Thermal degradation of metabolites in urine using multiple isotope-labelled internal standards for off-line GC metabolomics - effects of injector and oven temperatures

Emmanouil D. Tsochatzis\textsuperscript{a,b}, Caroline Nebel\textsuperscript{a}, Marianne Danielsen\textsuperscript{b,c}, Ulrik K. Sundekilde\textsuperscript{a,b}, Trine Kastrup Dalsgaard\textsuperscript{a,b,c,*}

\textsuperscript{a} Department of Food Science, Agro Food Park 48, Aarhus N 8200, Denmark
\textsuperscript{b} CIFOOD, Centre for Innovative Research, Aarhus University. Agro Food Park 48, 8200 Aarhus N, Denmark
\textsuperscript{c} CBIO, Centre of Circular Bioeconomy, Blichers Alle 20, Tjele 8830, Denmark

1. Introduction

Metabolomics has been defined as “the quantitative measurement of the dynamic multi-parametric response of a living system to pathological stimuli or genetic modification” [1,2]. For metabolomics, several analytical techniques can be applied, presenting specific advantages and disadvantages. The most used include nuclear magnetic resonance spectroscopy (1H NMR), direct infusion mass spectrometry (DI-MS), gas chromatography coupled to mass spectrometry (GC-MS), two-dimensional GC coupled to MS (GC × GC–MS), liquid chromatography coupled to MS (LC-MS), two-dimensional liquid chromatography (2D LC), capillary electrophoresis coupled to MS (CE-MS) and super-critical fluid chromatography coupled to MS (SFC-MS) [2-5].

GC–MS remains an analytical technique of preference for the analysis of volatile and semi-volatile analytes. However, this technique presents some known limitations: substances with polar nature, non-volatile, large/bulky and/or thermostable cannot be directly analysed with GC. Thus, an often-used approach is to rely on an extensive sample preparation steps that transform non-volatile compounds into volatile substances by following derivatization schemes (e.g., methoximation, silylation) prior to analysis.

The use of derivatization coupled with extensive sample preparation and typically long chromatographic analysis time, make GC–MS a rather low-throughput technique for metabolomic applications. However, an important advantage of GC–MS in this field, is that the identification of peaks (peak annotation or, more difficult, the structure elucidation of unknowns) is more straightforward than in LC–MS [2,5]. When using derivatization for metabolomics, it is crucial that all metabolites are...
Conditions of derivatization can vary considerably, with different reaction temperatures (e.g. 37 °C) and reaction times that can range from minutes to hours, as reported in applications [7] and research studies [10,11]. Furthermore, in an investigation of the derivatization conditions and the stability of derivatized biological samples, it was highlighted that the stability of individual metabolites varies over time, and special attention should be paid to the analysis time, where a maximum of 18 h is considered as safe, while there is also possibility of online trimethylsilyl (TMS) derivatization, within the same time frames prior to injection, in order to increase reproducibility and avoid stability issues among derivatized samples [11].

Furthermore, during GC–MS analysis high temperatures are applied. In the injection system temperatures can reach 300 °C, oven programs can start as low as 40 °C up to 300 °C and for the ionization for mass spectrometry analysis, temperatures of 230 °C are regularly applied in the source [12].

In GC-based metabolomics studies, molecular profiles are generated based on thermally stable molecules [13,14]. During the entire analytical process, small molecules and metabolites can be subjected to a wide range of temperatures, from low sample-storing temperatures (-80 °C, 4 °C) up to high thermal processing temperatures during derivatization reactions and, finally, the analysis with GC–MS. However, thermal processing during sample derivatization, vaporization and GC analysis may potentially lead to formation of degradation products of metabolites or de novo formation of compounds [12,15].

Although it is a well-known and accepted problem, there is limited knowledge concerning the evaluation of in situ thermal effects at elevated temperatures (inlet temperature, temperature ramps) on metabolites within a GC system, while using a limited number of internal standards, especially in case on non-automated GC metabolomics. Thus, the objective of this work was to evaluate the thermal effects of GC injector and oven temperature on urine metabolites, applying multiple and multi-class isotope-labelled internal standards and assess their stability for off-line GC metabolomics using a selected derivatization process of urine samples.

2. Materials and methods

2.1. Chemicals

MSTFA (2,2,2-Trifluoro-N-methyl-N-(trimethylsilyl)-acetamide) containing 1 %TMCS (trimethylchloro silane) (Thermo Scientific, USA). TMSCN (trimethylsilyl cyanide) 98% was purchased from Sigma Aldrich (Steinheim, Germany). A mix of 20 labelled amino acids “Cell Free” mix (20 amino acid) (13C, 97-99%; 15N, 97-99%), L-valine-2,3-d2, L-alanine-2,3,3,3-d4, myo-inositol-1,2,3,4,5,6-d6, Citric-2,2,4,4-d4 acid (98%), decanoic-1,13C acid (98%), lauric-1,13C acid (98%), myristic-1,13C acid (98%), palmitic acid-1,13C acid (98%), and stearic-1,13C acid (98%) were added to the mixture and stirred for 30 s using a vortex mixer. Solutions were extracted using an ultraturax T25 (IKA, Germany) at 10000 rpm for 10 min at room temperature and centrifuged for 10 min (14,000 × g, 4 °C). An aliquot (200 μL) of the supernatant were evaporated to dryness under vacuum at 35 °C using vacuum centrifuge (miVac, United Kingdom) and derivatized.

