ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

DETERMINATION OF 3-MONOIODOTYROSINE AND 3,5-DIIODOTYROSINE IN NEWBORN URINE AND DRIED URINE SPOTS BY ISOTOPE DILUTION TANDEM MASS SPECTROMETRY

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Table S1. Experimental chromatographic conditions applied for ^{15}N-MIT purification by semi-preparative liquid chromatography.

Chromatograph	Agilent Infinity 1260	
Column	AerisPeptide XB-C18 (Phenomenex)	
Column dimensions	250x4.6mm x5µm	
Temperature	25 °C	
Detection wavelenght	280 nm	
Injection volume	100 µL	
Flow	2 mL/min	
Phase A	0.1 % formic acid in water	
Phase B	ACN, 0.1 % formic acid	
Chromatography	Time (min)	- % B
	0	8
	5	8
	24	70
	29	70
	29.5	8
	49.5	8

Table S2. Experimental chromatographic conditions for the separation of creatinine, MIT and DIT in standard solutions and urine and DUS samples.

Chromatograph	Agilent Infinity 1290	
Column	Zorbax Eclipse C18	
Column dimensions	50 x 2.1mm x 1.6µm	
Temperature	25 °C	
Injection volume	10 µL	
Flow	0.4 mL/min	
Phase A	0.1 % formic acid in water	
Phase B	ACN, 0.1 % formic acid	
Chromatography	Time (min)	- % B
	0	2
	9	35
	10	80
	11	80
	12	2
	15	2

Table S3. Experimental mass spectrometric conditions for the measurement of creatinine, MIT and DIT in standard solutions and urine and DUS samples.

Mass spectrometer		Agilent 6460	
Ionization Source		Electrospray jet stream	
Ionization mode		Positive	
Gas temperature		250 °C	
Gas flow		9 mL/min	
Sheath gas T		400 °C	
Sheath gas flow		11 mL/min	
Nebulizer pressure		25 psi	
Capillary voltage		4000 V	
Nozzle voltage		1000 V	
	Transition	CE (V)	Fragmentor (V)
Creatinine	114→86	5	135
	115→87	5	135
	116→88	5	135
MIT	364→262	5	135
	365→263	5	135
	366→264	5	135
	367→265	5	135
DIT	490→388	10	135
	491→389	10	135
	492→390	10	135
	493→391	10	135

Calculations for Double-Spike IDMS to correct for MIT and DIT interconversion

The sample (s), containing natural abundance 3-monoiodotyrosine (MIT) and 3,5-diiodotyrosine (DIT), is spiked with a known amount of $^{15}N_1$-MIT (tracer 1, *t1*) and $^{13}C_2$-DIT (tracer 2, *t2*). Ideally, isotopically labelled ^{15}N-MIT and $^{13}C_2$-DIT should not contain ^{15}N-DIT and $^{13}C_2$-MIT, respectively. However, our model takes into account such possible contributions as described in the mass balances for the number of moles (N) of MIT and DIT in the mixture (m) of sample and labelled compounds:

$$N_m^{MIT} = N_s^{MIT} + N_{t1}^{MIT} + N_{t2}^{MIT} \tag{9}$$

$$N_m^{DIT} = N_s^{DIT} + N_{t1}^{DIT} + N_{t2}^{DIT} \tag{10}$$

where N_m^{MIT}, N_s^{MIT}, N_{t1}^{MIT} and N_{t2}^{MIT} are the number of MIT molecules in the mixture, sample, tracer 1 and tracer 2 (as impurity), respectively, and N_m^{DIT}, N_s^{DIT}, N_{t1}^{DIT} and N_{t2}^{DIT} are the number of DIT molecules in the mixture, sample, tracer 1 (as impurity) and tracer 2.

