Analytica Chimica Acta 1277 (2023) 341668

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Total analysis system for the determination of uremic toxins in human plasma based on bead injection solid phase extraction hyphenated to mass spectrometry

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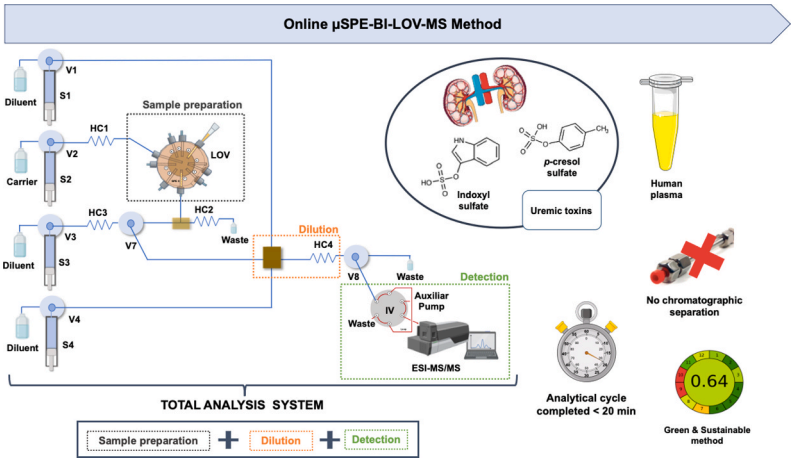
HIGHLIGHTS

- Total analysis system for uremic toxins determination in biomatrices.
- Fully automated μSPE-LOV-MS method for INDS and pCS determination in plasma.
- Analytical cycle completed in less than 20 min without chromatographic separation.
- Environmentally friendly methodology with reduced human intervention.
- Uremic toxins successfully determined in clinical plasma samples.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Automation
Lab-on-valve
Mass spectrometry

ABSTRACT

Indoxyl sulfate (INDS) and *p*-cresol sulfate (pCS) are two of the most relevant uremic toxins that are recognized to have an essential role in chronic kidney disease (CKD) progression and associated cardiovascular risk. Thus, it is crucial to accurately assess their circulating levels in the body. Aiming at establishing an analytical strategy for quantification of INDS and pCS in human plasma, an automatic on-line micro-solid-phase extraction (μSPE)

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https://doi.org/10.1016/j.aca.2023.341668
Received 11 April 2023; Received in revised form 4 July 2023; Accepted 26 July 2023
Available online 27 July 2023

On-line hyphenation
Indoxyl sulfate
p-Cresol sulfate

procedure hyphenated to tandem mass spectrometry (MS/MS) detection without previous chromatographic separation was herein developed. The bead injection (BI) concept was used to implement the μSPE procedure in the lab-on-valve (LOV) format. After studying the extraction conditions, the anion-exchange OASIS WAX sorbent beads (10 mg) and 99% ACN–H_2O (15:85, v/v)–1% (v/v) NH_4OH were chosen as sorbent and eluent, respectively, as they provided the highest analyte recoveries. Subsequently, the μSPE-BI-LOV system was hyphenated on-line to a MS/MS detector and the full analytical cycle, comprising sample preparation and analytes detection, was completed in <20 min. The developed μSPE-BI-LOV-MS methodology presented good linearity ($r^2 > 0.999$) for quantification of the target analytes at concentrations ranging from 18 to 360 μg mL^{-1} in plasma. LOQ values were 2 μg mL^{-1} for INDS and 7 μg mL^{-1} for pCS in plasma. Human plasma samples from healthy subjects and individuals with CKD were successfully analyzed using the developed approach. The proposed automatic methodology can be described as an eco-friendly strategy, with a favorable score of 0.64 after greenness evaluation using the AGREE metric.

1. Introduction

Indoxyl sulfate (INDS) and *p*-cresol sulfate (pCS) are two of the most representative, biologically relevant uremic toxins [1]. These are small molecules that have a lower renal clearance and can accumulate in the organism due to their high affinity for plasmatic proteins [1,2]. Due to this fact, their plasmatic levels are correlated with renal failure and chronic kidney disease (CKD) progression, with the subsequent increase of cardiovascular risk [2–4]. Considering the risk associated with the presence of higher concentrations of INDS and pCS in the organism, knowing their accurate circulating levels is critical. Liquid chromatography coupled to mass spectrometry detection (LC-MS) is the first-choice for determining INDS and pCS in biological samples [5]. However, some of the proposed methods in the literature do not allow the direct analysis of the target compounds in biological matrices and require extensive sample pretreatment procedures, e.g., deproteinization, including precipitation, centrifugation, and supernatant separation, to remove potential interfering species [5].

Regarding the several sample preparation techniques available, solid-phase extraction (SPE) is one of the most used approaches for sample preparation in bioanalysis. SPE is a versatile technique and can be easily miniaturized and automated using flow-based platforms, such as lab-on-valve (LOV). LOV is a suitable platform for the development of automatic on-line renewable/disposable micro-solid-phase extraction (μSPE) procedures exploiting the bead injection (BI) concept, and it has been used to determine numerous bioactive compounds in diverse matrices [6–12]. It consists of a micromachined device mounted atop a multi-position valve that enables a flexible fluidic manipulation of solid materials due to the mesofluidic dimension of the integrated channels and, thus, contributes to improved precision with accurate control over time events [13,14]. One of the main advantages of the μBI-SPE systems is the ability to remove the sorbent bed at the end of each assay and replace it with a fresh bead portion, which is especially useful for the analysis of complex matrices (e.g., biological samples) [8,10]. Indeed, when permanent/reusable sorbents are used, the extraction performance for subsequent samples can be hampered due to sorbent deterioration or carryover effects [15,16]. Furthermore, the LOV platform permits the on-line transport of solvents and allows the on-line coupling with different detection systems [12,13], such as mass spectrometry detection.

