Quantification of polar metabolites by GC MS-MS technique in urine samples from subjects with type 2 diabetes

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BACKGROUND AND AIM

The aim of the study was to optimize a new gas chromatography quantitative method to identify metabolic concentration of 34 amino acids and organic acid in urine samples from subjects with type 2 diabetes before and after the administration of two drugs with diuretic properties: Dapagliflozin (DAPA), an antihyperglycemic agent acting on renal glucose reabsorption and Hydrochlorothiazide (HCT).
The aim of this project is to evaluate the effect of treatment on metabolite concentrations and if changes are correlated with excretion of the metabolites of Di-2-ethylhexyl phthalate (DEHP) and Bisphenol A, two endocrine disruptor chemicals (EDC) widely used in industry and in a large range of daily life products.
To accomplish this I set up a GCMS method for the quantification of urinary metabolites.

METHODS

Amino acids and organic acids, due to their polar nature, require derivatization before gas chromatography analysis, but the high amount of urea can interfere with chemical derivatization. Further interferences are mainly related to the instrument, such as overload of the chromatographic column and coelution peaks. Urease solution (1 mg/mL in water) was prepared by using a lyophilized enzyme (Urease type III, Sigma- Aldrich® U1500). 100 uL of urease was added in 50 uL of urine sample into test tube and incubated for 1 h at 37 °C. After incubation 700 µL cold methanol were added and then samples were centrifuged 20 min at 13,000 rpm at 4 °C. The supernatant was evaporated to dryness under nitrogen at 40 °C. Two-step derivatization method was optimized. First 30 uL of Methoxyamine 20 mg/mL was added into test tube (30 min at 60 °C); after evaporating the residue to dryness under nitrogen at 40 °C, 30 uL di TBDMS e 70 uL di Acetonitrile were added and incubated for 1h min at 60 °C. Quantification was performed by labelled internal standards and normalized by urinary creatinine concentration.
This method was optimized for minimize chemical derivatization interference and the use of GC-MS/MS system with optimized MRM transition allows greater selectivity and sensitivity and minimizes chromatographic interference.

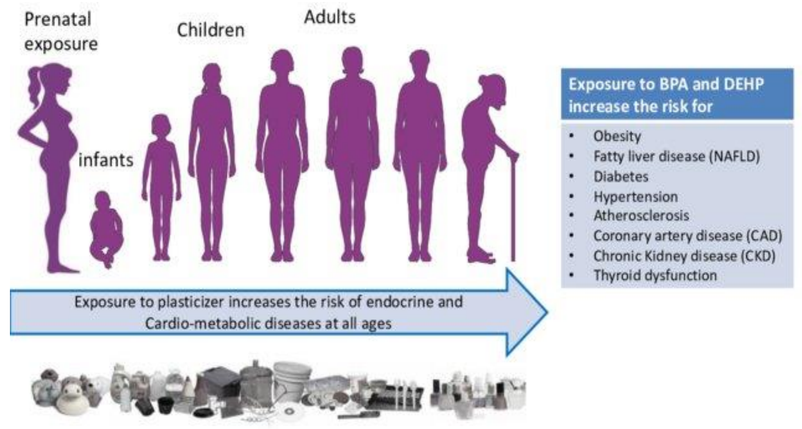

Fig1: Assessment of exposure to Di (2-ethylhexyl) phthalate (DEHP) metabolites and Bisphenol A (BPA) and its importance for the prevention of cardiometabolic diseases. (Carli F et al, Metabolites 2022)

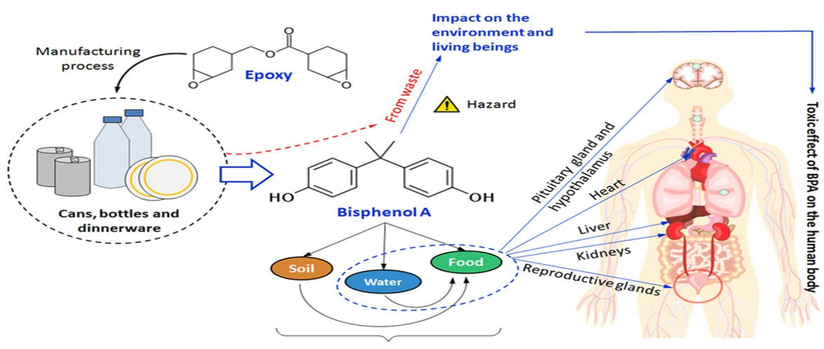

Fig2. The hazardous threat of Bisphenol A: Toxicity, detection and remediation (Tarafdara A et al J Hazard Mater 2022)

RESULTS

Table 3: Important features identified by t-tests

	Compounds	t.stat	p.value	-log10(p)	FDR
1	alpha-ketoglutaric acid	-2.8662	0.0056157	2.2506	0.1797
2	3-Hydroxybutyric acid	-2.1893	0.032228	1.4918	0.2374
3	Leucine	-2.1883	0.032307	1.4907	0.2374
4	Fumaric acid	-2.0921	0.040402	1.3936	0.2374
5	Methionine	-2.0805	0.041485	1.3821	0.2374

