Urine and saliva biomonitoring by HF-LPME-LC/MS to assess dinitrophenols exposure.

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Abstract

In this work, the determination of 2,4-, 2,5- and 2,6-dinitrophenols and the identification of some of their metabolites in human urine and saliva is proposed. A three phase hollow fiber based liquid phase microextraction prior to ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry allowed low detection and quantitation limits of the target analytes, as well as the investigation and tentatively identification of some metabolites by accurate mass full-spectrum measurements. The chromatographic separation was accomplished on an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7 µm particle size) at 25 ºC using water and acetonitrile (with 0.1 % (v/v) formic acid) 20:80 v/v as mobile phase, at a flow rate of 0.5 mL/min in isocratic elution mode for 5 min. Hollow fiber liquid phase microextraction was achieved at donor phase pH 2, acceptor phase pH 13 and dihexylether as supported liquid membrane. Under the optimal conditions, detection limits for 2,4-, 2,5- and 2,6-dinitrophenol, respectively, were 0.18 $\mu g \cdot L^{-1}$, 0.38 $\mu g \cdot L^{-1}$ and 0.14 $\mu g \cdot L^{-1}$ in urine samples and 0.32 $\mu g \cdot L^{-1}$, 0.67 $\mu g \cdot L^{-1}$ and 0.24 $\mu g \cdot L^{-1}$ in saliva samples. The proposed methodology was applied on urine and saliva samples from laboratory staff likely to be or not occupationally exposed to dinitrophenols, finding quantitative levels of 2,4- and 2,6-dinitrophenol and identifying some metabolites previously reported in literature.

Keywords*:* Hollow fiber liquid phase microextraction; HF-LPME; Liquid chromatography quadrupole time-of-flight; Dinitrophenols; Human urine, Human saliva.

1. Introduction

Dinitrophenols (DNPs) are compounds widely used in several industrial and human activities like dyes manufacturing, wood preservation, pesticides for agriculture, and explosives fabrication, among others. 2,4-dinitrophenol (2,4-DNP), 2,5-dinitrophenol (2,5-DNP) and 2,6-dinitrophenol (2,6-DNP) are the most common ones, being the 2,4-DNP the most widely used and consequently the more regulated. The United States Environmental Protection Agency (U.S. EPA) classified 2,4-DNP as priority pollutant [1] and 2,5-DNP and 2,6-DNP were established as toxic compounds for human health and safety by the United States Department of labor [2]. DNPs are considered an uncoupling of mitochondria that reduces the formation of high energy phosphate bonds, while consuming oxygen. This mechanism of action is called uncoupling of oxidative phosphorylation [3,4].

First studies about DNPs toxicity dates from the beginning of the First World War when they were used in the manufacturing of ammunition. 2,4-DNP was also used as herbicide, wood preserver, photographic developer and fungicide, and it was introduced in the 1930s in USA as a weight-reduction drug, hypothyroidism drug and for depressed metabolic states which was sold without doctor´s registration. However, it was discontinued in 1938, due to the reported adverse effects that include fatal poisoning [5-8]. Nowadays, it is easy to check its on-line sales as dietary supplement for body builders despite recent studies have reported its toxicity [9,10].The main route of DNP poisoning is ingestion, with the lethal dose of 4.3 mg/Kg, but also adverse effects by inhalation and skin contact have been described [5,10,11]. Poisoning symptoms are hyperthermia, tachycardia, flushed skin, urinary volume decrease, nausea, coma and finally death. Until 2012, 62 deaths have been described in the medical literature related to DNPs [5] and after that several new cases have been reported [6].

DNPs stimulate respiration, increases O_2 consumption and cause the loss of the pH gradient across the inner mitochondrial membrane. Therefore, it has been demonstrated that 2,4-DNP has potential for autoxidation, increasing the production of reactive oxygen species and reducing the mitochondrial membrane.

Abbreviations: DNP: Dinitrophenol; UHPLC-QTOF-MS: ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry; HF-LPME: hollow fiber based liquid phase microextraction.

Politi et al. [12] reported 2,4-DNP undergoes a metabolism pattern consisting in its reduction to 2-amino-4-nitrophenol, 2-nitro-4-aminophenol, and 2,4-diaminophenol and conjugation with glucuronic acid and sulphate to produce DNP metabolites such as 2,4-diaminophenol sulphate, 2-amino-4-nitrophenol glucuronide and 2,4-dinitrophenol glucuronide. The compound is excreted by urine, partially as its unchanged form and as its glucuronides and sulfate derivatives.

