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A NOVEL PLATFORM FOR AUTOMATED GC-MS BASED METABOLOMICS

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I. INTRODUCTION

We have developed a complex automated analytical platform called MetaboAuto for unattended GC-MS analysis of protic metabolites in body fluids and tissue extracts followed by automated data processing by an in-house developed Metabolite Cloud toolbox.

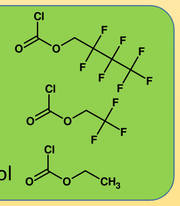
The simultaneous liquid-liquid microextraction (LLME) and alkylchloroformate (RCF) derivatization provides stable reaction products (esters, *N*-carbamates and (*S,O*)-carbonates) with greatly reduced polarity, immediatly measurable by GC-MS. Metabolite coverage is more than 200 metabolites.

The tested RCF reagents:

Heptafluorobutyl chloroformate (HFBCF)

Trifluoroethyl chloroformate (TFECF)

Ethyl chloroformate (ECF) and ethanol



The GC-MS metabolite profiling workflow:

II. AUTOMATED SAMPLE PREPARATION

A GC-MS instrument built-in or a stand-alone workstation developed from in-house components and by modification of an RTC autosampler corpus (CTC Analytics, Zwingen, Switzerland) have been used for sample preparation. The in-house programmed MetaboAuto workstation operations comprise sample aliquot transfers, addition of internal standards, reagents, vortexing, dilution, mixing, withdrawal of an organic layer, evaporation of solvents by nitrogen, exchange and cleaning of syringes followed by injection of the final sample extracts into a GC (and/or HPLC) chromatograph.

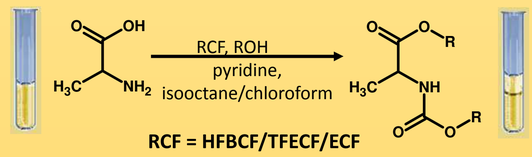
Manual sample preparation

Automated sample preparation

A) Tool station (2 exchangeable syringes - 100 µl syringe for sample work-up, 10 µl syringe for sample injection)
B) Wash station
C) Vortex station
D) Trayholder for samples, reagents, reaction vials
E) Injection port
F) GC with s/sls
G) Q-MS analyzer

ISQ Single quadrupole MS (Thermo Fisher Scientific)

Derivatization and liquid-liquid microextraction – general reaction scheme:



RCF = HFBCF/TFECF/ECF

DATA PROCESSING

The RCF-treated metabolites are efficiently separated and ionized by EI or isobutane PICI or APCI mass spectrometry and automatically processed by in-house SW platform called **Metabolite Mapper** which enables automatic metabolite identification against an in-house MS spectral database and quantitative determination of targeted metabolites.

Watch video here:
www.youtube.com

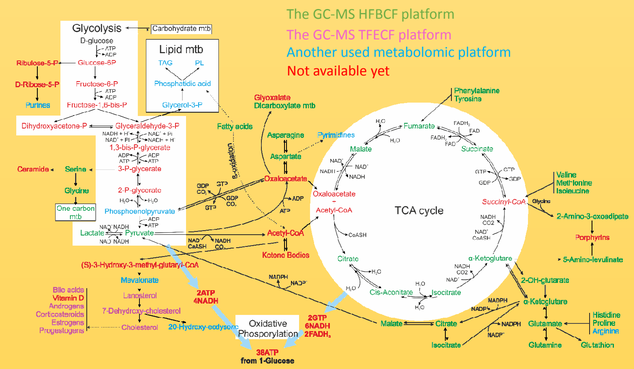
MetaboliteMapper in the MetCloud web interface

- Nominal data filtering, peak detection, peak alignment
- Grouping of features, component identification
- Analysis batch alignment
- High performance computing platforms
- Remote data processing and mining via the in-house MetCloud web interface

Reproducibility of automated (A) and manually (B) ECF-derivatization of urine for selected metabolites

(A)	Alanine	Valine	Leucine	Proline	Aspartate	Phenylalanine
Average	1.221	0.076	0.062	0.048	0.136	0.04
SD	0.014	0.003	0.003	0.002	0.007	0.002
RSD (%)	1.1	4.2	5.1	5.2	5.2	4.5
(B)	Alanine	Valine	Leucine	Proline	Aspartate	Phenylalanine
Average	1.04	0.328	0.528	0.84	0.168	0.191
SD	0.011	0.025	0.032	0.027	0.009	0.018
RSD (%)	1.1	7.6	6	3.2	5.2	9.5

Tab.1.: The average values are displayed like ratio between the instrument response for metabolites and appropriate ^{13}C labeled internal standards (n=10).



