RESEARCH PAPER

Isotopologue analysis of sugar phosphates in yeast cell extracts by gas chromatography chemical ionization time-of-flight mass spectrometry

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Abstract Metabolic flux analysis is based on the measurement of isotopologue ratios. In this work, a new GC-MS-based method was introduced enabling accurate determination of isotopologue distributions of sugar phosphates in cell extracts. A GC-TOFMS procedure was developed involving a two-step online derivatization (ethoximation followed by trimethylsilylation) offering high mass resolution, high mass accuracy and the potential of retrospective data analysis typical for TOFMS. The information loss due to fragmentation intrinsic for isotopologue analysis by electron ionization could be overcome by chemical ionization with methane. A thorough optimization regarding pressure of the reaction gas, emission current, electron energy and temperature of the ion source was carried out. For a substantial panel of sugar phosphates both of the glycolysis and the pentose phosphate pathway, sensitive determination of the protonated intact molecular ions together with low abundance fragment ions was successfully achieved. The developed method was evaluated for analysis of *Pichia pastoris* cell extracts. The measured isotopologue ratios were in the range of 55:1–2:1. The comparison of the experimental isotopologue fractions with the theoretical fractions was excellent, revealing a maximum bias of 4.6 % and an average bias of 1.4 %.

Keywords Isotopologue fractions · Gas chromatography · Time-of-flight mass spectrometry · Methane chemical ionization · Sugar phosphates · Yeast cell extracts

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Introduction

[13]C-based metabolic flux analysis (^{13}C-MFA) is an emerging approach in biotechnology. In this specific application, isotope tracers, like ^{13}C, are introduced into an organism, where the isotope label is distributed according to the tracer's metabolic fate and will consequently lead to a characteristic pattern of labelled metabolites. Conducting these labelling experiments will not only allow you to unravel non-obvious metabolic pathways and their regulation, but has also become a key tool in metabolic engineering of microorganisms to prove successful genetic manipulations [1–3].

Evidently, the accurate measurement of isotopologue distributions by NMR or mass spectrometry forms the basis for such experiments, which can be carried out at different levels of complexity—either the isotopologue distribution of proteinogenic amino acids are addressed or in more sophisticated strategies isotopologue distributions of free metabolites have to be assessed. In these cases, the isotopologues of sugar phosphates are of utmost importance, since they represent the starting point of metabolization of the labelled substrate.

Gas chromatography employing capillary columns in combination with mass spectrometry is prominently used in the

field of metabolomics due to its typically high separation efficiency and peak capacity. In combination with mass spectrometric detection systems, it represents a highly selective and sensitive methodology [4–7]. State-of-the-art GC-MS methods cover a wide range of analytes (e.g. organic acids, amino acids, sugars and sugar phosphates), which can be separated within a single chromatographic run after appropriate derivatization [8, 9].

Regarding the highly challenging analysis of sugar phosphates occurring in multiple isomers in the metabolic network, methods providing full coverage of all biologically important sugar phosphates in the pentose phosphate pathway are still missing. In the past, capillary electrophoresis (CE)-MS offered high separation efficiency, low sample consumption and selective detection covering a wide range of metabolites (as it is suitable for separation of cationic, anionic and neutral compounds), however, the limit of detection and limit of quantification posed severe limitations to the application of the method in quantitative metabolite profiling, thus also for the analysis of sugar phosphates [10–14]. The state-of-the-art LC-MS methods addressing quantification of sugar phosphates in biological samples [15, 16] involve mixed-mode, anion exchange, ion pairing or hydrophilic interaction liquid chromatography (HILIC). Moreover, reversed phase [17], porous graphitized carbon [18] and propylamine [19] chromatographic columns and specialized columns such as Nucleodex ß-OH [20, 21] were shown to be suitable for separation of at least a substantial number of the sugar phosphates. Han et al. introduced pre-column derivatization prior to separation on pentafluorophenyl core-shell particles with methylphosphonic acid as an efficient tailing-sweeping reagent to alleviate the typically poor retention on reversed phase chromatographic material [22]. Mixed-mode (HILIC-weak anion exchange) chromatography employing MS-friendly mobile phases combined with tandem mass spectrometry for phosphorylated compounds has been established by Hinterwirth et al. [23]. Ion pairing chromatography has been successfully carried out with different reagents and columns [24–27]. However, a major drawback of ion pairing chromatography is the irreversible contamination of mass spectrometry instrumentation by the ion pairing reagents. Ion exchange chromatography in combination with mass spectrometry has been applied successfully for profiling of ionic intermediates after eluent suppression. According to Wang et al., anion exchange chromatography in combination with mass spectrometry and with anion suppression covered a wider range of anionic metabolites than HILIC or ion pairing chromatography [28]. In this context, we have recently presented a method for strong anion exchange chromatographic separation of sugar phosphates with ICP-MS detection [29], which was used as the first chromatographic dimension for an LCxLC-ESI-MS/MS method circumventing the incompatibility of electrospray ionization with high buffer concentrations [30]. Although the method

provided the separation of some important sugar phosphates, others had to be excluded due to instability at the high pH of the mobile phase.

Hence, we dare to state that GC-MS remains unrivalled regarding separation power, selectivity and sensitivity in the case of sugar phosphates [31–35] and will prove to be the method of choice for the analysis of this metabolite panel.

In the context of ^{13}C-MFA, two ionization modes, i.e. electron ionization (EI) and chemical ionization (CI) are applicable for the ionization of the alkyloximated/silylated derivatives of sugar phosphates. As a drawback, EI leads to nearly complete fragmentation of the molecular ion and does therefore not allow the isotopologue-selective measurement of labelled metabolites generated in biological tracer experiments. Moreover, high-intensity fragment ions (e.g. m/z 73, 147, 299 and 315) containing no carbon backbone of the sugar phosphate molecule are generated by, e.g. rearrangement of the silyl-group and do not deliver the required isotopologue information for ^{13}C-MFA analysis. Contrarily, chemical ionization is regarded as a soft ionization process generating intact protonated molecule or $[M-CH_3]^+$ fragment ions, where a methylgroup of the trimethylsilyl is abstracted, and therefore allowing isotopologue-selective analysis. Different reagent gases for CI including methane, isobutane and ammonia have been investigated in the context of metabolomics [36]. The observed intensity of protonated molecular ions was at least ten times higher as compared to electron ionization and only a low degree of fragmentation and non-selective fragments were obtained. Accordingly, chemical ionization is to date predominantly used for identity confirmation of organic compounds [37, 38]. In the present work, a novel method employing capillary gas chromatography in combination with methane chemical ionization time-of-flight mass spectrometry (GC-CI-TOFMS) is presented for the separation of sugar phosphates and the determination of their isotopologue fractions in yeast cell extract samples. Important ionization parameters, i.e. emission current, electron energy, reagent gas pressure and source temperature were optimized. To the best of our knowledge, this is the first time that GC-CI-TOFMS was used for the determination of isotopologue fractions of sugar phosphates in cell extract samples from *Pichia pastoris*.

