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Fully Automatic In-Syringe Magnetic Stirring-Assisted Dispersive Liquid-Liquid Microextraction hyphenated to High Temperature Torch Integrated Sample Introduction System-Inductively Coupled Plasma Spectrometer with Direct Injection of the Organic Phase

Raquel Sánchez,[1] Burkhard Horstkotte,[2,3] Kateřina Fikarová,[2] Hana Sklenářová,[2] Salvador Maestre,[1] Manuel Miró,[2,3*] Jose-Luis Todolí[1*]

[1] University of Alicante, Department of Analytical Chemistry, Nutrition and Food Sciences, P.O. Box 99, 03080, Alicante, Spain

*Corresponding author. E-mail: jose.todoli@ua.es

[2] Charles University, Department of Analytical Chemistry, Faculty of Pharmacy, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic

[3] FI-TRACE group, University of the Balearic Islands, Department of Chemistry, Carreterra de Valldemossa km 7.5, 07122 Palma de Mallorca, Spain

*Corresponding author. E-mail: manuel.miro@uib.es

Abstract

A proof of concept study involving the on-line coupling of automatic dispersive liquid-liquid microextraction (DLLME) to ICP OES with direct introduction and analysis of the organic extract is herein reported for the first time. The flow-based analyzer features a Lab-In-Syringe (LIS) setup with an integrated stirring system, a Meinhard® nebulizer in combination with a heated single-pass spray chamber, and a rotary injection valve, used as on-line interface between the microextraction system and the detection instrument. Air segmented flow was used for delivery of a microliter fraction of the non-water miscible extraction solvent, 12 µL of xylene, to the nebulizer. All sample preparative steps including magnetic stirring assisted DLLME were carried out inside the syringe void volume as a size-adaptable yet sealed mixing and extraction chamber. Determination of trace level concentrations of cadmium, copper, lead, and silver as model analytes has been demonstrated by microextraction as diethyldithiophosphate (DDTP) complexes. The automatic LIS-DLLME method features quantitative metal extraction, even in troublesome sample matrices, such as seawater, salt, and fruit juices, with relative recoveries within the range of 94-103%, 93-100% and 92-99%, respectively. Furthermore, no statistically significant differences at the 0.05 significance level were found between concentration values experimentally obtained and the certified values of two serum standard reference materials.

Inductively coupled plasma (ICP)-based techniques are deemed the most universal atomic spectrometric techniques for metal assays as they enable detection of practically all metals and metalloids of the periodic table with excellent sensitivity, reproducibility and sample throughput. Besides, continuous improvements of instrumentation and software make ICP-based techniques user-friendly for routine analysis. However, limitations of instrumental robustness and background interferences in the analysis of high salt content solutions or samples with elevated organic load might jeopardize the reliability of the analytical method. In fact, the occurrence of this kind of matrices might deteriorate the nebulization efficiency, plasma electron density, and even lead to plasma torch shutdown. The sensitivity of ICP OES and ICP-MS based methods does not in some instances suffice for the detection of elements at trace level concentrations, as might be the case in environmental surveillance studies or health risk/exposure assessment. Several approaches have been developed to overcome or minimize these drawbacks, including sorbent-based analyte preconcentration,[1-3] the addition of oxygen to avoid carbon deposition, or the elimination of the sample matrix by electrothermal sample vaporization prior to sample injection into the plasma.[4,5]

With regard to sample handling strategies, liquid-liquid extraction (LLE) of hydrophobic metal or oxyanion complexes has proven to be a powerful pre-concentration and clean-up approach for trace metal analysis by graphite furnace (GFAAS) and flame atomic adsorption spectrometry.[6,7] In contrast, measurements by ICP-based techniques require generally in-line desolvation, solvent emulsification, or solvent dilution to yield steady nebulization conditions.[4,5] Few papers report on LLE with back-extraction of the target species into an aqueous phase as a front end to ICP detection.[8-11] This approach combines the advantages of LLE including salt removal and avoiding typical problems of on-line SPE (backpressure, filter blockage, etc.) along with eluate compatibility with the detector. However, both the operational time and, if automated, the instrumental complexity and effort, e.g. to yield reproducible solvent introduction and reliable phase separation, refrained this LLE mode from further development.[1,12,13]

As an alternative to matrix elimination, the use of a high efficiency micronebulizer in combination with a heated spray chamber, termed high temperature torch integrated sample introduction system (h-TISIS), has been reported for reliable ICP- assays of complex samples.[14,15] With the injection of a mere few microliters of sample, matrix effects have showed to become insignificant as the temperature of the spray chamber is set at 350°C for fuels and diverse acid digested environmental samples.[14,15] Moreover, direct analysis of hydrocarbon samples has also proven to be feasible.[14] Readers are referred to a series of reviews describing instrumental aspects and successful applications of this approach for metal/metalloid determination in organic matrices.[4,5]

This work was sparked by the consideration that such versatile sample introduction system could be hyphenated to automatic liquid-liquid microextraction for expedient analysis of organic extracts. In this context, the Lab-In-Syringe (LIS) concept[16,17] has gained considerable attention as a sample handling tool for straightforward and versatile batch-wise automation of liquid-phase based approaches. Taken as a sequel of the second generation of flow analysis, also called sequential injection analysis,[18,19] LIS is featured by carrying out the entire procedure in the void volume of the barrel of a gas-tight automated syringe pump operating as an enclosed mixing chamber. Of special impact is the integration of a magnetic stirring bar into the syringe for homogenous sample/reagent mixture and solvent dispersion.[20,21]

While there has been significant work harnessing flow-based approaches (mostly flow injection and sequential injection) for automated liquid-liquid extraction of metal species,[6,7,22-25] with potential implementation in microfluidic devices,[24,26,27] prior to on-line atomic spectrometric detection, reviewed elsewhere,[3,28,30] just few papers report on employing LIS, whose versatility has not been fully explored yet. LIS for metal assays has been merely coupled to atomic absorption spectrometric measurements, *namely*, mercury microextraction and cold vapor atomic absorption spectroscopy (AAS)[31,32] and more recently to non-dispersive liquid phase extraction of silver followed by GFAAS,[33] yet studies concerning on-line dispersive liquid-

liquid microextraction (DLLME) as a front-end microextraction approach to multi-elemental ICP OES/MS are still missing.

