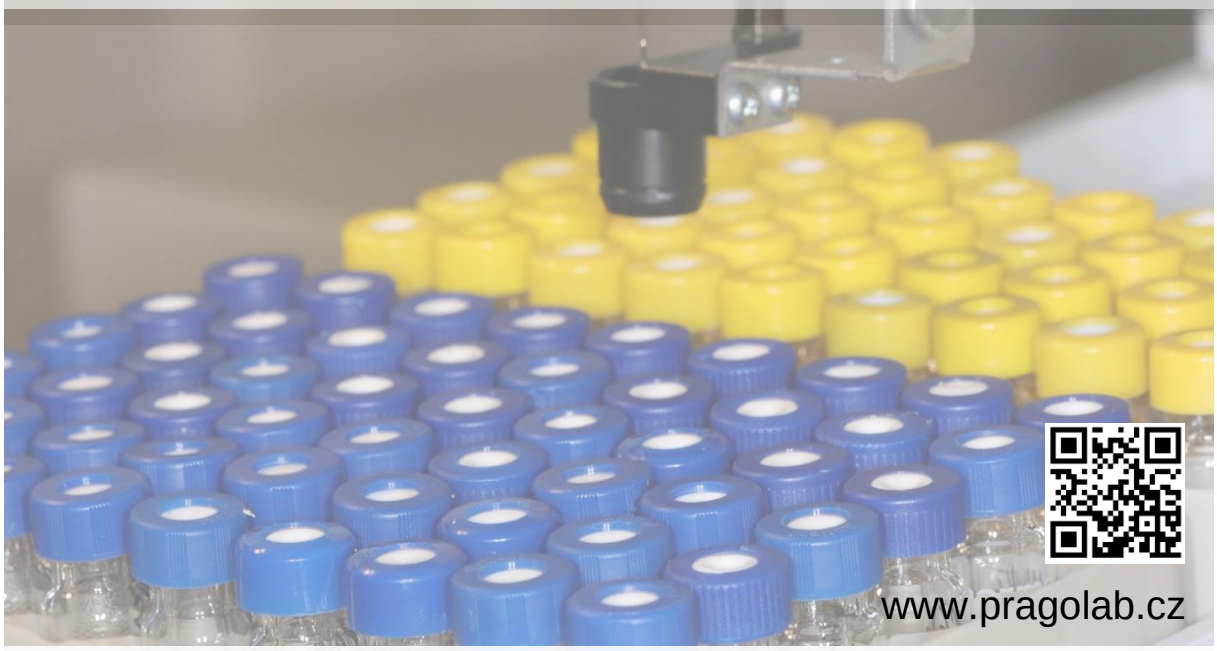
GC-MS Application Note

MetaboAuto® - Automated Sample Preparation Platform for on-line GC-MS Metabolomics

Metabolomic screening of more than 250 protic metabolites in biofluids and cell tissues



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METABO AUTO®

MetaboAuto® - Automated Sample Preparation Platform for on-line GC-MS Metabolomics

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Keywords: metabolomics, automatic sample preparation, alkyl chloroformate derivatization, liquid liquid microextraction, GC-MS, human serum/plasma, urine, cell tissue

Overview

- Validated automation analytical technology for determination > 90 metabolites and xenobiotics in human serum/plasma, urine and liver tissue.
- The developed sample preparation workflow involves immediate, in-situ derivatization with ethyl chloroformate in an aqueous biological matrix. The derivatized metabolites concomitantly migrate into an immiscible organic phase (simultaneous derivatization + liquid liquid microextraction analycal workflow).
- Metabolite coverage: amino acids, organic acids, small peptides, biogenic amines
- Automated sample preparation in stand-alone or on-line (on-deck) mode integrated with the autosampler function.
- Total analysis time in the on-line mode: < 25 min
- The sample preparation kit contains all necessary reagent media and consumables.
- Metabolite library containing EI mass spectra of > 250 protic metabolite derivatives and their structures that can be uploaded into the NIST® mass spectral library.
- Automated Qual/Quan utility for the Thermo Scientific (Xcalibur, TraceFinder) and Agilent (MassHunter) data processing software
- The sample work-up compatibility with LC-MS pipeline (MetaboAuto® for LC-MS).
- The MetaboAuto® platform extendable for the use of fluoroalkyl chloroformates and reagents containing stable isotopes (D3 and $13C_2$).
- The Operation Manual includes a video demonstrating the complete sample preparation workflow.
- Upgrade of the Phenomenex EZ:faast™ Amino Acid Analysis Kits

Introduction

Despite rapid progress in analysis of polar metabolites, their high-throughput, comprehensive quantitative analysis remains a challenging task. GC-MS represents a cost-effective and well-established technique in routine metabolomic analysis. However, the sample preparation requires derivatization of protic functional groups. Among the current methods, use of alkyl chloroformates (RCFs) such as the ethyl reagent (ECF) have been next to oximation-silylation protocols most established approach.

