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An estimation of the exposure to organophosphorus pesticides through the simultaneous determination of their main metabolites in urine by liquid chromatography–tandem mass spectrometry

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Abstract

A rapid method has been developed for the determination of 4-nitrophenol (PNP) (parathion and methyl-parathion metabolite) and 3-methyl-4-nitrophenol (3-Me-PNP) (fenitrothion metabolite) in human urine by coupled-column liquid chromatography combined with tandem mass spectrometry (LC–LC–MS/MS). The LC–LC–MS/MS approach allows the determination at sub-ppb level of free metabolites by injecting the urine directly into the system and the total metabolites after a simple enzymatic hydrolysis. The method has been validated, obtaining limits of detection of 0.1 and 0.2 μg/L for 4-nitrophenol and 3-methyl-4-nitrophenol, respectively. Additionally, a multi-residue LC–MS/MS method is proposed in order to evaluate the levels of other parathion and methyl parathion metabolites. This approach allows the simultaneous determination of dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), 4-nitrophenolsulphate and 4-nitrophenolglucuronide without tedious sample treatments. The applicability of both methods is demonstrated by applying them to various urine samples from an unexposed population and a grower who applied methyl parathion. The combination of both methods allows a general overview on the presence of different metabolites (free and conjugated) and the concentration ratios between them, giving useful information on organophosphorus pesticides metabolism and excretion.

Keywords: Organophosphorus pesticides; 4-Nitrophenol; 3-Methyl-4-nitrophenol

1. Introduction

Exposure to hazardous chemicals is a concern to the general population, and public health institutes are frequently asked to evaluate a community's or individual's risk of developing health problems from potential exposure to chemical contaminants in the environment. To assess risk, one needs to estimate the exposure. Measurements of environmental contaminants in air, water or soil can provide such estimates, but a better way to measure an individual's exposure to these contaminants is to directly determine those chemicals or their metabolites in body tissues or fluids, a technique sometimes called biological monitoring [1–3].

Parathion [*O*,*O*-diethyl *O*-(4-nitrophenyl) phosphorothioate], methyl-parathion [*O*,*O*-dimethyl *O*-(4-nitrophenyl) phosphorothioate] and fenitrothion [*O*,*O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate] are organophosphorus (OPs) insecticides that are toxic to mammals. The major toxic effect of OPs is the inhibition of esterases including acetylcholinesterase (ChE). For this reason, biological monitoring based on the inhibition of ChE has been used for many years to evaluate exposure in people applying pesticides [4].

After exposure and subsequent bodily absorption these pesticides are rapidly metabolised (Fig. 1). The main metabolites for these compounds are alkyl phosphates, such as dimethyl thiophosphate (DMTP) and dimethyl phosphate (DMP), and other more specific compounds, such as 4-nitrophenol (PNP) or 3-methyl-4-nitrophenol (3-Me-PNP), as well as the relative glucuronides and sulphates. The urinary dialkyl(thio)phosphates have been analyzed to reveal exposures to various OPs [5–8], however, alkyl phosphates are less specific as they are potential metabolites of most of the OPs. Therefore, the determination of alkyl phosphates gives global information about exposure

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to OPs compounds, but is rarely used to follow the exposure to an unique compound. The determination of PNP is more selective than that of alkylphosphates as it is released after exposure to only a few OPs (parathion, methyl parathion, and EPN). However, its appearance in urine could also be due to the exposure to a variety of industrial chemicals such as 4-aminophenol or PNP itself. In spite of these limitations, the urinary concentration of PNP and 3-Me-PNP could be monitored to show exposures to parathion and fenitrothion, respectively [9–11]. These phenolic groups are excreted in urine as conjugates with glucuronic acid or sulphuric acid producing the corresponding glucuronides and sulphates (Fig. 1). The release of nitrophenol from its conjugate forms is carried out by previous enzymatic hydrolysis.

Determination of urinary nitrophenols (free or conjugated) has been accomplished mainly by gas chromatography (GC) coupled either with an electron capture detector [9,12] or coupled to mass spectrometry (MS) [13]. Both approaches require an extensive sample preparation, involving formation of volatile derivatives, cleanup and sample concentration. When using GC–MS, the cleanup requirements are slightly simpler but the prederivatization steps are still necessary. To overcome this drawback, high performance liquid chromatography (HPLC) has been used, rendering faster and simpler methods [10,14,15]. More recently, LC–MS/MS methods have been developed [16,17] in order to determine pesticide metabolites in urine with the inherent advantages of this powerful technique (high sensitivity and selectivity, short analysis time), and that allowed reducing sample treatment. Recently, two LC–MS/MS methods have been published [18,19] for the determination of PNP in hydrolyzed urine. The first method allowed determination of concentration levels of 25 μg/L and used liquid–liquid extraction as the sample treatment. The second method, used off-line solid-phase extraction and lowered the limit of detection to 0.1 μg/L for PNP.

Regarding direct determination of conjugates in urine, the lack of glucuronide and sulphate-bound standards and the need for more sensitive and accurate measurements made their determination more difficult. However, a few applications have been described for conjugate metabolites of xenobiotics, such as morphine or naphthalene, in non-hydrolysed urine based on solid phase extraction followed by LC–MS/MS [20,21].

