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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ABSTRACT

High levels of 3-mono- and 3,5-diiodotyrosine (MIT and DIT, respectively) in urine have been related to iodotyrosine dehalogenase 1 deficiency, a type of congenital hypothyroidism. However, the determination of MIT and DIT in urine is not included in newborn screening programs performed in clinical laboratories to detect inborn errors of metabolism. We report here on the development of an analytical method for the determination of MIT and DIT in newborn urine and dried urine spots (DUS) by Liquid Chromatography Isotope Dilution tandem Mass Spectrometry (LC-IDMSMS). The development included the synthesis of ^{15}N-monoiodotyrosine and $^{13}C_2$-diiodotyrosine through the iodination of ^{15}N-tyrosine and $^{13}C_2$-tyrosine, respectively, using bis(pyridine)iodonium (I) tetrafluoroborate (IPy_2BF_4). Both labelled analogues were added at the beginning of the sample preparation procedure and used to develop both single- and double-spike LC-IDMS methods for the determination of MIT and DIT. The developed double spike methodology was able to quantify and correct possible MIT $\leftrightarrow$ DIT interconversions throughout the sample preparation, which was observed for concentrated urine samples but not for DUS. Suppression matrix effects on the absolute signals of MIT and DIT were observed in urine samples but did not affect the IDMS results as recoveries on urine samples at different dilution factors could be considered quantitative. Method detection limits were 0.018 and 0.046 ng·g^{-1} (limits of quantification 0.06 and 0.15 ng.g^{-1})by single-spike IDMS, for MIT and DIT, respectively, in the analysis of urine samples and 0.07 and 0.05 ng·g^{-1} (limits of quantification 0.23 and 0.17 ng.g^{-1}) for MIT and DIT, respectively, in the analysis of DUS. No significant differences were obtained for MIT concentrations in the analysis of the same newborn samples stored as liquid urine or DUS when the results were corrected for the creatinine content. Finally, 36 DUS samples from healthy newborns were analyzed and MIT was detected in all samples at low ng·mg^{-1}creatinine levels.

INTRODUCTION

Congenital hypothyroidism is the most common preventable cause of intellectual disability. Newborn screening (NBS) programs typically measure the concentration of thyrotropin stimulating hormone (TSH) in blood by inmunometric methods[1] alone or in combination with the hormone thyroxine (T4). Recently, a new type of congenital hypothyroidism was described[2] which can be missed by current NBS programs[3,4]: Iodotyrosine dehalogenase 1 deficiency (ITDD). This is a type of congenital hypothyroidism related to the failure of the thyroid enzyme iodotyrosine dehalogenase 1 (DEHAL1). This enzyme deiodinates 3-mono- and 3,5-diiodotyrosine (MIT and DIT, respectively) and recycles iodide for the efficient synthesis of thyroid hormones[3]. Thus, mutations in the gene encoding DEHAL1 (IYD, OMIM #612025) result in iodide deficiency, which is associated with hypothyroidism, gout and childhood mental and developmental disorders[5-7]. High levels of iodine in the form of MIT and DIT have been observed in patients with overt hypothyroidism due to DEHAL 1 malfunction[8,9]. For example, Afink *et al*.[8] found significantly higher concentrations of MIT and DIT in urine of two ITDD patients (concentration of 100.8 and 220.8 nM for MIT and 31.2 and 108.2 nM for DIT) versus control values of 2.6 ± 1.5 nM for MIT and 0.5 ± 0.1 nM for DIT, respectively.

Many NBS programs to detect inborn errors of metabolism involve the monitoring of metabolites in urine or dry urine spots (DUS) by mass spectrometry[10] and it would be ideal to include the determination of MIT and DIT in these programs to detect DEHAL 1 malfunction. However, only a few methods have been developed to determine MIT and DIT in biological fluids[8] and none of these procedures have been applied to study ITDD in neonates. Thus, the development of validated analytical methods for the determination of iodotyrosines (MIT and DIT) in urine or DUS would allow the inclusion of these metabolites in such programs.

Liquid Chromatography Isotope Dilution tandem Mass Spectrometry (LC-IDMSMS) has been described as the gold standard analytical methodology in thyroid hormone metabolism research due to their high sensitivity, specificity and reproducibility[11]. Reference measurement procedures for the determination of thyroid hormones in human serum based on IDMS have been developed and validated by the National Institute for Standards and

Technology (NIST)[12,13] and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)[14]. These methods have been also endorsed by the Joint Committee for Traceability in Laboratory Medicine (JCTLM)[15]. Thyroid hormone metabolites (THMs) have been determined in a variety of clinical samples using LC-MS[8,9,16-26] and GC-MS[27,28]. These methods reported different sample treatment procedures in order to: i) avoid ion suppression in the electrospray ionization source, ii) remove high abundant compounds which may interfere in the measurement and iii) enhance MS detection by means of preconcentration or derivatization. However, a recent review on THMs methods[3] stressed that, the lack of commercially available stable isotopically labelled internal standards for some THMs does not guarantee the proper correction of matrix effects and the reliable evaluation of extraction and/or derivatization efficiencies during sample preparation[13]. Isotopically labelled MIT and DIT have not been employed to develop analytical methods for these compounds and only the possible use of $^{13}C_9$-iodothyroxines has been indicated[3].

Iodine-containing compounds are known to be thermally unstable and suffer from photolysis. Recently, Hansen *et al.*[16] reported photolytic deiodination and transformation of 3,3',5-triiodothyronine to 3,3'-diiodothyronine and 3-iodothyronine under normal laboratory working conditions. For this reason, many researchers have tried to use a variety of antioxidants and their mixtures during preanalytical sample workup without clearly demonstrating benefits of such precautions. Hence, the possible interconversion of MIT and DIT during sample preparation and analysis will need to be studied. Analyte interconversion during chemical analysis has been studied using multiple spiking IDMS[29-33]. More recently, our research group has developed a double-spike method to evaluate creatine-creatinine interconversions during serum analyses[31] based on the differential labelling of both compounds ($^{13}C_1$ and $^{13}C_2$) allowing the detection and correction of interconversion reactions. On the other hand, the use of minimally ^{13}C- or ^{15}N-labelled compounds, previously characterized in terms of concentration and isotopic enrichment, reduces the occurrence of isotope effects and avoids the use of standard curves when multiple linear regression is applied[31,34].

To the best of our knowledge, only a few methods have been developed to determine MIT and DIT in biological fluids and none of these has been applied to study ITDD in neonates[8,9,25,26]. Those methods lack of an adequate internal standardization that may compromise the quality of the results and do not take into account the possibility of interconversion reactions. We present here the synthesis of differential, stable isotopically labelled ^{15}N-3-monoiodotyrosine (^{15}N-MIT) and $^{13}C_2$-3,5-diiodotyrosine ($^{13}C_2$-DIT) and their application to the development of an IDMS method to quantify their natural analogues in liquid urine and dried urine spot (DUS) of newborns. Furthermore, our method allows the application of double-spike IDMS equations to evaluate and correct possible MIT and DIT interconversions during chemical analysis. The method was applied to measure the levels of these compounds in neonatal urine and DUS samples, what could be considered the first step to assess the real prevalence of ITDD.