In this paper, in-syringe DLLME is explored for the first time as a “front-end” versatile microextraction platform for ICP-based detection. Diethyldithiophosphate (DDTP) is used as a selective chelating reagent on the basis of its ability of complexing metal species at the usual acidic pH values for sample conservation[34] as opposed to its carbamate counterparts, i.e. no additional buffering of sample is needed, which, in turn, make the analytical method straightforward (with no need of pH optimization) and less prone to blank contamination. As a consequence of the high stability constants of the DDTP chelates, even in strong acidic conditions, back-extraction methods with increasing of the acidity and/or the addition of competing metal species are proven inappropriate for quantitative recovery of DDTP complexed metals.[35,36] To tackle this issue, we have exploited h-TISIS as a viable interface for the direct injection of the metal containing organic extracts into the ICP system. With this interface, organic matrices are permitted whereby analyte dilution in the back-extraction solution in conventional liquid-phase microextraction approaches of trace metals is circumvented. Cadmium, copper, lead, and silver were chosen as model analytes and analyzed in varied environmental and food matrices.

Material and methods

Chemicals and samples

Ultrapure water was supplied by a three-step ion-exchange system Milli-Q, fed by reverse osmosis, Elix 3, both from Millipore (El Paso, TX, USA). Isopropanol and xylene (Panreac Química S.A., Barcelona, Spain) were employed for the cleaning of the syringe barrel and flow system prior to each extraction and as extraction solvent, respectively. Diethyldithiophosphate ammonium salt (DDTP, 95 %) was obtained from Sigma Aldrich (Saint Quentin Fallavier, France) and used as a chelating reagent, prepared in aqueous medium. 65% HNO_3 (Suprapur®, Merck KGaA, Darmstadt, Germany) was used to prepare washing solutions and acidify the standards and samples. An ICP multielement standard solution (Merck IV, Merck KGaA, Darmstadt, Germany) containing 1000 mg element per litre was used to prepare the standards by serial dilutions. Stock and standard solutions were prepared in 2 % (v/v) HNO_3. Organic multielement standards were prepared by dissolving a certified material (Conostan® S-21, Conoco Specialty Products, Inc., Ponca City, Oklahoma, USA) in xylene. In order to evaluate the reliability of the automatic system for handling complex matrices, a variety of real samples were analyzed: seawater, salt, salt without sodium, grape juice and apple juice. Salt and juice samples were bought in a local supermarket. Coastal seawater was collected in Alicante using pre-cleaned polyethylene flasks.The sample was taken at an approximately 50 cm depth and stored at 4ºC in the laboratory. Salt samples were prepared by dissolving 3.5 g of salt in 10 mL of Milli-Q water. All samples were filtered using 0.45 µm nylon syringe filters (Filter-Lab®, Filtros Anoia, Barcelona, Spain). Two certified lyophilized control serum samples (ClinChek® Controls, Recipe®, Munich, Germany) were used as quality control (QC) materials for evaluation of the trueness of the analytical method. Serum samples were reconstituted in 3.0 mL of ultrapure water with gentle mixing until complete dissolution of the lyophilised material.

Flow setup for automated DLLME

The system configuration for lab-in-syringe dispersive liquid-liquid microextraction (LIS-DLLME)-ICP OES assays is illustrated in Fig. 1 and a close up is presented in Fig S1. In all experiments, a MicroSIA device from FIAlab Instruments Inc. (Seattle, WA) was used to assemble the flow manifold. It integrates a 30 mm Stroke OEM low pressure Syringe Pump (SP, Cavro XCalibur) and an 8 port selection valve (SV, Vici Valvo) furnished with a PTFE rotor. The MicroSIA system contains two auxiliary supply ports of 5 and 24 V herein utilized for stirring activation and ICP triggering. The SP is furnished with a rotary head valve (HV) with three selectable ports (IN, OUT, and TOP) for tubing connections. A 5 mL-glass syringe (30 mm lift, 1.45 mm id, Tecan) was used for performing all solution handling including the DLLME procedure inside. A commercial PTFE covered magnetic stirring bar of 14 mm size

(4.5 mm diameter) was placed in the syringe barrel. To diminish the resulting dead volume at syringe emptying, the stirrer was flattened by sand papering to 3.5 mm height and made to length in order to fit snugly into the syringe. The stirrer was forced to spin at approximately 800 rpm by generating a rotating magnetic field outside the syringe (see Fig. 1 and Fig. S1). To this end, a pile of seven neodymium magnets (each 3 mm x 5 mm Ø) was hot-glued on top of a commercial cooling ventilator (12 VDC supply) serving as a cost-effective brushless motor (wings and protection removed). The motor was connected to the syringe piston bar so that the magnets were leveled with the stirring bar inside the syringe at any time. The motor was powered by the 5 V supply port of the MicroSIA and activated (generating a rotating magnetic field) by software control. By careful adjustment of this arrangement, stirring velocities exceeding 800 rpm were proven applicable

Lateral ports 2-6 of the SV (see Fig. 1) were connected to 2 % (v/v) HNO_3 (2), isopropanol (3) and 15 % (v/v) HNO_3 (8) for syringe chamber cleaning; extraction solvent (4), sample (5), and complexing reagent (6). Using a very short tube of PEEK piercing a wider silicone tube for drainage, port 1 allowed both syringe content discharge to waste during cleaning but also aspiration of air (see Fig. 1). Air inside the syringe enabled vortex formation by stirring, thus promoting solvent dispersion.