For TMSCN derivatization, 40 μL of methoxyamine hydrochloride in pyridine (20 mg mL⁻¹) were added to each GC vial, in pyridine) in order to protect aldehyde and ketone groups. Then solutions were mixed for 90 min (750 rpm) while incubated at 30 °C. After methoximation reaction, the samples were added 40 μL of trimethylsilylated (TMSCN) and shaking (750 rpm) for 60 min at 37 °C. After that, 40 μL of heptane were added to the mixture and stirred for 30 s using a vortex mixer (Fisher Scientific) before GC–MS analysis. The same approach (volumes, times, temperatures) was followed in case of MSTFA/1%TMCS, only by replacing the TMSCN (same volumes, times, temperatures) [6,10,16].

Derivatization protocols were reproduced three times per sample and each sample was analysed in duplicates, the same day, with the same analytical instrument and by the same analyst, under repeatability conditions. The two derivatization procedures were evaluated and assessed in terms of peak intensities, peak areas and number of derivatized metabolites, in respect to the ratio of the applied isotope-labelled internal standards.

2.2. Derivatization protocol

Standard mixtures of selected isotope labelled metabolites (deuterated, 13C, 15N) and a pooled urine sample from four different random urine samples, were prepared to be assessed. Two derivatization protocols were applied and tested with both the standards and urine, aiming to compare the selected derivatization protocols. These protocols were based in the one described in Nielsen et al. 2017 [10], which was based on the method reported by Khakimov et al. [6,10] and a slightly modified Fiehn GC/MS metabolomics using MSTFA/1% TMCS [7]. Two different silylation reagents were tested, namely TMSCN and MTSAF/1% TMCS, and resulting samples were applied to the same GC analytical procedure and oven programs. The first program was a short program with a total analysis time of 15 min, and an extended program with a total analysis time of 30 min (description in paragraph 2.3).

For both tested protocols, in 2 mL Eppendorf tube, an aliquot of 200 μL of urine were mixed with isotope-labelled internal standards (10 μL of 1 mg mL⁻¹ L-valine-2,3-d2, L-alanine-2,3,3,3-d4, 10 μL of 0.5 mg mL⁻¹ myo-inositol-d6 and 10 μL of 0.3 mg mL⁻¹ Citric-2,2,4,4-d4 acid in 1:1 water:methanol and 10 μL of 0.3 mg mL⁻¹ D-glucose1,2,3,4,5,6,7-d7, and 10 μL of palmitic acid-1,13C acid of 0.5 mg/mL, and stearic-1,13C acid of 0.5 mg/mL). Subsequently, 800 μL of methanol were added to the mixture and stirred for 30 s using a vortex mixer. Solutions were extracted using an ultraturax T25 (IKA, Germany) at 10000 rpm for 10 min at room temperature and centrifuged for 10 min (14,000 × g, 4 °C). An aliquot (200 μL) of the supernatant were evaporated to dryness under vacuum at 35 °C using vacuum centrifuge (miVac, United Kingdom) and derivatized.

For the GC–MS analysis, 40 μL of methoxyamine hydrochloride in pyridine (20 mg mL⁻¹) were added to each GC vial, in pyridine) in order to protect aldehyde and ketone groups. Then solutions were mixed for 90 min (750 rpm) while incubated at 30 °C. After methoximation reaction, the samples were added 40 μL of trimethylsilylated (TMSCN) and shaking (750 rpm) for 60 min at 37 °C. After that, 40 μL of heptane were added to the mixture and stirred for 30 s using a vortex mixer (Fisher Scientific) before GC–MS analysis. The same approach (volumes, times, temperatures) was followed in case of MSTFA/1%TMCS, only by replacing the TMSCN (same volumes, times, temperatures) [6,10,16].

Derivatization protocols were reproduced three times per sample and each sample was analysed in duplicates, the same day, with the same analytical instrument and by the same analyst, under repeatability conditions. The two derivatization procedures were evaluated and assessed in terms of peak intensities, peak areas and number of derivatized metabolites, in respect to the ratio of the applied isotope-labelled internal standards.

2.3. GC-qToF-MS

The samples (1 μL aliquot) were injected in split mode with a 1:10 flow into an Agilent Technologies 7890B gas chromatography system coupled to an Agilent Technologies 7200 Accurate-Mass Q-ToF mass spectrometer (Agilent Technologies, Waldbronn, Germany). Separation of the metabolites was performed on an HP-5MS capillary column coated with polyimide (20 m, 0.180 mm i.d., 0.18 μm film thickness; Agilent Technologies). The temperatures of the ion source was 230 °C, the mass spectral analysis was performed in scan mode with a quadrupole temperature of 150 °C and a fragmentation voltage of 70 eV. Solvent delay was adjusted accordingly for the two tested GC oven programs.

Two GC oven programs were tested: (1) a typical GC–MS metabolomics, so-called extended program (EP), where the initial temperature was 60 °C and held for 2 min, followed by a temperature ramp of 10 °C min⁻¹ up to a temperature of 320 °C, and then held for 5 min. The total analysis time was 34 min, and a solvent delay of 4 min was applied. The second program (2), the so-called short program (SP), was set at an initial temperature was 60 °C and held for 2 min followed by a temperature ramp of 30 °C min⁻¹ up to a temperature of 320 °C, and then held for 5 min. Total analysis time was 15.7 min. A solvent delay of 2 min was applied.

Two inlet temperatures of 240 °C and 270 °C were tested, whilst the remaining settings were kept the same (pressure of 21.4 psi, a gas
saver of 20 mL/min and 3 mL/min purge flow). The autosampler temperature was set to 8 °C, to avoid evaporation and retain stability of the derivatives.