EXPERIMENTAL

Reagents and materials

L-tyrosine labelled in one ^{15}N atom (^{15}N-tyrosine) and creatinine labelled in one ^{13}C (methyl group) were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-tyrosine labelled in two ^{13}C atoms ($^{13}C_2$-tyrosine, labelled in the positions 3 and 5 of the phenyl group) was purchased from Cambridge Isotope Laboratories (MA, USA). HBF_4 was acquired from Sigma-Aldrich. IPy_2BF_4 was synthesized and provided by the Selective Organic Synthesis Group at the Department of Organic and Inorganic Chemistry of the University of Oviedo. Natural abundance L-3-monoiodotyrosine and L-3,5-diiodotyrosine standards were purchased from Acros Organics (Geel, Belgium). Formic acid (FA), trifluoroacetic acid (TFA), sodium hydroxide and hydrogen chloride-1-butanol solutions were purchased from Sigma-Aldrich. Acetonitrile (ACN) Optima LC-MS grade was purchased from Fischer Scientific (Waltham, MA, USA). Ultrapure water was obtained from a Purelab Flex 4 system Veolia (France). Samples of frozen human urine and DUS from anonymous neonates were provided by the Laboratory of Metabolic Disorders of the Santiago de Compostela University

Hospital. DUS were stored in collection paper SS 903 (Schleicher & Schuell, Germany). Eppendorf tubes (1.5 mL) were purchased from Sigma-Aldrich.

Instrumentation

Chromatographic separations were carried out using an ultra-high-performance liquid chromatography (UPLC) system Agilent series 1290 (Agilent Technologies, Santa Clara, CA). The UPLC system was connected to a triple quadrupole mass spectrometer Agilent 6460 equipped with "jet-stream" electrospray ionization source operating in positive ionization mode. The preparative-LC system employed for the purification of the synthetized ^{15}N-3-monoiodotyrosine was an Agilent 1260 Infinity. A heating oven Heraeus® (Thermo, MA, USA) was used for stability experiments at 50 °C. An analytical balance model AB204-S (Mettler Toledo, Zurich, Switzerland) was used for the gravimetric preparation of all solutions. A centrifuge Micro STAR 17 from VWR (PA, USA) and an evaporator MiVac from Supelco (St. Louis, MO, USA) were used for the centrifugation and evaporation of the samples, respectively. A rotary Evaporator R-300 from Büchi (Flawil, Switzerland) was used for the evaporation of the solutions obtained during the synthesis of the labelled MIT and DIT when required. A pH-meter Basic 20 from Crison, (Barcelona, Spain) was used to adjust pH of the solutions (e.g., chromatographic mobile phases) and for the precipitation of the synthetized $^{13}C_2$-3,5-diiodotyrosine.

Procedures

Synthesis, purification and characterization of ^{15}N-monoiodotyrosine and $^{13}C_2$-diiodotyrosine

The reaction schemes for the synthesis of ^{15}N-MIT and $^{13}C_2$-DIT are shown in Figure 1. A mass of ca. 100 mg of ^{15}N-tyrosine was placed inside a 50 mL flask and 5 mL of dried ACN were added as reaction medium. Then, 0.37 mL of HBF_4 48 % (w/w) were added while stirring to dissolve tyrosine. Finally, 200 mg of IPy_2BF_4 were slowly added under N_2 atmosphere and a slight stirring. The reaction took place immediately and the color of the

solution turned to light yellow. Then, the solution was evaporated *in vacuo* (8 x 10^{-3} mbar) to purify the product from some by-products as iodine or pyridine and the dry residue was dissolved in a few milliliters of ACN 10 % (v/v) and TFA 0.1 % (v/v) aqueous solution. The isolation of ^{15}N-3-monoiodotyrosine from ^{15}N-3,5-diiodotyrosine (^{15}N-DIT) and ^{15}N-tyrosine impurities was carried out by semi-preparative HPLC as described in Table S1 of the electronic supplementary information. The isolated solid product was stored in dark at 4 °C under N_2 atmosphere.

For the synthesis of $^{13}C_2$-3,5-diiodotyrosine a similar procedure was applied with a larger amount, 490 mg, of IPy_2BF_4 added to 100 mg of the starting $^{13}C_2$-tyrosine. As in the other synthesis the reaction took place instantly and the color of the solution turned to orange due to the excess of iodinating reagent. The solution was evaporated *in vacuo* (8 x 10^{-3} mbar) to purify the product and the dry residue was dissolved in 5 mL of ultrapure water. Then, the solution was filtered, and the eluate was transferred to a flask. Separation and isolation of $^{13}C_2$-3,5-diiodotyrosine from $^{13}C_2$-3-monoiodotyrosine ($^{13}C_2$-MIT) and $^{13}C_2$-tyrosine impurities were carried out by isoelectric point precipitation at pH = 4.29[35]. To do so, 400 µL of NaOH 3M solution was added drop by drop under vigorous stirring until precipitation. The precipitate was filtered and washed with ultrapure water. Then, the precipitate was dissolved in 5 mL of HCl 1 % (w/w) aqueous solution and the precipitation was performed once again. After drying, the solid was preserved in dark at 4 °C under N_2 atmosphere.

The isotopic enrichment of the synthesized ^{15}N-MIT and $^{13}C_2$-DIT was calculated as described previously[36] and resulted in 99.13±0.06 % for ^{15}N-MIT and 99.10±0.02 % for $^{13}C_2$-DIT. The concentration of the labelled standards as well as the $^{13}C_2$-MIT concentration in the synthesized $^{13}C_2$-DIT and the ^{15}N-DIT concentration in the synthesized ^{15}N-MIT were determined by reverse IDMS using the natural abundance standards.

Sample preparation for urine and DUS samples

Urine samples stored at -20 °C were thawed on ice and centrifuged (14000 rpm) at 4 °C for 10 min. Then, ca. 0.1 g of a 100 µg·g^{-1} solution of $^{13}C_1$-creatinine and ca. 0.1 g of a 2.5 ng·g^{-1} mixture of $^{15}N_1$-MIT and $^{13}C_2$-DIT were added to ca. 0.2 g urine into 2 mL glass vials. The addition of sample and labelled standards was always controlled gravimetrically. After vortex

mixing, samples were evaporated and reconstituted with 100 µL butanol-HCl 3 M by vigorously shaken for 1 min. Then, vials were centrifuged (14000 rpm) for 5 min and the supernatant was transferred to 2 mL glass vial for derivatization at 70 °C for 30 minutes[37]. After derivatization, the solutions were evaporated under vacuum. Finally, the dry residue was reconstituted with 50 µL 0.1 % (v/v) FA, centrifuged to 14000 rpm for 5 min and the supernatant was submitted to chromatographic separation. The injection volume was 10 µL.

DUS samples stored at -20 °C were left to reach room temperature. Our method was designed to take 12 spots of $^1/_8$ inches per sample using a die cutter. Then, these spots were extracted with 300 µL H_2O in 1.5 mL Eppendort tubes by shaking at 700 rpm for 30 min at room temperature. Next, tubes were centrifuged at 15000 rpm for 3 min and 200 µL of the supernatant was taken and transferred to 2 mL glass vials. Following, ca. 0.1 g of ^{13}C-creatinine 100 µg·g^{-1} solution and ca. 0.1 g of individual ^{15}N-MIT and $^{13}C_2$-DIT 2.5 ng·g^{-1} solutions was added gravimetrically. Then, the samples were treated following the same procedure as that described above for liquid urine samples.