After tracer addition and homogenization with the sample, a fraction of MIT may convert into DIT, or vice versa, during any of the stages of sample preparation. If we assume that isotope equilibration takes place before the occurrence of any interconversion reaction we can establish new mass balance equations, (11) and (12), in which *F1* refers to the fraction of MIT transformed into DIT and *F2* to the fraction of DIT transformed into MIT:

$$N_m^{MIT} = (N_s^{MIT} + N_{t1}^{MIT} + N_{t2}^{MIT}) \times (1 - F1) + \left(N_s^{DIT} + N_{t1}^{DIT} + N_{t2}^{DIT}\right) \times F2 \tag{11}$$

$$N_m^{DIT} = \left(N_s^{DIT} + N_{t1}^{DIT} + N_{t2}^{DIT}\right) \times (1 - F2) + (N_s^{MIT} + N_{t1}^{MIT} + N_{t2}^{MIT}) \times F1 \tag{12}$$

These mass balance equations can be transformed into molar fractions (x) for each isotopic form ($x_s=N_s/N_m$, $x_{t1}=N_{t1}/N_m$ and $x_{t2}=N_{t2}/N_m$) using partial mass balances taken from equations (11) and (12), leading to equations (13), (14) and (15) for MIT and (16), (17) and (18) for DIT:

$$x_s^{MIT} = \frac{N_s^{MIT}(1 - F1) + N_s^{DIT} F2}{N_m^{MIT}} \tag{13}$$

$$x_{t1}^{MIT} = \frac{N_{t1}^{MIT}(1 - F1) + N_{t1}^{DIT} F2}{N_m^{MIT}} \tag{14}$$

$$x_{t2}^{MIT} = \frac{N_{t2}^{MIT}(1 - F1) + N_{t2}^{DIT} F2}{N_m^{MIT}} \tag{15}$$

$$x_s^{DIT} = \frac{N_s^{DIT}(1-F2) + N_s^{MIT}F1}{N_m^{DIT}} \quad (16)$$

$$x_{t1}^{DIT} = \frac{N_{t1}^{DIT}(1-F2) + N_{t1}^{MIT}F1}{N_m^{DIT}} \quad (17)$$

$$x_{t2}^{DIT} = \frac{N_{t2}^{DIT}(1-F2) + N_{t2}^{MIT}F1}{N_m^{DIT}} \quad (18)$$

The isotopic composition measured for each compound can be expressed as a function of three isotopic patterns: the natural abundance pattern, the isotopic pattern of the $^{13}C_2$ tracer (t2) and the isotopic pattern of ^{15}N tracer (t1). In this way we can establish the overdetermined systems of equations (19) and (20) for MIT and DIT, respectively. As indicated in the manuscript, by applying multiple linear regressions six molar fractions can be obtained (3 for MIT and 3 for DIT) which could be employed to solve equations (13) to (18):

$$\begin{bmatrix} A_1^{m,MIT} \\ A_2^{m,MIT} \\ A_3^{m,MIT} \\ A_4^{m,MIT} \end{bmatrix} = \begin{bmatrix} A_1^{s,MIT} & A_1^{t1,MIT} & A_1^{t2,MIT} \\ A_2^{s,MIT} & A_2^{t1,MIT} & A_2^{t2,MIT} \\ A_3^{s,MIT} & A_3^{t1,MIT} & A_3^{t2,MIT} \\ A_4^{s,MIT} & A_4^{t1,MIT} & A_4^{t2,MIT} \end{bmatrix} \times \begin{bmatrix} x_s^{MIT} \\ x_{t1}^{MIT} \\ x_{t2}^{MIT} \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \\ e_3 \\ e_4 \end{bmatrix} \quad (19)$$

$$\begin{bmatrix} A_1^{m,DIT} \\ A_2^{m,DIT} \\ A_3^{m,DIT} \\ A_4^{m,DIT} \end{bmatrix} = \begin{bmatrix} A_1^{s,DIT} & A_1^{t1,DIT} & A_1^{t2,DIT} \\ A_2^{s,DIT} & A_2^{t1,DIT} & A_2^{t2,DIT} \\ A_3^{s,DIT} & A_3^{t1.DIT} & A_3^{t2,DIT} \\ A_4^{s,DIT} & A_4^{t1,DIT} & A_4^{t2,DIT} \end{bmatrix} \times \begin{bmatrix} x_s^{DIT} \\ x_{t1}^{DIT} \\ x_{t2}^{DIT} \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \\ e_3 \\ e_4 \end{bmatrix} \quad (20)$$