Materials and methods

Chemicals

2-Phosphoglyceric acid disodium salt hydrate (2PG), 3-phosphoglyceric acid disodium salt (3PG), 6-phosphogluconic acid barium salt (6PGA), dihydroxyacetone phosphate lithium salt (DHAP), erythrose-4-phosphate sodium salt (E4P), fructose-6-phosphate disodium salt hydrate (F6P), glucose-6-phosphate dilithium salt (G6P), glyceraldehyde-3-phosphate

solution (3GAP), manose-6-phosphate disodium salt hydrate (M6P), mannitol-1-phosphate lithium salt (Mn1P), ribulose-5-phosphate sodium salt (Rb5P), ribose-5-phosphate disodium salt hydrate (R5P), xylulose-5-phosphate sodium salt (Xl5P) were purchased at Sigma Aldrich (Sigma Aldrich, Vienna, Austria) and sedoheptulose-7-phosphate lithium salt (S7P), was purchased from Carbosynth, UK. Stock standard solutions of all compounds were prepared by dissolving an appropriate amount of solid standard in LC-MS-grade water in amber vials. All single stocks were kept at −20 °C and were stable for a minimum of 3 weeks. The working standards were prepared daily in LC-MS grade water. Ethoxyamine hydrochloride was purchased from Sigma Aldrich, N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) in with 1 % trimethylchlorosilane (TMCS) from Thermo Scientific (Waltham, MA, USA). Ethoxyamine solution was prepared daily by dissolving an appropriate amount of solid substances in water-free pyridine (Sigma Aldrich). LC-MS grade water was used for standard and sample preparation. A mixture of n-alkanes from C_8 to C_{40} in hexane (Sigma Aldrich) was used for the calculation of modified retention indices.

Instrumentation

An Agilent 7200 GC-QTOFMS system (Agilent, Waldbronn, Germany) was used for the separation of sugar phosphates and analysis of their respective isotopologues. The system consisted of a G3440B Agilent 7890B network gas chromatograph equipped with a multimode injector and device backflush device and an Agilent G3851A orthogonal accelerator quadrupole time-of-flight mass spectrometer (QTOFMS) with removable ionization source (EI/CI ionization sources). A multipurpose sampler (MPSII, Gerstel, Germany) was used for automated two-step derivatization of the analytes and injection into the Agilent multimode injector. The sensitivity and accuracy of the mass spectrometer was checked before starting analysis with an internal calibrant solution containing perfluorotributylamine (PFTBA) and perfluoro-5,8-dimethyl-3,6,9-trioxydodecane (PFDTD) applying the mass calibration protocol of the system. The non-bleed septum and single tapered UltraInert Inlet Liners with O-ring pre-installed were changed after approximately hundred injections. The calibration of the mass axis of the TOFMS was programmed in each sequence after ten sample injections. Data acquisition and evaluation were carried out with MassHunter Acquisition and MassHunter Quantitative and Qualitative (Agilent Technologies, CA, USA), respectively.

Chemostat cultivation

The chemostat cultivations were performed in a 1.4-L benchtop bioreactor (DASGIP Parallel Bioreactor System, Germany) with a working volume of 400 mL. A 100-mL pre-culture medium (per litre: 10 g yeast extract, 20 g peptone, 10 g glycerol)

was inoculated from the working cell bank (750 μl cryostock of P. pastoris CBS7435) and grown at 28 °C and 150 rpm overnight. This culture was used for inoculation of the bioreactor at an optical density (OD600) of 1.0. After a batch phase of approximately 24 h the feed and harvest for the continuous chemostat cultivation were started. The cells were grown under glucose-limited conditions with a dilution rate of 0.1 h^{-1} for at least seven residence times before taking the sample. Three independent chemostat cultivations were performed. Temperature, pH and dissolved oxygen were maintained at 25 °C, pH 5.0 (adjusted with 8 M KOH) and 20 % (by controlling the stirred speed and inlet gas flow), respectively. The batch medium contained per litre: 39.9 g glycerol, 1.8 g citric acid, 12.6 g $(NH_4)_2HPO_4$, 0.022 g $CaCl_2 \cdot 2H_2O$, 0.9 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 2 mL Biotin (0.2 g L^{-1}), 4.6 mL trace salts stock solution. The pH was set to 5.0 with 32 % (w/w) HCl. The chemostat medium contained per litre: 55 g glucose $1H_2O$, 2.3 g citric acid, 21.75 g $(NH_4)_2HPO_4$, 0.04 g $CaCl_2 \cdot 2H_2O$, 2.5 g KCl, 1.0 g $MgSO_4 \cdot 7H_2O$, 2 g Biotin (0.2 g L^{-1}), and 2.43 g trace salts stock solution. The pH was set to 5.0 with 32 % (w/w) HCl. Trace salts stock solution contained per litre: 6.0 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4$ H_2O, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 5.0 g $FeSO_4 \cdot 7H_2O$, and 5.0 mL H_2SO_4 (95–98 % w/w).

Sampling and quenching

Approximately 50 mL of the fermentation broth was sampled into 200 mL of 60:40 methanol:water kept at approx. −30 °C for instantaneous quenching of the cellular metabolism. Subsequently, 2-mL aliquots of the quenched cell suspension (corresponding to approximately 10 mg cell dry weight) were filtered through cellulose acetate filters (0.45 μm, Sartorius BioLab Products) using two filtration units (Polycarbonate Filter Holder; Sartorius Lab Technologies Products) equipped with a vacuum pump in parallel. The cell pellet on the filter was washed once with cold quenching solvent before transfer to a 15-mL tube and stored at −80 °C until extraction. The exact biomass content of the sample aliquot was determined by drying five replicates of 2-mL quenched cell suspension to constant weight at 105 °C in pre-weighed glass tubes.

