Developing metabolomics for a systems biology approach to understand Parkinson's disease

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Chapter 2

LC-MS/MS analysis of the central energy and carbon metabolites in biological samples following derivatization by dimethylaminophenacyl bromide

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Journal of Chromatography A (2019)
Volume 1608, 20 December 2019, 460413

Abstract

Recent advances in metabolomics have enabled larger proportions of the human metabolome to be analyzed quantitatively. However, this usually requires the use of several chromatographic methods coupled to mass spectrometry to cover the wide range of polarity, acidity/basicity and concentration of metabolites. Chemical derivatization allows in principle a wide coverage in a single method, as it affects both the separation and the detection of metabolites: it increases retention, stabilizes the analytes and improves the sensitivity of the analytes. The majority of quantitative derivatization techniques for LC-MS in metabolomics react with amines, phenols and thiols; however, there are unfortunately very few methods that can target carboxylic acids at the same time, which contribute to a large proportion of the human metabolome. Here, we describe a derivatization technique which simultaneously labels carboxylic acids, thiols and amines using the reagent dimethylaminophenacyl bromide (DmPABr). We further improve the quantitation by employing isotope-coded derivatization (ICD), which uses internal standards derivatized with an isotopically-labelled reagent (DmPABr-D_6). We demonstrate the ability to measure and quantify 64 central carbon and energy-related metabolites including amino acids, N-acetylated amino acids, metabolites from the TCA cycle and pyruvate metabolism, acylcarnitines and medium-/long-chain fatty acids. To demonstrate the applicability of the analytical approach, we analyzed urine and SUIT-2 cells utilizing a 15-minute single UPLC-MS/MS method in positive ionization mode. SUIT-2 cells exposed to rotenone showed definitive changes in 28 out of the 64 metabolites, including metabolites from all 7 classes mentioned. By realizing the full potential of DmPABr to derivatize and quantify amines and thiols in addition to carboxylic acids, we extended the coverage of the metabolome, producing a strong platform that can be further applied to a variety of biological studies.

Background

Metabolomics, the younger sibling of genomics and proteomics, is a fast-evolving field which has established itself as a promising approach for understanding biological variations within a range of matrices in humans, animals, microbes and plants[1-8]. The quantitative profiling of metabolites in biological samples is challenging due to the vast number of metabolites, variation in physicochemical properties and the wide range of concentrations in samples. All of these factors result in large differences in the recovery, sensitivity and matrix interferences of these metabolites when analyzed by various methods. Nevertheless, recent advances in mass spectrometry have given scientists the ability to further understand the human metabolome and focus more closely on selected pathway analysis. When we study metabolic pathways, we experience the complexity as they can comprise of many chemical conversions and are intertwined, making a targeted assay with coverage of over 50 relevant metabolites highly beneficial for researchers in metabolism.

Mass spectrometry (MS) has the ability to identify and quantify the metabolome with current methods reaching sensitivities down to picomolar concentrations, even without any prior separation[9]. However, in the majority of cases, chromatography prior to MS is used to better address the challenges introduced by ion suppression, separation of isomers and in-source fragmentation. The three most common separation techniques, LC, GC and CE, have provided robust methodologies to better cover the human metabolome. Each of these techniques has been applied to numerous types of metabolites, and each technique has tailored advantages for specific types of metabolites. For example, UPLC-MS (RP & HILIC) provides coverage for a large proportion of the metabolome with the advantages of high-throughput, sensitivity, reliability and robustness[10]. Still, in LC-MS, metabolites can suffer from limited sensitivity, or poor separation of particularly polar metabolites. Quantification of metabolites with electrospray ionization (ESI)-MS can suffer from ion suppression[11]. This interference can be corrected for by using coeluting isotopically-labelled internal standards, which are of limited availability and excessive costs.

Methods have been developed to combat these problems using advanced separation techniques and also chemical derivatization, which is the focus of this article. Chemical derivatization can be used to increase the separation resolution, sensitivity or to stabilize the metabolites, resulting in an increased metabolic coverage of MS-based metabolomics methods. For instance, benzoyl chloride is used to derivatize catecholamines and their metabolites to prevent oxidation and increase sensitivity in LC-MS[12]. In a recent review, Higashi, Ogawa [13] summarise the current techniques that are used for derivatization and conclude that isotope-coded derivatization (ICD) has the ability to enhance quantification in LC-MS(/MS). ICD is the process of labelling metabolites in a first sample with an unlabelled derivatization reagent and then using an isotopically labelled reagent to derivatize the same metabolite standards in a neat solution, i.e. pure solvent. This mixture, when added to the sample, can act as the corresponding internal standard (IS) for all analytes of interest. The benefit of this technique is the ability to introduce an isotopically labelled equivalent for all metabolites regardless of chemical structure complexity, which corrects for eventual ion suppression. Approaches such as ICD are important during derivatization workflows to compensate for possible matrix effects as the native matrix is altered due to derivatization. However, having an IS for each metabolite provides a tool to adjust for matrix interferences independent of the starting matrices. ICD can provide a cost-effective alternative when stable isotope IS are not available while still enabling improved trueness and precision. In this way, the derivatization reaction method is exploited in an additional manner next to modifying the separation and ionization of metabolites.

Several studies have utilized a range of reagents, some taking advantage of the ICD strategy to improve the quantitative performance[12,14,15]. Typical examples are benzoyl chloride[12,14] and dansyl chloride[15] which both label amines, thiols, phenols and some alcohols. Another reagent, dimethylaminophenacyl bromide (DmPABr), has been applied previously to label carboxylic acid groups[16]. There were inconsistent reports about the reactivity of DmPABr. Guo, Li [16] reported that DmPABr reacts only with carboxylic acids (i.e., not amines and thiols), and in a follow-up study Peng and Li acknowledged that it reacts also with nucleophiles at certain reaction conditions[17]. However, to conform with the aims of their method, liquid-liquid extraction (LLE) was applied to reduce the interference from amino acids and

derivatives, by excluding them altogether. The need for a reliable method that combines labelling of the amine, thiol and carboxylic acid functional groups has been highlighted by previous papers that have required two separate derivatization methods (DmPABr and dansyl chloride) to achieve the same coverage[18].

In the current paper, we expand the utilization of the reagent DmPABr to simultaneously derivatize metabolites with carboxylic acid, amine and thiol functional groups. We did not apply LLE, and analyzed amino acids, N-acetylated amino acids, carnitines, and organic acids using LC-MS in positive ionization mode. We have examined and optimised the reaction conditions to reliably and repeatably derivatize a range of metabolites and analyze them in a single, highly sensitive quantitative method. The reaction mechanism is identical to that of the reagent phenacyl bromide with primary amines[19], secondary amines[20,21], thiols[22,23] and carboxylic acid-containing metabolites (derivatization example shown in Figure 2.1). First, we made adaptations to the method published by Guo, Li [16,]Peng, Li [17,]Stanislaus, Guo, Li [24] to improve the metabolite coverage to include a wide range of central carbon and energy-related metabolites. Then, we developed a targeted quantitative UPLC-MS/MS method to allow for the sensitive analysis of these metabolites in a single 10-minute analysis. The final applied method was successfully validated for linearity, precision, limits of detection (LOD) and quantification (LOQ). By applying this method to human urine and *in vitro* experiments using human pancreatic cancer cells (SUIT-2), we could confirm the broad applicability of this methodology and biological relevance for the scientific community.

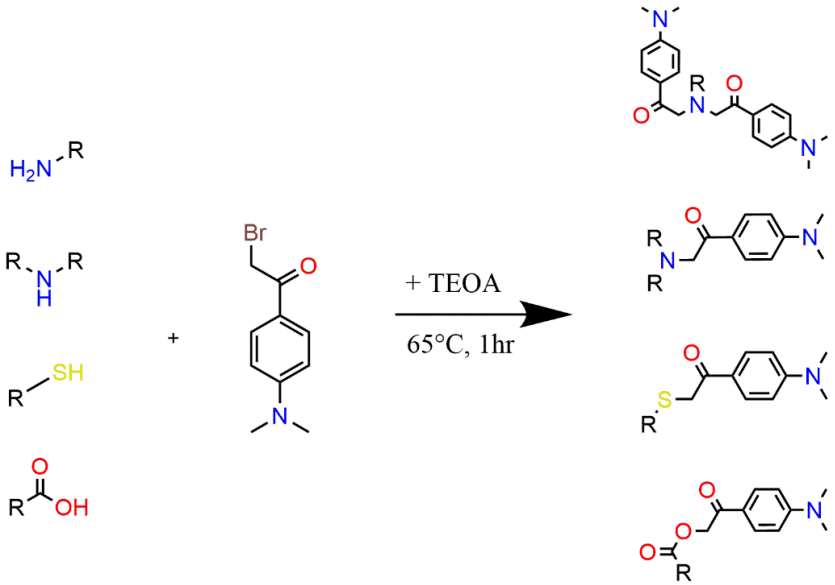

Fig 2.1. The derivatization reaction of DmPABr with the primary amine, secondary amine, thiol and carboxylic acid, respectively.

Materials and Methods

Chemicals

All of the chemicals were purchased from Sigma-Aldrich (St. Louis, USA) unless otherwise stated (abbreviations shown in Table 2.1). The LC-MS grade ACN was sourced from Actu-all Chemicals (Oss, The Netherlands) and de-ionized water was acquired using a Merck Milli-pore A10 purification system (Raleigh, USA). Stock solutions of 5 mg/mL Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val; 10mM NA-Ala, NA-Arg, NA-Asn, NA-Asp, NA-Cys, NA-Gln, NA-Glu, NA-Gly, NA-His, NA-Leu, NA-Lys, NA-Met, NA-Phe, NA-Pro, NA-Ser, NA-Thr, NA-Trp, NA-Tyr, NA-Val; 1 mg/mL AKG, CIT, FUM, ICIT, LAC, MAL, OXA, PYR, SUCC were made in 1:1 DMSO/DMF and stored at -80°C. Stock solutions of 2mg/mL CR; 1 mg/mL AA, AC, BTA, DEA, DDA, EA, EIA, FOR, OLA, OCA, PA, PPA, SA, UDA in 100% ACN (v/v) and stored at -80°C.

