Metabolomics of biofluids : from analytical tools to data interpretation

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Chapter 1

Gas chromatography/atmospheric pressure chemical ionization-time of flight mass spectrometry: analytical validation and applicability to metabolic profiling

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ABSTRACT

Gas Chromatography (GC)-Mass Spectrometry (MS) with Atmospheric Pressure (AP) interface was introduced more than 30 years ago but never became a mainstream technique, mainly because of technical difficulties and cost of instrumentation. A recently introduced multipurpose AP source created the opportunity to reconsider the importance of AP ionization for GC. Here, we present an analytical evaluation of GC/APCI-MS showing the benefits of soft atmospheric pressure chemical ionization for GC in combination with a Time of Flight (TOF) mass analyzer. During this study, the complete analytical procedure was optimized and evaluated with respect to characteristic analytical parameters, such as repeatability, reproducibility, linearity, and detection limits. Limits of detection (LOD) were found within the range from 11.8 to 72.5 nM depending on the type of compound. The intraday and interday repeatability tests demonstrate relative standard deviations (RSDs) of peak areas between 0.7%-2.1% and 3.8%-6.4% correspondingly. Finally, we applied the developed method to the analysis of human cerebrospinal fluid (CSF) samples to check the potential of this new analytical combination for metabolic profiling.

INTRODUCTION

There are different definitions of metabolomics. However, regardless of terminology and phrasing differences, any definition implies an enormous analytical challenges to cover a wide range of polarities, concentrations, and sizes of chemical entities composing the human metabolome. In response to this challenge, more and more efforts are directed toward cross-platform analysis and integration of data obtained on different analytical platforms. At the same time, a revision and modernization of proven technologies like, for example, gas chromatography (GC) is taking place. Since it was invented by Martin and James (1) in 1952, GC became one of the most important and widely applied techniques in modern analytical chemistry. Even before the term "metabolomics" was introduced, there were a number of published studies with GC as main analytical method, which could be described as metabolomics or metabolic profiling.(2) However, only with the introduction of fused-silica capillary columns at the end of 1980s, which significantly improved the separation quality of GC, and GC-MS instrumentation, GC turned into the one of the most effective techniques for large scale metabolic profiling.(3-8) GC-MS was the first analytical technique implemented in a real metabolic profiling workflow. It includes all steps from sample preparation to the compound identification and remains flexible because of a number of options in selection of mass analyzer and ionization techniques. There are several types of mass analyzers routinely used with GC systems, namely, ion trap (IT), single (Q) and triple-quadrupoles (QqQ), and time of flight (TOF). However, the characteristics of a TOF mass analyzer are most favorable for such application as metabolic profiling. Speed, sensitivity, resolving power, and multiplex detection are clear advantages over scanning instruments, such as quadrupoles. These performance factors make TOF mass analyzers almost ideal for metabolomics, especially in combination with GC.(9) Moreover, modern TOF analyzers provide a data quality sufficient for identification of metabolites using a combination of accurate mass, isotopic distribution, and retention time.(10;11)

Most of the commercial GC-MS systems use ionization under vacuum conditions: electron impact ionization (EI) and chemical ionization (CI).(12) EI is considered to be a hard ionization technique, meaning that the energy of the electrons is high enough to produce highly reproducible fragmentation patterns of small molecules. Characteristic fragmentation patterns make GC/EI-MS a powerful analytical technique for comparing the mass spectra of unknown substances to data sets of commercial and open source 70 eV EI mass spectral libraries. However, the fragmentation of the compounds is sometimes so strong that it impairs the structural significance of the parent ion. On the contrary, CI where ions are formed because of the reaction with reagent gas is a softer ionization

technique and energy transfer usually does not exceed 5 eV. Consequently, fewer fragments are formed and information about the precursor ion is preserved. Moreover, since the fragmentation pattern depends on the properties of the reagent gas, different structural information can be obtained from different reagent gases. Atmospheric pressure ionization sources (API), which are probably the key of the "overnight success" of MS detectors in analytical sciences because of coupling with liquid chromatography, are rarely used with GC instruments. The first APCI source for GC-MS was described more than 30 years ago by Horning *et al.*(13-16) Later, several papers were published in which the effluent from a GC is ionized at atmospheric pressure with an interface coupling the GC to a ^{63}Ni ion source of a mass spectrometer built for APCI gas-phase studies.(17-19) Revelsky *et al.*(20) and Schiewek *et al.*(21) have applied GC/APPI-MS for analyzing a wide variety of volatile organic compounds, and ESI has been successfully applied for ionization of gaseous analytes separated by GC.(22;23) Even so, GC/API-MS has never become widely used, in part because of the high costs of the custom instrumentation needed for these analyses, in part because of availability of commercial "plug and play" EI and CI GC systems. Recently, Schiewek *et al.* introduced a new multipurpose API source, which for the first time offers a "user friendly" and robust solution for a GC/APCI technique.(24) In the current manuscript, we present a detailed analytical evaluation of GC/APCI in combination with a TOF mass spectrometer. In addition to the detailed examination of the analytical performance (repeatability, reproducibility, linearity, and detection limits), we demonstrate the applicability of this technique for metabolic profiling of cerebrospinal fluid (CSF).

MATERIALS AND METHODS

Chemicals. A standard solution of 17 amino acids at 1 mM each in 0.1 M HCl was purchased from Sigma-Aldrich. 4-Nitrobenzoic acid, dopamine hydrochloride, and Phe-Gly hydrate were obtained from Fluka. Sarcosine, theophylline, caffeine, nortriptyline hydrochloride, hippuric acid, creatinine, 4-*O*-methyldopamine hydrochloride, homovanillyl alcohol, benzoic acid, uric acid, and 5-hydroxyindole-3-acetic acid were acquired from Sigma. Stock standard solutions of the 31 compounds under study were prepared in methanol at a concentration of 200 µM. N,O-bis(Trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS) and N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS) from Pierce (Rockford, IL, U.S.A.) were used as derivatization reagents. These reagents were used from freshly opened 1 mL bottles. Methoxyamine hydrochloride was purchased from Supelco. Methanol (HPLC grade) was acquired from Sigma-Aldrich and pyridine (>99%, ultrapure GC grade) was from Fluka.

Biological Samples. Human CSF samples were taken by lumbar puncture. The study was approved by the ethical committee at the Leiden University Medical Center. Samples were processed within 1 h, centrifuged at 300 × g to remove cells, aliquoted and stored at -80 °C until use.

Protein Precipitation and Metabolite Extraction. 250 µL sample aliquots were taken, 600 µL of cold extraction solvent (MeOH) were added, and the sample was shaken vigorously for 20 s. The samples were placed in an ice bath for 2 h, and then centrifuged at 20,800 rcf for 15 min. The liquid supernatant was collected and evaporated in a speed vacuum concentrator before derivatization.