ECF directly attacks primary; secondary amino-, hydroxy-, thiol- and carboxy groups providing respective carbamate, (thio)carbonate and ester derivatives with high yields in a two-phase aqueous-organic medium. In contrast to the oximation-silylation approach, the chloroformate reaction media can be applied directly, is-situ, to the aqueous biological matrix. During the reaction, the ECF excess is decomposed to carbon dioxide facilitating the sample mixing and less polar product formation proceeding in seconds. The derivatives are simultaneously transferred into an immiscible organic phase while undesired background sample matrix is thus efficiently eliminated so that even single quadrupole GC-MS instruments can be utilized for the metabolite analyses. Automation of the workflow enables an unattended 24h/7d sample preparation and improves validation parameters of the obtained quantification data. The MetaboAuto® robotic workstation involves a CTC PAL robotic tool exchanger arm and several modules that can be assembled, and their operation programmed according to the customer´s needs, **Fig.1.**

The MetaboAuto® platform mounted on a GC-MS system performs the following operations: transfer of liquid samples, sample dilution, separation of two layers of liquids by microcentrifugation, vortexing, and injection of the sample into the GC injector.

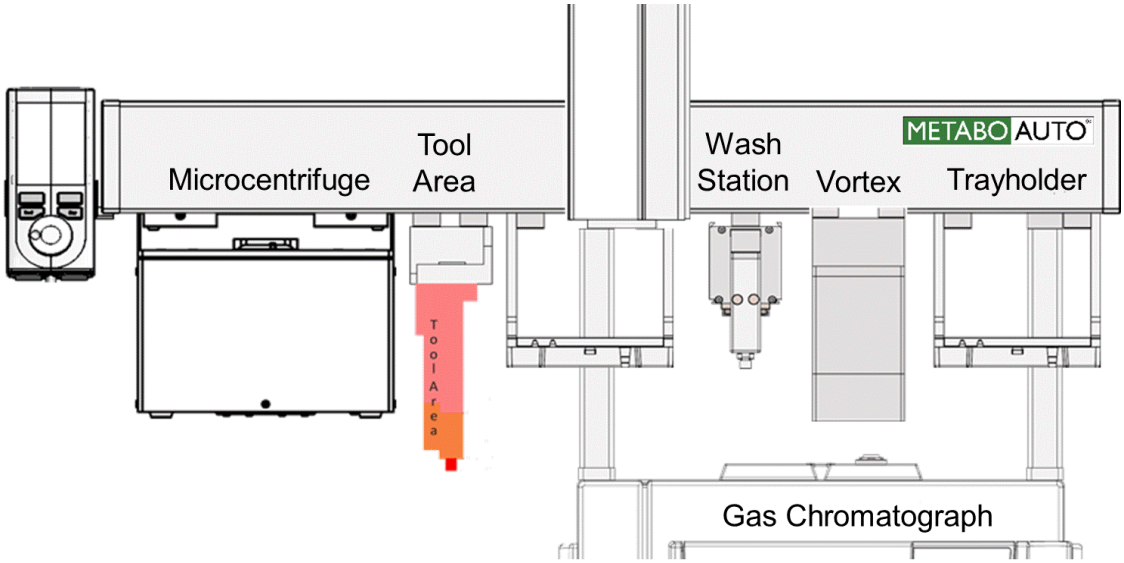

Fig. 1 Block diagram of the MetaboAuto® unit allowing automated sample preparation and injection into a GC-MS system: on-line (on-deck) version (from left), a microcentrifuge for liqud-liquid microextraction and obtaining an organic phase; a tool area for the storage and changing of sampling and dispensing syringe (volumes 10 µL, 100µL); a tray holder (for 2 x 54 reaction vial; 2 x 54 sample vial and 1 x 15 vials with reagent solutions); a Fast Wash Module for the used solvents (chloroform, isopropanol); a vortex module for mixing of a reaction sample medium.

Methods

Sample preparation

Protic metabolites possessing active hydrogens in various functional groups (carboxy-, amino-, hydroxy- and thiol-) can be measured after their ethyl chloroformate (ECF) labeling. **Fig. 2** shows the automated sample preparation workflow for GC-MS metabolomics of protic metabolites in diverse biological matrices. It involves 6 steps: (1) addition of an internal standard, (2) buffer 1, (3) catalytic medium, (4) chloroformate reagent 1, (5) buffer 2 and reagent 2 followed by transfer of organic phase into an autosampler vial and injection into a GC-MS system. Urine (25 µl) is worked-up online, while plasma/serum (25 µl) requires a preliminary parallel lipoprotein precipitation, centrifugation of the samples and the supernatant transfer into an autosampler vial. The cell tissue extraction (7 mg) is more complex and comprises a cell structure destruction by homogenization (e.g. with a TissueLyser, Qiagen) at -18 °C, sonification and centrifugation at 4 °C. The arising supernatant is further subjected to the automated sample preparation procedure. The complete process ensures transformation of protic metabolites in well-defined derivatives that are simultaneously extracted into an organic phase. Organelle and membrane residues, (lipo)proteins, sugars and most of the pyridine catalyst are thus efficiently eliminated and background noise minimized providing much cleaner sample extracts and lower GC-MS system contamination. The overall reaction scheme for the derivatization of tyrosine is shown in **Fig. 3.**

