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Department of Analytical Chemistry, Nutrition and Food Science

TANDEM MASS SPECTROMETRY COUPLED TO LIQUID CHROMATOGRAPHY WITH ON-LINE PRETREATMENT: DETERMINATION OF XENOBIOTICS IN FOODSTUFF AND BIOMARKERS IN BIOLOGICAL SAMPLES

PhD THESIS

SUMMARY IN ENGLISH

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1 AIM

This thesis focuses on the development of new analytical methods based on the coupling of mass spectrometry and high-efficiency separation techniques, such as capillary electrophoresis and high performance liquid chromatography. These couplings were applied to the resolution of problems of special interest as regards food safety and environmental health. Thus, the author was interested in the determination of toxic residues in foods and the assessment of endogenous and exogenous compounds used as biomarkers in biological samples.

The overall aim of the Thesis is the development of new methodologies that will meet the demands currently addressed in the field of Analytical chemistry such as the simplification of analytical procedures, both in the sample treatment steps, which are especially tedious and time-consuming, and in aspects related to separation-quantification and identification.

In particular the author aims to develop new strategies for sample treatment (extraction-preconcentration-clean-up) using sorbents formed of restricted access materials in on-line configurations that will facilitate or reduce the steps in the global process. Special attention is devoted to the methodological development of these sample treatment steps based on the use of these sorbents to be able, where possible, to perform automated sample treatment. These materials exclude macromolecules such as proteins and other polar substances thanks to a mechanism of molecular exclusion and their surface polarity. Accordingly, one of the objectives of the Thesis is to show that the use of these materials allows the automation of sample treatment with matrices of great complexity such as foods and biological samples since they provide suitable cleaning for later chromatographic or electrophoretic analysis.

A second aim is the study of new chromatographic mechanisms that will allow the number of analyzable compounds to be expanded. This aspect covers study of the chromatography of hydrophilic interactions for the separation of high-polarity compounds. In this section, the author performs a detailed study of the retention mechanisms that arise in hydrophilic interaction liquid chromatography (HILIC) when used in the separation of high-polarity

compounds. Of special relevance is the quantitative evaluation of the electrostatic contribution to the retention mechanism. A study is also made of the possible coupling of hydrophilic interaction liquid chromatography to reversed-phase chromatography (HILIC-RPLC) with a view to carrying out the simultaneous and automatic determination of endogenous compounds (usually high-polarity compounds) and of exogenous compounds used as biomarkers of exposure to xenobiotic compounds (of intermediate and low polarity).

In the different methods developed, mass spectrometric detection in different modes is used (quadrupole, triple quadrupole, ion trap) to optimize the detection and identification of analytes, studying the fragmentation processes and the transitions that can provide unequivocal identification.

Finally, an important point is that from the methodological point of view the Thesis is self-contained in its scope since the methodological innovations addressed focus on the establishment of rapid, sensitive and selective analytical methods that are also able to meet the criteria set by the legislation for validation as quantitative confirmatory methods in the analysis of compounds of interest. The compounds of interest in the different matrices studied in this Thesis can be divided into pollutants in foods (chapter 2) and biomarkers in biological samples (chapter 3).

Among the specific aims is the development of new automated and validated methodologies for the determination of different endocrine-disrupting agents in honey samples using chromatographic and electrophoretic methods. Another study encompassed in this section is the optimization of and automated method for the determination of several different benzimidazoles in milk samples.

A further avenue of enquiry addressed is the development of different methods of analysis for the separation, identification and quantification, at trace levels in urine samples, of biomarkers of exposure to exogenous compounds using reversed-phase chromatography (RPLC), and biomarkers of effect, or endogenous markers, using hydrophilic interaction liquid chromatography (HILIC) with a zwitterionic stationary phase The exogenous biomarkers studied are xenobiotic compounds of special relevance owing to their

wide use in agricultural and industrial activities. The endogenous biomarkers studied are related to alterations in DNA/RNA: nucleosides modified by methylation (1-methylguanine, 7-methylguanine, 9- methylguanine, 7- methylguanosine and 1-methyladenosine) and hydroxylation (8-hydroxiguanine, 8-hydroxyguanosine and 8-hydroxy-2'-deoxyguanosine). The method developed is applied to the analysis of samples from oncologic patients in order to determine the potential of these biomarkers in the diagnosis or monitoring of the progress of the oncologic disease.