Port IN on the syringe HV was connected to the central port of the SV via a 15 cm long holding coil (HC, PTFE tube, 1.0 mm i.d.). Port OUT was used to empty the syringe to waste without passing the HC. The TOP position was connected via a 20 cm transfer line (0.5 mm i.d.) to a low pressure (PEEK stator and rotor) six-port injection valve (IV) from Vici-Valco (Schenkon, Switzerland), used as interface between the LIS-based microextraction system and the ICP OES. A PEEK capillary of 8 cm (0.25 mm i.d.) was used as injection loop, the total injection volume including the valve rotor channel was estimated as 12 µL.

Instrumental control of the extraction system was done via USB using the open-source software Cocosoft, version 4.3 (FI-TRACE, University of the Balearic Islands).[37] The software is written in Python programming language and enables the use of variables, loops, routines, and conditionals, and communication via serial interface. Triggering of ICP OES activation and data registration was done by relay contact using the 24 V supply port of the MicroSIA instrument.

ICP OES measurements

An Optima 4300 DV Perkin-Elmer ICP OES spectrometer (Uberlingen, Germany) was used as detection instrument and the emission intensity signals were axially taken. The system was equipped with a 40.68 MHz free-running generator and a polychromator with an echelle grating. Table 1 summarizes the operational instrumental conditions.

A glass concentric nebulizer (TR-50-C3, Meinhard®, Golden, CA) was fitted to a 12 cm^3 glass single pass spray chamber (h-TISIS).[38] The h-TISIS was jacketed with a copper coil connected to a power supply so as to heating the chamber at will. Hereto, the coil temperature was programmed by means of a thermocouple attached to its surface (Desin Instruments, Barcelona, Spain).[14]

The solutions were delivered to the nebulizer by a peristaltic pump (Gilson Minipuls3 Model M312, Villiers-le-Bel, France) and a 0.19-mm i.d. PVC-based material with plasticizer (Tygon® R-3607, Ismatec, S.A.) tubing was employed.

An air-segmented flow injection methodology was selected to deliver sample volumes at the 5-15 µL level to the instrument. Air was continuously aspirated by means of a peristaltic pump. At a given time and precisely controlled by software, a sample plug was driven to the nebulizer using a carrier stream of air to avoid sample dispersion. Images of the injection of the analyte-containing organic phase into the ICP torch are compiled in Fig S2. With this system, oxygen was not needed to minimize background interferences in troublesome samples because of two facts: *(i)* the injected sample volume was a mere of a few microliters; and, *(ii)* the oxygen in the air stream continuously aspirated could boost the total carbon combustion. Therefore, negligible soot deposits were found throughout the present work.

Analytical protocol

The analytical workflows are given as supplementary materials (Tables S1 and S2). The DLLME protocol was started by cleaning the syringe with (1) isopropanol to remove any residues of the extraction solvent from the previous extraction, (2) 15% (v/v) HNO_3 and two times with 2% (v/v) HNO_3 to keep the syringe free from metal traces, and (3) with the corresponding sample solution, that is, 2%(v/v) HNO_3 for blank measurements or the sample solution itself from position 5 of the SV.

The in-syringe DLLME protocol is performed as follows: 250 µL of air (to promote vortex formation with the consequent solvent dispersion), 270 µL of xylene, 3600 µL of sample, a 20 µL air plug (to avoid contact between sample and chelating reagent in the HC), 250 µL of reagent solution, and a final volume of 180 µL air to empty the overall HC content into the syringe barrel were sequentially aspirated. Immediately before the aspiration of the extraction solvent, stirring at 800 rpm was activated. After an extraction time of 120 s, the stirring was deactivated for phase separation for 30 s, which allowed the xylene droplets to float and to coalesce. Eight repeated activations of the stirrer for a minimum time (< 1 s, not achieving the final stirring rate) were done to remove any xylene residues, which were stuck on the stirring bar.

In the final step, the organic phase was pushed at 80 µL s^{-1} towards the injection valve first to clean the transfer line and push out any residues from the previous injection to waste. Then, aliquots of the solvent (12 µL) were injected repeatedly into ICP OES by IV activation into the air flow carrying the injected volume to the h-TISIS at a delivery flow rate of 50 µL min^{-1}. Every organic extract was injected three times for assessing the repeatability of the ICP readouts. Finally, the aqueous syringe content was emptied to waste with the HV in position OUT.

Results and Discussion

Investigation of the h-TISIS-ICP OES operational conditions

Parameters related to the nebulization and ICP OES measurements including the injection volume of the organic phase, the nebulizer gas flow rate and the spray chamber temperature were evaluated. For injection volumes of xylene larger > 12 µL, the plasma was unstable and tended to shut down. The nebulizer gas flow rate was also optimized. The evaluated values were in the range of 0.15-0.40 L min^{-1}. It was verified that the optimum nebulizer gas flow rate in terms of sensitivity was 0.26 L min^{-1}. Higher flow rates might not ensure the quantitative evaporation of the solvent in the aerosol phase within the spray chamber because of the short residence times but lower flow rates might lead to excessively big aerosol droplets.

The effect of the evaporation chamber temperature on the analytical performance was also investigated. ICP OES signal intensities for Ag, Cd, Cu and Pb were thus recorded at h-TISIS temperatures ranging from 150 to 400 ºC. The h-TISIS spray chamber working at temperatures > 300ºC provided 8, 7 and 12 fold-peak height improvements with respect to those at room temperature for Ag, Cd, Cu and Pb, respectively (see Fig. 2). This was due to the enhancement of the aerosol solvent evaporation inside the chamber and, hence, of the analyte mass delivered to the plasma. The working temperature was set to 350ºC because, under these circumstances, non-spectral interferences by the solvent itself were practically neglegible.[14,15]

The signal obtained for organic standards with h-TISIS working at the optimum experimental conditions was compared with a conventional introduction system (*i.e.*, cyclonic spray chamber operating at room temperature). The nebulizer gas flow rate employed for the conventional system was 0.4 L min^{-1}. Table 2 shows that h-TISIS readouts were up to 13 fold improved as compared to those of the cyclonic spray chamber. Limits of detection (LODs) were determined according to the $3s_b$ criterion, where s_b was the standard deviation of ten consecutive blank measurements. As expected from the sensitivity data, the highest LODs (Table 2) were obtained for the conventional sample introduction system. It is however important to note that the discrepancies observed across the trends in LODs and the analytical readouts are attributed to the dependence of the spray chamber design upon the standard deviation of the background.