where A_i^s, A_i^{t1} and A_i^{t2} are the isotope distributions for each compound at the 4 measured masses. The isotopic distributions of in-cell molecular fragments, A_i^m, were measured at 4 different masses using the transitions 364→262, 365→263, 366→264, 367→265 for MIT and 490→388, 491→389, 492→390, 493→391 for DIT. This was performed by peak area integration of the corresponding chromatographic peak and dividing each peak area by the sum of all peak areas measured for the 4 MRM transitions. Once the molar fractions are computed, the interconversion factors *F1* and *F2* can be calculated by dividing equations (14) and (15) for MIT and equations (17) and (18) for DIT. In this way, we end up with equations (21) and (22) where the only unknowns are *F1* and *F2*:

$$F1\left(x_{t2}^{MIT}N_{t1}^{MIT} - x_{t1}^{MIT}N_{t2}^{MIT}\right) + F2\left(x_{t1}^{MIT}N_{t2}^{DIT} - x_{t2}^{MIT}N_{t1}^{DIT}\right) = \left(x_{t2}^{MIT}N_{t1}^{MIT} - x_{t2}^{MIT}N_{t1}^{MIT}\right) \quad (21)$$

$$F1\left(x_{t2}^{DIT}N_{t1}^{MIT} - x_{t1}^{DIT}N_{t2}^{MIT}\right) + F2\left(x_{t1}^{DIT}N_{t2}^{DIT} - x_{t2}^{DIT}N_{t1}^{DIT}\right) = \left(x_{t1}^{DIT}N_{t2}^{DIT} - x_{t2}^{DIT}N_{t1}^{DIT}\right) \quad (22)$$

Note that these equations are simpler when the tracers employed are pure, that is, N_{t2}^{MIT} =0 and N_{t1}^{DIT} =0. Once *F1* and *F2* are calculated, the original MIT and DIT amounts in the sample, N_s^{MIT} and N_s^{DIT}, corrected for the possible interconversion occurring during sample preparation and measurement, can be calculated from the ratios of equations (13) and (14) for MIT, and (16) and (18) for DIT. The final two equations with two unknowns, equations (23) and (24), provide the degradation corrected concentrations of MIT and DIT.

$$N_s^{MIT}(1-F1)+N_s^{DIT}F2=\frac{X_s^{MIT}}{X_{t1}^{MIT}}(N_{t1}^{MIT}(1-F1)+N_{t1}^{DIT}F2) \tag{23}$$

$$N_s^{MIT}F1+N_s^{DIT}(1-F2)=\frac{X_s^{DIT}}{X_{t2}^{DIT}}(N_{t2}^{DIT}(1-F2)+N_{t2}^{MIT}F1) \tag{24}$$

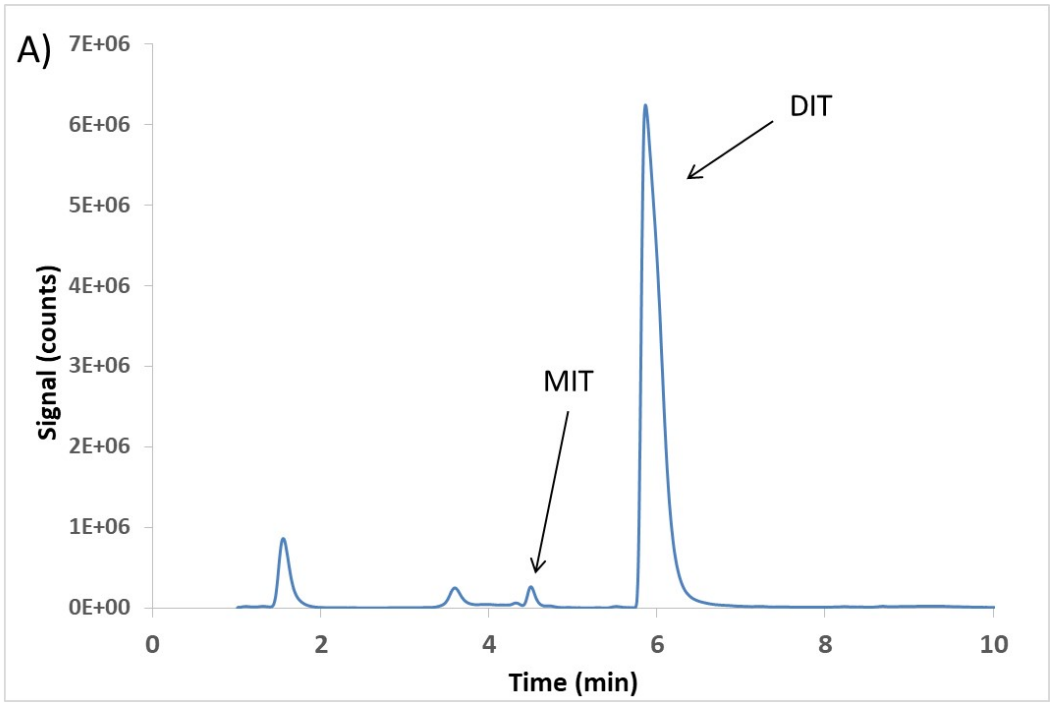
These equations will reduce to the conventional single-spike IDMS equations when the interconversion factors *F1* and *F2* are 0:

$$N_s^{MIT}=\frac{X_s^{MIT}}{X_{t1}^{MIT}}N_{t1}^{MIT} \tag{25}$$

$$N_s^{DIT}=\frac{X_s^{DIT}}{X_{t2}^{DIT}}N_{t2}^{DIT} \tag{26}$$

To convert from number of moles N_s to concentrations C_s we have to take into account the masses taken and the molecular weights of the compounds as indicated in the manuscript.

Figure S1. LC-ESI-MS chromatogram acquired in SCAN mode of the synthetized crude of $^{13}C_2$-DIT (A) before and (B) after purification by isoelectric point precipitation.



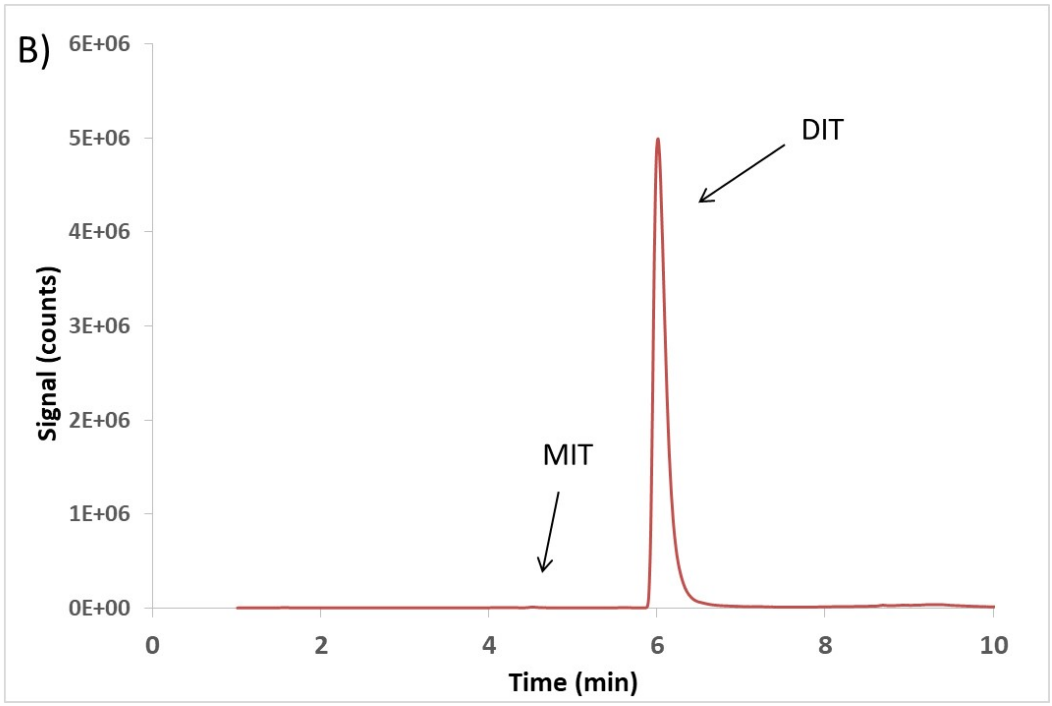


Figure S2. LC-ESI-MS chromatogram acquired in SCAN mode of the synthetized crude of ^{15}N-MIT (A) before and (B) after purification by semi-preparative LC.