LC-MS/MS analysis of the samples

The chromatographic separation of creatinine, MIT and DIT was carried out using a reverse-phase Zorbax C18 Eclipse Plus analytical column (2.1 mm x 50 mm and 1.8 µm particle size). LC-MS/MS analyses were performed in a gradient elution mode using an aqueous mobile phase A (0.1 % (v/v) FA) and an organic mobile phase B (0.1 % (v/v) FA in ACN). The experimental chromatographic conditions are summarized in Table S2 of the electronic supplementary information. MS/MS analysis was performed using positive-ion electrospray ionization and the selected reaction monitoring (SRM) mode. Instrumental parameters for the detection of the compounds were optimized by direct injection of 1 µg·g^{-1} standard solutions of natural abundance MIT and DIT dissolved in a mixture of mobile phases A and B (1:1). The data acquisition and treatment was carried out using an Agilent MassHunter Workstation ver. B.06.00. Experimental mass spectrometric conditions are shown in Table S3 of the electronic supplementary information.

IDMS procedures

The determination of MIT and DIT together with the creatinine content in urine was based on the Isotope Pattern Deconvolution approach described before[30]. For creatinine a simple two-pattern equation 1 was employed:

$$\begin{bmatrix} A_1^m \\ A_2^m \\ A_3^m \end{bmatrix} = \begin{bmatrix} A_1^s & A_1^t \\ A_2^s & A_2^t \\ A_3^s & A_3^t \end{bmatrix} \times \begin{bmatrix} x_s \\ x_t \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \\ e_3 \end{bmatrix} \qquad (1)$$

Where A^m, A^s and A^t are the relative abundances for three selected nominal masses (1, 2 and 3) for the mixture (m), the sample (s) and the tracer (t) respectively. An error vector e must be included to solve in equation (1) by multiple linear regression. The unknowns x_s and x_t, the molar fractions of sample and tracer in the mixture, are calculated by a simple matrix inversion procedure (using the LINEST equation in Excel). For the IDMS determination of creatinine the masses were: M, M+1 and M+2 respectively as the tracer was labelled with only one ^{13}C atom.

Due to the differential labelling between MIT and DIT and the presence of minor impurities of $^{13}C_2$-MIT in the $^{13}C_2$-DIT spike solution, the matrix calculations both for MIT and DIT was based on three isotopic patterns[30] and four MRM transitions for each compound. In brief, the isotopic composition of the mixture of sample and tracer was deconvoluted by linear regression using the equation 2:

$$\begin{bmatrix} A_1^m \\ A_2^m \\ A_3^m \\ A_4^m \end{bmatrix} = \begin{bmatrix} A_1^s & A_1^{t1} & A_1^{t2} \\ A_2^s & A_2^{t1} & A_2^{t2} \\ A_3^s & A_3^{t1} & A_3^{t2} \\ A_4^s & A_4^{t1} & A_4^{t2} \end{bmatrix} \times \begin{bmatrix} x_s \\ x_{t1} \\ x_{t2} \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \\ e_3 \\ e_4 \end{bmatrix} \qquad (2)$$

Where A^m, A^s , A^{t1} and A^{t2} are the relative abundances for four selected MRM transitions for the mixture (m), the sample (s) and the tracers ($t1$ for MIT and $t2$ for DIT respectively) and e refers to the error vector. The unknowns x_s, x_{t1} and x_{t2}, the molar fractions of sample and both tracers in the mixture, are calculated applying the equation for each compound (MIT and DIT independently).

Once the molar fractions were calculated the final concentrations of the three compounds in the sample were calculated using equations 3 to 5[30]:

241 $$C_s = C_t \times \frac{x_s}{x_t} \times \frac{m_t}{m_s} \times \frac{w_s}{w_t}$$ (3), in the creatinine chromatographic peak,

242 $$C_s = C_{t1} \times \frac{x_s}{x_{t1}} \times \frac{m_{t1}}{m_s} \times \frac{w_s}{w_{t1}}$$ (4), in the MIT chromatographic peak and

243 $$C_s = C_{t2} \times \frac{x_s}{x_{t2}} \times \frac{m_{t2}}{m_s} \times \frac{w_s}{w_{t2}}$$ (5), in the DIT chromatographic peak respectively.

244 Where C_s is the concentration of the analyte in the sample (unknown). C_t, C_{t1} and C_{t2} are the
245 known concentrations of the tracers for $^{13}C_1$-creatinine, $^{15}N_1$-MIT and $^{13}C_2$-DIT,
246 respectively; m_s, m_t, m_{t1} and m_{t2} are the masses taken from sample and the three tracers during
247 sample preparation while w_s, w_t, w_{t1} and w_{t2} are the molecular weights of the natural
248 abundance and labelled analytes respectively. In this initial calculation the possible
249 interconversion of MIT and DIT is not taken into account and the molar fractions x_{t2} in the
250 MIT peak and x_{t1} in the DIT peak are not used. This procedure is called "single-spike IDMS"
251 throughout the paper. As previously demonstrated,[30] using the proposed IDMS quantification
252 strategy the measurement of the isotopic distribution and the concentration of the internal
253 standard are the most important uncertainty sources contributing to the to the total combined
254 uncertainty.

255 For the evaluation of the possible interconversion of MIT and DIT during sample
256 preparation/analysis according to the process:

257 $$MIT \underset{F1 \rightarrow}{\overset{\leftarrow F2}{\rightleftharpoons}} DIT$$

258 A double-spike calculation procedure was applied based on that described before for
259 creatine-creatinine interconversion[31]. In this alternative calculation procedure, the molar
260 fractions x_{t2} in the MIT peak is used to compute the fraction of DIT converted to MIT during
261 sample preparation (factor *F2*) while the molar fraction x_{t1} in the DIT peak is used to compute
262 the fraction of MIT converted to DIT (factor *F1*). A complete explanation of the
263 mathematical equations for "double-spike IDMS" is given in the references[30,31]. We have
264 also included this information, specific for MIT and DIT, in the electronic supplementary
265 information. Basically, using the double-spike approach, we can compute in each sample the
266 corresponding interconversion factors *F1* and *F2* and the degradation-corrected
267 concentrations of MIT and DIT.

RESULTS AND DISCUSSION

Synthesis and purification of labelled iodotyrosines.

Isotopically labelled standards of MIT or DIT are not commercially available, thus, we attempted the synthesis of labelled MIT and DIT through the iodination of isotopically labelled tyrosine. The efficient control in the iodination of aromatic compounds at room temperature requires the use of specific reagents, such as IPy_2BF_4[38,39]. This reagent was used to iodinate commercially available ^{15}N-tyrosine and $^{13}C_2$-tyrosine, which were used as labelled starting material as described in Figure 1. Using two different labelled starting materials, a double spike strategy can be applied to correct for interconversions throughout the chemical analysis as described previously for creatine and creatinine[31].

For the synthesis of $^{13}C_2$-DIT, we used an excess of iodination reagent to ensure a high reaction yield (71 %) and low impurities of $^{13}C_2$-MIT. Final purification of $^{13}C_2$-DIT was carried out by isoelectric point precipitation at pH 4.30. Figure S1 of the electronic supplementary information shows a LC-ESI-MS chromatogram acquired in SCAN mode for the synthetized crude of $^{13}C_2$-DIT before and after purification by isoelectric point precipitation. The ratio of peak areas of MIT and DIT decreased from 1:60 to 1:90 after purification.