System configuration and evaluation of the analytical protocol

Our experimental setup features significant advances as compared to previous works in the field of LIS.[20,21] For example, the induction of solvent dispersion by stirring bar rotation did not require any additional "driving device" to generate a rotating magnetic field as reported previously.[20,21] As the syringe pump was placed here in common up-right orientation, the magnetic stirring bar had to move with the piston so that the motor was fixed to the piston bar to assure steady leveling of both motor and stirrer. To reach the required rotation rate of 800 rpm for solvent dispersion, the stirring bar had to turn smoothly inside the syringe. A 15 × 4 mm stirring bar was thus sandpapered to a 14 mm length (syringe inner diameter was 14.5 mm). Smaller stirring bars (e.g. 10 mm × 2 mm), potentially offering a lower dead volume, were not able to keep up with the required rotation rate but dangle inside the syringe. Due to the inertia of the liquid, the stirring bar is slowed down at the onset of stirring. Thus, a purpose-made control circuit was used for a slow turn-on of the inducing motor.[20] The motor then reached its final speed after approximately 5 s, which enabled synchronized rotation of the stirring bar.

Regarding the analytical protocol for in-syringe DLLME, the following two operational sequences for in-line sequential aspiration of solutions to the syringe were tested: 1: Air, extraction solvent, sample, air, DDTP reagent and air; and, 2: Air, sample, air, DDTP reagent, extraction solvent and air. The segmentation between the sample and the DDTP reagent was done to prevent complex formation already inside the holding coil and the potential sorption of the chelate onto the hydrophobic walls of the flow manifold, which would in turn jeopardize the precision and the analyte recovery and lead to carry-over effects. Air was further found to favor vortex formation with the consequent dispersion of the extraction solvent into tiny droplets. It was demonstrated that the first aspiration sequence was superior in terms of peak height (1.4-1.5 times higher signal) and thus was kept further on. Because the extraction solvent was the first solution introduced into the syringe, smaller droplets were formed, thus enhancing the surface area with the subsequent improvement of the extraction efficiency.

One disadvantage of the LIS-based extraction system herein proposed is the potential cross-over contamination because of the syringe void volume caused by the stirring bar along with the possibility of sorption of organic phase droplets onto the PTFE bar. Generally, the rinsing of the syringe after extraction is done in three steps; a first cleaning step with isopropanol, to remove organic solvent remnants; a second step with a concentration of nitric acid ranging from 2-15% (v/v) to remove metal leftovers and, finally, with the sample, in order to rinse the system with the sample matrix itself. However, the hydrophobic analyte complexes can further be retained in the tubing and injection valve, potentially leading to carry-over effects. To evaluate the effectiveness of several cleaning protocols (see Table S3), the concentrations of metals in three consecutive blank samples analyzed after a standard of 100 µg L^{-1} of Ag, Cd, Cu, and Pb were determined. Figure S3 shows the percentage of the Ag blank signals in consecutive injections with respect to that obtained at the 100 µg L^{-1} level. The rinsing protocol capitalizing upon 15% (v/v) HNO_3 provided the best performance because signals for the first extraction of the blank corresponded to only 5% of the signal obtained for the 100 µg L^{-1} standard. Similar results were found for Cd, Cu and Pb. In the remainder of washing protocols using 2-10% (v/v) HNO_3, the first blank signal amounted to as much as ca 20-95% of the initial Ag signal.

Selection of physical and chemical parameters

Volume of the extraction solvent, DDTP concentration and extraction time

The volume of the extraction solvent in the automatic LIS procedure is particularly important inasmuch as large volumes facilitate quantitative extraction efficiency while microvolumes (usually a few microliters) are preferable with respect to the improvement of preconcentration factors. Evaluation of the volume of xylene as extraction solvent was performed by comparison of the analytical readouts obtained for volumes in the range of 220 to 320 µL at the 100 µg L^{-1}

level. Larger solvent volumes were considered unacceptable for analyte enrichment while smaller volumes of solvent were unlikely to be applicable herein as the system's reliability is based on the premise that the solvent droplets coalesce to one phase so that introduction of droplets of the aqueous phase into the h-TISIS-ICP OES is circumvented. The ICP OES signals were normalized with respect to the maximum peak height (obtained with 270 µL). Figure S4 indicates that the normalized readouts increased with the volume of extraction solvent up to 270 µL, with repeatabilities in all instances better than 3%. Similar trends were found for peak area; hence, the analytical signal was taken as peak height throughout. Note that similar behavior was found for all the elements, therefore, Ag and Cd were selected as model analytes for further studies.

In DLLME, the higher the interfacial area between immiscible phases is the shorter the extraction time for attaining comparable extraction efficiencies. For a fixed stirring rate (*viz.*, 800 rpm), the effect of the stirring time was evaluated. The minimum extraction time to achieve pseudo-equilibrium conditions was estimated at the onset of the curvature of the regression line of the peak height against extraction time for which the analytical readouts approach to steady-state conditions. The pseudo-equilibrium conditions were reached at 60-65 s for all the elements under the experimental conditions indicated above. Moreover, it was observed that almost 100% (in absolute mass) of the analytes were extracted in the organic phase for stirring times of 100-120 s. For stirring times >100 s the influence of the extraction time was virtually negligible as the peak height remained practically unaltered. However, the intra-day precision improved with the extraction time, reaching RSD values lower than 5% at 120 s. An extraction time of 120 s was therefore chosen for the remaining work. The concentration of the extraction agent was also evaluated. Figure S5 indicates that peak heights increased with DDTP concentration up to 50 mmol L^{-1}, which was selected for the remainder of the experiments.