Metabolite	Abbreviation	Metabolite	Abbreviation
Alanine	Ala	N-acetylmethionine	NA-Met
Arginine	Arg	N-acetylphenylalanine	NA-Phe
Asparagine	Asn	N-acetylproline	NA-Pro
Aspartic acid	Asp	N-acetylserine	NA-Ser
Cysteine	Cys	N-acetylthreonine	NA-Thr
Glutamine	Gln	N-acetyltryptophan	NA-Trp
Glutamic acid	Glu	N-acetyltyrosine	NA-Tyr
Glycine	Gly	N-acetylvaline	NA-Val
Histidine	His	Alpha-Ketoglutaric acid	AKG
Isoleucine	Ile	Citric acids	CITS
Leucine	Leu	Fumaric acid	FUM
Lysine	Lys	Lactic acid	LAC
Methionine	Met	Malic acid	MAL
Phenylalanine	Phe	Oxaloacetic acid	OXA
Proline	Pro	Pyruvic acid	PYR
Serine	Ser	Succinic acid	SUCC
Threonine	Thr	Acetylcarnitine	AC
Tryptophan	Trp	Decanoylcarnitine	DC
Tyrosine	Tyr	Hexanoylcarnitine	HC
Valine	Val	Lauroylcarnitine	LC
N-acetylalanine	NA-Ala	Myristoylcarnitine	MC
N-acetylarginine	NA-Arg	Octanoylcarnitine	OC
N-acetylasparagine	NA-Asn	Palmitoylcarnitine	PC
N-acetylaspartic acid	NA-Asp	Propionylcarnitine	PPC
N-acetylcysteine	NA-Cys	Stearoylcarnitine	SC
N-acetylglutamine	NA-Gln	Arachidonic acid	AA
N-acetylglutamic acid	NA-Glu	Capric acid	DCA
N-acetylglycine	NA-Gly	Caprylic acid	OCA
N-acetylhistidine	NA-His	Dodecanoic acid	DDA
N-acetylisoleucine	NA-Ile	Oleic acid	OLA
N-acetylleucine	NA-Leu	Undecanoic acid	UDA
N-acetyllysine	NA-Lys	Creatinine	CR

Table 2.1. List of the abbreviations for the metabolites analyzed in this method

Derivatization reagent

The DmPABr reagent was purchased from BioConnect BV (Huissen, The Netherlands) and the internal standard DmPABr was synthesised following the published protocol by Guo, Li [16] using dimethyl sulphate-D_6 instead of dimethyl sulphate-$^{13}C_2$. The structure of the reagent was confirmed using nuclear magnetic resonance (NMR). Also, with reference to the paper from Guo, Li [16], it is noted that the stability of the metabolites after reaction with DmPABr lasts for up to 6 months in a solution, and does not alter quantitative results[24]. The DmPABr reagent was stored in ACN at -80°C to prevent the nucleophilic substitution reaction.

Method validation and biological application

Method optimization and validation

The following performance parameters were assessed on all 64 metabolites in triplicate. Method optimization started with the selection of an appropriate alkaline solution at a range of concentrations, comparing triethylamine (TEA at 0, 50, 100, 150, 250, 300, 500 and 750 mM) and triethanolamine (TEOA, at 0, 200, 400, 650, 700, 750, 800 and 1000 mM). The reaction time was assessed for the selected time points 0, 5, 15, 30, 45, 60, 90, 180 and 240 minutes using TEOA (750 mM) incubated for one hour at 65°C. Due to the ability of water to react with the reagent acting as a nucleophile the reaction was assessed in the presence of water at 0%, 20%, 40%, 60%, 80% and 100%. The final optimized method used 750 mM solution of TEOA for derivatization at 65°C for one hour in a shaking incubator. The method was characterised by a matrix-free 8-point calibration line, and by determining the carry-over by a solvent injection blank after injecting the highest calibration level (calibration point 7: supplementary material Table S6). The calibration experiment was replicated (n=5). Matrix effect (ME) is defined as "the direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample"[25]. The ME was calculated as the area of the internal standards in the neat solution against the area of the internal standard in the presence of the matrix. The method was also assessed for linearity of the calibration line (n=5) and LOD/LOQ. The LOD and LOQ were calculated using the following equations according to the ICH Q2R1 guidelines – σ being standard deviation of the signal in the blank injection:

$$LOD = (3.3 * \sigma) / slope$$
$$LOQ = (10 * \sigma) / slope$$

$$ME = (\text{Internal standard in neat solution} / \text{internal standard in matrix}) * 100$$

Urine validation samples

Urine from 10 healthy volunteers (aged 20-30) was collected and pooled and used for method optimization and validation. A volume of 10 µL of urine was transferred to an Eppendorf safe-lock vial (0.5 mL). The urine was dried in a Labconco SpeedVac (MO, United States). The dried content was reconstituted in 10 µL of DMSO/DMF to dissolve the remaining content. Then, 10 µL of triethanolamine (750 mM) was added to the vial, followed by 10 µL of DmPABr (82 mM). The sealed Eppendorf vial was placed into a shaking incubator for 60 minutes at 65°C to complete the derivatization. A total of 10 µL of formic acid (30 mg/mL) was added to the vial to quench the reaction with an additional 30 minutes in the shaking incubator. Then, 5 µL of DmPABr-D_6-labelled metabolites were then added (concentrations in supplementary material Table S6). Before vortexing, 45 µL of ACN was also added to the vial. The content was then transferred to an HPLC vial for analysis. The trueness and precision of the method was generated by using a pooled sample of urine collected from healthy urine donors. Samples were analyzed in repeated experiments on 3 separate days in replicates each day (n=5). Using this data, RSD calculations were performed to demonstrate the lack of variation in the derivatization conditions on separate days.

SUIT-2 oxidative stress analysis and validation

Human pancreatic cancer cells (SUIT-2) were cultured and placed into a 24-well plate, each containing $1*10^6$ cells in 0.4 mL of culture media. The SUIT-2 cells were exposed to 1 nM, 10 nM and 100 nM of rotenone for 3, 8 and 24 hours (n=3). The cells were washed with PBS and transferred into an Eppendrof tube. The aliquots were centrifuged at 500 rpm to sediment the cells and the cell media was removed. To the cells, cooled methanol (80% v/v) was added to the cells before probe sonification, followed by centrifugation at 13,000 rpm to produce a protein precipitation; the supernatant was transferred to an Eppendorf safe-lock vial (1.5 mL) without disturbing the pellet. A volume equivalent to $2.5*10^5$ cell supernatant was taken to total dryness in a speed vacuum concentrator. The following were added to the vial and vortexed between additions: 10 µL of DMSO/DMF (to first dissolve the dried

content), 10 µL of triethanolamine (750 mM) And 10 µL of DmPABr (82 mM). The sealed Eppendorf vial was placed into a shaking incubator for 60 minutes at 65°C to complete the derivatization. A volume of 10 µL of formic acid (30 mg/mL) was added to the vial to quench the reaction with an additional 30 minutes in the incubation. Finally, 5 µL of DmPABr-D_6-labelled metabolites were diluted in 45 µL of ACN and added to the vial. The content was then transferred to an HPLC vial for analysis.

LC-MS/MS analysis

Samples were analyzed by LC-MS using a Waters Acquity UPLC Class II (Milford, USA) coupled to an ABSciex QTrap 6500 series (Framingham, USA). The samples were run using scheduled multi-reaction monitoring (MRM) in positive mode with selected time windows. An injection of 1 µL was made per sample to minimise detector saturation and maintain desirable peak shape. The analytical column used was a Waters AccQ-tag C18 column (2.1mm x 100 mm, 1.8 µm, 180 Å), maintained at 60°C. Mobile phase solvent A was 0.1% v/v formic acid and 10 mM ammonium formate in water and mobile phase solvent B was 100% acetonitrile. Using the flow rate of 700 µL/min, the gradient profile is as follows: initial, 0.2% B; 1.5 min, 20% B; 4 min, 50% B; 6 min, 90% B; 10 min, 99.8% B; 13 min, 99.8% B; 13.1 min, 0.2% B and 15 min, 0.2% B. The last 6 minutes allow for column washing and equilibration prior to the next injection. The following parameters were used for the AB Sciex QTrap 6500 analysis (MRM transitions shown in Table S2 of the supplementary materials); electrospray ionization was used in positive mode at 4.5 kV. The gas temperature was 600°C. Automated peak integration was performed using AB Sciex MultiQuant Workstation Quantitative Analysis for QTrap; all peaks were visually inspected to ensure adequate integration.

Results & Discussion

Novel derivatization approach

DmPABr derivatization has been a successful method to support untargeted and targeted metabolomics platforms using ICD for carboxylic acid-containing metabolites. It was previously highlighted by Peng, Li [17] that DmPABr also reacts with the amine group of asparagine and labels it twice (once on the acid and once on the amine group), however the double labelled metabolite was reported to be the minor

peak compared to the single derivatized form. As demonstrated in this paper, DmPABr can react with an amine group (once or twice) via a nucleophilic reaction in a quantitative manner, which is useful for LC-MS analysis. In comparison to another common reagent in LC-MS/MS, benzoyl chloride[12,14], the reagent DmPABr offers a more versatile and universal solution for derivatization. This reaction, however, is slower and forms a more stable bond, as DmPABr has the ability to react next to the amine group. It also reacts with carboxylic acids without forming an unstable anhydride as the bromine is attached to a methyl group rather to the acyl group. Therefore, we studied the ability of DmPABr to react with multiple functional groups such as the amine, carboxy and thiol groups and to use it for the metabolomics analysis of urine and cell samples.