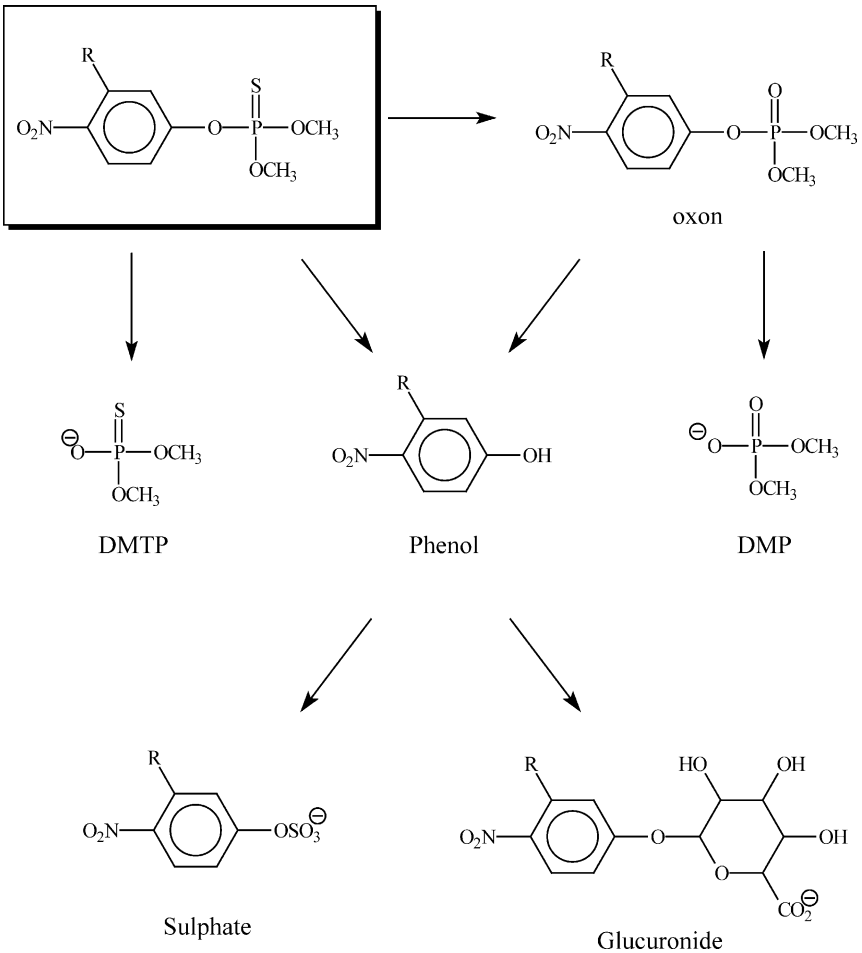

Fig. 1. Metabolism of methyl parathion (R=H) and fenitrothion (R=CH_3).

One of the main analytical challenges in the trace level analysis of biological samples is the correct quantification of analytes as a consequence of suppression or enhancement of the ionisation process in the electrospray interface (ESI) [22,23], as this leads to unacceptable errors. Different strategies can be followed to overcome this problem, mainly the use of adequate (isotope labelled) internal standard (if available) the dilution of sample (or extract), or calibration in matrix. In a previous paper [24], we proved that coupled-column liquid chromatography (LC–LC) was an efficient way to remove matrix interferences without any dilution; thus the combination of LC–LC with MS–MS allows the direct analysis of urine samples with excellent analytical characteristics. This has also been confirmed in the LC–LC–MS/MS determination of chlorpyrifos metabolite, 3,4,6-trichloro-2-pyridinol in human serum and urine [17].

In this paper, we make use of the hyphenated technique LC–LC–(ESI)–MS–MS for the direct determination of the free OP's metabolites PNP and 3-Me-PNP in human urine at sub-ppb levels. Additionally, a LC–MS/MS method is developed for the simultaneous determination of other metabolites as DMP, DMTP, PNP-sulphate and PNP-glucuronide in order to have a wider overview of human exposition to fenitrothion, parathion and methyl parathion. To our knowledge this is the first LC–MS/MS method proposed for the direct and simultaneous determination of methyl parathion conjugates and alkyl phosphates. The analytical methodology developed has been applied to urine samples collected from exposed and unexposed volunteers.

2. Experimental

2.1. Reagents and chemicals

PNP and 3-Me-PNP reference standards were purchased from Sigma (St. Louis, MO). 4-Nitrophenol-D_4 (D_4-PNP) was used as labelled internal standard, and was purchased from Cambridge Isotope Laboratories (Andover, MA). The conjugates 4-nitrophenol-β-D-glucuronide and 4-nitrophenolsulphate along with the enzymes β-D-glucuronidase and sulphatase were purchased from Sigma. DMP and DMTP were synthesized as previously reported [8] from dimethyl chlorothiophosphate (DMClTP) and dimethyl chlorophosphate (DMClP) purchased from Aldrich (Milwaukee, WI, USA).

HPLC-grade acetonitrile was obtained from Scharlab (Barcelona, Spain). LC-grade water was obtained by purifying demineralized water in a Nanopure II system (Barnstead Newton, MA, USA). Analytical grade formic acid (HCOOH, content >98%) was bought from Fluka (Buchs, Switzerland) and analytical grade hydrochloric acid (37%) was purchased from Merck (Darmstat, Germany). Tetrabuthylammonium acetate (TBA) was obtained from Sigma (St. Louis, MI, USA).

Standard stock as well as labelled internal standard (IS) solutions were prepared by dissolving 25 mg of the analyte in 50 mL of HPLC grade acetonitrile obtaining a final concentration of 500 μg/mL. For the LC–LC analysis, the stock solutions were diluted and mixed with LC-grade water.

2.2. Liquid chromatography

The triple quadrupole mass spectrometer was interfaced to an HPLC system based on a 233XL autosampler with a loop of 20 μL (Gilson, Villiers-le-Bel, France) and two pumps: an Agilent 1100 (Agilent, Waldbron, Germany) binary pump and a Waters Alliance 2690 (Waters, Milford, MA, USA) quaternary pump. The LC–LC set-up is shown in Fig. 2.

In the determination of PNP and 3-Me-PNP, the LC–LC separation was performed using a Discovery C18 5 μm, 50 mm × 2.1 mm (Supelco Bellfonte, PA, USA) as first separation column (C-1), with a mobile phase consisting of acetonitrile–0.01% HCOOH in water (M-1) at a flow rate of 200 μL/min. The second column (C-2) was an ABZ+ 5 μm, 100 mm × 2.1 mm (Supelco) with a mobile phase consisting of acetonitrile–water (M-2) at a flow rate of 200 μL/min.