In the last years, the direct coupling of flow systems with mass spectrometry detectors (FIA-MS) has been reported for the determination of endogenous compounds in various biomatrices [17–20]. High-throughput FIA-MS has been applied as a simpler alternative to conventional LC-MS methods, taking advantage of the capabilities of the selective detection in MS/MS equipment [21]. However, despite the numerous advantages associated to FIA-MS, the direct introduction of the sample into the MS detector leads to the need for an extensive sample preparation step before analysis, particularly for complex matrices (e.g., plasma) [21]. In this way, the use of a flow-based platform, such as LOV, that enables performing miniaturized sample preparation and is amenable to direct coupling to MS, has a high potential to implement fully automated and on-line analysis methods. Previous studies reported the use of the LOV platform for SPE processes, as well as the use of MS as a detection method for determining different bioactive compounds in various matrices, such as biological or environmental samples [10,12]. However, a direct coupling between LOV and MS has not been proposed as of yet, and chromatographic separations have always been performed before MS detection [10,12]. Having this in mind, the on-line coupling of a μSPE-BI-LOV flow system with tandem MS detection was explored in this work. Hence, a total analysis system was pursued to automate and accommodate all the necessary operations in a single mesofluidic platform for the quantification of INDS and pCS in plasma samples. To achieve this goal, a fully automated flow system was proposed for performing renewable μSPE combined with in-line dilution of sample extracts prior to the quantification of the target analytes in human plasma by tandem mass spectrometry. To the best of our knowledge, this is the first methodology to report a total analysis system for quantifying endogenous compounds in biomatrices using a mesofluidic platform (LOV) to perform in-valve sample preparation as a front end to a MS-based detection.

2. Materials and methods

2.1. Reagents and solutions

All chemical reagents were of analytical grade. Ammonium hydroxide (NH_4OH), formic acid, and acetonitrile (ACN, LiChrosolv LC-MS grade) were purchased from Merck KGaA (Darmstadt, Germany). 3-Indoxyl sulfate potassium salt was obtained from Sigma Aldrich (St. Louis, USA) and 3-indoxyl sulfate–d_4 potassium salt (IS-INDS, internal standard) was acquired from Toronto Research Chemicals Inc. (Toronto, ON, Canada), through LGC standards (Barcelona, Spain). *p*-Cresol sulfate ammonium salt and [$^2H_4$] - *p*-cresol sulfate ammonium salt (IS-pCS, internal standard) were purchased from Alsachim (Illkirch Graffenstaden, France). All solutions were prepared using ultrapure water (resistivity >18 MΩ cm) from the Arium water purification system (Sartorius, Göttingen, Germany).

Two commercial sorbents with 60 μm particle size (OASIS MAX and OASIS WAX) were purchased from Waters (Milford, MA, USA). The sorbents were obtained in cartridge format, dismantled, and the sorbent beads were saved in a clean glass container. For the preparation of the bead suspensions, 200 mg of sorbent were mixed with 2000 μL of conditioning solution (ACN-H_2O (50:50, v/v)).

Aqueous stock solutions of each analyte and internal standard were prepared at 1 mg mL^{-1} and kept at −20 °C. For SPE extraction (batch and at-line assays), intermediate solutions of each analyte at 40 μg mL^{-1} were prepared in water, and subsequently diluted in the same solvent to obtain working standard solutions at the required concentrations. For the chromatographic analysis of the SPE fractions from batch and at-line assays, two intermediate solutions containing 50 μg mL^{-1} and 2 μg mL^{-1} of each target analyte were prepared daily in mobile phase (ACN–H_2O (15:85, v/v) with 0.05% (v/v) formic acid). Furthermore, working standard solutions for the initial studies were prepared in the range of

0.02–1.0 μg mL^{-1} by diluting the intermediate solutions in mobile phase. For initial studies, the internal standards (IS-INDS and IS-pCS) were added to each standard solution and eluate, before chromatographic analysis, to attain a final concentration of 0.2 μg mL^{-1} each.

For the analysis of plasma samples using the proposed μSPE-BI-LOV-MS method, a matrix-matched calibration was implemented. Hence, calibration standards at five concentration levels (18, 90, 180, 270 and 360 μg mL^{-1}) were prepared by spiking a pool of healthy human plasma with different amounts of each target uremic toxin and processed by the developed method, without the addition of IS. Moreover, a blank unspiked sample (plasma without the addition of analytes) was also analyzed under the same conditions.

2.2. Chromatographic analysis

In the preliminary assays performed to establish the best conditions for the μSPE-BI-LOV procedure, fractions from critical steps (sample loading, washing and elution) were collected and analyzed by UHPLC-MS/MS.

A Nexera X2 UHPLC system comprised of two LC-30AD pumps, a DGU-20A5R degassing unit, a SIL-30AC autosampler, and a CTO-20AC oven was used to perform the chromatographic separation. Detection was performed using a triple quadrupole LCMS-8040 mass spectrometer with an electrospray ionization source (ESI) (Shimadzu Corporation, Kyoto, Japan). The UHPLC-MS/MS system was also equipped with an additional LC-20AD pump and a high-pressure injection valve FCV-20AH2 (Shimadzu Corporation), which were used to implement the on-line coupling of the LOV platform with the ESI-MS/MS system.

A BEH C18 column (100 × 2.1 mm, 1.7 μm; Waters) kept at 40 °C was used to achieve the analytes separation. Elution was carried out in isocratic mode at a flow rate of 0.2 mL min^{-1}, using a combination of 85% solvent A (water with 0.05% (v/v) formic acid) and 15% solvent B (ACN with 0.05% (v/v) formic acid). An injection volume of 0.2 μL was employed.

The mass spectrometer was operated in the negative ionization mode (ESI-). Data were acquired in the selected reaction monitoring (SRM)

