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Tandem focused ultrasound (TFU) combined with fast furnace analysis as an improved methodology for total mercury determination in human urine by electrothermal-atomic absorption spectrometry

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Abstract

A new sample preparation procedure based on tandem (that is, different diameter probe sonicators used in the same sample treatment) focused ultrasound (TFU) for mercury separation, preconcentration and back-extraction in aqueous solution from human urine has been developed. The urine is first oxidized with $KMnO_4$/HCl/focused ultrasound (6 mm probe). Secondly, the mercury is extracted and preconcentrated with dithizone and cyclohexane. Finally, the mercury is back-extracted and preconcentrated again with the aid of focused ultrasound (3 mm probe). The procedure allows determining mercury by electrothermal atomic absorption spectrometry with fast furnace analysis and calibration against aqueous standards. Matrix modification is provided by the chemicals used in the sample treatment. The procedure is accomplished with low sample volume (8.5 ml). Low volume and low concentration reagents are used. The sample treatment is rapid (less than 3 min per sample) and avoids the use of organic phase in the graphite furnace. The preconcentration factor used in this work was 14. The limit of detection and the limit of quantification in urine were, respectively, 0.27 and 0.9 $\mu g\,l^{-1}$. The relative standard deviation of aqueous standards ($n = 10$) was 4% for a concentration of 100 $\mu g\,l^{-1}$ and 5% for a concentration of 400 $\mu g\,l^{-1}$. Recoveries from spiked urine with inorganic mercury, methyl-mercury, phenyl-mercury and diphenyl-mercury ranged from 86 to 98%.

Keywords: Focused ultrasound; Mercury; Urine; ET-AAS

1. Introduction

Mercury and their compounds are toxic to humans because of their accumulation in living tissues, even in very small doses, causing many harmful effects. The determination of mercury in urine can give important information concerning human exposure to this metal [1].

Mercury in urine can be measured using potentiometry [2], gas-chromatography [3], cold vapour atomic fluorescence spectrometry [4], and by atomic absorption spectrometry with the cold vapour technique [5] (CV-AAS). Electrothermal atomic absorption spectrometry (ET-AAS) cannot be used for direct mercury determination in urine due to the high detection limit for mercury inherent to this technique (e.g., 4 $\mu g\,l^{-1}$ in our conditions) and to the low mercury content in urine of non exposed people (generally less that 1 $\mu g\,l^{-1}$). However, the simplicity of the methodology, the high sample throughput and the full automation are advantages of ET-AAS. In addition, the low sample and reagent volumes needed in this technique meet the requirements of the analytical minimalism concept outlined by Halls [6]. In order to circumvent the limitation of low mercury concentration in urine, different methodologies involving preconcentration procedures have been cited in literature [7–9]. In these procedures, an urine pre-treatment is mandatory previous mercury extraction in order to eliminate or diminish any interference caused by the organic matter present in the urine. Different approaches can be found in literature based on the use of high acid/s concentration/s and/or digestion in closed systems [7,9,10] which are time/reagent consuming. Recently [5] we have developed a new and fast sample treatment for mercury FI-CV-AAS determination in urine, which needs a 0.5% of $KMnO_4$ in

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HCl media (1 M) and the aid of focused ultrasound (FU), as outlined by Mason [11]. The organic mercury compounds tested were decomposed in less than 1 min and the urine matrix interferences were completely avoided after 5 min of ultrasonic energy irradiation. However, this method does not allow the determination of Hg(II) by ET-AAS in urine of due to the lower mercury content compared to the detection limit of the technique, as commented before.

In the liquid–liquid preconcentration of mercury, the analyte is transferred for an aqueous solution to an organic complexing phase with lower volume. Dithizone is one of the most common complexing agents used for mercury complexation [7], since the mercury is strongly bound to the dithizone sulphidric groups. Additionally, dithizone has been used successfully as stabilising agent in ET-AAS [12]. The liquid–liquid preconcentration of mercury from urine using dithizone and cyclohexane as organic phase has been successfully applied by Burrini and Cagnini [7]. The mercury was determined by ET-AAS using the organic phase, that is, the cyclohexane was directly introduced into the furnace. The direct analysis of organic solutions in electrothermal atomic absorption spectrometry (ET-AAS) has several drawbacks. There are environmental problems due to the volatilization of the organic phase and the volatilization also leads to an increase in the actual concentration of the analyte; a poor performance of the auto sampler droplet dispensing is observed and aqueous standards cannot be used for calibration [7]. In addition, Volynsky et al. [13] emphasized that the spreading of organic samples over the graphite furnace surface distorts the atomic absorption profiles, renders the analytical curve non-linear and decreases the sensitivity. Furthermore, according to Tserovsky and Arpadjan [14], the removal of organic liquids after their penetration into the graphite requires long pretreatment at high temperature. Hence, the volatile compounds would be lost at this stage.