~~DNPs have been mainly determined by liquid chromatography, gas chromatography, voltammetry and amperometry, capillary electrophoresis and ultraviolet-visible spectrophotometry [13-16].~~ DNPs first determinations in biological fluids were made using UV spectrophotometry [13]. Later, separation techniques have been widely used, mainly liquid chromatography using reverse phase chromatography with UV detector [14,15] and negative ionization mass spectrometry detection [12,16], however, Dejmkova et al. [17] proposed the amperometric detection as alternative for the determination of 2,4-DNP amino metabolites in spiked urine samples. Gas chromatography with mass spectrometry detection have also been reported in a method for serum determination from subjects using DNP as weight loss drug [8]. Capillary electrophoresis coupled to ESI mass spectrometry detection employing an ammonium acetate buffer modified with methanol was optimized by Teich et al. [18] to achieve the separation of nine nitrophenols, including 2,4-DNP, from atmospheric particles, however, non-quantifiable levels of dinitrophenols were found by authors. On the other hand, voltammetry employing anodic oxidation with a carbon paste electrode based on microbeads of glassy carbon have also been applied for the determination of 2,4-DNP and some of its main metabolites [17], but unfortunately the method showed important matrix interferences in urine samples.

Respecting extraction procedures, several different extraction methods like solid phase extraction, monolithic fiber solid phase microextraction, ultrasound-assisted emulsification microextraction and more recently dispersive liquid-phase microextraction or supramolecular solvent combined with dispersive solid phase extraction, among others have been used [14,17-22]. The use of hollow fiber liquid phase microextraction (HF-LPME) was first reported in 2001 for 2,4-DNP in seawaters [21]. Later, some other works have been published for the determination of several DNPs, being the procedures applied to their extraction from environmental [18,23-26] and biological [28-29] matrices, however none of them were nor applied in saliva neither reported the extraction of metabolites.

There are not literature reporting the determination of these three analytes all together in biological human samples. Politi et al. [12] determined the main phase I and II metabolites of 2,4-DNP in blood, urine, bile and gastric content human samples from a fatal case of poisoning and death. Miranda et al. [8] reported the cases of two individuals whose deaths were attributed to ingestion of 2,4-DNP. Also Zack et al. [29] reported the death of a man by consumption of 2,4- DNP for 2 months' time. Zhao et al. [30] also reported 16 cases of acute 2,4-DNP poisoning through occupational exposure due to ignoring the risk of poisoning.

In this paper the simultaneous determination of 2,4-, 2,5- and 2,6-DNPs in human urine and saliva was carried out by ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) detection after a three-phase hollow fiber based liquid phase microextraction (HF-LPME) step. Additionally, the identification of metabolites based on previously reported metabolic pathways [12,31] was investigated by accurate mass measurements using the high-resolution mass spectra obtained. The analysis of urine and saliva samples from an occupational exposed subject showed the presence of 2,4-DNP and 2,6-DNP and some of its main metabolites.

2. Materials and Methods

2.1 Chemicals and reagents

All chemicals were of analytical-reagent grade or better. All solutions and dilutions were prepared using ultrapure water from a Milli-Q^{TM} Plus (Millipore, Billerica, MA, USA) water purification system. 2,4-DNP, 2,5-DNP, 2,6-DNP and dihexyl ether were purchased from Fluka-Sigma–Aldrich (Madrid, Spain), sodium hydroxide, acetone, formic acid 98-100% Suprapur® and hydrochloric acid from Merck KGaA (Darmstadt, Germany), acetonitrile and water LC-MS Ultra ChromasolV® from Sigma-Aldrich (Madrid, Spain), 0.5 mM sodium hydroxide in 90:10 (v/v) 2-propanol:water and Leucine Enkephalin from Waters (Barcelona, Spain). Accurel®Q3/2 polypropylene hollow fiber (600 mm i.d., 200 mm wall thickness and 0.2 mm pore size) was purchased from Membrana (Wuppertal, Germany).

