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Dispersive liquid-liquid microextraction using a low transition temperature mixture and liquid chromatography-mass spectrometry analysis of pesticides in urine samples.

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Abstract

Biomonitoring is a potent tool to control the health risk of people occupationally and non-occupationally exposed. The latest trend in bioanalytical chemistry is to develop quick, cheap, easy, safe and reliable green analytical procedures to analyse a large number of chemicals in easily accessible biomatrices such as urine. In this paper, a new dispersive liquid–liquid microextraction (DLLME) procedure, conceived to treat urine samples and based on the use of a low transition temperature mixture (LTTM), was developed and validated to analyse twenty pesticides commonly used in farm practises. The LTTM was composed of choline chloride and sesamol in molar ratio 1:3 (ChCl:Ses 1:3); its characterization via differential scanning calorimetry identified it as an LTTM and not as a deep eutectic solvent due to the occurrence of a glass transition at -71 °C. The prepared mixture was used as the extraction solvent in the DLLME procedure, while ethyl acetate as the dispersing solvent. The salting out effect (50 mg mL^{-1} of NaCl in a diluted urine sample) improved the separation phase and the analyte transfer to the extractant. Due to the high ionic strength and despite the density of the LTTM (1.25 g mL^{-1}), the LTTM layer floated on the top of the sample solution after centrifugation. All extracts were analysed by high-performance liquid chromatography coupled to mass spectrometry. After optimization and validation of the whole method, lower limits of quantitation were in the range of 0.02 – 0.76 µg L^{-1}. Extraction recoveries spanned from 50 to 101 % depending on the spike level and analytes. Precision and accuracy ranges were 3-18% and 5-20%, respectively. The extraction procedure was also compared with other methods, showing to be advantageous for rapidity, simplicity, efficiency, and low cost. Finally, urine samples from ten volunteers were effectively analysed using the developed method.

Keywords: deep eutectic solvent; low transition temperature mixture; pesticides; urine; liquid chromatography; dispersive liquid-liquid microextraction.

1. Introduction

In occupational safety and health, urine is one of the most used matrices for the biomonitoring of workers because of reasons connected to logistics, costs, and non-invasiveness of the sampling method. Spot and h-24 samples can be collected in large quantities, allowing the further advantage of obtaining adequate enrichment factors during the sample pre-treatment step. The volume variability is the main drawback that, however, can be corrected by using creatinine as a neutral marker [1].

Pesticides are a perfect example of chemicals whose exposure can be experienced both directly (e.g., by farmworkers) and indirectly (e.g. by population) through their inhalation, skin contact and ingestion [2-4]. At present, unlike matrices such as food and environmental waters, only a modest number of methods has been developed for the determination of pesticides in urine, as it is well-illustrated in the review by Yusa et al. [5]. Even if urine consists primarily of water (up to 95%), high contents of urea (20-25 g L^{-1}) and sodium chloride (10-16 g L^{-1}) as well as other minor components such as proteins (less than 100 mg L^{-1}) make its analysis problematic [6]. The urine treatment is a crucial step to find a compromise among yields, cleanliness of the extract and enrichment factor. To this end, the simple "dilute-and-shoot" approach is the less adequate to reach the necessary sensitivity. The most common technique has been undoubtedly solid-phase extraction (SPE), performed offline [7,8] or online [9,10], using packed cartridges or disks [11]; sometimes, SPE has been combined with a preliminary step of hydrolysis [12] or with a derivatization step when separation was carried out by gas chromatography [13,14]. If the great advantage of SPE is the achievement of a high enrichment factor, weak points are time and costs when compared to those of liquid-phase microextraction techniques, such as dispersive liquid-liquid microextraction (DLLME) [15]. It is well-known that, in DLLME, an extraction solvent (immiscible with water) and, when necessary, a disperser (miscible with both the extractant and aqueous sample) are rapidly injected into the sample solution (individually or as a mixture) to form a cloudy solution; especially when a dispersant is unused, the extractant dispersion is favored by manual agitation, vortex, ultrasounds, etc. Usually, after centrifugation, the extraction solvent is settled on the bottom of the centrifuge tube due to its higher density (e.g. a chlorinated

solvent [16] or a low transition temperature mixture (LTTM)/deep eutectic solvent (DES) [17,18]). However, if a lighter extraction solvent is used, it is collected on the surface as a liquid layer or as a solid droplet after cooling [18]. The advantages of DLLME include ease of use, speed, good enrichment factor, and low environmental factor (E-factor) [19], while the disadvantages could be ascribed to the use of organic solvents even if in small amounts. Recently, LTTMs and DESs, which are defined as "drinkable solvents" for their innocuity, have been used in substitution of chlorinated solvents [17], sometimes without requiring the use of a disperser [20]. These are mixtures with a transition (melting for DESs and glass transition for LTTMs) occurring at a temperature significantly lower than the melting points of the individual starting components. Only mixtures that are liquid at room temperature are of interest within the analytical field. When treating aqueous samples, (environmental or biological fluids), the most used mixtures to perform DLLME are "quasi-hydrophobic" (usually the hydrogen bond acceptor (HBA) is the polar component, while the hydrogen bond donor (HBD) is poorly polar) or "hydrophobic" (both HBA and HBD are non-polar components) [21]. Thus far, in the specific case of pesticides, only a DES-based DLLME procedure has been developed for their recovery from urine [18]; in this case, a mixture composed of menthol and phenylacetic acid was used. The authors modified the classical procedure and assembled a simple but original tool to disperse the extractant and solidify it so to eliminate the centrifugation step.

