

Automated two step derivatization and liner exchange coupled to GC-TOF mass spectrometry

Tobias Kind¹, Oliver Lerch², Peter Tablack³, Carsten Denkert⁴, Oliver Fiehn¹

¹ UC Davis Genome Center, Davis, CA ² Gerstel GmbH, Germany ³ LECO GmbH, Germany ⁴ HU Charite Berlin, Pathology, Germany

Overview

Purpose

We here present a proof-of-concept study that automatic liner exchange dramatically improves data quality for GC/MS analyses due to limited carry over effects. The methodology was developed for blood plasma, however, we found that other human tissues were equally well extracted. We have applied this method to ovarian cancer types as a proof-of-principle study in order to find metabolic differences between cancer types that might be used for prediction of patient's survival likelihood.

Methods

A CTC Twin-Pal dual robotic autosampler installed by Axel Semrau GmbH (Germany) was used for derivatization and injection into the GC-TOF-MS. An OPTIC3 programmable temperature vaporization (PTV) injector from ATAS GL (Netherlands) was coupled to a direct thermal desorption (DTD) unit and connected to an Agilent 6890 gas chromatography oven with a Pegasus 3 time of flight (TOF) mass spectrometer from LECO (USA). The timing of the derivatization process (combining a methoximation and silylation step), the liner exchange and the injection sequence, was controlled with a special software scheduler (Axel Semrau GmbH). Mass spectral deconvolution and calculation of retention indices was accomplished by the ChromaTOF software (LECO). Statistical data evaluation was performed with Statistica Data Miner (Statsoft Inc., USA).

Results

Liner exchange and automatic derivatization resulted in low cross-contamination compared to classic Agilent injectors, specifically for unsaturated free fatty acids and aromatics. Blood plasma was used for validating the extraction method. Best results were achieved by -17°C cold isopropanol/water mixtures, 2:1, at a ratio of 400 µl solvent:30 µl blood plasma. This method was applied to ovarian cancer tissues.

From over 700 detectable peaks in these cancer tissues, about 130 metabolites were unambiguously identified from ovarian cancer tissue samples using a large retention index database and mass spectral matching. Together with several hundred unknown metabolites the result matrix was filtered with univariate methods and processed with multivariate statistics to characterize several ovarian cancer types.



Setup of a GC/TOF instrument with automatic two-step derivatization (red ovens) and liner exchange. Both liners and vials are crimped with magnetic caps for transport.

Method development

Extraction

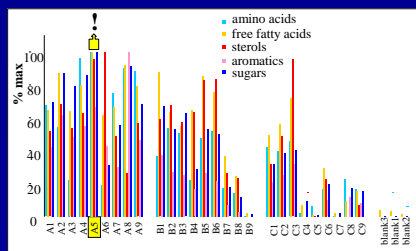
Three different variants of extraction solvent mixtures were tested for assessment of precision and comprehensiveness of metabolite profiling. These were isopropanol:water, acetone:water and the mixture best suitable for plant leaf profiling for GC/MS, a mixture of water:methanol:chloroform¹. Each of the methods was tested with three ratios of extraction volume : biosample amount, and with three mixture ratios between the solvents. In all cases, solvent mixtures were chilled to -17°C in order to precipitate proteins during extraction, and to stop all residual enzymatic activity. It was further found to be important to carefully degas all solvents. Otherwise, recovery of oxidizable compounds such as ascorbate, cysteine, α-tocopherol or glutathione were found significantly reduced or absent.

µl solvent	300	400	500
isopropanol/water 1:1	A1	A2	A3
isopropanol/water 2:1	A4	A5	A6
isopropanol/water 3:1	A7	A8	A9
isopropanol/acetone 1:1	B1	B2	B3
isopropanol/acetone 1:2	B4	B5	B6
isopropanol/acetone 1:3	B7	B8	B9
H ₂ O/MeOH/CHCl ₃ 5:30:5	C1	C2	C3
H ₂ O/EtOH/CHCl ₃ 2:6:2	C4	C5	C6
H ₂ O/MeOH/CHCl ₃ 2:6:2	C7	C8	C9

Test of 27 methods for 30 µl blood plasma extraction

Upper panel: outline of the experimental design for method development. From literature, it was found that acetone and isopropanol were the most frequently used solvents for metabolite extractions (or general protein precipitation) from blood plasma.

