DOI: 10.1002/sscp.202000025

RESEARCH ARTICLE

Critical considerations for trimethylsilyl derivatives of 24 primary metabolites measured by gas chromatography–tandem mass spectrometry

Benedikt Engel | **Peter Suralik** | **Martina Marchetti-Deschmann**

Institute of Chemical Technologies and Analytics, TU Wien, Vienna, Austria

Correspondence
Prof. Mag. Dr. Martina Marchetti-Deschmann, Institute of Chemical Technologies and Analytics, TU Wien, Getreidemarkt 9/164-IAC, 1060 Vienna, Austria.
Email: martina.marchetti-deschmann@tuwien.ac.at

Cellular reactions are very important for biological systems and understanding metabolic changes is therefore crucial. Here, a gas chromatography–tandem mass spectrometry method based on multiple reaction monitoring is presented, increasing specificity and sensitivity of the method. Twenty-four metabolites of the primary carbon metabolism were selected for method development as representatives of different analyte classes (carbohydrates and their phosphates, amino acids, organic acids). The derivatization strategy necessary for analysis includes trimethylsilyl derivatization or its combination with methoximation. Derivatization products and reaction kinetics were carefully studied and compared. It is shown in detail that mere silylation results in up to seven monosaccharide derivatives and that irregular derivatization products were observed for particular amino acids showing either one or two silylation products for the ε-amino groups. Additionally, it was found that there is not a defined endpoint for this reaction. Methoximation/silylation was also optimized and studied in detail (time, temperature, kinetics) and showed reduced complexity for the derivatization products. However, some metabolites exhibited significantly lower signal responses. Most interestingly, it was observed that in the presence of amino acids the derivatization products for monosaccharides are altered when compared to monosaccharide standards, as demonstrated by the detailed discussion of glucose derivatization in the presence of lysine.

KEYWORDS
gas chromatography, metabolites, methoximation, silylation, targeted metabolomics

1 | INTRODUCTION

Among the omics studies, metabolomics is the universal analysis of all metabolites in a biological system [1]. It completes the information received through genomics, transcriptomics, and proteomics by providing quantitative

Article Related Abbreviations: CE, collision energy; CID, collision induced dissociation; EI, electron ionization; G6P, glucose-6-phosphate; MeOx, methoximation; MRM, multiple reaction monitoring; MSTFA, *N*-methyl-trimethylsilyltrifluoroacetamide; TMS, trimethylsilylation

statements of low molecular weight analytes (<1800 Da), which express the metabolic status of a biological system [2]. The increasing number of publications in the field shows the importance of this research area and indicates that it is an important tool to study phenotypes and their changes [3].

There are two main approaches in metabolomics, targeted and untargeted analysis [4]. Each method has its inherent pros and cons that have to be considered for the whole analytical process. An untargeted approach is the analysis of the global metabolite profile of a sample, it produces an extensive amount of raw data, which makes identification and characterization of analytes a grand challenge. Due to different physicochemical properties and different abundance levels of these metabolites, a complete coverage is very difficult. In addition to that, sample preparation techniques are often biased toward certain analytes (e.g., polar versus nonpolar) and the sensitivity and/or selectivity of a chosen analytical technology limit the possibilities for untargeted approaches. In contrast, a targeted approach is the analysis of known, well-defined analytes, which makes method development less challenging, nevertheless still demanding because of the different nature of the analytes.

Chromatographic methods allow the simultaneous measurement of a large number of analytes and in this context, GC has the big advantage of a far better chromatographic resolution when compared to LC [5-7]. However, GC analysis very often demands derivatization of analytes prior to analysis to increase volatility by decreasing the polarity of analytes. Today, there are many different derivatization methods available to modify polar functional groups, i.e., alkylation, silylation, or acylation. In this work, a thorough investigation of trimethylsilylation (TMS) with *N*-methyl-trimethylsilyltrifluoroacetamide (MSTFA) is presented [8-11] as it gives the most volatile of the TMS acetamides [3]. MSTFA introduces trimethylsilyl groups into molecules with hydroxyl (-OH), carboxyl (-COOH), thiol (-SH), and/or amine groups (-NH_2) by replacing one or two active hydrogen atoms (-H) forming O-, S-, N^1- (one H replaced), or N^2- (two H replaced) TMS derivatives. Additionally, a systematic investigation of the combination of TMS with a prior methoximation (MeOx/TMS) using methoxyamine hydrochloride is presented. MeOx converts aldehyde and keto groups into oximes reducing by this the number of tautomeric forms due to limited rotation along the C=N bond (mainly syn- and anti-isomers) [12].

Furthermore, a method based on triple-quadrupole technology in combination with GC and electron ionization (GC–EI–MS/MS) is discussed. The separation of a biological sample through GC reduces the complexity and MS in general is more sensitive and selective than other detectors allowing high throughput analysis at relatively low costs [13,14]. Above this, the implementation of triple quadrupole technology as the latest technological advancement, has the benefit of producing highly characteristic fragment ions for each analyte by collision induced dissociation (CID) experiments of selected ions, often already characteristic fragments generated in the EI source. This can in the end be considered as a highly specific MS^3 experiment. However, when running experiments in the so-called multiple reaction monitoring (MRM) mode, care has to be taken about the selection of appropriate transitions (precursor/product ion pair) to achieve maximum specificity [15].

Here, we present a GC–EI–MRM method to detect 24 metabolites of the primary carbon cycle metabolism after MeOx/TMS derivatization. We give details on pitfalls occurring for data analysis resulting from derivatization and present LODs and LOQs for representative analytes, i.e., eight amino acids, four organic acids, three hexoses, three pentoses, two disaccharides, one sugar phosphate, and three sugar alcohols.

2 | MATERIAL AND METHODS

2.1 | Reagents and solvents

Glycine (≥99%), L-arginine (≥98%), L-cysteine (97%), L-glutamic acid (≥99.5%), L-lysine (≥98%), *N*-acetyl-L-glutamine (≥98%), L-norvaline (≥99%, used as internal standard), citric acid (≥99.5%), L-ascorbic acid (≥99.7%), α-ketoglutaric acid (≥99%), sodium pyruvate (≥99%), D-(-)-arabinose (≥99.8%), D-(-)-fructose (≥99%), D-(+)-glucose (≥99.5%), D-(+)-mannose (≥99%), D-(-)-ribose (≥99%), D-(+)-xylose (≥99%), myo-inositol (≥99%), D-lactose monohydrate (≥99.5%), D-glucose-6-phosphate sodium salt (≥98%, G6P), D-mannitol (≥99.5%), and D-sorbitol (≥99.5%) were obtained from Sigma–Aldrich (St. Louis, MO, USA). D-(-)-α-(4-Hydroxyphenyl)-glycine (≥99%), D-2-phenylglycine (≥99%), and sucrose (≥99.5%) were obtained from SERVA (Heidelberg, Germany). Pyridine (anhydrous, 99.8%) and methoxyamine hydrochloride (≤100%) were purchased from Sigma–Aldrich. MSTFA (≥99%, 1 mL vials) was obtained from Macherey-Nagel (Düren, Germany). Water used for this work was purified (18.2 MΩcm resistivity at 25°C) with a Milli-Q water purification system (Millipore, Billerica, MA, USA).