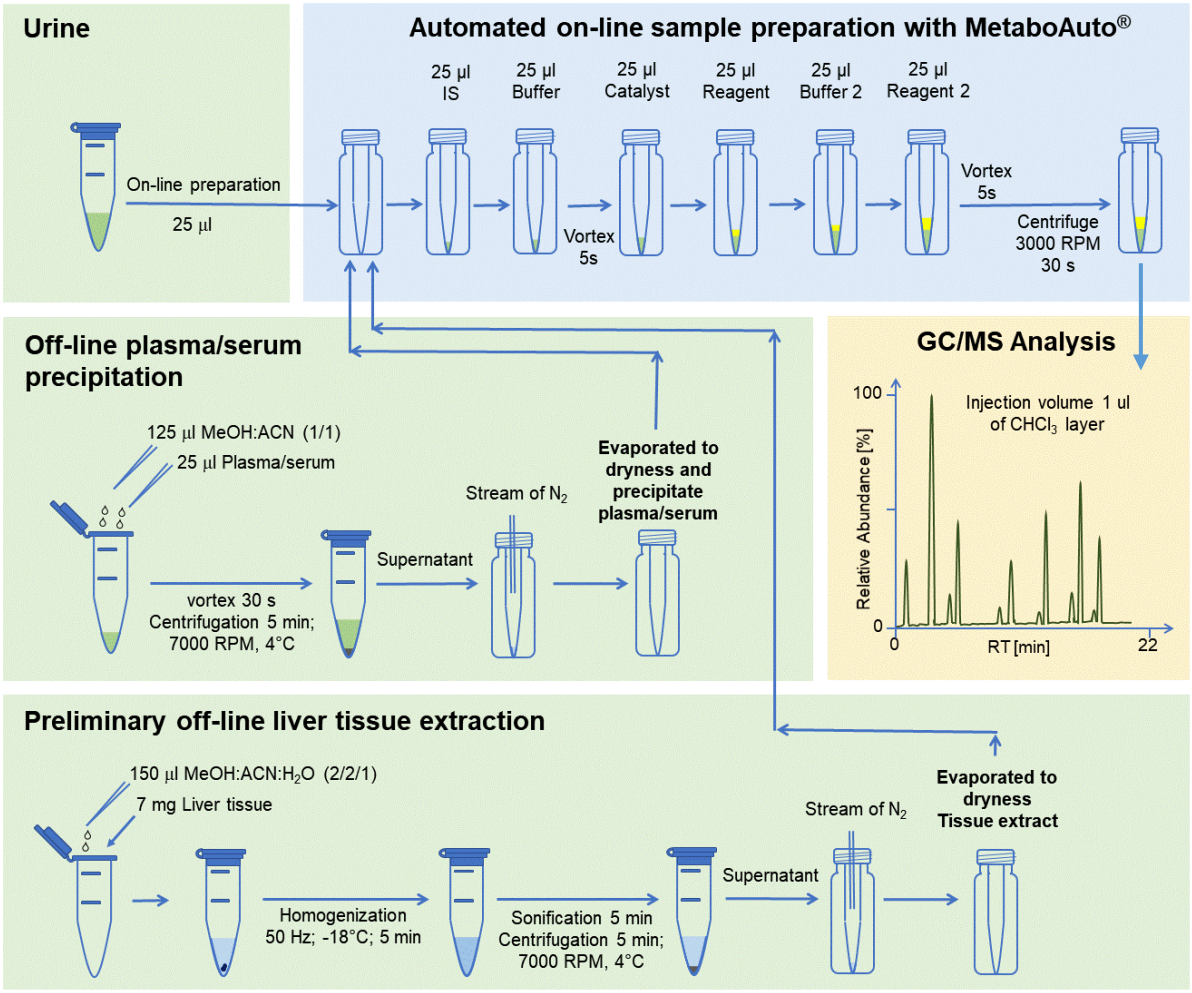

Fig. 2: Automated sample preparation for biological samples and manual presample off-line steps for plasma /serum and cell tissue prior to the developed automated on-line sampler preparation with MetaboAuto®.

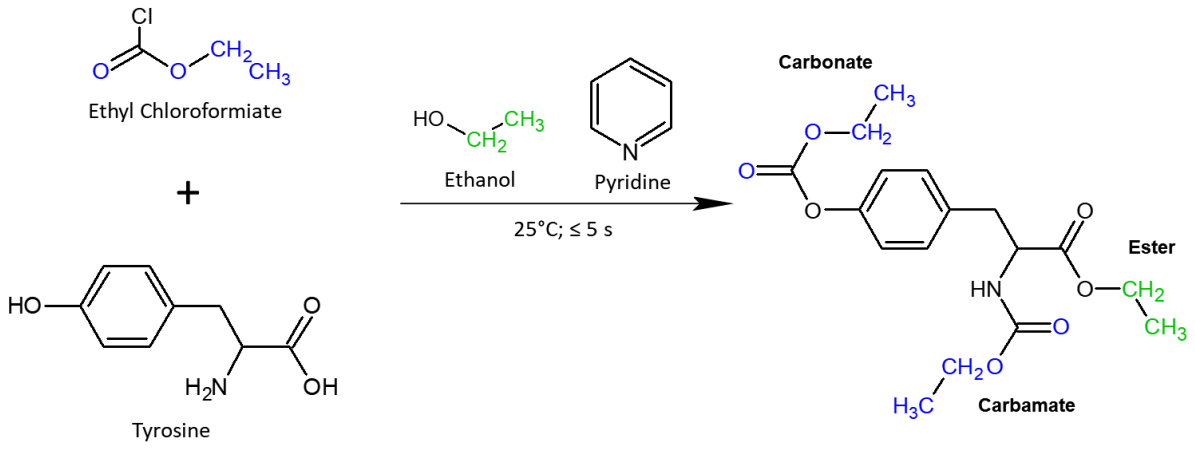

Fig. 3: The ECF – ethanol derivatization scheme demonstrated on the reaction with tyrosine.