Selection of metabolites and biological relevance

Utilizing the full capability of DmPABr allows us to extend from only derivatizing carboxylic acids to also targeting amines and thiols which broadens the applicability of the method significantly. To demonstrate this, we chose to measure central carbon and energy-related metabolites related to mitochondrial dysfunction as it requires the analysis of a broad range of chemically diverse metabolites. The key metabolites in aerobic respiration that are imperative for mammalian survival, such as α-ketoglutaric acid, citrates, succinic acid, fumaric acid, malic acid and oxaloacetic acid, are frequently used references to determine changes in mitochondrial function and cellular health in metabolomics studies in urine, plasma and *in vitro* models[26]. N-acetylation has been known to increase during mitochondrial dysfunction due to the elevation of acetyl-CoA. Therefore, N-acetylated amino acids reflect mitochondrial dysfunction and energy metabolism, and in addition are highly relevant in urine as they remove excess amino acids from the body[27,28]. Shifting of the energy balance from aerobic respiration to anaerobic respiration can also be noted by measurement of pyruvic acid and lactic acid which will both drastically increase [27]. An additional target group, acylcarnitines, was also selected, as it reflects energy processes, particularly following beta-oxidation and when fatty acids are transported into the mitochondria[27,29]. The last target group, fatty acids, is included to represent an alternative energy source by the mitochondria when sugars are inaccessible or depleted. Three common conditions that are often associated with mitochondrial

dysfunction are Parkinson's disease[30], Leigh's syndrome[27] and diabetes[31]; all of which hold extensive interest within the scientific community.

Optimization of reaction conditions

We investigated and optimised the method to analyze amines, thiols and carboxylic acids to inform on central carbon and energy metabolism. We have found three key factors that affect the derivatization of the functional groups mentioned above: alkalinity of reaction solution, reaction time, and the presence of water during the reaction. Figure 2.2A demonstrates that after 60 minutes the relative peak area did not increase significantly anymore, with high performance parameters (indicated in Table 2.2). This applied to all of the targeted functional groups: Ala (1° amine & carboxylic acids), NA-Asp (carboxylic acids), NA-Cys (thiol & carboxylic acid), PYR (α-keto acid) and AC (carboxylic acid). This derivatization time was considered acceptable in terms of metabolic coverage. We have utilized similar inert base catalysts as in previous articles published for DmPABr that target the carboxylic acid function group, which utilized either 750 mM triethanolamine (TEOA) [16] or 200 mM triethylamine (TEA) [24]. Variations in response using these bases are depicted in Figures 2.2B and 2.2C, leading to the selection of the appropriate conditions for derivatization of amine and thiol groups. Additional experiments indicated that 750 mM of TEOA was the optimum condition for consistent derivatization of metabolites in urine and cells (data not shown). TEOA (750 mM) also provided the most consistent derivatization indicated by identical values over the concentration range of 650 – 800 mM. TEOA was preferred because according to literature it causes less ion suppression in mass spectrometers, than TEA at the concentration tested [32].

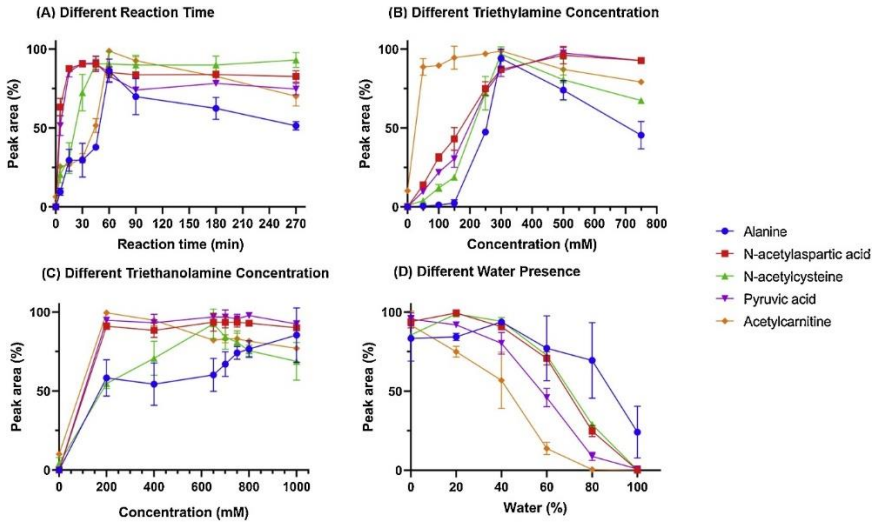

Figure 2.2. DmPABr reaction optimization shown for 5 metabolites (Ala – Blue; NA-Asp – Red; NA-Cys – Green; PYR – Purple; AC – Orange; n=3 per condition) representing the major classes selected in the method. (A) the effect of reaction time with 750 mM triethanolamine; (B) use of TEA as base; (C) use of TEOA as base; and (D) the reaction efficiency in the presence of water with 750mM triethanolamine. The data is presented as peak area normalized to the highest peak area.

Analyte	LODs (nM)	LOQs (nM)	Fit (R^2)	RSD (%)	Carryover (%)	Analyte	LODs (nM)	LOQs (nM)	Fit (R^2)	RSD (%)	Carryover (%)
Ala	44.8	134	0.9938	7.7	0.01	**NA-Met**	0.11	0.33	0.9867	4.4	0.003
Arg	10.3	30.8	0.9944	6	0.004	**NA-Phe**	0.27	0.82	0.9965	4.8	0.003
Asn	4.01	12	0.9965	3.5	0.01	**NA-Pro**	15.6	46.8	0.9969	12.5	0.05
Asp	11.4	34	0.9915	9.9	0.02	**NA-Ser**	5.55	16.6	0.9919	9.6	0.01
Cys	49.5	148.6	0.9834	6	0.01	**NA-Thr**	10.4	31.3	0.9987	3.7	0.01
Gln	18.5	55.5	0.9913	12.3	0.02	**NA-Trp**	2	6	0.9965	1.1	0.003
Glu	43.5	130.5	0.9941	2.1	0.01	**NA-Tyr**	0.12	0.37	0.9901	11.5	0.01
Gly	932.4	2797	0.9952	1.8	0.32	**NA-Val**	0.52	1.56	0.9932	13	0.002
His	379.4	1138	0.9956	4.1	0.01	**AKG**	29.7	89.1	0.9909	6.2	0.08
Ile	9.28	27.9	0.9956	7.9	0.01	**CITS**	1001	3003	0.9988	1.7	0.12
Leu	11.3	33.9	0.9942	7.5	0.01	**FUM**	69.5	208.5	0.9949	3.9	0.03
Lys	142.8	428.3	0.9959	6.3	0.02	**LAC**	2192	6578	0.9927	4	0.01
Met	4.33	13	0.9903	0.6	0.01	**MAL**	43.8	131.5	0.9979	1.6	0.01
Phe	25.6	76.9	0.9997	10.2	0.02	**OXA**	14	42	0.9950	1.7	0.02
Pro	22.1	66.4	0.9870	3.6	0.003	**PYR**	55.8	167.3	0.9926	2.4	0.004
Ser	506	1518	0.9917	3.3	0.01	**SUCC**	23.1	69.4	0.9997	3.8	0.02
Thr	47.6	142.8	0.9950	2.9	0.01	**AC**	0.36	1.09	0.9951	12.8	0.0001
Trp	5.33	16	0.9978	9	0.02	**DC**	0.3	0.91	0.9964	8	0.01
Tyr	6.87	20.6	0.9966	7	0.02	**HC**	0.61	1.82	0.9903	10.2	0.001
Val	11.7	35.6	0.9939	10.5	0.01	**LC**	0.9	2.7	0.9988	11.3	0.004
NA-Ala	4.21	12.6	0.9941	4.3	0.002	**MC**	0.5	1.5	0.9996	8.7	0.01
NA-Arg	0.16	0.47	0.9989	9.1	0.001	**OC**	1.32	3.98	0.9998	5.5	0.01
NA-Asn	0.27	0.81	0.9882	11	0.02	**PC**	0.91	2.72	0.9970	7.1	0.02
NA-Asp	4.32	13	0.9942	3	0.002	**PPC**	2.85	8.56	0.9985	8.5	0.0001
NA-Cys	34.5	103.4	0.9975	4.6	0.06	**SC**	0.25	0.75	0.9993	8.4	0.03
NA-Gln	0.94	2.83	0.9979	6.7	0.001	**AA**	4.45	13.4	0.9995	1	0.04
NA-Glu	3.65	10.9	0.9979	1.5	0.02	**OCA**	137	411	0.9992	4.2	0.02
NA-Gly	3.32	9.97	0.9971	4.5	0.001	**DCA**	104	314	0.9860	2.6	0.01
NA-His	0.16	0.48	0.9917	3.7	0.001	**DDA**	15.3	46	0.9912	4.5	0.01
NA-Ile	27.7	83	0.9961	1.1	0.001	**OLA**	185	555	0.9960	4.4	0.02
NA-Leu	30.4	91.2	0.9946	1.5	0.001	**UDA**	12.9	38.7	0.9985	4.2	0.008
NA-Lys	72.6	217	0.9912	4.9	0.028	**CR**	548.2	1644	0.9943	1.6	0.001

Table 2.2. Information relating to the carry-over, limit of detection (LOD), limit of quantification (LOQ) and linearity of an 8-point calibration line in aqueous solution. RSD was calculated using calibration point 4 (concentration shown in supplementary Table S2).

Of the options for synthesized isotopically labelled DmPABr, D_6 was used in place of $^{13}C_2$ on the amine residue of DmPABr, as utilized for high-resolution MS by Guo, Li [16]. With this, we have been able to introduce a mass difference of 6 Da, which is preferable for low-resolution MS compared to the previous addition of 2 Da, and less costly. The mass difference of 2 Da with the internal standard DmPABr-$^{13}C_2$ provided cross-talk interference in the triple-quadrupole MS with the metabolites labelled once, such as long-chain fatty acids and N-acetylated amino acids. However, this was not as detrimental to the metabolites labelled more than once (such as amino acids) as a mass difference gain of 18 Da was observed for metabolites such as Ala (labelled thrice) when using DmPABr-D_6.