A lower reaction yield (44 %) was obtained in the synthesis of ^{15}N-MIT. In this case, we used stoichiometric amounts of IPy_2BF_4 and applied a low stirring rate. However, a 2:3 ratio between ^{15}N-DIT and ^{15}N-MIT was obtained before purification. In this case, purification by isoelectric point precipitation was not possible, as we did not observe precipitation, probably because of kinetic factors. Therefore, purification was carried out by semi-preparative-HPLC. Figure S2 of the electronic supplementary information shows a LC-ESI-MS chromatogram acquired in SCAN of the synthetized crude of ^{15}N-MIT before and after purification by semi-preparative LC. The experimental chromatographic conditions applied for ^{15}N-MIT purification are summarized in Table S1 of the electronic supplementary information. A 1:350 ratio between ^{15}N-DIT and ^{15}N-MIT was obtained in the final purified product.

297

298 **Measurement of the isotope composition of natural abundance MIT and DIT by**
299 **LC-ESI-MS/MS and selection of the MRM transitions.**

300 Using the optimized chromatographic conditions of Table S2 in the electronic supplementary
301 information, the chromatographic retention times for derivatized MIT and DIT were 7.8 and
302 8.8 min, respectively, whereas creatinine eluted at 0.5 min. Urinary creatinine was measured
303 to normalize the concentrations of MIT and DIT found in urine samples and DUS. The
304 protonated molecular ion $[M+H]^+$ was selected as precursor ion for creatinine, MIT and DIT.
305 Product ions $[(M+H)-102]^+$, arising from the loss of C_4H_8, H_2O and CO by collision induced
306 dissociation (CID), were selected for MIT and DIT due to their high intensity. The most
307 abundant product ion for creatinine was the loss of CO. Collision energies and fragmentor
308 voltages of 5 V and 135 V were selected for MIT, respectively, whereas 10 V and 135 V
309 were selected for DIT. All Mass Spectrometry conditions are indicated in Table S3 of the
310 electronic supplementary information.

311 The measurement of the isotopic distribution of natural abundance compounds was studied
312 for the selected clusters to check the accuracy of the measurements injecting into the
313 LC-MS/MS system individual 1 $\mu g \cdot g^{-1}$ solutions of each compound. Theoretical isotope
314 distributions of in-cell fragment ions measured by MRM can be calculated by a suitable
315 MRM dedicated software such as IsoPatrn©[40] and compared with the experimental values.
316 Table 1 shows that, for natural abundance and labelled MIT and DIT, there is good agreement
317 between the experimental and theoretical isotopic compositions. The uncertainty of
318 experimental values is indicated as the standard deviation of n=5 independent LC-MS/MS
319 injections. Experimental abundances shown in Table 1 were employed in the IDMS
320 calculations.

321

322 **Calculation of the isotopic enrichment, concentration and purity of the synthesized**
323 **^{15}N-MIT and $^{13}C_2$-DIT.**

The application of IDMS with multiple linear regression using the characterized labelled analogues allows the direct determination of the analytes in a sample without resorting to calibration graphs[30,34] as indicated in the procedures. The successful application of this strategy requires the knowledge of the isotopic composition and the concentration of the labelled analogues. We have previously developed in our laboratory a procedure to calculate isotopic enrichments of labelled organic compounds[36] based on the experimental measurement of their isotopic distribution. For this purpose, individual 1 $\mu g \cdot g^{-1}$ solutions of both labelled standards were injected (n=5) into the LC-MS/MS system. The isotopic enrichments (^{13}C or ^{15}N abundance) obtained for the synthesized products were 99.13±0.06 % and 99.10±0.02 % for ^{15}N-monoiodotyrosine and $^{13}C_2$-diiodotyrosine, respectively. The concentration of the tracers was calculated by reverse IDMS using the natural abundance compounds as standards and it is described in the next section on stability studies. In addition, the purity of the labelled synthesized products was determined by calculating the concentration of ^{15}N-DIT and $^{13}C_2$-MIT impurities in ^{15}N-MIT and $^{13}C_2$-DIT, respectively. The percentages of the impurities in the final solid purified products were 0.07 % (w/w) and 1.32 % (w/w) for ^{15}N-MIT and $^{13}C_2$-DIT, respectively. These impurities were taken into account in the double-spike IDMS calculations as indicated by the equations given in the electronic supplementary information. The isotope compositions measured for the labelled compounds are also shown in Table 1 in comparison with the theoretical abundances. The agreement can be considered satisfactory for ^{15}N-MIT but not quite so for $^{13}C_2$-DIT where a 2 % contamination with natural abundance DIT is detected (2 % abundance for the transition 490→388 in $^{13}C_2$-DIT). Thus, the experimental abundances shown in Table 1 were employed in the IDMS calculations so this possible contamination was taken into account and corrected.

Stability studies.

Previous works have reported the use of antioxidants to suppress free radical oxidation and/or metal induced oxidation of iodine containing molecules[12,13]. Recently, Hansen *et al.* reported the degradation of several THMs under different pH conditions and their photolytic deiodination[16] and, on the other hand, Richards *et al.* reported no statistical difference in the

serum concentration of 4 THMs when applying three freeze/thaw cycles[17]. However, the stability of MIT and DIT solutions has not been studied so far. In our case we employed a reverse IDMS experiment to check the concentration and long-term stability of ^{15}N-MIT and $^{13}C_2$-DIT dissolved at 1 $\mu g \cdot g^{-1}$ levels in 0.1 % formic acid in water (v/v) and stored at 4 ºC in the dark. The results for a six-month stability study are shown in Figure 2 expressed as the percentage of the ratio of the measured concentration to the initial concentration. As can be observed, the results ranged between 93 and 104 % during the whole duration of the experiment indicating that the tracers were stable.

We also performed a different approach to study the stability of 1 $\mu g \cdot g^{-1}$ solutions of the in-house synthesized ^{15}N-MIT and $^{13}C_2$-DIT tracers based on guideline 111 of the Organization for Economic Co-operation and Development (OECD) to test the hydrolysis of compounds as a function of pH[41]. In our case, this experiment consisted on a five-day accelerated stability study at 50 ºC. To do that, three individual 1 $\mu g \cdot g^{-1}$ solutions of ^{15}N-MIT and $^{13}C_2$-DIT were prepared in different aqueous buffer solutions in amber vials. Ammonium formate 10 mM, ammonium bicarbonate 10 mM and ammonium acetate 10 mM were employed to obtain buffer solutions at pH 4.0, 7.0 and 9.0, respectively. These solutions were stored at 50 °C in the dark and their concentrations were measured by triplicate for a period of five days by reverse IDMS. Figure S3 shows the results as a function of the initial concentration. As can be observed, MIT degradation was not significantly affected by pH, whereas DIT showed a degradation factor around 20 % at acidic and neutral pH, what is in agreement with the results obtained by Hansen *et al*[16].

Matrix effects on MIT and DIT determination in urine by LC-MS/MS.