Instrumentation

MetaboAuto® enables robust, routine GC-MS metabolomic measurements on every instrument capable to communicate with a CTC PAL RTC equipment. The online sample preparation mode ensures metabolite profiling in less than 22 min and unattended simultaneous preparation of a next sample for another GC-MS run. Fresh sample extracts are thus prepared in the same time window improving further measurement uncertainties and method validation statistics. The obtained sample extract (1µl) is injected in a splitless mode, the metabolite derivatives are separated on a medium polar capillary column and after electron ionization detected in either a full scan (m/z 40-500 Da) or SIM (MRM) MS scan mode. A typical GC-MS instrument set-up is shown in **Fig. 4.**

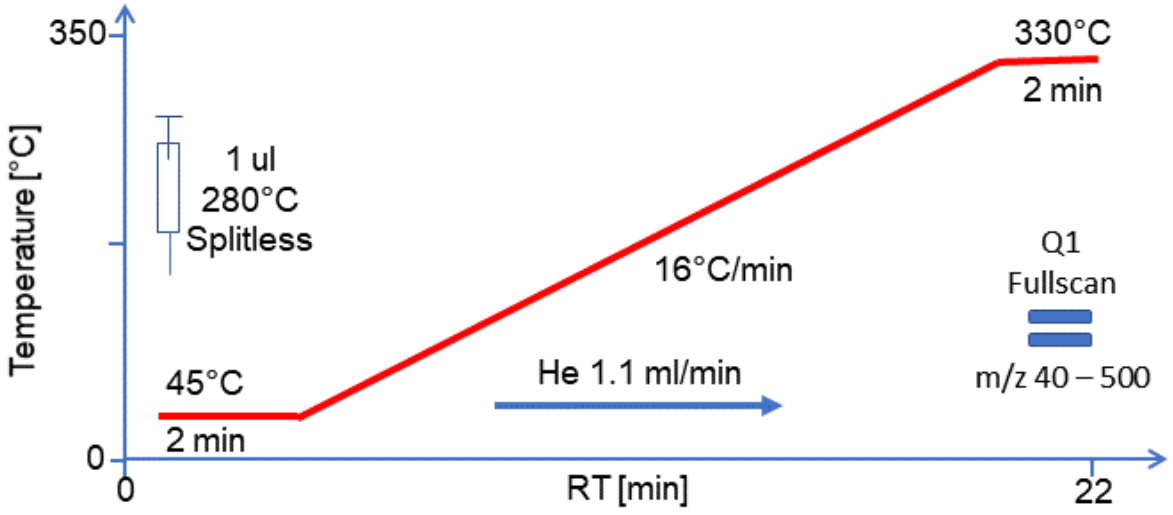

Fig 4: A typical GC-MS instrument set-up and temperature programming for profiling of more than 250 protic metabolites used for metabolite analysis after the MetaboAuto ® sample preparation.

Data processing

For the convenient data handling, the Metabo-Auto® platform is equipped with tools for the automatic metabolite identifications and their quantitative analysis. The metabolites (as the ECF-ethanol derivatives) are detected with aid of the Metabo-Auto® library retention time (RT) and EI mass spectral libraries which contains RT data and EI mass spectra of > 250 protic metabolites. Moreover, the library can be uploaded and directly used with a commercial NIST mass spectral library. This feature is illustrated in **Fig.5**, where a routine for the detection of tyrosine in the MetaboAuto® library is depicted.

Quantitative analysis of the detected protic metabolites is facilitated by the developed software utility which allows immediate analyte quantifications in the Thermo Scientific (Xcalibur, TraceFinder) or Agilent (MassHunter) data processing environment.