Derivatization. A speed vacuum concentrator or lyophilizer was used for drying the standard mixture (100 µL at 100 µM) and the CSF extracts to complete dryness. A mixture of 20 mg/mL of methoxyamine·HCl in pyridine was freshly prepared using an ultrasonicator. The dried samples were taken from store and warmed up to room temperature before starting derivatization. Methoxyamine + pyridine mixture (100 µL) was added to each GC vial, closing it immediately, and the samples were agitated for 2 min. Methoxyamination was performed at 40 °C for 60 min. After the addition of the derivatization reagent containing 1% TMCS as the catalyst (100 µL) the solution was vortexed again for 2 min. Trimethylsilylation reaction was performed at 40 °C for 30 min. A minimum of 2 h equilibration time was necessary before sample injection.

GC-MS Analysis. The derivatized samples (1 µL) were applied by splitless injection with a programmable CTC PAL multipurposesampler (CTC Analytics AG, Zwingen, Switzerland) into an Agilent 7890A GC (Agilent, Palo Alto, U.S.A.) equipped with a HP-5-MS column (30 m, 0.25 mm ID, 0.25 µm film thickness). Injection programs included sequential washing steps of the 10 µL syringe before and after the injection, and a sample pumping step for removal of small air bubbles.

The injection temperature was set at 250 °C. Helium was used as carrier gas at a constant flow rate of 1 mL/min through the column. For every analysis splitless injection time was 60 s and after this the injector was purged at 20 mL/min flow rate. The column temperature was initially kept at 70 °C for 5 min and then raised at 5 °C/min over 42 min to 280 °C and held for 10 min.

The GC transfer line to the mass spectrometer was kept at 280 °C. The APCI source and MS were operated in positive mode, temperature and flow rate of the dry gas (nitrogen) were 250 °C and 5.00 L/min, respectively. The APCI vaporizer temperature was 450 °C; the pressure of the nebulizer gas (nitrogen) was set to 2 bar, and the voltage of the corona discharge needle was 2000 nA. Capillary voltage was set at -1000 V and the end-plate offset at -1000 V.

As a detector an orthogonal-accelerated TOF mass spectrometer (oaTOF-MS) MicroTOF (Bruker Daltonik, Bremen, Germany) was used. The polarity of the APCI interface and all the parameters of TOF MS detector were optimized using the area of the MS signal for the metabolites included in the standard mixture and the chromatographic resolution as analytical parameters. The position of the column in the transfer line, the transfer line temperature, the flow rate and pressure of nebulizer gas (nitrogen), the vaporizer temperature, voltages in the corona, source and ion transfer settings: all those parameters were optimized empirically. These are essential for optimal performance of an instrument but may vary from instrument to instrument.

Data were acquired for mass range from 50 to 1000 m/z with a repetition rate of 1 Hz. DataAnalysis 4.0 software (Bruker Daltonik) was used for data processing. The SmartFormula tool within DataAnalysis was used for the calculation of elemental composition of compounds; it uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (Sigma-Value) for increased confidence in the suggested molecular formula.(11)

The instrument was calibrated externally using an APCI calibration tune mix. Because of the compensation of temperature drift in the mass spectrometer, this external calibration provided consistent mass values for a complete experimental sequence. Moreover, an additional internal calibration was performed using cyclic-siloxanes, a typical background in GC-MS.(25;26)

Linearity and Sensitivity. Linearity of the detector response (TOF-MS) was verified with standard solutions containing the 31 analytes under study at 5 different concentration levels over the range from the quantification limit to 100 μM. Each point of the calibration graph corresponded to the mean value from three independent replicate injections. Calibration curves were obtained for each standard by plotting the standard concentration as a function of the peak area obtained from GC/APCI-TOF MS analyses. The sensitivity of the analytical procedure was calculated by defining the limits of detection (LOD) and quantification (LOQ) for the individual analytes in standard solutions according to the IUPAC method.(27) The smallest concentration that could be detected with a reasonable certainty for our analytical procedure (LOD) was considered S/N = 3, while LOQ was S/N = 10.

Precision and Accuracy. The precision of the analytical procedure described was measured as repeatability and reproducibility. Quality control (QC) samples were tested in six replicates (at an intermediate concentration value of the calibration curve) and

calculated with calibration curves obtained daily. The precision of the analytical procedure was expressed as the relative standard deviation (RSD). The intra- and interday repeatability in the peak areas was determined as the RSD obtained for six consecutive injections of each metabolite at an intermediate concentration value of the calibration curve, carried out within the same day and on three different days.

RESULTS AND DISCUSSION

Selection of Derivatization Conditions. BSTFA (+1% TMCS) and MSTFA (+1% TMCS) were used as derivatization reagents. They react with a range of polar compounds by replacing active hydrogen in alcohols, amines, carboxylic acids, and so forth. To find optimal derivatization conditions, we studied effects of derivatization time and temperature and the concentration ratio of the derivatization reagent to the concentration of pyridine/methoxyamine.

Regardless of the derivatization reagent, changing the reagent to pyridine/methoxyamine ratio from 0.8:1.2 until 1.2:0.8 did not affect peak areas of the test mixture significantly. Thus, the ratio 1:1 was chosen for further experiments. The effect of the derivatization time on peak areas was most significant in the interval between 10-30 min. Starting from 30 min incubation peak areas remained constant and further increase of derivatization time had little impact on data quality (Supplementary Materials, Figure S1). Thus, to reduce the error and shorten time, 30 min was selected as derivatization time. The influence of temperature on peak areas was minimal, at least in the evaluated interval between room temperature and 80 °C. However, at 40 °C we observed more compounds with just one TMS derivative. Thus, the final derivatization protocol consisted of a methoxyamination step (40 °C for 60 min) and subsequent trimethylsilylation (MSTFA + 1% TMCS, at 40 °C for 30 min).

The stability of derivatized samples is an important factor for large scale metabolomics temperature and performed analysis in equal time intervals between 0 and 72 h. Data proved to be rather consistent from 0 to 65 h. However, data collected on later time points demonstrated steadily increasing variability. Nevertheless, to avoid any possible risk of derivatization-dependent variability, material should preferably be processed within the first 48 h.

GC/APCI-TOF MS Analysis of Standard Mixture. A standard mixture consisting of 31 compounds was used for the general test of performance and evaluation of analytical parameters. The compounds were selected with the aim to cover a range of polarities and molecular weights of the metabolites typically reported as components of body fluids. Table 1 represents our test mixture grouped in different chemical families, such as amines, amino

acids, organic acids, alcohols, xanthines, compounds with indole or imidazole groups, and one dipeptide.

Table 1. Compounds Included in the Standard Mixture

amino acids	alanine
	arginine
	aspartic acid
	cysteine
	glutamic acid
	glycine
	histidine
	isoleucine
	leucine
	lysine
	methionine
	phenylalanine
	proline
	serine
	threonine
	tyrosine
	valine
	sarcosine
organic acids	benzoic acid
	hippuric acid
	4-nitrobenzoic acid
alcohols	homovanillyl alcohol
xanthines and related coumpounds	caffeine
	theophylline
	uric acid
compound with indoles group	5-hydroxyindole-3-acetic acid
amines	nortriptyline hydrochloride
Compounds with hydroxyl and amine groups	dopamine hydrochloride
	4-O-Methyldopamine hydrochloride
compounds with imidazol groups	creatinine
dipeptides	Phe-Gly hydrate

Figure 1 represents a combined extracted ion chromatogram (EIC) of the standard mixture recorded with optimum GC and MS settings.