The complexity of urine samples is highly variable[42] so creatinine concentration in urine is used in clinical diagnosis as an adjustment factor to give relative substance concentrations in urine[43]. Hence, matrix effects in the ESI source must be carefully study for each target compound evaluating not only the initial amount of urine taken and the injection volume but also the matrix load based on their creatinine content. For the evaluation of the initial sample amount, 2 mL of a pooled urine sample, containing a creatinine concentration of 1.3 mg g^{-1},

were spiked with 50 ng of both MIT and DIT (natural abundances). Different amounts (50, 100, 150, 200 and 250 mg) of this urine sample were taken and analyzed following the sample preparation procedure described in the experimental section using a constant injection volume of 1 μL. Figure S4A of the electronic supplementary information shows an increase in the MIT and DIT signal up to 200 mg of urine. Then, the signal decreased for both compounds when analyzing 250 mg of the urine sample.

Taking a fixed sample amount of 200 mg, we evaluated the signals obtained for different injection volumes (1, 2, 5, 10, 20 μL). The variation of signals for MIT and DIT are presented in Figure S4B of the electronic supplementary information. These results showed that 10 and 20 μL provided the highest signal for both MIT and DIT. Therefore, 10 μL were selected as the optimum injection volume.

Finally, five different dilutions of another urine sample, dilution factors of 24, 10, 5, 2 and 1 (undiluted), were analyzed to study the signal variation with the dilution factor fixing the sample amount (200 mg) and the injection volume (10 μL). Each dilution was spiked with the same amount MIT and DIT to yield a final concentration of 2.5 ng g^{-1} after dilution. Figure S5 of the electronic supplementary information shows that both MIT and DIT signals increased for the more diluted urine samples. This was expected, as the dilution factor is correlated with the amount of coeluting compounds that may eventually compromise the ionization efficiency at the retention times of MIT and DIT. We expected that the use of labelled internal standards would compensate for this variable suppression effect as discussed in the next section.

Recovery studies for the analysis of liquid urine samples.

Recovery studies were performed in the same pooled human urine sample at different dilution factors (24, 10, 5, 2, undiluted). After dilution, the samples were fortified with natural abundance MIT and DIT at three different levels (0.25, 0.625 and 1.25 ng·g^{-1}) and analyzed as described in the procedures. The results obtained for MIT and DIT for the different fortifications and dilution factors assayed, both by single-spike IDMS and double-spike IDMS, are shown in Table 2. These recovery values are obtained from the slope of the linear

412 plot of “found concentration” vs “added concentration” while the uncertainty values
413 correspond to the standard error of the slopes obtained. The endogenous MIT and DIT
414 concentration of the undiluted urine sample was established from the intercept of the plots
415 and the dilution factor applied and was, on average, 2.0 $ng \cdot g^{-1}$ MIT and 0.50 $ng \cdot g^{-1}$ DIT for
416 the undiluted urine sample containing 1.3 $mg \cdot g^{-1}$ creatinine. As it can be seen in Table 2
417 recovery values can be considered quantitative for MIT and DIT at all dilution factors except
418 for MIT determination in the undiluted urine. There seems to be no effect of the calculation
419 mode (single-spike or double-spike) except for MIT determination in the undiluted urine (34
420 and 43 % recovery respectively) where a deiodination factor *F1* of ca. 19 % was measured
421 by double-spike IDMS. Regarding interconversion factors, no MIT iodination (*F2*, formation
422 of ^{15}N-DIT) was detected in any of the samples. This was expected, as iodination reactions
423 are unlikely to occur. Instead of that, deiodination of DIT (*F1*, formation of $^{13}C_2$-MIT) was
424 only detected in the undiluted urine (19 %) as indicated above.

425 It is worth noting here that the recovery results for undiluted urine, particularly for MIT, are
426 probably underestimated due to statistical reasons. The endogenous concentration of MIT in
427 the undiluted urine sample (2.0 $ng \cdot g^{-1}$) was too high for the selected additions (0.25, 0.625
428 and 1.25 $ng \cdot g^{-1}$) and so the uncertainty of the slope in the representation “found vs added”
429 was around ±20 % (standard error of the slope) both by single-spike and double-spike for
430 MIT. Better values are obtained for a dilution factor of 2 but still the uncertainties of the
431 recoveries for MIT were quite high (8-10 %). As the deiodination factor *F1* start to be
432 noticeable for a dilution factor of 2 (3.7 %) and increased for the undiluted urine sample it
433 was decided not to repeat the experiments with higher additions of MIT and to recommend
434 a dilution factor of 5 for the liquid urine samples to assure quantitative recoveries and avoid
435 interconversion reactions taking place. In that way, single-spike IDMS calculations can be
436 performed avoiding more complicated data treatment procedures such as double-spike
437 IDMS.

438

439 **Recovery studies for the analysis of dried urine spots (DUS)**

When analyzing DUS the sample amount taken in each spot depends on the amount and distribution of the urine impregnated on the surface as well as the number and dimension of the spots taken for analysis. Therefore, concentration values of MIT and DIT in DUS need to be expressed always as relative to the creatinine content, as the exact amount of urine sample taken in each analysis is unknown and variable. Additionally, the content of matrix in the extracted DUS samples is much lower than in the original urine due to the dilution effect of the extraction process. Recovery studies were carried out by fortifying two newborn urine samples with MIT and DIT at three different levels (between 0.15 and 0.90 $ng \cdot g^{-1}$). Filter paper sheets SS 903 were impregnated with the fortified samples and dried for 3 h at room temperature in the dark and extracted as described in the Experimental section. The main difference compared to the procedure applied for liquid urine samples is that the labelled analogues were added to the isolated supernatant after extraction of the urine from the spot. Figure 3 shows a representative LC-MS/MS chromatogram acquired in the MRM mode of a fortified DUS sample spiked with 0.60 $ng \cdot g^{-1}$ of both MIT and DIT (in the original urine sample). As it can be observed, for the three transitions indicated for each compound, adequate signal to noise ratios are obtained allowing the right integration of the corresponding chromatograms. Table 3 shows the recovery values obtained for the analysis of the DUS by using both double-spike IDMS and single-spike IDMS calculations. For this experiment, two samples containing 1.4 and 2.7 ng $MIT \cdot mg^{-1}$ creatinine and 3.1 and 5.0 ng $DIT \cdot mg^{-1}$ creatinine, respectively, were fortified at different levels with MIT and DIT up to 14 ng $MIT \cdot mg^{-1}$ creatinine and 17 ng $DIT \cdot mg^{-1}$ creatinine, respectively. As can be observed, the recoveries obtained ranged from 104 to 132 % both by double-spike IDMS and single-spike IDMS for the two samples evaluated. Recoveries and reproducibility of recovery values are marginally better by single-spike IDMS. Regarding interconversion factors, all values were, in all cases, close to 0 (average values are shown in Table 3 for double-spike calculations) which means that no interconversion was detected in DUS which is an advantage in comparison with liquid urine samples at high matrix content.