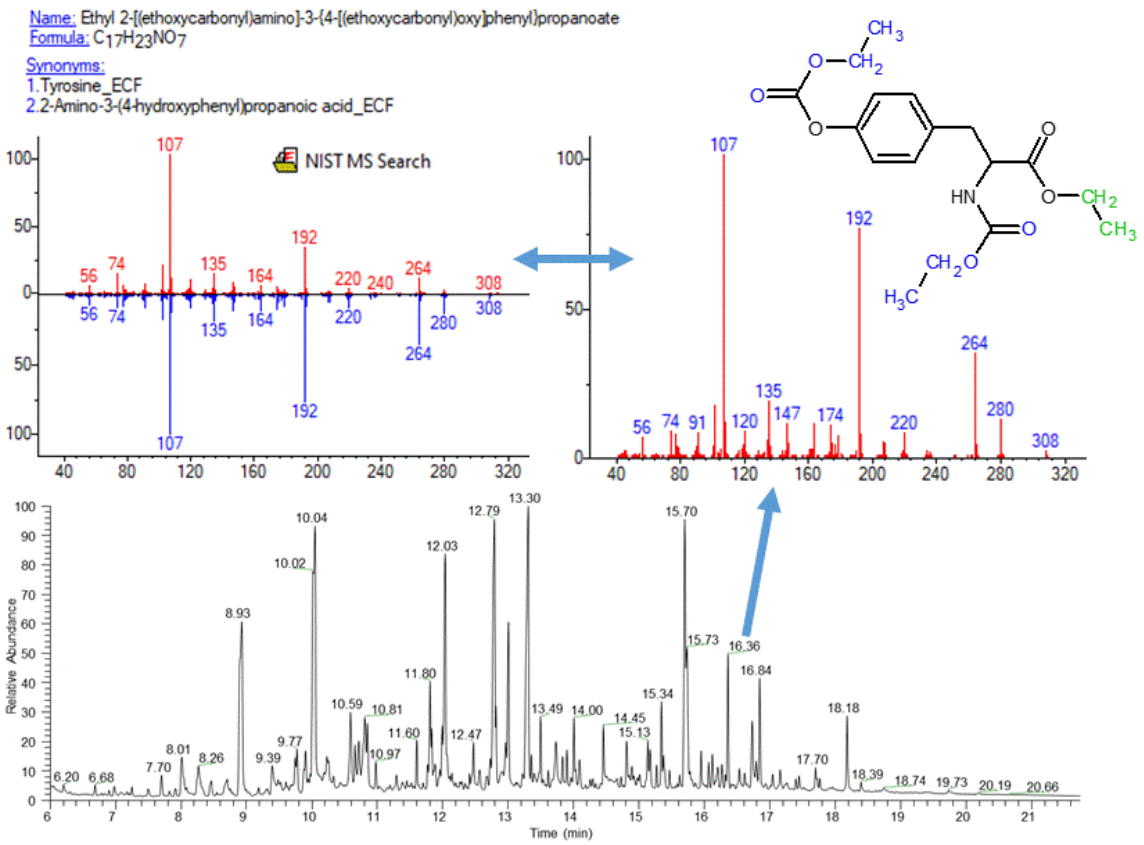

Fig. 5: Inspection of the EI spectrum of the tyrosine metabolite in human urine. The sample was on-line worked-up with the MetaboAuto® sample preparation workstation, measured by a single-quadrupole GC-MS and the detected metabolites identified by means of the MetaboAuto® EI mass spectral library fully implemented into the NIST mass spectral library environment.

Results

The MetaboAuto® platform represents a new, original technology for comprehensive metabolomic GC-MS analysis of analytes in urine, plasma and tissue extracts. On-line (on-deck) use of the MetaboAuto® platform enables unattended 24h/7d, automated sample preparation, GC-MS measurement,data processing for more than 250 metabolites and xenobiotics in the complex biological matrices. Systematic profiling in a 25 µl volume of a pooled human urine and serum (a pooled sample, n=10) revealed the capability to quantify more than 100 and 70 analytes, respectively, using a single quadrupole MS full scan detection. Similarly, presample extraction of 7 mg of pork liver tissue enabled automated profiling of more than 70 protic using the automated MetaboAuto® sample preparation and GC-MS analysis (a pooled tissue extract, n=5). For all the detected metabolites, the relative standard deviation not exceeding 20 % was a criterion for the acceptance in the metabolite quantification list. The metabolite list used for the described MetaboAuto® testing is summarized in **Table 1**.

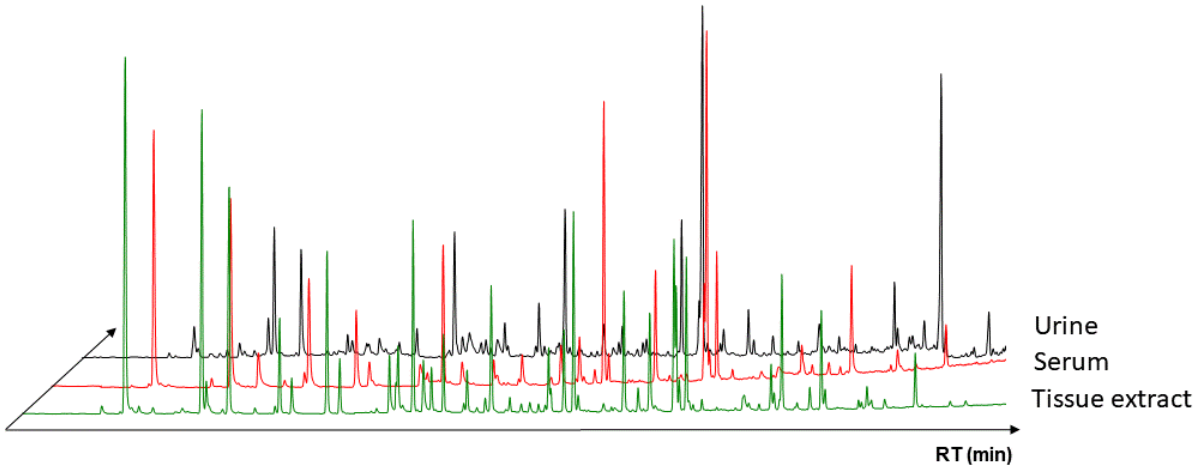

Fig. 6: Typical clean TIC GC-MS chromatograms obtained by the MetaboAuto® sample preparation and single quad GC-MS metabolomic analysis of an examined urine, blood plasma and pork liver extracts.