200 pg/µL Leucine Enkephalin was monthly prepared in acetonitrile:water (1:1 v/v) with 0.1% formic acid for lock mass correction. Mass spectrometer calibration was achieved by infusing a sodium formiate solution prepared as follows: 100 µL of aqueous NaOH

0.1 M and 200 µL formic acid 10% (v/v) to a final volume of 20 mL acetonitrile-water (80:20 v/v).

Aqueous working solutions of the studied compounds were daily prepared by adequate dilutions from methanolic 100 µg mL^{-1} stock solutions. The acceptor solution at pH 13 was prepared as follows: 700 µL of NaOH 2 M was diluted to 50 mL with water.

2.2 Liquid Chromatography conditions

Xevo G2-S QTOF mass spectrometer (Waters, Micromass, Manchester, UK) coupled to an Acquity ultra-performance liquid chromatography system (Waters, Milford, MA, USA) equipped with an electrospray interface (ESI) was used. The separation was performed by UHPLC with a conditioned autosampler at 10°C, using an Acquity UHPLC BEH C18 analytical column (50 mm × 2.1 mm i.d., 1.7 µm particle size) (Waters, Milford, MA, USA). The column temperature was maintained at 25ºC. The mobile phase used for MS and MS/MS experiments consisted of water (solvent A) and acetonitrile (with 0.1 % (v/v) formic acid) (solvent B) and an isocratic elution 80% A and 20% B for 5 min at 0.4 mL/min flow rate. For MS^E experiments the gradient elution program started with an isocratic step at 95% A for 10 min, followed by a gradient to 80% A in 2.0 minutes and subsequent isocratic step for 8.0 minutes. After that, a gradient step form 80% A to 5% A was applied in 3.0 min and it was maintained at 5% A for 15 min, returning then to initial conditions (95% A) in 2.0 minutes. Two minutes were waited before injections to re-equilibrate the column. The injection volume was 5 µL.

2.3 Mass detection conditions

All MS and MS/MS experiments were operated in negative ionization and resolution mode. The nebulisation gas (nitrogen, supplied by a high purity nitrogen generator Nitrogen Zefiro 35 LC-MS, Cinel-gas, Italy) was set at 900 L/h and the cone gas (nitrogen) to 52 L/h, desolvation temperature was set to 400 ºC and source temperature to 100 ºC. The microchannel plate detector potential was set to 2925 V. All analyses were performed using an independent reference spray (LockSpray) to ensure accuracy and reproducibility. Leucine Enkephalin solution infused at a flow rate of 10 µL/min was used for lock mass correction through the lockspray needle every 30 s for 0.30 s, performing on-going correction of the exact mass of the analyte. For MS spectra, data were collected within 100-800 *m/z* at a scan time of 0.3 seconds and were averaged over 10 spectra min^{-1}. The capillary and cone voltages were set to -2.4 kV and 12 V, respectively.

For MS/MS experiments, the following conditions were used: cone voltage 20 V; collision energy 20 eV and argon as collision gas (99.995%, Praxair). The same precursor ion 183.0042 $[M-H]^-$ was selected for the three DNPs using the quadrupole analyser and subsequently the fragment ions (109.0171, 123.0090 and 153.0074) were analysed in the TOF analyser.

MS^E procedure was applied for metabolite identification. Positive and negative ionization modes were used for metabolites search. UHPLC–QTOF-MS^E detection collects data using two scan functions providing fragment ion information without precursor ion selection. Two functions collected within 100-800 *m/z* at different collision energies were acquired: the low energy function was obtained at 0V to obtain accurate mass data for intact precursor ions, and the high-energy function was obtained using a collision energy ramp range from 15 to 30 V to obtain product ions.

2.4 Data processing

The data operating software was MassLynx™ version 4.1 (Waters). For MS and MS/MS experiments the data processing was carried out using the software ChromaLynx™ XS (Waters). The accurate mass of deprotonated molecules was determined based on average spectra obtained in full scan mode. A target analysis was chosen for which a identify method was created with the retention time, accurate mass and chemical formula of DNPs. To confirm the presence of compounds in urine and saliva samples, the exact mass of data reported from ChromaLynx™ were obtained setting the parameters as follows: target retention time tolerance (min): 0.100; mass tolerance: 0.010 Da; mass accuracy for positive identification: 0.002 Da; range of time: 0.0-5.0 min and range of mass: 0.0-400.0. Additionally, the extracted ion chromatograms for each compound were also used to confirm their presence.