Lower panel: comprehensiveness of extraction with respect to average recovery of 68 known metabolites, divided to five general compound classes. It was found that any protocol involving chloroform led to dramatic decreases in metabolite recoveries, arguably caused by co-precipitation with proteins.

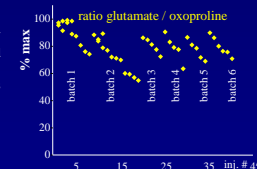


Instrumentation

Derivatization

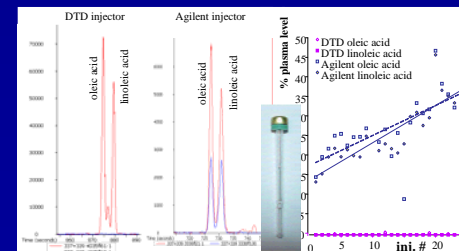
Profiling primary metabolites by GC/MS involves two reaction steps: first a methoximation of carbonyl groups is applied to prevent decarboxylation of α-oxo acids and also to prevent cyclization of monosaccharides. Secondly, a silylation reaction is employed to exchange all acidic protons in a mild and complete way in order to increase volatility. With this procedure, compounds up to trisaccharides and including sugar monophosphates can be profiled. However, primary amines have two reactive protons, each reacting with different kinetics. Consequently, absolute peak heights will be different for these compound classes (including amino acids!), depending on the actual waiting time on the autosamplers, if the derivatization is carried out in batches in a manual way. We have therefore optimized and programmed an automatic reaction schema, with two CTC-PAL arms and a 250 ml syringe for addition of reagents, and a 10 ml syringe for addition of internal standards and injection.

Figure: Manual derivatization of 45 identical samples, derivatized in six batches. The ratio of glutamate to oxoproline declines linearly with the injection number for each batch. With automatic derivatization, each sample is injected after identical derivatization reaction times.



Automatic liner exchange

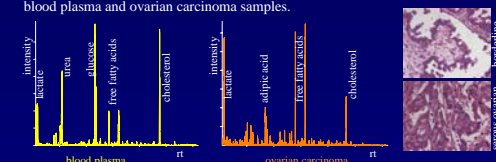
In metabolomics, complex samples are injected which may still contain matrix or involatile material. This material (e.g. membrane lipids) will gradually accumulate by each injection, giving rise to cross-contaminations due to partial decomposition. Below, 25 consecutive injections are compared with automatic liner exchange (DTD) and without (Agilent s/s injector). Genuine free fatty acid levels for ion trace m/z 337:339 are compared for blood plasma (red graph) and for the 25th blank injection (blue graph). Zero cross contamination is observed if automatic liner exchange is employed.



Application

Discrimination of ovarian cancer types

Ovarian cancer biopsies from 75 patients were investigated by GC/TOF with the aim to find biomarkers that could generate novel hypotheses of metabolic aberrations with respect to tumor types, FIGO stages, survival rates, grading, response to chemotherapy and other medical metadata. Below, chromatograms of blood plasma and ovarian carcinoma samples.

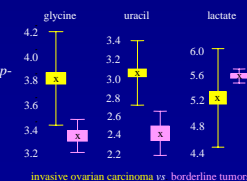


Multivariate clustering

Mucinous, endometrial and clear cell ovarian carcinoma were clearly separated by Principle Components Analysis. However, inherent variability in invasive serous ovarian carcinoma and borderline tumors was larger than between-group variance, and no unsupervised clusters were detectable (data not shown).

Univariate statistics

Despite the large variance in invasive and borderline tumors, univariate analysis resulted in a number of metabolites that were differentially controlled at very low *p*-values that even fit the hard Bonferroni-thresholds. Box-whisker plots of log-transformed data are given for mean, standard errors and standard deviations. None of the 16 detectable free fatty acids were significantly different.



Conclusions

With automatic liner exchange, fractionation of complex samples into lipophilic and polar fractions can be avoided. This enables quantification free fatty acids and sterols concomitant with sugars and amino acids. Blood plasma extraction procedures have been validated. Statistical investigations of ovarian carcinoma tissues promise better understanding of metabolic errors in this disease.

Reference

¹ Weckwerth W, Wenzel K, Fiehn O (2004) Process for the integrated extraction, identification and quantification of metabolites, proteins and RNA to reveal their core-regulation in biochemical networks. *Proteomics* 4, 78-83