Other adaptations of the derivatization procedure were also evaluated, including the total elimination of the aqueous content prior to derivatization with DmPABr to improve reaction efficiency and decrease reaction variability. This was expected to be needed to create a quantitative method, unlike the previous published method that focused on identification. This variability and poor labelling efficiency may arise from the ability of water to act as a nucleophile under basic conditions (deprotonation). It has previously been noted that as little as 5% water content during the derivatization reaction has the ability to reduce the labelling efficiency by hydrolysing DmPABr [23]. It was also reported that the presence of water competes with the metabolites to react with DmPABr as a nucleophile and thus hydrolysing the reagent [33]. We investigated whether a low percentage of water would interfere with the labelling of the amine group. Figure 2.2D shows that the presence of 40% of water did not significantly change the derivatization efficiency. This may be explained by Stanislaus, Guo, Li [24], where the increase in DmPABr concentration from 20 mg/ml[16] to 40 mg/ml (excess reagent) resulted in 2-fold higher rate of the Sn2 reaction, ensuring completion of the reaction. The presence of water above 20% showed a decline in derivatization efficiency and in the presence of 100% water, the reaction is severely compromised. Therefore, we chose to conduct the reaction without the

presence of water to avoid the complications due to possible hydrolysis of the reagent.

Targeted LC-MS/MS method

The aims of the chromatographic method were to combine high-throughput analysis with sensitive measurement of a wide range of chemical classes. After derivatization with DmPABr, polar compounds which are hardly retained in RP could be retained and separated, hence eliminating the need for HILIC separation. Moreover, derivatization with DmPABr allows sensitive and universal analysis in positive ionization mode, instead of in two ionization modes. By using DmPABr, we introduce the tertiary amine group (Figure 2.3 that improves ionization hence enhances signals. Further improvement in intensity of measured metabolites can be gained by careful selection of MRMs. In Figure 2.4 A and B we illustrate that the metabolites that are labelled on the carboxylic acid show common and prominent fragments of 180.0 Da or 134.1 Da. These product fragments are ideal when measuring metabolites such as those involved in the TCA cycle as they lack nitrogen and are difficult to analyze in positive ionization mode without labelling. For metabolites which are labelled multiple times, such as amino acids, the fragments 180.0 Da and 134.1 Da are usually present (Fig. 2.4B) but are not selected as their signal is lower. Instead, a higher mass product ion giving a better signal is often seen. This results from derivatization twice on the amine group, and once on the acid moiety, yielding more sensitive fragments like 319.2 Da and 366.2 Da observed for arginine & alanine in Figure 2.4 C and 2.4 D, respectively. Having a common fragmentation pattern reduces the specificity of metabolite species but with adequate chromatography, this issue can be negated. Additionally, a qualifier transition can also be set which will provide a unique fragmentation pattern to identify the specific metabolite but this will provide a lower sensitivity due to more MS/MS events. For the complete method, the labelling pattern and the chosen MRM transitions are detailed in supplementary Table S2.

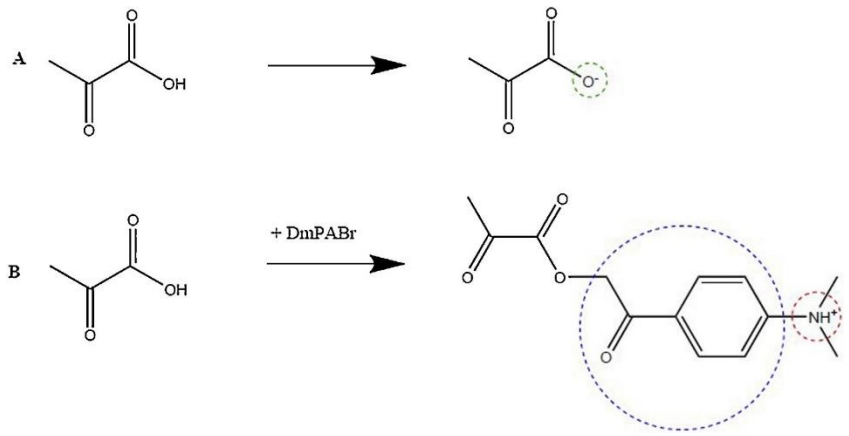

Figure 2.3. Demonstration of MS analysis of pyruvic acid: (A) in conventional negative ionization mode, holding a negative charge on the oxygen (green circle); (B) following DmPA labelling, producing a highly ionisable group (tertiary amine – red circle) and a higher retention group (blue circle).

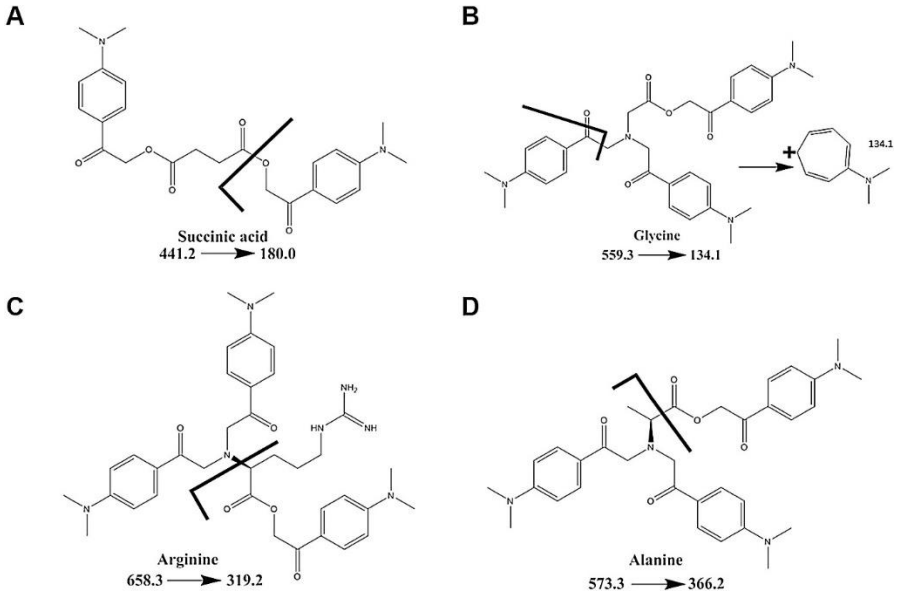

Figure 2.4. The common fragment formation of 180.0 Da & 134.1 Da: the product ion of the derivatization label and metabolite-specific product ions.

To demonstrate the applicability of the method on biological samples, SUIT-2 cell extracts were subjected to derivatization and analysis, resulting in wide representation of various chemical classes (Figure 2.5). The figure demonstrates that owing to strong retention of polar analytes (PYR, Gly) all 64 metabolites are detected in one run in positive ionization mode only within 8.4 min (latest elution, of arachidonic acid, AA). The derivatization leads to unique retention profile, such as the close elution between undecanoic acid (derivatized once) and leucine (derivatized three times), yet with baseline resolution between the isomers Ile and Leu. Another critical pair of isomers, CIT and ICIT present a common challenge in chromatography, and are not baseline resolved here (see Fig.2.5), therefore are reported as total citrates (CITS). In contrast, good separation was observed for N-acetylated amino acids, many of which elute early. The first peak to elute was creatinine (Cr) which is often used to normalize and report metabolites in urine[34]. DmPABr can successfully derivatize creatinine, unlike the reagents utilized in commercially-available kits, that quantify non-derivatized creatinine (Biocrates AbsoluteIDQ® p180 Kit; Waters AccQ-Tag™).

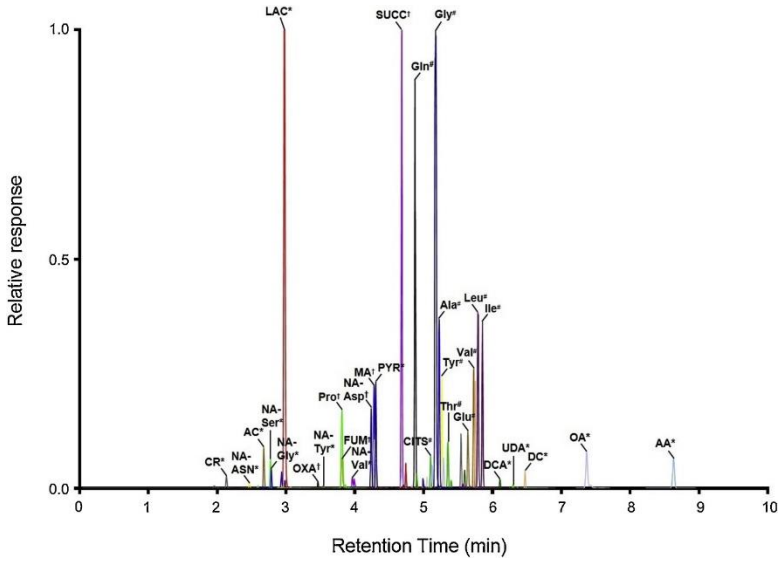

Figure 2.5. LC-MS/MS analysis of 64 metabolites after DmPABr derivatization in SUIT-2 cells. DmPA labelling pattern is also included (= labelled once; † = labelled twice; # = labelled thrice)*

Method performance in neat solutions

The methods performance incorporates the derivatization efficiency and the instrumental response. The method was validated for 64 metabolites that were deemed to be biologically relevant to assess the central energy and carbon metabolism. Using the ICD strategy, each metabolite had its corresponding DmPABr-D_6 internal standard to correct for ion suppression. This resulted in linear calibration lines for all metabolites in neat solutions (Table 2.2). All metabolites, including the amino acids which are derivatized by reacting 2-5 times with DmPABr showed a satisfactory linear calibration ($R^2 > 0.99$) except for Cys ($R^2 = 0.98$) and Pro ($R^2 = 0.98$). The RSD for all metabolites in neat solution was recorded below 12.8% also shown in Table 2.2.

