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Highly Sensitive GC/MS/MS Method for Quantitation of Amino and Nonamino Organic Acids

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ABSTRACT: Metabolite profiling methods are important tools for measurement of metabolite pools in biological systems. While most metabolite profiling methods report relative intensities or depend on a few internal standards representing all metabolites, the ultimate requirement for a quantitative description of the metabolite pool in biological cells and fluids is absolute concentration determination. We report here a high-throughput and sensitive gas chromatography/tandem mass spectrometry (GC/MS/MS) targeted metabolite profiling method enabling absolute quantification of all detected metabolites. The method is based on methyl chloroformate derivatization and quantification by spiking samples with metabolite standards separately derivatized with deuterated derivatization reagents. The traditional electron impact ionization is replaced with positive chemical ionization since the latter to a much larger extent preserve the molecular ion and other high molecular weight fragments. This made it easier to select unique MS/MS transitions among the many coeluting metabolites. Currently, the novel GC/MS/MS method comprises 67 common primary metabolites of which most belong to the groups of amino and nonamino organic acids. We show the applicability of the method on urine and serum samples. The method is a significant improvement of present methodology for quantitative GC/MS metabolite profiling of amino acids and nonamino organic acids.

Biological research is heavily influenced by technological advancements. Metabolomics, the comprehensive analysis of metabolite pools, has over the past decade had a rapid development due to an increased focus in the research community and also due to instrumental developments enabling more sensitive and precise analysis.[1] The field of metabolome analysis poses particular technical challenges due to the large chemical variety, rapid turnover, chemical instability and dynamic range of the metabolites. As a consequence, comprehensive analysis of the metabolome has to be approached by combining several different analytical techniques.[2] There are two core technological platforms used for metabolome analysis: mass spectrometry (MS) and nuclear magnetic resonance (NMR), both with excellent and complementary application areas for the analysis of metabolite pools.[3,4] MS instruments have very high sensitivity and are most frequently used in combination with separation techniques as liquid and gas chromatography (LC and GC, respectively) since biological extracts are far too complex for direct analysis on the MS instrument.[5] Both GC/MS and liquid chromatography (LC)/MS have proven important in analyzing various metabolite classes.[6−8] GC/MS has been regarded as the golden standard in metabolite analysis due to high chromatographic resolution and very reproducible chromatography, in addition to standardized electron impact ionization conditions enabling establishment of searchable mass spectra libraries.[9] Metabolite derivatization based on silylation agents (e.g., *N*-methyltrimethylsilyltrifluoroacetamide (MSTFA)) has been most popular.[10−12] However, other derivatization methods are also in frequent use, e.g., methyl chloroformate (MCF) derivatization as this method have high sensitivity and stability toward the amino acid and nonamino organic acid metabolite groups.[13−15] One of the main challenges in LC/MS based metabolite analysis has been to establish chromatographic conditions for separation of low molecular weight and highly charged analytes as they are not retained by standard reverse phase (RP) chromatography. This has been counteracted by usage of ion-pair reagents in the mobile phase.[16,17] Alternatively, the introduction of hydrophilic interaction (HILIC) LC-columns (and lately improvement in HILIC column technology) have significantly advanced the field of LC/MS based metabolomics.[18,19] The current status in MS-based metabolomics is that a comprehensive metabolite profiling of a biological sample requires the use of several different GC/MS and LC/MS methods.[2] Therefore, it is a challenging task to characterize the metabolome as several instrumental configurations need to be available in a metabolomics laboratory.