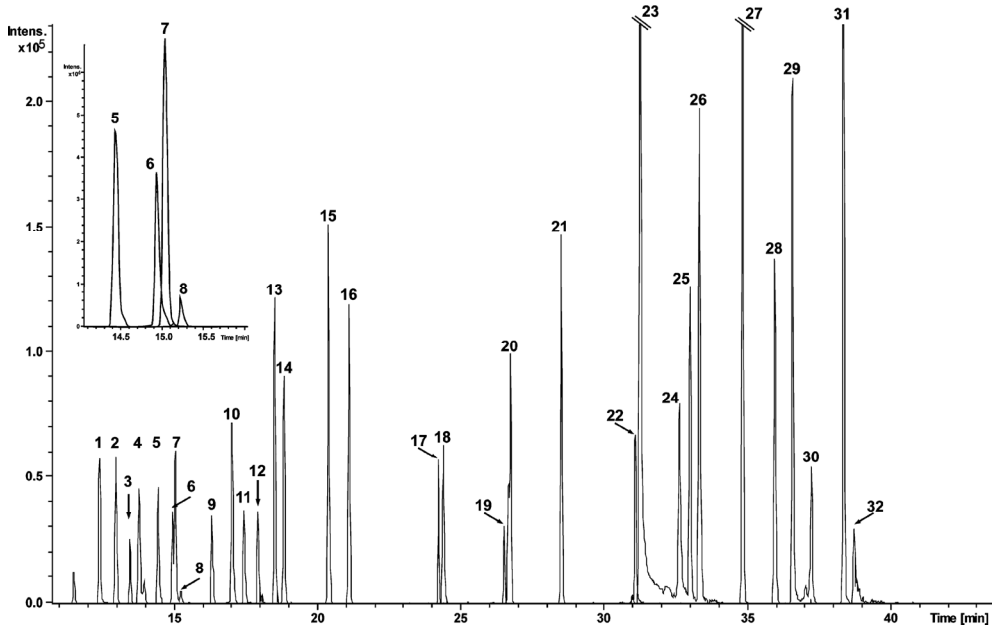

Figure 1. Extracted ion GC/APCI-TOF MS chromatograms of the 32 features corresponding to 25 compounds of the standard mix (100 μM). Numbering of compounds corresponds to Table 2.

With an analytical window of approximately 30 min, we observed 32 peaks, which could be assigned to 25 compounds. Table 2 shows all analytes detected, with their formula, retention time, measured and theoretical m/z, error (mDa) and sigma value. All values were calculated from samples with concentrations close to the LOQ; nevertheless the mass position error remained within 1.0 mDa and high quality sigma fit values (<10 mSigma) were obtained for all compounds.

However, the same table (Table 2) demonstrates that we failed in detecting a few components of our test mixture, namely, three amino acids (arginine, cysteine, and histidine), one organic acid (4-nitrobenzoic acid), homovanillyl alcohol, and creatinine. The thermal instability of amino acids, especially arginine and cysteine, is a known problem and has already been addressed in literature.(28;29) In addition, treatment with silylation reagents, even under the mild conditions generally employed in metabolite profiling, can lead to chemical conversion.(30) For example, arginine can be converted to ornithine. When studying metabolic processes in detail, particularly where the intermediate compounds may be reactive or unstable, one should always be aware of such possibilities when interpreting the results. If there is any doubt, alternative derivatization procedures for specific functional groups should be considered.

Table 2. Forms of the Different Compounds Included in the Standard Mixture (at a Concentration Close to LOQ) Detected with GC/APCI-TOF MS Method.

peak ID	compound	formula (peak found)	retention time	m/z experimental	m/z calculated	error (mDa)	mSigma value
1	Valine+1TMS+H	$C_8H_{20}NO_2Si$	12.4	190.1256	190.1258	0.21	3.4
2	Alanine+2TMS+H	$C_9H_{24}NO_2Si_2$	13	234.1338	234.134	0.2	5.1
3	Glycine+2TMS+H	$C_8H_{22}NO_2Si_2$	13.5	220.1181	220.1184	0.31	4.6
4	Sarcosine+2TMS+H	$C_9H_{24}NO_2Si_2$	13.8	234.1338	234.134	0.21	4.4
5	Leucine+1TMS+H	$C_9H_{22}NO_2Si$	14.4	204.1414	204.1414	0	1.8
6	Proline+1TMS+H	$C_8H_{18}NO_2Si$	14.9	188.1108	188.1101	-0.7	2.2
7	Isoleucine+1TMS+H	$C_9H_{22}NO_2Si$	15	204.1409	204.1414	0.49	1.8
8	Uric acid+3TMS+H	$C_{14}H_{29}N_4O_3Si_3$	15.2	385.1545	385.1542	0.31	3.4
9	Valine+2TMS+H	$C_{11}H_{28}NO_2Si_2$	16.3	262.1656	262.1653	-0.29	6.1
10	Benzoic acid+1TMS+H	$C_{10}H_{15}O_2Si$	17	195.087	195.0877	0.7	1.8
11	Serine+2TMS+H	$C_9H_{24}NO_3Si_2$	17.4	250.129	250.1289	-0.1	1.6
12	Leucine+2TMS+H	$C_{12}H_{30}NO_2Si_2$	17.9	276.1813	276.181	-0.3	8.9
13	Isoleucine+2TMS+H	$C_{12}H_{30}NO_2Si_2$	18.5	276.1802	276.181	0.8	6.4
14	Glycine+3TMS+H	$C_{11}H_{30}NO_2Si_3$	18.8	292.1578	292.1579	0.09	4.7
14	Serine+3TMS+H	$C_{12}H_{32}NO_3Si_3$	20.4	322.1681	322.1684	0.29	5.1
16	Threonine+3TMS+H	$C_{13}H_{34}NO_3Si_3$	21.1	336.1834	336.1841	0.71	2.9
17	Methionine+2TMS+H	$C_{11}H_{28}NO_2SSi_2$	24.2	294.1376	294.1374	-0.2	1.3
18	Aspartic acid+3TMS+H	$C_{13}H_{32}NO_4Si_3$	24.4	350.1631	350.1634	0.32	1.6
19	Glutamic acid+3TMS+H	$C_{14}H_{34}NO_4Si_3$	26.6	364.1786	364.179	0.4	5.5
20	Phenylalanine+2TMS+H	$C_{15}H_{28}NO_2Si_2$	26.7	310.1653	310.1653	0	5.8
21	Phenyl-Gly+H	$C_{11}H_{15}N_2O_3$	28.5	223.108	223.1077	-0.29	3.3
22	Hippuric acid+1TMS+H	$C_{12}H_8NO_3Si$	31.1	252.1047	252.105	0.3	1.8
23	Caffeine+H	$C_8H_{11}N_4O_2$	31.2	195.0835	195.0836	0.1	3.2
24	Theophylline+1TMS+H	$C_{10}H_{17}N_4O_2Si$	32.6	253.1116	253.1115	-0.1	2.9
25	Lysine+4TMS+H	$C_{18}H_{47}N_2O_2Si_4$	33	435.2699	435.2709	1	2.5
26	Tyrosine+3TMS+H	$C_{18}H_{36}NO_3Si_3$	33.3	398.1999	398.1998	-0.12	5.5
27	4-Methyldopamine hydrochlor+3Si+H	$C_{18}H_{38}NO_2Si_3$	34.8	384.2199	384.2205	0.61	4.2
28	Dopamine hydrochlor+4TMS+H	$C_{20}H_{44}NO_2Si_4$	35.9	442.2448	442.2444	-0.39	4
29	Uric acid+4TMS+H	$C_{17}H_{37}N_4O_3Si_4$	36.5	457.1939	457.1937	-0.18	9.1
30	Phenyl-Gly+2TMS+H	$C_{17}H_{31}N_2O_3Si_2$	37.2	367.1869	367.1868	-0.11	5.6
31	5-hydroxyindole-3-acetic+3TMS+H	$C_{19}H_{34}NO_3Si_3$	38.3	408.1842	408.1841	-0.08	7.7
32	Nortriptyline hydrochlor+H	$C_{19}H_{22}N$	38.7	264.1744	264.1747	0.29	9.2