Effect of the acid and counter ion on the extraction procedure

The effect of the acid nature and counter ions on the extraction efficiency of target metals was evaluated. Hence, a cohort of six standards was prepared with the same metal concentration but with increasing concentrations of strong acids (HCl or HNO_3) to evaluate the potential salting-out effects and metal complexation. The matrix composition was: 0.21, 0.51 or 1.03 mol L^{-1} in HNO_3 or HCl. According to previous researchers,[21] the effect of the two counter anions as interfering species for DDTP extraction was not statistically significant (Fig. S6). With respect to the acidity of the sample matrix, a loss of signal intensity was observed at the concentration level of 1.03 mol L^{-1} regardless of the acid nature. For nitric acid, 6% and 12 % signal losses were observed for Ag and Cu, respectively. On the other hand, a 7% loss of peak height was observed in both cases for 1.03 mol L^{-1} HCl.

Analytical method performance

Under the selected experimental conditions, a linear correlation of peak height against analyte concentration in aqueous medium subjected to automatic DLLME was observed. The calibration was performed using six concentration levels in aqueous phase from 0.4 up to 11 µg L^{-1} with an injection volume of 12 µL of organic phase. Coefficients of determination (R^2) higher than 0.9991 were obtained for five inter-day calibration curves. As a benchmark of inter-day precision, relative standard deviations were 5, 7, 4, and 8 % for the slopes of the calibration curves of Ag, Cd, Cu, and Pb, respectively. Moreover, no outlying measurements (> three times the standard error of the slope) were found. LODs were calculated according to the $3s_b$ criterion (n=10), and in all instances were lower than 0.1 µg L^{-1}. LOQs were 0.16, 0.14, 0.14 and 0.21 µg L^{-1} for Ag, Cd, Cu, and Pb, respectively. Repeatability values for six consecutive analysis of a 2.0 µg L^{-1} aqueous standard were 3.1, 4.0, 2.8 and 3.9 % for Ag, Cd, Cu and Pb, respectively.

An alternative calibration method was also tested. In this case, organic standards (12 µL) were introduced directly to the ICP OES following the air-segmented injection methodology described above. Organic standards were prepared using xylene as a diluent of the certified reference material Conostan® S-21. Coefficients of determination (R^2) higher than 0.9993 were obtained for five calibration curves within the concentration range spanning from 5-170 µg/L on 5 subsequent days. The inter-day precision in terms of sensitivity was similar to that of the procedure with aqueous standards followed by DLLME. Notwithstanding the deterioration in sensitivity (see Table 3) as the organic standards in this second external calibration method are not subjected to preconcentration, LOQs were not proportionally increased because of the deterioration of the blank repeatability values for the LIS-DLLE method. Repeatability values for six consecutive analysis of a 25 µg L^{-1} organic standard were were 2.1, 3.4, 2.7 and 4.2 % for Ag, Cd, Cu and Pb, respectively.

The preconcentration factor was obtained as the ratio of the slope of the straight line regression following the automatic LIS extraction procedure to that obtained by direct injection of organic standards into h-TISIS-ICP OES. Table 3 compiles the sensitivities of both calibration curves. The nominal pre-concentration factor was estimated from the ratio of the sample volume (3.60 mL) to that of the organic solvent (270 µL), that is, 13.3. Table 3 shows that the experimentally obtained pre-concentration factors were similar to the nominal value, thus signalling that the extraction efficiency for all the metals was close to 100%.

The entire automatic LIS procedure, including mixing of the sample and reagents, extraction, phase separation, measurement and system cleaning, lasted ca. 375 s, which gives rise to a sample throughput of 9 h^{-1}. The cleaning protocol using 1.2 mL of isopropanol lasted 15 s. Shortening of the rinsing time could most likely be effected by replacing the rotary valve by a low-dead volume stainless steel stator and rotor so as to minimize carry-over effects.

Analysis of real samples

With the aim of validating the extraction methodology, five real samples including seawater, salt, salt without sodium, grape juice and apple juice were analyzed by LIS-DLLME. To this end, a given aliquot was spiked with 2.0 µg L^{-1} of a multi-elemental solution in the aqueous phase. Consequently, the analytical concentration in the organic phase after the preconcentration step was around 25 μg L^{-1}. Note that the non-spiked samples were also analyzed. Original metal concentrations are summarized in Table S4.

Table 4 (right) lists the relative recoveries for Ag, Cd, Cu and Pb, which were close to 100% in all the cases. It can therefore be concluded that additive or multiplicative matrix effects for any of the tested samples, even for typically not applicable samples of high salt content, were insignificant. Recovery values were also calculated using a calibration curve obtained by direct injection of the organic standards into the ICP (see Table 4 left). In this case, the concentration of the organic standards was divided by the preconcentration factor and used as X-axis data with the ICP OES readouts as Y-axis for direct analysis of the spike recoveries in the aqueous phase. Experimental results compiled in Table 4 demonstrated that both external calibration methods provide comparable metal recoveries for all the samples with troublesome matrices. It is important to point out that there is no need to subject the aqueous standards to the DLLME procedure to get reliable results as the target metals regardless of the matrix composition were quantitatively extracted in the organic phase.

For further QC/QA assessment, two serum reference materials, differentiated by the level of metal concentration, were analyzed by LIS-DLLME. For further QC/QA assessment, two serum certified reference materials (CRM), differentiated by the level of metal concentration, were analyzed by LIS-DLLME. Statistical assessment of experimental data for the CRMs was done by comparison of the difference between the certified and the measured values against the associated expanded uncertainty (U_Δ) because the number of accepted sets of data is not provided in the CRM report. The absolute difference (Δ_m) between the mean measured value (c_m) and the mean certified value (c_{CRM}) is calculated according to equation 1. The combined uncertainty (u_Δ) was calculated, based on equation 2, from the uncertainty of the certified value (u_{CRM}) and the standard deviation (s_m) of the experimental data. The expanded uncertainty U_Δ for a confidence level of approximately 95 % is obtained by multiplying the combined uncertainty (u_Δ) by a coverage factor (k) equal to 2 (Equation 3). To evaluate the method performance, Δ_m was compared against U_Δ. Because Δ_m is in all cases $< U_\Delta$, no statistically significant differences were found at the 95% level between the values obtained experimentally and the certified concentrations for any of the target elements (see Table 5 and Table S5).