To process MS^E data obtained, MetaboLynx™ XS software was used. The software compares extracted ion chromatograms of samples with a control sample, reporting ions and chromatographic signals that would match with possible metabolites, according to metabolic routes of interest previously defined by the user (*expected metabolites*) or not (*unexpected metabolites*). From the accurate mass obtained by averaged spectra in the low energy survey scan a possible elemental composition is proposed and the corresponding biotransformation pathway assigned. In this survey, only the expected metabolites search was realized.

2.5 Extraction procedure

400µl of saliva or 700µl of human urine were directly diluted in 50 ml of deionized water, pH was adjusted to 2 (using HCl) and submitted to the HF-LPME extraction procedure described below.

Hollow fibers (13 cm length) were washed with acetone in an ultrasonic bath and dried. The fiber was first immersed in dihexyl ether for 10 s to impregnate the pores and rinsed with water on the outside by placing it into an ultrasonic bath for 20 s to remove the excess of organic solvent. Then, the lumen of the prepared fiber was filled with 30 µL of aqueous acceptor phase (pH 13) using a HPLC syringe. Both open ends of the fiber were closed by means of a hot soldering tool and adhesive tape. After that, the fiber was placed on the glass beaker and the portion that contains the acceptor phase was immersed in 50 mL of sample solution (pH 2, adjusted with HCl) placed in a 60 mL glass beaker. The sample was stirred for 20 min with a magnetic stirrer (ANS-00/1 Science Basic Solutions (Rubí, Barcelona, SPAIN) at 300 rpm. Once the extraction is completed, the fiber was removed from the sample solution and one of the ends was cut to collect the acceptor phase using a HPLC syringe, which was transferred to a conic micro insert to be injected into the UHPLC system.

3. Results and discussion

3.1 LC-MS conditions

Initially, a depth study of the ionization and fragmentation of the DNPs was carried out for each compound individually. DNPs were detected in negative ionization mode (ESI^-) and optimal parameters were optimized by direct infusion of each analyte in aqueous solution as a first step and then corrected under the optimal mobile phase composition. Several ionization conditions were studied including the use of formic acid, ammonium formiate, acetonitrile and methanol as modifiers to evaluate the enhancement/suppression on the ionization produced by their presence. Considering the similar structure (**Fig. 1**) and the same *m/z* values for the three studied compounds, a previous separation by UHPLC was needed before mass spectrometry detection. Different mixtures of organic solvents (acetonitrile and methanol) and aqueous solvents with additives such as acetic acid, formic acid and ammonium salts, under isocratic and gradient elution profiles, were tested in order to achieve an efficient separation of DNPs. Acetonitrile as organic modifier and formic acid as additive to aqueous mobile phase resulted to provide the better

separation and peak shapes, therefore acetonitrile-formic acid (0.1 % v/v) aqueous solution was confirmed as the mobile phase. For MS and MS/MS acquisitions, a satisfactory separation was achieved with an isocratic elution in 5 min (see obtained chromatograms (**Fig. S1**) and spectra (**Figs. S2 and S3**) in the Electronic Supplementary Material (ESM)).

For confirmation purposes, MS/MS conditions were also evaluated at several cone voltages and collision energies under the optimum tune conditions previously obtained for full MS acquisitions, looking for a reproducible and sensitive fragmentation pattern for each compound. Under the optimum conditions described in section 2.3, the spectra showed different fragmentation pattern for each compound (see **Fig. S3**). Table 1 shows the optimum collision energies as well as elemental composition of all the fragment ions obtained in MS/MS experiment. As it can be seen, 2,4-DNP showed the ion fragment *m/z* 109.0171 as the most intense, followed by the fragments *m/z* 123.0090, 137.0109 and 153.0074. On the other hand, 2,5 and 2,6-DNP exhibit a slightly different fragmentation pattern, showing 5 fragments ions, of which *m/z* 153.0074, 137.0109 and 123.0090 match with those of 2,4-DNP, though their ratio is completely different, being the most intense *m/z* 153.0074 in the case of 2,6-DNP and *m/z* 123.0090 for 2,5-DNP.