In this paper, a DLLME procedure was developed to extract a wide number of pesticides from urine, using an LTTM composed of chlorine chloride (ChCl) and sesamol (Ses) in a molar ratio 1:3 (ChCl:Ses 1:3) followed by HPLC coupled to mass spectrometer analysis. In the optimization step, particular attention was devoted to obtaining an accurate phase separation and to avoid problems related to proteins, even if occurring at low concentration. Once optimized, the method was validated and compared with conventional DLLME and SPE approaches, proving to offer best results. Finally, it has been also able to find pesticide traces in volunteers not professionally exposed.

2. Experimental

2.1. Chemicals, materials, and solutions

The analytical standards, all with a purity greater than 98%, were purchased from Aldrich–Fluka–Sigma S.r.l. (Milan, Italy). **Table S1** in the supporting information shows the physicochemical characteristics of the 20 pesticides: acetamiprid, azoxystrobin, boscalid, buprofezin, chlorpyrifos, chlorpyrifos-methyl, clofentezine, dodine, hexythiazox, imidacloprid, methoxyfenozide, myclobutanil, penconazole, propiconazole, pyraclostrobin, pyridaben, pyriproxyfen, spirotetramat, tebuconazole, tebufenpyrad. Acetonitrile, methanol, acetone, toluene, ethyl acetate, choline chloride (ChCl), sesamol (Ses) were obtained from Sigma-Aldrich S.r.l.; 2-amino-1-methyl-1H-imidazol-4-ol, commonly known as creatinine, was purchased from the same company. Ultrapure water was produced from a Milli-Q water generator (Millipore, Bedford, MA, USA).

Individual stock solutions were prepared by dissolving weighed amounts of the analyte standards and creatinine in appropriate solvents: acetonitrile for clofentezine, toluene for pyraclostrobin, and methanol for creatinine and the other analyte standards. Urinary creatinine is a chemical by-product generated from muscle metabolism, whose excretion is independent of urine flow; for this reason, it is used to correct for analyte concentration in urine and to make the result representative of the exposure to a chemical [22]. The concentration of all the stock solutions was 1 mg mL^{-1}, except that of pyraclostrobin and creatinine which was 0.5 mg mL^{-1}. Working composite standard solutions were obtained by diluting the individual ones with methanol at concentrations depending on the purpose. All standards and solutions were stored at 4°C.

2.2. Biological samples

Urine samples were collected daily in 50-mL falcons from healthy voluntary donors of both sexes, aged between 20 and 50 years (ten samples overall). A pool of urine from the different donors (~ 50 mL) was also collected and then subsampled to be used for method optimization and validation. All urine samples were iced and stored at -18 °C till their analysis.

All participants provided informed consent, and the study and the sample protocol were approved by the Ethics Committee of the University of Rome "Università Campus Bio-Medico di Roma" (Prot. 83/20 OSS ComEt CBM).

2.3. Preparation of ChCl:Ses 1:3 mixture

The first step for the preparation of ChCl:Ses 1:3 was to dry ChCl in a muffle oven at 80 °C for 24 h. Then, 1.000 g of ChCl and 2.983 g of Ses were quickly weighed (OHAUS DV215CD Discovery Semi-Micro and Analytical Balance 81 g/210 g capacity, 0.01 mg/0.1 mg readability) in a 25-mL weighing bottle and blended with a spatula. After closing the weighing bottle, the solid mixture was heated at a temperature of about 50 °C for 5 min. Finally, it was allowed to cool at room temperature. About 3 mL of an amber viscous liquid was obtained and utilized to perform about 30 extractions.

2.4. Extraction procedure

The photos in **Fig. 1** depict the main steps of the extraction procedure. Three mL of urine were diluted with 2 mL of Milli-Q water and added with 250 mg of NaCl to obtain a concentration of 50 mg mL^{-1}. The extraction solvent (100 µL of ChCl:Ses 1:3) and the dispersing solvent (400 µL of ethyl acetate) were drawn with individual Hamilton micro-syringes and sequentially injected into the sample. The formation of a cloudy solution was supported by vortexing the sample for 1 minute. Afterwards, the emulsion was centrifuged at 10000 rpm for 10 min. Due to the high ionic strength of the aqueous sample, the ChCl:Ses 1:3 mixture floated on the top of the solution and could be taken with a micro-syringe (100 µL). Finally, after being transferred to a vial, 2 µL was injected for the HPLC-MS analysis.