Extraction of sugar phosphates

The metabolite extraction procedure was adapted from Neubauer et al. [39]. In brief, 4 mL of pre-heated extraction solvent (75 %v/v ethanol) was poured onto the filter. After vortexing for approx. 20 s, the samples were incubated at 85 °C in a water bath for 3 min with intermediate mixing. After rapid cooling-down on dry ice, the samples were centrifuged at 4000×g for 10 min at −20 °C. The supernatant was decanted into pre-cooled 15-mL tubes. The ethanolic extracts were stored on at −80 °C.

Automated just in time online derivatization of sugar phosphates

Direct silylation of sugar phosphates leads to the occurrence of a large number of different chromatographic peaks caused by the presence of different isomers of each sugar phosphate, related to cyclic and open-chain structures as introduced by Harvey et al. [44]. In addition, the trimethylsilyl (TMS) derivative of ketose and aldose phosphates is unstable. By introducing an alkyloximation step prior to silylation, the carbonyl group is protected. For the protection of the ketogroups already during sample preparation [9], 10 μL of ethoxyamine hydrochloride in water-free pyridine c(EtOX)=19 mg mL^{-1} was added to all sample aliquots and standard ethoximation before drying. The solutions were then dried to complete dryness in a vacuum centrifuge operating at low pressure (below 1 mbar). The two-step derivatization procedure was modified for the MPS2 based on the derivatization parameters published by Koek et al. [5]. For ethoximation, the dried sample was reconstituted in 18 μL ethoxyamine in water-free pyridine (18.7 mg mL^{-1}) and incubated at 40 °C for 90 min Subsequently 42 μL MSTFA with 1 % TMS, was added and the samples were incubated at 40 °C for 50 min for silylation. The derivatized samples were put at 4 °C for approx. 4 min for sample cool down and where then injected by the MPS2.

Determination of retention indices

Because of the low sensitivity of alkane retention index compounds in CI, electron ionization was used to calculate modified retention indexes (gradient temperature retention indices). The retention index mixture was prepared by diluting the Sigma Aldrich retention index standard containing C_8–C_{40} alkanes in 18 μL ethoxyamine in water-free pyridine and 42 μL of MSTFA with 1 % TMCS and injected into GC-EI-TOFMS. The modified retention indices were calculated as described elsewhere [40].

Analysis of sugar phosphates by GC-CI/EI-TOFMS

The sugar phosphate derivatives were separated on a nonpolar Optima 1MS Accent analytical column (Macherey-Nagel, Germany, 60 m×0.25 mm i.d., 0.25 μm film thickness, 100 % dimethylpolysiloxane stationary phase), which was protected by a deactivated nonpolar guard column (5 m× 0.32 mm i.d., Agilent). Helium (5.0, Linde Austria) was used as carrier gas at a constant flow rate of 1.3 mL min^{-1}. GC injection of 1.0 μL aliquots of sample solution was performed in hot split/splitless mode (splitless time 1 min, 250 °C). The following GC temperature gradient was used: 70 °C for 1 min, 20 °C min^{-1} to 190 °C, 5 °C min^{-1} to 225 °C, 3 °C min^{-1} to 260 °C and 20 °C min^{-1} to 310 °C (hold for 1 min). The solvent delay was set to 7 min. Back-flushing was employed

for 3 min at 310 °C after each chromatographic run. A more detailed information and discussion regarding derivatization and separation of sugar phosphates on the nonpolar Optima 1MS Accent column is in preparation [41].

Chemical ionization was performed with methane (for GC-MS, 5.0, Linde Austria) as reagent gas. For internal standardization during optimization of the source for chemical ionization, meso-erythritol was chosen as internal standard. Optimum CI source parameters were 200 eV electron energy and 40 % methane gas flow (equal to 2.0 mL min^{-1}) at 150 °C. The emission current was kept at 10 μA. The TOFMS was set to a mass acquisition range of 70–900 m/z. For electron impact ionization, the ion source temperature and emission current applied to the filament were set to 220 °C and 35 μA, respectively. Electron energy was kept at 70 eV. With both ionization modes, the TOFMS was operated in 2 GHz-EDR mode (2 GHz extended dynamic range) in order to extend the linear dynamic range.

Isotopologue distribution calculation

In order to evaluate the method regarding the trueness of the determined isotopologue fractions in real matrices, five independently prepared yeast cell extracts were analysed applying the conditions described above. Isotopologue fractions were calculation according to Eq. 1.

$$IF_i = \frac{A_i}{\sum_{i=0}^{n} A_i} \tag{1}$$

where, IF_i is the measured isotopologue fraction i and n is the number of carbon atoms in the metabolite backbone. A_i represents the peak areas of the respective isotopologues obtained by integration of the extracted ion chromatograms (±50 ppm mass extraction window). The trueness (bias) of the results was obtained by subtracting the natural isotopologue fractions (calculated via envipat open-source software [42]) from the experimental values.