There are two main error sources in a metabolite profiling experiment. The first is loss of metabolites during the sample processing steps (quenching, extraction, and concentration), while the second is analytical errors in a chosen analytical platform. The first challenge is frequently correlated by adding a few heavy labeled internal standards to the quench solution and using the assumption that all other metabolites within the same chemical group are lost at the same extent. Addition of a true internal standard for each metabolite might not be practical for all metabolites, due to price or necessity of custom synthesis. Several groups have developed a more advanced protocols, e.g., Wu et al. developed an isotope dilution strategy for accurate quantification of intracellular metabolites using uniformly ^{13}C-labeled cell

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extracts as internal standards.[20] Uehara et al. reported a similar approach using isotope labeled *E. coli* extracts for comparison of mammalian cell metabolomes.[21] Bennet and co-workers addressed this using *E. coli* grown on solid medium with U−^{13}C-glucose as the sole carbon source and used naturally labeled metabolites as internal standards.[22] However, physiological studies are most often performed in liquid culture which often precludes the use of expensive isotopically labeled substrates.

The second error source, analytical errors, are introduced during analysis as the performance of a GC/LC/MS instrument varies with time (i.e., within a sequence of analysis, during maintenance intervals where the ion source can become contaminated, after tuning where the detector sensitivity is changed, derivatization step for GC, etc). The usage of isotope labeled cell extracts added prior to extraction can be used to eliminate these variations. However, this kind of internal standard might not be suitable for all analytical purposes since there will be batch to batch variations and some metabolites of interest might not be present in the isotope cell extract or only present at very low concentration, making it less robust and useful as an internal standard. Therefore, methods for eliminating solely instrumental analytical errors are also highly requested, for example, in studies of samples that can readily be analyzed without any preprocessing steps such as urine and serum. Derivatization of metabolites makes possible introduction of heavy labeling for all known metabolites through the use of a labeled derivatization agent, as has been shown for both GC/MS and LC/MS.[23−26] Here, we present a novel approach based on GC/MS/MS and the use of isotopically labeled MCF derivatization agents and apply this novel method for profiling of amino acid and nonamino organic acid containing metabolites in two human body fluids, urine and serum. The method enables absolute concentration determination of 67 metabolites and can also be expanded with new metabolites that readily can be derivatized with MCF. Importantly, the method also makes interlaboratory comparisons more credible.

MATERIALS AND METHODS

Derivatization and GC/MS/MS. Derivatization followed the protocol developed by Villas-Boas and co-workers[13] Urine (50 μL) was derivatized directly while a protein precipitation step was included for the serum (100 μL serum samples were diluted with four volumes of methanol, kept on ice for 30 min, centrifuged 10 min at maximum speed in an Eppendorf centrifuge, supernatant transferred to a new tube and vacuum-dried, and finally reconstituted directly with 1 M NaOH used in the derivatization protocol. The GC/MS QqQ system was an Agilent 7890A series GC system coupled with an Agilent 7000B QqQ MS. The GC was operated in constant pressure mode (20 psi) with helium as the carrier gas and using d_5-glutamate as a standard for retention time locking of the method. A J&W Scientific DB-5MS column (30 m long with 10 m guard column, 0.25 mm inner diameter, 0.25 μm film thickness) was used. The inlet had a pressure set to 20 psi, temperature of 290 °C, and injection (1 μL) was performed in the pulsed splitless mode. The GC oven was kept at 40 °C for 2 min before raising to 320 °C using a linear gradient of 30 °C/min and finally kept at 320 °C for 4 min resulting in a 15.5 min total run time. The MS transfer line temperature was set to 280 °C. Methane reagent gas was used for positive chemical ionization.

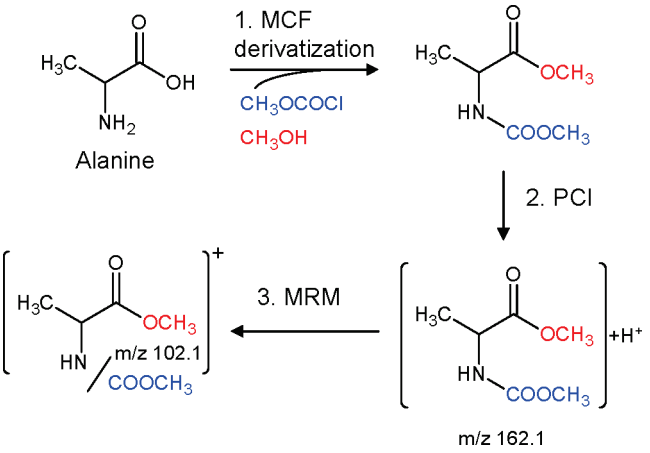

Figure 1. The three steps in MCF derivatization of alanine with subsequent positive chemical ionization and MS/MS detection. Step 1 shows that the carboxylic and amino groups are differently labeled, the former from the solvent methanol while the latter from MCF. Step 2 shows the generation of the protonated ion species of alanine generated during the ionization. Step 3 shows the established unique MRM transition enabling a highly sensitive and selective detection of alanine.