Analyte concentrations were normalized towards creatinine concentration as follows (equation 1):

$$C_{normalized} = \frac{C_{sample}\ in\ \mu g\ L^{-1}}{C_{creatinine}\ in\ g\ L^{-1}}$$

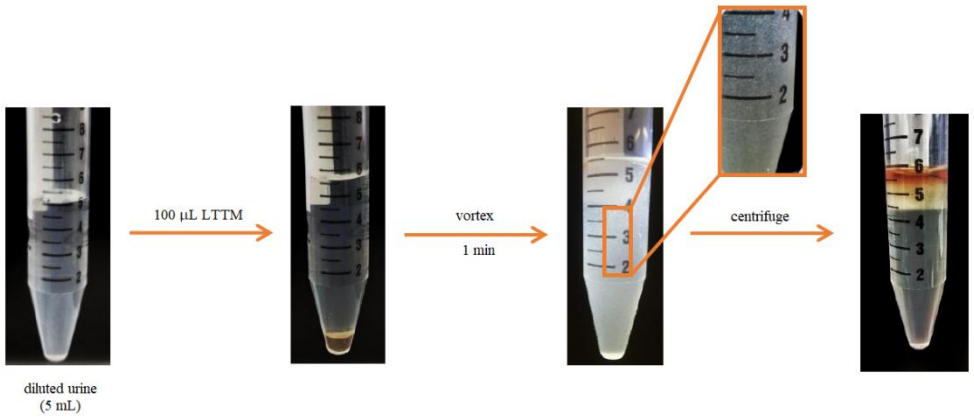

Fig. 1. Photos illustrating the several steps of the DLLME procedure

2.5. Creatinine determination

Since creatinine concentration in urine is very high (up to 0.4- 3.0 g L^{-1} [23]), 50 µL of urine was diluted with Milli-Q water in a 50-mL volumetric flask; then, a 2-µL volume was directly injected for the HPLC-MS analysis. Considering the high dilution ratio applied (1:1000), the matrix effect was negligible and the concentration of creatinine in real samples was calculated by means of external calibration.

2.6. High-performance liquid chromatography-tandem mass-spectrometry

The extracts were analysed by means of a HPLC series 200 binary pump equipped with an autosampler (Perkin Elmer, Norwalk, CT). The column was an X-Terra C18 (2.1 x 150 mm; 3.5 µm), protected by a guard column (Waters, Milford, Massachusetts, USA). The mobile phase was composed of water (phase A) and acetonitrile (phase B), both being 5 mM in formic acid. The analyte separation was conducted applying a flow rate of 0.200 mL min^{-1} and eluting in linear gradient: phase B was increased from 35% to 100% in 16 min and, then, held at 100% for 4 min, for a total run time of 20 min. The autosampler needle device was washed with the phase B after each injection.

The determination of creatinine was accomplished in a separate run by using the same column and mobile phases in isocratic conditions, maintaining 65% of phase A for 5 min at the flow of 0.200 mL min^{-1}.

The detection was performed by an API 4000 Qtrap mass spectrometer (AB SCIEX, Foster City,CA, U.S.A.) equipped with an electrospray source using the following settings: capillary voltage +5000 V, air nebulizer gas 2 L min^{-1}, air drying gas at 350 °C and 20 L min^{-1}, nitrogen curtain gas 5 L min^{-1}, nitrogen collision gas 4 mTorr. The full width at half maximum (FWHM) was set at m/z 0.7 ± 0.1 in each mass-resolving quadrupole to operate with a unit resolution. Chromatograms were acquired using the Scheduled MRM™ Algorithm (Analyst® Software 1.6), which allows many MRM transitions to be recorded without compromising the data quality. To this end, two scheduled multiple reaction monitoring (SMRM) transitions were selected per analyte and detected only in the corresponding retention window (tr ± 60 s); a total of 40 ion currents were recorded with a target scan time of 2 s and a pause time of 5 ms. All LC–MS parameters, useful for identification and quantification, are listed in **Table S2**. **Fig. S1** shows the LC-SMRM chromatogram of a human urine sample spiked pre-extraction with the analytes at a level corresponding to their lower limit of quantitation level.

2.7. Differential scanning calorimetry (DSC)

A Mettler Toledo DSC STAR822e instrument (MettlerToledo, Greifensee, Switzerland) was used to characterize the thermal properties of ChCl:Ses 1:3. About 2 mg of sample was rapidly weighed in an aluminium pan and sealed to avoid water absorption. The temperature program was set as follows: cooling from 20 to −100 °C and then heating up to 25 °C, using a scanning rate of 10 °C min^{-1}. The furnace was purged by flowing dry nitrogen at a flow rate of 30 mL min^{-1}.

3. Results and discussion

3.1 Characterization of the ChCl:Ses 1:3 mixture

3.1.1 Density measurement

One mL of ChCl:Ses 1:3 was weighed on a microbalance (see paragraph 2.3 for the balance model). Density was calculated as the mean of three replicates at 23 °C:

(1) $\rho = m/V = 1.25 \pm 0.01 g\ mL^{-1}$

This result is in accordance with literature's density values of other mixtures [24] and with our experimental observations, i.e. sedimentation of the mixture after the centrifugation step in a water sample.

3.1.2. Differential scanning calorimetry (DSC)

Fig. 2 shows the thermogram of ChCl:Ses 1:3. In both profiles, an intense glass transition is observed both in the cooling and heating runs. The glass transition temperature (Tg), taken at the midpoint (vertical bar), is - 71°C. The presence of this kind of transition and the absence of both a crystallization and melting make it possible to classify the ChCl:Ses 1:3 mixture as a LTTM.