Results and discussion

Optimization of chemical ionization source parameters

In this work, methane and ammonia were tested as reagent gases for ionization of sugar phosphates with CI. The spectra obtained with ammonia as reagent gas revealed less fragmentation of the derivatized sugar phosphates, whereas the intensities of the protonated molecules in the mass spectra were similar to those observed with methane (data not shown). However, due to safety considerations, methane was preferred for further optimization. Using methane, the three dominant reactive species formed by ionization of the reagent gas are
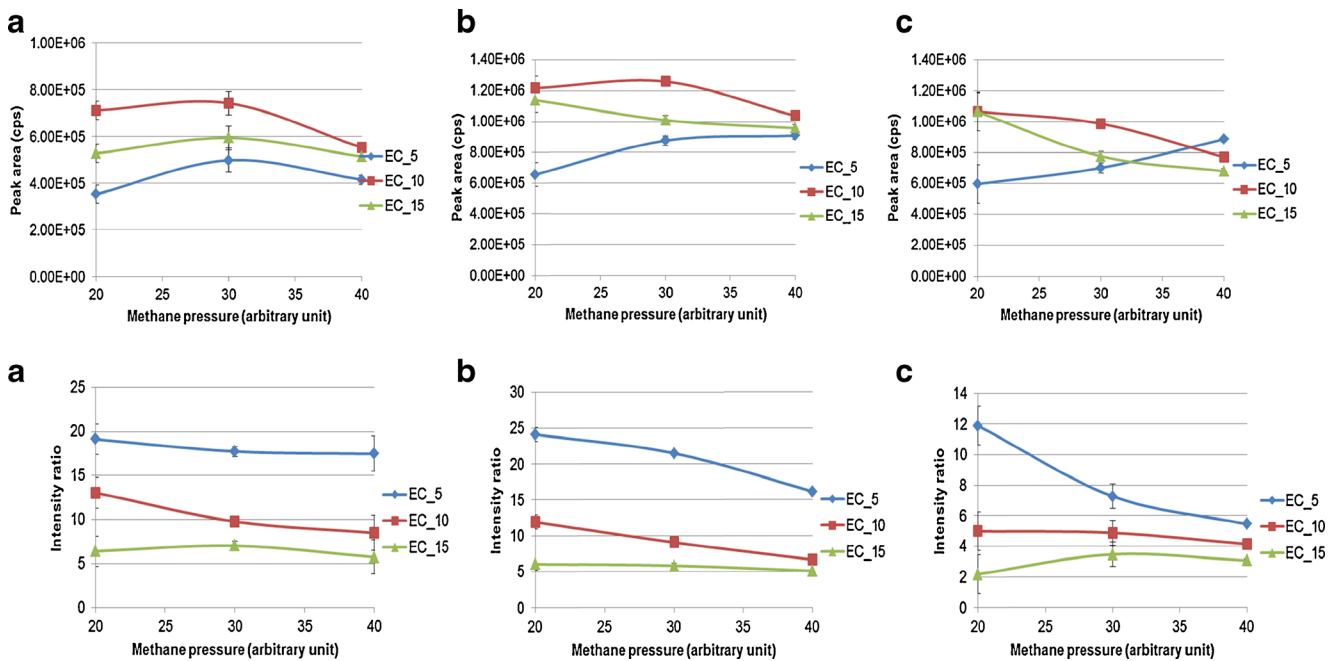
Fig. 1 Dependence of sensitivity and degree of fragmentation of (*a*) 3PG, (*b*) R5P and (*c*) G6P on the emission current and methane pressure. The *upper graphs* show the measured intensity of the protonated molecules, whereas the *lower graphs* depict the intensity ratio of the protonated molecules vs. the derivatized phosphate ion (EC_5, EC_10 and EC_15 correspond to emission currents of 5, 10 and 15 μA)

CH_5^+ (*m/z* 17.0386), $C_2H_5^+$ (*m/z* 29.0381) and $C_3H_5^+$ (*m/z* 41.0393). Three crucial parameters of the CI source requiring optimization are the reaction gas pressure, the ion source temperature and the emission current applied to the CI filament [43]. For monitoring, the influence of reaction gas pressure on the ratio of $C_2H_5^+$ and $C_3H_5^+$, the methane flow rate was

stepwise increased from 10 to 50 % (corresponding to 0.5 to 2.5 ml min^{-1} methane gas flow) at a source temperature of 150 °C (see below). CH_5^+ could not be monitored, as the mass range is restricted to *m/z* 20 in the TOFMS software. The reagent gas pressure was optimized for positive chemical ionization (PCI) in a way that the abundance of the $C_3H_5^+$ ion

Table 1 Mass accuracy and limit of detection (LOD) of protonated molecules of derivatized sugar phosphates measured by GC-TOFMS after chemical ionization utilizing methane as reaction gas

No.	Protonated molecular ions	Chemical formula of ions	Modified retention indeces	Calculated *m/z*	Experimental *m/z* mean±sd (n=5)	Error (mDa)	Mass accuracy (ppm)	LOD (μM) (n=5)
1	[PEP_3TMS+H]$^+$	$C_{12}H_{30}O_6PSi_3^+$	1500.9	385.1082	385.1108±0.0005	2.6	6.8	0.4
2	[DHAP_1Ethox_3TMS+H]$^+$	$C_{14}H_{37}NO_6PSi_3^+$	1700.1, 1800.0	430.1661	430.1673±0.0004	1.2	2.8	0.4
3	[3GAP_1Ethox_3TMS+H]$^+$	$C_{14}H_{37}NO_6PSi_3^+$	1700.6, 1700.7	430.1661	430.1671±0.0004	1.0	2.3	0.5
4	[2PG_4TMS+H]$^+$	$C_{15}H_{40}O_7PSi_4^+$	1700.9	475.1583	475.1606±0.0005	2.3	4.8	0.8
5	[3PG_4TMS+H]$^+$	$C_{15}H_{40}O_7PSi_4^+$	1800.1	475.1583	475.1598±0.0005	1.5	3.1	0.6
6	[E4P_1Ethox_4TMS+H]$^+$	$C_{18}H_{47}NO_7PSi_4^+$	1800.7, 1800.9	532.2162	532.2155±0.0013	−0.7	−1.3	0.2
7	[R5P_1Ethox_5TMS+H]$^+$	$C_{22}H_{57}NO_8PSi_5^+$	2101.0, 2200.1	634.2663	634.2690±0.0008	2.7	4.3	0.1
8	[Rb5P_1Ethox_5TMS+H]$^+$	$C_{22}H_{57}NO_8PSi_5^+$	2101.0, 2100.6	634.2663	634.2659±0.0008	−0.4	−0.6	0.1
9	[Xl5P_1Ethox_5TMS+H]$^+$	$C_{22}H_{57}NO_8PSi_5^+$	2101.0, 2100.8	634.2663	634.2661±0.0008	0.2	0.3	0.2
10	[F6P_1Ethox_6TMS+H]$^+$	$C_{26}H_{67}NO_9PSi_6^+$	2399.9, 2400.0	736.3164	736.3176±0.0011	1.2	1.6	0.1
11	[G6P_1Ethox_6TMS+H]$^+$	$C_{26}H_{67}NO_9PSi_6^+$	2400.2, 2400.6	736.3164	736.3193±0.0011	2.9	3.9	0.1
12	[M6P_1Ethox_6TMS+H]$^+$	$C_{26}H_{67}NO_9PSi_6^+$	2400.1, 2400.5	736.3164	736.3177±0.0013	1.3	1.7	0.1
13	[Mn1P_6TMS+H]$^+$	$C_{27}H_{72}O_9PSi_7^+$	2400.3	767.3293	767.3311±0.0008	1.8	2.3	0.2
14	[6PGA_6TMS+H]$^+$	$C_{27}H_{70}O_{10}PSi_7^+$	2400.9	781.3086	781.3089±0.0007	0.3	0.4	0.5
15	[S7P_1Ethox_7TMS+H]$^+$	$C_{30}H_{77}NO_{10}PSi_7^+$	2600.6, 2600.8	838.3664	838.3641±0.0025	−2.3	2.7	0.1