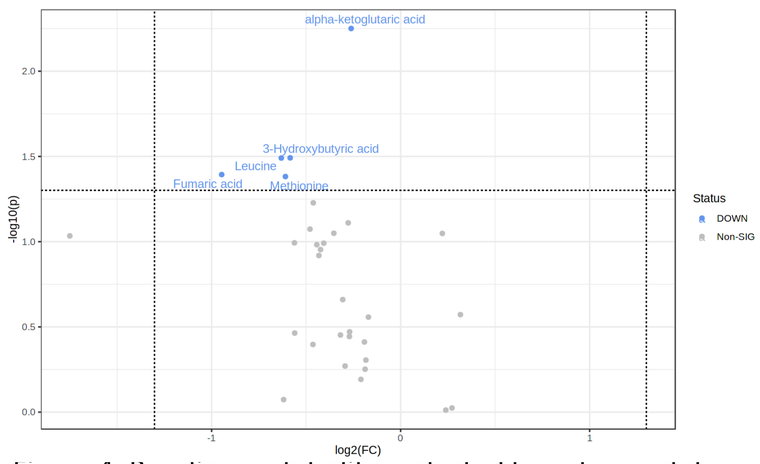

Figure 4: Baseline metabolites selected by volcano plot with fold change>1 for high/low exposure to phthalates and t-tests threshold <0.1.

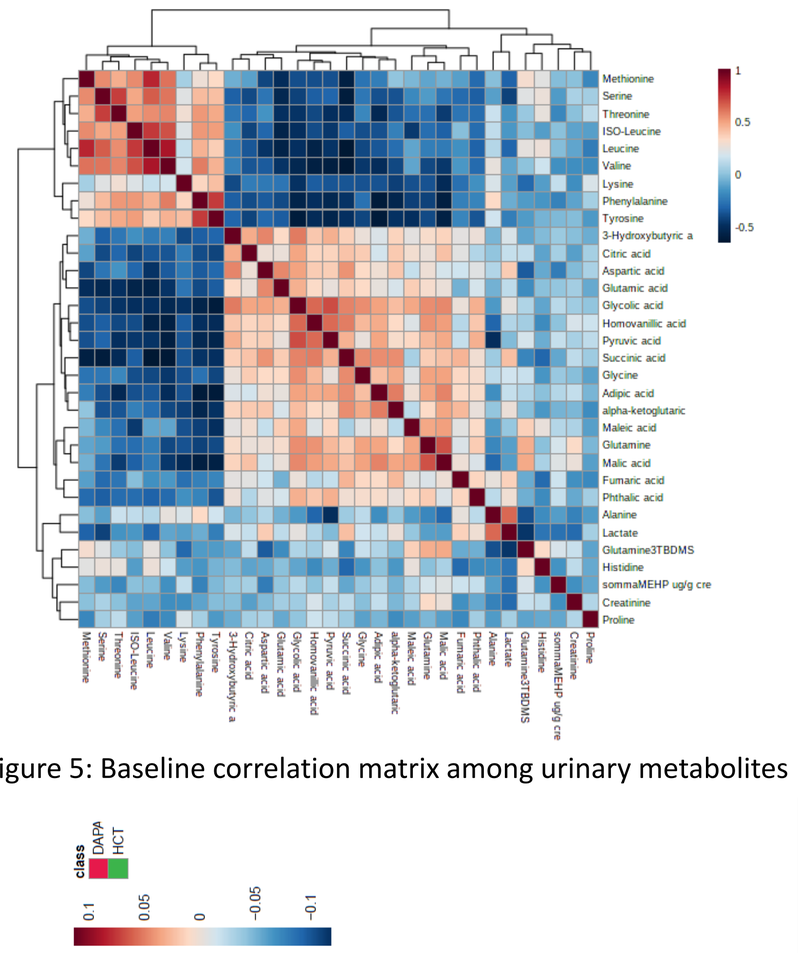

Figure 5: Baseline correlation matrix among urinary metabolites

Anthropometric Characterstics

	DAPA		HCT		HCT vs DAPA
	BASALE	POST	BASALE	POST	P value
N	18	18	16	16	ns
Età	59.8±2.0		62.2±1.9		ns
BMI Kg/m²	32.1±1.6	31.7±1.6*	28.8±1.2	29.0±1.1	ns
AST U/l	17.5±1.3	17.7±1.5	20.1±1.5	19.4±1.4	ns
ALT U/L	23.5±2.9	24.1±3.3	25.6±1.8	24.1±1.9	ns
GGT U/L	27.4±5.0	25.4±4.1	32.4±6.8	30.1±5.0	ns
Colest. TOT mg/dl	170.2±6.8	168.59±5.31	164.2±5.2	162.2±7.1	ns
LDL mg/dl	106.9±7.5	104.5±6.2	99.3±4.5	98.1±4.9	ns
HDL mg/dl	50.0±3.7	50.5±4.4	55.0±4.1	51.1±3.5	ns
TRIGL. mg/dl	124.1±11	138±16	102±11	109±12	ns
GLUCOSE mg/dl	145±8	139±7.3	125±7	131±12	ns
Ur. Creatinine urinary mg/dl	106±11	70±5*	91±7	92±9	0.04