The emerging interest in fast methodologies forces to bear in mind the following items: (1) rapid sample preparation procedures based on Green Chemistry (e.g., methodologies based on the application of ultrasonic energy) and (2) fast thermal programs when working with ET-AAS. With respect to fast thermal programs, the use of matrix modifiers and/or the decrease of the organic matter content in aqueous solutions introduced into the furnace are needed to avoid (i) mercury volatilization and (ii) the pyrolysis stage, if possible.

In the present work we have developed a new and fast sample treatment for the separation, preconcentration and back-extraction of mercury from human urine and its subsequent determination by ET-AAS. The proposed method entails a new concept in the application of ultrasonic energy, the tandem focused ultrasound (TFU), where more than one ultrasonic tip is used in the sample treatment. Focused ultrasound has been cited in literature as useful tool in the separation of trace metals and metalloids from biological solid matrices [15], in the acceleration of metal fractionation by sequential extraction schemes [16] and in the selective oxidation of physicochemical forms of elements for speciation [17]. The back extraction in aqueous solution allows the use of simple aqueous standards for calibration, which simplify the procedure and speeds the analysis. The preconcentration is regarded to increase the Hg(II) content above the detection limit of ET-AAS.

2. Experimental

2.1. Apparatus

A Branson Sonifier 150 ultrasonic cell disruptor-homogeniser (63 W, 22.5 kHz, Branson Ultrasonics Corporation, USA) equipped with a 3-mm and a 6-mm titanium microtip was used. Ultrasonic energy irradiation was fixed at any desired level using a power setting in the 40–70% range with the 6-mm micro-tip and a 10% with the 3-mm micro-tip. The Sonifier 150 has a digital LCD display which provides a continuous read-out of the watts delivered to the end of the probe (range 5–12 W in this work). A Shimadzu UV-2501 spectrophotometer was used to record the effectiveness of the sample treatment. Mercury absorbance was measured with a Varian (Cambridge, UK) atomic absorption spectrometer model SpectrAA-300 plus equipped with a graphite furnace and an autosampler. Zeeman background correction was used. A mercury hollow-cathode lamp operated at 4 mA was used as a radiation source. The mercury analytical line at 253.7 nm and a slit width of 0.5 nm were used for measurements. Pyrolytic graphite-coated graphite tubes with L'vov platform were used. The electrothermal program is presented in Table 1.

A special autosampler cup was developed in this work, with a conical-shaped bottom and a capacity of 2.5 ml (Fig. 1).

2.2. Reagents

Since a preconcentration procedure was developed special care was taken in order to choose the highest pure reagents available in the market. Milli-Q ultrapure water was used throughout. $KMnO_4$ pro analyse (maximum 0.000005% Hg, N 105084), sodium oxalate pro analyse (N 106557), and cyclohexane pro analyse (N 109666) were purchased from Merck (Darmstadt, Germany). Dithizone pro

Table 1
Thermal program for Hg

	Stage						
	1	2	3	4	5	6	7
Temperature (°C)	85	95	120	120	1800	1800	2100
Furnace time (s)	5	40	30	20	1	4	2
Gas flow (l min^{-1})	3	3	3	3	0	0	3
Read command	–	–	–	–	Yes	Yes	–

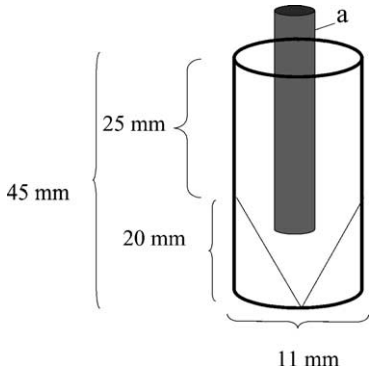

Fig. 1. Special autosampler cup: (a) ultrasonic probe.

analyse (N 33154), HCl ACS (N 30721), were purchased from (Riedel-de Häen, Seelze, Germany). Palladium nitrate atomic absorption modifier solution was purchased from Perkin-Elmer (N BO 190 635).