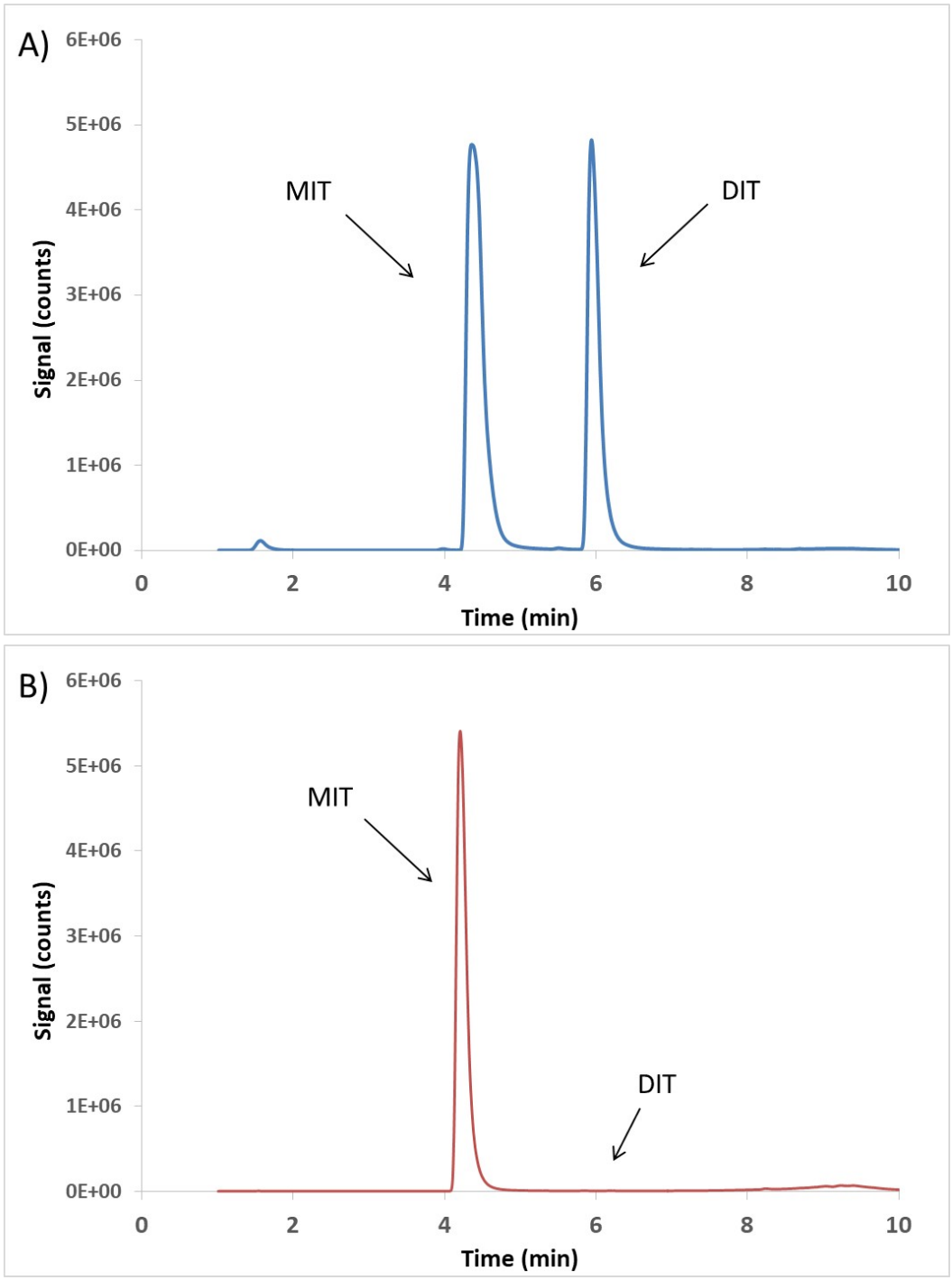


Figure S3. Stability study for A) ^{15}N-MIT and B) $^{13}C_2$-DIT MIT in 1 µg·g^{-1} solutions stored at 50 ºC in pH 4, 7 and 9 over five days.