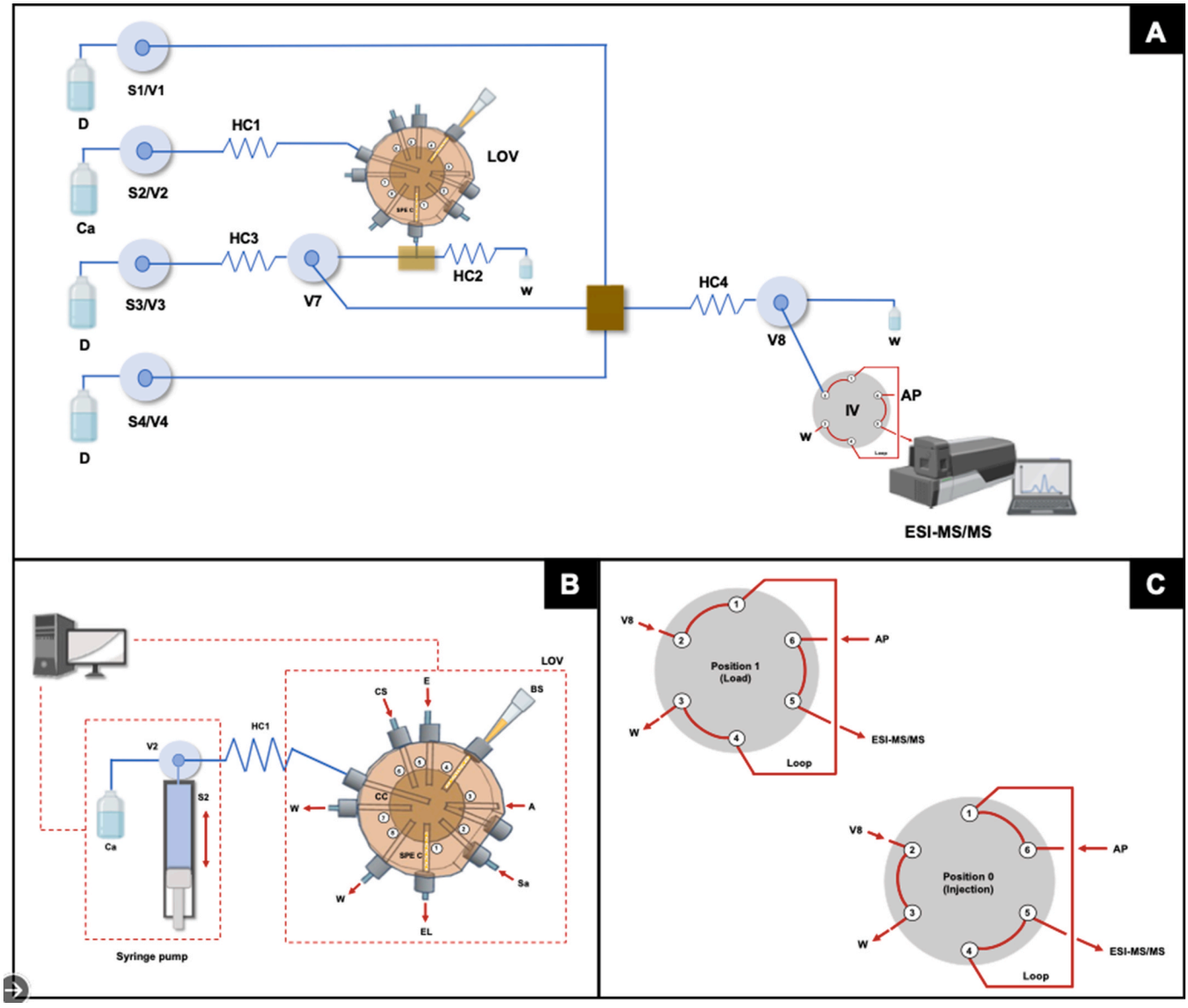

Fig. 1. Schematic illustration of (A) the on-line μSPE-BI-LOV-MS system configuration; (B) the μSPE-BI-LOV system; and (C) the hyphenation to ESI-MS/MS through a 6-port high pressure injection valve. A, air; AP, auxiliary pump; BS, bead suspension; Ca, carrier; CC, central channel; CS, sorbent conditioning solution; D, diluent; EL, eluate; E, eluent; HC, holding coil; IV, injection valve; Sa, sample; SPE C, SPE column; S, syringe; V, three-way solenoid valve; W, waste. For simplicity, the syringes, with a capacity of 10 mL (S1 and S4) and 2.5 mL (S2 and S3), were not illustrated.

mode (INDS, *m/z* 212.80 > 80.00, *m/z* 212.80 > 131.95; pCS, *m/z* 187.70 > 107.00, *m/z* 187.70 > 80.05). The internal standards were monitored at *m/z* transitions 216.80 > 80.00, 216.80 > 135.90 for IS-INDS and 191.80 > 111.05, 191.80 > 79.95 for IS-pCS. The first mentioned *m/z* transition was employed for quantification, whereas the second was used for identity confirmation. MS operating parameters were defined as follows: Nebulizing gas (N_2, 3 L min^{-1}), drying gas (N_2, 15 L min^{-1}), heat block temperature (400 °C), desolvation line temperature (200 °C), detector voltage (1.88 kV) and collision gas (argon, 230 kPa). The LabSolutions software version 5.60 SP2 (Shimadzu Corporation) was applied for peak detection and quantification.

2.3. Analytical instrumentation and flow network

The manifold configuration and the different components of the flow system proposed for the extraction and determination of the target analytes are schematically represented in Fig. 1.

A multisyringe pump (Crison Instruments, Allela, Spain) equipped with two syringes (Hamilton, Bonaduz, Switzerland) of 2.5 mL (syringes S2 and S3) and two syringes (Hamilton) of 10 mL (syringes S1 and S4) was used as the propulsion unit. Syringes S1, S3 and S4 were filled with diluting solution (ACN–H_2O (15:85, v/v)) whereas syringe S2 was filled with carrier solution (water). The access to the solution reservoirs (position off) or the flow network (position on) was controlled by a three-way commutation valve (NResearch, Caldwell, NJ, USA) placed at the head of each syringe. The flow setup also integrated two extra commutation valves (V7 and V8) (NResearch) for in-line dilution and further coupling to the detection system (Fig. 1A).

The multisyringe pump was attached to a customized LOV device (Ideia.M, Porto, Portugal) fabricated from chemically resistant polyetherimide [6]. A central channel and eight peripheral ports (1.5-mm i.d.) were included in the LOV device, which was incorporated atop a Crison multi-position selection valve (Model VA 2 S4). The central channel of the LOV system was connected to a 2.5 mL glass syringe (S2) via a holding coil (HC1), and to the eight peripheral ports, one at a time.

The extraction system consisted of a μSPE column located at port 1 of the LOV, packed with 10 mg of sorbent. A polypropylene frit of 1-mm thickness and with 35 μm pore diameter (MoBiTec, Göttingen, Germany, ref #M523515) was inserted between the outlet of port 1 and a polyetheretherketone (PEEK) nut to retain the sorbent in the channel of the device. The bead suspension container was a pipette tip (1 mL) connected to a PEEK nut fitted into port 4 of the LOV. Polytetrafluorethylene (PTFE) tubing (Omnifit, Cambridge, UK) with 0.8-mm i.d. and 1.5-mm i.d. was used, respectively, to connect the LOV ports to the solution reservoirs, and for the connections to solution flasks and syringes and HC1 (with a 3.5 mL capacity). Hence, the configuration of peripheral LOV ports (Fig. 1B) was as follows: (1) μSPE column; (2) sample; (3) air; (4) bead suspension container; (5) eluent; (6) conditioning solution; (7) and (8) waste.

For the in-line dilution setup (Fig. 1A), the port 1 of the LOV was attached to a tee connection of polyetherimide, that, in turn, was connected to an additional holding coil (HC2, PTFE tubing of 0.8-mm i.d, 1.5 mL capacity) used as eluate reservoir, and an external solenoid commutation valve (V7) that helped driving the flow toward the desired direction (off: to eluate reservoir; on: to in-line dilution system). This commutation valve (V7) was connected to the multisyringe pump (syringe S3) through a holding coil of 1 mL capacity (HC3). Furthermore, V7 was also connected to a PEEK cross confluence placed before other external commutation valve (V8). This cross confluence allowed performing the dilution of the eluate, due to its connection with syringes S1 and S4, which were filled with the diluent solution (Fig. 1A).