An inorganic mercury stock standard solution (Merck, $1\,g\,l^{-1}$) was used. A methyl-mercury stock standard solution ($100\,mg\,l^{-1}$) was prepared from methyl-mercury chloride (Riedel-de Häen) by dissolving the appropriate amount of the solid in ultrapure water. Stock standard solutions ($100\,mg\,l^{-1}$) of phenyl-mercury and diphenyl-mercury were prepared from the corresponding chloride salts (Riedel-de Häen) by dissolving the appropriate amount of the solid in methanol (Merck). All stock standard solutions were stored in a refrigerator at 4 °C and protected from light. Working standard solutions were prepared just before use by appropriate dilution of the stock standard solution.

2.3. Specimen collection

Exogenous contamination was avoided cleaning all the plastic bottles used for specimen collection with HNO_3 10% v/v. The bottles were then rinsed gently with ultrapure water and dried at room temperature. Urine specimens were collected each day of analysis in clean plastic bottles and acidified with HCl (1 ml of concentrated HCl to ca. 250 ml of urine). Optimisation of parameters was performed with 24 h urine. The urine was taken from a female volunteer, healthy student (22 years old). When necessary, for comparative purposes, urine from other non-exposed students was also used.

2.4. Preconcentration procedure

2.4.1. Urine oxidation

In previously decontaminated polyethylene tubes (50 ml capacity), 50 mg of $KMnO_4$, 8.5 ml of urine and 1 ml of concentrated hydrochloric acid were introduced. Finally 0.5 ml of water was added or, when necessary, 0.5 ml of mercury standard to check recoveries. Polyethylene tubes were immersed in an ice-bath and each sample was irradiated with ultrasound by using the 6-mm microtip during 1 min at a power setting of 40% (7–8 W delivered as digital LCD displayed). The urine oxidation was considered complete when a colourless solution was obtained.

2.4.2. Mercury extraction and preconcentration

Complexing reagent: a saturated solution of dithizone was prepared in cyclohexane by dissolving 0.0125 g of dithizone in 50 ml of cyclohexane. The solution was filtered. A characteristic green colour solution is formed. This solution should be maintained in a well cleaned closed vessel and protected from light. If the colour of the solution changes to any other than green a new solution should be prepared since photochemical reactions between dithizone itself and organic solvents has been described in literature [18].

Ten milliliters of the oxidized sample (step 1) was introduced into a 25 ml volumetric flask. Then 2 ml of complexing reagent was added and the flask was shaken vigorously during 15 s. Milli-Q water was added to the volumetric flask until the organic phase was at ca. 1 cm from the volumetric flask neck. Finally, 1 ml of of the organic phase was loaded with an automatic pipette and transferred into a special autosampler cup (Fig. 1).

2.4.3. Mercury back-extraction and second preconcentration

To the autosampler cup with the organic phase (step 2), 150 μl of 3.7×10^{-3} M $KMnO_4$ and 150 μl of 2 M HCl were added. Then, focused ultrasonic energy was applied with the 3-mm microtip during 15 s. The organic phase was taken from the autosampler cup and the aqueous phase was allowed to stand. The aqueous sample is ready to be analysed.

The whole procedure allows to concentrate the mercury by a factor of ca. 14 in less than 3 min.

3. Results and discussion

3.1. Thermal program

The thermal program was optimised for determination of mercury in aqueous standards since the final solution after the preconcentration and back-extraction procedure was an aqueous solution. The study was carried out with three different matrix modifiers: Palladium nitrate (0.043 M in 2.4 M HNO_3), $KMnO_4$/HCl (1.85×10^{-3} M/1 M), and a mixture of palladium nitrate and $KMnO_4$/HCl (0.0043 M/1.85×10^{-3} M/1 M). An additional study was carried out in order to know how the amount of modifier could affect the mercury signal. Fig. 2 shows both studies.