Metabolome coverage measured by the developed GC-MS platforms

III. APPLICATIONS

GC–MS PROFILING IN URINE AND HUMAN PLASMA

Fluorinated alkyl chloroformate (FCF) reagents are highly reactive and capable to transform the target protic functional groups under pyridine catalysis without the necessity of excess of the corresponding alcohol. From the examined set of 150 diagnostic urinary metabolites (**Fig. 1**) with HFBCF, a single product was provided by 119 metabolites (79%), two and more derivatives were yielded by 27 metabolites (18%) and 2 metabolites (uric acid, urea) remained untouched. Between-run precision and accuracy was at all analytes between 0.7-19 %, 81-120 %, respectively.

The developed protocols were applied to GC–MS analysis of 100 morning urines obtained from healthy patients where 108 metabolites relevant to the major metabolite pathways were clearly detected in a 40 µl sample aliquot. **[4]**

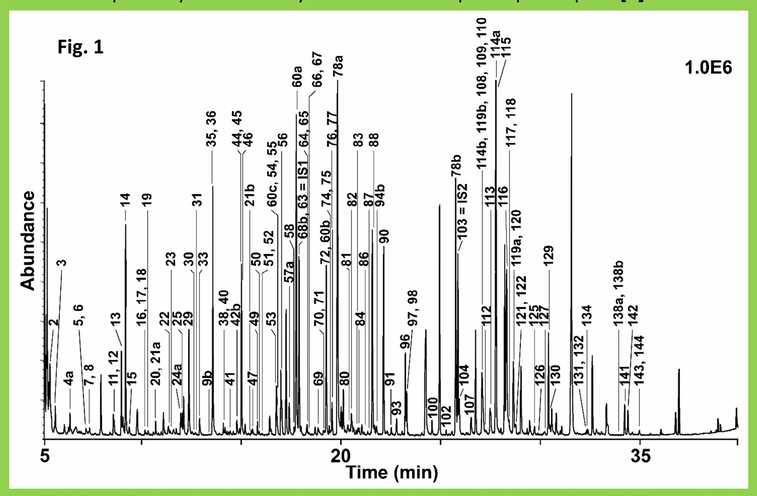
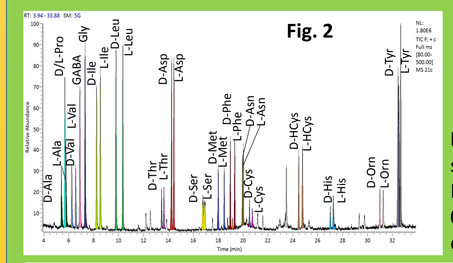


Fig. 1 TIC-GC-MS of real urine sample (40 µl) with identified (see **[4]**) HFBCF-metabolites

A similar protocol was earlier succesfully applied to GC-MS analysis of biomarkers related to folate and cobalamin status in human serum. **[1]**

CHIRAL AND ACHIRAL GC–MS PROFILING

HFBCF derivatization allows the chiral GC–MS analysis of all proteinogenic amino acid enantiomers, except D,L-arginine, D,L-cystine (not eluted) and D,L-proline (not separated) on a Chirasil-Val capillary column.



HFBCF-treated *L*-(+)-alanine

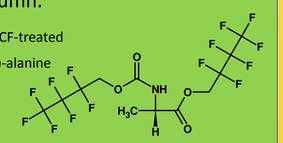
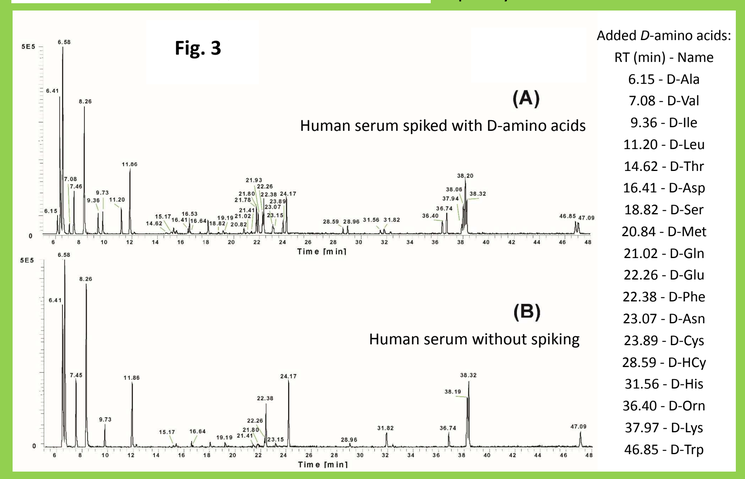


Fig.2 TIC GC–MS separation of selected amino acids as the HFBCF derivatives on a 25 m × 0.25 mm; 0.12 µm i.d. Chirasil-Val capillary column.