The hyphenation with the MS detector was accomplished through a rotary 6-port high pressure injection valve (IV) operating in two positions (load (1) and injection (0), Fig. 1C) that was positioned between an additional commutation valve (V8, Fig. 1A) and the detector. This valve was used to drive the flow from the in-line dilution system towards the MS detector (on position) or to the waste (off position), sending only selected portions, at low flow rates, to the IV low bore channels. The load position of IV allowed filling the injection loop (volume of 100 μL), made of 0.75-mm i.d. PEEK tubing (VICI, Valco Instruments, Schenckon, Switzerland), while switching it to the injection position permitted the automatic injection of the diluted eluate into the MS detector.

Quick Basic 4.5 (Microsoft, Redmond, WA, USA) and LabSolutions software version 5.60 SP2 (Shimadzu Corporation) were used to define and implement all the steps of the analytical process. The first mentioned software permitted to control the direction and speed (flow rate) of piston movement on the multisyringe apparatus, the position of solenoid commutation valves, and the selection of the different ports in LOV. The second software was used for the selection of the position of the injection valve (IV) and to data acquisition in MS detection.

2.4. Analytical procedure of the on-line μSPE-BI-LOV-MS

The mechanized analytical procedure consisted of five operations: (1) sorbent conditioning and in-line formation of the μSPE column; (2) sample loading and matrix removal; (3) sample elution; (4) dilution and transport of the eluate to MS for analysis; (5) sorbent removal. The established analytical method is briefly described in the following subsections, and information is presented in detail in Table S1.

2.4.1. Sorbent conditioning and in-line formation of the μSPE column

The syringe pump (S2) was set to aspirate 550 μL of the sorbent conditioning solution (ACN-H_2O (50:50, v/v)) from port 6. After that, 125 μL of this solution were injected towards the beads reservoir (port 4) to resuspend the sorptive material so as to obtain a homogeneous dispersion. Thereafter, 100 μL of the bead suspension was aspirated into the HC1 and pushed forward to fill the SPE column channel (port 1) entirely, ensuring the repeatability of the formation of the SPE column for each assay [22]. After, the column was immediately perfused with the sorbent conditioning solution and carrier (water). The excess of beads was removed by propelling 700 μL of carrier through port 8 (waste).

2.4.2. Sample loading and matrix removal

For sample loading, 1000 μL of standard or sample were aspirated from port 2 into the HC1 following the aspiration of an air plug (350 μL). A portion of the HC1 content (1200 μL) was loaded through the SPE column (port 1) at a flow rate of 0.5 mL min^{-1}, which enabled sample loading into the sorbent, retention of the target analytes, and removing of sample by air. The flow rate applied for the loading step (0.5 mL min^{-1}) was set as low as possible in order to maximize the contact between the sample and the sorbent while also avoiding the occurrence of higher pressure in the flow system [10,23]. After that, the SPE column was washed with 500 μL of water (washing solution) at a flow rate of 1 mL min^{-1} to further remove the sample matrix, particularly proteins present in plasma, and the non-retained species.

2.4.3. Sample elution

First, an air plug (300 μL) was aspirated through port 3 to prevent the dispersion of the eluent into the carrier solution. After that, the elution was carried out at a flow rate of 0.5 mL min^{-1} to maximize the interaction between the eluent and the sorbent, using 1000 μL of the eluent solution (99% ACN–H_2O (15:85, v/v)–1% (v/v) NH_4OH). The eluate was maintained in HC2 for the next step of the procedure.

2.4.4. Dilution and transport of the eluate to MS for analysis

For the in-line mixing, 400 μL of the eluate stored in HC2 were aspirated through a tee connection and a solenoid valve (V7) towards the HC3. After flow reversal, 250 μL of the eluate were pumped through the dilution system, and a portion (50 μL) was mixed in-line with 400 μL of the diluent (ACN–H_2O (15:85, v/v)) propelled by syringes S1 (200 μL) and S4 (200 μL) at the cross confluence, resulting in a ratio of 1:9

(Fig. 1A). Finally, through activation of a solenoid valve (V8), 250 μL were pushed, and the diluted eluate was loaded into the injection loop (100 μL) of the IV (position 1, Fig. 1C). Thereafter, the position of the IV was changed to inject the diluted eluate present in the loop, corresponding to ca. 10 μL of the initial eluate volume, into the mass spectrometer for MS/MS analysis (position 0, Fig. 1C), using the diluent as a carrier. The analyses were performed in the negative ionization mode (ESI-) and data were acquired in SRM mode as indicated above. At 6 min after injection, the IV was returned to position 1. Finally, the dilution setup was washed with diluent solution to remove the leftovers of eluate in the system.

2.4.5. Sorbent removal

First, 1500 μL of the sorbent conditioning solution, previously kept in the HC1, was used to wet the beads packed into port 1. After that, the beads were aspirated back into the HC1 at a higher flow rate (5 mL min^{-1}) and then disposed of to waste (port 8). Subsequently, the LOV column port and the injection loop of IV were cleaned with 300 μL of carrier and diluent solutions, respectively, at a flow rate of 0.4 mL min^{-1}. After this step, the system was prepared to process the following sample.

2.5. Application of the method to human plasma samples

The study complied with the 1964 Helsinki Declaration and all subsequent revisions and followed the generally accepted norms of good clinical practices. Furthermore, the study was approved by the local Ethics Committee (Ethics Committee for Health of *Centro Hospitalar Universitário de São João*/Faculty of Medicine of University of Porto, reference no. CES 87-15). The signed informed consent of each participant was previously obtained. Briefly, the samples were divided in two groups: i) samples from patients with chronic kidney disease (CKD) undergoing peritoneal dialysis and ii) samples from healthy control individuals who were living kidney donors (before organ donation). Immediately after collection, blood was centrifuged at 3,892×*g* for 15 min at 4 °C and the plasma was immediately frozen and stored at −80 °C until further analysis.

Before the μSPE procedure in the LOV manifold, all plasma samples (20 μL) were diluted with water (1:20 or 1:200, depending on the assays performed) and filtered through a Corning® (New York, USA) syringe filter (regenerated cellulose, pore size of 0.2 μm), to remove microbial cells and suspended particles. In preliminary studies with plasma samples, the eluates resulting from the batch SPE and at-line μSPE-BI-LOV assays were acidified with formic acid to obtain a 0.1% (v/v) formic acid/0.1% (v/v) formate buffer and centrifugation was performed at 12,100×*g* during 8 min at room temperature. The resulting supernatants were loaded to the autosampler (injection volume of 0.2 μL) for UHPLC-MS/MS analysis.