Chemicals and Synthesis of Deuterated MCF. All nonlabeled metabolite standards and 20% phosgene solution in toluene were purchased from Sigma-Aldrich. Deuterated metabolite standards and deuterated methanol were purchased from Cambridge Isotope Laboratories. The following procedure provided poor yield of d_3-MCF, and attempts to improve the procedure have not been pursued by us. Evidently, the use of toluene should be avoided. Phosgene is an insidious poison that should only be used in a ventilated hood and with caution. Deuterated methanol (35 g, 0.97 mol) was added over 1 h to a 20% phosgene solution in toluene (500 mL, 1.02 mol) at 0 °C over 1 h. The hydrochloric gas generated was neutralized by leading it through a cooled aqueous sodium hydroxide solution. The reaction mixture was stirred for 5 h under nitrogen atmosphere before warmed to room temperature. Nitrogen purge for 2 h was used to remove hydrochloric gas and unreacted phosgene, after which the product was isolated by distillation. The d_3-MCF was collected at 70−76 °C (uncorrected) as a ∼1:1 mixture with toluene (12.2 g), giving a total yield of approximately 6%. ^{13}C NMR spectrum of synthesized d_3-MCF was recorded on a Bruker Avance DPX 400 MHz instrument, fitted with a 5 mm dual probe to verify the expected product formation. Characterization by NMR; δ_C (100.6 MHz, $CDCl_3$) 57.3 (septet, J = 22.8 Hz, OMe), 151.5 (C═O).

RESULTS AND DISCUSSION

Method Development Strategy. MCF reacts with amino and carboxylic acid groups. The resultant derivative is finally extracted into chloroform. This is a significant advantage compared to silylation since no unreacted derivatization agent will be transferred and injected into the GC/MS instrument as it does for silylation. Another advantage is that the carboxylic acid and amino groups are differentially labeled; the former group is finally modified through nucleophilic attack by the solvent, usually methanol, while the latter retain the methylformate group from MCF. This is exemplified for alanine in Figure 1, step 1. Thus, it can be used to identify functional groups of unknown analytes by