Analysis of creatinine by GC requires rather selective conditions, which are optimal only for creatinine itself and a few related compounds. Creatinine can be converted, for instance, to the ethyl ester of N-(4,6-dimethyl-2-pyrimidinyl)-N-methylglycine,(31) or derivatized

with trifluoroacetic anhydride,(32) although the last one has been mainly analyzed by HPLC. The same is true for 4-nitrobenzoic acid or homovanillyl alcohol. In general, we can conclude that those two compounds are analyzed more properly by HPLC.

At a first glance, the few compounds "missing" from our test mixture might be considered as serious drawback of the total workflow. However, metabolic profiling workflows always imply a compromise between analytical limitations of the methods and their applicability. Even more, as Fiehn *et al.*(33) formulated in their validation criteria for metabolite profiling protocols, comprehensiveness is more important than inclusion of a certain metabolite, and the overall dynamic range for the majority of the compounds is more important than the detection limit for one specific substance. Thus, we measured compounds belonging to nine different chemical families within one experiment (chromatogram). Moreover, the correct elementary composition of measured compounds was calculated from data acquired at levels close to the LOQ(11;34). Considering the chromatographic behavior, mass accuracy, and isotopic distribution, the described method could distinguish between isomers (i.e., Alanine/Sarcosine; Isoleucine/ Leucine).

Analytical Parameters. Calibration curves were obtained for each standard by plotting the standard concentration as a function of the peak area obtained from GC/APCI-TOF MS analyses. The parameters of the calibration functions, LOD, calibration range, correlation coefficient, precision, and accuracy are summarized in Table 3.

To calculate the calibration functions and LOD's, we took the EIC of the most intense or base peak in the mass spectrum for each compound in the standard mixture. If the compound was represented by more than one silylated form, the one with higher linearity in the calibration range was used for calculation of analytical parameters. For example, in the case of glycine, we used glycine+3TMS+H; for isoleucine, isoleucine+1TMS+H; for leucine, leucine+1TMS+H; for serine, serine+3TMS+H; for valine, valine+1TMS+H; for uric acid, uric acid+4TMS+H; and in the case of Phe-Gly hydrate, we used Phe-Gly+H. The results summarized in Table 3 indicate that the GC/APCI-TOF MS method is a reliable approach for the analysis of a wide range of compounds. LODs were found within the range from 11.8 to 72.5 nM depending on the type of compound. To the best of our knowledge, these LOD values are considerably lower than the normal values previously described in literature for the determination of this kind of compounds by GC-MS.(29;35-37) Still, the brief overview of the values reported in literature (Supplementary Materials, Table S1) for more "classical" GC-MS systems shows how difficult it is to do a fair comparison with APCI-GC. LOD and LOQ values usually reported in the studies targeted to one or two classes of the metabolites. On the contrary, our standard mixture was designed to mimic a profiling condition and includes compounds belonging to nine different chemical families.

Table 3. Analytical Parameters for the GC/APCI-TOF MS Method Described (Positive Polarity).

Compounds	LOD (μM x 10^{-3})	LOQ (μM x 10^{-3})	calibration range (μM)	calibration curve[a]	r^2	considered ion	repeat. intra day[b]	repeat. inter day[b]	reproducibility[c]	accuracy[d]
Valine+1TMS+H	28.5	95		y = 88131x - 770846	0.9311	190.1256	1.23	5.16	5.98	95.7
Alanine+2TMS+H	48.7	162.3		y = 51606x - 1007331	0.9136	234.1338	1.51	4.1	6.39	96.4
Sarcosine+2TMS+H	55.1	183.7		y = 45585x + 1523067	0.921	234.138	1.9	3.76	7.01	98.3
Leucine+1TMS+H	24.7	82.3		y = 101739x - 1476560	0.902	204.1414	0.95	5.37	7.55	102.1
Proline+1TMS+H	72.5	241.7		y = 34612x + 45712	0.9694	188.1108	0.73	5.05	6.5	101.6
Isoleucine+1TMS+H	25.3	84.3		y = 99186x - 1232551	0.9841	204.1409	1.87	6.37	8.78	99.5
Benzoic acid+1TMS+H	39.4	131.3		y = 63729x + 201338	0.9196	195.087	1.65	4.74	7.04	98.5
Glycine+3TMS+H	25.2	84		y = 99579x + 1491920	0.9757	292.1578	1.23	6.01	6.57	99.2
Serine+3TMS+H	36.2	120.7		y = 69450x - 719359	0.9814	322.1681	2.09	4.22	6.88	96.1
Threonine+3TMS+H	35	116.7		y = 71798x - 170943	0.9233	336.1834	1.87	4.11	7.09	95.5
Methionine+2TMS+H	45.4	151.3		y = 55355x - 619794	0.9867	294.1376	1.11	5.01	6.44	98.1
Aspartic acid+3TMS+H	38.2	127.3		y = 65717x + 213067	0.9338	350.1631	0.89	5.55	6.01	98.7
Glutamic acid+3TMS+H	48.1	160.3	QL-100	y = 52232x - 760373	0.9391	364.1786	1.09	4.11	6.55	97.3
Phenylalanine+2TMS+H	32.6	108.7		y = 76927x - 488942	0.9057	310.1653	1.21	4.09	6.32	98.3
Phenyl-Gly+H	18.5	61.7		y = 135673x + 2391638	0.9052	223.108	1.56	5.65	7.11	96.1
Hippuric acid+1TMS+H	16.6	55.3		y = 151185x - 2387767	0.962	252.1047	1.76	6.01	6.21	98.2
Caffeine+H	11.8	39.3		y = 212078x - 1766041	0.967	195.0835	1.44	5.98	6.09	98.1
Theophylline+1TMS+H	14.5	48.3		y = 172904x - 4035222	0.9309	253.1166	1.32	4.89	6.81	96.1
Lysine+4TMS+H	22.2	74		y = 112802x - 853604	0.9372	435.2699	1.78	5.01	7.45	97.7
Tyrosine+3TMS+H	19.1	63.7		y = 131596x - 1252913	0.9334	398.1999	1.66	5.22	8.9	96.3
4-Methyldopamine+3TMS+H	17.5	58.3		y = 143581x + 2238266	0.9785	384.2199	1.56	5.76	7.91	95.9
Dopamine+4TMS+H	18.7	62.3		y = 134231x + 1841824	0.976	442.2448	1.01	4.33	6.56	100.8
Uric acid+4TMS+H	23.8	79.3		y = 105543x - 1922989	0.9372	457.1939	0.91	4.52	8.32	99.7
5-Hydroxyindole-3-	17	56.6		y = 147645x + 709932	0.9339	408.1842	0.9	4.25	8.76	98.4
Nortriptyline+H	65.9	219.7		y = 38120x - 758169	0.9665	264.1744	1.81	5.11	6.04	96.4