3. Results and discussion

3.1. Development of the μSPE-BI-LOV method

The main challenge in the pre-treatment of plasma samples is the elimination of interferents, particularly proteins, whose presence could lead to matrix effects and compromise the sensitivity of the method. The dilute-and-shoot technique is not effective for sample preparation of this type of biomatrices because it normally requires performing a preliminary step of protein precipitation and does not allow an efficient clean-up of the matrix [24]. Thus, an automated μSPE methodology has been proposed as an alternative for the clean-up of plasma samples for subsequent MS analysis.

One of the advantages of μSPE systems is the small amount of sorbent required, but care must be taken when establishing the working range for retained analyte mass, which should be kept <1%. In order to ensure a ratio between the mass of analyte and the mass of solid material that was adequate to attain quantitative determinations, the selected target mass range of each analyte was 0.05–2.5 μg, considering the use of 10 mg solid material per extraction. Consequently, initial studies were performed with plasma samples diluted 1:20 while on-line hyphenation targeted higher plasmatic concentrations, employing 1:200 dilution.

3.1.1. Selection of the sorbent material and the eluent

Initial studies for the selection of the sorbent and the eluent were performed under batch SPE mode, using 60 mg cartridges of two different sorbents (OASIS MAX and OASIS WAX). The target analytes bear similar physicochemical properties, both containing an ionizable sulfate group that is deprotonated for pH values between 1 and 14 (Figs. S1 and S2). Thus, the negatively charged species of these analytes prevail and the efficiency of their extraction is not affected by the pH. Taking this into account, the sorbents that exhibit a mixed mode reversed phase/anionic exchange mechanism with the presence of charged groups (quaternary ammonium groups for OASIS MAX and piperazine groups for OASIS WAX) that permit to retain negatively charged compounds, were chosen. However, OASIS MAX was discarded because of the strong anionic exchange nature of the sorbent so that both analytes were strongly retained by the sorbent (Table S2), but not eluted even at pH < 2 [25,26].

The presence of piperazine groups in the backbone of OASIS WAX allows weak anionic exchange with analytes which are anionic during extraction over the entire pH range, such as INDS and pCS [25]. Different eluent compositions were tested (Table S2). The eluents containing water and NH_4OH in their composition (eluents 4, 5 and 6) provided the highest extraction recoveries for both compounds (98.4–108.0%) (Fig. 2, Table S2) because of the uncharged nature of the sorbent under alkaline conditions. The use of aqueous eluents is also pivotal because low recovery values (<52%) were obtained when the eluent was composed only by ACN and NH_4OH (eluent 3) (Fig. 2, Table S2) because of the aprotic nature of the solvent that does not boost the acid-base properties of the ammonia and thus the piperazine moieties remained mostly charged. Thus, the eluent selected for further experiments was eluent 4 (Table S2), a mixture of 99% ACN–H_2O (15:85, v/v)–1% (v/v) NH_4OH.

Next, the best conditions established in batch assays (OASIS WAX as the sorbent and a mixture of 99% ACN–H_2O (15:85, v/v)–1% (v/v) NH_4OH as the eluent) were tested under the μSPE-LOV format using 10 mg of sorbent. The recovery values were similar to the values verified throughout the batch SPE assays, for both analytes. The conditions used for aqueous standards were also applied to pooled healthy human plasma fortified with the target analytes (4 μg mL^{-1} of each analyte) and

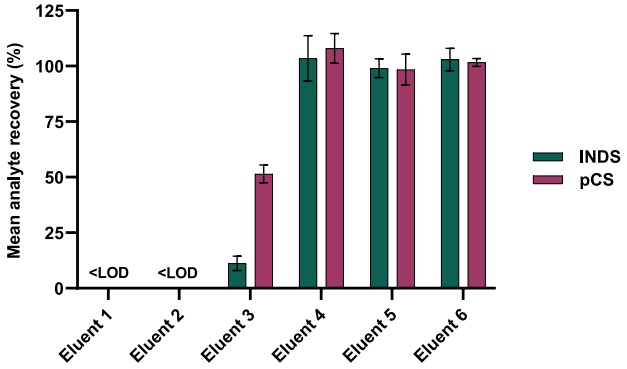

Fig. 2. Effect of eluent composition in the recovery of INDS and pCS in batch SPE assays. Eluent 1: ACN; Eluent 2: ACN-H_2O (75:25, v/v); Eluent 3: 99% ACN - 1% (v/v) NH4OH; Eluent 4: 99% ACN-H_2O (15:85, v/v) - 1% (v/v) NH_4OH; Eluent 5: 99% ACN-H_2O (50:50, v/v) - 1% (v/v) NH_4OH; Eluent 6: 99% ACN-H_2O (75:25, v/v) - 1% (v/v) NH_4OH.

diluted 1:20 before extraction by the μSPE-LOV system. In comparison to the standards prepared in water, the recovery values were lower for the fortified plasma matrix (102.8 ± 5.9% (standard) *vs* 74.9 ± 2.8% (plasma) for INDS; 95.7 ± 3.6% (standard) *vs* 83.0 ± 2.7% (plasma) for pCS) (Fig. 3), but still acceptable for bioanalysis if matrix matching standards are chosen for quantification in samples [27,28].

Having in mind the on-line hyphenation of automatic μSPE-BI-LOV with MS detection and considering the maximum recommended concentration of NH_4OH (0.1%, v/v) to be used in MS systems without jeopardizing analytical performance, the influence of this additive in the analyte recoveries was further evaluated. In comparison to the recoveries obtained with the eluent containing 1% (v/v) of NH_4OH, lower recovery values were attained when only 0.1% (v/v) of NH_4OH was added to the eluent (see Fig. 3). Additionally, for plasma matrix, a low repeatability was observed for the eluent with 0.1% (v/v) of NH_4OH, particularly for pCS (±30.8%). Thus, a higher percentage of NH_4OH (1%, v/v) was deemed necessary for efficient releasing of the target analytes from the sorbent and attain high recovery values. Considering the experimental findings and the application to plasma, the eluent composition was maintained as 99% ACN–H_2O (15:85, v/v)–1% (v/v) NH_4OH and the implementation of a dilution strategy prior to MS analysis was thus envisaged.

3.1.2. Study of the elution profile

A study of the elution profile of the target analytes was performed at-line for aqueous standards containing 0.5 μg mL^{-1} of each target analyte and plasma fortified with 4 μg mL^{-1} of each analyte (diluted 1:20 before extraction) using the μSPE-BI-LOV system. Different volumes (200–1500 μL) of the selected eluent were propelled and the elution profile was assessed by representing the mean of analyte recovery (%) *vs* eluent volume (Fig. S3).

