Improvements in Quantitative and Qualitative Metabolic Profiling Using a Novel Atmospheric Pressure GC Source Coupled to High-Resolution TOF-MS Analysis

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Authors:

C.J. Wachsmuth[1], K. Dettmer[1], P.J. Oefner[1], C. Gebhardt[2], V. Tellstroem[2], A. Barsch[2], T. Arthen-Engeland[2]

[1]Universitiy of Regensburg, Regensburg, Germany
[2]Bruker Daltonik GmbH, Bremen, Germany

Overview:

A new GC-APCI source coupled to a high resolution orthogonal TOF-MS was used for the GC/MS analysis of reference standards, derivatized metabolite standards and cell culture supernatant samples. Compared to earlier results [1] we observed improved analytical performance regarding sensitivity and analytical working range. Furthermore, an increased number of compound peaks could be assigned.

Introduction:

GC/MS is one of the fundamental analytical techniques presently used in metabolomics. Most of the work is based on electron ionization (EI-) MS reference libraries of known, derivatized compounds. While EI is the common analytical standard for GC/MS, high resolution GC-APCI-MS became more prominent in the last few years because it enables the identification of unknown analytes [1-2]. The soft APCI ionization preserves the molecular ion information and opens the doors to the world of unidentified metabolites which could not yet be identified due to missing library data or missing standards.

In the present study, we used a new GC-APCI II source. The source consists of an atmospheric pressure chemical ionization source chamber equipped with a corona discharge needle assembly and a flexible heated GC transfer line. While other GC-APCI sources use a fixed GC-transfer line design the GC-APCI II source allows easier handling of the GC and a quicker exchange of the chromatography type, e.g. from GC to LC source and GC to LC chromatographic system (and vice versa). Compared to earlier designs, the GC-APCI II source was further developed in the following respect:

- The whole source was designed much tighter to exclude uncontrolled gas exchange with the outside air and to control the water content in the ion source.
- The APCI vaporizer heater used in the previous design is omitted within the GC-APCI II ion source, thus suppressing gas turbulences due to a high vaporizer gas flow.
- The heat required for the APCI process is generated in close vicinity to the ionization region and is preserved there by shielding the ionization region from the outer source area.
- All gas flows in the ion source were optimized to reduce turbulences and to guide the GC eluent flow into the ion source.

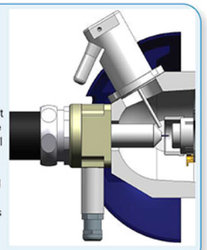

Fig. 1: Scheme of the GC-APCI II ion source.

The GC-effluent is directed from the GC transfer line (left) into the APCI region of the ion source. Mechanical design, gas flows, heat distribution and electrical fields are optimized for efficient AP chemical ionization and ion transfer into the MS inlet.
The GC-APCI II source is equipped with an automatic calibration gas module that flushes calibration gas into the APCI region.

Finally this source supports also a mass calibration module which allows automatic MS calibration by injecting PFTBA calibration gas into the ion source chamber during each GC/MS run.

Methods:

Cell culture supernatants of pancreatic cancer cells (MiaPaCa-2 cells, N=3) as well as pure metabolite standards were methoximated and trimethylsilylated. One µl of each sample was injected into the GC.

GC/MS analysis was performed using a Bruker 450-GC with PAL Combi-xt Autoinjector and a micrOTOF orthogonal TOF mass spectrometer or an impact HD Q-TOF-MS (both Bruker Daltonics). The GC was operated with a 30 m Restek Rxi-5ms capillary column (0.25 mm ID, 0.25 µm film thickness), operated at 1 ml/min constant helium flow and a GC oven temperature program at 50°C (1 min) - 8°C/min - 300°C (15 min). Data were acquired from 50 - 1000 m/z at 3 spectra per second, operated in the positive ionization mode. Spectra were externally calibrated using PFTBA as calibration gas injected automatically into the APCI source at the beginning of each MS run. The DataAnalysis software (Bruker Daltonics) was applied for peak detection and data evaluation.

