

In-time TMS derivatization and GC/MS determination of Sugars, Organic Acids and Amino Acids for High Throughput Metabolomics Studies

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ABSTRACT

Metabolomics aims to identify the changes in endogenous metabolites of biological systems in response to intrinsic and extrinsic factors in clinical, food and nutrition, and environmental based research. Gas chromatography-mass spectrometry (GC-MS) instruments are among the most commonly used in metabolomics studies, due to their high separation efficiency and good reproducibility compared to other platforms. This is primarily due to the robust, reproducible, and selective nature of the technique, as well as the large number of well-established commercial libraries and authentic metabolite standards available to researchers. However, metabolomics-based samples require chemical modifications/derivatization before GC-MS analysis. When processing large batches (> 20-40 samples), several potential issues can arise in the derivatization process. Sample-to-sample reproducibility for polar metabolites can vary significantly throughout sequences of large batches when all samples are derivatized simultaneously. This challenge lies predominantly in the time differences between when the samples are derivatized, and when the first and all subsequent samples are injected into the GC-MS. This issue is further exacerbated as the sample batch size becomes larger. Here we show how a GERSTEL MultiPurpose Sampler (MPS) with automatic tool exchange coupled to an Agilent 5977 GC-MSD can be used to overcome such challenges

and improve the quality and reliability of the generated metabolomics data.

INTRODUCTION

Previous GERSTEL Application Notes (AN/2014/08 and AN/2015/01) have shown automated ultrasonic-assisted liquid extraction and filtration, and automated sample preparation using an MPS Dual Head autosampler within metabolomics studies. As stated in previous application notes, metabolomics aims to identify the changes in endogenous metabolites in biological systems in response to intrinsic and extrinsic factors. Metabolomics studies focusing on small molecules (MW: ≤ 600 Da) routinely apply GC-MS analysis due to the availability of established protocols for the analysis of sugars, amino acids, sterols, hormones, catecholamines, hydroxyl acids, fatty acids, aromatics and many other intermediates of primary metabolism (after Fiehn, 2016). A prerequisite for GC-MS based metabolomics, especially for polar compounds, is derivatization to reduce analyte polarity and increase thermal stability and volatility. Molecules containing carboxylic acids, alcohols, amines, and thiols can be derivatized either by alkylation, acylation, or silylation. The most commonly used approach in metabolomics that improves mass fragmentation and chromatographic resolution comprises a two-step derivatization protocol. The first step involves

methoximation with methoxyamine hydrochloride (MOX), which protects carbonyl moieties of keto acids and sugars before the step of silylation with BSTFA or MSTFA. Metabolomics studies that focus on larger ‘small molecules’ (MW: 600 - 1700 Da) tend to employ liquid chromatography-mass spectrometry (LC-MS). LC-MS based approaches do not require sample derivatization as part of its workflow.

GC-MS sample preparation using a common MOX + BSTFA/MSTFA derivatization protocol has always been a bottleneck in the upstream metabolomics workflow. Conventional approaches are time consuming, and commonly used heat blocks and ThermoMixer®s are limited in terms of their capacity for processing large sample batches (i.e., sample holding capacity is limited to 20-24 samples). Additionally, due to a large variation between biological samples, the number of experimental or technical replicates in metabolomics research is increasing and the need for more samples is considered more important when compared with other “-omics” fields. This has resulted in a significant increase in the number of overall samples in a typical metabolomics study, which if analyzed by GC-MS, all require derivatization before sample injection. The widely used incubation method generally takes about 120-180 minutes for derivatization to complete for a batch of 20-24 samples (Beale et al., 2018). If limited to one ThermoMixer®, the time needed to prepare 100’s of samples can be significant. Although techniques such as microwave heating incubation have reduced the time required to about 10 minutes (3 minutes reaction + 7 minutes cooling) (Beale et al., 2018), the problem of post-derivatization degradation remains: The time elapsed from derivatization to GC introduction varies greatly from the first to the last sample, determined by the GC cycle time and the position of each sample in the sequence table. The result is a different degree of metabolite degradation within the vial from sample to sample and potentially the production of multiple derivatization products for the one metabolite (i.e., 1x trimethylsilyl (TMS), 2x trimethylsilyl (2TMS) and 3x trimethylsilyl (3TMS) derivatives). This can pose challenges in downstream data processing. Also, many laboratories cannot afford the capital outlay to invest in such microwave technologies that can facilitate these time savings. In cases where more than one conventional ThermoMixer is used (i.e., sample processing throughput is more than 20 samples), the metabolite richness will decrease in later samples within the GC-MS sequence. In larger batches, this time delay between the first and last sample, which