2.2 | Metabolite mixture

Aqueous stock solutions of every standard were prepared at 10 mg/mL with the exception of D-2-phenylglycine and

L-glutamic acid for which 2 mg/mL stock solutions were used due to poor solubility in water. A mixture of the 24 metabolites was prepared using these stock solutions resulting in a concentration of 416.7 µg/mL for each analyte except D-2-phenylglycine and glutamic acid where a concentration of 83.3 µg/mL was obtained.

2.3 | Sample derivatizations

Prior to derivatization the samples had to be dried because of moisture sensitivity of the reagents and the derivatives. Therefore, 5 µL of each standard or 50 µL of the metabolite mixture were pipetted into 200 µL glass inserts (Macherey-Nagel, Düren, Germany). Five microliters of L-norvaline (5 mg/mL in water) were added with a micro syringe as internal standard. Subsequently, samples were dried using a vacuum centrifuge (UniEquip, Planegg, Germany) and placed into 1.5 mL glass vials (Macherey-Nagel, Düren, Germany).

2.4 | Trimethylsilylation

Dried samples were dissolved in 50 µL pyridine and 50 µL MSTFA were added under a slight nitrogen stream to remove oxygen from the system. The reaction conditions were optimized during this work; in the end, optimized conditions were 37°C for 30 min for which the samples were placed in a constant temperature oven after sealing the glass vials with crimp caps. The derivatized samples could be measured by GC–MS/MS directly after cooling of the reaction vial.

2.5 | Methoximation in combination with trimethylsilylation

The second derivatization method was carried out with freshly prepared methoxyamine hydrochloride in pyridine (20 mg/mL). For this, 50 µL of the reagent were added to dried samples. Reaction conditions were optimized during this work; in the end, 37°C over 90 min in a temperature oven were considered optimum. After this, TMS was carried out by adding 50 µL of MSTFA to the samples; reaction at 37°C for 30 min after sealing the glass vials with crimp caps. Samples were measured by GC–MS/MS directly after cooling the reaction vial.

2.6 | GC–MS/MS analysis

Derivatized samples were analyzed using a Shimadzu GC2010 (Kyoto, Japan) gas chromatograph, coupled to a TQ8040 triple quadrupole mass spectrometer. A Zebron ZB50 fused silica column (30 m × 0.25 mm × 0.25 µm, Phenomenex, CA, USA) with a diphenyl dimethyl polysiloxane (50:50) phase was used. Linear velocity (36.7 cm/s) was used as flow control mode, which resulted in a carrier gas (helium 5.0) flow rate of 1.0 mL/min in the column. The initial column temperature in the oven was 70.0°C and was held for 5 min after injection followed by a 5°C/min ramp to reach 245°C, held again for 1 min, then a quick increase to 310°C followed (heat rate 250°C/min) that was held for 5 min (total run time 46.26 min). One microliter of the samples was injected via a deactivated inlet liner (Shimadzu, Kyoto, Japan) at a split ratio of 1:10 and 230°C. The interface temperature was set to 250°C. The mass spectrometer was equipped with an EI ion source (70 eV, rhenium filament, 200°C) operated in positive ionization mode. Ions were recorded after a solvent cut time of 5 min, using the Q3 scan for method development and molecular identifications or by using the MRM mode for quantitative analyses. MRM was run with a loop time of 0.3 s, a minimum dwell time of 11 ms and a maximum of six ions measured simultaneously (details below). Argon 5.0 was used as collision gas and collision energies (CEs) were optimized for each analyte using the vendor provided software, GCMSsolution version 4.20 (Shimadzu). All data were acquired using the same software and structures were identified based on similarity search against the NIST11 and Wiley9 mass spectral libraries, respectively. In case of missing database entries all mass spectra were interpreted manually for structure confirmation.

2.7 | Multiple reaction monitoring

MRM optimization was carried out using the Smart MRM tool provided with the vendor's software. In brief, for each sample the signals obtained in a Q3 scan was integrated after confirmation of substance identification. Subsequently, product ion scans from characteristic fragment ions of each analyte were recorded with different CEs ranging from 3 to 45 V (3 V steps). CEs producing most abundant and highly specific transitions were chosen for each analyte.

2.8 | Analytical parameters

To determine calibration functions, 14 dilutions (1:10 to 1:7500) were prepared from the stock solution containing all 24 compounds giving analyte concentrations from 15.43 nM to 0.56 mM. D-2-Phenylglycine and L-glutamic acid were limited by their solubility and were included in the calibration between 7.35 nM and 0.22 mM. Limit of

detection and limit of quantification (LOD/LOQ) calculations were based on the calibration function using only signals with a S/N above 3. LODs were reported as 3.3-times and LOQs as 10-times the absolute standard error of the regression function ($S_{y/x}$).

3 | RESULTS AND DISCUSSION

The aim of this work was the investigation of 24 selected metabolites of the primary carbon metabolism by means of GC and MRM after optimized derivatization conditions. The selection of analytes represents four biologically important metabolite classes: sugars, organic acids, sugar phosphates, and amino acids. Derivatization methods were optimized first using one metabolite per class. After that, calibration functions, LODs, and LOQs were determined for the mixture of the 24 analytes.

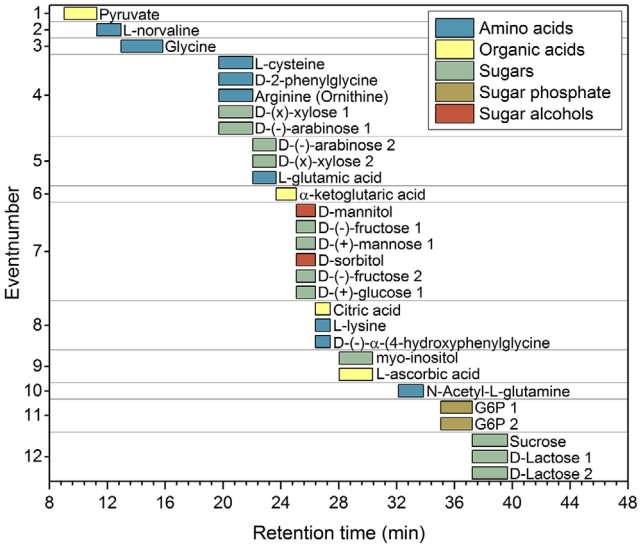

FIGURE 1 Time segments of the final GC-MRM-MS method for 24 metabolites after MeOx/TMS derivatization

3.1 | MRM optimization

Measuring analytes in MRM mode after EI can be considered as a highly specific measurement of analytes as fragment ions generated in the EI source are selected and further fragmented by CID corroborating analyte identity. The crucial benefit of this technique is significantly reduced S/N ratios. Moreover, it allows to differentiate substances in chromatographically overlapping peaks using either the respective parent ions (different molecular weights) or fragment masses (isobars).

As shown in Supporting Information Table S1, for most of the investigated substances, parent and fragment ions were highly characteristic, except for the investigated sugars where all isomers have the same parent and in most of the cases also identical fragment ions differing only in their respective intensities. For monosaccharides, it was essential to achieve optimal separation by GC. Excellent separation and MS identification based on MRM transitions could be achieved for most of these analytes, except furanoses, in particular arabinose, ribose, and xylose, could not be separated in an optimal manner. Every optimization step for these pyranoses however resulted in disadvantageous separations of all the other analytes and so for further method development, the decision was made to remove Ribose from the mixture (coelution with Arabinose and Xylose, further details see Section 3.4).