Method limits of detection and quantification

The limits of detection (LOD) were calculated as three times the standard deviation of six water blanks analyzed by the proposed IDMS procedures as no samples free of MIT and/or DIT could be obtained. The detection limits obtained were 0.019 and 0.045 $ng \cdot g^{-1}$ by double-spike IDMS and 0.018 and 0.046 $ng \cdot g^{-1}$ by single-spike IDMS, for MIT and DIT respectively. The limits of quantification were calculated as ten times the standard deviation of six blanks obtaining 0.06 and 0.15 $ng.g^{-1}$ for MIT and DIT, respectively. No interconversion reactions were detected in the blanks with similar detection limits regardless of the calculation procedure employed. Afink *et al*.[8] reported LODs of 0.2 $pmol \cdot g^{-1}$ for MIT and DIT in aqueous solutions (which corresponds to 0.061 and 0.087 $ng \cdot g^{-1}$ for MIT and DIT respectively). Han *et al*.[25] measured MIT and DIT in aqueous solutions by Liquid Chromatography coupled to Inductively Coupled Plasma Mass Spectrometry (LC-ICP-MS) reaching marginally better LODs than those reported by Afink (0.19 $pmol \cdot g^{-1}$ and 0.17 $pmol \cdot g^{-1}$ for MIT and DIT, respectively). In conclusion, our method offers LODs between 2 and 3 time lower than those reported previously[8,25].

The limits of detection for the analysis of DUS were calculated analyzing n=6 independent blanks. The blank analyses were carried out with non-impregnated filter paper sheets and following the indicated sample preparation procedure. Each blank value was obtained from the average of n=3 independent LC-MS/MS injections and the limit of detection was calculated as three times the standard deviation of the concentrations found in the blanks. The limits of detection were 0.07 and 0.05 $ng \cdot g^{-1}$ in the extract for MIT and DIT, respectively and the limits of quantification were 0.23 and 0.17 $ng.g^{-1}$ for MIT and DIT, respectively.

Analysis of newborn urine and DUS samples

Comparison of the analysis of liquid urine vs DUS

The single-spike IDMS methodology was applied to the determination of MIT and DIT in 14 newborn urine samples stored both as liquid urine and DUS. Samples were taken from the Newborn Screening Program of the University Hospital of Santiago de Compostela (Spain) in the first days of life of newborns, stored at -20 °C until their determination. This work was carried out in accordance with the ethical standards of the University Hospital of Santiago de

Compostela and the "World Medical Association Declaration of Helsinki¨ for the ethical principles for Medical Research involving human subjects. In order to properly compare the results obtained in both type of samples, MIT and DIT were referred to the urinary creatinine concentration and the results expressed as $ng \cdot mg^{-1}$ creatinine. MIT was present in all samples, however, only a few DUS sample provided DIT values above the limit of detection so no comparison was possible for DIT. One sample (sample 4) provided abnormally low creatinine concentrations, both in liquid urine and DUS, and was eliminated from the comparison. Figure 4 shows the comparison of the results obtained for both type of the samples for MIT. As can be observed, the results obtained in both type of samples are in good general agreement taking into account the standard deviation of three independent replicates. Please note that these measurements require 2 independent determinations per sample (MIT and creatinine) increasing the uncertainty of the comparison. Furthermore, the results of the validation regarding the recovery (Table 2 and Table 3) cover the results shown in Figure 4.

Repeatability, intraday and interday variability in the measurement of MIT in newborn DUS samples

Three DUS samples containing different levels of MIT were measured to evaluate the repeatability (several injections of the same extract), the intraday reproducibility (several independent sample preparations and injections in the same day) and the interday variability repeating the experiment on different days. The results obtained are collected in Table 4. For the two more concentrated samples (DUS 1 and 2) repeatability varies between 1.0 and 16.6 % (average 7.0 %, n=18) while intraday variability varies between 2.0 and 13.3 % (average 7.5 %, n=6). However, interday variability increases to ca. 15 % for both samples indicating that this last factor could be the main source of uncertainty. Regarding sample DUS 3, containing a lower concentration of MIT, the uncertainty is about a factor of two higher than for the other two samples. According to the literature[8], levels of MIT in ITDD patients should be about 50 times higher than in controls so the variability of the measurements observed for healthy newborns in Table 4 should be more than adequate to detect ITDD in newborn screening programs.

MIT levels in newborn DUS samples

A total of 38 DUS samples were measured for MIT and DIT to investigate the levels in healthy newborns and their variability. MIT was detected in all samples while DIT was only detected clearly in 4 out of the 38 measured DUS samples. Two samples containing anomalous low creatinine levels were eliminated from the dataset. The results obtained for MIT in 36 samples are shown in Figure 5. The median of all values was 3.4 ng MIT·mg^{-1} creatinine with a total range between 0.4 and 26.4 ng MIT·mg^{-1} creatinine. Again, the results of the validation regarding the recovery using DUS (Table 3) cover most of the results shown in Figure 5.

These values could give an initial idea of the levels of MIT in healthy newborns but more measurements would be required to establish reference control levels and to check the real prevalence of ITDD in newborns.

CONCLUSIONS

The synthesis of isotopically labelled MIT and DIT has enabled the development of a IDMS methodology capable of quantifying both iodinated amino acids in newborn urine samples and MIT in DUS samples. Using a different isotopic label in each compound we have developed a double spike strategy to correct and quantify the interconversion between both compounds throughout the analytical methodology. DIT deiodination has been observed in concentrated liquid urine samples but no deiodination was apparent after 5 times dilution. For DUS samples no interconversion reactions were detected. Method detection limits were 0.018 and 0.046 ng·g^{-1} by single-spike IDMS, for MIT and DIT, respectively, in the analysis of urine samples and 0.07 and 0.05 ng·g^{-1} for MIT and DIT, respectively, in the analysis of DUS. So, a single-spike IDMS methodology was proposed for the determination of MIT and DIT in urine samples and DUS. Good agreement was obtained for MIT concentrations when analyzing the same samples stored as liquid urine and DUS allowing the study of the levels of MIT in DUS from healthy newborns. Unfortunately, the limit of detection was not low

556 enough to detect DIT in most of the DUS samples measured. The proposed methodology can
557 be a suitable tool to perform the first cross-sectional study to estimate the real prevalence of
558 ITDD in neonates what, at the same time, opens the possibility for the implementation of
559 MIT and DIT determination in Newborn Screening (NBS) programs.

560

561 **CONFLICTS OF INTEREST**

562 There are no conflicts to declare.

563

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FIGURES AND TABLES

Figure 1. Reaction schemes for the synthesis of: A) ^{15}N-3-monoiodotyrosine and B) $^{13}C_2$-3,5-diiodotyrosine.