is sometimes >24 hours, can cause considerable degradation and changes in chromatographic data. Additionally, the manual preparation of replicates can result in higher relative standard deviation (RSD) between replicate and non-replicate samples (Zarate et al., 2017).

Here we describe an automated in-time sample derivatization protocol typically used in metabolomics studies, aimed at reducing post derivatization metabolite degradation by enabling samples to be analyzed ‘in-time’ (when needed, and at a consistent post derivatization time point for all samples). Thus, improving metabolite reproducibility via the use of the MPS tool exchange, where different syringes are utilized for specific tasks that are aimed to reduce the total number of transfers, eliminate reagent carry over and volume transfer errors.

EXPERIMENTAL

Instrumentation:

Recommended GERSTEL MPS robotic Pro modules and minimum configuration (Figure 1):

- Maestro Version 1.5.3.67 or newer.
- GERSTEL MPS robotic Pro 120 cm (minimum) rail.
- 3-Position Tool Park Station.
- Universal Syringe Module (USM) with 10 µl syringe (100111-017-00 or 100111-018-00).
- USM with 100 µL syringe (100111-004-00).
- Tray Holder with 3x VT-40 (40 x 2 mL vial) aluminum racks (015587-000-00); configured as a virtual tray (120 x 2 ml vial rack).
- Peltier cooled stack, three drawer unit with 6x VT-54 (54x 2 mL vial) plastic racks (100100-038-NS). Peltier stack unit plumbed with nitrogen to keep the cooler unit dry. Temperature set at 4°C (Peltier stack is optional).
- Agitator (100100-006-00) with inserts for 2 mL Vials (093631-002-00). Agitator set at a suitable temperature.
- Fast wash station (100100-032-00) with two 1 L wash solvent bottles; one bottle of hexane and one bottle of acetone:ethyl acetate (50:50).

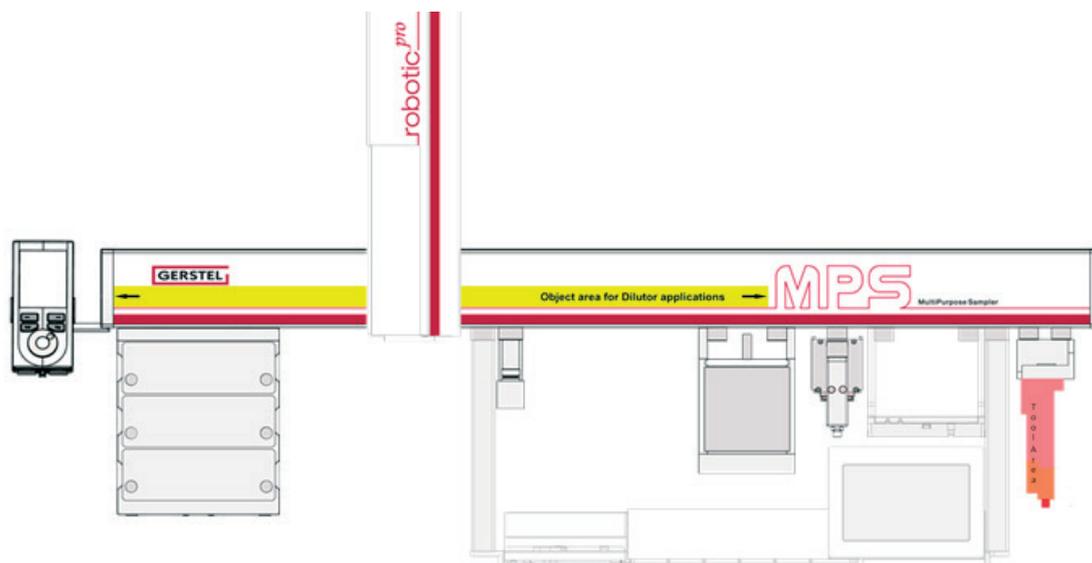


Figure 1: GERSTEL MPS robotic Pro workstation configuration.