All metabolites were separated by GC within 48 min and a maximum of three transitions were identified for each analyte. The most intensive transition was used for quantification while two others were considered as qualification transitions. Figure 1 shows 12 MRM events found to be optimal for the 48 min GC separation to monitor all 24 metabolites with a maximum of six analytes measured per MRM event.

3.2 | TMS of metabolites by the use of MSTFA

TMS is a common derivatization strategy and many different protocols in terms of sample volumes, temperature, and derivatization time are available. To establish a methodology valid for future biological samples, an internal standard, norvaline, which gave a constant signal over time, was added already at this stage of method development to correct for sample losses or enrichments eventually occurring during sample preparation. Areas normalized against norvaline are reported throughout the rest of this publication.

Although most of the analytes give one distinct signal in chromatography and a mass spectrum matching to the respective spectrum of the database, it was noticed that glycine and lysine were each converted into two derivatives. Both amino acids showed TMS at the COOH-group (O-TMS), but differences for the NH_2-groups as identified by database search and manual spectral interpretation. Figure 2A and B shows the mass spectra with the proposed structures for the two potential derivatives of glycine (9.00 and 13.58 min), one having a single (N^1-TMS) and the other having a double (N^2-TMS) silylation at the α-NH_2-group. Due to the fact that lysine also exhibits two NH_2-groups for the side chain, four derivatives were expected. Interestingly, lysine yielded only two derivatives (23.64 and

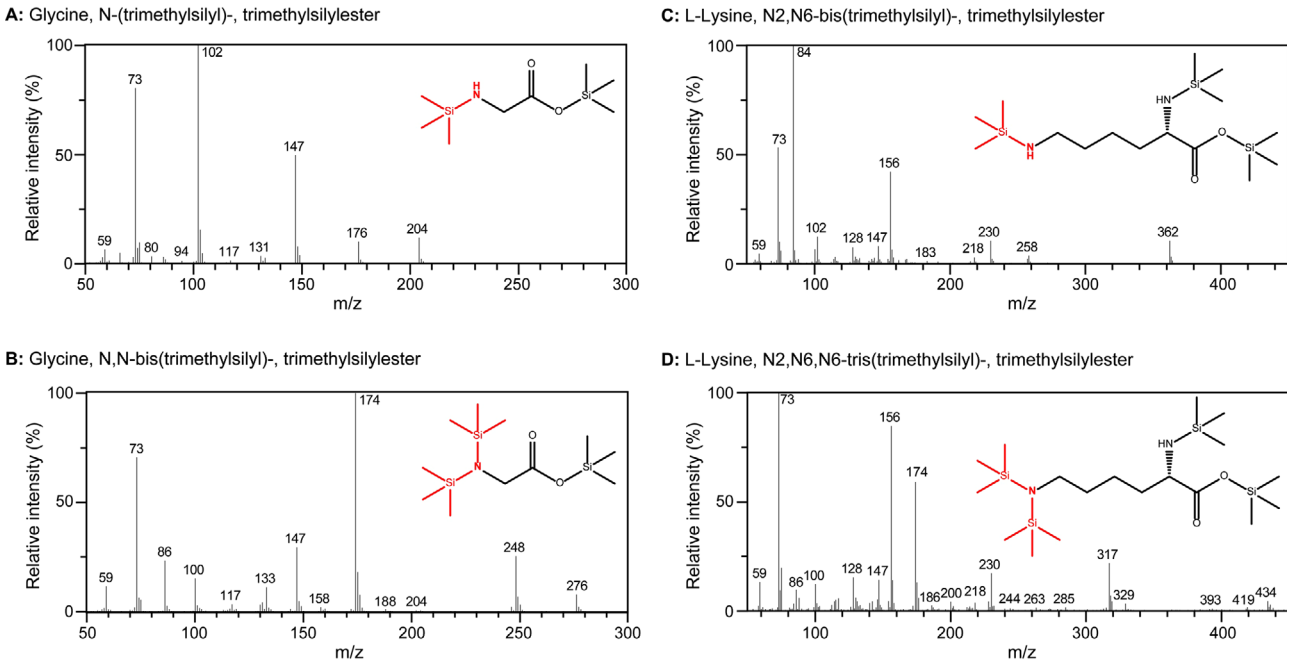

FIGURE 2 Mass spectra and chemical structures of glycine and lysine derivatives after TMS derivatization. The corresponding retention times in GC analysis using the parameters outlined in the method section are: (A) 9.00 min, (B) 13.58 min, (C) 23.64 min, and (D) 27.25 min. Structure elements in red indicate the NH_2-groups giving two derivatization products

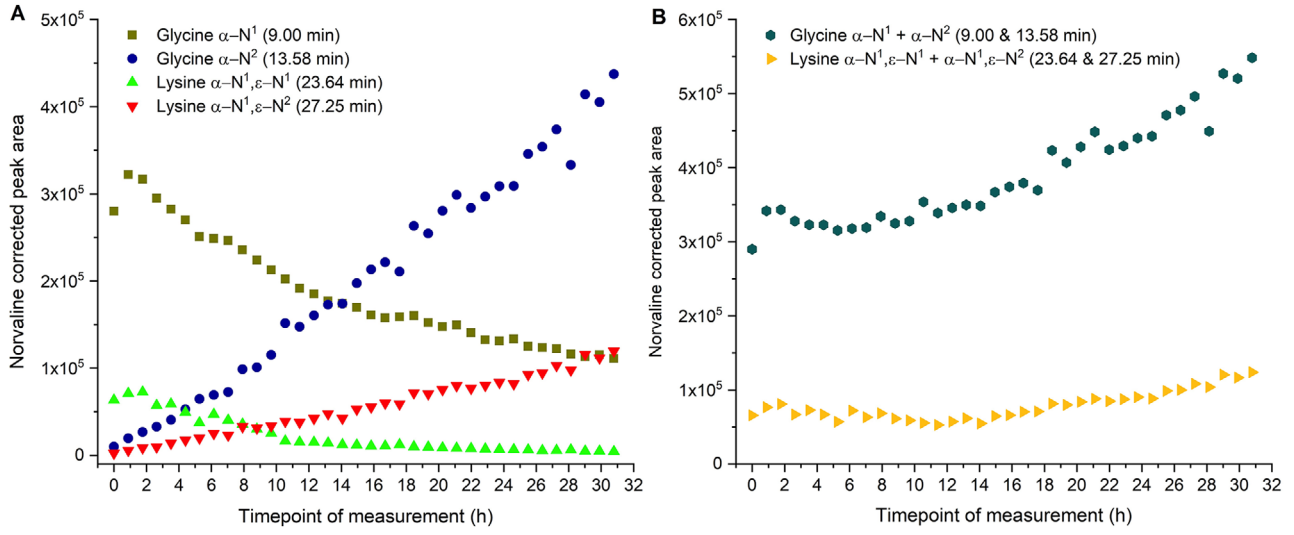

FIGURE 3 Kinetic study of glycine and lysine derivatization over 32 hours using TMS. (A) Peak areas of the four obtained derivatives and (B) sum of peak areas for both glycine and lysine derivatives. N^1 and N^2 represent the TMS derivatization at the corresponding α- or ε-NH_2-groups. All areas are corrected against norvaline (internal standard)

27.25 min; Figure 2C and D), both with a single silylation at the α-NH_2-group but again differing in the silylation grade of the ε-NH_2-group. The formation of two derivatization products can be explained by (a) sequential and slow reactions of the H-atoms of the α-NH_2-group of glycine and the ε-NH_2-group of lysine and (b) steric hinderance inhibiting consecutive derivatization of the α-NH_2-group of lysine. To ensure completeness of reaction, kinetic studies were carried out. Twelve identical samples, containing 10 mg/mL glycine and lysine each, were prepared. The development of peak areas of the derivatives over 32 h of silylation was monitored (Figure 3). It was observed that the TMS reaction did not stop and that the end point was not reached even after 30 h. The peak areas for the N^2-TMS derivative

increased constantly over time, whereas the peak area of the N^1-TMS derivative (α-N^1 in the case of glycine and ε-N^1 in the case of lysine) decreased. Furthermore, it has to be stated that the sum for all derivatives was not constant, but increased by 64 and 62% for glycine and lysine, respectively (Figure 3B).