Table 1: The metabolite list examined for the testing of the MetaboAuto® sample preparation workstation hyphenated with a GC-EI/MS instrument (ISQ, ThermoScientific). The profiled metabolites, their KEGG number and method reproducibility expressed as RSD (%) for analytes detected in the examined three biological matrices (human urine, serum and pork liver tissue). A pooled human urine, serum and pork liver sample (n=10 each) was prepared by the robotic workstation (n=45 for each sample matrix) and was accomplished within 60 h.

		Reproducibility (RSD, %)		
The Profiled Analyte	KEGG	Serum	Urine	Liver
(*E*)-hexadec-7-enoic acid	NA	8	NA	5
(*Z*)-5-dodecenoic acid	NA	NA	NA	NA
(*Z*)-hexadec-7-enoic acid	NA	NA	NA	NA
1,2,3-Propanetricarboxylic acid	NA	NA	NA	NA
1,3-Diaminopropane	C00986	16*	NA	NA
11-Eicosenoic acid	C16526	23	NA	7
1-Aminecyclopropane-carboxylic acid	C01234	NA	NA	NA
1-Piperidine carboxylic acid	NA	NA	6	NA
2,2-Dimethylsuccinic acid	NA	NA	NA	NA
2,3-Diaminopropanoic acid	C03401	NA	10	NA
2,4-Diaminobutyric acid	C03283	NA	NA	NA
2,6-Diaminopimelic acid	C00666	NA	NA	NA
2,6-Pyridinedicarboxylic acid	NA	NA	NA	NA
2-Aminoadipic acid	C00956	NA	5	4
2-Aminobutyric acid	C02356	5	7	4
2-Aminoheptanoic acid	NA	NA	NA	NA
2-Aminoisobutyric acid	C03665	NA	NA	NA
2-Aminooctanoic acid	NA	NA	NA	NA
2-Aminopimelic acid	NA	NA	NA	NA
2-Furoic acid	C01546	NA	NA	NA
2-Hydroxy(phenyl)acetic acid	C01983	NA	9	NA
2-Hydroxy-2-methylpropionic acid	C21297	NA	13	NA
2-Hydroxy-3-methylbutyric acid	NA	8	22*	8
2-Hydroxyadipic acid	C02360	NA	NA	NA
2-Hydroxybutyric acid	C05984	3	16	23
2-Hydroxydecanoic acid	NA	NA	NA	NA
2-Hydroxyglutaric acid	C01087	58*	14*	9*
2-Hydroxyisocaproic acid	C03264	NA	NA	NA
2-Hydroxyoctanoic acid	NA	NA	NA	NA
2-Hydroxysebacic acid	NA	NA	10	NA
2-Hydroxyvaleric acid	NA	NA	NA	NA
2-Isopropylmalic acid	C02504	NA	NA	NA
2-Methylglutaric acid	NA	NA	NA	NA
2-Oxoadipic acid	C00322	NA	37	NA
2-Oxobutyric acid	C00109	NA	NA	NA
2-Oxoglutaric acid	C00026	NA	20*	NA
2-Oxohexanoic acid	C00902	6	19	21
2-Oxooctanoic acid	NA	NA	NA	NA
2-Phenoxypropionic acid	NA	NA	NA	NA
2-Phenylacetic acid	C07086	NA	17	NA
2-Phenylbutyric acid	NA	NA	NA	NA
3,4-Dihydroxymandelic acid	C05580	NA	NA	NA
3,4-Dihydroxyphenylacetic acid	C01161	NA	8	NA
3-Alanine	C00099	NA	13	5
3-Aminobutyric acid	NA	NA	17	NA
3-Aminoisobutyric acid	C05145	NA	8	NA
3-Hydroxyadipic acid	NA	NA	9	NA
3-Hydroxydecanoic acid	NA	NA	NA	NA
3-Hydroxydodecanoic acid	NA	NA	NA	NA
3-Hydroxyisobutyric acid	C06001	35*	NA	NA
3-Hydroxyisovaleric acid	C20827	57	8	NA
3-Hydroxyphenylacetic acid	C05593	NA	NA	NA
3-Hydroxyproline	C05147	8	18	5
3-Hydroxypropionic acid (dimer)	C1013	NA	NA	NA
3-Hydroxyvaleric acid	NA	NA	NA	NA
3-Chlorotyrosine	NA	NA	NA	NA
3-Iodotyrosine	NA	NA	NA	NA