2.4. GC-qToF MS metabolomics data

Raw GC-qToF-MS data were deconvoluted using Mass Hunter Unknown Analysis software (Quantitative Analysis, version B.07.00/Build 7.0.457.0, Agilent Technologies) and metabolite identification using the NIST11 library. Raw data from all samples were transferred into Mass Profiler Software (MPP) (Agilent, USA) for retention time alignment, peaks filtration as well as for generation of volcano plots and multivariate analysis. Normalization of the peak area was realized using the total area of the applied isotope-labelled internal standards, namely L-valine-d2, L-alanine-d6, myo-inositol-d6, citric-2,2,4,4-d4 acid and D-glucose-d7, and palmitic acid-1,2,13C acid of 0.5 mg/mL, and stearic-1,13C acid as highlighted in paragraph 2.2.

2.5. Statistical analysis

All identified factors were compared with 1-way analysis of variance (ANOVA) with Minitab software (Minitab Inc) after checking for normal distribution and homogeneity of variances (Bartlett test). After processing, integrated peak areas for the detected analytes data were analyzed by Agilent MPP for multivariate data analysis. Before statistical analysis, data were visually examined for outliers (Hotelling’s T2 value > 95% confidence limit) in principal component analysis (PCA).

3. Results and discussion

3.1. Derivatization

One of the advantages of GC-MS is its versatility among different brands, although special attention shall be taken regarding the derivatization of non-volatile metabolites, to perform it in a similar, and if possible, an automated and reproducible way. However, the effect of thermal degradation of substances can occur and it shall be taken into consideration, prior analysis. Hence, a focus was given to reported methods, a generally extensively applied one (MSTFA/1% TMCS; Agilent Fiehn GC metabolomics) and a second one on that is not that less extensively used (TMSCN). A special care during sample preparation was taken in case of TMSCN due to the generation of the toxic hydrogen cyanide by-product [6], operating under well-ventilation and isolated place. Therefore, the application of combined isotope-labelled internal standards can support the applicability and repeatability of this important step within GC-based metabolomics.

Hence, these two derivatization methods were applied to pooled urine samples, using a mixture of selected isotope-labelled internal standards. They were compared in terms of signal intensity of the derivatized molecules, and the potential effects on the GC system (dirty liners; injection systems), together with reproducibility (Fig. 1). By applying a MSTFA/1% TMCS derivatization, significant higher intensities of metabolites were observed compared to the trimethyl silyl cyanide (TMSCN) derivatization (Fig. 1, volcano plot). To evaluate these effects, the extended GC program and an inlet temperature of 240 °C were applied for both derivatization protocols. The results are in accordance with previous studies [6,10,11].

Unsupervised principal compound analysis (PCA) showed a clear separation of the TMSCN derivatization against the MSTFA/1% TMCS derivatization along PC1, presenting a variation of 31.97%, indicating a clear difference in either number or level of the detected/derivatized metabolites. The PCA results and the volcano plot highlights significantly differentiated identified metabolites, which are being up-regulated in case of the MSTFA/1 %TMCS. The plot is organized with mass-to-charge ratio (m/z) on the y-axis and by retention time on the x-axis. The fold change (≥1.5) at the retention times of the amino acids (3–6 min), showed to represent the vast majority of the statistically different (p-value < 0.01) metabolites that seemed to report higher intensities with the MSTFA/1 %TMCS and therefore it was selected for the subsequent studies (Fig. 1). Repeatability of the analysis was very good, where in both cases, for the same metabolites, relative standard deviation (RSD, %) was below 20% for all identified and analysed metabolites. However, main goal of this test was not to identify differences among applied derivatization strategies nor the optimization of the method, which eventually exist and are unavoidable. A large number of derivatization reagents can be used in GC-metabolomics, i.e., BSTFA, BSTFA/1% TMCS that have been studied and compared and previously reported [6,10,11]. However, from our results we can conclude that the
derivation with MSTFA/1%TMCS can be realized in repeatable conditions, and with high derivatization efficiency. In addition, the use of TMSCN did not show any added-value nor higher derivatization efficiency, thus its use shall be avoided also considering its associated toxicity.

TMSCN has been reported to be a more efficient agent when compared with MSTFA (not including 1% TMCS), with or without methoximation [6], where the TMSCN derivatization (with no methoximation) was more sensitive than MSTFA (though no TMCS was included) and with higher signal intensities [6]. In the present study, the intensities of the majority of the identified analytes were significantly higher with MSTFA/1%TMCS than with TMSCN. Both results can be tentatively explained considering the combined use of 1% TMCS and pyridine from the methoximation. This is a key point, as both processes can act as catalyst and increase the yields of derivatization [7,11,17]. In addition, the use of methoxime hydrochloride in pyridine, can further support the derivatization reaction. Thus, pyridine, apart from being a common solvent, supports as a promoter of reactions with TMCS [9,17]. It captures protons and acts as an additional derivatization catalyst by increasing nucleophilicity [7]. Hence, the combination of MSTFA with 1% TMCS (the latter as catalyst) can enhance the derivatization of moderately hindered or slowly reacting compounds, in the presence of pyridine.