Determination of conjugates and alkyl phosphates was carried out by using a Kromasil C18 100 mm × 2.1 mm (Sharlab, Barcelona, Spain), as the only chromatographic column with a mobile phase consisting of 0.01% HCOOH in methanol–water at a flow rate of 300 μL/min.

2.3. Mass spectrometry

A Quattro LC (quadrupole–hexapole–quadrupole) mass spectrometer with a orthogonal Z-spray–electrospray interface (Micromass, Manchester, UK) was used. Drying gas as well as nebulising gas was nitrogen generated from pressurized air in a NG-7 nitrogen generator (Aquilo, Etten-Leur, NL). The nebuliser gas flow was set to approximately 80 L/h and the desolvation gas flow to 800–900 L/h. Infusion experiments were performed using a Model 11 single syringe pump (Harvard, Holliston USA), directly connected to the interface.

For operation in the MS/MS mode, collision gas was Argon 99.995% (Carburos Metalicos, Valencia, Spain) with a pressure of 1×10^{-3} mbar in the collision cell. Capillary

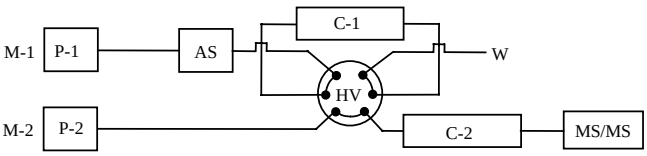

Fig. 2. HPLC set-up for coupled-column LC. AS: sample injector with 20 μL loop; HV: six-port high-pressure valve; P-1: isocratic LC pump; P-2: isocratic LC pump; C-1: first separation column; C-2: second separation column; M-1 and M-2: mobile phases on C-1 and C-2, respectively; MS/MS: tandem mass spectrometer detector; PC: data system; W: waste. Conditions: Section 2.

voltages of 3 kV were used in negative ionization mode. The interface temperature was set to 350 °C and the source temperature to 120 °C. Optimized cone voltages, collision energies and SRM transitions are shown in Table 1. Dwell times of 0.2 s/scan were chosen for all compounds except for DMP (0.5 s/scan) due to the lower sensitivity of this analyte.

Masslynx NT v 3.5 (Micromass, Manchester, UK) software was used to process the quantitative data obtained from calibration standards and urine samples.

2.4. Sample procedure

Urine samples were collected from non-exposed healthy volunteers and from a farmer who applied methyl parathion. For the determination of free PNP and 3-Me-PNP, 50 μL of a 1 mg/L solution of labelled IS were added to 1 mL of untreated urine. Then, 20 μL were directly injected in the LC–LC–MS/MS system. For the determination of free metabolites released from conjugates 1 mL urine was buffered with 50 μL acetic acid/ammonium acetate (pH 5.5) and 50 μL of labelled internal standard solution were added. Then it was hydrolyzed overnight with 50 units of β-D-glucuronidase/sulphatase at 37 °C. Finally, 20 μL of the hydrolyzed mixture were injected in the LC–LC system.

Direct determination of conjugates and alkyl phosphates was carried out taking 500 μL urine that was previously diluted three-fold with 0.5% HCOOH solution, then, adding 75 μL of TBA 500 mM and injecting 50 μL in the LC–MS/MS system.

2.5. LC–LC procedure

The mobile phase M-1 was acetonitrile–0.01% HCOOH in water (25:75, v:v). Acetonitrile–water (65:35, v:v) was used as mobile phase M-2 (Fig. 2). Both mobile phases were used at a flow of 200 μL/min.

A volume of 20 μL was injected onto C-1. After clean up during 3 min of M-1, C-1 was switched on-line with C-2 during 90 s to transfer the fraction (300 μL) containing the analytes (free PNP and 3-Me-PNP) to C-2.

Table 1
Mass spectrometry optimized parameters for the determination of analytes and internal standards

Compound	Type[a]	Precursor ion (*m*/*z*)	Cone (V)	Collision energy (eV)	Product ion (*m*/*z*)
PNP	Q	138	35	17	108
	q	138	35	20	92
D_4-PNP	Q	142	35	17	112
	q	142	35	20	96
3-Me-PNP	Q	152	35	17	122
	q	152	35	20	107
PNP-glucuronide	Q	314	20	15	138
	q	314	20	15	113
PNP-sulphate	Q	218	20	15	138
	q	218	20	35	108
DMP	Q	125	30	25	79
	q	125	30	20	63
DMTP	Q	141	25	17	126
	q	141	25	17	96

[a] Q: quantitative transition, q: confirmatory transition.

2.6. Validation study

The precision (expressed as a relative standard deviation in percentage) was evaluated, within day, by determining the analytes in spiked samples at two different levels ($n = 5$ at each concentration level). The calibration curve was obtained by analyzing standard solutions in triplicate at eight concentrations between 0 and 100 μg/L.

Recoveries (quantified value/spiked value) were obtained using three different urine samples each spiked at two concentration levels and processed as samples. These experiments were performed in quintuplicate.

The limit of quantification (LOQ) was taken as the lowest concentration level validated, for which adequate recoveries (between 70 and 110%) and precision (R.S.D. < 15%) were obtained for both transitions. The limit of detection (LOD), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, was estimated when the signal was three times the background noise from the chromatograms at the lowest analyte concentration assayed.

3. Results and discussion

3.1. MS optimization

The full-scan mass spectra and the MS/MS spectra of all analytes were obtained from infusion of 5 μg/mL 50:50 acetonitrile:water solutions of each compound at a flow of 10 μL/min. The MS spectra of all analytes showed a strong signal as $[M - H]^-$ using electrospray interface. The PNP, 3-Me-PNP and D_4-PNP MS/MS spectra showed the same behavior as a previously reported method [18], with an abundant fragment ion optimised with collision energy of 17 eV. This is produced by the loss of NO, which is a characteristic of nitrophenols derivatives. In order to obtain a confirmatory transition, the collision energy was increased to 22 eV. Then, a minor fragment due to the loss of NO_2 was obtained.