3.2 Hollow Fiber-Liquid Phase Microextraction

HF-LPME (urine and saliva samples) was carried out according to the optimal conditions proposed by Villar et al. [26] for environmental samples with a slight modification in the sample dilution performed, as described in section 2.5. Under these conditions, a high preconcentration was achieved. Enrichment factors (Efs) were calculated as the corresponding relation between final concentration in acceptor phase and initial concentration in donor phase of each analyte. No significant differences were found between the values obtained in urine and saliva, with average Efs of 118 ± 3, 107 ± 5 and 200 ± 3 for 2,4-, 2,5- and 2,6-DNP, respectively. The enrichments obtained in saliva and urine were higher for 2,4 and 2,6-DNP than those reported by Villar et al. (54 and 55 respectively) [26].

To the best of our knowledge, only three works have been previously reported [27-29] involving the LPME determination of nitrophenols (only 2,4-DNP) in biological samples (plasma); the authors used a dynamic LPME that implies the use of two syringe pumps assembled to the hollow fiber using a much more cumbersome device than the one used

in this work, achieving, after an extraction time of 20 min approximately, a limit of detection (LOD) of 50 $\mu g \cdot L^{-1}$ for 2,4-DNP, which is higher than the one obtained in this work.

3.3 Analytical figures of merit

Analytical parameters of the method were evaluated in terms of linearity, repeatability, intermediate precision, limits of detection and quantification (LOQ) and trueness according to EURACHEM (European Analytical Chemistry) guide [32].

Linearity response was studied using DNPs standard solutions within the range 1-500 $\mu g \cdot L^{-1}$, in triplicate. Calibration curves were constructed using a least-square linear regression analysis and the selected HF-LPME conditions were applied to standard aqueous pH 2 solutions containing different DNPs concentrations which were extracted through a Q3/2 polypropylene hollow fiber supporting dihexyl ether as liquid membrane. Aqueous pH 13 solutions were used as acceptor phase and the extracts obtained were analyzed according to the proposed UHPLC procedure obtaining correlation coefficients of 0.996 for 2,4-DNP and 0,998 for 2,5-DNP and 2,6-DNP (table 2).

Method LODs and LOQs were calculated as the minimum concentration of analyte giving peaks whose signal to noise ratios are 3 and 10, respectively, taking into account the different dilutions in urine and saliva samples. The detailed results are depicted in table 2, therefore LOQs for urine samples were within 0.46-1.27 $\mu g \cdot L^{-1}$ and within 0.81-2.18 $\mu g \cdot L^{-1}$ for saliva samples. Moreover, LODs for 2,4-, 2,5- and 2,6-DNP in urine were 0.18, 0.38 and 0.14 $\mu g \cdot L^{-1}$ respectively, while in saliva were in the range 0.24-0.67 $\mu g \cdot L^{-1}$. Few works have been published on determination of DNPs in biological samples, mainly in blood, serum and plasma [8, 12, 17, 27-31, 33], in fact, there are no references for their determination in saliva samples, although some of these works also reported levels in urine samples [8, 12, 17, 29, 33]. However, they are mainly focused on the determination of 2,4-DNP and most of them propose detection limits in the order of $mg \cdot L^{-1}$ so the sensitivity values achieved in this work represent a great advantage for detection and quantification at low concentration levels.

Repeatability (intraday) and intermediate precision, expressed as the relative standard deviation, were evaluated by submitting standard solutions to the HF-LPME procedure. Three different concentration levels of each compound (5, 50 and 500 $\mu g \cdot L^{-1}$) were analyzed, in one single day for repeatability (in quintuplicate) and one day per week

among two months for intermediate precision (in triplicate). Repeatability showed relative standard deviations in the range 1.3-3.8% for urine and 1.5-8.9% for saliva, while for intermediate precision results were within 2.5-8.9 % and 3.8-7.1% for urine and saliva samples, respectively (table 2).

Accuracy was evaluated by recovery assays on spiked samples, calculated as percentage of extracted compound at a low level of concentration for each kind of matrix and compound. Spiked urine and saliva samples at 5, 50 and 500 $\mu g \cdot L^{-1}$ (in triplicate) were submitted to the microextraction procedure described in 2.5 section. Recoveries obtained were in the range 75-80% (± 2.1) for urine samples and in the range 74-80% (± 1.3) for saliva samples.