In accordance with Moros et al., the results of the selected derivatization (MSTFA/1% TMCS) indicated stability up to 18 h, (Figure S1). An additional observation was that the derivatization with TMSCN resulted in a brownish coloured solution that seemed to affect the lifetime of the GC liner (Figure S1, supplementary material) and consequently, it also affected the repeatability of the analytical signals. Thus, the optimized MSTFA/1%TMCS method has been selected and used further for the metabolomics study of the selected standards and for the analysis of the pooled urine sample. Thus, the application of multiple labelled IS for GC metabolomics it is highly preferable, in order to facilitate high repeatability in case of the off-line GC metabolomics. It shall be highlighted that a typical derivatization protocol, such as reported by Fiehn et al. introduces and includes the application of a single internal standard (myristic acid-d_{27}) [16]. However, these types of analytes (either isotope-labelled or not fatty acids) are rather stable, as already reported by Moros et al. and as presented in the current study. Moreover, it is well known that O-silylated derivatives are more stable than the N-silylated analogues and both hydrogens of the primary amino group can be replaced and the substitution reaction highly depends on the reaction conditions and purity of reagents [16,18,19]. Therefore, and especially in non-automated procedures, the use of multiple and multi-group internal standards is important to assess these phenomena and validate the derivatization process (repeatability, reproducibility), especially for either O-silylation or N-silylation.

3.2. Stability of isotope-labelled internal standards

After selecting the most efficient derivatization protocol, a study of the stability of the selected isotope-labelled internal standards was realized. Within this framework, in this work we studied the presence of multiple isotope-labelled internal standards, during derivatization, in order to evaluate the repeatability of the derivatization process and to eliminate potential random errors. The use of a single isotope-labelled internal standards is recommended, namely myristic acid-d_{27}, in a generic GC-metabolomics workflow [16], which however does not cover the potential effect to different chemical groups. Thus, initial tests using selected isotope labelled amino acids (alanine, valine), fatty acids, organic acids, sugars (glucose) and sugar alcohol (myo-inositol) revealed a statistically significant degradation only in case of amino acids and particularly in case of the deuterated amino acids alanine-d_{4} and valine-d_{2}. In particular, alanine-d_{4} was most affected. However, an effect on valine was also observed, though it was considered as negligible (RSD < 11%). The remaining analytes, such as isotope-labelled sugars, sugar alcohols and fatty acids, showed a decrease, though negligible, presenting an RSD below 15% (Table 1). Hence, concentration differences (ΔC, %) of all studied deuterated standards injected with the two different inlet temperatures (270 °C, 240 °C) after optimized derivatization, are given in Table 1. Statistical analysis (p = 0.05) revealed statistical significant differences, only in cases of alanine-d_{4} and valine-d_{2} among the two inlet temperatures, in both GC temperature ramp programs.

Nevertheless, the selection of isotope-labelled internal standards shall cover a significant range of groups of metabolites, and a stability study verified their potential use. Hence, in this study, and based on the reported results from Moros et al. [11], we selected several compounds to be evaluated for isotope-labelled internal standards, including amino acids (valine, alanine), fatty acids (octanoic, decanoic, lauric, palmitic, stearic), organic acids (citric acid), sugars (D-glucose) and sugar alcohols (myo-inositol). Results indicated that compounds derivatized with both derivatization agents were stable with no statistical difference (1-way ANOVA; p > 0.05) up to 16 h after derivatization, and especially with the MSTFA/1 % TMCS agent (Figure S2), indicating that MSTFA can be used to evaluate and ensure the repeatability of the process. Furthermore, the adjustments of the operating condition of GC–MS between batches, together with the required sample preparation (derivatization) can be corrected by combining internal standard together with a quality control sample (QC) [2,6,7,17].

Following the general accepted GC-metabolomics protocols, and as already reported, a robust, quantitative GC–MS metabolomic workflow therefore requires automated timed on-line sample preparation [8]. Hence, this a desired scenarion, which however not all laboratories can invest to support, thus alternative and acceptable approaches are desired in case of non-automated strategies (off-line).

It is well known that O-silylated derivatives are more stable than the N-silylated analogues and both hydrogens of the primary amino group can be replaced and the substitution reaction highly depends on the reaction conditions and purity of reagents [16,18,19]. Therefore, and especially in non-automated procedures, the use of multiple and multi-group internal standards is important to assess these phenomena and validate the derivatization process (repeatability, reproducibility), especially for either O-silylation or N-silylation.

3.3. Inlet temperature

In order to assess the thermal degradation of metabolites during GC metabolomics, isotope labelled (deuterated, 13C, 13C/15N) standards were used to cover the entire range of potential analytes. The samples were analysed with two different inlet temperatures (240 °C and 270 °C) and with two GC oven programs (short 16 min vs extended 30 min). The selection of these two inlet temperature ranges was based on existing GC-metabolomics protocols [7,8]. Higher temperatures might facilitate better evaporation of heavier metabolites (270 °C). Lower temperatures might result in dirty liners that subsequently will affect sensitivity and repeatability [17]. Therefore, following the general procedures for GC-metabolomics and without risking dirtying the liner, we selected temperatures above 240 °C. All the samples were derivatized with the optimized protocol (section 3.1) employing a mixture of MSTFA/1% TMCS, after methoximation. The obtained chromatograms are presented in Fig. 2.