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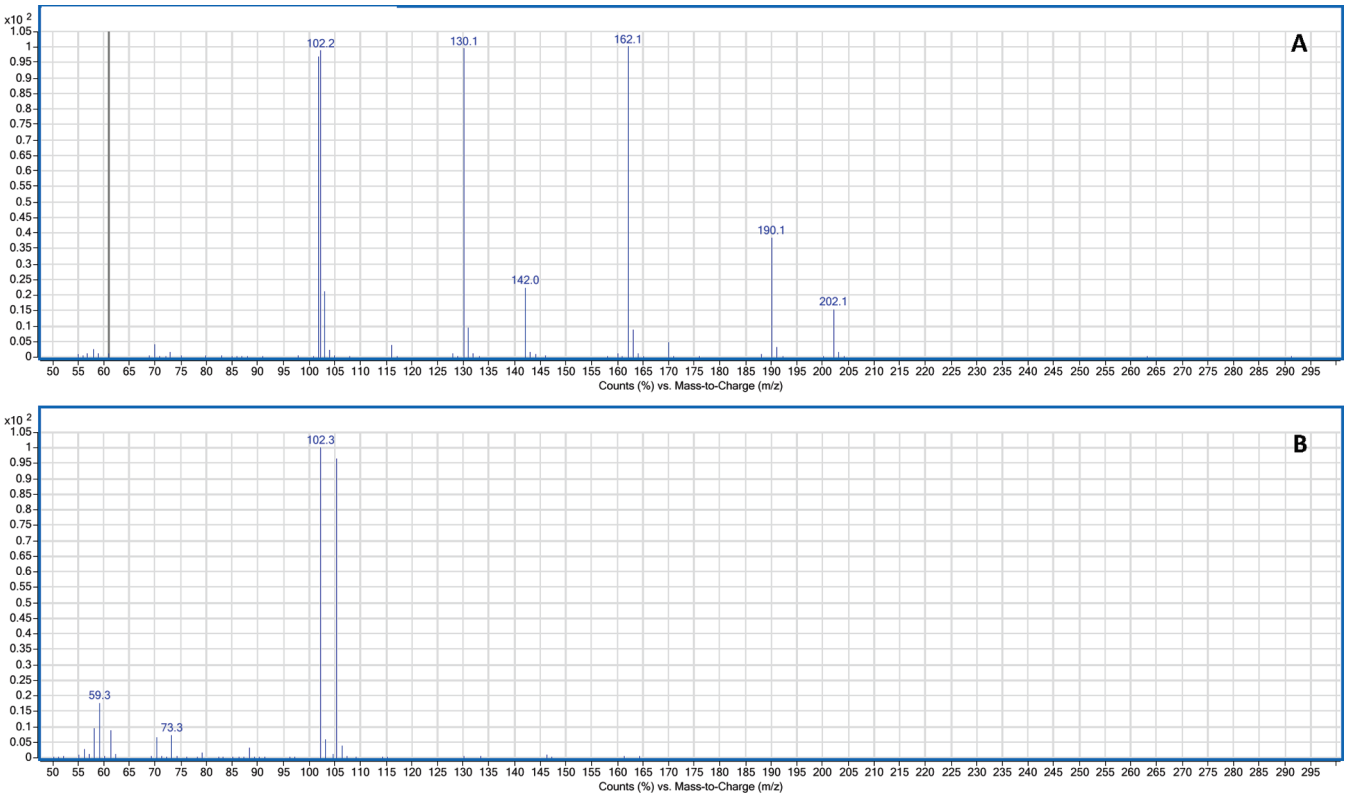

Figure 2. PCI (A) and EI (B) scans of MCF derivatized alanine.

dissolving a parallel in deuterated methanol. If the resulting mass spectrum has a three mass unit shift for some fragments, then the unknown contains a carboxylic acid group. Furthermore, this can also be taken advantage of and used to introduce internal standards for all tested metabolites. If both deuterated methanol and deuterated MCF are used, then the method will cover all metabolites that are amenable to MCF derivatization with subsequent detection in a GC/MS analysis. The only requirement is that the fragmentation mass spectrum contains a fragment that has retained one or both of the derivatized group so that the coeluting deuterized internal standard can be readily separated by mass from the metabolite in the sample. Deuterated MCF is not ready available for European laboratories (available for U.S. and Canadian laboratories though) but can easily be produced in a standard organic chemistry laboratory. It is recommended to use pure phosgene gas and not a 20% phosgene solution in toluene as the toluene seemingly forms an azeotrope with MCF during the distillation purification process.