N-acetylated amino acids also showed good analytical performance similar to their free amino acid counterparts. Table 2.2 also shows that the carry-over of the method was negligible (<0.05%). Looking at limits of detection (which are affected by the derivatization process itself), N-acetylated amino acids have a very low LOD, as recorded for NA-Asp (4 nM), NA-Cys (34 nM) and NA-Phe (0.3 nM), which are sufficient for their analysis in urine and cells. N-acetylated amino acids are used as the transport mechanism to excrete excess amino acids (particularly in the urine) that occur in relatively low concentrations when compared to free amino acids in urine [28,35]. We have circumvented the issues of limited dynamic range of the detector in order to allow good quantitation of a wide concentration range of metabolites. As shown in Table 2.2, the LOD of Gly, His, Ser, CITS and LAC, were higher compared to the other metabolites in this method. This is due to intentional choice of less sensitive MRM channel, to reduce the signal and prevent detector saturation, counteracting the high physiological concentrations in urine or cells. Another intervention to prevent detector saturation took place, namely non-optimal ionization spray voltage throughout (4.5 kV vs. the optimal 5.5 kV). The application of this method to various matrices could benefit from tailoring the MS parameters as well as sample handling to improve the LOD.

Method performance in urine and cells

We applied the quantitative DmPABr method to urine and SUIT-2 cells (Tables 2.3-2.4). Table 2.3 shows the endogenous concentration of the metabolites measured in

urine from healthy males, after normalization to creatinine (measured in the same method). A total of 57 compounds were detected and quantified in urine. The compounds that were not detected include some carnitines and medium chain fatty acids as they do not occur or occur in low concentrations in healthy urine. All of the amino acids and N-acetylated amino acids that have been studied fall within the expected concentrations curated in HMDB[3]. Urine was assessed for intra-day and inter-day variability. Amino acids such as Ala, Ile, Trp had very low intra- and inter-day variability (all below 10%) and N-acetylated amino acids including NA-Asp, which is crucial for neurological studies, had an intra-day and inter-day variability of 2.2% and 5.4%, respectively. Creatinine had an intra-day and inter-day variability of 4.5% and 7.4%, respectively, which provides consistent normalization factor, if desired. Overall, the amino acids had a higher derivatization variability than other classes, which is probably due to the second step of derivatization on the 2° amine requiring more energy, compared to the single reaction with the carboxylic acid group.

Analyte	Urine Concentration (µmol/mmol creatinine)	Intraday precision (%)	Interday precision (%)	Matrix effect (%)	Analyte	Urine Concentration (µmol/mmol creatinine)	Intra-Day precision (%)	Inter-Day precision (%)	Matrix effect (%)
Ala	3.81	4.7	5.1	92.3	NA-Met	0.03	2.3	4	37.7
Arg	0.32	5.2	9.5	68.45	NA-Phe	0.05	3.1	4.2	51.7
Asn	1.11	4.9	7	74	NA-Pro	1.31	29.3	23.5	31
Asp	0.04	18.9	16.5	95	NA-Ser	0.25	2.4	4.1	66.5
Cys	11.5	12.9	21	91.2	NA-Thr	1.7	3.2	3.6	53.9
Gln	3.67	22.8	18.5	83.5	NA-Trp	0.2	3.6	3.7	45.5
Glu	0.12	5.5	6.4	81	NA-Tyr	0.047	5	9.8	36.8
Gly	136	1.4	4.2	67	NA-Val	2.07	3.7	13.2	16.6
His	76.3	5.2	5.7	42.7	AKG	0.5	10.6	12.8	71.3
Ile	0.23	6.7	7.7	80.3	CITS	7.47	13.1	11.5	73.9
Leu	0.52	6.4	7	85	FUM	0.13	6.7	7.7	144.4
Lys	1.22	23.1	28	42.2	LAC	0.89	3.3	8.9	43.5
Met	0.11	7.7	11.9	78.2	MAL	0.13	7.1	7.4	42.8
Phe	1.35	4.1	9.2	80.2	OXA	0.3	3.9	6.6	57.2
Pro	0.11	6.4	6.7	129.5	PYR	0.17	11.5	10.2	50.3
Ser	4.43	7.3	15.5	72.1	SUCC	5.31	2.7	3.4	39.4
Thr	1.21	6.3	16.1	70.2	AC	1.93	4.2	5	83.38
Trp	1.27	7.7	7.6	77.3	DC	0.001	9.5	7.8	93.1
Tyr	1.33	5.4	6.8	85	HC	ND	N/A	N/A	77.9
Val	0.62	5	7.3	78.9	LC	ND	N/A	N/A	83.6
NA-Ala	0.54	3.6	6.7	43.4	MC	ND	N/A	N/A	91.9
NA-Arg	0.67	1.3	1.5	67.5	OC	0.007	5.3	5	58.2
NA-Asn	2.01	7.7	7.4	54.8	PC	ND	N/A	N/A	96.3
NA-Asp	2.29	2.2	5.4	55.1	PPC	0.07	2.2	2.8	88.7
NA-Cys	1.45	4.1	10.4	27.4	SC	ND	N/A	N/A	95.4
NA-Gln	1.26	5.5	6.4	34.7	AA	0.02	9.4	13.5	103.6
NA-Glu	0.67	3.6	3	62.3	OCA	ND	N/A	N/A	66.7
NA-Gly	0.13	3.6	7.1	33.3	DCA	ND	N/A	N/A	90.7
NA-His	1.28	3	4.8	63.9	DDA	0.008	10.9	24.1	83.2
NA-Ile	0.14	11.5	10.3	46.7	OLA	0.006	11.2	16.3	95.8
NA-Leu	0.13	4.9	6.4	47.6	UDA	0.004	21.4	39.1	98.3
NA-Lys	0.3	24	22	60	CR*	N/A	4.5	7.4	38.1

** Creatinine was used for normalization*

Table 2.3. Method performance in urine of healthy men aged 18-30 to calculate concentration, intraday and interday precision calculated as %RSD. ND = Not Detected, N/A= Not Applicable

The application of the method to cells was conducted by measuring untreated SUIT-2 cells. The cells were assessed for intra-day variability and not inter-day due to practical considerations (Table 2.4). All 64 metabolites were detected from the intracellular environment in cell lysate. This provided an excellent readout on the energy state of the cells using metabolites involved in the TCA cycle (i.e., CITS, FUM & SUCC) and glycolysis (PYR & LAC).

Analyte	SUIT-2 Conc. (fmol/mg)	Intra-Day precision (%)	Matrix effect (%)	Analyte	SUIT-2 Conc. (fmol/mg)	Intra-Day precision (%)	Matrix effect (%)
Ala	408	4.9	121.1	NA-Met	0.022	1.8	77.6
Arg	243	10.3	75.8	NA-Phe	0.063	10	78.3
Asn	173	6.7	40.7	NA-Pro	0.111	9.6	78.6
Asp	56.8	5.7	75.1	NA-Ser	13.1	7.2	73.8
Cys	166	30.8	98.6	NA-Thr	5.66	5.7	74.2
Gln	2360	10.1	75.3	NA-Trp	0.034	10.8	52.7
Glu	474	16.8	83.3	NA-Tyr	0.126	6.5	87
Gly	2970	3.4	87.4	NA-Val	0.034	11.7	86.3
His	467	3.7	62.5	AKG	7.75	10	93.7
Ile	277	8.7	90.7	CITS	214	9.8	97
Leu	333	9.7	72.1	FUM	245	12.7	131.8
Lys	130	28.5	78.4	LAC	846	2.4	72.5
Met	127	18.7	87.9	MAL	114	3.8	75.1
Phe	309	9	70.2	OXA	18.2	13.6	75.4
Pro	206	10.3	114.5	PYR	191	1.4	78.1
Ser	270	24.9	105.1	SUCC	249	13.8	60.6
Thr	401	31.1	74.1	AC	12.1	13.8	86.4
Trp	59.7	10.7	97.8	DC	0.006	23.3	109.5
Tyr	284	7.7	66	HC	0.033	18.6	81.5
Val	307	10.8	76.4	LC	0.01	9.7	85.6
NA-Ala	3.05	3.7	82.1	MC	0.099	8	94.1
NA-Arg	0.699	8.6	74.2	OC	0.011	19.9	68
NA-Asn	159	9.8	64.8	PC	0.195	11.9	98.4

Analyte	SUIT-2 Conc. (fmol/mg)	Intra-Day precision (%)	Matrix effect (%)	Analyte	SUIT-2 Conc. (fmol/mg)	Intra-Day precision (%)	Matrix effect (%)
NA-Asp	56.6	2	76.7	PPC	0.379	7.4	91.2
NA-Cys	50.5	12	54.4	SC	0.067	9.4	96.5
NA-Gln	8.55	8.6	81.1	AA	0.457	19.5	98.2
NA-Glu	0.964	2.1	71	OCA	2.57	24.8	67
NA-Gly	3.75	5.6	53.3	DCA	1.16	27.3	93.7
NA-His	0.318	19.6	81.1	DDA	1.64	13	83
NA-Ile	0.126	8	56.1	OLA	6.84	14.7	88.1
NA-Leu	0.217	14	55	UDA	0.669	25.6	88.9
NA-Lys	0.045	31.5	84.2	CR	305	11.6	81.7

Table 2.4. Method performance in SUIT-2 cell to calculate concentration per mg and intra-day variability

Matrix effect was calculated for both urine and cell samples. The matrix effect was significant during the early eluting peaks such as the N-acetylated amino acids in urine. However, the matrix interferences were not as high during the analysis of cells. The presence of a matrix effect shows the importance of using the ICD technique to provide an internal standard for all metabolites.