[a] A (peak area)= a + b × C (μM) for five points (n = 5).
[b] RSDs values (%) for peak areas corresponding to each compound; measured from three injections of each analyte within the same day (intra-) and on three different days (inter-).
[c] RSDs values (%) from two consecutive injections with two different technicians and within two different days.
[d] The accuracy of the assay is the closeness of the test value obtained to the nominal value. It is calculated by determining trueness and precision. (%Recovery, %RSD).

Moreover, a proper comparison of APCI and EI/CI could only be done if data are obtained on the same mass analyzer type, with the same sample preparation and derivatization strategies. At the end, the output still will not be 100% conclusive. We see as more beneficial the strategy, which will explore complementarities of both methods, combining high quality MS data generated under APCI condition with highly reproducible fragmentation spectra of EI.

Finally, we calculated the two most important parameters for evaluation of the precision of the analytical procedure: repeatability and reproducibility. In terms of repeatability; calculated RSDs did not exceed 6.37%. Reproducibility was determined by calculation the RSDs values (%) from two consecutive injections with two different technicians and within two different days and it did not exceed 8.90%.

Applicability of GC/APCI-TOF MS for Metabolic Profiling in Biological Samples. A human CSF pool was extracted, dried, derivatized, and analyzed by GC/APCI-TOF MS as described above (see Materials and Methods). At first, we compared the chromatograms of the human CSF with those obtained for the standard mixture. Confirmation of compounds identity was accomplished by comparing retention time, mass position, and isotopic pattern of standards and sample.

Figure 2A shows the metabolic profile of human CSF as base peak chromatogram. The observed complexity and richness of the chromatogram demonstrates the potential of the method. In Figure 2B we show several EICs of metabolites, which were identified in the CSF. Several of them were assigned using only mass position and isotopic distribution. Supplementary Materials, Figure S2 shows an example of such assignment for N-acetyl-aspartate.

Table 4 contains information concerning the compounds of our standard mixture found in the human CSF (formula, retention time, experimental m/z and theoretical, mass error and sigma value). Even in this case of analyzing an extremely complex biological sample, the accurate measurements (very low mass error) and the isotopic distribution evaluation (sigma value) obtained by TOF MS could confirm the identity of the analytes.

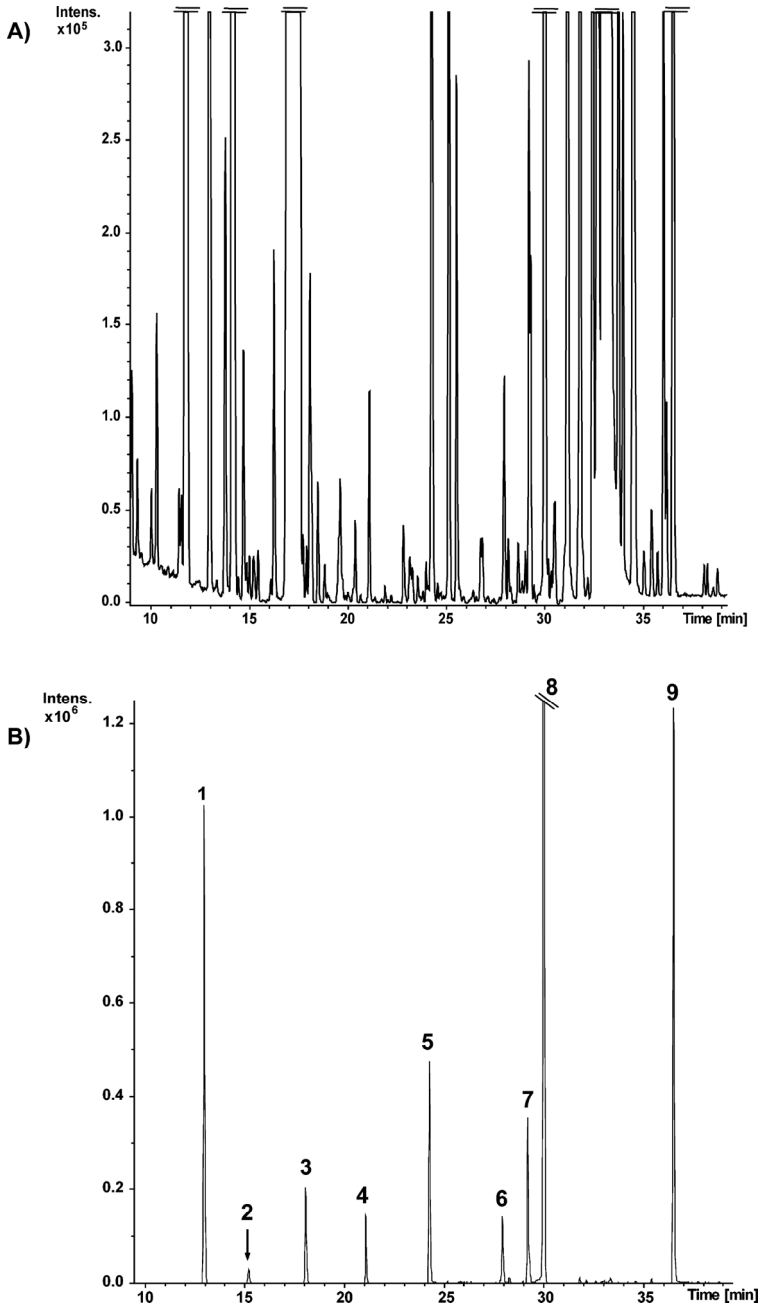

Figure 2. GC/APCI-TOF MS analysis of CSF sample: (A) Base peak chromatogram of the derivatized CSF sample. (B) EICs of several identified metabolites; peaks 1 (Glycine), 2 (Uric acid), 4 (Threonine) assigned with help of standards, peaks 3 (Glycerol), 5 (Pyroglutamic acid), 6 (N-acetyl-aspartate), 7 (Ribitol), 8 (Glutamine), 9 (Glucose) assigned using mass position and isotopic pattern.