Biological extracts are very complex, and coelution of metabolites is common even for long GC/MS runs. The triple quadrupole (QqQ) mass spectrometer offers significant advantages both for selectivity and sensitivity compared to the single quadrupole (SQ) instruments. The use of SQ instrument would be particularly challenging for this method using so many deuterated internal standards as it will not be possible to differentiate between fragments without the derivatization groups which significantly will limit the selection of unique qualifier ions that is important in traditional electron ionization GC/MS analysis. In this method, we choose to combine QqQ MS detection with positive chemical ionization (PCI) rather than the traditional electron impact (EI) ionization. The advantage of PCI is the retaining of the molecular ion and other relative high molecular weight fragments compared to EI. Figure 2 shows mass spectra for PCI and EI scans of MCF derivatized alanine. It is obviously easier to establish unique multiple reaction monitoring (MRM) transitions using PCI than EI as more potential precursor ions are generated during the former ionization mode. This increases the selectivity and decreases the problem of coeluting metabolites. In addition, preliminary investigations revealed higher sensitivity of PCI for the early eluting organic acids (data not shown). The ionization process and subsequent fragmentation in the collision cell of the QqQ MS is shown for alanine in Figure 1, steps 2 and 3, respectively. The chosen MRM quantification transition for alanine in the method is shown in Table 1. The MRM transition for MCF-alanine (162.2 → 102.2) is easily separated by the QqQ MS instrument from the MRM transition of the MCF-d_3-alanine sampling standard (MRM 165.2 → 105.2) and the d_3MCF/d_3MeOH-alanine analytical internal standard (MRM 168.2 → 105.2). The two transitions of d_3MCF-MeOH-alanine and MCF-d_3MeOH-alanine have the same precursor ion (165.2) but different product ions (102.2 and 105.2, respectively). These

Table 1. PCI MRM Transitions of Alanine and Deuterated Alanine Dissolved in Either Methanol or Deuterated Methanol and Derivatized with Either MCF or Deuterated MCF

	M_w	CI	MRM quant transition
MCF-alanine	161.1	162.1	162.1 → 102.1
MCF-d_3 alanine	164.1	165.1	165.1 → 105.1
d_3MCF-alanine	164.1	165.1	165.1 → 102.1
MCF-d_3MeOH-alanine	164.1	165.1	165.1 → 105.1
d_3MCF-d_3MeOH-alanine	167.1	168.1	168.1 → 105.1

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Table 2. Overview of All Metabolites Included in the MCF PCI MRM GC/MS/MS Method with Retention Time, MRM Transition (i.e., PIS, precursor ion, and Tg, target ion), Optimized Collision Energy, Limit of Detection, and MRM Transition for the Internal Standard[a]