TMS trimethylsilyl group (−Si(CH$_3$)$_3$), *Ethox* ethoxylamine group (=NOCH$_2$CH$_3$)

was 20 % higher than that of the $C_2H_5^+$ ion. Otherwise, increased fragmentation would lead to low signal intensities of the protonated molecules due to increased fragmentation. As the proton affinities of $C_2H_5^+$ and $C_3H_5^+$ are higher than that of CH_5^+, these ions give the highest possible yield of the protonated molecules as fragmentation is less favoured.

However, the application of the optimized reaction gas settings to the analysis of derivatized sugar phosphates revealed the need of further compound specific optimization. A mixture containing a 3PG, a tetrose phosphate (E4P), a pentose phosphate (R5P), hexose phosphate (G6P) and sedoheptulose 7 phosphate was used to find an optimum compromise

Fig. 2 Experimental mass spectra of ribose-5-phosphate obtained **a** in CI mode with 20 %, **b** in CI mode with 40 % of methane gas flow and **c** in EI mode at 70 eV. The *m/z* values given in the spectra represent the theoretical (exact) monoisotopic masses of the detected ions. The measured accurate mass values and are given in Table 1
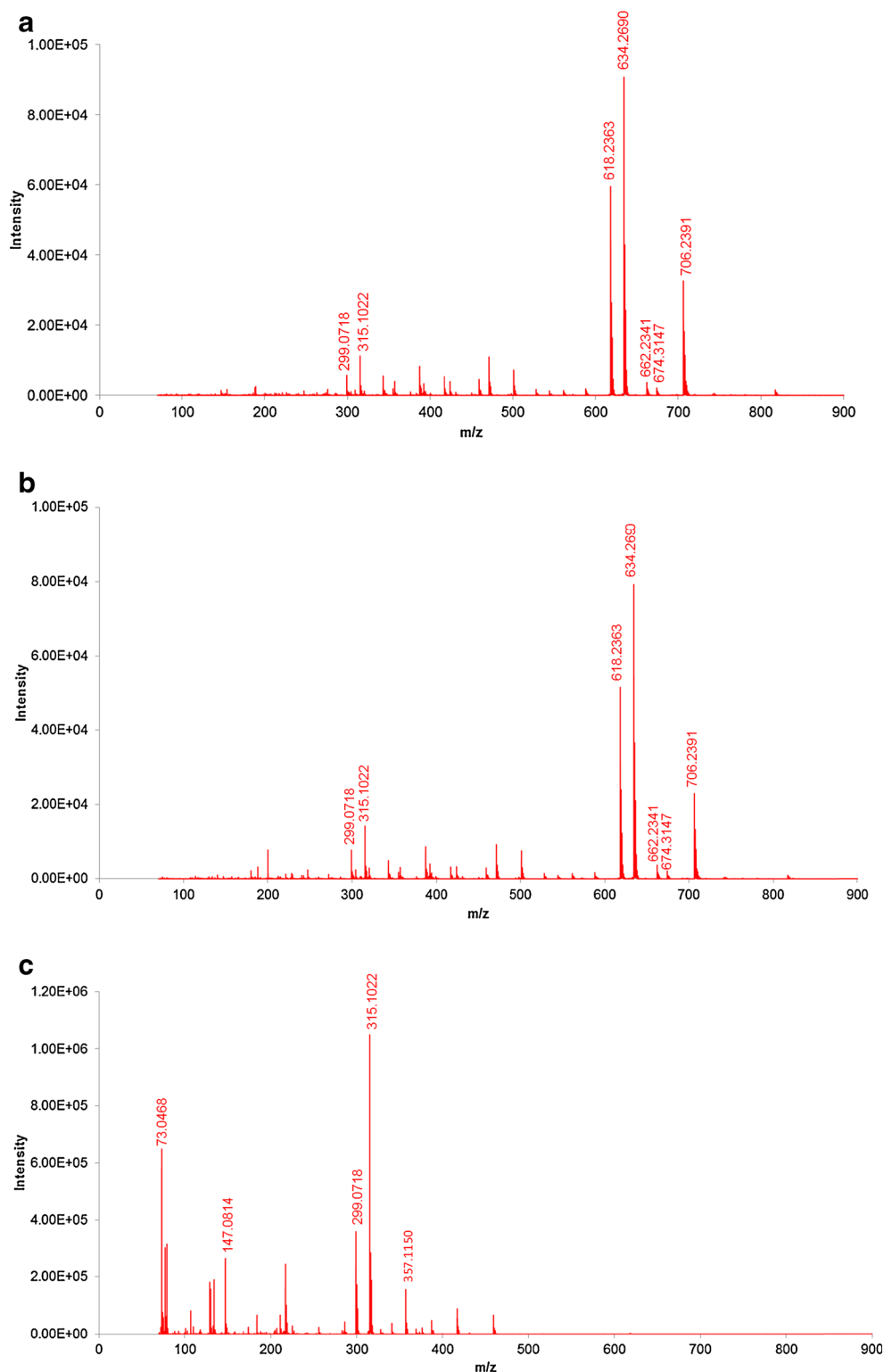
regarding the settings of the source parameters mentioned above. For this purpose, the intensity ratios of the protonated molecular ion vs. the phosphorous containing base peak present in EI mass spectra (m/z 315.1022 ($C_9H_{20}O_4PSi^+$) or 299.0718 ($C_8H_{17}O_4PSi^+$), according to Harvey and Horning [44]) was calculated for the different source settings (see Fig. 1).