Table 4. Compounds Included in the Standard Mixture Found in Human CSF Samples

compound	formula (peak found)	retention time	*m/z* experimental	*m/z* calculated	error (mDa)	mSigma value
Valine+1TMS+H	$C_8H_{20}NO_2Si$	12.4	190.1245	190.1258	1.29	5.2
Alanine+2TMS+H	$C_9H_{24}NO_2Si_2$	13	234.133	234.134	1	4.5
Glycine+2TMS+H	$C_8H_{22}NO_2Si_2$	13.1	220.1171	220.1184	1.3	5.1
Sarcosine+2TMS+H	$C_9H_{24}NO_2Si_2$	13.8	234.1338	234.134	0.21	2.7
Leucine+1TMS+H	$C_9H_{22}NO_2Si$	14.4	204.1404	204.1414	1	4.1
Isoleucine+1TMS+H	$C_9H_{22}NO_2Si$	15	204.1422	204.1414	-0.79	2.7
Uricacid+3TMS+H	$C_{14}H_{29}N_4O_3Si_3$	15.2	385.153	385.1542	1.19	3.7
Valine+2TMS+H	$C_{11}H_{28}NO_2Si_2$	16.3	262.1653	262.1653	0	5.3
Benzoicacid+1TMS+H	$C_{10}H_{15}O_2Si$	17	195.0869	195.0877	0.8	8.4
Serine+2TMS+H	$C_9H_{24}NO_3Si_2$	17.4	250.129	250.1289	-0.1	1.9
Leucine+2TMS+H	$C_{12}H_{30}NO_2Si_2$	17.9	276.1815	276.181	-0.49	10.1
Isoleucine+2TMS+H	$C_{12}H_{30}NO_2Si_2$	18.5	276.1823	276.181	-1.3	6.4
Glycine+3TMS+H	$C_{11}H_{30}NO_2Si_3$	18.8	292.1574	292.1579	0.5	9.3
Serine+3TMS+H	$C_{12}H_{32}NO_3Si_3$	20.4	322.1689	322.1684	-0.52	5.2
Threonine+3TMS+H	$C_{13}H_{34}NO_3Si_3$	21.1	336.1861	336.1841	-1.98	5.6
Methionine+2TMS+H	$C_{11}H_{28}NO_2SSi_2$	24.2	294.138	294.1374	-0.59	7.4
Asparticacid+3TMS+H	$C_{13}H_{32}NO_4Si_3$	24.4	350.1631	350.1634	0.32	1.6
Phenylalanine+2TMS+H	$C_{15}H_{28}NO_2Si_2$	26.7	310.1663	310.1653	-0.99	6.9
Phenyl-Gly+H	$C_{11}H_{15}N_2O_3$	28.5	223.1082	223.1077	-0.49	3.3
Hippuricacid+1TMS+H	$C_{12}H_8NO_3Si$	31.1	252.1047	252.105	0.3	1.8
Caffeine+H	$C_8H_{11}N_4O_2$	31.2	195.0835	195.0836	0.1	3.5
Theophylline+1TMS+H	$C_{10}H_{17}N_4O_2Si$	32.6	253.1116	253.1115	-0.1	2.9
Lysine+4TMS+H	$C_{18}H_{47}N_2O_2Si_4$	33	435.2697	435.2709	1.22	2.5
Tyrosine+3TMS+H	$C_{18}H_{36}NO_3Si_3$	33.3	398.1999	398.1998	0.12	5.5
Uricacid+4TMS+H	$C_{17}H_{37}N_4O_3Si_4$	36.5	457.1949	457.1937	-1.19	9.1
5-hydroxyindole-3-acetic+3TMS+H	$C_{19}H_{34}NO_3Si_3$	38.3	408.1834	408.1841	0.69	7.7
Nortriptyline+H	$C_{19}H_{22}N$	38.7	264.1734	264.1747	1.29	9.2

In total, our method was capable to determine more than 300 compounds with different isotopic features in the CSF sample. As commented before, the identity of some of those peaks could be corroborated by the standards included in our mixture, but in other cases, we used mass position and isotopic distribution to achieve the identification of the analytes present in the CSF according to their molecular formula. Some examples are included in Figure 2B, where we have shown the EICs of silylated forms of uric acid, glycerol, pyroglutamic acid, N-acetyl-aspartate, ribitol, glutamine, and glucose. The values of mass error and sigma value for the mentioned compounds were excellent, showing the capability of our GC/APCI-TOF MS method to confirm the identity of an important number of metabolites which can be found in CSF samples. However, being strict we should

discriminate between assignments validated by data from the standard mixture and those which were made solely based on sigma value calculation. If in the first case the reference to standard makes an assignment almost 100% correct, the second one is the best guess possible on the basis of available data. In Figure 3 we have shown MS spectra produced by GC/APCI-TOF MS for some compounds found in human CSF. Included compounds belong to different chemical families: amino acids, xanthines, organic acids, indoles and amines.

Valine was detected as valine+1TMS+H (m/z 190.1245), according to the reaction described above $[M+H]^+$ (in the current case $[M+1TMS+H]^+$), observing mainly the mentioned m/z signal and not its fragments. In the case of glycine and aspartic acid, the main peak in the spectrum was the amino acid+3TMS+H. Because of in source-fragmentation, some fragments were also observed. A neutral loss of 72.0387 appears after losing one of the trimethylsilane (TMS) groups, more precisely -OH replacement with -$OSi(CH_3)_3$, (=$[C_3H_8Si]$), trimethylsiloxane. The loss of two TMS groups should lead to $[M-2TMS+H]^+$, resulting in a loss of 144.0785. Moreover, for glycine we detected a fragment produced for the loss of 82.0495, and for aspartic acid, another one after losing 118.1170. The last one could be the result of losing one TMS group and three CH_3 groups. One of the xanthines, theophylline, showed in its spectrum $[M+1TMS+H]^+$ and also $[M-1TMS+H]^+$ with low intensity in comparison with $[M+1TMS+H]^+$. [5-Hydroxyindole- 3-acetic acid+3TMS+H]$^+$ was the peak we found in CSF for the compound containing an indole moiety. Again 72.0389 for the loss of one TMS group, 144.0801 for the loss of 2TMS, and 118.1171 for the loss of one TMS group and three CH3 groups were observed. The amine nortriptyline hydrochloride showed up as $[M+H]^+$ without undergoing any fragmentation.

As commented before, Table 4 includes only a small fraction of compounds detected in CSF. We have detected more than 300 distinct features even using very strict peak finding criteria. This fact in combination with the here presented analytical characteristics (LODs, repeatability, and reproducibility) demonstrates the potential of GC/APCI-TOF MS for metabolic profiling. In other words, this analytical procedure might indeed be a valuable addition to the “metabolomics toolbox”.