GC-MS: An Agilent Technologies 7890B GC system coupled with an Agilent Technologies 5977B MSD was used (Agilent Technologies, Mulgrave, VIC, Australia). The GC-MS system was fitted with 2×15 m DB-5MS UI columns, 0.25 mm internal diameter and 0.25 μm film thickness (Agilent Technologies), with a mid-point backflush program. Injections (1.0 μL) were performed in 1:10 split mode. The GC-MS method was locked to myristic-d27 acid (RT 15.44 minutes). GC conditions are based after Fiehn (2016). Data acquisition and spectral analysis were performed using MassHunter GC/MS Acquisition software (B.07.06.2704) and the Qualitative Analysis software (Version B.07.00) of MassHunter workstation. Identification of the compounds was performed according to the Metabolomics Standard Initiative (MSI) chemical analysis workgroup (Fiehn et al., 2007, Sansone et al., 2007, Sumner et al., 2007). In addition, the workflow here enables identification of unknowns using retention indices based on a mixed FAMES standard.

Sample preparation:

Required equipment (Kinesis and Agilent Technologies) and reagents (all Sigma Aldrich, unless specified):

- BSTFA + 1% TMCS.
- MOX reagent (methoxyamine hydrochloride in anhydrous pyridine).
- FAMES standard (C4-C30 even carbon saturated FAME mix).
- Myristic-d27 acid (Cambridge Isotope Laboratories).

- GC vial with fused small volume insert (sample vials).
- GC vial with suitable small volume inserts.
- Magnetic vial cap (either screw or crimp top cap) with PTFE/Silicon/PTFE sandwich septa.

The following protocol is sufficient for preparing 80 vials for metabolomics profiling. This number can be extended or reduced, depending on the sample sequence/batch size. The Peltier stack can be used to replenish reagents on the universal tray holder virtual rack, remove analyzed samples and transfer new samples to be derivatized, this would simply need to be modified in the prep-sequence within the Maestro software.

Preparation of reagents and samples:

1. Transfer the MOX reagent, using either the MPS Robotic Pro (automated; using a third tool not listed here and fitted with a 500 μL syringe) or a transfer pipette, into 16 pre-labeled MOX vials.
2. Transfer the BSTFA reagent, using either the MPS Robotic Pro (automated; using a third tool not listed here and fitted with a 500 μL syringe) or a transfer pipette, into 16 pre-labeled BSTFA vials.
3. Place MOX vials in the virtual tray positions 106 – 120 (Figure 2).
4. Place the BSTFA vials in the virtual tray positions 91 – 105 (Figure 2).
5. Place the FAME standard vials (or a combination of standards) in the virtual tray positions 81 – 90 (Figure 2).

- Dry sample extracts in the GC vials with fused inserts, dried either under a stream of nitrogen or using a SpeedVac unit (not described here). Dried samples are to be placed in positions 1- 80 on the virtual tray (Figure 2).