Following the preliminary results for the isotope-labelled analytes, especially amino acids and after assessing their degradation potential within different inlet temperatures, pooled urine sample was also analysed with the selected GC temperature programs. Volcano plots were generated to visualize significant statistical differences (p < 0.05) and their magnitude of change (fold change; FC) among identified metabolites in the real urine samples (Fig. 3, Table S2). In case of the inlet temperature effects, the volcano plot revealed a significant effect mainly to AA and especially to L-alanine, L-lysine, L-glycine, L-valine and L-proline, confirming the results obtained with the labelled amino acids.
Moreover, sugars (D-glucose, D-galactose, D-mannose, D-erythritol, trofuranose), sugar alcohols (myo-inositol, ribitol) and acids (lactic acid, butanoic acid, oxalic acid) were not that affected, presenting absolute values of FC lower than 1.5, and not being statistically different. In addition to amino acids, also the glucose oxime, trihydroxybutyric acids, oleic acid and (Z,Z)-9,12-Octadecadienoic acid were mostly affected.

A similar effect of potential thermal degradation of small organic molecules has been reported by Lu et al., where the importance of the injection step, prior GC metabolomics analysis was presented [15]. Moreover, potential thermal degradation of analytes that could be accelerated by degradation products arising from an unclean injector or the sample itself has been highlighted [15,18]. However, Lu et al. did not differentiate these effects nor clustered them according to chemical groups or metabolites (i.e. amino acids, sugars, fatty acids) [15]. To the best of our knowledge, only Fang et al. evaluated the thermal degradation of small molecules within metabolomics, following a fundamental different approach compared to this study. They reported these effects in plasma, after derivatizing and heating (different temperatures and time combinations) and by analysing with LC-MS [12]. Hence, there is a need to assess in situ the behaviour of metabolites and organic compounds, within a GC inlet system as well as within typical GC analysis (fast or slow temperature ramping). Furthermore, heating at 250 °C in the GC inlets is for a very short time period (<30 s) and not high as 300 s as in the LC-MS study [12].

Compared to LC-MS, the injection step is considered more challenging in GC. The sample might undergo thermal degradation, during introduction to the inlet system. Moreover, Lu et al. highlighted that certain non-volatile metabolites, such as phosphatidylcholines, can degrade in the GC injector, cross contaminate samples, and lead to undesired catalytic degradation of analytes of interest [15]. In addition, another point of interest, is the loss of trimethylsilyl groups at high temperatures, and our results are in accordance with what has been reported for GC metabolomics [12,15]. Hence, O-trimethylsilyl groups are more stable and robust for GC measurement of sugars, phosphates, and hydroxyl- acids, while N-trimethylsilyl groups are less stable, as in case of amines and amino acids, already highlighted by Lu et al. [15].

| Labelled metabolite          | Short program (SP) | Extended program (EP) |
|------------------------------|--------------------|-----------------------|
|                              | ΔC_{270-240} (%)    | p-value                             | Abundance | ΔC_{270-240} (%)    | p-value | Regulation |
| Alanine-d_{3}                 | -15.1              | <10^{-7}                | lower     | -32.1              | <10^{-5}  | lower     |
| Valine-d_{2}                  | -6.8               | <10^{-7}                | lower     | -10.3              | <10^{-5}  | lower     |
| Octanoic-1-{^{13}}C- acid     | -8.7               | >0.05                  | -         | -6.9               | >0.05     | -         |
| Decanoic-1-{^{13}}C-acid      | -8.3               | >0.05                  | -         | -7.9               | >0.05     | -         |
| Lauric-1-{^{13}}C acid        | -7.9               | >0.05                  | -         | -8.6               | >0.05     | -         |
| citric acid-2,2,4,4-d_{4} acid| -5.3               | >0.05                  | -         | -2.3               | >0.05     | -         |
| D-(+)-glucose-d_{2}           | -9.5               | >0.05                  | -         | -2.4               | >0.05     | -         |
| D-(−)-glucose-d_{2}           | -5.9               | >0.05                  | -         | -0.7               | <0.05     | -         |
| Palmitic-1,2-{^{13}}C acid    | 6.0                | >0.05                  | -         | 4.9                | >0.05     | -         |
| Myo-inositol-d_{6}            | -1.9               | >0.05                  | -         | -2.1               | >0.05     | -         |
| Stearic-1-{^{13}}C acid       | 4.6                | >0.05                  | -         | 2.6                | >0.05     | -         |

Note 1: Non statistically significant (p > 0.05).

Note 2: ΔC_{270-240} (%): Per cent concentration difference between inlet temperature 270 °C and 240 °C.

Table 1: Concentration differences of deuterated standards injected with the two studied inlet temperatures, and tested with the two GC temperature ramps, after derivatization with MSTFA/1% TMCS.

Fig. 2. GC-chromatogram of A: {^{13}}C/{^{15}}N amino acids, short program (short program, overlaid inlet temperatures); B: {^{13}}C-fatty acids, myoinositol-d_{6} and deuterated amino acids IS (short program, overlaid inlet temperatures); C: {^{13}}C/{^{15}}N amino acids extended program overlaid inlet temperatures; D: {^{13}}C-fatty acids, myoinositol-d_{6} and deuterated amino acids IS (extended program, overlaid inlet temperatures); (two inlet temperatures: —240 °C ; — 270 °C).
Furthermore, derivatization temperatures should be kept as moderate as possible (below 40 °C), possibly by extending the derivatization times, to avoid having potential degradation reactions [2,7,8,12].

In the present study, glutamate was found to be in very low concentrations, probably due to the potential degradation within 30 s, which is aligned with previous reported research for thermal degradation (at 250 °C; Fang et al. 2015 [12]) of amino acids [12,20]. Another substance of interest was citric acid-d₄, which belongs to the group of small organic acids that is known to be thermally unstable. Citric acid has been reported to degrade after only 60 s of heating at standard inlet temperatures (250 °C). Our work suggested that by keeping the derivatization and inlet temperatures low, this degradation can be avoided, as citric acid could be analysed with both GC programs and inlet temperatures tested, with no statistical differences.