As shown in Fig. 1, the intensity of the protonated molecular ions of 3PG, R5P and G6P revealed a maximum at an emission current of 10 µA at all selected methane gas flows. At 40 % methane, an emission current of 5 µA showed an improvement regarding sensitivity for high masses only. Independent of the methane pressure, extensive fragmentation was observed when applying higher emission currents on the filament. Considering the extent of fragmentation, it became evident that an emission current of 5 µA would be preferable, but as the precision of isotopologue ratio analysis is strongly depending on sensitivity (ion counting statistics), an emission current of 10 µA was selected as optimum value.

The collision probability between the analyte molecules and the reagent gas increases with gas pressure. As a consequence, the number of proton transfer reactions also increases, especially for high mass analytes. On the other hand, a high pressure of methane in the ion source will lead to lower transmission as observed by Schoots et al. [42]. Fragmentation was lower at lower flow rates, but with the drawback of low sensitivity. Over the investigated mass range, the highest sensitivity was obtained at a methane gas flow of 30 %. Since measurement precision was significantly higher at 40 % than at 30 % methane, we selected 40 % as optimum value.

Temperature and electron energy are additional source parameters affecting the degree of fragmentation and the intensity of protonated molecules. As fragmentation of the derivatized sugar phosphates increased with temperature, an ion source temperature of 150 °C was set as an optimum value. Even lower ion source temperatures were avoided in order to circumvent contamination of the ion source and the quadrupole analyser system. Optimization of electron energy was performed using the internal calibrant solution of the instrument. Changes of electron voltage affected the intensities of fragment ions and protonated molecule to the same degree. According to Gross [45], electron energy should not be lower than 200 eV. As higher voltages did not improve sensitivity, the electron voltage was set to this minimum value, which is also beneficial regarding filament life time. All remaining source parameters, e.g. repeller and ion focusing voltages were first optimized with the automated tuning procedure. Repeller voltage was then further optimized via manual tuning using the internal PFDTD calibrant solution following the suggestions of Abate et al. [37].

Methane chemical ionization mass spectra of derivatized sugar phosphates

In time-of-flight mass spectrometry, accurate mass measurement of protonated molecular ions enables identity confirmation of unknown compounds. Moreover, selectivity is significantly improved, as very narrow mass extraction windows can be used for generation of extracted ion chromatograms. The average mass accuracies obtained for five independent measurements for the investigated sugar phosphates are listed in Table 1. The TOFMS system employed within this work showed an excellent mass accuracy below 7 ppm (external mass calibration).

Representative mass spectra of R5P are depicted in Fig. 2a–c. With CI (2a, 2b), the protonated molecule [M+H]$^+$ with m/z 634.2690 and [M-CH$_3$]$^+$ with m/z 618.2363 represented the most abundant ions. Additionally, an ion at m/z [M+73.0486]$^+$ was observed in the mass spectrum especially in the case of analytes with high molecular mass. This ion represents an adduct with the trimethysilylium ion which is formed in the source, as a consequence of the large excess of

Fig. 3 Extracted ion chromatograms of a 25 µM mixture of selected sugar phosphates separated on an Optima 1MS Accent capillary GC column after two-step derivatization combining ethoxymation and trimethylsilylation (50 ppm mass extraction window)
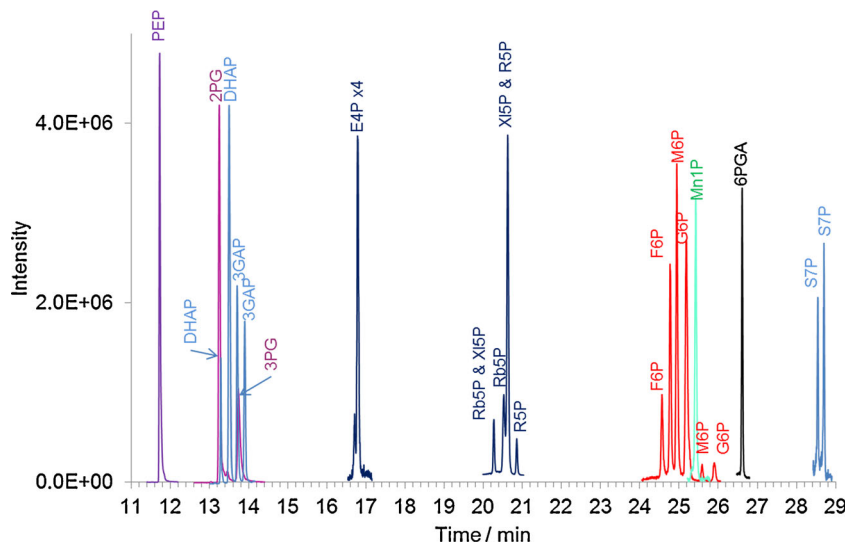
Table 2 Trueness (bias) and precision of isotopologue fractions (IF_{exp}) of sugar phosphates in yeast cell extracts (*P. pastoris*)