As depicted in Fig. S3A, the elution profile of both analytes in aqueous standard solutions was very similar, with recoveries >79% for all the tested volumes. Nevertheless, the highest analyte recoveries were obtained with elution volumes ranging from 500 to 1500 μL (94.1–102.8% for INDS and 86.6–95.7% for pCS). On the other hand, as shown in Fig. S3B, the analytes exhibited a different elution behavior when present in plasma matrix. For INDS, the elution profile presented a sharp increase from 200 to 500 μL of eluent volume, followed by a slight reduction when using a volume of 1000 μL for analytes desorption. On the other hand, the obtained recovery values for pCS were similar for all the tested eluent volumes. Despite the matrix-depending elution behavior, the higher recovery values of both analytes were obtained using the same eluent volume, *i.e.*, 500 μL (analyte recovery of 78.3 ± 2.7% for INDS and 92.3 ± 3.5% for pCS).

3.2. Design of the on-line μSPE-BI-LOV-MS system

After defining the experimental conditions of the μSPE-BI-LOV setup and procedure (see section 3.1), the next step was the implementation of the hyphenation strategy between the LOV and the MS detector. The on-line transport of the eluate to the MS detector was a challenging task due to the need of matching the eluate composition with the maximum amount of NH_4OH that is recommended in the MS system. Therefore, a dilution step before MS detection was necessary. To implement this step, in-line dilution of the eluate was automatically performed prior to MS detection (Fig. 1A). In fact, the mixing factor can be set at will by taking advantage of the computer controlled and the multichannel operation of the multisyringe pump. Hence, an in-line mixing of the eluate with ACN–H_2O (15:85, v/v) (1:9) was implemented, allowing to achieve a final concentration of 0.11% (v/v) of NH_4OH in the eluate to make it compatible with MS analysis. Considering this, the proposed setup presents valuable features, namely, the capacity to dilute only a portion of the eluate, control of the dilution factor, and, consequently, a reduction in the volume of reagents required for this stage along with a reduction in the volume of generated waste.

The analysis of plasma samples was conducted using the final setup and the elution volume (500 μL) yielding the highest recovery values in plasma after at-line analysis. However, an incomplete elution of the target analytes was observed, confirmed by a second elution fraction (500 μL) containing 20% of the total recovered amount. Increasing the elution volume to 1000 μL provided good recovery values (91–100%). Therefore, an elution volume of 1000 μL was selected for the on-line assays.

3.3. Analytical performance of the on-line μSPE-BI-LOV-MS method

The analytical performance of the proposed method was evaluated by the determination of linear range, trueness, repeatability, reproducibility, and limit of quantification (LOQ) in accordance with the European Medicines Agency (EMA) and International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines [27,29].

Since the target analytes are endogenous compounds of plasma, blank matrix free from INDS and pCS is not available. Hence, the calibration curves and the following quantification of the two analytes in

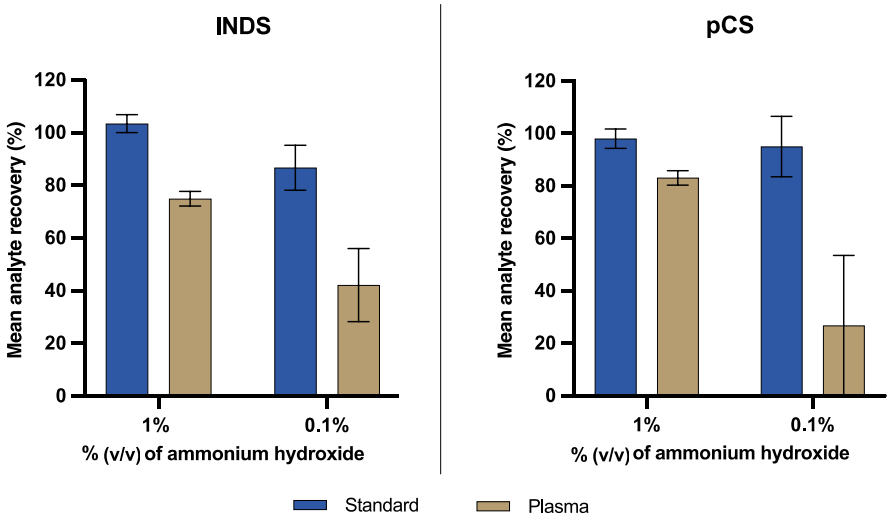

Fig. 3. Influence of the percentage of ammonium hydroxide added to the eluent composed by 15% ACN, in the recovery of the target analytes from aqueous standards and plasma.

plasma samples were implemented using the background subtraction strategy [28]. The calibration curves for INDS and pCS were linear over the tested concentration range (18–360 μg mL^{-1} in plasma, Fig. S4) with correlation coefficients >0.999. Furthermore, the recovery values at five concentrations levels (18, 90, 180, 270 and 360 μg mL^{-1}) presented deviations <15% from the nominal value, meeting the requirements of EMA and ICH guidelines. The recovery values ranged from 91.9 to 106.9% for INDS, and from 97.3 to 108.0% for pCS. The precision, represented as CV, was evaluated for plasma standards at two concentration levels (18 and 360 μg mL^{-1}) and did not exceed 15% for both intra-day and inter-day analysis of the two target analytes, thus complying with the limits recommended by guidelines [27,29]. The intra-day precision (repeatability) was ≤1.1% for INDS and ≤4.6% for pCS, and the inter-day precision (reproducibility) was ≤14.7% for INDS and ≤14.9% for pCS.

The LOQ was determined as the concentration equal to 20% of the endogenous levels (background concentration) [27,28], and the values obtained were 2 μg mL^{-1} for INDS and 7 μg mL^{-1} for pCS.

3.4. Application of the method to human plasma samples

The proposed μSPE-BI-LOV-MS method was applied to the analysis of the target analytes content in human plasma samples obtained from two healthy control subjects (samples 1 and 2) and two CKD patients (samples 3 and 4). The results obtained for two independent analyses of each sample are presented in Table 1 along with illustrative chromatograms of INDS and pCS in plasma from a healthy control subject and from a CKD patient (Fig. 4). A system based on four identification points (1 precursor ion and 2 product ions) [30] was applied for confirmatory analysis of INDS and pCS. The relative abundance of qualifier (q) and quantifier (Q) ions was determined in matrix-matched standards and samples with the maximum allowed tolerances of ±20% for INDS as the q/Q value was ≥50% and ±30% for pCS as the q/Q value was within the range 10–20% [30]. Matrix-matched standards presented mean ion ratio values of 66.3 ± 4.5% (INDS) and 11.0 ± 0.4% (pCS) whereas the ion ratio values in samples ranged from 55.6 ± 3.3% to 63.5 ± 3.2% for INDS and from 10.3 ± 0.6% to 11.7 ± 0.6% for pCS (Tables S3 and S4). These results evidenced that ion ratio values complied with the tolerance limits in all samples and thus permitted to confirm the presence of INDS and pCS.