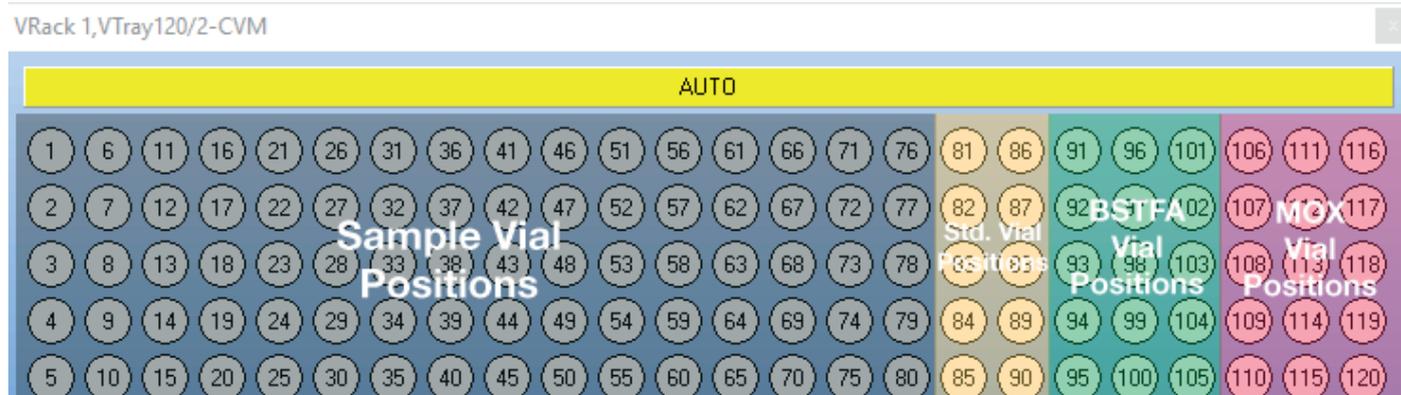


Figure 2: Sample and reagent positions on the Universal Tray Holder configured as a virtual tray.

In-time derivatization prep-sequence

In Maestro, a prep-sequence was created comprising the steps/actions listed below, noting that the intention was to replicate the standard two-step derivatization protocol typically used in offline sample preparation:

- Adding MOX reagent to each sample to prepare oximes of steroids and keto acids before silylation. MOX derivatization typically consists of agitation/mixing while incubating samples for the appropriate period of time.
- Adding BSTFA reagent to each sample prepares trimethylsilyl (TMS) derivatives. BSTFA derivatization typically consists of agitation/mixing while incubating samples under the appropriate conditions.

Note: Typically, samples are left to reach room temperature before being injected onto the GC-MS for metabolomics analysis. To ensure this is standardized, a “holding” period of 60-120 minutes is nominally set, to be determined by the analyst.

Prep-sequence functions:

- Transfer MOX reagent from the MOX reagent vial to the sample vial using the 100 μ L syringe tool, ensuring the syringe is washed in both hexane and the acetone:ethyl acetate wash solvents before and after each transfer. MOX reagent is to be added using the Accurate Add feature in Maestro, allowing for 10% of the MOX reagent to be lost to waste.
- Move sample vial with MOX from tray to agitator and mix under the appropriate conditions.
- Move sample vial with MOX from agitator, to tray. Note: This move step is optional, as the BSTFA reagent addition described in the next step can be done directly through the top of the agitator cover. However, there is a pause to agitation while this transfer occurs.
- Transfer BSTFA reagent to the sample vial using the 100 μ L syringe tool, ensuring the syringe is washed in both hexane and the acetone:ethyl acetate wash solvents. BSTFA is to be added using the Accurate Add feature, allowing for 10% of the BSTFA reagent to be lost to waste.
- Transfer FAME standard to the sample vial using the 10 μ L syringe tool, ensuring the syringe is washed in both hexane and the acetone:ethyl acetate wash solvents. The FAME standard is to be added using the Accurate Add feature, allowing for 10% of the FAME standard to be lost to waste.
- Move sample vial with MOX + BSTFA + FAME standard from tray to agitator, and mix under the appropriate conditions. Note, this move step is not needed if the BSTFA and FAME standard are added through the agitator cover.

7. Move sample vial with MOX + BSTFA from agitator to tray.
8. Leave the derivatized sample on the tray for 60-120 minutes.
9. Change syringe tool from 100 μ L syringe to 10 μ L syringe, inject 1 μ L of sample (or as per the GC method).

Note: Dedicating the 100 μ L syringe for reagent transfers minimizes the number of septum punctures per transfer. For example, using a 10 μ L syringe to transfer the MOX, BSTFA and FAMES reagents equates to 7 vial punctures before the sample is picked up for injection. While, the use of PTFE/Silicon/PTFE septa ensures sample integrity is maintained, minimizing the number of transfers and septum piercings will ultimately result in reducing syringe volume transfer errors, potential degradation of the septa and possible deterioration of the vial contents.