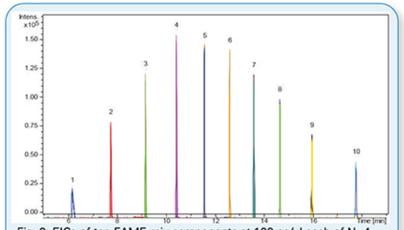

Fig. 2: EICs of ten FAME mix components at 100 pg/µl each of N=4 subsequent GC/MS replicate runs at impact HD (Δ **EIC = 2 mDa)**

Results:

The general GC-APCI II performance was checked with an even-numbered, saturated straight-chain fatty acid methyl ester (FAME) mix (Sigma-Aldrich No. 49453-U), diluted by a factor of 10^{-4}. This mix is an ideal GC/MS test mix because it tests the GC performance (peaks shapes, retention times, reproducibility) of underivatized volatile and semi-volatile components and the sensitivity of selected components. Fig. 2 shows four subsequent, overlaying GC/MS runs of the FAME test mix including ten GC peaks of the individual FAME components within each run analyzed with an impact HD Q-TOF. The FAMEs are eluting as sharp and overlaying GC peaks with good retention time stability, small peak widths (FWHM) below 2 s for the mid-eluting FAMEs and excellent reproducibility. Analytical results are presented in Table 1 and address chromatographic parameters, such as retention times stability, GC peak widths, area reproducibility and S/N values (as an indicator for sensitivity). The 2nd part of the table shows mass reproducibilities and average mass deviations of the FAMEs analyzed: most of the mass deviations are below 1 mDa; only the smallest C6-FAME has a slightly higher mass deviation from the calculated mass of its protonated molecular ion due to the lack of calibration masses in the low mass range. This was improved later by applying higher CID voltages to generate more fragmentation ions from PFTBA calibration gas and therefore obtain more calibration signals [3].

The FAME test mix was used to confirm proper analytical operation of the system before the analysis of derivatized metabolite standards and the assessment of the quantitative performance. For that purpose, a master mix containing 43 metabolite standards covering a variety of chemical classes, such as amino and organic acids as well as sugars and alcohols, was serially diluted over a range of 0.002-1000 µM. Twelve stable isotope-labeled internal standards were included in the study for acquisition of calibration curves, which were obtained as previously reported [1].

As presented in Fig. 3 for a derivatized metabolite standard at a concentration of 31.25 µM, GC-APCI II/TOF-MS yielded sharp and narrow peaks. Analytes not having been resolved chromatographically could further be distinguished by the accurate mass of their quasi-molecular ion. Quantification results of annotated metabolites are shown in Table 2 for GC-APCI "I" and II ion sources. For each of these compounds an internal standard had been included in the study. As a result, lower limits of quantification were better in case of GC-APCI II and the analytical range was notably increased to approximately three orders of magnitude.

Furthermore, from the analysis of derivatized cell culture supernatants of MiaPaCa-2 cells about twice as many detected peaks were obtained with the novel ion source. In fact, 362 versus 180 peaks were extracted from GC-APCI II and GC-APCI I data, respectively, using the dissect peak finding algorithm with an internal S/N threshold of 20. Several of the metabolites only extracted from the GC-APCI II data are listed in Table 3 including figures of merit obtained from their identification.

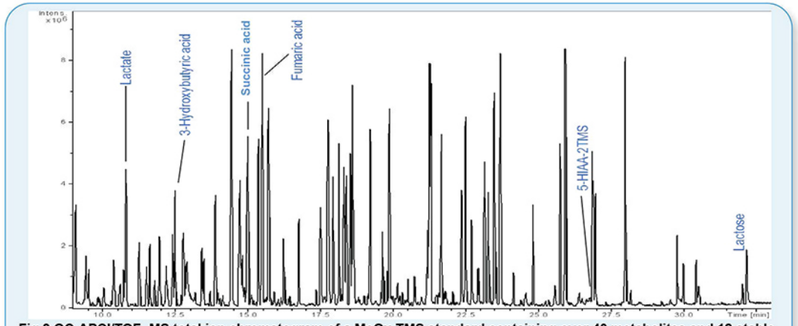

Fig.3 GC-APCI/TOF- MS total ion chromatogram of a MeOx-TMS standard containing over 40 metabolites and 12 stable isotope labed standards. (5-HIAA: 5-hydroxyindoleacetate, TMS: trimethylsilyl group)

Standard Compound	GC-APCI II linear range [µM]	GC-APCI II Order of Magnitude	GC-APCI I linear range [µM]	GC-APCI I Order of Magnitude
Lactic acid	0.1 - 187	3.2	2 - 750	2.6
3-Hydroxy-butyric acid	0.1 - 250	3.3	0.1 - 187	2.9
Succinic acid	0.1 - 46	2.7	0.3 - 94	2.6
Fumaric acid	0.4 - 187	2.7	0.5 - 250	2.7
5-HIAA + 2TMS	0.4 - 46	2.0	1 - 187	2.3
Lactose	0.1 - 375	3.9	4 - 125	1.5

Table 2: Comparison of quantification results for the novel GC-APCI II source and previous results using GC-APCI I source [1].