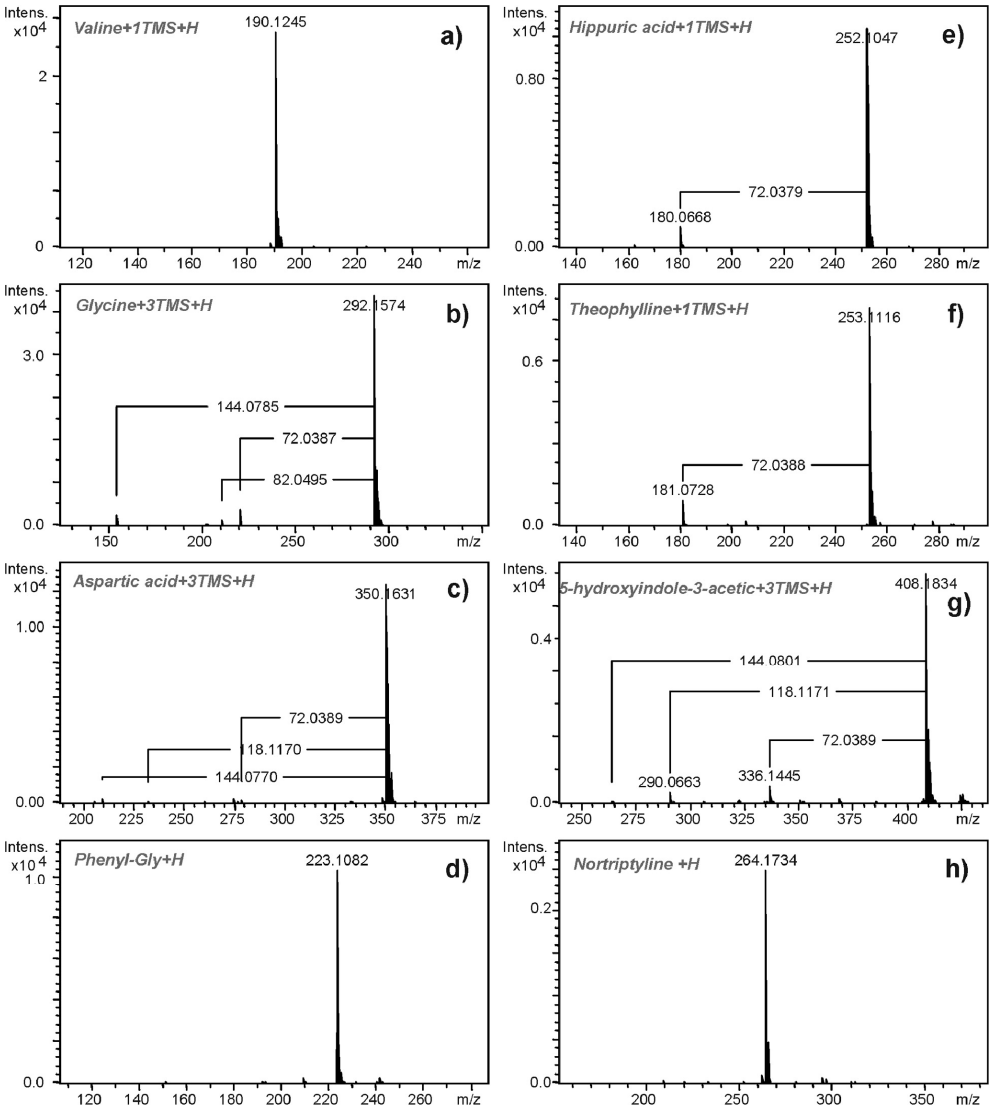

Figure 3. Typical APCI MS spectra of silylated compounds from different chemical families: amino acids (a–c), dipeptide (d), organic acid (e), xanthine (f), indole (g), and amine (h).

CONCLUSIONS

EI and CI are the ionization techniques conventionally used in GC-MS, both operating under vacuum condition. EI mass spectra are mainly characterized by numerous fragments produced during the high energy ionization process, while the CI mass spectra exhibit both the protonated molecules and intense fragment ions. Commercial and in-house database

mass spectral libraries can then be used to identify the separated compounds or at least give structural clues to support the identification process. Here, we present an alternative to the classical GC-MS methods, namely, gas phase APCI as interface in combination with orthogonal TOF-MS. A very sensitive and accurate GC/APCI-TOF MS method was developed for the automated analysis of metabolites in biological samples. At present, the analytical evaluation of the method was made by using amino acids, organic acids, alcohols, xanthines, indoles, dipeptides, compounds with imidazole groups, amines, and analytes with hydroxyl and amine groups, demonstrating that 25 analytes of the 31 present in our mixture can be reliably determined. Excellent repeatability was obtained, with relative standard deviations (RSDs) of peak areas between 0.7% and 2.1% in the intraday study, and between 3.8% and 6.4% in the interday study.

Analysis of CSF has demonstrated a rich chromatographic pattern consisting of hundreds of features. The high quality of the spectra creates an opportunity to make structural assignments of metabolites based on mass position and isotopic distribution. However, the use of more advanced mass analyzers such as hybrid quadrupole TOF will be beneficial to resolve more difficult cases and support identification by fragmentation data. In summary, GC/APCI-TOF MS is an analytical procedure, which combines the best of chromatography with one of the most robust MS interfaces, and as such, it has a potential to become one of the standard methods in metabolic profiling.

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SUPPLEMENTARY MATERIALS

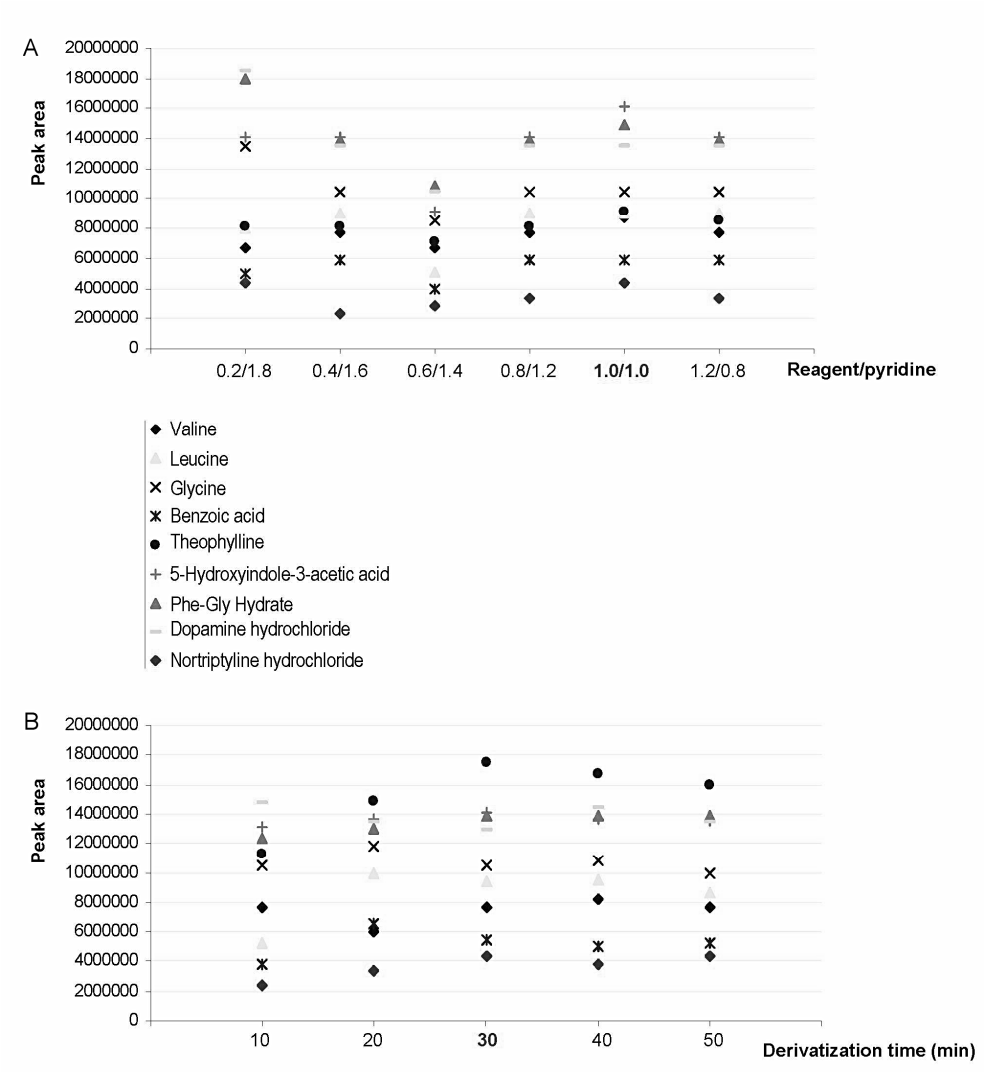

Figure S1. Effect of derivatization conditions on peak area of several metabolites included in the standard mixture. A) volume ratio of derivatization reagent (µL) and pyridine (µL); B) derivatization time.