In relation to conjugates, the full-scan mass spectra of 4-nitrophenyl glucuronide and 4-nitrophenyl sulfate showed a strong signal as $[M - H]^-$ as well as other cone fragments. The MS/MS glucuronide spectrum shows the fragmentation of the conjugate in the aglycone (4-nitrophenol, *m*/*z* 138) and in the glucuronidate (*m*/*z* 175). The loss of CO_2 and H_2O from the glucuronic acid yielding the corresponding fragment at *m*/*z* 113 $[Glu\text{-}CO_2\text{-}H_2O\text{-}H]^-$ can also be seen. These transitions can be used for confirmatory purposes. In the case of the sulfate, the main fragmentation corresponded

to the conversion to the aglycone (*m*/*z* 138), although the loss of NO from the aglycone (*m*/*z* 108) was also observed when the collision energy was increased to 30 eV.

Both the alkyl phosphates presented two important fragments. In the case of DMTP (*m*/*z* = 141) they are due to the loss of CH_3 (*m*/*z* = 126) following by a loss of formaldehyde (*m*/*z* = 96). For DMP (*m*/*z* = 125), the loss of dimethyl ether produces the fragment at *m*/*z* = 79 and the loss of two methoxyls generates the other fragment observed (*m*/*z* = 63).

Precursor and product ion, cone voltage and collision energies optimised for quantification and confirmation transitions of metabolites and conjugates are shown in Table 1.

3.2. Free and total phenolic metabolites determination

In a first approach, PNP and 3-Me-PNP were chosen as biomarker of exposure to parathion and fenitrothion, respectively. In a previous work [24], we showed that LC–LC is a good approach for automated removal of interferences after direct injection of human urine. The results slightly improved by using D_4-PNP as labelled IS, which was found to be an appropriate IS for both analytes. In this way, the determination of free metabolites was performed by directly injecting urine samples containing D_4-PNP into the LC–LC–MS/MS system.

However, when we applied this LC–LC–MS/MS method to hydrolyzed urine, poor recoveries were obtained (around 60%). These recoveries were corrected for PNP by using the deuterated internal standard, but this correction was not efficient in the case of 3-Me-PNP. These differences could be explained by the hydrolysis process, as large amounts of various hydroxy compounds can be released after hydrolysis (e.g. nitrophenols) inhibiting the ionisation of the analytes and decreasing the recoveries. In order to avoid this effect, an improvement of the chromatographic process in the second column is necessary in order to enhance the resolution between analytes and co-transferred (from C-1 to C-2) interferences and minimizing the potential suppression effects in the electrospray source. After testing different gradients and compositions, an isocratic mobile phase 35:65 (water:acetonitrile) was chosen as M-2 for the chromatographic separation in C-2, as a compromise between speed and good recoveries.

Finally, the selected LC–LC conditions were set at 75:25 0.01% HCOOH:acetonitrile mobile phase (M-1) and at 35:65 water:acetonitrile mobile phase (M-2). A low concentration of HCOOH in M-1 was mandatory in order to enhance the clean up, as glucuronides present in urine elute as unretained compounds in the Discovery column when a small amount of acid is added into the mobile phase [17]. Under these conditions, PNP and 3-Me-PNP were correctly quantified in both direct and hydrolyzed urine using calibration with standards in solvent.

Standard curves showed excellent linearity in the range 0.5–100 μg/L for both compounds ($r > 0.995$, residuals < 15%) when either external or labelled internal standards were used. Typical chromatograms for 1 μg/L standard solutions and for urine samples (blank and spiked at 1 μg/L) are shown in Fig. 3, where the high sensitivity and selectivity can be observed. However, it was difficult to obtain a real blank sample as most of non-exposed volunteers presented small concentration levels of PNP, which was confirmed by an additional transition. Under this situation, the spiked samples were blank subtracted in order to obtain the recovery values. The LOD estimated from these chromatograms were 0.1 and 0.2 μg/L for PNP and 3-Me-PNP, respectively.

Precision and recoveries of the method are reported in Table 2. In the table, it can be seen that the relative standard deviations were lower than 8% and recoveries were between 87 and 105% for the quantitative transition selected. The LC–LC approach rendered an efficient clean up allowing the correct quantitation of both metabolites in difficult matrices as hydrolyzed human urine using external standard calibration with aqueous solutions. In this way, the use of expensive labelled internal standards (if available) is not required. Obviously, when we used D_4-PNP in combination with this effective cleanup step, the results were excellent as

Table 2
Validation study ($n = 5$) of the developed procedure for the determination of 4-nitrophenol and 3-methyl-4-nitrophenol in urine (three samples at two levels)

Urine	1 (μg/L)				50 (μg/L)			
	External calibration		IS (D4-PNP)		External calibration		IS (D4-PNP)	
	Q[a]	q[a]	Q	q	Q	q	Q	q
PNP								
1	89 (5)[b]	87 (7)	94 (4)	101 (5)	87 (5)	83 (7)	102 (3)	100 (2)
2	100 (6)	98 (6)	98 (3)	99 (4)	99 (2)	98 (4)	102 (3)	101 (3)
3	102 (5)	89 (5)	100 (4)	92 (3)	97 (4)	97 (2)	103 (2)	104 (3)
3-Me-PNP								
1	101 (6)	97 (12)	97 (3)	106 (10)	89 (5)	89 (6)	92 (5)	94 (5)
2	104 (4)	106 (9)	96 (4)	94 (9)	93 (2)	94 (5)	98 (2)	96 (4)
3	102 (4)	110 (13)	105 (2)	107 (7)	92 (3)	94 (3)	99 (3)	101 (3)

[a] Q: quantitative transition, q: confirmatory transition.
[b] Percentage recovery and relative standard deviation in brackets.