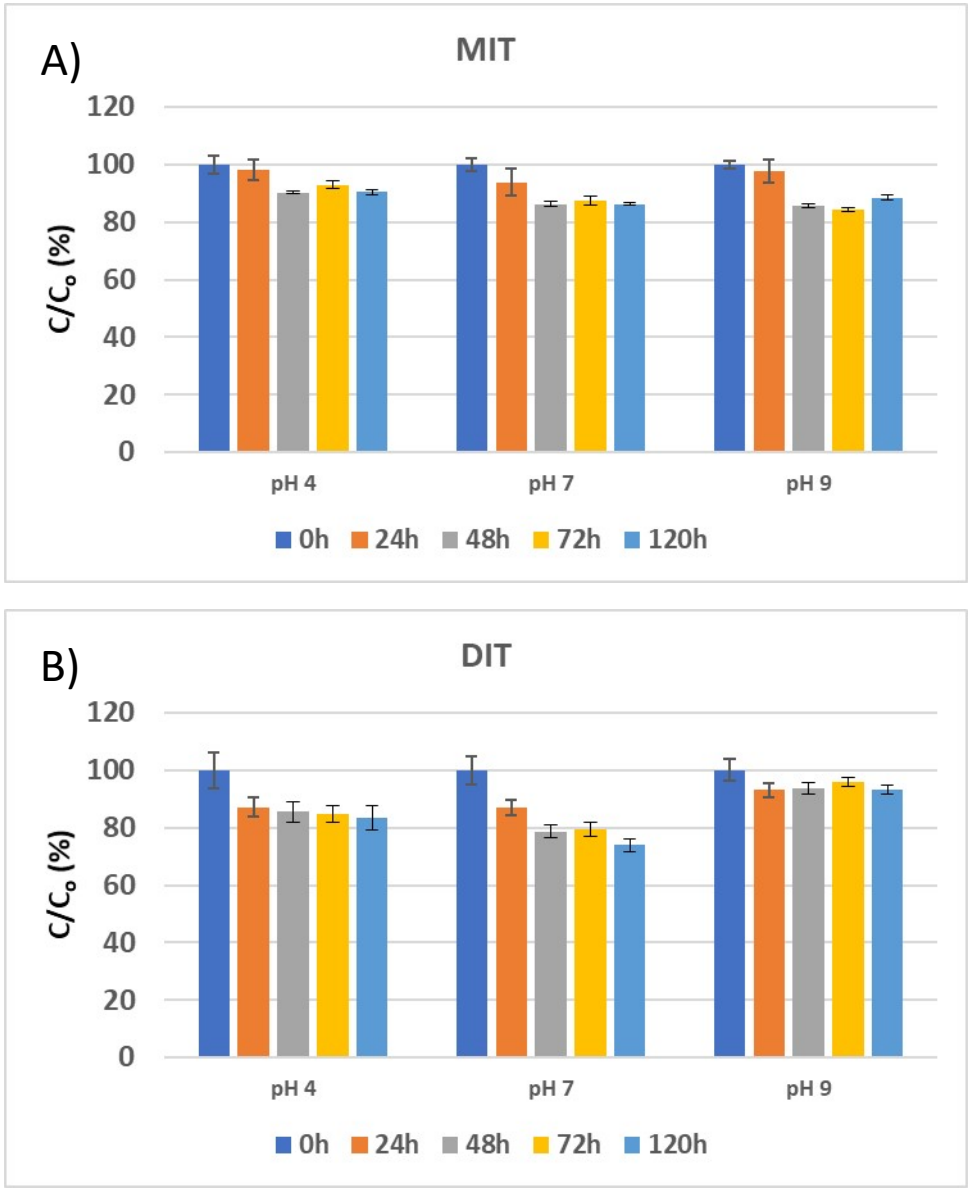
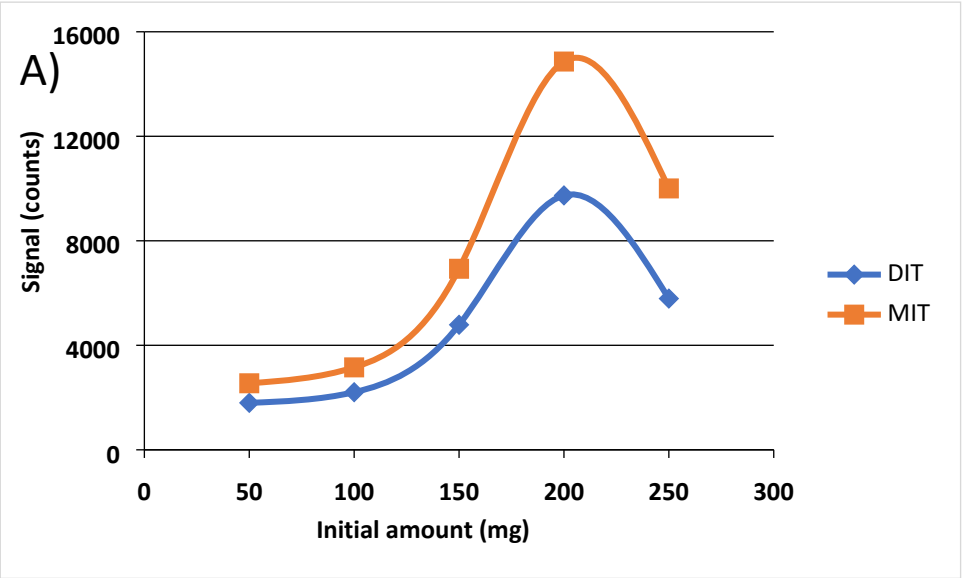