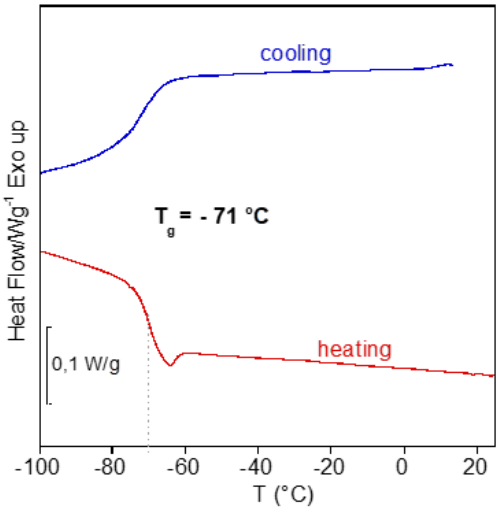

Fig. 2. DSC thermogram of ChCl:Ses 1:3; scan rate 10 °C min-1.

ChCl:Ses 1:3 was used for the first time in another work [25] aimed at the extraction of Sudan I from food samples. Nevertheless, it was erroneously identified as a DES instead of a LTTM.

3.2 Optimization of the DLLME extraction

The parameters known to be critical for DLLME were studied in order to optimise the extractive yield of the method: nature and volume of the extracting solvent and dispersing solvent; volume, ionic strength and pH of the aqueous sample; mode and time to assist dispersion. All experiments were carried out in triplicate using 5 mL of diluted urine (3 mL of human urine + 2 mL of Milli-Q water) spiked with analytes at 1 ng mL^{-1}.

3.2.1 Dispersing solvent selection and volume optimization

The dispersing solvent must be miscible both with the extraction solvent and with water so to assist the formation of the emulsion (cloudy solution). THF, ethyl acetate, isopropanol, acetonitrile and acetone were tested as dispersing solvents. The experiments were carried out as follows: 100 µL of ChCl:Ses 1:3 and 500 µL of a dispersant were added to a volume of 5-mL of diluted urine; the solution was stirred for some minutes and then centrifuged to evaluate the volume of the extract. Among all used solvents, only THF and ethyl acetate allowed one to withdraw volumes of LTTM suitable for the subsequent instrumental analysis (around 100 µL), probably due to their higher logP values (see **Table S3** reporting logP, miscibility with water and toxicity of the selected solvents). Being less toxic, ethyl acetate was selected for the following experiments. In order to find the optimum, different volumes were tested: 200, 250, 300, 350, 400, 500, 600, 800 µL. Since the different volume of LTTM that could be taken depended on the dispersant volume, all extracts were brought to the same final volume by the addition of methanol (Vf =100 µL) to make an objective comparison of the yields. The highest extraction efficiency was obtained using 400 µL of ethyl acetate, while volumes less than 350 µL allowed one to recover volumes not reproducible and/or not enough for subsequent instrumental analysis. It has been observed that without the use of the dispersing solvent, the LTTM is completely dissolved in the urine sample after vortexing the sample. On the other hand, using more than 500 µL of dispersing solvent, the volume of the final extract was much more than 100 µL, probably due to the partial mixing of the dispersant in the LTTM **(Fig. 3a).**

3.2.2 Optimization of the extraction solvent volume

Keeping the volumes of the sample (5 mL) and the dispersing solvent (400 µL) unchanged, it was checked whether an increase in the extractant volume (100 µL, 200 µL) resulted in a substantial improvement of the analyte recovery. The optimization of this factor was done considering the consequent variation in the enrichment factor, inversely proportional to the volume of the withdrawn extract. To make a reliable comparison, the two series of experiments (three replicates for each condition) were carried out bringing the extracts to the same final volume with methanol (200 µL). After LC-MS analysis, it was verified that the yield is better using the smaller quantity of extraction solvent, i.e. 100 µL of ChCl:Ses 1:3. This phenomenon finds explanation in the variation of the ratio between the volumes of the extraction solvent and dispersing solvent. In fact, using 200 µL of ChCl:Ses 1:3, a 400-µL volume of ethyl acetate is not enough to form a dispersion as fine as that obtained with 100 µL of extractant; in this way, because a smaller contact surface between aqueous sample and extractant is generated, the analyte mass transfer is less effective and affects the recovery. **(Fig.3b).**

3.2.3 Optimization of the sample volume

Since urine is a complex biological matrix, it was necessary to dilute it with water in order to improve the phase separation during the DLLME procedure and minimize the matrix effect. The optimization was aimed to define both the sample volume and the dilution ratio. Regardless the dilution ratio, the treatment of sample volumes greater than 5mL leads to increase the volumes of extractant and dispersant necessary for the stabilization of the ternary mixture, but this strategy is in contradiction with the aims of microextraction techniques focused to minimizing wastes of solvents. **Fig.3c** depicts both extractive yield and S/N ratio obtained using different dilution ratios while maintaining the final sample volume equal to 5 mL. As can be seen, the optimum is 3 mL of urine diluted with 2 mL of MilliQ water.