$$\Delta_m = |c_m - c_{CRM}| \quad \text{Equation 1}$$

$$u_\Delta = \sqrt{s_m^2 + u_{CRM}^2} \quad \text{Equation 2}$$

$$U_\Delta = k\, u_\Delta \quad \text{Equation 3}$$

Conclusions

In this work, a novel approach capitalizing on a portable flow setup has been proposed for the first time for the coupling of automatic in-syringe magnetic stirring-assisted dispersive liquid–liquid microextraction to ICP spectrometry for direct analysis of metal laden organic extracts using an h-TISIS-based total sample consumption system. With this miniaturized sample introduction system, negligible matrix effects were observed in the analysis of carbon-containing matrixes. Because of the high stability constants of DDTP-metal chelates, back-extraction to aqueous phase for conventional ICP measurements in the aqueous phase is proven unfeasible. Using a univariate optimization strategy suitable experimental conditions were found for DLLME-h-TISIS-ICP OES detection of trace level concentrations of target elements in troublesome samples with enrichment factors of ca. 13. Limits of detection found for two distinct calibration procedures were: 0.05, 0.04, 0.04 and 0.06 μg L^{-1} for Ag, Cd, Cu and Pb (extraction procedure) and 0.07, 0.09, 0.06 and 0.10 μg L^{-1} for Ag, Cd, Cu and Pb (direct injection of standards) respectively, allowing its successful application to the analysis of certified serum materials and spiked environmental samples and beverages. Efficiencies of extraction were close to 100 % with repeatabilities usually down to 8%. Therefore, external calibration can be streamlined by direct injection of organic standards into the h-TISIS-ICP detector system with no need to subject them to the extraction procedure. Further work is underway to expand the scope of the hyphenated LIS-DLLME-h-TISIS-ICP system for detection of bioaccessible metals, metalloids and organometallic compounds in complex foodstuff and soil extracts.

Supplementary Information. Additional experimental data and information includes (i) Images of the flow setup and plasma characteristics, (ii) Readouts of cleaning procedures and operational steps, (iii) Effect of volume of organic phase on the analytical readouts, (iv) Effect of chelating reagent concentration on the analytical readouts, (v) Effect of acid type and concentration on the analytical readouts, (vi) Detailed analytical procedure and cleansing protocol, (vii) Concentration of targeted species in the real samples and (viii) Statistical analysis of experimental data for CRM.

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Table 1. Operating conditions of the ICP OES furnished with h-TISIS for injection of organic samples

Variable	Value
Injected sample volume [µL]	12
Nebulizer gas flow, Q_g [L min^{-1}]	0.26
Outer gas flow [L min^{-1}]	15
Intermediate gas flow [L min^{-1}]	1.0
Rf power [kW]	1.35
Integration time [ms]	25
Sampling time [s]	1
Plasma viewing mode]	Axial
Temperature spray chamber [ºC]	350
Elements and Wavelengths [nm]	Ag 328.068 Cd 228.802 Cu 324.752 Pb 220.353

Table 2. Peak height and LODs obtained for the h-TISIS compared against those obtained for the conventional system.*

	h-TISIS$^{\Phi}$			Conventional system$^{\Phi}$			Peak height$^{(h\text{-}TISIS)}$/ Peak height$^{(Conventional)}$	LOD$^{(Conventional)}$/ LOD$^{(hTISIS)}$
	Peak height	RSD (%)	LOD ($\mu g\ L^{-1}$)	Peak height	RSD (%)	LOD ($\mu g\ L^{-1}$)		
Ag	6.1×10^{5}	2.4	0.6	5.0×10^{4}	11.2	2.3	12	4
Cd	1.4×10^{4}	7.2	0.4	1.3×10^{3}	9.5	3.6	11	10
Cu	8.1×10^{5}	2.7	0.5	6.1×10^{4}	1.6	1.9	13	4
Pb	1.4×10^{4}	4.6	0.4	1.4×10^{3}	10.3	2.1	10	5

* Metal concentration: 100 $\mu g\ L^{-1}$ in xylene. Injected volume: 12 µL. Q_g (h-TISIS): 0.26 $L\ min^{-1}$, Q_g (Conventional system): 0.40 $L\ min^{-1}$.
$^{\Phi}$ 10 replicates.

477 **Table 3**. **Slopes of the calibration curves by the automatic LIS-DLLME procedure and the**
478 **direct injection of organic standards along with the experimental pre-concentration**
479 **factors**

	Slope – Aqueous standards - LIS-DLLME procedure (L μg^{-1})	**Slope – Organic standards - Direct injection (L μg^{-1})**	**Pre-concentration factor**
Ag	1.1×10^5	8.1×10^3	13.6
Cd	1.7×10^3	0.13×10^3	13.1
Cu	7.9×10^4	5.9×10^3	13.4
Pb	1.9×10^3	0.14×10^3	13.5

480

481

Table 4. Relative recoveries (%) for complex samples using the LIS-DLME-h-TISIS-ICP OES system

	*Standards: Direct injection**								*Standards: Extraction procedure*[#]							
Samples	**Ag**		**Cd**		**Cu**		**Pb**		**Ag**		**Cd**		**Cu**		**Pb**	
	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)
Seawater	94	1.4	96	1.1	103	0.5	95	0.6	95	1.4	97	1.1	103	0.5	96	0.6
Salt A	98	1.1	99	0.6	95	0.2	94	0.3	99	1.1	100	0.6	97	0.2	95	0.3
Salt B (Without Na)	96	1.2	98	1.1	96	1.1	93	2.0	97	1.2	100	1.1	97	1.1	94	2.0
Apple juice	98	0.9	95	1.1	97	1.2	94	1.0	99	0.9	96	1.0	98	1.2	96	1.0
Grape juice	97	0.3	92	2.0	97	1.1	97	0.7	97	0.3	93	2.0	98	1.1	98	0.7

* The standards were prepared in xylene and directly injected in triplicate into the h-TISIS-ICP OES without the use of the extraction procedure.