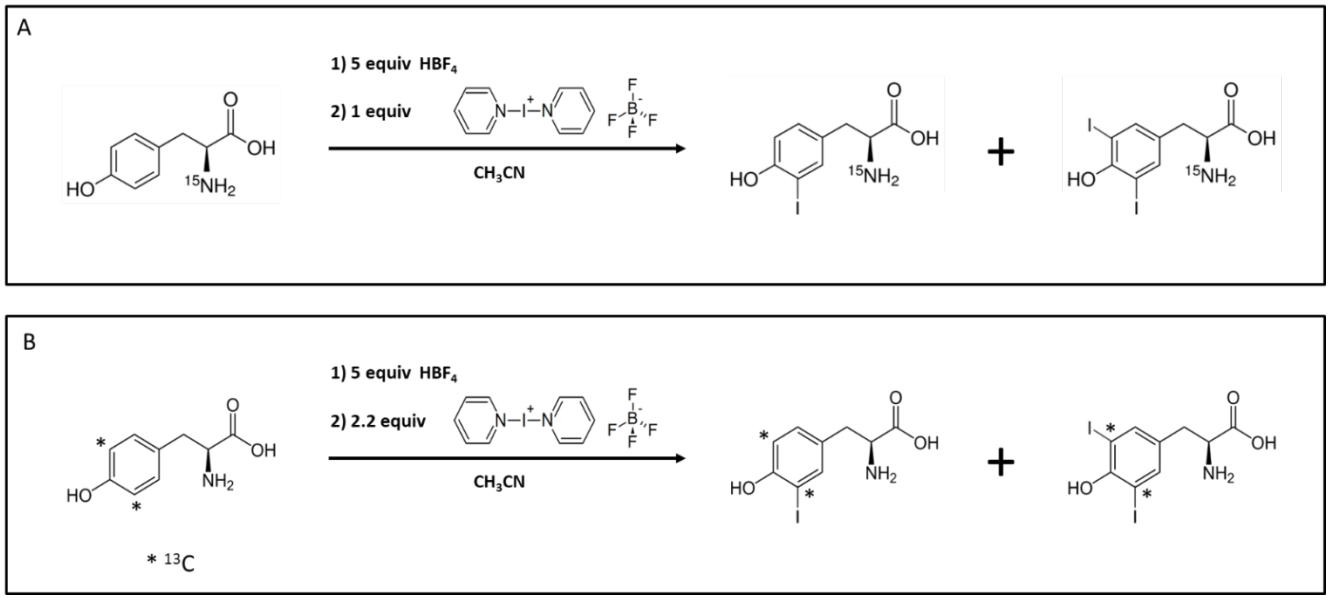

Table 1. Comparison of theoretical and experimental isotopic distribution for natural abundance (Nat) and labelled MIT and DIT.

Compound	Transition	Theoretical value	Experimental value	Standard deviation (n=5)
Nat-MIT	364→262	0.91104	0.91142	0.00016
	365→263	0.08345	0.08311	0.00017
	366→264	0.00521	0.00526	0.00003
	367→264	0.00025	0.00025	0.00000
^{15}N-MIT	364→262	0.00823	0.00777	0.00006
	365→263	0.90686	0.90900	0.00050
	366→264	0.07973	0.07838	0.00047
	367→265	0.00494	0.00485	0.00012
Nat-DIT	490→388	0.91115	0.91164	0.00034
	491→389	0.08335	0.08287	0.00032
	492→390	0.00525	0.00524	0.00001
	493→391	0.00025	0.00024	0.00000
$^{13}C_2$-DIT	490→388	0.00008	0.02132	0.00024
	491→389	0.01668	0.01885	0.00018
	492→390	0.91906	0.90019	0.00048
	493→391	0.06418	0.05963	0.00034

Figure 2.- Long-term stability of individual ^{15}N-MIT and $^{13}C_2$-DIT at 1 µg·g^{-1} solutions stored in 0.1 % formic acid at 4 °C and dark expressed as the percentage of the ratio of the measured concentration and the initial concentration.

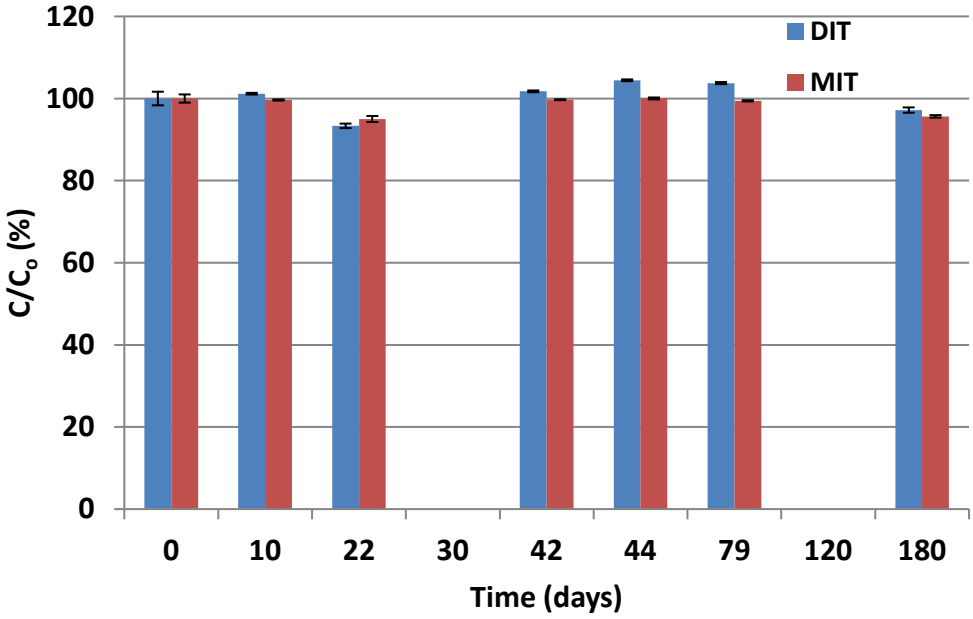

Table 2. Recoveries values obtained in the analysis of a liquid human urine samples fortified at 0.25, 0.625 and 1.25 $ng.g^{-1}$ of MIT and DIT at different dilution factors. The recovery is calculated as the slope of the representation of found *vs* added concentrations for the different dilution factors. The undiluted urine contained 1.3 $mg.g^{-1}$ creatinine. The average interconversion factors F1 (% DIT deiodination) and F2 (% MIT iodination) are also given in the table for the double-spike calculations. Uncertainty values correspond to the standard error of the slope.

Calculation mode	Dilution factor	MIT Recovery (%)	DIT Recovery (%)	F1 (%) DIT→MIT	F2 (%) MIT→DIT
Single-spike IDMS	24	99.7 ± 0.7	98.6 ± 1.2	-----	-----
	10	102.2 ± 1.3	105.0 ± 1.9	-----	-----
	5	102.5 ± 1.6	97.2 ± 4.3	-----	-----
	2	88.8 ± 8.2	88.7 ± 1.7	-----	-----
	Undiluted	33.9 ± 20.6	90.0 ± 8.4	-----	-----
Double-spike IDMS	24	102.7 ± 4.2	97.6 ± 9.6	-3.6	0.9
	10	104.5 ± 0.9	95.2 ± 3.9	-2.7	1.0
	5	101.5 ± 1.3	98.0 ± 3.8	-0.6	-4.7
	2	87.4 ± 10.2	92.7 ± 4.2	3.7	-0.4
	Undiluted	43.0 ± 18.7	94.0 ± 4.9	18.7	-0.3

Figure 3. LC-MS/MS chromatograms acquired in the MRM mode of a fortified DUS sample spiked with 0.60 $ng.g^{-1}$ for both MIT and DIT (in the original urine sample).