3.2.4. Ionic strength

It was observed that using the diluted urine samples, the phase separation between the settled LTTM and the aqueous sample was hindered by the formation of a suspension at the bottom of the test tube. For this reason, two different approaches were tested: *i*) filtration of the spiked urine before the extraction or *ii*) addition of salt in the spiked sample before the extraction. Syringe filters based on PTFE, PVDF, WATMAN, and cellulose acetate were tested, but all of them showed an affinity for the analytes and reduced drastically the recovery. Regarding the addition of salt to the sample, the effect of NaCl concentrations ranging from 5 to 100 mg mL^{-1} on the recovery yields was examined. The lower concentration able to improve the phase separation was 50 mg mL^{-1}: at this salt concentration, the density of the aqueous sample increases to such an extent that the LTTM layer floats on the top of the sample solution instead of settling on the bottom of the centrifuge tube.

3.2.5 Dispersion medium and extraction time

The performance of both vortex and sonicator agitation was evaluated at different times (1, 2, 4 min). Comparing the mean value of the chromatographic peak areas, the best yield was obtained by vortexing for 2 min (**Fig.3d**). The high density of the extraction solvent could be the cause of the inefficiency of the sonicator to produce a fine dispersion in the times tested.

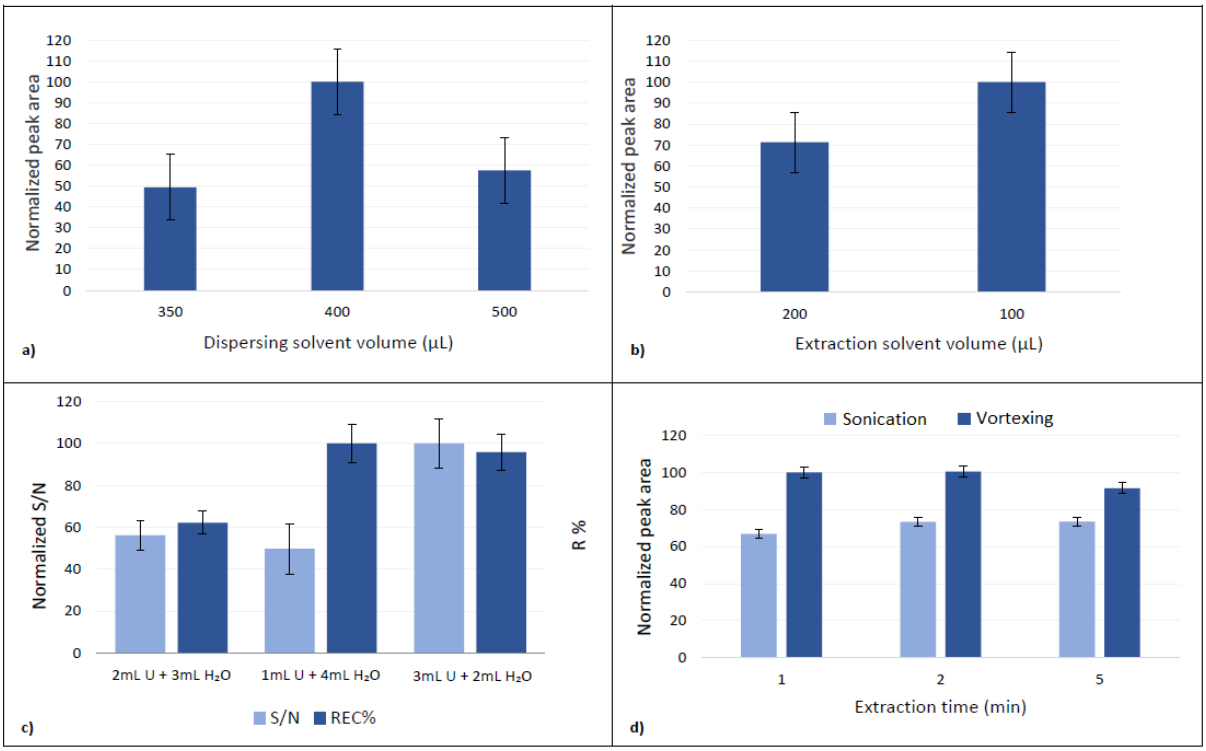

Fig. 3. a) Volume optimization of the dispersing solvent; b) Volume optimization of the extractant (ChCl:Ses 1:3); c) Optimization of the dilution factor of urine as compromise between best recovery and S/N ratio; d) Comparison between sonication and vortexing at different times of agitation. Since an analogous trend was observed for all analytes, the average of their normalized chromatographic areas, as well as the average of the corresponding normalized S/N were taken into account. Normalization was performed attributing 100% to the highest absolute values.