Method application in cells to reflect energy metabolism

Exposing cells to rotenone is a well-established method to induce mitochondrial dysfunction by blocking complex I of the electron transport chain. This allows us to simulate disorders such as Parkinson's disease and Leigh's syndrome. Application of the method to SUIT-2 cells exposed to 100 nM rotenone showed the most changes in the central metabolism when compared to 1 nM and 10 nM (data shown in supplementary Figures S4 & S5 for 8 and 3 hour rotenone exposure). Out of the 64 metabolites, 28 showed significant changes including metabolites from all of the 7 classes (additional data shown in supplementary Table S3). The top ten most distinguishing metabolites were: Asn ($p = 0.0001$); AKG ($p = 0.0001$); Pro ($p = 0.0004$); CITS ($p = 0.0004$); PYR ($p = 0.0004$); OXA ($p = 0.001$); FUM ($p= 0.002$); AC ($p = 0.002$); SUCC ($p = 0.002$) and MC ($p = 0.003$) as shown in Figure 2.6. The two metabolites with the largest fold change, CITS and AKG, coincide with the shutdown of the TCA cycle by rotenone inhibition of complex I of the electron transport chain [36]. The same reduction was seen in SUCC and FUM but to a lesser extent. Interestingly, we also identified changes in the N-acetylated amino acids such as NA-Glu ($p = 0.01$), NA-Ala ($p = 0.003$). N-acetylation of amino acids has been documented in mitochondrial dysfunction but has not been extensively studied due to difficulty with analysis[27,37,38]. The results obtained with our novel method demonstrate its potential in studying the role of central carbon and energy metabolism such as mitochondrial dysfunction and Parkinson's disease[39].

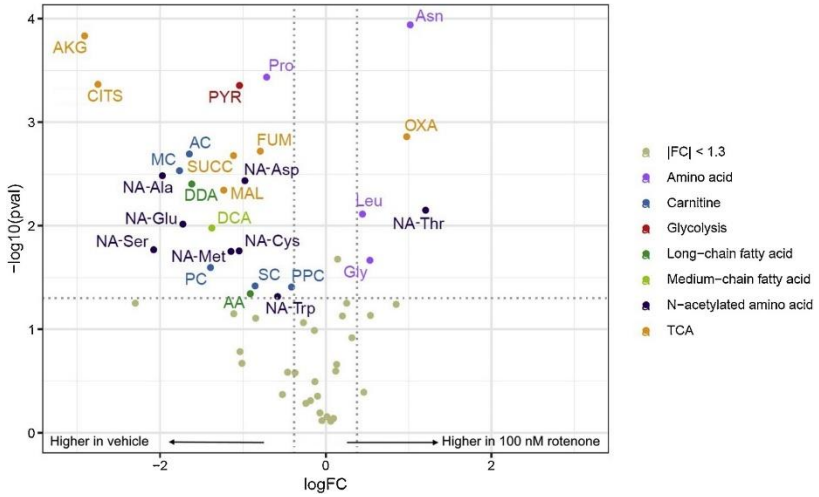

Figure 2.6. Volcano plot of SUIT-2 cells exposed to 100 nM rotenone for 24 hours vs control. All of the metabolites involved in the method show biological changes across all classes once treated with rotenone (amino acids – light purple; carnitines – blue; glycolysis – red; long-chain fatty acids – dark green; medium-chain fatty acid – light green; N-acetylated amino acids – dark purple; and TCA metabolites – orange).

Conclusion

The presented work expands the metabolite coverage of DmPABr by implementation of changes to the reaction conditions. Actually, the derivatization of several functional groups including carboxylic acids, primary amines, secondary amines and thiol groups was achieved in a consistent and robust way for the first time using DmPABr. This vastly improves the coverage of the method allowing for a higher proportion of the human metabolome to be targeted. We have demonstrated that using DmPABr derivatization to its full ability allows us to create a single RPLC-MS/MS analysis within 10 min acquisition time using only positive ionization mode. Since we used a targeted metabolomics method employhing internal standards which were derivatized with stable isotope-labelled reagent, we can report each metabolite reliably with its absolute concentration. The great versatility of this approach was demonstrated by quantification of urine metabolites (normalized to DmPABr-derivatized creatinine). Applying the method to SUIT-2 cells exposed to

rotenone showed significant changes in almost 50% of the metabolites covered in this method, including common TCA and glycolysis metabolites and not-so-commonly studied N-acetylated amino acids. Understanding and documenting these biological and biochemical changes in the brain could prove invaluable for future research into neurodegenerative diseases, and requires investigation with a precise and robust quantitative analytical approach. A computational approach towards the prediction of derivatization of metabolites, and the prediction of retention for new metabolites, will further support the method application to cover a wider range of metabolites in complex matrices.

Acknowledgements

The author expresses thanks to: Jaco van Veldhoven for support during the synthesis of DmPABr-D_6; Alisa L. Willacey for advice and guidance during the finalization of the study and Alida Kindt for statistical support. This project was supported by the SysMedPD project, which has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement no, 668738.

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Supplementary information

Table S1: List of abbreviations

Metabolite	Abbreviation	Metabolite	Abbreviation
Alanine	Ala	N-acetylmethionine	NA-Met
Arginine	Arg	N-acetylphenylalanine	NA-Phe
Asparagine	Asn	N-acetylproline	NA-Pro
Aspartic acid	Asp	N-acetylserine	NA-Ser
Cysteine	Cys	N-acetylthreonine	NA-Thr
Glutamine	Gln	N-acetyltryptophan	NA-Trp
Glutamic acid	Glu	N-acetyltyrosine	NA-Tyr
Glycine	Gly	N-acetylvaline	NA-Val
Histidine	His	Alpha-Ketoglutaric acid	AKG
Isoleucine	Ile	Citric acids	CITS
Leucine	Leu	Fumaric acid	FUM
Lysine	Lys	Lactic acid	LAC
Methionine	Met	Malic acid	MAL
Phenylalanine	Phe	Oxaloacetic acid	OXA
Proline	Pro	Pyruvic acid	PYR
Serine	Ser	Succinic acid	SUCC
Threonine	Thr	Acetylcarnitine	AC
Tryptophan	Trp	Decanoylcarnitine	DC
Tyrosine	Tyr	Hexanoylcarnitine	HC
Valine	Val	Lauroylcarnitine	LC
N-acetylalanine	NA-Ala	Myristoylcarnitine	MC
N-acetylarginine	NA-Arg	Octanoylcarnitine	OC
N-acetylasparagine	NA-Asn	Palmitoylcarnitine	PC
N-acetylaspartic acid	NA-Asp	Propionylcarnitine	PPC
N-acetylcysteine	NA-Cys	Stearoylcarnitine	SC
N-acetylglutamine	NA-Gln	Arachidic acid	AA
N-acetylglutamic acid	NA-Glu	Capric acid	DCA
N-acetylglycine	NA-Gly	Caprylic acid	OCA
N-acetylhistidine	NA-His	Dodecanoic acid	DDA
N-acetylisoleucine	NA-Ile	Oleic acid	OLA
N-acetylleucine	NA-Leu	Undecanoic acid	UDA
N-acetyllysine	NA-Lys	Creatinine	CR

Table S2. Target analyte list with MRM parameters for the AB Sciex Qtrap 6500 and labelling pattern including the number of DmPA-tags per class.

Metabolites	Rentention time (mins)	Precusor (m/z)	Product (m/z)	Collision Energy (eV)	Labelling pattern			
					1° Amine	2° Amine	Thiol	Acid
CR	2.12	275.1	134.1	40	1	0	0	0
NA-Arg	2.35	378.2	180	30	0	0	0	1
NA-Asn	2.46	336.2	134.1	25	0	0	0	1
NA-Gln	2.54	350.2	134.1	25	0	0	0	1
NA-Ser	2.62	309.1	180	20	0	0	0	1
AC	2.64	365.1	134.1	35	0	0	0	1
NA-Gly	2.74	279.1	180	15	0	0	0	1
NA-Thr	2.82	323.2	180	20	0	0	0	1
PPC	2.94	379.2	134.1	35	0	0	0	1
NA-Ala	2.99	293.1	180	15	0	0	0	1
LAC	2.99	252.0	180	5	0	0	0	1
NA-Lys	3.3	511.2	134.1	25	2	0	0	1
NA-Pro	3.35	319.2	70	30	0	0	0	1
NA-Tyr	3.41	385.2	180	15	0	0	0	1
NA-His	3.42	520.2	271.2	45	0	0	0	1
OXA	3.46	455.0	134	35	0	0	0	2
NA-Met	3.65	353.3	180	15	0	0	0	1
NA-Val	3.68	321.2	180	15	0	0	0	1
Pro	3.8	438.1	289.1	25	0	1	0	1
FUM	3.8	439.1	134	30	0	0	0	2
HC	3.95	421.4	140.1	35	0	0	0	1
NA-Leu	4.04	335.2	180	15	0	0	0	1
NA-Trp	4.07	408.3	180	15	0	0	0	1

Metabolites	Rentention time (mins)	Precusor (m/z)	Product (m/z)	Collision Energy (eV)	Labelling pattern			
					1° Amine	2° Amine	Thiol	Acid
NA-Ile	4.08	335.2	180	15	0	0	0	1
NA-Phe	4.12	369.2	180	15	0	0	0	1
NA-Asp	4.23	498.1	180	30	0	0	0	2
PYR	4.28	250.1	180	15	0	0	0	1
MAL	4.31	457.1	134.1	35	0	0	0	2
NA-Cys	4.34	486.2	134.1	35	0	0	1	1
NA-Glu	4.35	512.2	134.1	35	0	0	0	2
His	4.38	639.2	244.2	15	2	0	0	1
OC	4.6	449.1	134.1	35	0	0	0	1
SUCC	4.68	441.1	134	30	0	0	0	2
Arg	4.71	658.2	319.2	35	2	0	0	1
Gln	4.91	630.2	340.2	30	2	0	0	1
Asn	4.94	616.2	339.2	30	2	0	0	1
AKG	5.1	469.2	180	30	0	0	0	2
CITS	5.1	676.1	180	10	0	0	0	3
DC	5.11	477.5	140.1	35	0	0	0	1
Ser	5.15	589.2	408.2	10	2	0	0	1
Gly	5.2	559.2	134.1	10	2	0	0	1
Ala	5.23	573.2	366.2	20	2	0	0	1
Tyr	5.28	665.2	458.2	30	2	0	0	1
Thr	5.31	603.2	422.2	25	2	0	0	1
Cys	5.4	766.2	470.2	25	2	0	1	1
LC	5.54	505.5	140.1	35	0	0	0	1
Lys	5.56	476.6	134.1	30	4	0	0	1
Trp	5.56	688.2	340.2	30	2	0	0	1
Asp	5.6	778.2	392.2	30	2	0	0	2
Met	5.62	633.2	426.2	25	2	0	0	1