		Reproducibility (RSD, %)		
The Profiled Analyte	KEGG	Serum	Urine	Liver
3-Methoxytyramine	C05587	NA	NA	NA
3-Methylcrotonylglycine	C20828	NA	10	NA
3-Methylglutaconic acid	NA	NA	NA	NA
3-Methylglutaric acid	NA	11	15	9
3-Methylhippuric acid	NA	NA	NA	NA
3-Nitrotyrosine	NA	NA	NA	NA
3-Nitrotyrosine	NA	NA	NA	NA
3-Phenyllactic acid	NA	NA	6	NA
3-Phenylpropionic acid	C05629	NA	NA	NA
4-Aminobenzoic acid	C00568	NA	NA	NA
4-Aminobutyric acid	C00334	NA	22	NA
4-Hydroxybenzoic acid	C00156	NA	NA	NA
4-Hydroxycinnamic acid	C00811	NA	11	NA
4-Hydroxymandelic acid	C11527	NA	8	NA
4-Hydroxyphenylacetic acid	C00642	94*	5	NA
4-Hydroxyphenylglycine	C12323	NA	NA	NA
4-Hydroxyphenyllactic acid	NA	NA	7	NA
4-Hydroxyphenylpyruvic acid	C01179	86*	5	NA
4-Methoxyphenylacetic acid	NA	NA	NA	NA
4-Methylpentanoic acid	C21399	NA	NA	NA
4-Phenylbutyric acid	C21793	NA	NA	NA
5-Aminolevulinic acid	C00430	NA	7	4
5-Aminovaleric acid	C00431	NA	NA	NA
5-Hydroxyindolylacetic acid	C05635	NA	10	NA
5-Hydroxypipecolic acid	NA	12	NA	15
5-Methoxytryptamine	C05659	NA	NA	NA
8,11,14-Eicosatrienoic acid	C03242	23	NA	8
9,12,15-octadecatrienoic acid	C06427	NA	NA	NA
9-*Z*-Myristoleic acid	C08322	NA	NA	NA
Adipic acid	C06104	NA	7	NA
Alanine	C00041	4	5	3
Alanylglycine	NA	25*	NA	8
Alloisoleucine	C21096	14*	13	NA
Aminomalonic acid	C00872	NA	NA	NA
Arachidic acid	C06425	NA	17*	8
Arachidonic acid	C00219	NA	NA	NA
Asparagine (nitrile)	C00152	4	7	4
Aspartic acid	C00049	9	12	6
Azelaic acid	C08261	NA	6	11
Benzoic acid	C00180	49*	15	10*
Cadaverine	C01672	NA	NA	NA
Caffeic acid	C01197	NA	NA	NA
Cinnamic acid	C10438	NA	NA	NA
cis-Aconitic acid	C00417	11	6	NA
Citraconic acid	C02226	NA	NA	NA
Citramalic acid	C00815	5	13	5
Citric acid	C00158	6	5	10
Cystathionine	C02291	NA	14	15
Cysteamine	C01678	12*	14*	7*
Cysteamine	C01678	NA	NA	NA
Cysteine	C00097	14*	13	19
Cysteinylglycine	C01419	NA	NA	NA
Cystine	C00491	NA	NA	NA
Decanoic acid	C01571	7	22	12*
Docosahexaenoic acid	C06429	19	NA	11
Docosanoic acid	C08281	NA	NA	NA
Dodecanoic acid	C02679	6	10	6

The Profiled Analyte	KEGG	Reproducibility (RSD, %) Serum	Urine	Liver
Dopamine	C03758	NA	12	NA
Eicosapentaenoic acid	C06428	NA	NA	NA
Elaidic acid	C01712	30	NA	6
Ethylmalonic acid	NA	NA	7	NA
Ferulic acid	C10470	NA	15	NA
Fumaric acid	C00122	16	15	8
Fumarylacetone	NA	NA	NA	NA
Gentisic acid	C00628	NA	NA	NA
Glutamic acid	C00026	8	7	3
Glutamine	C00064	26*	19*	13*
Glutaric acid	C00489	18	9	6
Glycine	C00037	6	5	3
Glycolic acid	C00160	NA	26*	NA
Glycylproline	NA	NA	10	8
Heptadecanoic acid	NA	NA	9	NA
Heptanoic acid	C17714	NA	NA	NA
Hexacosanoic acid	C21931	NA	NA	NA
Hippuric acid	C01586	81*	7	4
Histamine	C00388	NA	NA	NA
Histidine	C00135	17	8	11
Homocysteine thiolactone	NA	NA	NA	6*
Homogentisic acid	C00544	NA	32	NA
Homoserine	C00263	NA	NA	NA
Homovanillic	C05582	NA	6	NA
Cholesterol	C00187	NA	NA	NA
Imidazoleacetic acid	C02835	NA	NA	NA
Indol-3-propionic acid	NA	NA	NA	NA
Indole-2-carboxylic acid	NA	NA	NA	NA
Indolylacetic	C00954	18	23	NA
Isocitric acid	C00311	36	7	6
Isoleucine	C00407	6	10	4
Isovaleric acid	C08262	NA	NA	NA
Itaconic acid	C00490	NA	8	NA
Kainic acid	C12819	NA	NA	NA
Kynurenic acid	C01717	NA	25	NA
Kynurenine	C00328	NA	NA	NA
Lactic acid	C00186	2	10	1
Laurylamine	NA	NA	NA	NA
Leucine	C00123	6	8	4
Levulinic acid	NA	NA	NA	NA
Linoleic acid	C01595	10	NA	5
Lysine	C00047	4	5	4
Maleic acid	C01384	10	9	5*
Malic acid	C00149	14	27*	5
Malonic acid	C00383	NA	NA	NA
Mandelic acid	C01984	NA	NA	NA
Methionine	C00073	5	6	5
Methionine_sulfone	NA	13	9	13
Methylmalonic acid	C02170	NA	NA	NA
Methylsuccinic acid	NA	NA	7	NA
Muconic acid	C02480	NA	NA	NA
Myristic acid	C06424	7	9	10
N(2)-Acetyllysine	C12989	NA	NA	NA
N(6)-Acetyllysine	C02727	NA	12	NA
N(6)-Carboxymethyllysine	NA	NA	NA	NA
N(6)-Methyllysine	NA	NA	NA	NA
***N*-Acetylaspartic acid**	NA	6	7	5
N-Acetylglutamic acid	NA	NA	NA	NA
N-Acetylglycine	NA	NA	NA	NA