3.3 Method validation and matrix effect evaluation

The method validation was conducted by evaluating a series of quantitative and qualitative parameters including recovery, enrichment factor, within-run and between-run precision, within-run and between-run accuracy, lower limit of quantification (LLOQ), sensitivity, linearity, matrix effect and selectivity. **Tables 1** and **2** summarize the figures of merit of the quantitative validation parameters. Quantitative analysis was performed by the external calibration method since blank samples were available (see paragraph 2.2). The calibration curves were constructed spiking nine 5-mL aliquots of diluted blank urine with increasing concentrations of the target analytes (LLOQ level and 1, 15, 30, 45, 60, 75, 100 µg L^{-1}). The extraction procedure is that described in section 2.4. The peak area ($A_{analyte}$) was plotted versus the spike level (µg L^{-1}) by applying the least-square method ($y = mx + q$ as regression model). As it can be seen from **Table 1**, determination coefficients (R^2) obtained are greater than 0.99 for all the analytes. For each analyte, the evaluation of the matrix effect (ME) was

performed comparing the slope of the matrix-matched calibration curve with the slope of the calibration curve in solvent. The first one was built extracting blank urine samples spiked post-extraction with the analytes, while the second one was obtained spiking 100-µL aliquots of ChCl:Ses 1:3 with increasing concentrations of analytes; the volume of all calibrators was brought to the same value with methanol. Each slope was calculated as mean of three replicates. The following equation was used for the ME evaluation:

$$ME = \frac{slope\ of\ matrix\ matched\ calibration\ curve - slope\ of\ calibration\ curve\ in\ solvent}{slope\ of\ calibration\ curve\ in\ solvent} \cdot 100$$

Matrix effect percentages are listed in **Table 1**. As can be seen, the matrix effect causes signal suppression for all analytes that, however, is moderate or negligible for most of them. This is probably the result of the urine dilution as well as the selective extraction performed by ChCl:Ses 1:3. Only for imidacloprid, clofentazine and chlorpyrifos-methyl a suppression of about -30% was observed , probably in relation to the co-elution of interfering compounds at their corresponding retention times. The calibration curve for creatinine (endogenous substance) was built by means of external calibration instead of standard addition method because of the high dilution ratio applied. To this end, five 50-µL aliquots of Milli-Q water (instead of 50-µL aliquots of urine) were diluted in 50-mL volumetric flasks and spiked with the creatinine standard solution so to obtain the following concentrations: 0.0625 mg L^{-1}, 0.125 mg L^{-1}, 0.625 mg L^{-1}, 1.25 mg L^{-1}, 2.5 mg L^{-1}; considering a 1:1000 dilution ratio, such concentrations correspond to g L^{-1} spike levels in the pure urine (see **Table 1** for the creatinine calibration curve and R^2). In order to verify the reliability of the quantification approach for creatinine, some samples were also analysed by applying the standard addition method, obtaining a good agreement between the results attained with both methods; this outcome was also confirmed by the similar slope exhibited by the two calibration curves (negligible matrix effect). The use of the external calibration approach allows one to save time since the standard addition method requires to construct a curve for each real sample analysed.

Table 1. Linear regression parameters and matrix effect

Analyte	regression equation		R^2	Matrix effect
	$m \pm s_m t_{(0.05;6)}$	$q \pm s_q t_{(0.05;6)}$		(%)
Imidacloprid	9.28 ± 0.29	- 4.26 ± 0.18	0.9983	- 30
Acetamiprid	15.0 ± 0.4	- 9.45 ± 0.21	0.9985	- 13
Dodine	27.6 ± 0.7	- 20.6 ± 0.8	0.9994	- 9.1
Spirotetramat	48.5 ± 0.9	55.0 ± 0.3	0.9983	- 10
Azoxystrobin	42.4 ± 0.9	65.2 ± 0.5	0.9975	0
Myclobutanil	35.6 ± 0.7	20.7 ±0.3	0.9994	- 9.1
Boscalid	18.1 ± 0.4	- 3.82 ± 0.36	0.9958	- 2.3
Tebuconazole	38.5 ± 0.7	8.03 ± 0.25	0.9969	- 7.0
Methoxyfenozide	109 ± 1	90.7 ± 0.9	0.9991	- 20
Penconazole	19.4 ± 0.5	- 12.9 ± 0.2	0.9974	- 9.3
Propiconazole	25.2 ± 0.5	21.3 ± 0.6	0.9990	- 11
Pyraclostrobin	102 ± 1	123 ± 1	0.9964	- 3.3
Clofentezine	13.6 ± 0.4	13.4 ± 0.2	0.9953	- 30
Chlorpyrifos-methyl	1.79 ± 0.13	- 0.11± 0.04	0.9971	- 26
Buprofezin	332 ± 2	348 ± 3	0.9988	- 1.7
Tebufenpyrad	18.6 ± 0.6	11.0 ± 0.2	0.9983	- 8.1
Pyriproxyfen	125 ± 1	84.3 ± 0.9	0.9986	- 13
Chlorpyrifos	2.44 ± 0.22	1.38 ± 0.18	0.9983	- 14
Hexytiazox	28.3 ± 0.6	- 1.37 ± 0.09	0.9997	- 9.1
Pyridaben	164 ± 1	145 ± 1	0.9987	- 13
Creatinine	2236 ± 5	77.9 ± 0.8	0.9988	0