Note: Washing syringes using the fast wash module enables each syringe to be rinsed with varying polarity solvents, using a larger volume and at a fast wash flow rate compared to conventional wash bottle stations.

Figures 3 and 4 illustrate the prep-sequence steps within the prep-sequence editor and the graphical overview of these steps for visualizing the pre-sequence process.

PrepBuilder 1 - D:\MassHunter\GCMS\1\Tunes\Def_Meta_Prep.prp

Prep. Settings
Vial Range: 1-5

Prep. Action Settings
Action: PREP
Method: Ahead, Extensive - Prep Ahead as far as possible
Syringe: 10ul 57mm LS 4
Source: [Dropdown] Vial: AUTO
Destination: [Dropdown] Vial: AUTO

Buttons: Add, Insert, Replace, Delete, Clean

Action	Method / Value	Source	Vial	Destination	Vial	Misc
PREP Vials 1-5	Ahead, Extensive					
ADD	Add MOX Large syringe	VRack 1.VTray120/2-CVM	120	VRack 1.VTray120/2-CVM		100ul 57mm LS 1
MOVE		VRack 1.VTray120/2-CVM		Agitator 1.AgiTray6/2-CVM		100ul 57mm LS 1
MIX	Mix for x min					
MOVE		Agitator 1.AgiTray6/2-CVM		VRack 1.VTray120/2-CVM		100ul 57mm LS 1
ADD	Add BSTFA Large syringe	VRack 1.VTray120/2-CVM	105	VRack 1.VTray120/2-CVM		100ul 57mm LS 1
ADD	Add FAME Small syringe	VRack 1.VTray120/2-CVM	85	VRack 1.VTray120/2-CVM		10ul 57mm LS 4
MOVE		VRack 1.VTray120/2-CVM		Agitator 1.AgiTray6/2-CVM		100ul 57mm LS 1
MIX	Mix for Y min					
MOVE		Agitator 1.AgiTray6/2-CVM		VRack 1.VTray120/2-CVM		10ul 57mm LS 4
WAIT	Wait z min					
INJECT	Metabolomics_split_RTLM	VRack 1.VTray120/2-CVM		Front_MMI		10ul 57mm LS 4
END						
PREP Vials 6-10	Ahead, Extensive					
ADD	Add MOX Large syringe	VRack 1.VTray120/2-CVM	120	VRack 1.VTray120/2-CVM		100ul 57mm LS 1
MOVE		VRack 1.VTray120/2-CVM		Agitator 1.AgiTray6/2-CVM		100ul 57mm LS 1
MIX	Mix for Y min					
MOVE		Agitator 1.AgiTray6/2-CVM		VRack 1.VTray120/2-CVM		100ul 57mm LS 1
ADD	Add BSTFA Large syringe	VRack 1.VTray120/2-CVM	105	VRack 1.VTray120/2-CVM		100ul 57mm LS 1
ADD	Add FAME Small syringe	VRack 1.VTray120/2-CVM	85	VRack 1.VTray120/2-CVM		10ul 57mm LS 4
MOVE		VRack 1.VTray120/2-CVM		Agitator 1.AgiTray6/2-CVM		100ul 57mm LS 1
MIX	Mix for Y min					
MOVE		Agitator 1.AgiTray6/2-CVM		VRack 1.VTray120/2-CVM		10ul 57mm LS 4
WAIT	Wait z min					
INJECT	Metabolomics_split_RTLM	VRack 1.VTray120/2-CVM		Front_MMI		10ul 57mm LS 4
END						
PREP Vials 11-15	Ahead, Extensive					
ADD	Add MOX Large syringe	VRack 1.VTray120/2-CVM	120	VRack 1.VTray120/2-CVM		100ul 57mm LS 1
MOVE		VRack 1.VTray120/2-CVM		Agitator 1.AgiTray6/2-CVM		100ul 57mm LS 1
MIX	Mix for x min					

Buttons: Edit Sample Table, OK, Cancel, Help

Figure 3: PrepBuilder, Prep sequence editor within Maestro software, with the steps and sequence needed for in-time derivatization in metabolomics studies.