It was also observed that all analyzed monosaccharides were converted into several products, all separable by GC–MS. In particular, pentoses yielded five derivatives except for arabinose showing only four. All hexoses yielded seven derivatives giving by this the highest number of derivatives. An explanation for this is the free aldehyde group allowing the formation of furanose and pyranose hemiacetals besides open-chain derivatives. Details for glucose are discussed exemplarily: after TMS, glucose showed seven peaks that were separated by GC within a rather narrow time window from 25.92 to 28.46 min (Supporting Information Figure S1). An exact structure/retention time correlation could not always be achieved. For the α- and β-1,2,3,5,6-pentakis-*O*-(trimethylsilyl)glucofuranose (structure I), the mass spectra observed for peaks 1 and 2 are similar and the signals clearly baseline separated (25.92 and 26.34 min). The same is true for α/β-1,2,3,4,6-pentakis-*O*-(trimethylsilyl)glucopyranose (structure II), which had the highest probability for identifications when searching the databases (3, 4, 5, 6, and 7 at 26.76, 26.99, 27.41, 27.94, and 28.46 min). Additionally, 2,3,4,5,6-pentakis-*O*-(trimethylsilyl)glucose (structure III) could be obtained as the most probable structure for the peaks eluting at 26.76 and 28.46 min (peaks 3 and 7). Purified derivatives were not available during this study making unambiguous peak assignment/confirmation of mass spectra impossible. Structures were denoted solely based on database searches giving mass spectral similarities (≥93%, except 82% for the peak at 25.92 and 26.34 min, and 83% for the peak at 27.41 min). The kinetics of derivatization was studied in detail also for monosaccharides (Figure 4) and a rapid decrease of five peak areas was observed, while the portion of two other derivatives were increasing over the first 10 h. Nevertheless, this reaction was almost complete after 20 h showing a further increase of only 6% (peak at 26.76 min) and 7% (peak at 28.46 min). It can be stated that TMS continues over the first hours and is reaching its endpoint at about 10 h. The final products that could be obtained were pyranose hemiacetals of glucose or the open-ring chain form, respectively.

In summary, TMS is difficult to keep constant for monosaccharides and some amino acids. It produces many derivatives and has no clear endpoint making quantification challenging. Although the obtained derivatives for each monosaccharide were baseline separated, the resulting high number of derivatives for all studied saccharides is highly unfavorable as such derivates can co-elute or have same MRM transitions making identification and quantification impracticable.

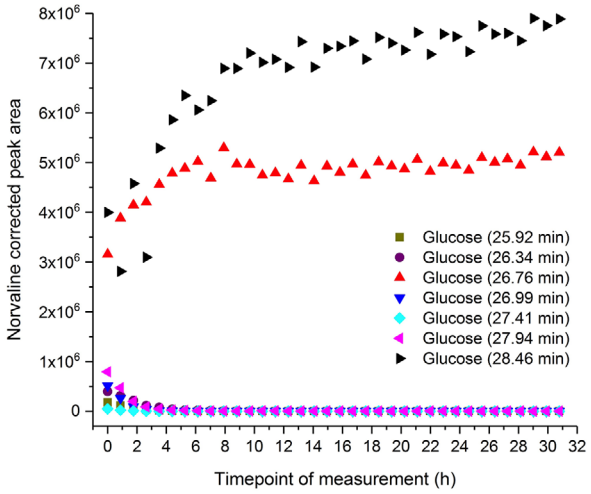

FIGURE 4 Kinetic study of glucose derivatization over 32 hours using TMS. All areas are corrected against norvaline (internal standard)

3.3 | Silylation in combination with preceding methoximation (MeOx/TMS)

To overcome isomerization and other reaction by-products, MeOx was introduced as published previously by Roessner et al. [10]. This step has the big advantage that aldehyde- and ketone-groups of reducing sugars are protected and uncontrolled ring formation during TMS is prevented by stabilizing carbonyl moieties in the β-position [10,16]. The only fact that has to be considered is, that MeOx gives two different stereoisomers in the case of monosaccharides: syn- (Z) and anti- (E) isomers [11,17], regardless of whether a D- or L-monosaccharide is the original analyte. By this, the number of signals per monosaccharide was significantly reduced, but retention time shifts had to be considered for the new products.

Derivatization conditions were optimized for five selected metabolites being candidates for each compound class (sugars, organic acids, sugar phosphates and amino acids), i.e., glucose, fructose, ketoglutaric acid, G6P, and cysteine. The optimization process started with a MeOx reaction at 30°C for 90 min that was combined with TMS at 37°C for 30 min [10]. Optimization was evaluated according to characteristic mass spectra and GC performance (peak shape, area). Fixed TMS conditions (30 min, 37°C) were first combined with varying MeOx conditions. Three different reaction times (30, 90, and 150 min) and temperatures (room temperature RT, 45, and 65°C) were