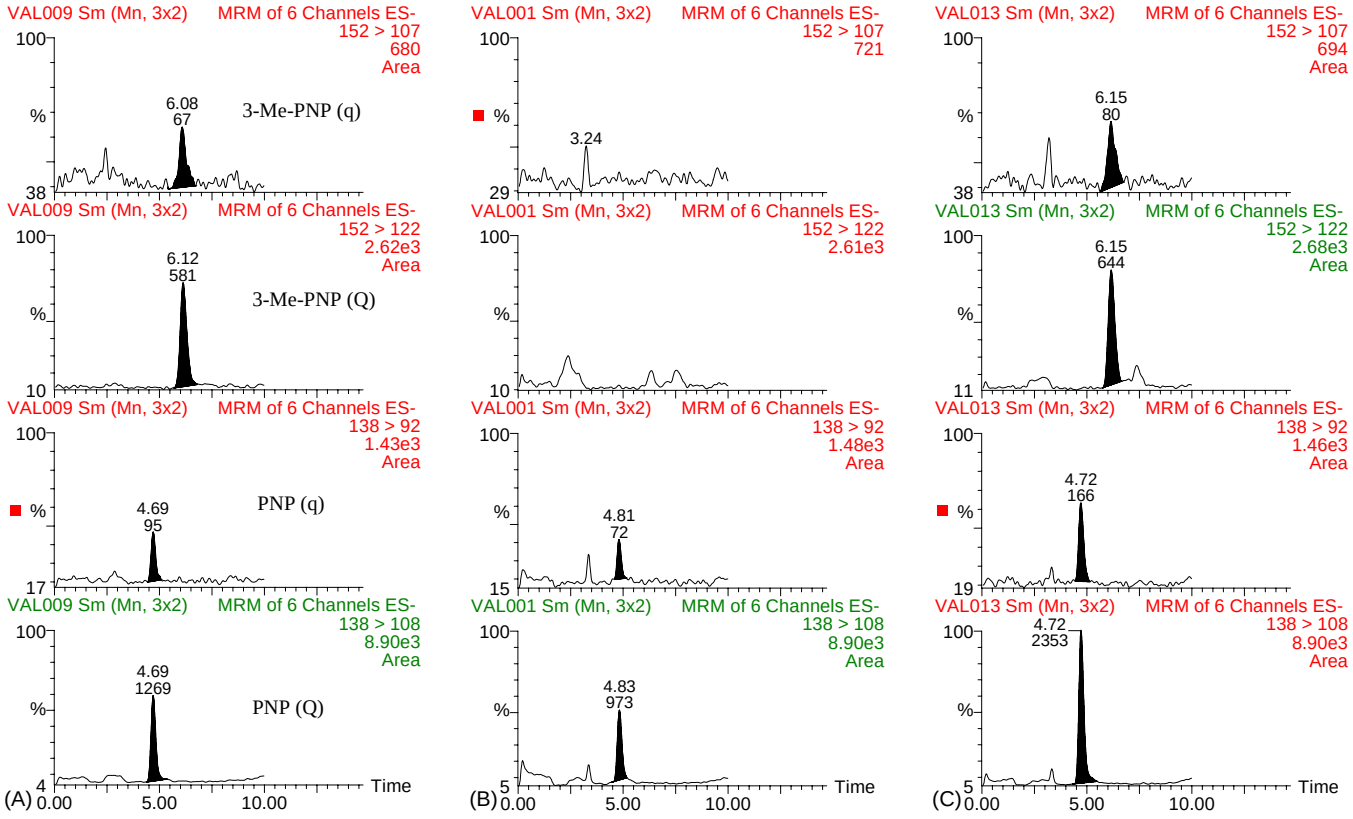

Fig. 3. LC–LC–MS/MS chromatograms for quantitative (Q) and confirmatory (q) transitions of PNP and 3-Me-PNP for (A) 1 μg/L standards, (B) urine "blank" sample and (C) urine spiked at 1 μg/L (peak annotation: top, retention time; bottom, area).

regard both precision (CV < 5%) and accuracy (92–105%), even in the case of 3-Me-PNP.

Information given in Fig. 3 and Table 2 shows that both quantification and confirmation MS/MS transitions can be detected at the lowest level assayed with satisfactory precision and accuracy. Therefore, 1 μg/L was established as LOQ.

3.3. Conjugates and alkyl phosphates determination

The method developed above allowed the rapid and accurate determination of free and the total phenolic metabolites concentration (the sum of free metabolites and those released after hydrolysis) but did not show information about other metabolites or conjugates of OPs selected. In order to get this valuable information, a new approach was evaluated by determining directly the glucuronide and sulphate conjugates as well as the alkyl phosphates related with the parent compounds. Methyl-parathion was the pesticide chosen as a model compound, and therefore PNP-Glucuronide, PNP-Sulphate, DMP and DMTP should be determined.

Regarding alkylphosphates, in a previous work [8] we developed a method for urine analysis using ion-pair formation as an efficient way to obtain sufficient retention using reverse phase material. In order to obtain adequate chromatographic peaks, we were forced to add the ion-pair reagent, TBA, also to the sample. However, this high content of TBA inhibited the MS response, avoiding the detection of DMP. Later, in a recent work [25] we developed a method for the fungicide fosetyl, an alkylphosphonate. Enough retention time, good peak shape and better sensitivity were observed by pre-forming the ion pair only in the sample vial to be injected in the LC–MS system, avoiding the use of TBA in the mobile phase. This last approach was selected in the present paper in order to simultaneously determine PNP-glucuronide, PNP-sulphate, DMTP and allowed us to include DMP as well. Moreover, dilution of the sample and addition of acid to both, the mobile phase and the sample was advisable to obtain a correct peak shape. The optimized method involved a three-fold dilution with water 0.5% HCOOH and the addition of TBA (25 mM in sample).