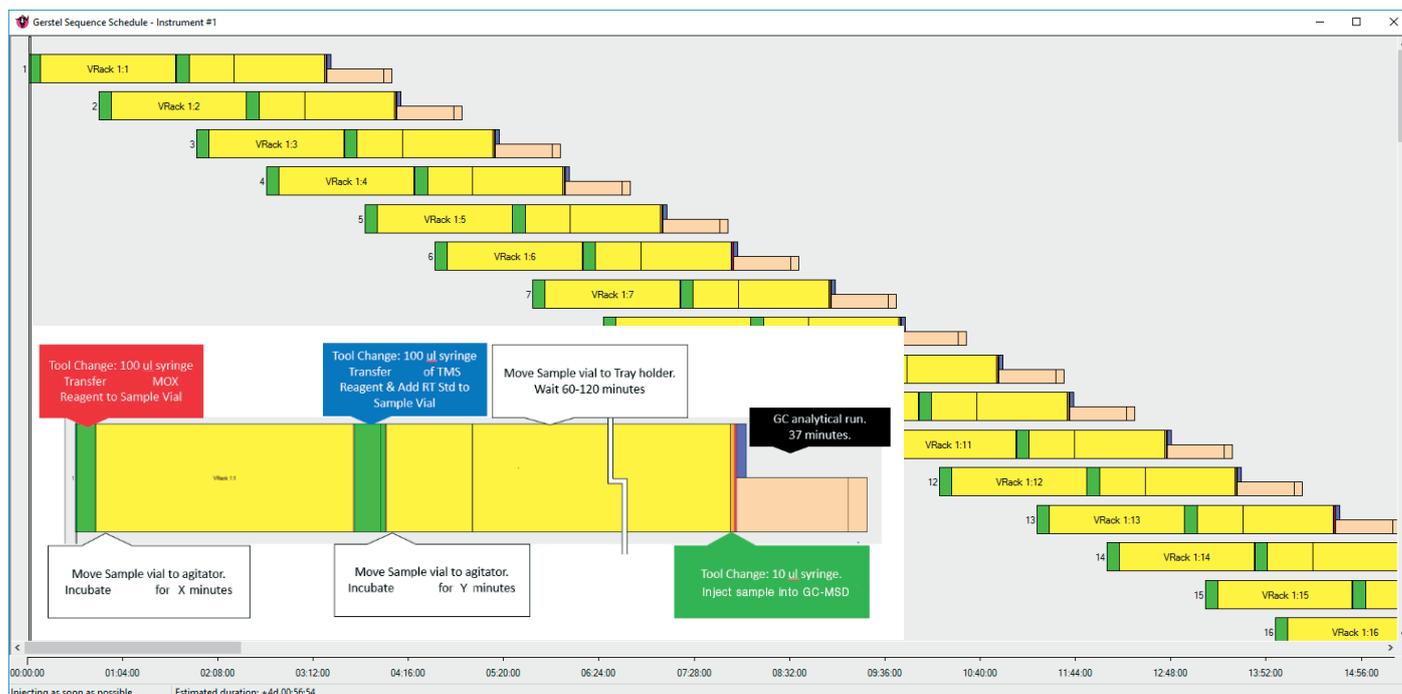


Figure 4: Maestro PrepSequence Scheduler overview of the created prep-sequence for 1 sample (insert) and a visualization of the prep-sequence for multiple samples using the prep-ahead loop.

To illustrate the application of the in-time derivatization protocol, overlapped using the prep-ahead sequence feature of Maestro, a mixed standard of authentic and isotopically labelled standards was used. The table below lists the standards used in the metabolomics mix. The derivatization protocol implemented on the GERSTEL MPS robotic Pro was compared with a conventional offline approach, where samples are agitated and incubated using a ThermoMixer®. The offline approach is limited to 24 samples per batch.

