalysis [25]. Fructose is a ketone and thus is reduced less readily than the aldehydes and this probably accounts for the poorer precision obtained for this analyte. Thus is accurate quantification of fructose were required it would be better to employ a stable isotope labelled form of fructose as an internal standard. The limits of detection for the method were obtained from calibration curves obtained for the four common hexoses with the concentrations as follows: 0, 0.02, 0.04, 0.06, 0.08 and 0.14 μg. The reagent blanks contained no peaks for the sugars. Using the linest function in Excel applied to the calibration lines in order to obtain the standard deviation of the blank (S_B) and taking the LOD to be the blank 3x S_B gives limits of detection as follows: glucose 0.011 μg, galactose 0.0199 μg, mannose 0.0142 μg, fructose 0.0337 μg. These purely statistical estimates are on the cautious side because as shown in the blanks are clear while the signal to noise values for 0.02 μg amounts of the sugars are still infinite. In the case of high resolution trap mass spectrometers there is often very little background noise when narrow detection ranges are entered particularly if large number of mass excessive atoms such as deuterium are present in a molecule giving it a distinctive fractional mass increase over the nominal mass.

4. Discussion

Separation of sugar isomers is by LC methods difficult and has not often been achieved for even a small set of isomers like the four common hexoses. Capillary GC-MS methods have higher chromatographic resolving power than LC-MS methods and can be applied to separation of sugar isomers. However, even when using capillary GC separation sugar isomers elute closely and this is complicated by the fact that the commonly used oximation procedures produce two peaks for each aldose [4–6]. The electron impact fragmentation patterns for derivatised sugars obtained from GC-MS analysis are not very informative and are thus not conducive to uncovering the presence of new sugars whereas under positive ion ESI conditions most of the ion current is associated with the molecular ion giving better specificity and sensitivity. As can be seen in Fig. 5 derivatisation with $^2H_5$-aniline reveals the previously unknown complexity of the sugar profile in human urine. It is becoming increasingly evident that the microbiome in the human gut can carry out a range of enzymatic transformations of the dietary polysaccharides and monosaccharides [26,27]. Thus the pattern of sugars in urine has the potential to reveal the status of the microbiome and could be useful in the diagnosis and treatment of diseases such as irritable bowel disease to which this method will now be applied.

The ability of the tagging method to uncover further unknown patterns is illustrated in the human brain extracts shown in Fig. 6 there is at least one additional unknown hexose. In addition elevated levels of a pentose were observed in the bipolar brains although not quantified. The elevated level of the pentose mirrored the elevation in the levels pentitols observed in our earlier paper [21].

Since high-resolution mass spectrometry is used in combination with a tag containing the five deuterium atoms it is possible to be very sure of the identity of such unknowns although without a standard the exact isomer remains unknown. There are eight possible aldohexoses alone and the method described in this paper provides a sensitive and specific mechanism for probing sugar metabolism as part of a metabolomics work flow for uncovering biomarkers in clinical and biological samples. The method is now being applied to metabolomic profiling of sugars urine samples from patients with irritable bowel disease.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2017.01.038.

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