### 3.4. GC oven programs

In order to further investigate the effect of GC inlet temperatures on amino acid stability, a total of 15 ¹⁵N/¹³C labelled amino acids were analyzed. For all the labelled amino acids, the differences in their concentration (ΔC, %) were calculated. Sample were injected with the two studied inlet temperatures after methoximation and derivatization with MSTFA/1% TMCS (Table 2). Thus, it can be observed that in all cases a decrease of amino acids concentration is observed due to inlet temperature or GC oven programs, ranging from −51.9% for leucine to −12.0% for asparagine for the short program, while for the extended program ranged from −69.1% for leucine to −6.9% for threonine. All differences were statistically significant (p < 0.05), except in case of methionine (both programs), asparagine, aspartic acid (short programs) and for threonine (extended program).

The results of both GC temperature programs were comparable, although the higher inlet temperature presented more relevant degradation effects. As it can be extrapolated from Table 2, differences in concentration between −20% and +20%, can be considered as acceptable, although the analysis was targeted and in this case the threshold shall be typically of ±15% having an acceptable maximum of ±20% [11,21]. The latter value comes from specific acceptance criteria have been set for targeted analysis (e.g., ICH, EMAS, or the US FDA) as well as in case of validation of bioanalytical methods [11,21-25]. However, it is important to underline the fact that although the current study explicitly refers to GC metabolomic’s thermal degradation, identical or similar thermal effects can occur also to other hyphenated techniques (e.g., liquid chromatography) coupled to MS, for metabolomics. Thus, in an electrospray ionization (ESI) source, temperatures exceeding 250 °C are applied, which may lead to identical degradation effects to those found with GC, or even larger [26,27]. Therefore, it is clear that the thermal degradation is of overall importance in the fields of MS-based metabolomics [12].

For the assessment and evaluation of the GC temperature ramps, in combination with the different inlet temperatures, the pooled urine sample was analyzed and the differences observed were evaluated by a volcano plot among the identified metabolites in the tested urine sample (Fig. 4, Table S3). In case of the inlet temperature effects and as already reported in case of GC inlet, the volcano plot revealed a number of different metabolites, where the majority of them were the amino acids, presenting a FC significantly higher than 1.5. Within this framework and in correlation to what was observed with the isotope labeled metabolites, the amino acids affected were mainly L-alanine, L-lysine, L-glutamic acid, L-valine and L-proline, confirming the results obtained with the labelled amino acids. Moreover, sugars, sugar alcohols and acids were not highly affected, presenting absolute values of FC lower than 1.5, and not being statistically different. The GC temperature results are in accordance with the GC inlet results.

### Table 2

| Target metabolite | Short method tᵢ (min) | ΔC²₇₀⁻²₄₀ (%) | Extended method tᵢ (min) | ΔC²₇₀⁻²₄₀ (%) |
|-------------------|-----------------------|----------------|--------------------------|----------------|
| Glycine           | 4.18                  | −12.5⁺         | 5.66                     | −21.0⁺         |
| Alanine           | 4.29                  | −12.3⁺         | 5.93                     | −37.8⁺         |
| Valine            | 4.51                  | −14.0⁺         | 6.52                     | −36.6⁺         |
| Leucine           | 4.58                  | −51.9⁺         | 6.67                     | −69.1⁺         |
| Isoleucine        | 4.70                  | −18.9⁺         | 6.96                     | −31.9⁺         |
| Proline           | 5.20                  | −38.9⁺         | 7.60                     | −57.1⁺         |
| Methionine        | 5.39                  | −12.5⁻         | 8.84                     | −16.7⁻         |
| Serine            | 5.53                  | −16.0⁺         | 9.18                     | −23.7⁺         |
| Phenylalanine     | 5.64                  | −23.2⁺         | 9.65                     | −32.2⁺         |
| Threonine         | 5.77                  | −48.6⁺         | 10.00                    | −6.9⁻          |
| Aspartic acid     | 5.91                  | −13.9⁻         | 10.37                    | −22.5⁻         |
| Asparagine        | 6.35                  | −12.0⁻         | 11.57                    | −31.4⁻         |
| Glutamic acid     | 6.73                  | −51.0⁺         | 12.78                    | −29.7⁺         |
| Lysine            | 7.84                  | −43.4⁺         | 15.95                    | −28.4⁺         |
| Tyrosine          | 7.90                  | −23.7⁺         | 16.11                    | −19.6⁺         |

ΔC²₇₀⁻²₄₀ (%): Concentration difference in percent between inlet temperature 270 °C and 240 °C. Note: Values marked with asterisk (⁺) are statistical significant at 95 % CI level (p < 0.05).

¹ Based on peak areas at temperatures of 270 °C and 240 °C.
The metabolic profiling study of urine and respective PCA (Figure S3), clearly shows that short and fast ramp GC programs, together with different inlet temperatures, significantly affected the metabolic profile of the urine sample. This effect was most pronounced for amino acids, which were within retention times from 4 up to 8.0 min. Negligible effects, though slightly higher signal intensities were observed for fatty acids and sugars in the lower inlet temperatures (240 °C) and the low ramp GC program (10 °C min⁻¹; extended). In case of the short program, regardless of inlet, higher temperature ramps (e.g. 30 °C min⁻¹) will expose the compounds to high temperatures for a significant time period affecting overall the metabolic profile, including also fatty acids and sugars, and not only for the amino acids [12]. In this case, a degradation of the derivatized substances can occur, together with potential formation of new compounds or breakdown/degradation products, or even chemical reaction between derivatized metabolites [12]. Thus, the latter programs (short GC programs; high temperature ramps) should be avoided in case of GC metabolomics and metabolic profiling.