As shown in Table 1, INDS and pCS were detected in all analyzed samples. The concentration values obtained for INDS ranged from 2.2 μg mL^{-1} in healthy controls to 71 μg mL^{-1} in CKD patients. On the other hand, the determined concentrations for pCS ranged from 33 μg mL^{-1} in healthy controls to 241 μg mL^{-1} in CKD patients. A comparison between the concentration values of INDS and pCS in healthy controls and disease patients showed that the content of target analytes was, on average, ca. 27 and 6 times higher in the CKD patients for INDS and pCS, respectively. These results are in accordance with studies that describe the association between a progressive accumulation of these compounds and the development of CKD [31,32]. Hence, the differences observed between controls and patients reinforced the importance of these two uremic toxins as potential biomarkers to define the stage of renal failure and disease progression.

Table 1
Quantification of INDS and pCS in plasma samples collected from healthy donors (control) and CKD patients analyzed using the developed on-line μSPE-BI-LOV-MS method.

Samples[a]	Concentration of the target analyte (μg mL^{-1})[b]	
	INDS	pCS
Sample 1	2.2 ± 0.2[c]	34.1 ± 0.8
Sample 2	2.5 ± 0.7[c]	33 ± 2
Sample 3	54 ± 6	129 ± 13
Sample 4	71 ± 3	241 ± 9

[a] Samples 1 and 2 were collected from healthy donors (control), and samples 3 and 4 from CKD patients.
[b] Each value corresponds to the mean ± standard deviation.
[c] Concentration value is below the lower calibration standard (18 μg mL^{-1}) and above the limit of quantification.

3.5. Comparison with other analytical methods and evaluation of method greenness

The comparison of the developed strategy with previously reported methods for the determination of the target analytes in plasma samples using MS detection is shown in Table 2. Compared to previously reported methods [33–36,38–41], which required chromatographic separation and normally lasted >50 min with exception of the methods using 96-well plates for sample preparation [33–35], the proposed method offers significant advantages. In fact, by eliminating the need for chromatographic separation and allowing to complete the whole analysis of the two target analytes in <20 min, the developed on-line μSPE-BI-LOV-MS method makes the process faster and more efficient. In terms of analysis time, the method described by Ahmed et al. [36] took a similar time compared to the proposed method (<20 min) to complete the whole analysis. However, this method only allows the determination of one analyte (INDS) with the implementation of chromatographic separation, whereas the proposed methodology permits the simultaneous analysis of two analytes without the need for previous chromatographic separation. The LOQ values obtained in the current work were in the low μg mL^{-1} range (≤7 μg mL^{-1} for both analytes), in contrast to other alternatives that could determine the target analytes at ng mL^{-1} levels (Table 2). However, since the concentrations of the target analytes expected in plasma samples are at the high μg mL^{-1} level (particular in individuals with disease), a lower LOQ is not required. The miniaturized and automated features, the high analysis throughput, and the elimination of the chromatographic separation step outbalance the higher LOQ values of the proposed methodology.

As shown in Table 2, SPE is not the first-choice method for sample preparation and its application has been rarely reported for extraction of uremic toxins [5]. Oda et al. [33] reported the use of a microSPE plate for the analysis of the target compounds that allowed simultaneous processing of 96 samples in shorter time (<15 min), but additional steps to the sample pre-treatment were necessary, resulting in a more laborious sample preparation procedure with the need of more human intervention. In contrast, the method proposed in this work allows the combination of the sample preparation step with the additional steps (e.g., dilution of the extracts), which are performed automatically and on-line, resulting in considerable reduction in the need of operator intervention and, consequently, a decrease in experimental errors.

The use of green and sustainable methodologies is the current trend in the development of analytical procedures. Thus, the AGREE metric was used to assess the greenness of several methods (Table 3, Figs. S5 and S6) [37]. The strategy proposed here shows a green performance and is the most environmentally friendly. As can be seen in Table 3, the proposed strategy (method 9) exhibited an overall score of 0.64 out of 1.0 when overall scores ≤0.51 were achieved for the other methods (methods 1–8). The main features contributing to the greenness of the new method were the sample amount in the μL scale (20 μL) (criteria 2), the reduced number of steps performed (criteria 4), the automated and miniaturized procedure (criteria 5), and the non-application of derivatization agents (criteria 6). Furthermore, the implementation of a total on-line procedure that includes sample preparation and detection analytical steps in a single assembly (criteria 1 and criteria 3), the use of low organic solvent amounts, and the limited use of health-hazardous compounds (criteria 11) also contributed to the greenness of this novel method. Additionally, it is important to refer that only three over twelve criteria evaluated presented lower scores (<0.4), namely the volume of waste generated (10.5 mL per sample) (criteria 7), the energy consumption (criteria 9) and the non-use of reagents from renewable

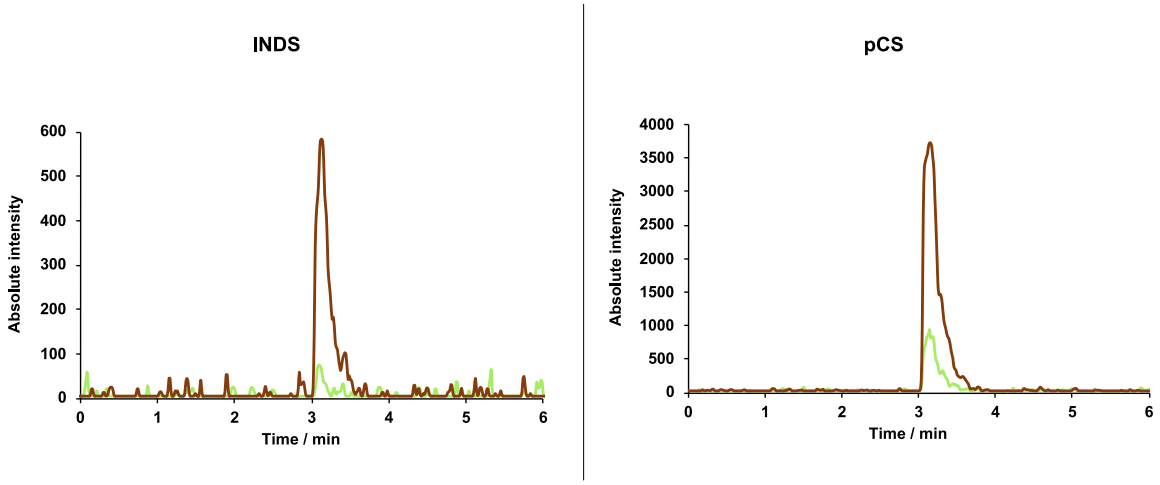

Fig. 4. Representative chromatograms for INDS and pCS in plasma samples from a healthy control subject (green line) and a CKD patient (brown line), obtained in SRM mode, using the developed on-line μSPE-BI-LOV-MS system. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

Table 2
Comparison of the proposed on-line μSPE-BI-LOV-MS method with previously reported methods using mass spectrometry detection for the determination of target analytes in plasma samples.