[#] The standards were prepared in Ultrapure water, then analyte extraction was performed into xylene (in triplicate) and, finally, a small volume of each extract (in triplicate) was injected into the h-TISIS-ICP OES

Table 5. Concentrations for the reconstituted certified serum samples as obtained by the automatic LIS-DLLME procedure

	Serum - Level I						Serum - Level II[Φ]					
	Ag		Cd		Cu		Ag		Cd		Cu	
	Mean ($\mu g\ L^{-1}$)	s ($\mu g\ L^{-1}$)	Mean ($\mu g\ L^{-1}$)	s ($\mu g\ L^{-1}$)	Mean ($\mu g\ L^{-1}$)	s ($\mu g\ L^{-1}$)	Mean ($\mu g\ L^{-1}$)	s ($\mu g\ L^{-1}$)	Mean ($\mu g\ L^{-1}$)	s ($\mu g\ L^{-1}$)	Mean ($\mu g\ L^{-1}$)	s ($\mu g\ L^{-1}$)
Extraction procedure*	9.29[¥]	0.09	2.2[¥]	0.01	0.775[¥]	0.002	47.3[Φ]	0.2	4.62[Φ]	0.01	1.23[Φ]	0.01
Direct injection[#]	9.49	0.09	2.2	0.02	0.781	0.003	47.5	0.2	4.63	0.01	1.22	0.02
Certified value*	9.85	2.00	2.28	0.47	0.801	0.122	48.0	9.8	4.54	0.93	1.34	0.20

*The standards were prepared in Ultrapure water, and analyte extraction was performed into xylene (in triplicate). A small volume of the extract (in triplicate) was injected into the h-TISIS-ICP OES.

[¥] The calibration was performed using seven concentration levels of aqueous standards ranging from 0.3 up to 11 $\mu g\ L^{-1}$.

[Φ] The calibration was performed using eight concentration levels of aqueous standards ranging from 1 up to 15 $\mu g\ L^{-1}$. For Ag determination, the sample was 1:4 diluted with Ultrapure water.

[#] The standards were prepared in xylene and directly injected in triplicate into the h-TISIS-ICP OES without applying the extraction procedure. The calibration was performed using ten concentration levels of organic standards ranging from 0.5 up to 170 $\mu g\ L^{-1}$.

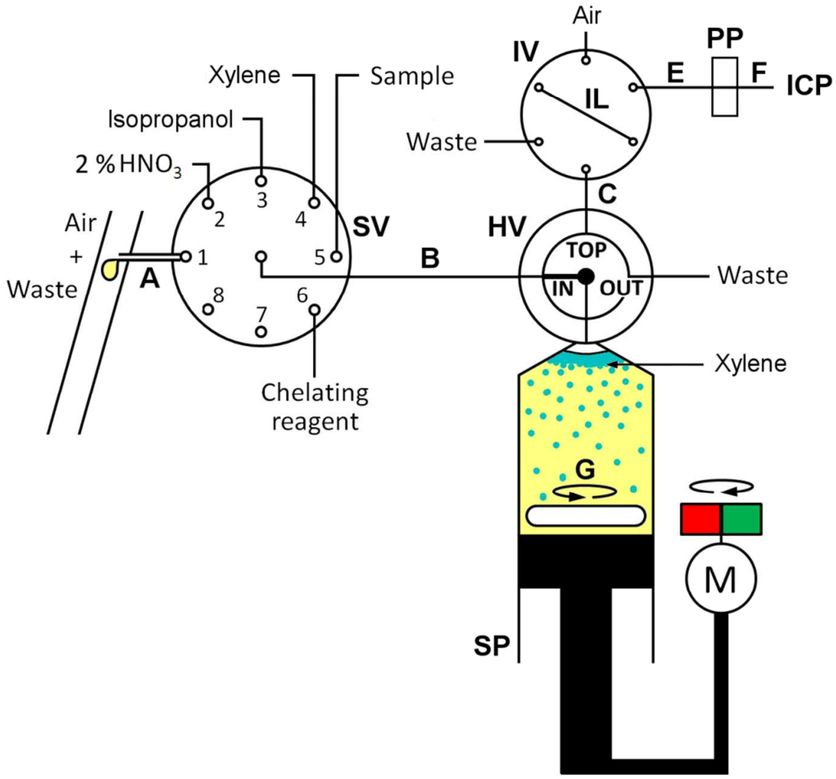
* The standard deviation was estimated as the combined standard uncertainty with a coverage factor of 1.96 at the 95% confidence level.

Figure captions

Figure 1. Outline of the automatic and miniaturized LIS-DLLME system. HV – Head valve (of syringe, positions IN, OUT, and TOP), IV – Injection valve, IL – Injection loop, 8 cm, 0.25 mm i.d., M – DC motor, PP – Peristaltic pump, SP – Syringe pump, SV – Selection valve. Tube dimensions: A – 5 cm, 0.8 mm i.d., B – 15 cm, 1.0 mm i.d., C – Transfer line 20 cm, 0.5 mm i.d., E – 20 cm, 0.25 mm i.d. (PEEK), F – red-orange peristaltic/elastic tube, 40 cm, 0.16 mm i.d., G – Magnetic stirring bar.