3.4 Analysis of human saliva and urine samples

Saliva and urine samples from external volunteers and from our laboratory staff (whose informed consent was obtained) during the development of this work were regularly collected and analyzed. Samples were classified as follows: saliva and urine samples from (a) external volunteers (sampled individuals, m=3), (b) laboratory staff (m=5) and (c) laboratory staff working directly with DNPs (m=2). Urine samples were collected with an interval time 1-2 hours after saliva samples. A total of 30 samples were randomly taken, in order to control the safety standards among the laboratory staff. The results obtained are summarized in table 3.

2,5-DNP was not found in any of the samples analyzed, but unexpectedly 2,4-DNP (655 ± 4.3 $ng \cdot L^{-1}$) and 2,6-DNP (detected) were found in an urine sample from an individual corresponding to (c) group; 2,4-DNP was also found in a saliva sample from the same individual at 56 ± 5.2 $ng \cdot L^{-1}$ level. Checking the working conditions of the laboratory staff, we verified that both samples belonged to an individual who had manipulated the compounds without wearing the complete individual protection equipment, concretely the adequate protective mask.

To our knowledge, there are not previous evidence of the presence of 2,5- and 2,6-DNPs in urine and saliva samples by either ingestion or occupational exposure, however, 2,4-DNP can easily enter body through inhalation, ingestion or skin [11], being oral ingestion is the main cause of poisoning described [10, 12, 34] in bibliography. Therefore, Polti et al. [12] and Miranda et al. [8] found 14.3 and 53 $mg \cdot L^{-1}$, respectively, in urine samples from deaths attributed to 2,4-DNP poisoning by ingestion. Zack et al. [29] reported levels

of 95.3 $mg \cdot L^{-1}$ in urine and found also high concentrations in several organs, on another death case report after ingestion. On the other hand, W.D.L. Smith [35] found a urine concentration of 52.3 $mg \cdot L^{-1}$ after three days of occupational exposure and 34.4 $mg \cdot L^{-1}$ nine days after having been exposed. The levels found, in general, are much higher than the founded in this work, since they corresponded to cases of fatal poisoning. The high sensitivity achieved in this work would allow the detection of cases in which the exposure had not been prolonged.

3.5 Identification of metabolites

Considering the high potential that high resolution mass spectrometry offers, a tentative identification of the presence of some metabolites was investigated using MS^E experiments as described in section 2.3 by positive and negative ionization modes. The obtained low energy and high energy chromatograms were processed by Metabolynx™ software. Most of the metabolic activity of DNPs have been attributed to liver and intestinal microsomal enzymes. Previous studies of their biotransformation have identified that the main metabolic pathway is DNPs nitroreduction to the corresponding monoreduced aminonitrophenols and, to a lessen extend, the diaminophenols forms. Aditionally, several conjugated metabolites including glucuronidation, 2-amino-4-nitrophenol ethereal sulfate, and 2-amino-4-acetylaminophenol have also been proposed [30]. Therefore, the following routes were selected for these searches: phase I metabolites: reduction ($+H_2$), hydroxylation (+O), hydration ($+H_2O$), 2xhydroxylation ($+O_2$), nitroreduction ($-O_2+H_2$) and dinitroreduction ($-O_2+H_2$); and phase II metabolites: sulfate conjugation ($+SO_3$), hydroxylation+sulfation, acetylation ($+C_2H_2O$) and glucuronidation ($+C_6H_8O_6$).

All the 30 samples collected were analyzed according to the procedures described in section 2, after that, data obtained were processed as described in section 2.4, selecting the metabolic pathways detailed above. The results obtained are summarized in Table 4, a positive identification of several metabolites was obtained in just one urine sample out of the 30 samples analyzed, which corresponded to the individual described in section 3.4. As can be seen in the table 4, the diamino-reduced metabolites of both 2,4- and 2,6-DNP were identified, as well as the conjugates of the glucuronide acid of the parent compounds 2,4- and 2,6-DNP and of the metabolite 2-amino-4-nitrophenol and the sulfate conjugate of both parent compounds. On the bases of the metabolites identified, dinitroreduction, glucuronidation and sulfation were the main pathways by which DNPs

were metabolized. This is in accordance with previously reported data in literature [31,12] and additionally, it demonstrates that the HF-LPME method proposed is also usable for the extraction of some of the main metabolites of DNPs, however their quantification was not carried out.