Analyte	IF	$Area_{meas}$ (counts)					IF_{exp} (%)					IF_{exp} (%) Mean±SD	IF_{theor}	Bias
		A1	A2	A3	A4	A5	IF_{A1}	IF_{A2}	IF_{A3}	IF_{A4}	IF_{A5}			
PEP	MH+0	346,637	65,424	226,565	273,028	228,519	70.7	68.4	70.5	70.2	70.1	70.0±0.9	69.8	−0.2
	MH+1	87,342	17,577	57,515	70,443	59,290	17.8	18.4	17.9	18.1	18.2	18.1±0.2	20.0	1.9
	MH+2	56,539	12,670	37,479	45,352	38,338	11.5	13.2	11.7	11.7	11.8	12.0±0.7	10.3	1.7
DHAP	MH+0	24,398	29,372	23,414	16,097	22,034	65.5	67.3	66.6	66.5	65.3	66.2±0.8	67.9	1.7
	MH+1	8627	10,681	8324	5899	8307	23.1	24.5	23.7	24.4	24.6	24.1±0.6	21.4	−2.7
	MH+2	4252	3585	3438	2195	3387	11.4	8.2	9.8	9.1	10.0	9.7±1.2	10.7	1.0
3GAP	MH+0	13,950	16,381	4204	9461	6332	68.1	64.3	63.2	65.2	61.9	64.5±2.3	67.9	3.4
	MH+1	4304	6677	1590	3576	3017	21.0	26.2	23.9	24.6	29.5	25.0±3.1	21.4	−3.7
	MH+2	2243	2436	858	1474	883	10.9	9.6	12.9	10.2	8.6	10.4±1.6	10.7	0.3
2PG	MH+0	24,398	308,351	1,350,332	1,930,537	150,7778	65.5	65.5	65.7	65.1	65.6	65.5±0.2	63.0	−2.5
	MH+1	8627	102,464	450,628	664,754	508,567	23.1	21.8	21.9	22.4	22.1	22.3±0.5	23.5	1.3
	MH+2	4252	59,662	253,190	367,961	280,741	11.4	12.7	12.3	12.4	12.2	12.2±0.5	13.4	1.2
3PG	MH+0	15,140	53,031	78,312	54,705	53,625	63.3	64.6	64.8	64.1	64.8	64.3±0.6	63.0	−1.3
	MH+1	5605	18,665	26,386	19,156	18,895	23.4	22.7	21.8	22.4	22.8	22.7±0.6	23.5	0.9
	MH+2	3187	10,447	16,084	11,470	10,280	13.3	12.7	13.3	13.4	12.4	13.0±0.4	13.4	0.4
E4P	MH+0	4377	11,294	8382	7153	8916	56.2	58.4	55.5	54.4	59.7	56.8±2.2	58.8	2.0
	MH+1	1919	4700	3831	3591	3362	24.6	24.3	25.3	27.3	22.5	24.8±1.7	24.1	−0.7
	MH+2	1078	2655	2232	1680	1918	13.8	13.7	14.8	12.8	12.8	13.6±0.8	13.3	−0.2
	MH+3	420	693	668	722	737	5.4	3.6	4.4	5.5	4.9	4.8±0.8	3.7	−1.1
R5P	MH+0	32,513	19,340	19,894	21,125	26,844	50.6	50.4	50.8	49.3	49.4	50.1±0.7	51.4	1.3
	MH+1	15,909	9124	9460	10,695	13,654	24.8	23.8	24.2	25.0	25.1	24.6±0.6	26.0	1.4
	MH+2	10,503	6226	6557	7301	9191	16.3	16.2	16.7	17.1	16.9	16.7±0.4	15.7	−1.0
	MH+3	3792	2452	2340	2682	3145	5.9	6.4	6.0	6.3	5.8	6.1±0.3	5.3	−0.8
	MH+4	1549	1255	920	1007	1490	2.4	3.3	2.3	2.4	2.7	2.6±0.4	1.7	−0.9
Rb5P	MH+0	17,335	18,284	19,139	24,548	18,261	52.6	53.7	52.9	54.4	54.8	53.7±1.0	51.4	−2.3
	MH+1	8379	8265	8311	10,944	7420	25.4	24.3	23.0	24.3	22.3	23.8±1.2	26.0	2.1
	MH+2	4904	4866	6215	6528	5113	14.9	14.3	17.2	14.5	15.3	15.2±1.2	15.7	0.4
	MH+3	1658	1995	1796	2476	1743	5.0	5.9	5.0	5.5	5.2	5.3±0.4	5.3	−0.1
	MH+4	711	612	722	626	779	2.2	1.8	2.0	1.4	2.3	1.9±0.4	1.7	−0.2
Xl5P	MH+0	20,139	17,949	20,571	20,139	20,389	48.1	48.7	48.9	48.1	47.6	48.3±0.5	51.4	3.1
	MH+1	10,883	7990	10,599	10,883	10,704	26.0	21.7	25.2	26.0	25.0	24.8±1.8	26.0	1.2
	MH+2	7588	7820	7450	7588	8317	18.1	21.2	17.7	18.1	19.4	18.9±1.4	15.7	−3.3
	MH+3	2367	2243	2621	2367	2535	5.7	6.1	6.2	5.7	5.9	5.9±0.3	5.3	−0.7
	MH+4	866	824	837	866	896	2.1	2.2	2.0	2.1	2.1	2.1±0.1	1.7	−0.3
F6P	MH+0	20,064	17,232	9449	20,064	11,615	47.3	46.1	47.8	47.3	46.1	46.9±0.8	45.1	−1.9
	MH+1	10,268	9143	4998	10,268	6326	24.2	24.5	25.3	24.2	25.1	24.7±0.5	27.1	2.5
	MH+2	6913	6032	2872	6913	4255	16.3	16.1	14.5	16.3	16.9	16.0±0.9	17.7	1.6
	MH+3	3441	2937	1537	3441	1851	8.1	7.9	7.8	8.1	7.3	7.8±0.3	6.9	−0.9
	MH+4	1282	1383	724	1282	944	3.0	3.7	3.7	3.0	3.7	3.4±0.4	2.5	−0.9
	MH+5	445	627	169	445	209	1.0	1.7	0.9	1.0	0.8	1.1±0.3	0.7	−0.4
G6P	MH+0	282,001	150,139	194,381	286,637	241,382	49.9	49.2	49.3	49.7	50.1	49.7±0.4	45.1	−4.6
	MH+1	135,924	73,514	96,461	139,723	116,261	24.1	24.1	24.5	24.2	24.1	24.2±0.2	27.1	2.9
	MH+2	86,763	46,856	60,243	88,734	72,822	15.4	15.4	15.3	15.4	15.1	15.3±0.1	17.7	2.4
	MH+3	36,829	20,378	26,109	38,196	31,667	6.5	6.7	6.6	6.6	6.6	6.6±0.1	6.9	0.3
	MH+4	16,304	9409	11,790	17,016	13,943	2.9	3.1	3.0	2.9	2.9	3.0±0.1	2.5	−0.4
	MH+5	6900	4566	5062	6831	5673	1.2	1.5	1.3	1.2	1.2	1.3±0.1	0.7	−0.6
M6P	MH+0	14,201	17,935	9732	14,404	11,007	45.7	46.2	46.6	46.8	46.1	46.3±0.4	45.1	−1.2