Fig. 2A shows the pyrolysis curves for mercury (8000 pg) aqueous standards with the different modifiers used in this work. The atomization temperature during the optimization of the dry/pyrolysis study was 1600 °C whereas the drying temperature was 120 °C during the atomization study (pyrolysis step was omitted). As can be seen, the signal inten-

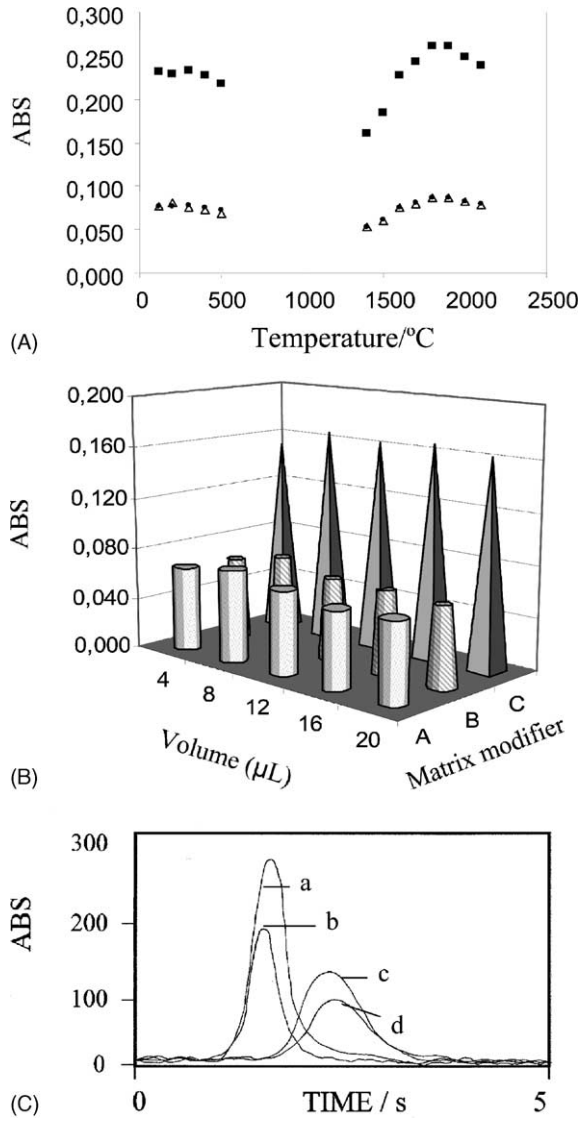

Fig. 2. (A) Ashing and atomisation curves for Hg (8×10^3 pg) in an aqueous standard solution: (■), 10 μl of $KMnO_4$/HCl (1.85×10^{-3} M/1 M); (●), 5 μl of palladium nitrate (10.000 pg ml^{-1}); (△), 10 μl of $KMnO_4$/HCl (1.85×10^{-3} M/1 M) and palladium nitrate (1000 pg ml^{-1}). (B) Mercury signal as a function of the matrix modifier quantity: (A) $KMnO_4$/HCl (1.85×10^{-3} M/1 M); (B) palladium nitrate (0.043 M in 2.4 M HNO_3); (C) $KMnO_4$/HCl (1.85×10^{-3} M/1 M) and palladium nitrate (0.0043 M). (C) Mercury absorbance shapes for: (a) and (b) aqueous Hg(II) standard (8000 pg) and treated sample (6664 pg), respectively. $KMnO_4$/HCl modifier for both; (c) and (d) aqueous Hg(II) standard (c: 8000 pg, d: 4000 pg) with palladium nitrate (c) and palladium nitrate/$KMnO_4$/HCl (d) modifier.

sities for Hg in aqueous solution were much higher (ca. 3 times) with the $KMnO_4$/HCl modifier than with any of the two other modifiers tested. This signal enhancement in electrothermal atomization caused by the action of the $KMnO_4$ is in agreement with the data previously reported by Welz et al. [19]. The mercury is stabilized by the $KMnO_4$, preventing losses up to 300 °C which again is according to the works of Welz et al. [19]. There was not a great difference in drying the sample in the range 120–300 °C, so the drying temperature of 120 °C was selected. On the other hand, the best sensitivity was achieved at the atomization temperatures of 1800 and 1900 °C. The atomization temperature selected was 1800 °C. Fig. 2A also shows that the palladium nitrate modifier may stabilize the Hg, but the sensitivity is three times lower than with the $KMnO_4$/HCl modifier. Surprisingly a mixture of palladium nitrate and $KMnO_4$/HCl did not provide better results than palladium alone. The Pd modifier may trap some components that served as carriers in its absence or the Pd may react with the $KMnO_4$ hindering its role as a modifier. For mercury in the treated sample (e.g., sample taken after the complete oxidation, preconcentration and back-extraction procedure), results showed similar pyrolysis/atomization curves, that is, the same sensitivities and absorption profiles that the ones presented in Fig. 2.