A recent publication [3] on the toxicological profile of various DNPs, reports as the main route of metabolism, the reduction of nitro groups generating the metabolites 2-amino-4-nitrophenol, 4-amino-2-nitrophenol and 2,4-diaminophenol. Furthermore, the authors describe that both 2,4-DNP and its main metabolites are excreted in urine. Even so, the evidence on absorption and distribution by exposure to DNPs in humans is very scarce or almost null. In our work, the presence of none of the metabolites indicated above was found, however, it was identified the presence of conjugated derivatives, which would be consistent with the reported in the cited work in which it is proposed that these metabolites may be conjugated with glucuronic acid or sulfate prior to excretion in the urine. Only Polti et al. [12], have reported the presence of these metabolites in samples in a case of fatal death by poisoning after ingestion.

Regarding 2,5- and 2,6-DNP, there is no information available about their absorption, distribution or elimination, although it has been suggested [3] that 2,4- and 2,6-DNP are not eliminated as rapidly as other DNP isomers.

Old reports [36,37] show evidence of absorption after inhalation of 2,4-DNP and even suggest the presence of metabolites in urine. On the other hand, more recent studies [11, 38] provide information on cases of death by poisoning after contact and inhalation, although these studies do not provide any quantitative or qualitative information on the presence of DNPs or their related compounds.

4. Conclusions

It has been demonstrated that the proposed HF-LPME/UHPLC-QTOF-MS method allows the highly sensitive determination of 2,4-, 2,5- and 2,6-DNPs. Additionally, the processing of MS^E experiments data through Metabolynx^{TM} software confirmed that some of their corresponding metabolites were also extracted.

The proposed method has been satisfactory applied to the determination of DNPs in human urine and saliva samples from our laboratory staff. 2,4 and 2,6-DNP were found in one urine sample, as well as 2,4-DNP was also quantified in one saliva sample. Both samples corresponded to the same individual, so the presence of these compounds might

have been due to an occupational exposure while working with DNPs standards without the complete individual protection equipment. Additionally, some of the main metabolites of 2,4- and 2,6-DNP were identified in just one urine sample. According to the levels found in the samples from this individual, it seems clear that the greatest absorption capacity by inhalation goes in the following: 2,4-, 2,6- and 2,5-DNP.

The potential of the proposed procedure for DNP's metabolites analysis using Metabolynx^{TM} software from data from MS^{E} experiments has also been demonstrated.

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Compliance with ethical standards

All urine samples were obtained from volunteers from whom informed consent was obtained.

Conflict of interest. The authors declare that they have no conflict of interest

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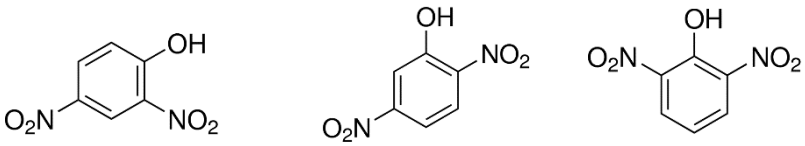
Figure 1.



2,4-DNP 2,5-DNP 2,6 DNP

Figure Caption

Fig. 1. Structure of 2,4- Dinitrophenol, 2,5-Dinitrophenol and 2,6-Dinitrophenol

Captions of tables

Table 1. Retention times, full MS and MS/MS fragments ions of DNPs

Table 2. Method figures of merit

Table 3. Results obtained on 30 samples from volunteers. (*see text for details*)

Table 4. Identification of metabolites in urine and saliva samples by MS^E

Table 1. Retention times, full MS and MS/MS fragments ions of DNPs.

Compound	Elemental Composition	Monoisotopic mass	extracted m/z (precursor)	t_r(min)	Fragment ions	
					m/z	Elemental Composition
2,4-DNP	$C_6H_4N_2O_5$	184.0120	183.0042	2.74	109.0171	C_6H_8NO
					123.0090	$C_6H_6NO_2$
					137.0109	$C_6H_5NO_3$
					153.0074	$C_6H_6N_2O_3$
2,5-DNP	$C_6H_4N_2O_5$	184.0120	183.0042	3.10	153.0074	$C_6H_6N_2O_3$
					125.0118	$C_6H_8NO_2$
					137.0133	$C_6H_5NO_3$
					107.0118	C_6H_5NO
					123.0090	$C_6H_6NO_2$
2,6-DNP	$C_6H_4N_2O_5$	184.0120	183.0042	2.19	123.0090	$C_6H_6NO_2$
					125.0118	$C_6H_8NO_2$
					137.0133	$C_6H_5NO_3$
					109.0171	C_6H_8NO
					153.0074	$C_6H_6N_2O_3$