Analyte	Sample preparation	Chromatographic separation?	Total procedure time per sample (min)[b]	LLOQ/LOQ	Reference
pCS	Protein Precipitation	Yes	>50	1 ng mL^{-1}[a]	[38]
INDS	Protein Precipitation	Yes	<20	0.1 μg mL^{-1}	[36]
INDS	SPE using OASIS WAX μelution plate	Yes	<10[c]	0.05 μg mL^{-1}	[33]
pCS INDS	Protein Precipitation using a 96-well Sirocco™ plasma protein filtering plate	Yes	<10[c]	24 ng mL^{-1} (pCS) 19 ng mL^{-1} (INDS)	[34]
pCS INDS	Protein Precipitation	Yes	>60	2.7 nmol mL^{-1} (pCS) 1.9 nmol mL^{-1} (INDS)	[39]
pCS INDS	Protein Precipitation	Yes	>50	NA	[40]
pCS INDS	Protein Precipitation using a 96-well Sirocco™ plasma protein filtering plate	Yes	<15[c]	0.005 μg mL^{-1} (pCS) 0.01 μg mL^{-1} (INDS)	[35]
pCS INDS	Protein Precipitation	Yes	>300	0.05 μg mL^{-1} (pCS) 0.05 μg mL^{-1} (INDS)	[41]
pCS INDS	On-line μSPE-LOV-MS using OASIS WAX	No	<20	2 μg mL^{-1} (pCS) 7 μg mL^{-1} (INDS)	This work

NA- Not available.
[a] The value corresponds to the LOD.
[b] Total procedure time: including sample preparation and analytical determination time.
[c] Estimated value considering the time for sample preparation divided by 96 samples plus the time for LC-MS analysis

sources (criteria 10). On the other hand, the main criteria contributing to the non-greenness of the previously reported methods were sample treatment (criteria 1), device positioning (criteria 3), automation and miniaturization of the procedure (criteria 5), energy consumption (criteria 9), source and toxicity of reagents (criteria 10 and 11). It is important to highlight that some of the criteria contributing to the non-greenness of the previous works (criteria 1, 3 and 5) were, in contrast, relevant features that contributed to the greenness of the proposed methodology.

4. Conclusions

An automatic μSPE-BI-LOV-MS method has been developed and successfully applied to the quantification of two uremic toxins (INDS and pCS) in plasma samples. To our knowledge, this is first time that a fully automatic and renewable μSPE procedure integrated within a LOV platform that was coupled on-line with tandem MS detection without implementing a chromatographic separation step has been used for the determination of endogenous compounds in biological samples. The proposed method can be regarded as a total analysis system once the extraction, dilution, and quantification steps were carried out on-line in a single instrumental assembly. Moreover, minimal human intervention was required with the use of the proposed flow system, yielding determinations with good precision.

The hyphenation of μSPE-BI-LOV with MS detection combines the flexibility, automation, miniaturization, and simplification granted by the mesofluidic platform with the selectivity, high sensitivity and robustness associated to MS detection, resulting in a competitive and efficient analytical system. Due to the high selectivity of tandem mass spectrometry detection, no previous chromatographic separation was

Table 3
Comparison of greenness of the previously reported methods using mass spectrometry detection for the determination of the target analytes in plasma samples with the developed on-line μSPE-BI-LOV-MS method based on AGREE metric results.

Criteria	Method*								
	1	2	3	4	5	6	7	8	9
1. Sample treatment									
2. Sample amount									
3. Device positioning									
4. Sample preparation stages									
5. Automation and miniaturization									
6. Derivatization									
7. Waste									
8. Analysis throughput									
9. Energy consumption									
10. Source and reagents									
11. Toxicity									
12. Operator's safety									
Overall score	0.46	0.51	0.49	0.46	0.49	0.48	0.51	0.46	0.64
Reference	[38]	[36]	[33]	[34]	[39]	[40]	[35]	[41]	This work

* The colors in table range from red (not green method) to green (green method)

required, reducing the analysis time, and increasing sample throughput. Indeed, the method permitted the simultaneous analysis of the two target analytes in 18 min, including sample treatment. In addition, this strategy leads to a more environmentally friendly and safer approach compared to previous methods, representing a significant advancement towards quantification of the target analytes, especially in innovation of sample treatment strategies. Despite the application for analysis of only two analytes, sample treatment for determination of a larger number of analytes with different physical-chemical properties is envisaged. Finally, the hyphenation strategy herein presented bears potential to be applied to other biomatrices, including non-invasive samples.

CRediT authorship contribution statement

Sara R. Fernandes: Methodology, Investigation, Validation, Writing – original draft. **Luisa Barreiros:** Conceptualization, Formal analysis, Supervision, Funding acquisition, Writing – review & editing. **Benedita Sampaio-Maia:** Resources, Writing – review & editing. **Manuel Miró:** Supervision, Project administration, Writing – review & editing. **Marcela A. Segundo:** Conceptualization, Supervision, Resources, Project administration, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

Data will be made available on request.

Acknowledgements

This work received financial support from PT national funds (FCT/MCTES, Fundação para a Ciência e a Tecnologia and Ministério da Ciência, Tecnologia e Ensino Superior) through the projects UIDB/50006/2020, UIDP/50006/2020 and 2022.06012.PTDC. S. R. Fernandes thanks FCT and ESF (European Social Fund) through Norte 2020 (Programa Operacional Regional Norte) for her PhD grants (SFRH/BD/130948/2017 and COVID/BD/152406/2022). L. Barreiros acknowledges funding from FCT through program DL 57/2016 – Norma transitória. M. Miró acknowledges financial support from the Spanish Ministry of Science and Innovation (MCIN) and Spanish State Research Agency (AEI) through project PID2020-117686RB-C33-R (MCIN/AEI/10.13039/501100011033).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2023.341668.

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