	Metabolite	Supplier	Cat. Number	CAS	Conc. (mg/mL)	Solvent
1	L-Valine	Sigma Aldrich	V-0500	72-18-4	10	Water
2	Succinic acid	Supelco (Sigma Aldrich)	47264	110-15-6	10	Water
3	L-Methionine	Sigma Aldrich	M-9625	63-68-3	10	Water
4	4-Hydroxyproline	Sigma Aldrich	H6002	51-35-4	10	Water
5	Salicylic acid	Sigma Aldrich	84210-100G	69-72-7	10	Water
6	α-Ketoglutaric acid	Sigma Aldrich	K1128-25G	328-50-7	10	Water
7	Shikimic acid	Supelco (Sigma Aldrich)	47264	138-59-0	10	Water
8	Citric acid	Supelco (Sigma Aldrich)	47264	77-92-9	10	Water
9	L-Lysine	Sigma Aldrich	L-5626	657-27-2	10	Water
10	Myristic acid	Sigma Aldrich	70079-5G	544-63-8	10	Chloroform
11	Stigmasterol	Sigma Aldrich	47132	83-48-7	10	Chloroform
12	d-Glucose- ¹³ C	Cambridge Isotope Laboratories	CLM-1396-10	110187-42-3	10	Water
13	Glycine- ¹³ C	Cambridge Isotope Laboratories	CLM-422-5	20110-59-2	10	Water
14	Glutamine (amide- ¹⁵ N)	Cambridge Isotope Laboratories	NLM-557-PK	59681-32-2	10	Water

Table 1: Metabolite stock solution

As illustrated in Table 2, RSDs for the mixed metabolomics standard improved significantly when implementing the offline (batch) derivatization protocol on the GERSTEL MPS robotic Pro using in-time sample preparation. This is further highlighted in the quantification/recovery assessment of these metabolites at 250 ppb (Figure 5).

Compound	Off-line (batch)	In-time (GERSTEL)
	Calculated RSD (n=20)	
Glycine- ¹³ C	21.7%	5.6%
L-Valine	9.5%	3.2%
Succinic acid	9.5%	3.5%
Salicylic acid	17.5%	7.6%
L-Methionine	9.9%	3.9%
4-Hydroxyproline	15.6%	5.1%
alpha Ketoglutaric acid	21.3%	7.6%
L-Lysine	25.8%	9.4%
Glutamine- ¹⁵ N amide	32.7%	8.1%
Shikimic acid	5.9%	4.9%
Citric acid	10.9%	3.8%
Myristic acid	2.9%	2.6%
d-Glucose- ¹³ C	9.5%	8.9%
Palmitic acid- ¹³ C	6.2%	5.9%

Table 2: Comparison between RSDs achieved using an offline batch derivatization protocol and using the same protocol implemented on the GERSTEL MPS robotic, based on just-in-time/in-time sample preparation in PrepAhead mode.