Fig. 2B shows the sensitivities for Hg (4000 pg) aqueous standards with different matrix modifier amounts. As can be seen, for each modifier there was no significant difference among sensitivities although their amount was allowed to vary by a factor of 5.

Fig. 2C shows absorption profiles for aqueous standard and sample solution with the three different matrix modifier using the optimum thermal program summarised in Table 1. (a) and (b) correspond to a Hg aqueous standard (8000 pg) and to the sample after oxidation, preconcentration and back-extraction (6664 pg) respectively, when using the $KMnO_4$/HCl modifier. As can be noted, the profiles are virtually equal, hence indicating the helpfulness of the back-extraction procedure in order to use calibration with aqueous standards. It should be also pointed out that the presence of any other component of the urine, as consequence of the methodology described in the oxidation procedure, has no significant effect in the absorbance profile. The absorbance profiles (c) and (d) correspond to a Hg aqueous standard, (c) (8000 pg) with palladium nitrate as modifier and (d) (4000 pg) with palladium nitrate and $KMnO_4$/HCl as modifier. As can be seen, both shapes are similar and, when comparing with (a) and (b) absorbance profiles, it can be noted that there is a delay in the atomization (ca. 1 s), which may be explained as consequence of the stabilization provided by the palladium modifier.

3.2. Mercury preconcentration

3.2.1. Urine oxidation

The degradation of organomercuarials in human urine with the aid of $KMnO_4$, in conjunction with other chemical reagents, has been previously cited in the literature [20]. Combination of $KMnO_4$ and high focused ultrasonic energy allows improving those procedures previously reported involving $KMnO_4$ for degradation of organomercurials in human urine, ensuring a fast degradation rate for both, organic matter and organomercurials without the need of any other chemical reagent [5].

For a successful mercury recovery it is recommended that, after urine degradation, the solution should not present (i)

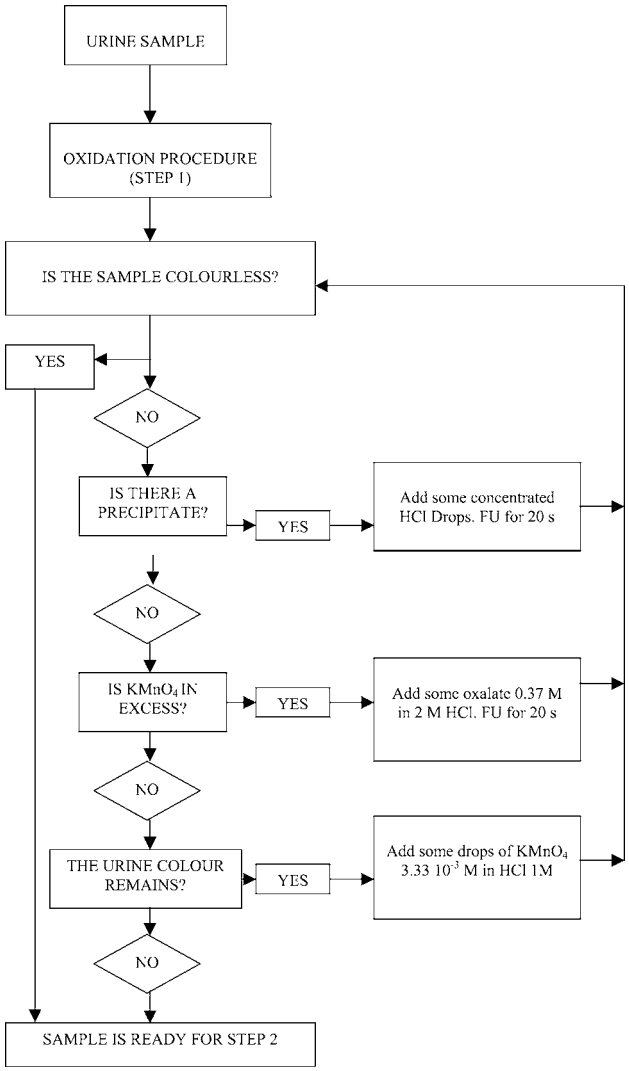

Fig. 3. Step 1: oxidation procedure.

precipitate (MnO_2), (ii) colour from the permanganate and (iii) yellow colour from the urine. In the presence of a dark precipitate, some drops of concentrated hydrochloric acid should be added. If the permanganate is in excess, some drops of oxalic acid 4% m/v in HCl 2 M should be added with sonication for ca. 10–20 s.