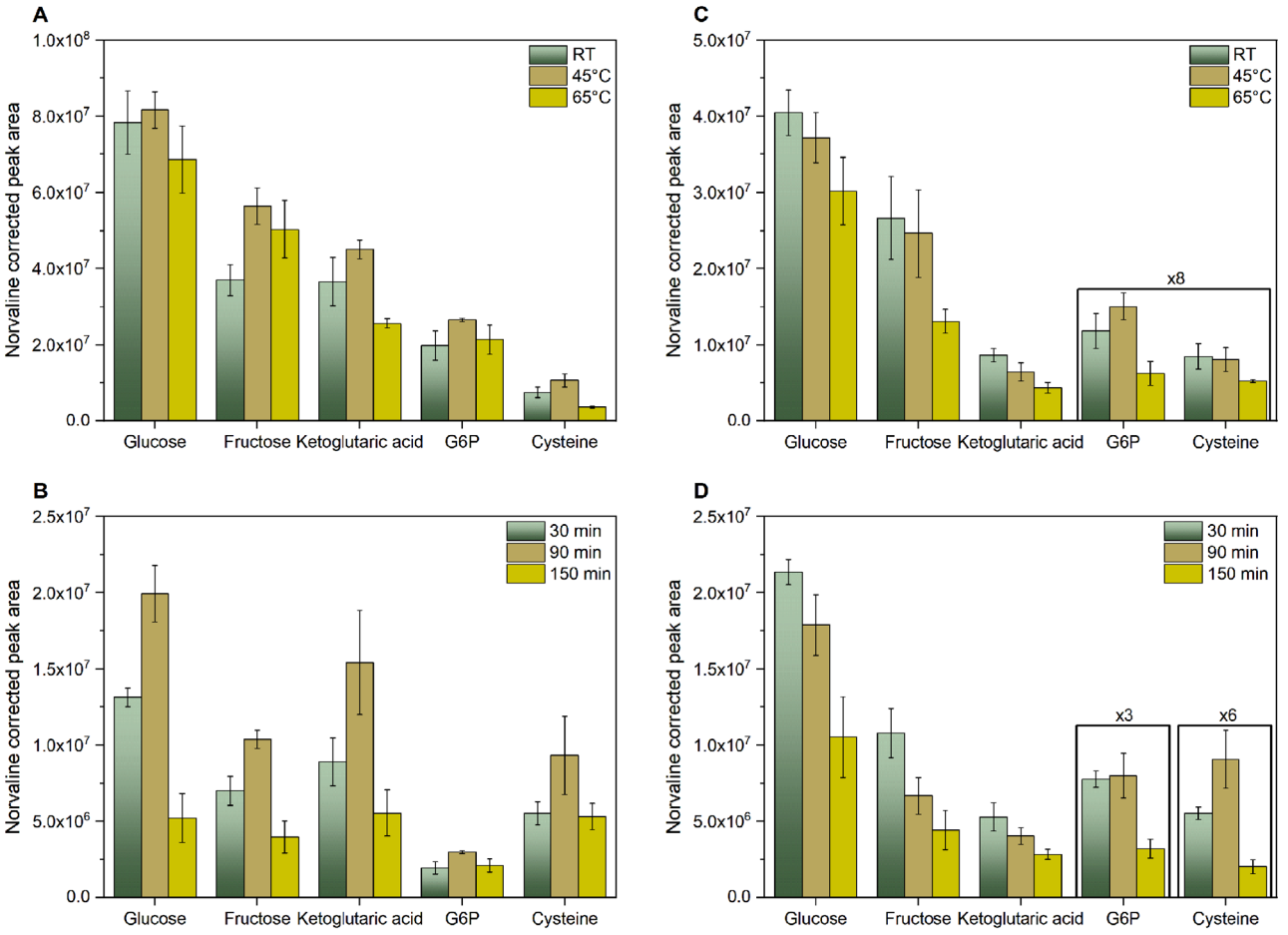

FIGURE 5 Comparison of derivatization conditions for five selected metabolites, glucose, fructose, ketoglutaric acid, G6P and cysteine. Peak areas for (A) different temperatures and (B) derivatization durations for MeOx (TMS reaction conditions constant at 37°C for 30 min). TMS reaction outcomes are compared for (C) different temperatures and (D) reaction durations while MeOx conditions were constant (37°C, 90 min). Error bars represent absolute errors of three technical replicates. All areas are normalized against norvaline, G6P and cysteine results are magnified

systematically changed. First, the three different reaction temperatures were investigated using a fixed reaction time of 90 min and subsequently the three reaction times were examined, while the optimized temperature was used. The optimization of the TMS reaction was carried out the same way using the optimized conditions for the MeOx step, a fixed reaction time of 30 min during the temperature optimization and subsequently varied reaction times at a fixed temperature (Figure 5).

For MeOx, a derivatization temperature of 45°C yielded the best results (Figure 5A). Especially fructose, α-ketoglutaric acid, and cysteine guided the decision making process as significantly higher reaction yields were achieved at this temperature. Regarding TMS, the optimum temperature was RT, except in the case of G6P where 45°C produced the most abundant signal (+27% compared to RT; Figure 5C). The aim of the optimization was to get settings that allow the use of one constant temperature oven to have a quick and easy sample preparation. Therefore, a compromise was made by performing all further derivatization reactions at 37°C.

The optimization of the MeOx reaction times were found to show an optimum at 90 min (Figure 5B). In the case of TMS, maximum peak areas were obtained already after 30 min for glucose, fructose, and α-ketoglutaric acid (Figure 5D). G6P showed similar peak areas after 30 and 90 min of derivatization. Only TMS of cysteine yielded the significantly higher signals after 90 min. Nevertheless, peak areas obtained after 30 min were well acceptable showing the lowest absolute standard deviation. Thus, reaction times of 90 min for MeOx and 30 min for TMS were selected as final parameters, representing the optimal values for all selected substances with reasonable compromises. In summary, the final parameters for the MeOx/TMS derivatization were as follows (literature values in parentheses): MeOx at 37°C for 90 min (30°C,

90 min) followed by TMS at 37°C for 30 min (37°C, 30 min) [10,18,19].

Kinetics for the optimized MeOx/TMS derivatization were again investigated. Although still two signals were observed for glycine, namely a α-N^1- and α-N^2-TMS derivative (9.00 and 13.58 min), more stable signals over time were gained, showing that the derivatization was complete after the first 4 h (Supporting Information Figure S2A). The observed peak areas in combination with standard deviations of only 11.90% and 2.90% for the α-N^1-TMS and the α-N^2-TMS signal gave only one product for lysine after 10 h (RSD of 3.30%), namely the α-N^1-/ε-N^2-TMS derivative (Supporting Information Figure S2A). Increasing the derivatization time to 10 h seems to be beneficial for this derivative, but shows less favorable results for the remaining substances (two exceptions: glucose and glycine). Moreover, such long reaction times increase the total time of analysis. Thus, it can be concluded that the presence of methoxyamine hydrochloride stabilizes reaction products giving better reproducibility for metabolite quantification.

In respect to the multiple hexose TMS derivatives, it was found that beside the positive effect of significantly reducing the chromatographic complexity the observed peak areas were also much more constant over time after MeOx (Supporting Information Figure S2B). This was confirmed by the relative standard deviation for the two glucose derivatives observed after MeOx/TMS (instead of 7 after mere TMS), 4.25% (26.68 min) and 3.95% (26.95 min).

However, an outstanding observation has to be mentioned. Two additional glucose derivatives were formed in the presence of lysine, but not glycine. Derivatization of mere glucose formed two expected isomeric products eluting at 26.68 and 26.95 min. In the presence of lysine, two additional peaks at 26.80 and 28.49 min were present. Database search indicated that these peaks can be the α- and β-form of 1,2,3,4,6-pentakis-*O*-(trimethylsilyl)-D-glucopyranose that is the cyclic form of the derivatized hexose. It can be assumed that a competitive oxidation reaction in the presence of lysine is allowing the open ring form to reform its respective hemiacetal and thus preventing MeOx of the carbonyl group of glucose. However, these signals are only minor (1.35% of main form) and can be neglected for more complex biological samples.

3.4 | Analysis of 24 metabolites of the primary carbon cycle applying the optimized MeOx/TMS method

Optimized MeOx/TMS conditions were applied in the investigation of a mixture containing all 24 metabolites. First, retention times and chromatographic performance were studied (Supporting Information Table S1) and the obtained structures of the derivatized analytes were identified based on similarity search (≥80%) against the NIST11 and Wiley9 spectral mass libraries (Supporting Information Table S2). Databases did not contain spectra for the glycine and glutamine derivatives, α-(4-hydroxyphenyl)-glycine and *N*-acetyl-L-glutamine, but manual spectra interpretation confirmed the proposed structures. Monosaccharides were expected to exhibit two separated peaks for respective isomers and GC separation was achieved accordingly for all investigated pentoses (arabinose, xylose) and hexoses (fructose, glucose, mannose), for which mass spectra were identical. However, the intensity ratio of the two isomers was different for the individual monosaccharides (first peak: second peak): arabinose (~1:5), fructose (~1:1), xylose (~1:6), glucose (~6:1), and mannose (~5:1). Exemplary structures and mass spectra are shown in Figure 6 for a pentose (arabinose) and two hexoses (glucose and fructose). The peak ratios can be explained by the varying structures, i.e., number of carbon atoms and especially the position of the carbonyl function. In addition to the investigated monosaccharides also lactose and sucrose, both disaccharides, and G6P, a phosphorylated hexose, were studied. The formation of Z and E isomers (Supporting Information Figure S3) resulted in two peaks with an intensity ratio of ~4:1 for G6P eluting at 35.66 and 35.84 min and lactose eluting at 39.14 and 39.36 min. In the case of lactose, it was hypothesized that one of the two monosaccharide building blocks gets methoximated and is remaining in its open form, while the other one remains cyclic while getting trimethylsilylated. This hypothesis could be confirmed by the obtained mass spectra. Sucrose on the other hand only resulted in the formation of one derivative (38.62 min) showing TMS at eight OH-groups and no MeOx (Supporting Information Figure S3).