		MRM-transition		limit of detection	urine		serum	
metabolite	rt (min)	sample/internal standard	collision energy	(pmol/μL)	μmol/L	std dev (%)	μmol/L	std dev (%)
(±)-3-methyl-2-oxovalerate	6.6	127.0−67.0/130.0−67.0	15	0.1	3	1	2	1
4-methylvalerate	6.3	131.0−43.0/134.0−43.0	20	0.1				
malonic acid	6.3	133.0−69.0/139.0−69.0	20	0.4	6	3	1	1
pyruvate/methylpyuvate	6.5	117.0−57.0/123.0−60.0	10	0.5	70	2	4	7
fumarate	7.0	145.0−85.0/151.0−88.0	15	0.1	2	3		
lactate/methylglyoxal	7.0	163.0−59.0/169.0−62.0	15	0.4	172	6	1401	1
succinate	7.0	115.0−59.0/118.0−62.0	15	0.2	13	1	3	2
citraconate/itaconate	7.4	127.0−59.0/130.0−62.0	15	0.1	22	1		
benzoate	7.5	137.0−77.0/140.0−77.0	25	0.2	1	2	2	3
citramalate	7.6	117.0−43.0/120.0−43.0	15	0.5	31	5		
alanine	7.7	162.0−102.0/168.0−105.0	10	0.5	408	5	153	5
glycine	7.7	116.0−44.0/154.0−47.0	15	0.7	1189	1	92	1
O-acetyl-L-serine	7.7	102.0−42.0/105.0−42.0	10	1.0	247	7	8	2
nicotinate	7.8	138.0−78.0/141.0−78.0	25	0.4	1	3		
phenylacetate	8.0	151.0−91.0/154.0−91.0	10	0.1	3	3		
2-aminobutyrate	8.2	116.0−57.0/119.0−60.0	15	0.3	6	1	3	5
salicylate	8.2	153.0−121.0/156.0−121.0	15	1.0	11	4	8	3
β-alanine	8.2	130.0−44.0/133.0−47.0	15	0.2	8	6	1	6
adipate	8.4	143.0−73.0/146.0−76.0	15	0.4	2	4	2	2
valine	8.5	130.0−71.0/133.0−74.0	20	0.5	20	2	64	5
α-ketoglutarate	8.5	175.0−55.0/146.0−55.0	15	1.0				
2-isopropylmalate	8.6	145.0−43.0/148.0−43.0	25	0.3				
β-hydroxypyruvate	8.6	175.0−75.0/181.0−81.0	10	1.0	108	1	3	9
α-ketoadipate	8.9	157.0−69.0/160.0−62.0	15	3.0				
leucine	8.9	144.0−43.0/210.0−47.0	15	0.8	12	8	53	4
isoleucine	9.0	144.0−69.0/147.0−36.0	25	1.0	9	7	22	6
γ-aminobutyrate	9.0	176.0−69.0/182.0−62.0	25	1.0				
threonine	9.1	160.0−56.0/135.0−56.0	15	0.3	134		no data	
malate	9.1	145.0−85.0/151.0−81.0	15	0.8	5	4		
oxaloacetate	9.1	143.0−69.0/149.0−69.0	25	1.3				
L-homoserine	9.2	132.0−30.0/135.0−61.0	15	1.1				
proline	9.2	188.0−84.0/194.0−87.0	25	0.7	5	8	50	4
aspartate	9.6	160.0−75.0/166.0−81.0	25	0.7	5	8	7	3
citrate	9.6	175.0−101.0/181.0−62.0	15	0.6	4406	9	8	6
5-aminovalerate	9.7	158.0−55.0/161.0−55.0	20	0.3	2	5		
anthranilate	10.1	178.0−90.0/181.0−90.0	25	0.3	2	6		
serine	10.1	160.0−100.0/166.0−103.0	15	0.5	316	3	35	2
allantoin	10.1	146.0−42.0/152.0−45.0	10	0.7				
glutamate	10.3	202.0−98.0/208.0−101.0	30	0.5	26	9	67	5
N-acetyl-L-glutamate	10.5	218.1−84.0/224.1−84.0	10	1.0	10	5		
methionine	10.5	162.0−61.0/165.0−61.0	10	0.4	4	1	7	4
hydroxyproline	10.5	144.0−82.0/147.0−85.0	10	0.8	20		no data	
β-methylamino-L-alanine	10.7	249.1−98.0/258.1−116.0	25	0.7				
2-oxobutyrate	10.8	259.1−67.0/265.1−95.0	30	1.0				
cysteine	10.9	192.0−89.0/198.0−62.0	15	0.9	149	7	6	1
isocitrate	10.9	217.1−75.0/226.1−81.0	15	0.7	147	6		
phenylpyruvate	11.0	205.1−115.0/208.1−62.0	15	0.8				
putrescine	11.0	173.0−55.0/176.1−55.0	25	0.7				
phenylalanine	11.0	178.0−128.0/181.0−128.0	20	0.3	37	3	32	6
hippurate	11.0	194.0−105.0/197.0−105.0	10	0.2	428	2		

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Table 2. Continued

		MRM-transition		limit of detection	urine		serum	
metabolite	rt (min)	sample/internal standard	collision energy	(pmol/μL)	μmol/L	std dev (%)	μmol/L	std dev (%)
4-imidazoleacrylate	11.3	211.1−107.0/217.1−110.1	20	0.12	4	3		
2,4-diaminobutyrate	11.3	217.1−70.0/223.1−73.0	25	0.2	1	8		
cadaverine	11.5	187.0−69.0/190.0−69.0	25	0.5				
4-aminobenzoate	11.6	210.1−91.0/216.1−94.0	20	0.4				
histamine	11.8	196.0−68.0/199.0−68.0	25	1.0	312	3	3	3
p-coumarate	11.9	237.1−133.0/243.1−136.0	20	0.5				
ornithine	12.0	231.1−128.0/237.1−131.1	10	0.5	9	1	16	4
N-glycyl-L- proline	12.5	213.1−70.0/216.1−70.0	25	0.3	4	5		
lysine	12.5	245.1−142.0/251.1−145.0	10	0.4	47	1	66	4
ferulate	12.8	267.1−176.0/273.1−176.0	20	0.6	5	1		
histidine	12.8	286.1−150.0/295.1−153.1	25	1.0	880	4	59	3
tyrosine	13.3	252.1−158.0/258.1−161.0	20	0.4	75	1	21	6
2,6-diaminopimelate	13.4	200.1−140.0/206.1−143.0	10	0.5				
tryptophane	14.3	245.1−185.0/248.1−185.2	15	0.7	39	1	16	4