Table 2. Method figures of merit.

	linearity (%)	Ef	Linear Range*	LOD*	LOQ*	Repeatability (%RSD)**			Intermediate precision** (%RSD)			% Recoveries** average (standard deviation)		
				urine/saliva	urine/saliva	urine/saliva			urine/saliva				urine/saliva	urine/saliva
						5*	100*	500*	5*	100*	500*	5*	50*	500*
2,4-DNP	99.9	115	LOQ-500	0.18/0.32	0.61/1.06	2.3/8.9	1.9/4.1	1.6/4.1	3.5/6.2	2.5/3.8	2.6/4.3	80 (0.4)/74 (0.8)	79 (1.2)/76 (2.2)	80 (3.2)/80 (4.8)
2,5-DNP	98.7	102	LOQ-500	0.38/0.67	1.27/2.22	2.2/3.0	1.3/2.1	2.4/3.2	5.1/4.1	4.2/5.6	4.4/7.1	75 (1.5)/78 (4.1)	80 (1.9)/77 (1.1)	79 (1.9)/75 (4.0)
2,6-DNP	99.9	197	LOQ-500	0.14/0.24	0.46/0.81	3.8/7.4	1.9/1.5	2.1/7.1	8.9/6.2	6.3/5.1	4.2/5.6	77(2.1)/80 (0.4)	79 (1.1)/78 (0.4)	74 (0.8)/80 (5.2)

* $\mu g\ L^{-1}$

**all determinations by triplicate except repeatability by quintuplicate

Table 3. Results obtained on 30 samples from volunteers. (*see text for details*)

Analyte	t_r(min)	m/z_{exp}	m/z_{theo}	Error ppm	Molecular formula	Urine*	Saliva*
2,4-DNP	2.74	183.0046	183.0042	2.18	$C_6H_4N_2O_5$	+ (1/30)	+ (1/30)
2,5-DNP	3.10	183.0045	183.0042	1.64	$C_6H_4N_2O_5$	-	-
2,6-DNP	2.19	183.0045	183.0042	1.64	$C_6H_4N_2O_5$	+ (1/30)	-

*positive detection from total of volunteers

Table 4. Identification of metabolites in urine and saliva samples by MS^E.

Compound	t_R (min)	m/z_{exp}	m/z_{theo}	Error ppm	Molecular Formula	Urine*	Saliva*
2,4-Dinitrophenol	10.53	183.0045	183.0042	1.09	$C_6H_4N_2O_5$	+ (1/30)	+ (1/30)
2,6-Dinitrophenol	6.86	183.0044	183.0042	6.01	$C_6H_4N_2O_5$	+ (1/30)	-
2,4-Diaminophenol	1.91	123.0547	123.0558	3.21	$C_6H_8N_2O$	+ (1/30)	-
2,6-Diaminophenol	1.72	123.0547	123.0558	-1.60	$C_6H_8N_2O$	+ (1/30)	-
2,4-Dinitrophenol sulfate	2.85	263.0928	262.9610	-2.65	$C_6H_4N_2O_8S$	+ (1/30)	-
2,6-Dinitrophenol sulfate	2.31	263.0938	262.9610	1.13	$C_6H_4N_2O_8S$	+ (1/30)	-
2-Amino-4-nitrophenol glucuronide	1.72	329.0291	329.0621	1.21	$C_{12}H_{14}N_2O_9$	+ (1/30)	-
2,4-Dinitrophenol glucuronide	1.47	359.0719	359.0363	1.94	$C_{12}H_{13}N_2O_{11}$	+ (1/30)	-
2-Amino-6-nitrophenol glucuronide	1.28	329.0290	329.0621	0.90	$C_{12}H_{12}N_2O_{11}$	+ (1/30)	-
2,6-Dinitrophenol glucuronide	1.08	359.0715	359.0363	0.83	$C_{12}H_{13}N_2O_{11}$	+ (1/30)	-

*positive detection from total of volunteers