Table 2 (continued)

Analyte	IF	Area_meas (counts)					IF_exp (%)					IF_exp (%) Mean±SD	IF_theor	Bias
		A1	A2	A3	A4	A5	IF_A1	IF_A2	IF_A3	IF_A4	IF_A5			
	MH+1	7887	9663	5011	7173	6013	25.4	24.9	24.0	23.3	25.2	24.5±0.9	27.1	2.6
	MH+2	5353	6386	3487	5019	3799	17.2	16.5	16.7	16.3	15.9	16.5±0.5	17.7	1.2
	MH+3	2107	2984	1440	2234	1681	6.8	7.7	6.9	7.3	7.0	7.1±0.4	6.9	−0.2
	MH+4	1001	1225	815	1409	868	3.2	3.2	3.9	4.6	3.6	3.7±0.6	2.5	−1.2
	MH+5	535	615	421	552	506	1.7	1.6	2.0	1.8	2.1	1.8±0.2	0.7	−1.2
S7P	MH+0	10,831	20,994	8976	15,011	13,278	40.2	39.8	39.2	39.7	39.3	39.6±0.4	40.2	0.6
	MH+1	7505	15,038	6491	10,322	9747	27.8	28.5	28.4	27.3	28.9	28.2±0.6	28.0	−0.1
	MH+2	4805	10,041	4676	7351	6434	17.8	19.0	20.4	19.5	19.0	19.2±0.9	19.6	0.5
	MH+3	2548	4157	1976	2753	2877	9.4	7.9	8.6	7.3	8.5	8.4±0.8	8.7	0.3
	MH+4	1280	2567	761	2331	1445	4.7	4.9	3.3	6.2	4.3	4.7±1.0	3.5	−1.2

bias=IF_exp−IF_theor; isotopologues which are not reported where below the limit of detection. Mn1P and 6PGA where not detected in the samples

derivatization reagent [46]. Ions which are typically formed in EI (Fig. 2c), i.e. the phosphorus-based nonspecific fragments at m/z 315.1022 ($C_9H_{20}O_4PSi^+$) and at 299.0718 ($C_8H_{17}O_4PSi^+$) and unspecific fragments from trimethylsilylation, i.e. $(CH_3)_3Si^+$ at m/z 73.0468 and $(CH_3)_3SiOSi^+(CH_3)_2$ at m/z 147.0814 showed extremely low abundance in the CI spectra. Ions with masses higher than those of the protonated molecules are $[M+C_2H_5]^+$ at m/z $[M+29.0386]^+$ and $[M+C_3H_5]^+$ at m/z $[M+41.0386]^+$. However, the intensities of those signals were significantly lower than those of the protonated molecules.

Separation and detection of derivatized sugar phosphates via GC-CI-TOFMS

An aqueous sugar phosphate standard containing 25 μM of each compound was dried, derivatized and analysed according to a procedure developed by Troyer et al. [41] using the optimized conditions for GC-CI-TOFMS. As can be seen in Fig. 3, the chromatographic method provides excellent separation of all compounds of interest for at least one of the *cis*- and *trans*-isomers with the exception of Xl5P. The Xl5P isomers co-elute with R5P and Rb5P. However, as the ratio of the two derivatization products is constant, the area of Xl5P could be calculated as proposed by Cipollina et al. [8]. Additionally, the panel of substances amenable to GC methods is generally limited due to poor derivatization yields and instability of derivatives [9], hampering, e.g. the analysis of glucose-1-phosphate and fructose-1, 6-biphosphate, which would be of biotechnological interest.

Isotopologue analysis and determination of isotopologue fractions

As sugar phosphates are important primary compounds of the central carbon metabolism both their selective chromatographic separation and isotopologue-selective detection for determination of isotopologue fractions in ^{13}C tracer experiments is highly desirable. As a matter of fact, the accuracy, i.e. trueness and uncertainty, of isotopologue ratio analysis significantly affects all further data processing in the context of ^{13}C metabolic flux analysis. Table 2 summarizes the isotopologue distributions determined for selected sugar phosphates in yeast cell extracts. Theoretical isotopologue fractions were calculated by isotope distribution calculator tool from envipat open-source software [42] and compared with the experimental values. As can be seen in Table 2, the experimental isotopologue fractions (IF_exp) of all analysed sugar phosphates were in excellent agreement with the theoretical values (IF_theor). The average bias was 1.4 % with a maximum value of 4.6 %. The range of the accurately determined isotopologue ratios is directly reflecting the linearity of the method. According to our results, the method is capable to determine isotopologue ratios in the range of 55:1–2:1 (Table 2). The LODs given in Table 1 were calculated following the novel EURACHEM validation guideline and are representing the lower limit of the working range [47]. Repeatability of five independent replicates was below 1 % standard deviation in most cases. This result can be attributed to counting statistics and peak integration repeatability, but is, in contrast to quantitative analysis, independent of derivatization repeatability. Higher values can be explained by the low abundance of the respective metabolites in the yeast cell extracts.

Conclusion

The presented GC-CI-TOFMS method providing modified retention indices, high mass accuracy and accurate isotopologue distribution for sugar phosphates shows a high potential for ^{13}C-based metabolic flux analysis. Compared to EI, the low degree of fragmentation and simpler mass spectra

of CI are advantageous especially in isotopologue ratio measurement.

As a general drawback of derivatization, the addition of silicon and carbon atoms demands for correction of the isotopologue distribution before implementation in metabolic flux modelling. Current developments include the programming and validation of a software correcting for the contribution of the natural abundance of the isotopes entered via the derivatization procedure. For this purpose, an object-oriented software tool will be developed using the programming language Perl. It relies on data caching in the form of lookup tables. The use of lookup tables significantly reduces the execution time for the computation of the multinomial distributions and significantly improves the overall performance of the software. The data obtained after this correction step can be directly imported into dedicated software products for MFA such as OpenFLUX2 [48] or 13CFLUX2 [49].

In order to obtain information on the position of the ^{13}C labels, future developments will aim at isotopologue-selective fragmentation via GC-CI-QTOFMS.

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