The Profiled Analyte	KEGG	Reproducibility (RSD, %) Serum	Urine	Liver
N-Acetylmethionine	C02712	NA	NA	NA
N-Glutarylglycine	NA	NA	NA	NA
Nicotinic acid	C00253	NA	NA	9
N-Isovaleroylglycine	NA	NA	NA	NA
N-Lauroylalanine	NA	6	8	4
N-Methylalanine	NA	6	6	3
N-Methylaspartic acid	NA	NA	NA	7
N-Methylisoleucine	NA	NA	NA	NA
N-Methylvaline	NA	NA	NA	NA
N-Oleoylglycine	NA	NA	NA	NA
Nonadecanoic acid	C16535	NA	NA	NA
Nonanoic acid	C01601	NA	NA	NA
O-Acetylserine	C00979	NA	NA	NA
Octacosanoic acid	C21933	NA	NA	NA
Octanoic acid	C06423	NA	NA	NA
Ornithine	C00077	4	9	7
Palmitic acid	C00249	8	27	5
Pentadecanoic acid	C16537	NA	NA	NA
Pentanoic acid	C00803	NA	NA	NA
Phenylalanine	C00079	7	5	4
Phenylethylamine	C02455	NA	NA	NA
Phenylglyoxylic acid	C02137	NA	NA	NA
Phenylpropionylglycine	NA	NA	NA	NA
Phenylpyruvic acid	C00166	27	20	NA
Picolinic acid	C10164	NA	NA	10
Pimelic acid	C02656	NA	8	NA
Pipecolic acid	C00408	NA	NA	NA
Proline	C00148	4	8	4
Prolylhydroxyproline	NA	NA	NA	NA
Putrescine	C00134	14*	NA	9*
Pyroglutamic acid	C01879	NA	NA	NA
Quinolinic acid	C03722	NA	NA	NA
Salicylic acid	C00805	NA	13	NA
Sarcosine	C00213	11	10	4
S-Benzylmercapturic acid	NA	NA	NA	NA
Sebacic acid	C08277	NA	NA	NA
Selenomethionine	C05335	NA	NA	NA
Serine	C00065	10	8	4
S-Methylcysteine	NA	NA	NA	NA
Suberic acid	C08278	NA	NA	NA
Succinic acid	C00042	7	7	4*
Succinylacetone	NA	NA	NA	NA
Synephrine	C04548	NA	NA	NA
Syringic acid	C10833	NA	NA	NA
Tartaric acid	C00898	NA	NA	NA
Tartronic acid	C02287	NA	NA	NA
Tetracosanoic acid	C08320	NA	NA	NA
Thioproline	NA	NA	9	4
Threonine	C00188	5	15*	4
trans-Urocanic acid	C00785	13	7	6
Tridecanoic acid	C17076	NA	NA	NA
Tryptamine	C00398	NA	NA	NA
Tryptophan	C00078	4	5	4
Tyramine	C00483	NA	10	NA
Tyrosine	C00082	7	9	7
Undecanoic acid	C17715	NA	NA	NA
Valine	C00183	6	7	4
Vanillic acid	C06672	8	NA	7
Vanillylmandelic acid	C05584	NA	8	NA

* No internal standard was used; NA: the metabolite data were not acquired; the validated analytes in bold.

Conclusion

- MetaboAuto® is a user-friendly cost-effective sample preparation workstation suitable for automated GC-MS profiling of > 250 protic metabolites in animal and human metabolomic studies.
- On line, on-deck sample preparation ensures analysis of a fresh sample extract directly before GC-MS instrumental analysis.
- Quantification of more than 90 metabolites and xenobiotics in the tested urine (25 µL volume) human serum (25 µL) and in the pork liver tissue extract (7 mg).
- MetaboAuto® EI mass spectral library covering retention data and EI mass spectra for > 250 metabolites and xenobiotics fully operating in the NIST mass spectral library environment.
- Data processing utility for the Thermo Scientific Xcalibur, TraceFinder and Agilent MassHunter qualitative and quantitative data analysis software is an integral part of the robotic sample preparation package.

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