[a] Results from analysis of a sample urine and serum with the newly developed GC/MS/MS method. The average is calculated from four parallels, and the error is given as percentage standard deviation.

transitions are not used in this method, but they are shown since they illustrate the strength of the MCF method in identifying functional groups in unknown metabolites.

Development and Application of the GC/MS/MS Method for Analysis of Urine and Serum Samples. An overview of the method settings for all the amino and or carboxylic acid containing metabolites included in the method is presented in Table 2. The protonated molecular ion $[M + H]^+$ was identified for over 50 metabolites, and it was chosen as the precursor ion for 16 metabolites. Of the 67 metabolites, it was only for less than 10 metabolites not possible to select the base peak in the mass spectrum for MRM transition due to the fact that it was the same for the nonlabeled and the deuterized metabolite (i.e., the base peak did not retain any derivatized group). The chromatographic run takes 16 min, which permits a throughput of three runs per hour when a 4 min postrun column backflush period is added (backflushing significantly reduces the instrument maintenance intervals, which can be quite frequent when injecting dirty and complex metabolite extracts). A standard solution covering all metabolites (approximately 1.5 mM concentration for each metabolite) and derivatized with MCF is used for making a four point standard calibration curve of each metabolite. In addition, the second highest standard concentration (approximately 0.5 mM) is also dissolved in deuterated methanol and derivatized using d_3-MCF. The d_3-derivatized standard solution is spiked in all MCF derivatized standard dilution samples and in all real samples (50 and 150 μL, respectively) enabling internal standardization for all metabolites. The linear range of the method spans from the highest concentration in the standard mixture to the limit of quantitation, which varies significantly for the various metabolites. The standard curves for all individual metabolites are forced through zero, and the best fit of either linear or power regression are used (R^2 values better than 0.98 for all metabolites presented in Table 2). The limit of quantitation is also dependent on the ratio between sample and internal standard solutions as the deuterated methanol contain one per thousand unlabeled methanol. Therefore, background noise for estimation of the limit of quantitation was determined using a blank sample spiked with a deuterated internal standard mixture. A few metabolites represented a challenge as a unique MRM transition could not be established. Leucine and isoleucine almost coelute, but the two metabolites can be separated through the use of other MRM transitions for verification purposes (transitions not shown in Table 2). Methyl-pyruvate and pyruvate yield the same product during MCF derivatization, hence the former is excluded in an analytical run. However, through selective usage of deuterated methanol, they can be separated in a dedicated target analyses aimed for these two metabolites only. This shows the challenge of running comprehensive metabolite profiling methods with parallel identification of a large number of metabolites.

Even though unique transitions can be established among all metabolites included as standards, real biological extracts might contain other (and unknown) metabolites that might influence the result. Therefore, urine and serum were chosen as biological samples for testing of the method, as these two fluids requires less processing steps than the analyzing of intracellular metabolites. Four parallels were run and average and standard deviation is given in Table 2. The variation in the data originates with the derivatization and the GC/MS analysis. In general, the percentage standard deviation is below 10% in these real sample backgrounds. Out of the 67 metabolites included in the method, 47 metabolites were found and quantified in the urine sample and 32 in the serum sample. The concentrations found were in the expected order of magnitude for many high abundance metabolites, e.g., alanine concentration in serum was determined to 152 μM, while data in the literature ranged from 150 to almost 600 μM.[27−29] Clearly, serum and urine concentrations of metabolites vary a lot and, as expected, there is also a large abundance variation between various metabolites. The latter underlines the importance of a large dynamic range of the MS detector in addition to high sensitivity. By running these two biological fluids and several microbial extracts (data not shown), we did not experience for any metabolite a contaminant analyte that interfered with the analysis. However, since PCI retaining many large fragments are used, there are for most metabolites other potential precursor ions present at almost the same intensity as the ones chosen in this original setup. It was experienced that the dwell time should not be lower than 10 ms for the highest instrumental