Metabolites	Rentention time (mins)	Precusor (m/z)	Product (m/z)	Collision Energy (eV)	Labelling pattern			
					1° Amine	2° Amine	Thiol	Acid
OCA	5.65	306.2	180	20	0	0	0	1
Glu	5.68	792.2	585.2	35	2	0	0	2
Val	5.75	601.2	394.2	30	2	0	0	1
Phe	5.77	649.2	442.2	30	2	0	0	1
Leu	5.83	615.2	408.2	30	2	0	0	1
Ile	5.87	615.2	408.2	30	2	0	0	1
MC	5.92	533.6	140.1	35	0	0	0	1
DCA	6.08	334.3	180	20	0	0	0	1
PC	6.26	561.6	140.1	35	0	0	0	1
UDA	6.27	348.3	180	20	0	0	0	1
DDA	6.44	362.3	180	20	0	0	0	1
SC	6.55	589.6	140.1	35	0	0	0	1
OLA	7.34	444.5	180	20	0	0	0	1
AA	8.58	474.5	180	20	0	0	0	1
CR-IS	2.11	281.1	140.1	40	1	0	0	0
NA-Arg-IS	2.35	384.2	186	30	0	0	0	1
NA-Asn-IS	2.4	342.2	140.1	25	0	0	0	1
NA-Gln-IS	2.48	356.2	140.1	25	0	0	0	1
NA-Ser-IS	2.56	315.1	186	20	0	0	0	1
AC-IS	2.64	371.1	140.1	35	0	0	0	1
NA-Gly-IS	2.7	285.1	186	15	0	0	0	1
NA-Thr-IS	2.79	329.2	186	20	0	0	0	1
NA-Ala-IS	2.94	299.1	186	15	0	0	0	1
PPC-IS	2.94	385.2	140.1	35	0	0	0	1
LAC-IS	2.95	258.1	186	15	0	0	0	1
NA-Pro-IS	3.3	325.2	76	30	0	0	0	1
NA-Lys-IS	3.3	523.2	140.1	25	2	0	0	1

Metabolites	Rentention time (mins)	Precusor (m/z)	Product (m/z)	Collision Energy (eV)	Labelling pattern			
					1° Amine	2° Amine	Thiol	Acid
NA-Tyr-IS	3.36	391.2	186	15	0	0	0	1
NA-His-IS	3.4	365.2	140.1	45	0	0	0	1
OXA-IS	3.43	467.1	140	35	0	0	0	2
NA-Met-IS	3.6	359.3	186	15	0	0	0	1
NA-Val-IS	3.61	327.2	186	15	0	0	0	1
Pro-IS	3.78	450.1	295.1	25	0	1	0	1
FUM-IS	3.78	451.1	140	30	0	0	0	2
HC-IS	3.95	427.4	140.1	35	0	0	0	1
NA-Leu-IS	3.96	341.2	186	15	0	0	0	1
NA-Ile-IS	4	341.2	186	15	0	0	0	1
NA-Trp-IS	4.03	414.3	186	15	0	0	0	1
NA-Phe-IS	4.08	375.2	186	15	0	0	0	1
NA-Asp-IS	4.2	510.1	186	30	0	0	0	2
PYR-IS	4.24	256.1	186	15	0	0	0	1
MAL-IS	4.24	469.1	140	35	0	0	0	2
NA-Glu-IS	4.29	524.2	140.1	35	0	0	0	2
His-IS	4.34	657.2	250.2	40	2	0	0	1
OC-IS	4.6	455.0	140.1	35	0	0	0	1
NA-Cys-IS	4.65	498.2	140.1	35	0	0	1	1
SUCC-IS	4.65	453.1	140	30	0	0	0	2
Arg-IS	4.7	676.2	331.2	35	2	0	0	1
Asn-IS	4.86	634.2	351.2	30	2	0	0	1
Gln-IS	4.87	648.2	352.2	30	2	0	0	1
AKG-IS	5.07	481.2	186	30	0	0	0	2
CITS-IS	5.07	694.1	186	40	0	0	0	3
Ser-IS	5.08	607.2	420.2	25	2	0	0	1
DC-IS	5.11	483.5	140.1	35	0	0	0	1

Metabolites	Rentention time (mins)	Precusor (m/z)	Product (m/z)	Collision Energy (eV)	Labelling pattern			
					1° Amine	2° Amine	Thiol	Acid
Gly-IS	5.13	577.2	140.1	30	2	0	0	1
Ala-IS	5.18	591.2	378.2	25	2	0	0	1
Tyr-IS	5.23	683.2	470.2	30	2	0	0	1
Thr-IS	5.25	621.2	434.2	25	2	0	0	1
Cys-IS	5.4	395.6	154.2	25	2	0	1	1
Lys-IS	5.5	491.6	140.1	30	4	0	0	1
Trp-IS	5.51	706.2	352.2	30	2	0	0	1
Asp-IS	5.54	802.2	410.2	30	2	0	0	2
LC-IS	5.54	511.5	140.1	35	0	0	0	1
Met-IS	5.56	651.2	438.2	25	2	0	0	1
Glu-IS	5.6	810.2	597.2	35	2	0	0	2
OCA-IS	5.65	312.21	186	20	0	0	0	1
Val-IS	5.69	619.2	406.2	30	2	0	0	1
Phe-IS	5.72	667.2	454.2	30	2	0	0	1
Leu-IS	5.76	633.2	420.2	30	2	0	0	1
Ile-IS	5.82	633.2	420.2	30	2	0	0	1
MC-IS	5.92	539.6	140.1	35	0	0	0	1
DCA-IS	6.08	340.3	186	20	0	0	0	1
PC-IS	6.26	567.6	140.1	35	0	0	0	1
UDA-IS	6.27	354.3	186	20	0	0	0	1
DDA-IS	6.44	368.3	186	20	0	0	0	1
SC-IS	6.55	596.7	140.1	35	0	0	0	1
OA-IS	7.34	450.5	186	20	0	0	0	1
AA-IS	8.58	480.5	186	20	0	0	0	1

Table S3. Concentration changes between the SUIT-2 cell vehicle control and cells exposed to 100 nM rotenone. The values were used to create the volcano plot shown in figure 6.

Metabolites	Vehicle mean conc.	100 nM Rotenone mean	LogFC	pval
Asn	6.61	7.63	1.02	0.0001
AKG	3.44	0.535	-2.91	0.0001
Pro	12.5	11.8	-0.713	0.0004
CITS	-0.209	-2.96	-2.75	0.0004
PYR	8.44	7.40	-1.04	0.0004
OXA	4.17	5.15	0.979	0.001
FUM	12.8	12.0	-0.789	0.002
AC	1.64	-0.00632	-1.65	0.002
SUCC	6.63	5.52	-1.11	0.002
MC	-5.87	-7.64	-1.76	0.003
NA.Ala	4.41	2.44	-1.97	0.003
NA.Asp	6.74	5.77	-0.98	0.004
DDA	11.3	9.70	-1.62	0.004
MAL	8.29	7.06	-1.23	0.005
NA-Thr	1.24	2.45	1.21	0.007
Leu	10.2	10.7	0.444	0.008
NA-Glu	6.03	4.31	-1.72	0.01
DCA	9.46	8.09	-1.37	0.01
NA-Ser	7.01	4.93	-2.08	0.02
NA-Cys	9.13	8.08	-1.04	0.02
NA-Met	-1.75	-2.90	-1.14	0.02
Tyr	8.44	8.59	0.145	0.02
Gly	-2.37	-1.84	0.535	0.02
PC	-4.16	-5.55	-1.39	0.03
SC	-1.60	-2.45	-0.852	0.04
PPC	0.822	0.408	-0.412	0.04
AA	-0.645	-1.56	-0.91	0.045

Metabolites	Vehicle mean conc.	100 nM Rotenone mean	LogFC	pval
NA-Trp	1.39	0.811	-0.580	0.048

Figure S4. SUIT-2 cells exposed to rotenone for 8 hours. The plots show four metabolites (A: PYR, B: MAL, C: PC, and D: SUCC), at four conditions: vehicle control, 1 nM rotenone, 10 nM rotenone and 100 nM rotenone. ns = not significant.