This method presented good linearity ($r > 0.99$, residuals < 30%) and adequate LOD for all analytes. However, the direct injection of urine samples spiked at concentration levels between 10 and 50 μg/L, did not lead to satisfactory recoveries, as can be seen in Table 3, due to the matrix effect of the urine components. The less retained compounds showed signal suppression meanwhile the most retained exhibited an increase in the response. In order to compensate this effect, an average correction factor was calculated for each metabolite (see Table 3), which allowed us to perform the semiquantitative analysis of samples, obtaining valuable

Table 3
Validation study ($n = 3$) of the developed procedure for the LC–MS/MS determination of methyl-parathion metabolites

Compound	Urine 1	Urine 2	Urine 3	LOD (μg/L)	Correction factor[b]
DMTP	80 (5)[a]	104 (4)	116 (6)	5	1
DMP	53 (4)	37 (7)	78 (9)	20	1.8
PNP-glucuronide	53 (5)	50 (11)	53 (3)	10	1.9
PNP-sulphate	157 (5)	174 (1)	153 (3)	5	0.62

[a] Percentage recovery and relative standard deviation in brackets.
[b] Obtained from the mean recoveries.

information about the ratio between different conjugated forms.

3.4. Application to real samples

The LC–LC method developed in this paper was successfully applied to evaluate the exposure to methyl parathion in a volunteer grower who applied this organophosphorus insecticide. Additionally, urine samples of a group of eleven unexposed volunteers were also analysed. In order to ensure the quality of the process several quality controls (QC) were injected between samples; QCs consisted of urine samples spiked with nitrophenol at two levels (1 and 50 μg/L). Additionally, urine samples spiked with nitrophenol glucuronide and sulphate were injected in order to check the yield of the hydrolysis step. Results were accepted as all QCs recoveries obtained were between 80 and 120%.

Urine samples from the grower were collected before and after application of methyl parathion. As Fig. 4A shows, the concentration of free PNP in the urine sample collected before application was very low (around 1 μg/L), and this concentration increased to 6.3 μg/L after application (Fig. 4C). As regards the total phenol determination (i.e. after hydrolysis of urine), the sample collected before application of methyl parathion (Fig. 4B) presented a peak corresponding to 8.5 μg/L of PNP, which increased up to 81 μg/L after exposure (Fig. 4D). The measured concentrations are in the range of other data reported previously for people who lived in residences illegally sprayed with methyl-parathion [18] but they are significantly lower (10-fold lower) than those reported for occupational exposures [3]. The lower concentration found in our work could be due to an improvement in the protection used by the grower provoked by the increased awareness of dangers about this subject in the last decades.

In relation to unexposed population, around 80% of the analyzed samples showed a small peak for PNP. We could quantify 30% of them as they presented a concentration higher than 1 μg/L (maximum of 4 μg/L). These results agree with those reported by Hill et al. [2], who also found concentrations above 1 μg/L in 41% of samples from unexposed people. They suggested that this low level might be related to the ingestion of acetaminophen.

As regards 3-Me-PNP, it was detected in the grower's urine samples collected before and after application of methyl-parathion although at low concentration levels. Only one of the samples from unexposed population contained 3-Me-PNP, at a concentration close to the LOQ of the method (1 μg/L).

Additional information was obtained by application of the multi-residual method to the urine samples of the grower and selected unexposed people who presented the highest PNP concentration. The results are summarized in Table 4, where the free PNP was obtained by the LC–LC method applied to

Table 4
Data obtained after analysis of urine samples from a grower before and after methyl-parathion application and from an unexposed volunteer by using the analytical methodology developed in this paper

Compound	Before application			After application[a]			Unexposed volunteer		
	μg/L	μM	Ratio (%)	μg/L	μM	Ratio (%)	μg/L	μM	Ratio (%)
DMTP	10	0.07	100	47	0.33	45	31	0.22	100
DMP	N/D	N/D		52	0.41	55	N/D	N/D	
Total		0.07			0.74			0.22	
PNP-glucuronide	N/D	N/D		43	0.14	18	N/D	N/D	
PNP-sulphate	16	0.07	93	139	0.62	77	5.1	0.023	94
PNP (free)[a]	0.9[b]	0.006	7	6.3	0.04	5	0.2[b]	0.0014	6
Total (sum)		0.08			0.80			0.024	
LC–LC method									
Total PNP (experimental)	8.5	0.06		81	0.58		4.2	0.03	

N/D: not detected. Estimated value, as it is lower than LOQ (1 μg/L).
[a] About 20 h later.
[b] Obtained by LC–LC method for non-hydrolyzed urine.