It should be stressed that when $KMnO_4$ is not enough to oxidize the organic matter present in the urine the characteristic yellow urine colour remains and methyl-mercury recovery is not complete. Therefore, the yellow colour can be used as a simple test to check the complete degradation of organic matter and organic-mercurials. If the yellow colour from urine has not totally disappeared, a controlled amount of permanganate should be added until a colourless solution is observed. A guide for the oxidation procedure is given in Fig. 3.

The urine oxidation procedure was developed under FU in the time scale of 0.5–8 min. The recovery of the spiked mercury varied between 91 and 97% in all the time scale studied, and 1 min. was chosen as optimum time. To the best of our knowledge a time as short as 1 min as previous sample treatment in the separation and preconcentration of mercury in urine has never been cited in the literature.

3.2.2. Mercury extraction and preconcentration

The separation of mercury from the oxidised urine (step 1) was performed with dithizone in cyclohexane. Theoretical calculations ([18], p. 43) predict that the percentage extraction will increase with excess of reagent, hence, the dithizone was prepared in excess. To check the remain mercury content in the urine sample after extraction, organic phase was completely discarded and a new extraction with dithizone in cyclohexane was performed followed by the back-extraction. The results did not show appreciable absorbance values, that is, there was no evidence of residual mercury.

Following indications given by Burrini et al. [7], the separation was performed shaking vigorously the two phases during 15 s. Longer shaking times did not provide better results, however we chose 30 s as optimum time in this step. This short time is one of the advantages of this procedure. Other point of major concern is the fact that there is no need for pH sample modifications in order to extract the mercury.

Although the organic complexing solution may also be used for the mercury determination, avoiding the back extraction, some problems were found in this work that make its utilization useless. Firstly, a poor performance was observed when the sample was introduced into the graphite furnace. It was important ensure that the droplet was properly dispensed into the furnace but due to the physical characteristics of the organic phase, when the drop on the capillary tip was being formed, very often the organic phase climbed the outside of the capillary walls, leading to a non-acceptable analytical performance. Thus, when working with the organic phase, it was necessary to observe the formation of the drop on the capillary tip and readjust the height of the capillary, when necessary, ensuring that the droplet always touched the bottom of the graphite tube before the injection was completed. The control of the drop deposition into the furnace by the operator was therefore time consuming, and even with all the precautions a maximum of 20–30% of bad depositions were observed. Secondly, the evaporation of the organic phase, that is the cyclohexane, led to an increase in the concentration of the mercury whilst the sample is standing in the autosampler, since after 30 min, the sample lost a 50% of its weight by evaporation. Additionally, the calibration with aqueous standards was not possible, as was also previously reported by Burrini et al. [7]. In order to avoid the drawbacks mentioned before, it was decided to add a back-extraction procedure.

3.2.3. Mercury back-extraction and second preconcentration

The microvolume used in the back-extraction procedure (300 μl) was chosen based on the advantages described by Casarek et al. [21].

In order to speed the procedure, the back-extraction using $KMnO_4$ was done in an autosampler cup specifically

Table 2
Validation of the proposed methodology

	ET−AAS value[a] ($X \pm ts/\sqrt{n}$ ($\mu g\,l^{-1}$))	R.S.D. (%)	FI−CV−AAS value[a] ($X \pm ts/\sqrt{n}$ ($\mu g\,l^{-1}$))	R.S.D. (%)	t_{exp} ($t_{crit} = 2.45$)
Volunteer I	4.7 ± 0.4	6	4.8 ± 0.4	6	0.5
Volunteer II	4.5 ± 0.6	9	4.9 ± 0.1	2	2

[a] Average value ± confidence interval ($n = 4$) for $P = 0.05$.