For each analyte, absolute LLOQ was estimated as the spike level detectable and quantifiable with a signal-to-noise ratio of 5 (five replicates). To this end, 5-mL blank samples of diluted urine were fortified pre-extraction with the analytes at decreasing concentrations until to meet the described requirements. Once the spike level was experimentally verified, the average value of LLOQ was calculated preparing five replicates. LLOQs were in the range 0.02-0.76 μg L^{-1} (**Table 2**)

To calculate recovery and within-run precision, five 5-mL aliquots were spiked with the pesticides pre-extraction at two concentration levels corresponding to LLOQ and 10 LLOQ; another aliquot was spiked post-extraction with the same nominal concentrations of the analytes. Good recovery values were obtained for most analytes; in few cases at the lower spike level, yields were around 50%, but

they were acceptable since the LLOQ of the method was very low. The enrichment factor (EF) was calculated according to the following equation:

$$EF = \frac{C_{analyte\ in\ the\ final\ extract}}{C_{analyte\ in\ the\ urine\ sample}}$$

The procedure was able to reach EFs spanning from 17.7 to 28.5 (see **Table 2)**. The within-run precision, described as relative standard deviation (RSD), was in the range of 3-18% (LLOQ) and 3-15% (10 LLOQ) for major part of the analytes (**Table 2)**. The between-run precision was evaluated as the RSD of three different analytical sessions, each of which involved 5-replicates at LLOQ and 10 LLOQ; its values were equal to or less than 15% (10LLOQ) and 20% (LLOQ).

The accuracy was calculated as the relative deviation between the mean measured concentration and the actual one, following the formula:

$$Accuracy\% = \frac{C_{spiked} - C_{measured}}{C_{spiked}} * 100$$

Replicates (N=5) were conducted at the same spike levels as precision and recovery. The within-run accuracy was in the range 11-20% (LLOQ) and 5-15% (10LLOQ) (see **Table 2**). The between-run accuracy was calculated in three different analytical sessions with values equal to or less than 15% (LLOQ) and 20% (10LLOQ).

Table 2. LLOQ, enrichment factor, recovery, precision, and accuracy

ANALITES	LLOQ (µg/L)	Enrichment factor[a]	Recovery (Within-run precision)[b] % (%)		Within-run Accuracy[b] %	
			Spike levels		Spike levels	
			LLOQ	**10LLOQ**	**LLOQ**	**10LLOQ**
Imidacloprid	0.22	28.5	101 (10)	89 (10)	19	15
Acetamiprid	0.28	23.3	83 (9)	72 (4)	11	15
Dodine	0.37	24.6	89 (7)	75 (3)	20	15
Spirotetramat	0.03	20.6	72 (4)	65 (8)	14	5
Azoxystrobin	0.07	23.4	85 (7)	74 (7)	20	15
Myclobutanil	0.08	21.3	65 (8)	77 (9)	20	7
Boscalid	0.1	21.6	75 (9)	69 (9)	20	15
Tebuconazole	0.03	22.5	71 (6)	79 (7)	20	9
Methoxyfenozide	0.04	21.8	75 (3)	71 (8)	14	12
Penconazole	0.2	25.7	83 (8)	88 (7)	19	15
Propiconazole	0.11	21.0	67 (16)	73 (9)	11	15
Pyraclostrobin	0.03	19.8	60 (10)	71 (6)	10	10
Clofentezine	0.22	22.5	78 (7)	68 (8)	20	15
Chlorpyrifos-methyl	0.76	24.5	80 (14)	83 (15)	16	14
Buprofezin	0.03	17.7	53 (4)	64 (15)	19	15
Tebufenpyrad	0.06	20.4	60 (10)	76 (15)	11	10
Pyriproxyfen	0.02	19.1	50 (7)	82 (8)	19	15
Chlorpyriphos	0.27	22.1	69 (18)	98 (12)	14	5
Hexytiazox	0.08	19.2	61 (8)	84 (11)	20	15
Pyridaben	0.02	18.3	60 (9)	62 (11)	20	7

[a] The enrichment factor has been reported as mean values of data obtained for spiking levels at LLOQ and 10LLOQ;
[b] Recovery, precision and accuracy were calculated preparing 5 replicates at each spike level.

3.4 Comparison with classical extraction procedures

Since the methods reported in the literature involve pesticides different from those selected in this work, the real potential of the proposed DLLME technique was evaluated by comparing it with two classical procedures, optimized in our lab: a conventional DLLME relying on the use of chloroform as an extracting solvent, and an SPE procedure based on OASIS-HLB cartridges. The evaluation was performed considering time, costs, solvent consumption, materials, and recovery. For the comparison, 9 blank samples were spiked at 1 ng mL^{-1} and extracted with the three procedures (three replicates per each procedure). **Table S4** resumes the extraction conditions for the three techniques. SPE is the technique providing the best yields (79% on average), but at the expense of longer times (55 min of total extraction) and a higher consumption of solvents (19 mL). On the other hand, the DLLME procedure based on the use of ChCl:Ses 1:3 is advantageous over the classical one in terms of yields (70% *vs* 63%, on average), extraction time (12 min *vs* 23 min) and negligible toxicity of the solvents

used. The less time employed using ChCl:Ses 1:3 is due to the direct injection of the extract in the chromatographic system. These advantages combined with the good yields make our procedure suitable for routine survey where, besides analysis time and simplicity of the procedure, also cheapness and the improved green character have a considerable relevance.