Figure 3: Anthropometric Characterstics
mean± standard error; Wilcoxon test; (basale vs post)
mean± standard error; Mann-Withney; (post DAPA vs post HCT)
*p value<0.05

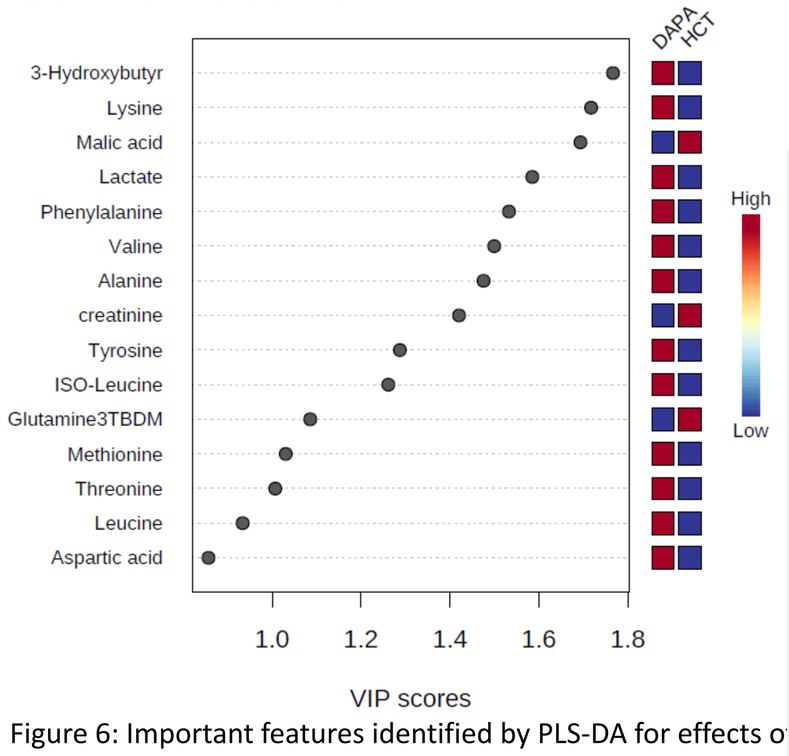

Figure 6: Important features identified by PLS-DA for effects o DAPA vs HCT. The colored boxes on the right indicate the relat concentrations of the corresponding metabolite in each group under study

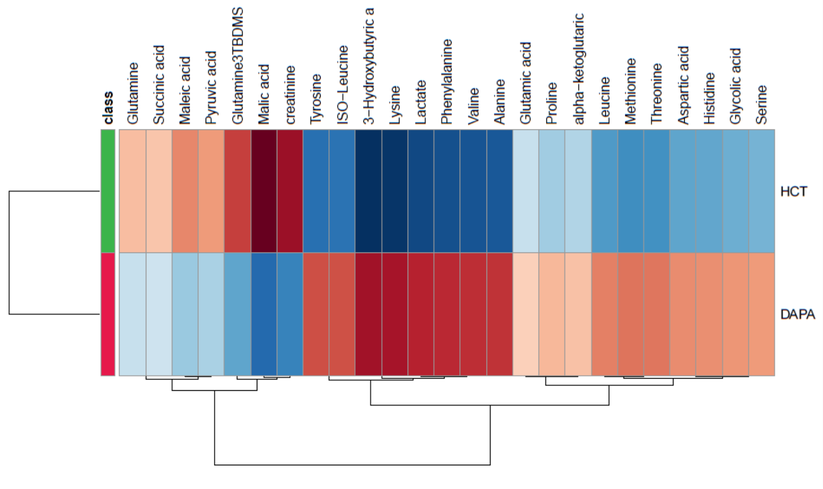

Figure 7: heatmap of fold changes in concentrations of the top 25 metabolites in DAPA vs HCT (using Metaboanalyst software).

CONCLUSION

Preliminary analyses of urinary metabolites quantified by a new GC-MS/MS method with labelled internal standards highlighted that:
1) four urinary metabolites are different among subjects with higher vs lower exposure to phthalates at baseline
2) the different effects of DAPA vs HCT on urinary metabolite excretion.

Reference
[1] Palmas, et all, Urine metabolome analysis by gas chromatography–mass spectrometry T (GC–MS): Standardization and optimization of protocols for urea removal and short-term sample storage, Clinica Chimica Acta, 2018, 236-242
[2] Mengozzi, et all High exposure to phthalates is associated with HbA1 c worsening in type 2 diabetes subjects with and without edentulism: a prospective pilot study, Diabetology & Metabolic Syndrome volume,2022, 14
[3] Kaspar et all, Urinary amino acid analysis: A comparison of iTRAQ–LC–MS/MS, GC–MS, and amino acid analyzer, Journal of Chromatography B, 2009, 1838-1846.