developed for this treatment. $KMnO_4$/HCl/focused ultrasound destroys the Hg complexes with dithizone, leading to the Hg(II) back extraction in the aqueous solution. The characteristics of the autosampler cup are depicted in Fig. 1. While any type of vessel can be used to hold the sample, the shape of the vessel is often determined primarily by the volume to be processed. For small volumes, such as in this case, the smallest diameter vessel that allows the probe to be inserted without risk of touching the sides of the vessel must be chosen. This minimized diameter raises the height of the liquid sample exposing a greater surface area to the external cooling bath for more effective heat transfer. Fig. 1 also shows a characteristic conical-shaped bottom. This type of shape raises the liquid level without increasing volume, thereby allowing the probe to be inserted more deeply into the process sample. Lowering the probe into the solution it avoids aerosoling and foaming since both generally occur when the probe tip is not immersed deep enough into the solution. Aerosoling and foaming have the effect of "de-coupling" the probe from the process sample. When this happens there is a change in sound or fluctuating readings are observed on the power meter. The back-extraction ($20\,\mu g\,l^{-1}$) was investigated under different sonication times ranging 5–30 s. Results showed that a minimum time of 10 s was required in order to achieve the total mercury recovery. The time selected as optimum was 15 s.

3.3. Analytical figures of merit

Calibration was performed with a series of Hg(II) standards. Sensitivity (m) was the slope value obtained by least-square regression analysis of calibration curves based on peak height measurements. The equation ($n = 5$) for the calibration curve was as follows:

$$Y = (32 \times 10^{-6} \pm 2 \times 10^{-6})(\mathrm{Hg}) + (92 \times 10^{-7} \pm 5 \times 10^{-7})$$

where Y is peak absorbance and (Hg) is the mercury mass deposited in the furnace in pg. For these conditions, the correlation coefficients of the calibration curves, r^2, was 0.999 within the investigation calibration range ($12.7-150\,\mu g\,l^{-1}$). The slope for the standard addition method was $m = (34 \times 10^{-6} \pm 3 \times 10^{-6}$, $n = 3$), similar to the calibration curve within the experimental error, according to the Student's t-test for a 95% confidence level. The linear range of the calibration curve ranged from the quantification limit up to $400\,\mu g\,l^{-1}$. The limit of detection (LOD), equal to $3.8\,\mu g\,l^{-1}$, was defined as $3\,s\,m^{-1}$, s being the standard deviation corresponding to 10 blank injections and m the slope of the calibration graph. The quantification limit (LOQ), defined as $10\,s\,m^{-1}$, was $12.7\,\mu g\,l^{-1}$. The LOD and LOQ in urine were 0.27 and $0.9\,\mu g\,l^{-1}$, respectively, due to the concentration factor of 14. The relative standard deviation (R.S.D.), estimated from aqueous standards (10 replicates) and calculated at concentrations of 100 and $400\,\mu g\,l^{-1}$ was, 4 and 5%, respectively.

3.4. Determination of mercury in spiked and no spiked urine

The feasibility of the TFU was checked by determining the mercury content in spiked ($20\,\mu g\,l^{-1}$) and non spiked urine samples. Inorganic mercury and organic mercury compounds were used in this study. Previously research developed in our laboratory had demonstrated that the couple $KMnO_4$/focused ultrasound was able to decompose methyl-mercury, phenyl-mercury and diphenyl-mercury [5]. The obtained recoveries ($n = 3$) were between 91 and 97% for inorganic mercury, 87–96% for methyl-mercury, 89–98% for phenyl-mercury and 86–95% for diphenyl-mercury. The sample treatment proposed was finally validated as follows: firstly, spiked urine samples ($5\,\mu g\,l^{-1}$) were subjected to the sample treatment described in the experimental section, and subsequently the mercury was measured by ET−AAS. Secondly, the mercury content of the same samples was measured by FI−CV−AAS after the sample treatment described in reference [5]. Results are shown in Table 2.

4. Conclusions

We have developed a new and fast sample treatment that entails the use of focused ultrasound in tandem, that is, the utilization of more than one ultrasonic tip in the same sample treatment. The $KMnO_4$ used in the urine oxidation and in the back-extraction step also acts as matrix modifier in the electrothermal determination of mercury, which in conjunction with the final back-extraction in aqueous solution makes possible to achieve the following items:

(i) Fast sample treatment: a sample can be ready in less than 3 min.
(ii) Green Chemistry: Few chemical reagents in low concentration and low volume.
(iii) Preconcentration by a factor of 14.

(iv) Pyrolysis stage omitted.
(v) Additional introduction of matrix modifier omitted.
(vi) Aqueous standard calibration.

In addition, the preconcentration procedure can be used in conjunction with others techniques such as CV-AAS, CV-AFS or ICP-MS.

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