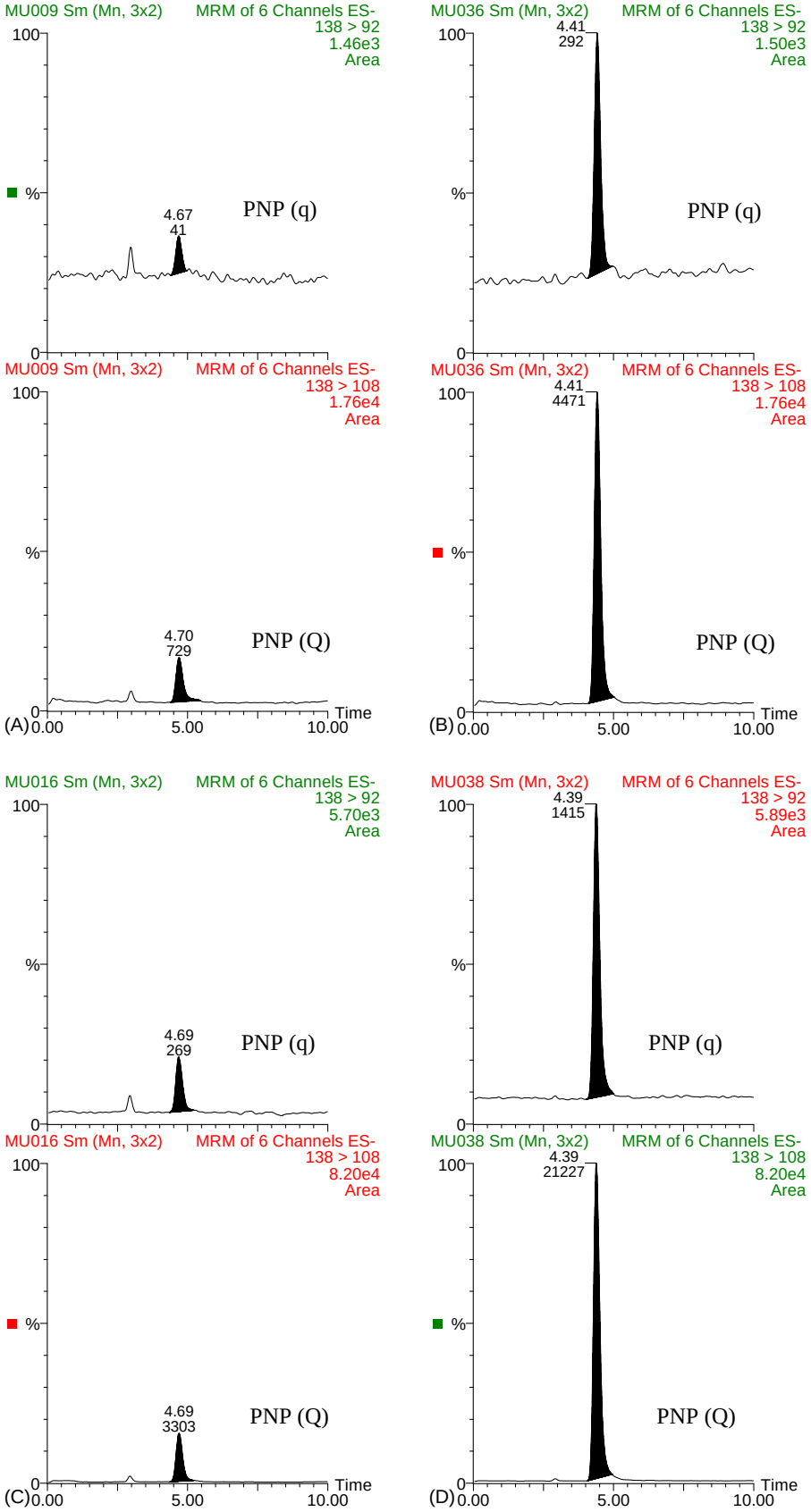

Fig. 4. Chromatograms obtained from urine of a grower who applied methyl parathion: (A) free PNP; (B) total PNP in the sample collected before application; (C) free PNP; (D) total PNP in the sample collected after application.