Figure S4. Signal obtained for MIT and DIT in a urine sample containing a creatinine concentration of 1.3 $mg \cdot g^{-1}$ A) when analyzing different sample amounts and B) when injecting different volumes into the LC-MS/MS system.



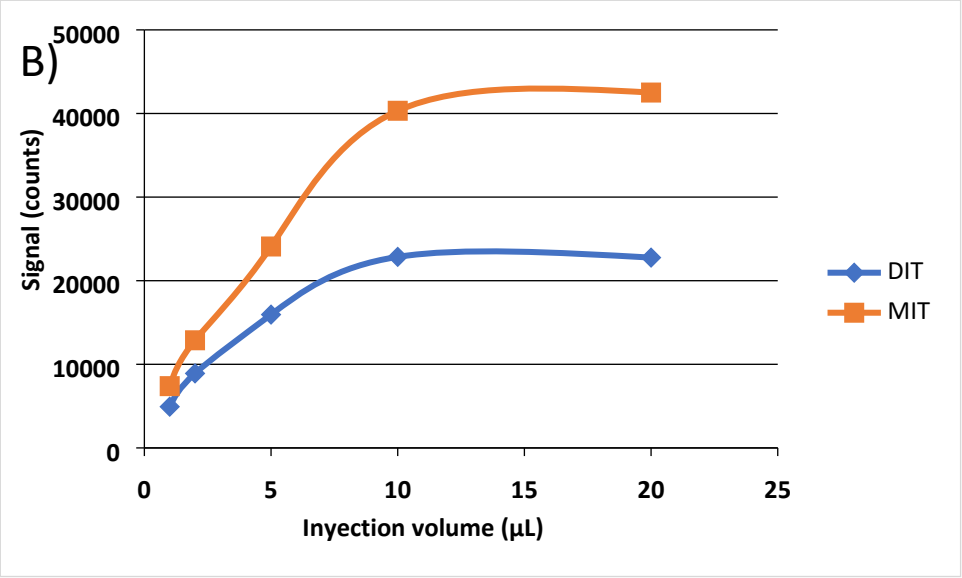


Figure S5. Effect of the dilution factor of the urine samples on the absolute signal for MIT and DIT (dilution factor 1 = undiluted). MIT and DIT were spiked to the diluted urine samples to a constant concentration of 2.5 $ng \cdot g^{-1}$.