As already mentioned above, two peaks for glycine were obtained: N^1-TMS and N^2-TMS. These two derivatives were also present after TMS derivatization (Figure 2A and B). Lysine formed one derivative having a single silylation at the α- and a double silylation at the ε-NH_2 group (α-N^1,ε-N^2-TMS), and all other investigated amino acids formed one derivative having a single silylation at the NH_2-group. Cysteine additional was silylated at the SH-group yielding a N^1,O,S-TMS derivative.

The investigated organic acids were each converted to one derivative having all OH-groups silylated: ascorbic acid (O,O,O,O-TMS at 29.64 min), citric acid (O,O,O,O-TMS at 26.91 min), α-ketoglutaric acid (O,O-TMS at 23.20 min), and pyruvate (O-TMS at 9.62 min). The latter two additionally showed MeOx. Exemplary the structure and mass spectrum of the α-ketoglutaric acid derivative is shown in Supporting Information Figure S4.

The total ion chromatogram of the GC–MRM–MS separation of all 24 metabolites is shown in Figure 7. As

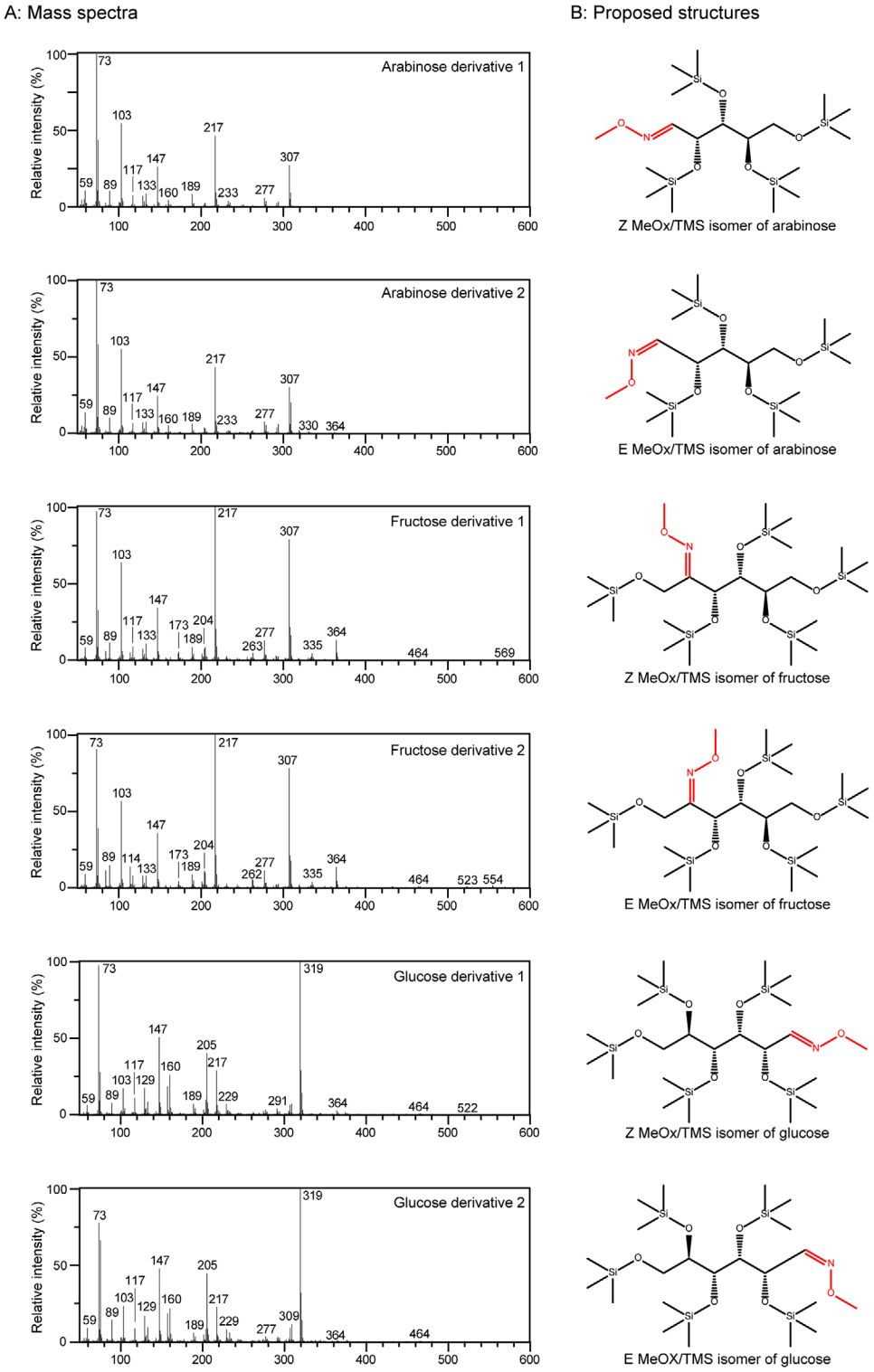

FIGURE 6 (A) Mass spectra and (B) structures of E- and Z-MeOx/TMS isomers of arabinose, fructose, and glucose. An accurate alignment of structures and mass spectra is not possible at this point due to the lack of purified standards

it can be seen, the chromatographic resolution for all compounds was very good, providing baseline separation for many peaks, except for xylose/arabinose (peaks 8 and 9) and fructose/mannose/sorbitol (peaks 15, 16, and 17). Due to co-elution, mannose/glucose exhibited only one signal, i.e., mannose overlaps with sorbitol (26.30 min) and glucose overlaps with citric acid (26.91 min). Although the molecular weights (MWs) of mannose and sorbitol