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performance. Since GC/MS peaks are not wide, the cycle time should at least be 3 cycles per second to obtain a ideal integration and accurate quantification. This implies that only 15 metabolites (i.e., 30 MRM transitions each with 10 ms dwell time) should be included in each time segment of the method. This can be challenging with many coeluting metabolites, and our strategy based on the experience by developing this method is that each new extract should be preanalyzed with the global method, and MRM transitions of metabolites not detected for each particular extract (i.e., bacterial, fungi, human cells, and body fluids) should be removed from the MRM list enabling longer dwell time/shorter cycle times for the metabolites to be quantified. The standard errors for the urine and serum samples in Table 2 would probably be smaller if dedicated urine and serum MRM methods with fewer transitions were applied, respectively,

Potential of the New PCI GC/MS/MS Method. This GC/MS/MS method is a further development of the well recognized GC/MS scan method for analyzing amino acids and organic acids established by Villas-Boas and co-workers.[13,14] Through the usage of PCI it was possible to establish unique MRM transitions for both MCF-derivatives and d_3MCF-d_3MeOH-derivatives of all metabolites, also taking into consideration that many metabolites coelute due to the steep temperature gradient enabling analysis of 3 samples per hour. A less expensive alternative to deuterated MCF and methanol is ethyl chloroformate (ECF) and ethanol as this combination also allows derivatization and detection of amino and organic acids.[30] However, since the ECF derivative will become heavier, they will, to varying degrees, elute later than the MCF derivatives. However, even though not tested in our laboratory, ECF derivatized metabolites as internal standards should in principle be better than a few selected heavy labeled standards. In fact, if EI is the only choice for ionization, then retention time separation of the metabolite and internal standard might be preferable as the methyl formate group from MCF and the methyl group from methanol often are not retained in the main fragments in the EI mass spectra. This method is an alternative to use of ^{13}C-labeled metabolites prepared from extraction of the ^{13}C-carbon source grown biomass. Hence the advantage is that the expensive ^{13}C-carbon source is not needed and all metabolites in the MRM method will be present at the same high concentration while this might not be the situation for an internal standard mixture made from a biological extract, especially if a bacterial ^{13}C-labeled extract is used as an internal standard for mammalian extracts.

Metabolite profiling of known metabolites are definitely challenging from an analytical point of view, and several analytical methods must be applied for a comprehensive coverage of the main and most abundant metabolites in a biological extract. However, by operating a mass spectrometer in scan mode, we frequently observe large peaks in the chromatogram that cannot be assigned to a known metabolite. This is the situation both for GC and LC based chromatography and points to another major challenge in metabolome research, i.e., the identification of unknowns. The present study shows the strength of the MCF method as amino and carboxylic groups are differentially derivatized. This can be used to identify functional groups in unknown metabolites even on a unit mass resolution GC/MS quadrupole instrument. However, a high mass accuracy MS instrument in combination with subsequent filtering of brutto formulas to select the most likely and chemically correct molecular formulas would be more advantageous.[31]

The field of metabolomics still face major technical challenges, not only analytically as this newly developed GC/MS/MS method addresses but also at the critical steps of quick inactivation of the metabolism to preserve the intracellular state at time of sampling and the later sample processing, including metabolite extraction and concentration, which are also sources of errors. This method enables absolute quantification of amino and carboxylic group containing metabolites in any kind of prepared sample; hence, it is also an important contribution to the focus of interlaboratory comparisons and standardization of results within the metabolomics community.

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