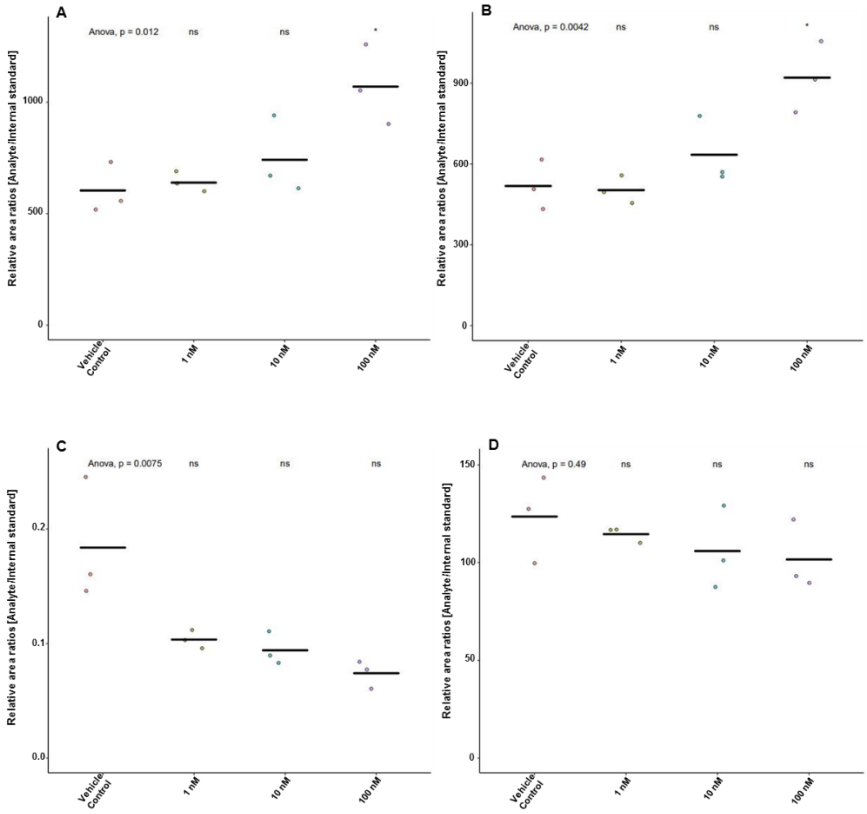

Figure S5. SUIT-2 cells exposed to rotenone for 3 hours. The plots show four metabolites (A: PYR, B: MAL, C: PC, and D: SUCC), at four conditions: vehicle control, 1 nM rotenone, 10 nM rotenone and 100 nM rotenone. ns = not significant.

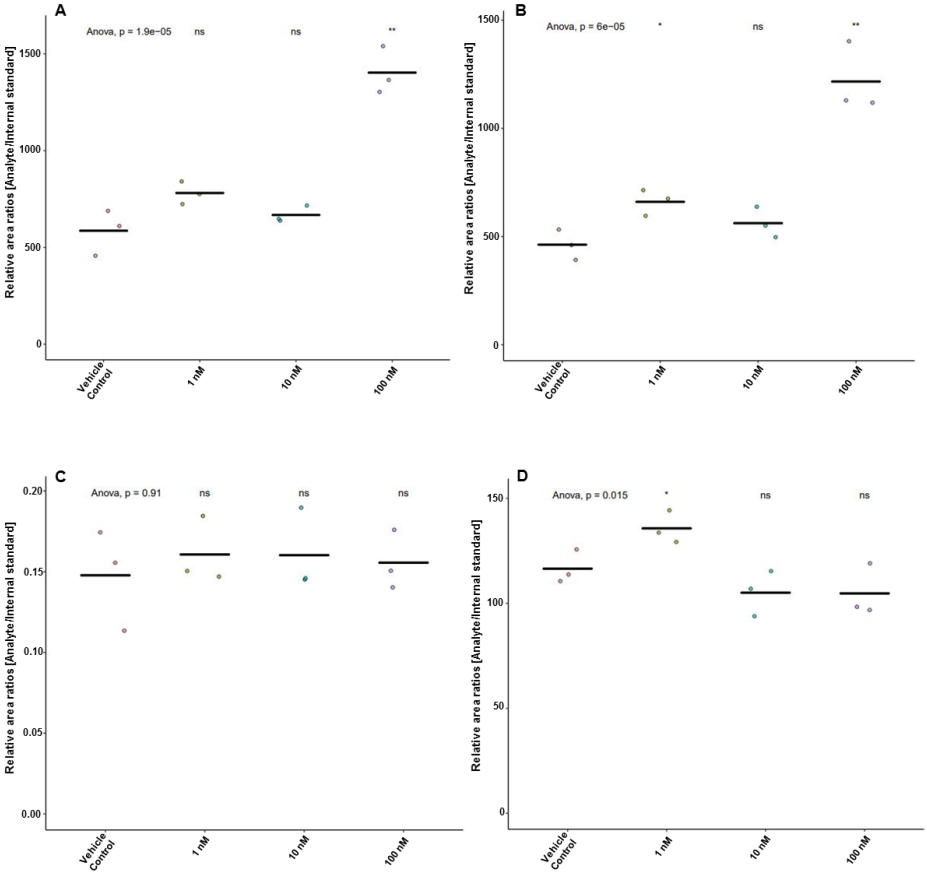

*Table S6. Metabolite standard concentrations (μM), utilized for calibration plots. * concentration of the internal standard.*

Metabolites	Cal 1	Cal 2	Cal 3*	Cal 4	Cal5	Cal 6	Cal 7
Ala	9.29	18.57	37.14	74.29	148.57	297.14	594.29
Arg	1.16	2.32	4.64	9.29	18.57	37.14	74.29
Asn	4.18	8.36	16.71	33.43	66.86	133.71	267.43
Asp	2.32	4.64	9.29	18.57	37.14	74.29	148.57
Cys	13.93	27.86	55.71	111.43	222.86	445.71	891.43
Gln	13.93	27.86	55.71	111.43	222.86	445.71	891.43
Glu	0.93	1.86	3.71	7.43	14.86	29.71	59.43
Gly	69.64	139.29	278.57	557.14	1114.29	2228.57	4457.14
Ile	0.93	1.86	3.71	7.43	14.86	29.71	59.43
Leu	1.39	2.79	5.57	11.14	22.29	44.57	89.14
Lys	6.96	13.93	27.86	55.71	111.43	222.86	445.71
Met	0.46	0.93	1.86	3.71	7.43	14.86	29.71
Phe	2.79	5.57	11.14	22.29	44.57	89.14	178.29
Pro	0.23	0.46	0.93	1.86	3.71	7.43	14.86
Ser	13.93	27.86	55.71	111.43	222.86	445.71	891.43
Thr	5.57	11.14	22.29	44.57	89.14	178.29	356.57
Trp	2.79	5.57	11.14	22.29	44.57	89.14	178.29
Tyr	3.71	7.43	14.86	29.71	59.43	118.86	237.71
Val	1.86	3.71	7.43	14.86	29.71	59.43	118.86
His	27.86	55.71	111.43	222.86	445.71	891.43	1782.86
NA-Ala	0.31	0.62	1.23	2.46	4.93	9.86	19.71
NA-Gly	0.15	0.29	0.58	1.16	2.32	4.65	9.3
NA-Ile	0.07	0.15	0.3	0.6	1.2	2.39	4.79
NA-Leu	0.07	0.15	0.29	0.58	1.16	2.33	4.66
NA-Pro	0.04	0.07	0.14	0.28	0.57	1.13	2.26
NA-Val	0.14	0.27	0.55	1.1	2.19	4.38	8.76
NA-Phe	0.03	0.06	0.13	0.25	0.5	1.01	2.01
NA-Trp	0.05	0.11	0.22	0.44	0.87	1.74	3.49
NA-Tyr	0.03	0.06	0.11	0.22	0.45	0.9	1.79
NA-Asp	0.74	1.49	2.97	5.94	11.89	23.78	47.56
NA-Glu	0.25	0.51	1.01	2.03	4.06	8.11	16.23
NA-Arg	0.27	0.53	1.07	2.14	4.28	8.55	17.11

Metabolites	Cal 1	Cal 2	Cal 3*	Cal 4	Cal5	Cal 6	Cal 7
NA-His	0.07	0.14	0.27	0.55	1.1	2.19	4.39
NA-Lys	0.14	0.27	0.54	1.09	2.18	4.36	8.72
NA-Ser	0.12	0.23	0.47	0.94	1.87	3.74	7.49
NA-Thr	0.31	0.62	1.23	2.46	4.93	9.86	19.71
NA-Cys	0.4	0.81	1.61	3.22	6.45	12.9	25.79
NA-Met	1.44	2.89	5.78	11.55	23.11	46.22	92.43
NA-Asn	1.03	2.05	4.1	8.21	16.42	32.83	65.66
NA-Gln	0.51	1.01	2.03	4.05	8.1	16.2	32.4
PYR	0.7	1.39	2.79	5.57	11.14	22.29	44.57
AKG	2.32	4.64	9.29	18.57	37.14	74.29	148.57
MAL	0.46	0.93	1.86	3.71	7.43	14.86	29.71
LAC	4.64	9.29	18.57	37.14	74.29	148.57	297.14
CIT/ICIT	58.04	116.07	232.14	464.29	928.57	1857.14	3714.29
SUCC	1.86	3.71	7.43	14.86	29.71	59.43	118.86
FUM	0.23	0.46	0.93	1.86	3.71	7.43	14.86
OXA	0.46	0.93	1.86	3.71	7.43	14.86	29.71
AC	0.93	1.86	3.71	7.43	14.86	29.71	59.43
OC	0.02	0.05	0.09	0.19	0.37	0.74	1.49
PPC	0.22	0.45	0.89	1.79	3.57	7.14	14.29
OCA	0.46	0.93	1.86	3.71	7.43	14.86	29.71
DCA	0.46	0.93	1.86	3.71	7.43	14.86	29.71
DDA	0.01	0.03	0.06	0.11	0.22	0.45	0.89
UDA	0.05	0.09	0.19	0.37	0.74	1.49	2.97
DC	0.22	0.45	0.89	1.79	3.57	7.14	14.29
HC	0.22	0.45	0.89	1.79	3.57	7.14	14.29
LC	0.22	0.45	0.89	1.79	3.57	7.14	14.29
MC	0.22	0.45	0.89	1.79	3.57	7.14	14.29
PC	0.22	0.45	0.89	1.79	3.57	7.14	14.29
SC	0.22	0.45	0.89	1.79	3.57	7.14	14.29
OLA	0.23	0.46	0.93	1.86	3.71	7.43	14.86
AA	0.05	0.09	0.19	0.37	0.74	1.49	2.97
CR	29.02	58.04	116.07	232.14	464.29	928.57	1857.14

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