3.5. Analysis of real samples

The validated method was applied to the analysis of samples taken from ten volunteers. The samples were analysed both for the pesticides (section 2.4) and creatinine (section 2.5) and the $C_{normalized}$ was calculated for the positive samples. Most turned out to be free of analytes, but one of them resulted to be positive for three pesticides: acetaprimid (4.33 μg $g^{-1}_{creatinine}$), azoxystrobin (0.03 μg $g^{-1}_{creatinine}$) and pyraclostrobin (0.03 μg $g^{-1}_{creatinine}$); these levels were ascribable to diet, i.e. ingestion of residues occurring in fruits and vegetables. **Fig. 4** shows the related chromatogram. As can be seen from this preliminary survey, the method has proved to have the adequate selectivity and sensitivity to confirm and quantify the selected pesticides also in urine of people not professionally exposed.

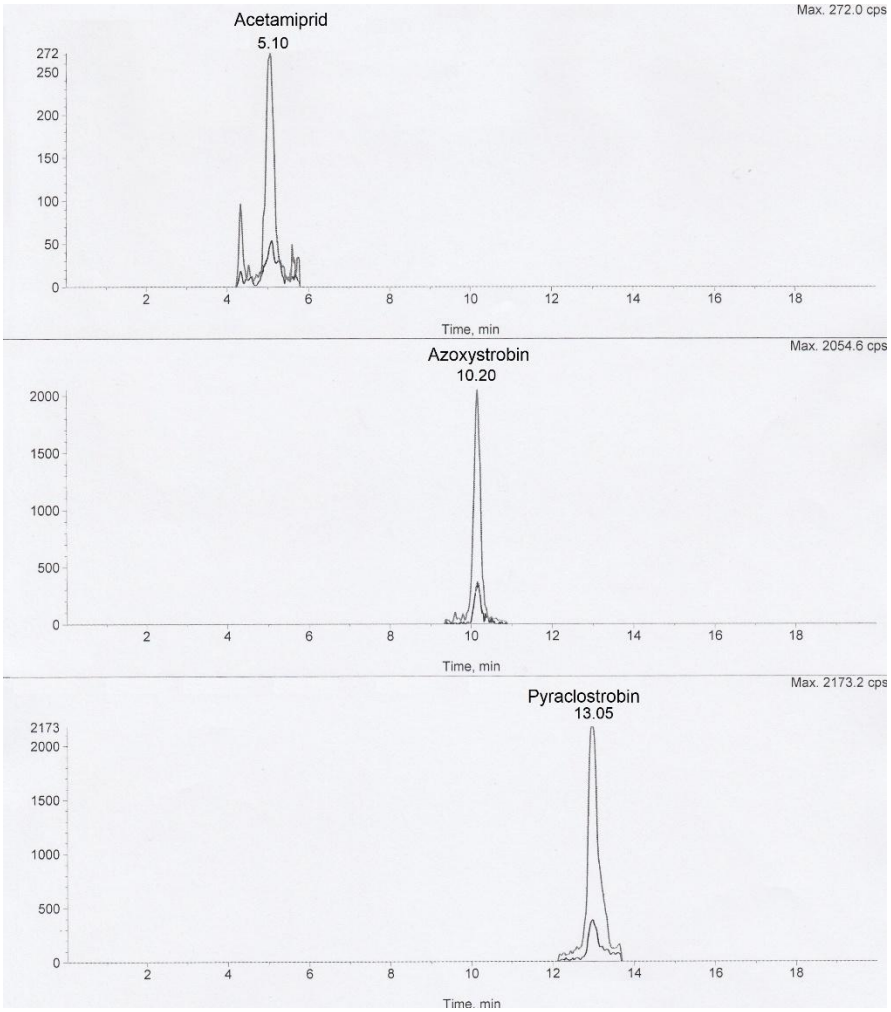

Fig.4. Ion currents of the pesticides extracted from the LC-SMRM chromatogram of a real urine sample. The measured concentrations were: acetaprimid (4.33 µg $g^{-1}_{creatinine}$), azoxystrobin (0.03 µg $g^{-1}_{creatinine}$) and pyraclostrobin (0.03 µg $g^{-1}_{creatinine}$); creatinine concentration in this sample was 0.3 g L^{-1}.

4. Conclusion

DESs and LTTMs have attracted considerable attention as green-solvents able to replace classical molecular solvents in many analytical methods. In this paper, ChCl:Ses 1:3 was used for the first time as an extraction solvent in a DLLME procedure to isolate twenty pesticides from human urine samples. This procedure exhibits the typical advantages of the microextraction techniques combined with those arising from using a DES. Even if DLLME is typically applied to treat water samples, it has been opportunely modified so to process urine samples. Compared with other techniques, the method here proposed shows a good competition in extractive yields for most of the selected analytes,

a much lower cost for single extraction, high speed and a lower impact on environment and operator health. These are all requirements which make it very appealing for clinical surveys.

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