Table S1. Some relevant information about other GC-MS methods previously published for the determination in biological samples of the same compounds as those under study in the current paper or other similar analytes.

Compounds under study	Derivatization reaction	Instrumentation used	Detection limit	Sample analyzed	Other comments	Reference
Organic acids, amino acids, sugars, polyols, purines, pyrimidines and other compounds are simultaneously analyzed and quantified	BSTFA with 1% TMCS	A Hewlett-Packard GC–MSD (HP6890/MSD5973) and a Shimadzu QP5000 GC–MS were used for GC–MS measurement	-	Human urine	Pilot study for screening of 22 target diseases in newborns conducted in Japan is described. The diagnostic procedure consists of the use of urine or filter paper urine, preincubation of urine with urease, stable isotope dilution, and GCMS.	J. Chromatogr. B 2001, 758, 3-25
Buprenorphine and norbuprenorphine	Pentafuoropropionic anhydride	HP 5890 GC with a 5971A mass selective detector	LOQ buprenorphine=0.05 ng/ml; for Norbuprenorphine=0.1 ng/ml	Human plasma	The method could be used to explore the pharmacokinetic/pharmacodynamic relationship of buprenorphine and norbuprenorphine	European J. Pharmaceutics and Biopharmaceutics 2001, 51, 147-151
Amino acids	Ethyl chloroformate	GC-MS Hewlett-Packard 5890 series II GC and 5971 A MS	0.5-1.0 µg/ml	Human urine	Several derivatisation reagents used. Threonine, serine, asparagines, glutamine, arginine not derivatized by using any tested reagent.	J. Chromatogr. B 2002, 776, 49-55
Organic acids and glycine conjugates	BSTFA with 1% TMCS	Hewlett-Packard HP5890A GC coupled to HP5970B mass-selective detector	0.4-200 nmol/l	Amniotic fluid	12 metabolites simultaneously quantified	J. Inherit. Metab. Dis. 2004, 27, 567-579
Global approach (hundreds of molecular features detected)	BSTFA with 1% TMCS	Agilent 6890 GC with FID and 5973MSD MS	-	Human urine samples	GC as complementary tool to NMR	Rapid. Commun. Mass Spectrom. 2006, 20, 2271-2280
Antidepressants and their active metabolites	Heptafluorobutyrylation	HP 6890 GC system with HP 5973 mass-selective detector	5.0-12.5 ng/ml (EI and positive CI)	Plasma	Validation of GC coupled to MS by CI and EI sources. CI offered advantages in selectivity and sensitivity	J. Chromatogr. A 2007, 1176, 236-245
Amino acids, organic acids, sugars…goblal approach	1st step: methoximation. 2nd step: MSTFA with 1% TMCS	Finnigan GC (Thermo Finnigan, USA) coupled with mass spectrometry (TRACE DSQ)	26 selected compounds could be detected at S/N ≥ 3 when the urine dilution was 0.02 (v/v, urine/urine +water): it could be defined as LOD	Rat urine	Forty-nine endogenous metabolites were separated and identified in GCMS chromatogram, of which 26 identified compounds were selected for quantitative analysis	J. Chromatogr. B 2007, 854, 20-25

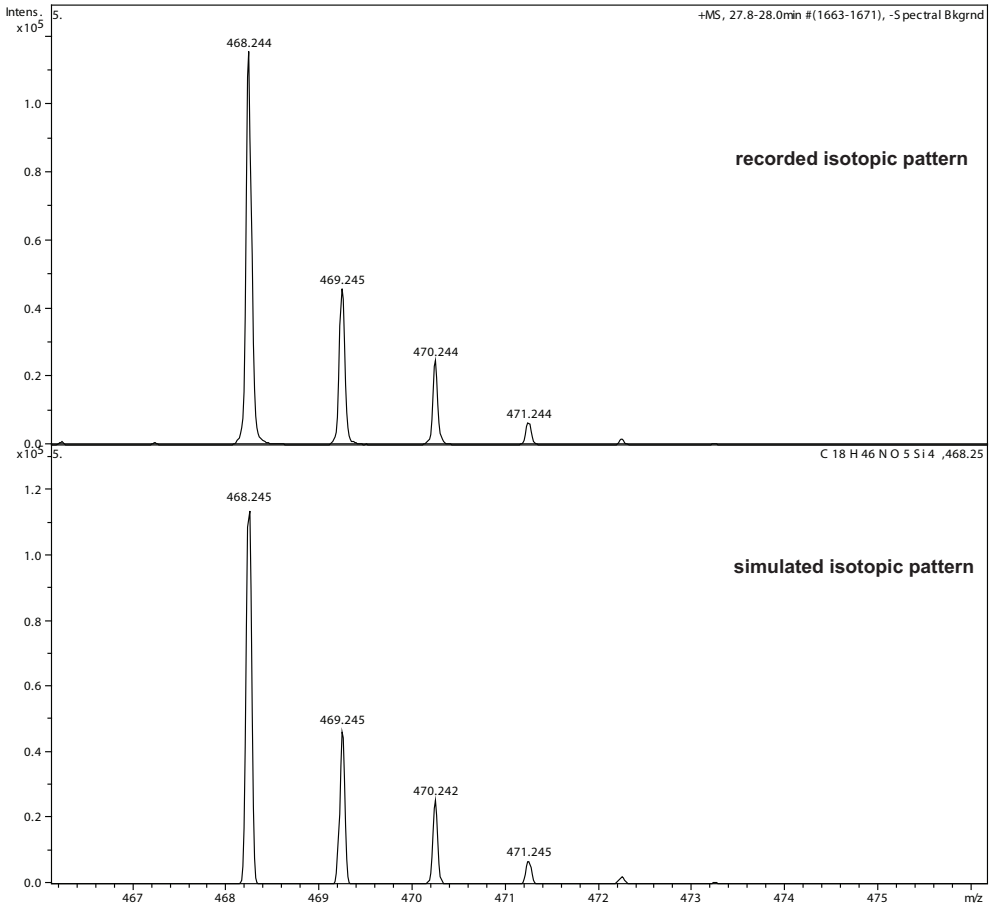

Figure S2. Assignment of N-acetyl-aspartate using accurate mass and isotopic distribution information.