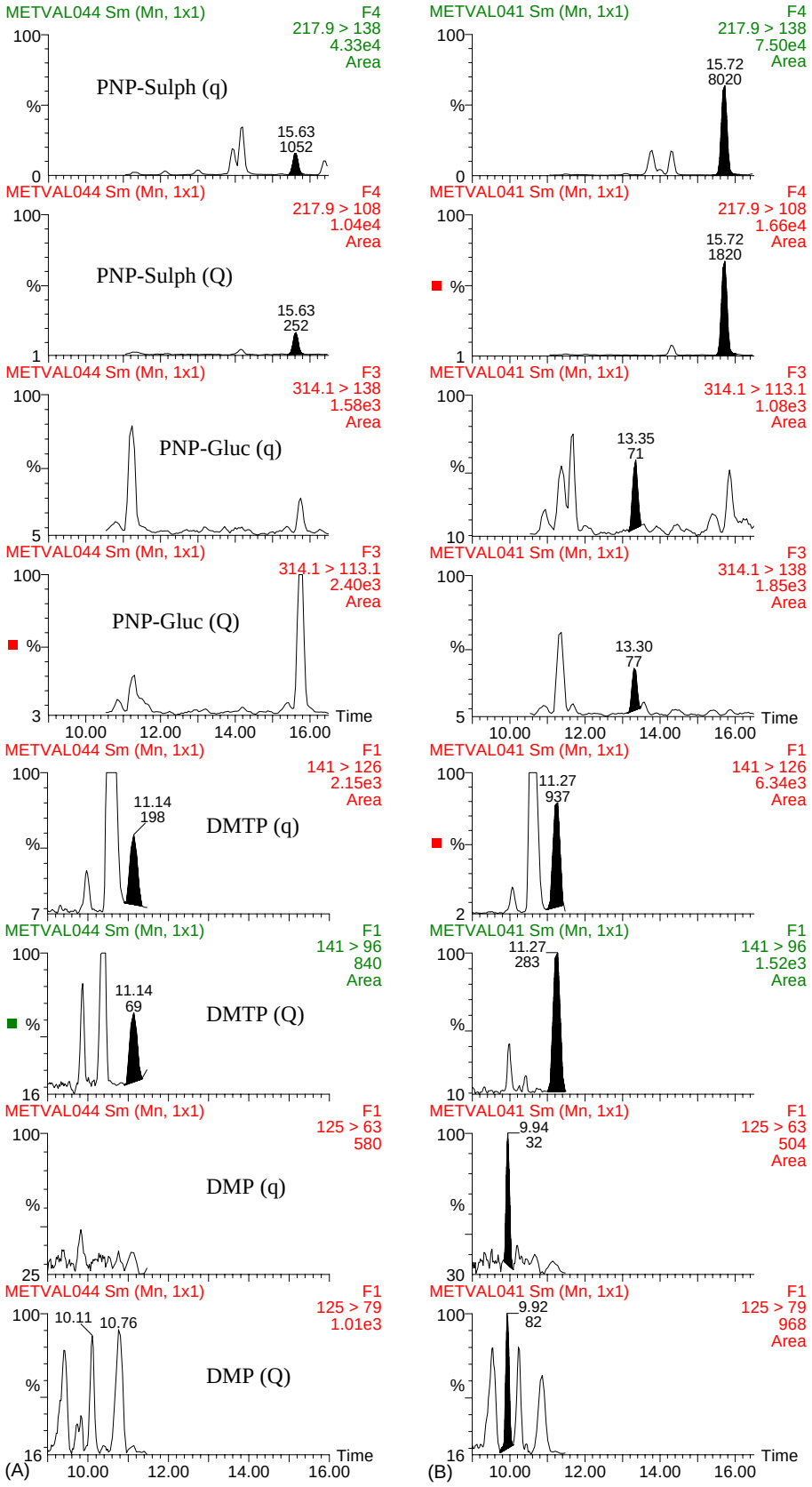

Fig. 5. Chromatograms corresponding to the quantification (Q) and confirmation (q) transitions for all methyl parathion metabolites determined in urine of a grower: (A) before application and (B) after application.

non-hydrolyzed urine. It can be observed that besides free PNP, small levels of PNP-sulphate and DMTP were present before application. After exposure to methyl-parathion a significant increase in all the metabolite concentrations was observed. With these results it seems that the main way to excrete PNP is as sulphate conjugate achieving 77% of the total PNP; the glucuronide represents about 18% and 5% remains as free metabolite. As regards alkyl phosphates, the distribution of phosphate and thiophosphate after exposition to methyl-parathion was almost identical. Obviously, in order to established the significance of this finding, more analysis of exposed population to methyl-parathion should be conducted. Additionally, the level of total alkyl phosphates (in μM) agrees with the total concentration of PNP, confirming that both were released from methyl parathion metabolism for exposed pesticide applicator. However, in the case of the unexposed population (see Table 4), the correlation between both total alkylphosphates and PNP fails, possibly due to the low level exposures and the non-specific sources that could contribute to a larger fraction of the total metabolite concentration measured. The major alkylphosphate metabolite present in urine from unexposed population was DMTP in agreement with recent published data [26].

Table 4 shows the total concentration obtained (μM) as the sum of different PNP-related metabolites determined by the LC method with the value achieved after urine hydrolysis and applying the LC–LC method. In spite of the semi-quantitative character proposed for the LC method, the results were comparable. Therefore, the multi-residual LC–MS/MS method could be an interesting tool in order to evaluate the ratio between different metabolite forms.

Fig. 5 shows the LC–MS/MS chromatograms for all individual metabolites in the grower urine sample, before and after application of methyl-parathion. We can observe the importance of the confirmatory transitions, as all selected transitions (both quantitative and confirmative) were very noisy in comparison with typical MS/MS chromatograms, as for example those of PNP (see Fig. 4). This fact can be explained by the relatively low specificity of the transitions selected. Thus, all the glucuronides or sulphates present in the sample with an aglycone of *m*/*z* 138 would be detected under these transitions, except for the 218 > 108 transition (PNP-sulphate, Q) which leads to the cleanest chromatograms due to its higher specificity. In the case of alkyl phosphates, the transitions involving losses of either alkyl groups or ethers are also rather unspecific. Therefore, it is necessary to select confirmatory transitions in order to avoid false positives. The use of both transitions together with the occurrence of the peak at the correct retention time allows the analyte confirmation in samples.

To obtain more data about the excretion of PNP, the most contaminated samples from unexposed volunteers were also analyzed with the multi-residual LC–MS/MS method. In all cases the only metabolite found was PNP-sulphate. Evidently, more samples from exposed and unexposed population should be analyzed to confirm these findings, but from this data we could conclude that the main way to excrete PNP in humans is as sulphate conjugate.

4. Conclusion

This work has shown that LC–LC–MS/MS is a rapid, sensitive and selective technique for the determination of PNP and 3-Me-PNP (the main metabolites of the organophosphorus pesticides parathion, methyl-parathion and fenitrothion) in human urine. Sample preparation is not necessary for free metabolites (direct analysis in urine) and it is very simple for conjugates. Additionally, the automated clean-up offered by the LC–LC system (a run time of only 7.5 min) makes this procedure rapid. Good precision and recoveries are obtained, with detection limits below 0.2 μg/L. As well, the use of (labelled) internal standard is not necessary, as the clean up performed by the LC–LC technique is efficient for the removal of interferences that could suppress the analytes ionization in the electrospray source employed. The method developed could be suitable for monitoring the exposure to selected OPs pesticides, although it would require its application to a larger sample set to demonstrate the stability over time.

Additionally, a semiquantitative LC–MS/MS approach has been developed for the simultaneous rapid determination of alkyl phosphates and PNP conjugates. The method allows to estimate concentration levels around 5–20 μg/L for all the analytes without tedious sample pre-treatment, as it only requires a three-fold dilution of urine sample with 0.5% HCOOH and the subsequent addition of TBA before injecting in the LC–MS/MS system. Nevertheless, the limitations regarding both reproducibility and sensitivity reduce the applicability of this analytical approach in the field of quantitative analysis mainly due to matrix differences between urine samples, but still provide useful information in those cases of exposed population.

The exposure to methyl-parathion has been easily and efficiently evaluated through the analysis of its metabolites in urine by combining both methods. Free and total PNP in urine are determined by the LC–LC–MS/MS procedure, meanwhile the LC–MS/MS multi-residual method gives a global overview on the ratio of different conjugates metabolites. Data obtained show that the main metabolite excreted in urine is the PNP-sulphate.

The analytical approach developed in this paper could easily be applied to other xenobiotics and would provide valuable information on their distribution in human urine and their excretion. In this way, biological monitoring of human exposure to chemicals could be efficiently directed towards the most appropriate metabolites.

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