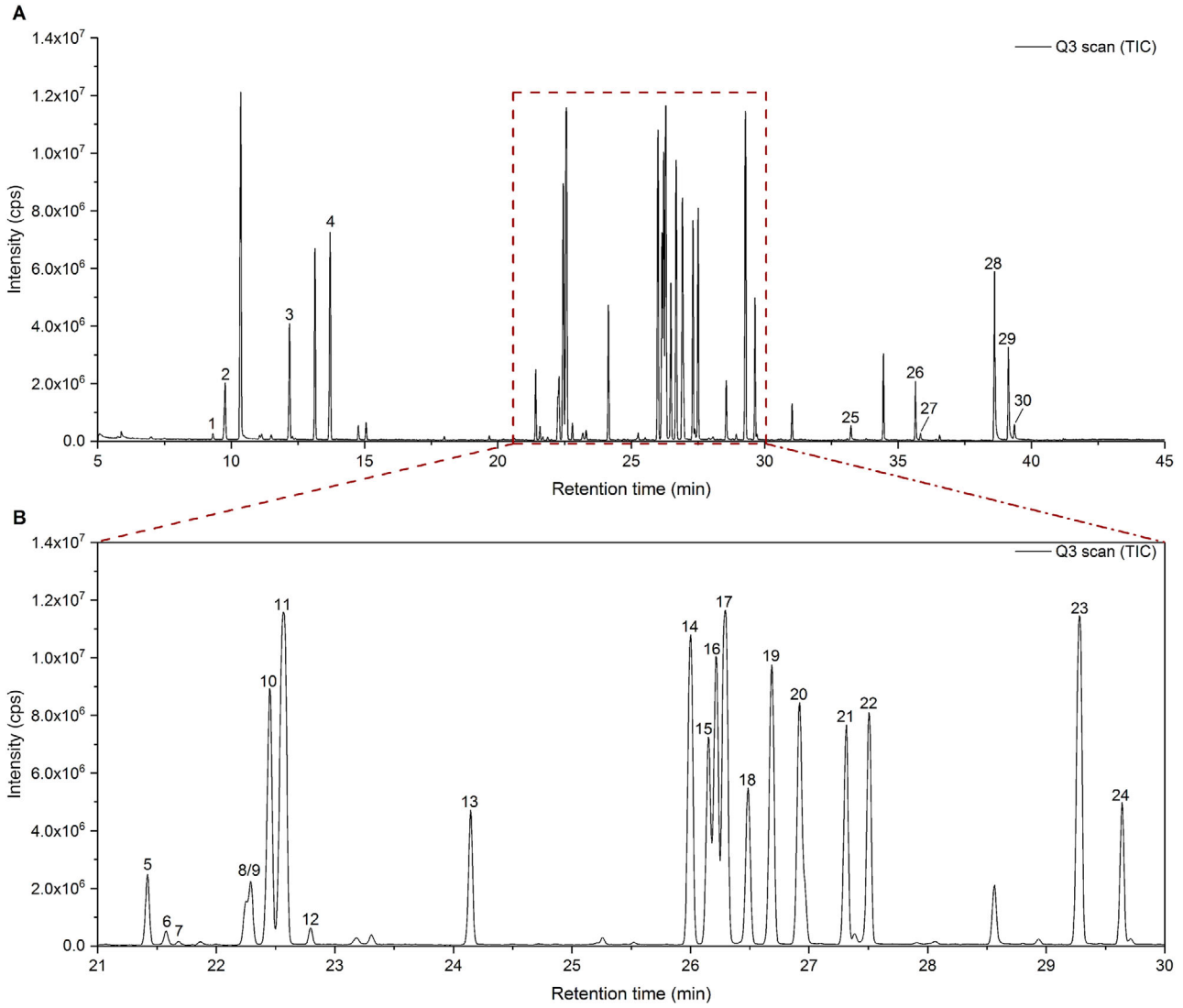

FIGURE 7 GC–MS chromatogram of a mixture of 24 metabolites belonging to the primary carbon metabolism. (A) Complete chromatogram, 5.00–45.00 min. (B) Zoom into the important time range between 21 and 30 min. Peak assignment: 1, glycine (N^1,O-TMS); 2, pyruvate (MeOx O-TMS); 3, norvaline (N^1,O-TMS); 4, glycine N^2, O-TMS; 5, cysteine (N^1,O,S-TMS); 6, 2-phenylglycine (N^1,O-TMS); 7, ornithine (α-N^1,δ-N^1,O-TMS); 8, xylose (MeOx tetrakis-O-TMS, 1Z/1E); 9, arabinose (MeOx tetrakis-O-TMS, 1Z/1E); 10, arabinose (MeOx tetrakis-O-TMS, 1Z/1E); 11, xylose (MeOx tetrakis-O-TMS, 1Z/1E); 12, glutamic acid (N^1,bis-O-TMS); 13, α-ketoglutaric acid (MeOx bis-O-TMS; 14, mannitol (hexakis-TMS); 15, fructose (MeOx pentakis-O-TMS, 1Z/1E); 16, mannose (MeOx pentakis-O-TMS, 1Z/1E); 17, sorbitol (hexakis-TMS); 18, fructose (MeOx pentakis-O-TMS, 1Z/1E); 19, glucose (MeOx pentakis-O-TMS, 1Z/1E); 20, citric acid (tetrakis-TMS); 21, lysine (α-N^1,ε-N^2,O-TMS); 22, α-(4-hydroxyphenyl)-glycine (N^1,O-TMS); 23, myo-inositol (hexakis-O-TMS); 24, ascorbic acid (tetrakis-TMS); 25, N-acetyl-glutamine (N^2,O-TMS); 26, G6P (MeOx hexakis-TMS, 1Z/1E); 27, G6P (MeOx hexakis-TMS, 1Z/1E); 28, sucrose (octakis-TMS); 29, lactose (MeOx octakis-TMS 1Z/1E); and 30, lactose (MeOx octakis-TMS 1Z/1E)

derivatives are different, 570.10 and 615.26 g/mol, respectively, the obtained precursor and fragment ions, thus monitored MRM transitions, are identical, so a separation by GC–MRM–MS was not possible. In contrast to that the co-eluting glucose (MW of the derivative: 570.11 g/mol) and citric acid (MW of the derivative of 408.67 g/mol) showed different transitions and thus differentiation by MS was possible. Nevertheless, quantification was possible for all compounds by using the second isomer of glucose (peak 19 at 26.68 min) and mannose (peak 16 at 26.22 min).

At this point, it has to be considered that the presence of mannose influences the signal of sorbitol. In the case of C5 sugars, ribose could not be separated from arabinose and xylose. The precursor and fragment ions are the same and the GC–MRM–MS method did not allow separation and quantification of single compounds.

TABLE 1 LODs, LOQs, and R^2 values for 24 representative metabolites of the primary carbon metabolism determined by GC–MRM

Compound	LOD (µM)	LOQ (µM)	R^2
Glycine	30.05	91.06	0.9974
L-Arginine	128.06	426.87	0.9875
L-Cysteine	4.64	14.05	0.9911
L-Glutamic acid	4.22	12.78	0.9921
L-Lysine	37.82	114.61	0.9875
N-Acetyl-L-glutamine	13.77	41.71	0.9966
D-(-)-α-(4-Hydroxyphenyl)-glycine	18.10	54.83	0.9925
D-2-Phenylglycine	0.77	2.32	0.9905
Citric acid	1.25	3.77	0.9883
L-Ascorbic acid	3.52	10.68	0.9939
α-Ketoglutaric acid	5.21	15.80	0.9837
Pyruvate	0.42	1.27	0.9959
D-(-)-Arabinose	1.00	3.02	0.9949
D-(-)-Fructose	3.98	12.05	0.9921
D-(+)-Glucose	0.26	0.80	0.9971
D-(+)-Mannose	0.80	2.42	0.9847
D-(-)-Ribose	–	–	–
D-(+)-Xylose	1.22	3.69	0.9928
Myo-inositol	2.50	7.57	0.9962
D-Lactose	2.35	7.13	0.9887
Sucrose	0.57	1.73	0.9917
D-Glucose-6-phosphate 1	6.88	20.86	0.9927
D-Glucose-6-phosphate 2	4.91	14.88	0.9990
D-Mannitol	3.02	9.16	0.9948
D-Sorbitol	3.73	11.30	0.9921