Furthermore, the observed differences between the two studied temperature programs in Fig. 3, could be tentatively explained by the significant signal suppression obtained in case of the fast ramp program. Not even a fast MS detector such as a TOF can avoid signal saturation. The latter was not observed when using an extended program, where the chromatography delivers more efficient separation of metabolites.

The metabolic profile revealed a decrease of peak intensities (only for the fast ramp GC program), for the amino acids, compared to the slow ramp GC program. Furthermore, regarding inlet temperature, glycine, leucine, alanine, isoleucine, proline and valine presented a concentration decrease (ΔC, %) of 25–37% in case of the short program, while for the extended programs decreases ranged from 15 to 25%, which can be considered within the acceptable criteria [21,22,26,28]. For the extended GC program, no significant effects have been observed in case of fatty acids and sugars, and other metabolites (i.e. urea, uric acid, hydroxybutyric acid, myo-inositol, glucose, galactose, lactic acid, glycerol) whereas in the short program the difference was statistically significant (Table S3).

However, even with the studied temperatures, there still is a potential of certain metabolites to degrade at elevated temperature (240 °C) in urine, even at minimal exposure times (30 s). That is the case of the tri- and di-organophosphates (e.g., adenosine triphosphate and adenosine diphosphate), and certain nucleosides and nucleotides that can be transformed into purine derivatives [12]. Furthermore, it can be concluded that the use of more than one internal standard, representing different chemical groups (i.e. fatty acids, sugars, sugar alcohols) will facilitate analysis and eventually minimizing the effects and variations due to thermal stability phenomena [15,17].

4. Conclusions

A study of the potential thermal degradation during GC metabolomics analysis of urine was performed to assess the in situ effects of thermal degradation of small molecules/metabolites within a GC system. Heating of samples within a GC system, inlet or oven programs, significantly affected the metabolic profile, with high temperatures specifically degrading all studied amino acids. Thus, fast ramp oven programs and in combination with higher inlet temperatures, should be avoided as they promote MS saturation. These observations were confirmed by metabolic profiling of urine, where no statistical effects of GC temperatures were observed for organic acids, fatty acids, sugars and sugar alcohols, while significant differences were observed for amino acids. Finally, higher derivatization temperatures should be avoided in order to minimize potential further degradation of metabolites.

Funding

This research was funded by Pelsdylraftgiftsfonden
CRediT authorship contribution statement

Emmanouil D. Tsochatzis: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Project administration. Caroline Nebel: Conceptualization, Methodology, Data curation, Writing – review & editing. Marianne Danielsen: Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing. Ulrik K. Sundekilde: Methodology, Writing – review & editing, Project administration. Trine Kastrup Dalsgaard: Conceptualization, Methodology, Writing – review & editing, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.122902.