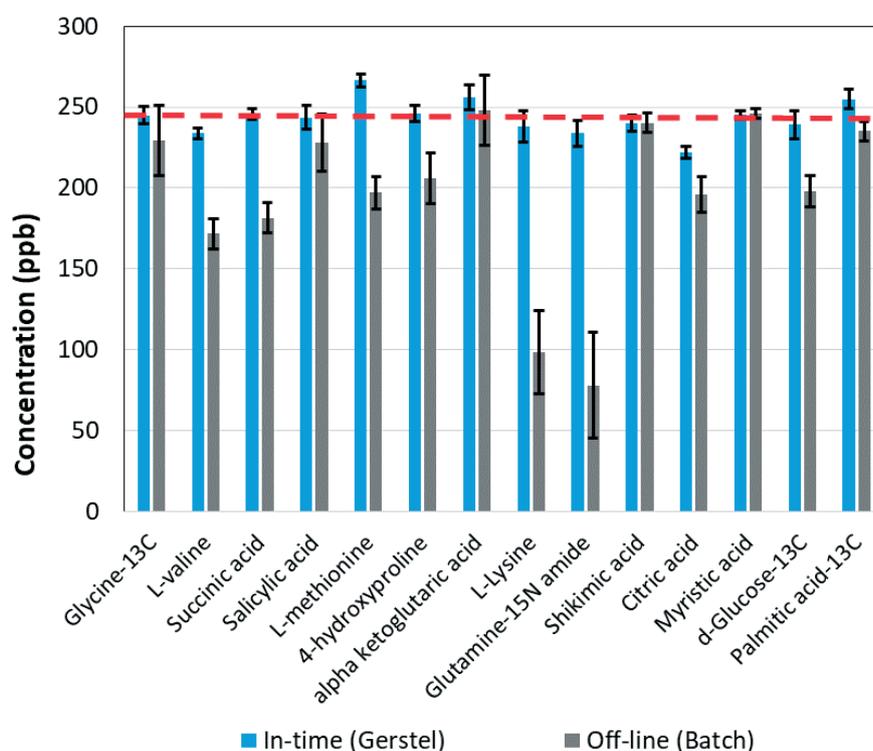


Figure 5: Quantification/recovery assessment for metabolites at 250 ppb using in-time (blue bars) and off-line/batch (grey bars) derivatization. RSD values are listed in table 2.

CONCLUSIONS

In this work, we have demonstrated an application that facilitates increased sample throughput in metabolomics studies. While the protocol here has scope for holding and preparing upward of 80 samples, with the use of the Peltier cooled stack with multiple sample trays, this can be increased to 200-300 samples. The workflow can be achieved and set up in Maestro, where sample movements from the Peltier stack to the virtual tray for sample derivatization, and vice versa, are defined. Noting that instrument maintenance and performance activities will still have to be managed to ensure ongoing reliable analysis, this can be achieved even for less than perfectly clean samples by incorporating the GERSTEL Automated Liner Exchange (ALEX) option. Furthermore, automated sample preparation and handling minimizes errors and problems relating to sample-to-sample batch derivatization differences and time differences between sample preparation and -injection. Using the GERSTEL MPS robotic Pro, individual samples are derivatized at a defined point in time (in-time) before injection into the GC-MS system, significantly reducing metabolite degradation throughout the sequence. As observed here, sample RSDs were significantly improved compared to offline approaches. This is most likely a combination of the in-time sample derivatization capability of the GERSTEL MPS robotic Pro, but also partly resulting from the use of precision 'Accurate add' reagent transfers, using specific large syringes for all the reagent transfers and minimizing the number of septum punctures. Lastly, the automated protocol described here has been observed to be economical in terms of reagent use and labor time, with a total reagent volume reduction and significant technician time savings compared to batch protocols (Table 3).

Number of samples	Off-line (Batch)		In-time (GERSTEL MPS)	
	Manual sample preparation time*	GC analysis time [#] , derivatization time NOT included	Manual sample preparation time [^]	GC analysis time [#] , derivatization time included
1	3.5 h	0.6 h	30 min	4.1 h
25	3.5 h	23.2 h (~1 day)	30 min	26.4 h (~1 day)
50	7.0 h	46.5 h (~2 days)	30 min	49.5 h (~2 days)
75	10.5 h (> 1 day)	69.7 h (~3 days)	30 min	72.8 h (~3 days)
100	14.0 h (2 days)	93.0 h (~4 days)	60 min	95.9 h (~4 days)
200	28.0 h (>3 days)	186 h (~8 days)	60 min	189 h (~8 days)

Table 3: Time required for offline batch derivatization and for GC/MS analysis compared with the time required for in-time derivatization and GC/MS analysis implemented using the Gerstel MPS and Maestro prep-sequence in PrepAhead mode.

Note:

*The manual sample preparation time for the Off-line (batch) approach is the time needed to manually prepare and derivatize samples for GC-MS analysis. This includes reagent transfers, incubations, placing vials on the sample trays, and setting up the sample sequence and instrument for analysis.

[^]The manual sample preparation time for the In-Time (GERSTEL) approach is the time needed to prepare reagent vials, place vials on the sample trays, and set up the sample sequence and instrument for analysis.

[#] The GC analysis time for the off-line protocol is the analytical run time only. The GC analysis time for the in-time protocol (GERSTEL) is the sample derivatization preparation time and the GC analysis time combined.

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