3.5 | Determination of LOD and LOQ

Most of the analytes showed excellent linear responses with *R*š > 0.99 (17 substances), a few only between 0.98 and 0.99 (six substances) and the resulting LODs, LOQs, and *R*š values are shown in Table 1. Analytical parameters for the organic acids could be determined without issues since all compounds were baseline separated. The co-elution of citric acid with one of the glucose isomers made analyses more tedious, yet possible. As reported above, two signals were observed for the sugar derivatives, but, most importantly, it was validated that the ratio of the obtained isomers remained constant for all concentration levels and therefore either the sum of the peak areas or only one peak could be considered for LODs and LOQs. In this study, ribose was excluded from the metabolite mixture since both of its derivatives co-eluted either with one derivative of arabinose or one of xylose. Both fructose peaks (26.15 and 26.49 min) could be obtained in the final chromatogram and yielded almost identical regression parameters and limits. Therefore, the final reported values represent averages resulting in an RSD of 0.03% for R^2 and 1.8% for LOD and LOQ. Arabinose and xylose were quantified by using the more intense peaks at 22.45 and 22.57 min for high sensitivity measurements. As described above two signals were also observed for glycine and the disaccharide lactose and again the more intensive was used for quantification (glycine 13.65 min, lactose 39.14 min). In the case of glucose (26.68 min) and mannose (26.22 min), the only peak observed was of course used for data evaluation. Both isomers observed for G6P at 35.65 and 35.84 min showed same performance characteristics, thus both peaks were used for evaluation.

In conclusion, the found LODs/LOQs can be considered as very satisfactory, since biologically relevant concentrations of metabolites in biological systems are very often in the low µM to a few hundred mM range [20], which is easily covered by the developed methodology.

4 | CONCLUDING REMARKS

GC coupled to EI-MS/MS showed to be a reliable platform for targeted metabolomics. The MRM technique allows highly sensitive and selective measurements by reducing background signals and thus increasing S/N. Although derivatization makes analysis more vulnerable to errors due to an increased number of preparation steps, the MeOx/TMS derivatization strategy allowed the analysis of a broad range of substance classes at biologically relevant LOD levels, i.e. 10^{-1} to 10^{-6} M.

Detailed studies of derivatization mechanisms and kinetics showed that derivatization is highly reproducibile allowing comparison between biological samples exhibiting very low expression levels changes. This will in the end provide a method fit for metabolomics applications targeting key analytes of the primary carbon metabolism and therefore energy levels of biological systems. However, it has to be noted that in complex samples co-elution can alter the determined absolute concentration levels, as observed for the measurement of arabinose/xylose in the presence of ribose or the altered derivatization behavior of glucose in the presence of lysine but not glycine.

ACKNOWLEDGEMENTS

We gratefully thank Shimadzu Austria for supporting this work by the loan of the TQ8040 and establishing the “Metabolomic and Bioprocess Analytics” Laboratory. Furthermore, we thank Prof. Günter Allmaier at TU Wien for his support and valuable discussions.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

ORCID

Martina Marchetti-Deschmann https://orcid.org/0000-0002-8060-7851

REFERENCES

1. Fiehn O. Metabolomics - the link between genotypes and phenotypes. Plant Mol. Biol. 2002;48:155-71.
2. Zhou B, Xiao JF, Tuli L, Ressom HW. LC-MS-based metabolomics. Mol. Biosyst. 2012;8:470-81.
3. Dettmer K, Aronov PA, Hammock BD. Mass spectrometry-based metabolomics. Mass Spectrom. Rev. 2007;26:51-78.
4. Roberts LD, Souza AL, Gerszten RE, Clish CB, metabolomics T. Curr. Protoc. Mol. Biol. 2012;98:30.32.31-30.32.24.
5. Pasikanti KK, Ho PC, Chan EC, Gas chromatography/mass spectrometry in metabolic profiling of biological fluids. J. Chromatogr. B 2008;871:202-11.
6. Koek MM, Muilwijk B, van der Werf MJ, Hankemeier T. Microbial metabolomics with gas chromatography/mass spectrometry. Anal. Chem. 2006;78:1272-81.
7. Chan EC, Pasikanti KK, Hong Y, Ho PC, Mahendran R, Raman Nee Mani L, Chiong E, Esuvaranathan K. Metabonomic profiling of bladder cancer. J. Proteome Res. 2015;14:587-602.
8. Villas-Boas SG, Mas S, Akesson M, Smedsgaard J, Nielsen J. Mass spectrometry in metabolome analysis. Mass Spectrom. Rev. 2005;24:613-46.
9. Fiehn O, Kopka J, Dormann P, Altmann T, Trethewey RN, Willmitzer L. Metabolite profiling for plant functional genomics. Nat. Biotechnol. 2000;18:1157-61.
10. Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L. Simultaneous analysis of metabolites in potato tuber by GCMS. Plant. J. 2000;23:131-42.
11. Gullberg J, Jonsson P, Nordstrom A, Sjostrom M, Moritz T. Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of Arabidopsis thaliana samples in metabolomic studies with gas chromatography/mass spectrometry. Anal. Bioanal. Chem. 2004;331:283-95.
12. Fiehn O, Kopka J, Trethewey RN, Willmitzer L. Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadrupole mass spectrometry.pdf. Anal. Chem. 2000;72:3573-80.
13. Glassbrook N, Beecher C, Ryals J. Metabolic profiling on the right path. Nat. Biotechnol. 2000;18:1142-3.
14. Villas-Boas SG, Smart KF, Sivakumaran S, Lane G A. Alkylation or Silylation for Analysis of Amino and Non-Amino Organic Acids by GC-MS? Metabolites 2011;1:3-20.
15. de Hoffmann E. Tandem mass spectrometry: A primer. J. Mass Spectrom. 1996;31:129-37.
16. Yi L, Shi S, Yi Z, He R, Lu H, Liang Y. MeOx-TMS derivatization for GC-MS metabolic profiling of urine and application in the discrimination between normal C57BL/6J and type 2 diabetic KK-Ay mice. Anal. Methods 2014;6:4380.
17. Peterson G. Gas-chromatographic analysis of sugars and related hydroxy acids as acyclic oxime and ester trimethylsilyl derivatives. Carbohydr. Res. 1974;33:47-61.
18. Tsugawa H, Bamba T, Shinohara M, Nishiumi S, Yoshida M, Fukusaki E. Practical non-targeted gas chromatography/mass spectrometry-based metabolomics platform for metabolic phenotype analysis. J. Biosi. Bioeng. 2011;112:292-8.
19. Welthagen W, Shellie RA, Spranger J, Ristow M, Zimmermann R, Fiehn O. Comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry (GC × GC-TOF) for high resolution metabolomics: biomarker discovery on spleen tissue extracts of obese NZO compared to lean C57BL/6 mice. Metabolomics 2005;1:65-73.
20. Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD, Absolute metabolite concentrations and implied enzyme active site occupancy in Escherichia coli. Nat. Chem. Biol. 2009;5:593-9.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Engel B, Suralik P, Marchetti-Deschmann M. Critical considerations for trimethylsilyl derivatives of 24 primary metabolites measured by GC-MS/MS. *Sep Sci plus*. 2020;3:407–418.
https://doi.org/10.1002/sscp.202000025