Added *D*-amino acids:
RT (min) - Name
6.15 - D-Ala
7.08 - D-Val
9.36 - D-Ile
11.20 - D-Leu
14.62 - D-Thr
16.41 - D-Asp
18.82 - D-Ser
20.84 - D-Met
21.02 - D-Gln
22.26 - D-Glu
22.38 - D-Phe
23.07 - D-Asn
23.89 - D-Cys
28.59 - D-HCy
31.56 - D-His
36.40 - D-Orn
37.97 - D-Lys
46.85 - D-Trp

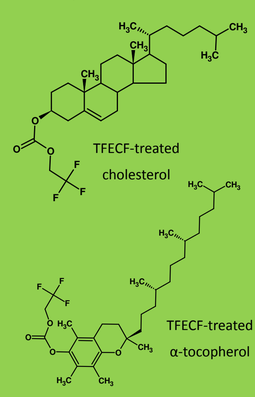
Fig. 3. TIC GC–MS chromatogram of the chiral amino acid analysis obtained from the serum extract. **[2]**

TOCOPHEROLS AND STEROIDS IN HUMAN PLASMA

The developed protocol demonstrates capability of the RCFs to derivatize **alicyclic hydroxyls in steroids and tocopherols** metabolites for GC–MS with excellent reaction rates, highest reaction yields, minor reagent consumption and easy conjunction with LLME methods.

LOQ: sterols 0.05 µg/ml
tocopherols 0.15 µg/ml
Within-run precision: 0.9 – 19.5 %
Between-run precision: 0.2 – 19.0 %
Accuracy: 82 – 115 %
Recovery: 90 – 110 %

The new method was validated for the determination of 6 diagnostic non-cholesterol sterols and four main tocopherols in **human serum and in amniotic fluid**. It was succesfully applied to GC-MS profiling in 40 woman sera and amniotic fluids, the result are well-comparable with those reported by other authors. **[3]**



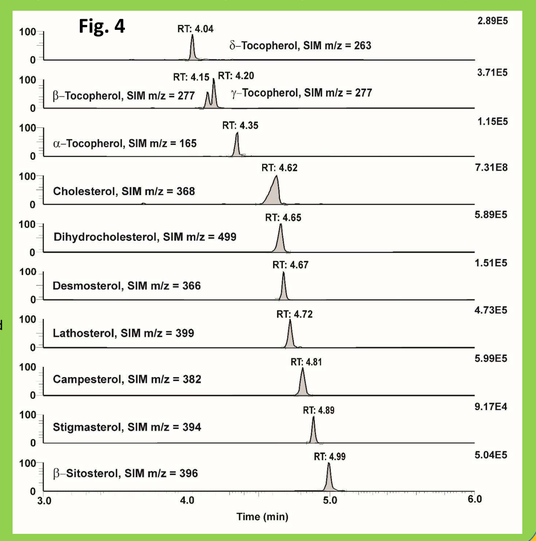


Fig. 4. Characteristic SIM GC–MS chromatograms of steroids and tocopherols detected in serum of a pregnant woman

IV. CONCLUSION

The novel metbolomic analytical platform was successfully applied to the GC-MS analysis of protic metabolites in human plasma [1-3] and urine [4]. Novel heptafluorobutyl chloroformate (HFBCF) and trifluoroethyl chloroformate (TFECF) based sample preparation protocols have enabled GC-MS analysis of 30 protic plasma metabolites involved in one carbon metabolism [1], 35 amino acid enantiomers in plasma [2], 16 plasma steroids, sterols and tocopherols [3] and 132 amino-carboxylic metabolites in urine [4].

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4. Hušek P., Švagera Z., Hanzlíková D., Řimnáčová L., Zahradníčková H., Opekarová I., Šimek P., J. Chromatogr. A (2016) 1443 211-232.

[1] [2] [3] [4]

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