References

[1] G. Theodoridis, H.G. Gika, I.D. Wilson, LC-MS-based methodology for global metabolite profiling in metabolomics/metabolomics, TrAC Trends Anal. Chem. 27 (3) (2008) 251–260, https://doi.org/10.1016/j.trac.2008.01.008.
[2] G. Theodoridis, H.G. Gika, I.D. Wilson, Mass spectrometry-based holistic analytical approaches for metabolite profiling in systems biology studies, Mass Spectrom. Rev. 30 (5) (2011) 884–906, https://doi.org/10.1002/mas.20306.
[3] G.A. Theodoridis, H.G. Gika, E.J. Want, I.D. Wilson, Liquid chromatography-mass spectrometry based global metabolite profiling: A review, Anal. Chim. Acta 711 (2012) 7–16, https://doi.org/10.1016/j.aca.2011.09.042.
[4] A. Alonso, S. Marsal, A. Julià, Analytical Methods in Untargeted Metabolomics: State of the Art in 2015, Front. Bioeng. Biotechnol. 3 (2015), https://doi.org/10.3389/fbioe.2015.00023.
[5] K. Segers, S. Declerck, D. Mangelings, Y.V. Heyden, A.V. Eeckhaut, Analytical techniques for metabolomic studies: a review, Bioanalysis 11 (24) (2019) 2297–2318, https://doi.org/10.4155/bio-2019-0034.
[6] B. Khakimov, M.S. Motawia, S. Bak, S.B. Engelsen, The use of trimethylsilyl cyanide derivatization for robust and broad-spectrum high-throughput gas chromatography-mass spectrometry based metabolomics, Anal. Bioanal. Chem. 405 (28) (2013) 9193–9205, https://doi.org/10.1002/acs.jafc.201373414.
[7] Agilent Technologies, Agilent G1676AA Fiehn GC/MS MetabolomicsRTL Library, (2013).
[8] O. Fiehn, Extending the breadth of metabolite profiling by gas chromatography coupled to mass spectrometry, TrAC Trends Anal. Chem. 27 (3) (2008) 261–269, https://doi.org/10.1016/j.trac.2008.01.007.
[9] Sigma Aldrich, The Use of Derivatization Reagents for GC, (2020), https://www.sigmaaldrich.com/technical-documents/articles/reporter-us-the-use-of-derivatization.html.
[10] S.D. Nielsen, B. Amer, K. Blaabjerg, T.K. Dalsgaard, R. Jessen, B. Petrat-Melin, M.K. Rasmussen, H.D. Poulsen, J.F. Young, Whole Milk Increases Intestinal ANGPTL4 Expression and Excretion of Fatty Acids through Feces and Urine, J. Agric. Food Chem. 65 (2) (2017) 281–290, https://doi.org/10.1021/acs.jafc.6b0413510.1021/acs.jafc.6b0413510.
[11] G. Mores, A.C. Chantziaraanou, H.G. Gika, N. Raikos, G. Theodoridis, Investigation of the derivatization conditions for GC-MS metabolomics of biological samples, Bioanalysis 9 (1) (2017) 53–65, https://doi.org/10.4155/bio-2016-0224.
[12] M. Fang, J. Ivansicovic, H.P. Benton, C.H. Johnson, G.J. Fatti, L.T. Hoang, W. Uritchonthai, M.E. Kurczy, G. Siudak, Thermal Degradation of Small Molecules: A Global Metabolomic Investigation, Anal. Chem. 87 (21) (2015) 10935–10941, https://doi.org/10.1021/acs.analchem.5b03003.
[13] S. Kerrigan, M. Savage, C. Cavazos, P. Bella, Thermal Degradation of Synthetic Cathinones: Implications for Forensic Toxicology, J Anal Toxicol. (2015) bkv099, https://doi.org/10.1093/jat/bkv099.
[14] M.L. Gonzalez, M. Carnicero, R. de la Torre, J. Ortuino, J. Segura, Influence of the injection technique on the thermal degradation of cocaine and its metabolites in gas chromatography, J. Chromatogr. B Biomed. Sci. 664 (2) (1995) 317–327, https://doi.org/10.1016/0165-2337(94)00084-M.
[15] W. Lu, X. Su, M.S. Klein, L.A. Lewis, O. Fiehn, J.D. Rabinowitz, Metabolite Measurement: Pitfalls to Avoid and Practices to Follow, Annu. Rev. Biochem. 86 (1) (2017) 277–304, https://doi.org/10.1146/annurev-biochem-061516-044952.
[16] Agilent Technologies, Agilent Fiehn 2013 GC/MS Metabolomics RTL Library - User Guide, (2013).
[17] M. Khosadadi, M. Pourfarzam, A review of strategies for untargeted urinary metabolomic analysis using gas chromatography–mass spectrometry, Metabolomics 16 (2020) 66, https://doi.org/10.1007/s11306-020-01687-x.
[18] O. Fiehn, G. Wohlgemuth, M. Schulz, T. Kind, D.Y. Lee, Y. Lu, S. Moon, B. Nikolau, Quality control for plant metabolomics: reporting MSI-compliant studies: Quality control in metabolomics, Plant J. 53 (2008) 691–704, https://doi.org/10.1111/j.1365-313X.2007.03387.x.
[19] H. Kanani, P.K. Chrysanthopoulos, M.I. Klapa, Standardizing GC-MS metabolomics, J. Chromatogr. B 871 (2) (2008) 191–201, https://doi.org/10.1016/j.jchromb.2008.04.049.
[20] N. Sato, A.T. Quintain, K. Kang, H. Daimon, K. Fujie, Reaction Kinetics of Amino Acid Decomposition in High Temperature and High-Pressure Water, Ind. Eng. Chem. Res. 43 (13) (2004) 2517–2522, https://doi.org/10.1021/ie020733w.
[21] C. Virgiliu, I. Sampsonidis, H.G. Gika, N. Raikos, G.A. Theodoridis, Development and validation of a HILIC-MS/MS multitargeted method for metabolomics applications: Liquid Phase Separations, Electrophoresis 36 (2015) 2215–2225, https://doi.org/10.1002/elps.201500088.
[22] H.G. Gika, G.A. Theodoridis, J.E. Wingate, I.D. Wilson, Within-Day Reproducibility of an HPLC–MS-Based Method for Metabolomic Analysis: Application to Human Urine, J. Proteome Res. 6 (2007) 3291–3303, https://doi.org/10.1021/pr070183p.
[23] T. Sangster, H. Major, R. Plumb, A.J. Wilson, I.D. Wilson, A pragmatic and readily implemented quality control strategy for HPLC-MS and GC-MS-based metabolomic analysis, Analyst 131 (10) (2006) 1075, https://doi.org/10.1039/b604498k.
[24] European Commission, Directorate General for Health and Food Safety, Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed., SANTE/11813/2017. (2017) 46.
[25] B. Magnusson, U. Ornemark, Euracchem Guide: The Fitness for Purpose of Analytical Methods - A Laboratory Guide to Method Validation and Related Topics, 2nd ed., Euracchem, 2014, https://www.euracchem.org/images/stories/Guides/pdf/MV_guide_2ed_EN.pdf (accessed October 7, 2020).
[26] O. Begou, H.G. Gika, I.D. Wilson, G. Theodoridis, Hypenitated MS-based targeted approaches in metabolomics, Analyst 142 (17) (2017) 3079–3100, https://doi.org/10.1039/C7AN01018K.
[27] G. Theodoridis, H.G. Gika, I.D. Wilson, Mass spectrometry-based holistic analytical approaches for metabolite profiling in systems biology studies: MS METABOLOMICS, Mass Spectrom. Rev. 30 (5) (2011) 884–906, https://doi.org/10.1002/mas.20306.
[28] S. Naz, M. Vallejo, A. García, C. Barbas, Method validation strategies involved in non-targeted metabolomics, J. Chromatogr. A 1353 (2014) 99–105, https://doi.org/10.1016/j.chroma.2014.04.071.