N=4 replicates # FAME	RT [min]	SD RT [s]	RSD RT %	FWHM [s]	RSD FWHM %	Average SN	Reproducibility Area RSD %	exper.average m/z [Da]	SD (m/z) [mDa]	Mass Deviation [mDa]
C 7 H 15 O 2	6.2	0.11	0.03	3.82	2.77	201	1.32	131.10793	0.21	1.27
C 9 H 19 O 2	7.8	0.10	0.02	1.60	1.06	705	1.51	159.13854	0.08	0.58
C 11 H 23 O 2	9.2	0.10	0.02	1.38	0.58	1360	1.59	187.16936	0.06	0.10
C 13 H 27 O 2	10.4	0.11	0.02	1.31	1.07	1687	1.45	215.20067	0.04	0.11
C 15 H 31 O 2	11.6	0.11	0.02	1.28	0.78	1126	1.23	243.23142	0.10	0.44
C 17 H 35 O 2	12.6	0.11	0.01	1.25	0.80	1193	1.12	271.26326	0.10	0.10
C 19 H 39 O 2	13.6	0.12	0.01	1.34	0.75	1454	0.80	299.29460	0.06	0.15
C 21 H 43 O 2	14.6	0.12	0.01	1.50	1.61	1373	1.07	327.32591	0.06	0.15
C 23 H 47 O 2	15.9	0.16	0.02	1.82	1.92	974	1.61	355.35703	0.08	0.02
C 25 H 51 O 2	17.7	0.26	0.02	2.36	1.61	610	2.05	383.38822	0.08	0.13

Table 1: Statistical results of 4 replicate GC/MS runs of the FAME test mix at 100 pg/µl for each component at impact HD: reproducibility of retention time, of GC peak half-widths, of area, average S/Ns and experimentally determined masses including their standard deviations SD and average mass deviation are shown.

Metabolite	RT [min]	Experimental m/z [Da]	Mass Deviation [mDa]	mSigma value	S/N
2-Hydroxybutyric acid+2TMS+H	12.06	249.1328	0.9	7.7	69
Thymine+2TMS+H	16.5	271.1278	1.5	3.1	262
Alanine+3TMS+H	16.88	306.1718	1.7	8.1	129
Glutamic acid+2TMS+H	18.07	292.1375	2	8.2	221
Phenylpyruvic acid+1MeOx+1TMS+H	19.22	266.1189	1.8	7.6	75
Palmitoleic acid+1TMS+H	24.61	327.2687	2.6	7.2	133

Table 3: Metabolites identified that corresponded to dissect compounds that were only extracted from GC-APCI II data. MeOx, group introduced by methoximation.
All data were generated using a micrOTOF. (mSigma values < 15 indicate perfect isotopic fidelity)

Conclusions:

Here we reported the application of a novel GC-APCI II ion source for the investigation of standards and samples for metabolomics using a micrOTOF and an impact HD Q-TOF-MS.

We demonstrated improved GC-APCI II/TOF-MS performance compared to a former GC-APCI design:

- Of underivatized FAMEs regarding retention time stability, GC peak shapes, peak area reproducibility and S/N values. For nearly all of the FAME components mass deviation and mass reproducibility at impact HD were determined below 1 mDa.
- Improved quantification results for a set of selected derivatized metabolite standards : the analytical (linear) working range was increased to about 3 orders of magnitude at linearities $R^2 > 0.99$.
- Better lower limits of quantification (LLOQs) for most of the derivatized metabolite standards due to reduced chemical background in the ion source.
- About twice as many extracted peaks in cell culture supernatant samples, with several identified metabolites that had not been revealed from the analysis of GC-APCI I data.

References:

[1] C.Wachsmuth et al.; Anal.Chem. 83 (2011) 7514
[2] A. Carrasco-Pancorbo et al.; Anal.Chem. 81 (2009) 10071
[3] T. Arthen-Engeland, A. Holle et al.; ASMS 2014, Poster 2032