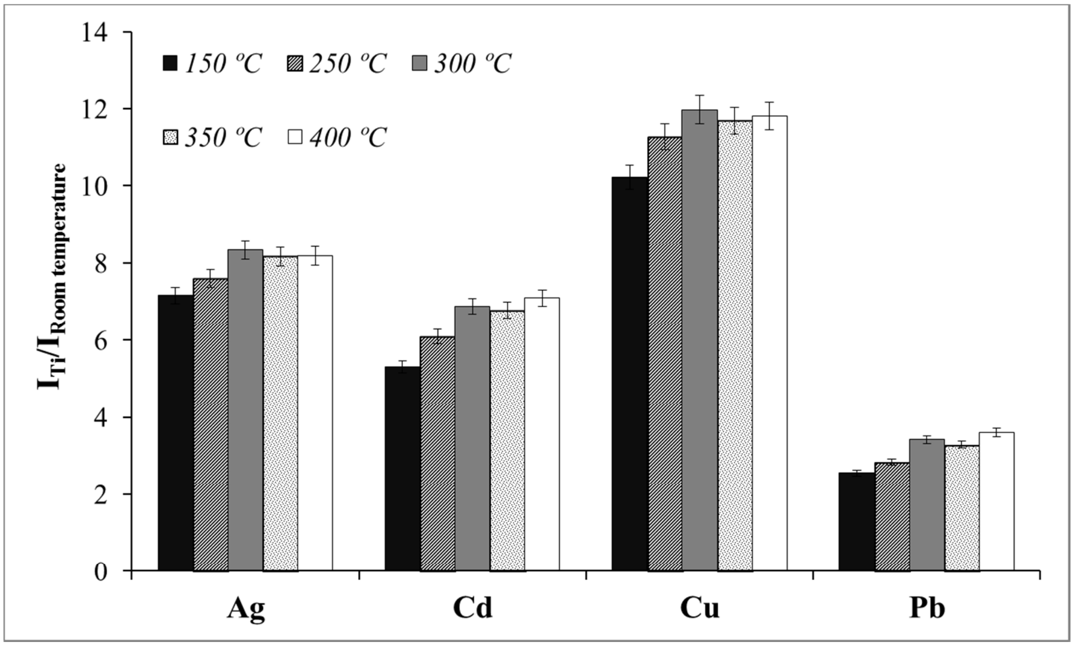
Figure 2. Normalized peak height with respect of that obtained at room temperature for different analytes and h-TISIS temperatures. Metal concentration: 100 μg L^{-1}. Injected volume: 12 μL xylene. Q_g: 0.26 L min^{-1}.

Figure 1



Air
IV
PP
E
F
ICP
IL
Xylene
Sample
Isopropanol
Waste
2 % HNO3
3
2
4
C
Air
+
Waste
A
1
5
SV
HV
B
TOP
IN
OUT
Waste
8
6
7
Chelating reagent
Xylene
G
M
SP

Figure 2


14
12
10
8
6
4
2
0
150 ºC
250 ºC
300 ºC
350 ºC
400 ºC
I_Ti/I_Room temperature
Ag
Cd
Cu
Pb

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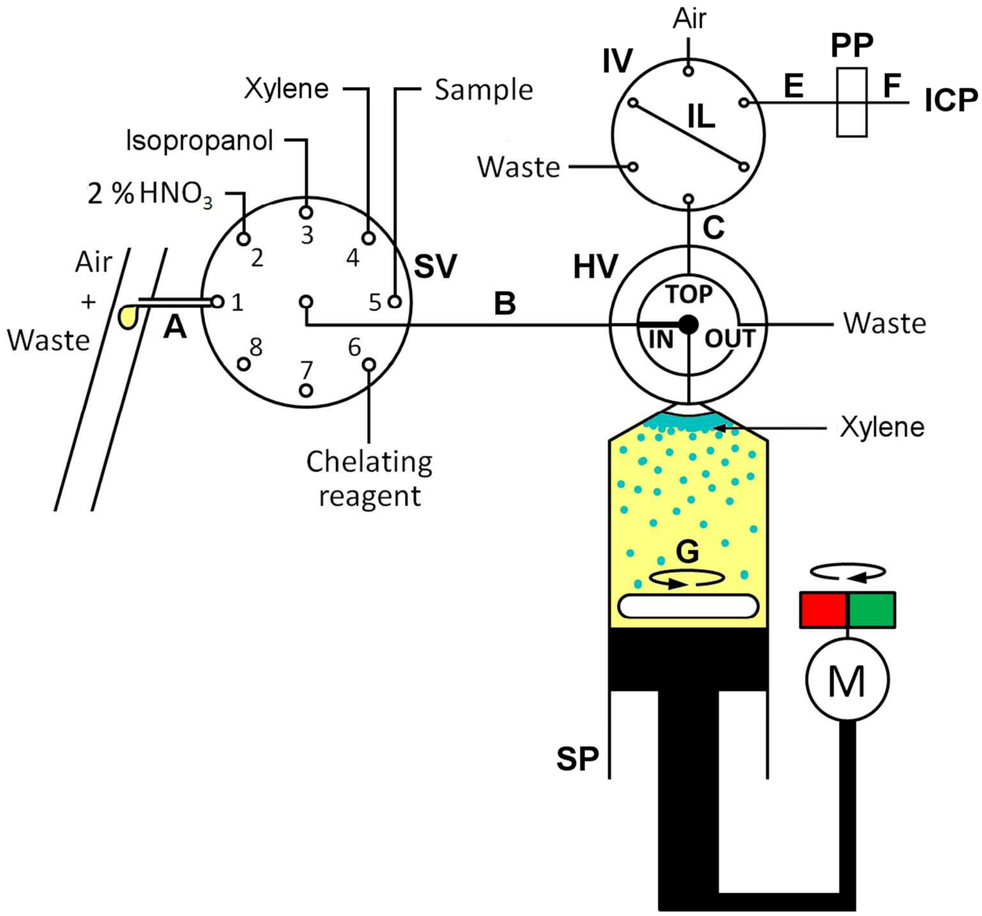

Figure 1

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Figure 2

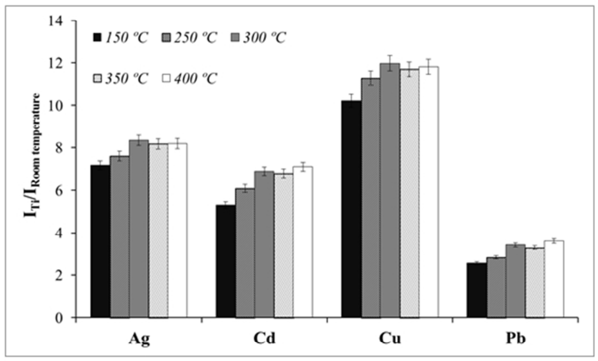

Figure 2

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