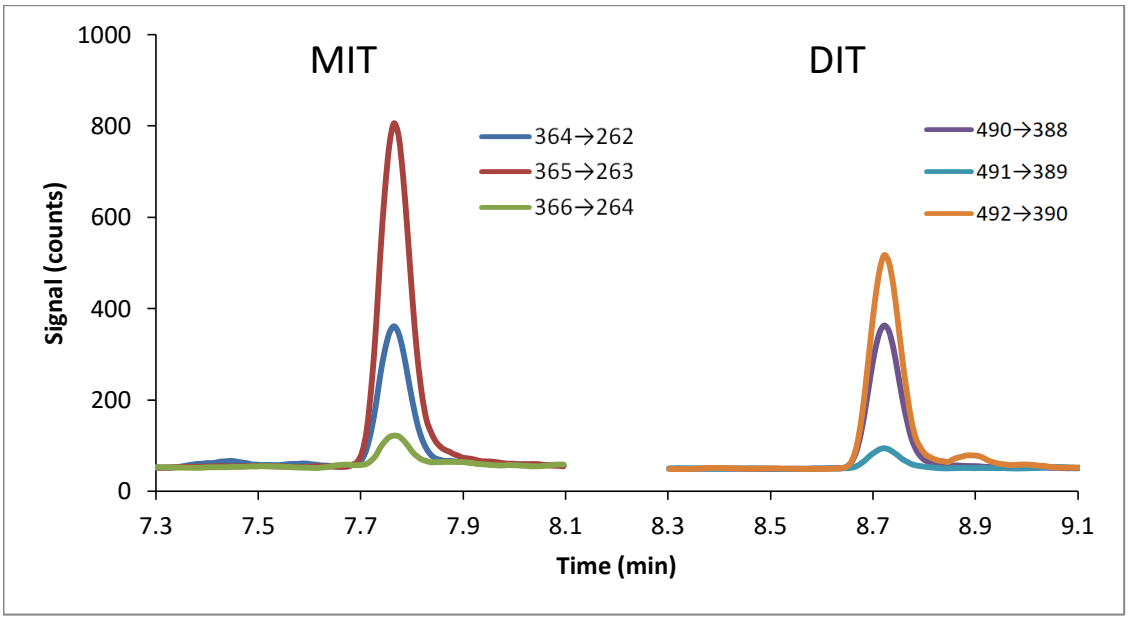

Table 3. Recoveries values obtained for MIT and DIT in the analysis of two fortified dried urine spots samples. Uncertainty values as in Table 2.

Calculation mode	Creatinine in the extract (μg g^{-1})	MIT Recovery (%)	DIT Recovery (%)	F1 (%) DIT→MIT	F2 (%) MIT→DIT
Single-spike IDMS	45.5	112.9 ± 3.6	104.9 ± 6.2	-----	-----
	85.6	113.1 ± 7.7	131.6 ± 17.9	-----	-----
Double-spike IDMS	45.5	117.6 ± 4.2	104.1 ± 6.1	-5.0	0.0
	85.6	120.9 ± 8.2	132.7 ± 18.6	-4.0	0.0

Figure 4. Results obtained in the determination of MIT in paired liquid urine and DUS samples of 14 neonates (ng MIT.mg^{-1} creatinine). Sample 4 provided abnormally low creatinine values and was eliminated from the comparison. Error bars indicate SD of the measurements (n=3 independent replicates).

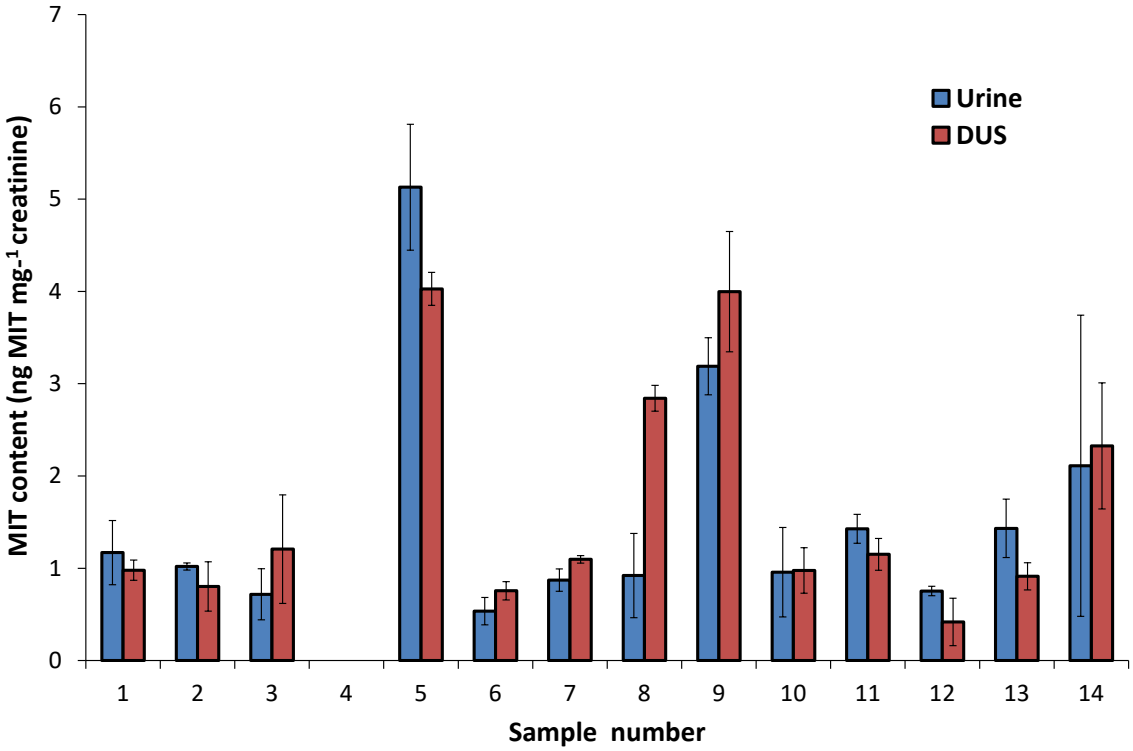

Table 4. Repeatability, intraday and interday variability in the determination of MIT in DUS samples. For each replicate 3 injections were performed and the uncertainty is given as SD of the different measurements.

		DUS 1		DUS 2		DUS 3	
		Concentration (ng MIT mg^{-1} creatinine)	RSD (%)	Concentration (ng MIT mg^{-1} creatinine)	RSD (%)	Concentration (ng MIT mg^{-1} creatinine)	RSD (%)
Day 1	Replicate 1	4.40±0.62	13.9	5.05±0.65	12.9	0.92±0.14	15.2
	Replicate 2	4.32±0.11	2.8	4.10±0.35	8.7	1.14±0.19	15.9
	Replicate 3	4.80±0.05	1.0	4.97±0.16	3.5	1.66±0.22	13.8
Average Day 1		4.51±0.27	5.8	4.70±0.52	11.2	1.25±0.38	30.4
Day 2	Replicate 1	5.18±0.27	5.1	4.97±0.11	2.4	1.28±0.05	3.8
	Replicate 2	4.45±0.54	12.3	3.85±0.14	3.6	1.06±0.11	11.3
	Replicate 3	4.59±0.49	10.9	4.15±0.19	4.9	1.14±0.16	14.4
Average Day 2		4.75±0.38	8.1	4.32±0.57	13.3	1.17±0.11	9.4
Day 3	Replicate 1	3.37±0.19	5.7	3.66±0.14	3.4	0.87±0.14	14.1
	Replicate 2	3.50±0.11	3.3	3.94±0.65	16.6	0.65±0.11	21.4
	Replicate 3	3.39±0.19	5.2	3.64±0.33	8.9	0.95±0.11	12.1
Average Day 3		3.42±0.05	2.0	3.75±0.16	4.6	0.84±0.14	17.9
Total Average		**4.23±0.65**	**15.5**	**4.26±0.57**	**13.6**	**1.09±0.27**	**26.1**

Figure 5. Concentrations found for MIT (ng MIT mg^{-1} creatinine) in 36 healthy newborn DUS samples ordered by increasing concentration. Error bars indicate SD of the measurements (n=3 independent replicates).