2 DETERMINATION OF XENOBIOTIC COMPOUNDS IN FOODSTUFF

2.A. DETERMINATION OF ENDOCRINE DISRUPTORS IN HONEY

RESEARCH ARTICLE I

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TECHNICAL NOTE

A confirmatory method for the determination of phenolic endocrine disruptors in honey using restricted-access material–liquid chromatography–tandem mass spectrometry

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Abstract The present work describes the development and validation of an analytical method based on liquid chromatography (LC), coupled with tandem mass spectrometry (MS/MS) that allows the determination and confirmation of several endocrine-disrupting chemicals (EDCs) in honey. The EDCs studied were nine phenols of different nature: chlorophenols (2,4-dichlorophenol, 2,4,5-trichlorophenol, and pentachlorophenol), alkylphenols (4-*tert*-butylphenol, 4-*tert*-octylphenol, and 4-*n*-octylphenol) bisphenols (bisphenol-A and bisphenol-F), and 4-*tert*-butylbenzoic acid. The method incorporates a restricted-access material (RAM), coupled on-line to the LC-MS/MS system, which allows direct injection of the matrix into the RAM-LC-MS/MS system. The optimized method developed, RAM-LC-MS/MS, was applied to fortified honey samples, affording detection limits in the 0.6–7.2 ng g^{-1} range, calculated for a signal-to-noise ratio of 3. In addition, the method was validated as a quantitative confirmatory method according to European Union Decision 2002/657/EC. The validation criteria evaluated were linearity, repeatability, reproducibility, recovery, decision limits, detection capabilities, specificity, and ruggedness. Repeatability and within-laboratory reproducibility were evaluated at two concentration levels, being ±11% or below at 20 ng g^{-1}. The decision limits (CC_α) and detection capabilities (CC_β) were in the 1.7–12.6 and 2.8–21.6 ng g^{-1} range, respectively.

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Keywords Endocrine-disrupting chemicals · Phenolic compounds · Honey · Liquid chromatography–tandem mass spectrometry · Validation according to 2002/657/EC · Restricted-access materials

Introduction

Honey is a healthy natural product with excellent nutritional properties. Since it is a product consumed worldwide, it is evident that human health should be taken into consideration in regards to its consumption. Owing to its sweetness, color, and different flavors, honey is often used as a sugar substitute, as is an ingredient, or used as a natural preservative in hundreds of manufactured foods. From the chemical point of view, honey is a complex mixture of sugars. Its composition depends strongly on the plant species from which the nectar or honey dew has been collected as well as on other factors such as environmental conditions and climate [1].

To ensure the safety and quality control of honey, it is necessary to perform the analysis of chemical contaminants in honey in order to assure that this natural product does not contain toxic residues in quantities that might imply a risk for consumers. Accordingly, sensitive, selective, fast, and inexpensive analytical methods for food analysis are continuously under development.

The analysis of hazardous compounds at trace level in complex matrices includes a procedure of sample preparation, which can cover analyte isolation, trace enrichment, and further cleanup to remove matrix interferents. The sample treatment techniques frequently employed for the isolation and enrichment of chemical contaminants from honey are liquid–liquid extraction (LLE) [2, 3], solid-phase extraction (SPE) [4–6], solid-phase microextraction (SPME) [7] and matrix solid-phase dispersion (MSPD)

[8]. Other analytical procedures reported involve supercritical fluid extraction (SFE) [9], ultrasound-assisted extraction (UAE) [10] or pressurized liquid extraction (PLE) [11].

Restricted-access materials (RAMs) are porous chromatographic supports specifically designed to allow solid–liquid extraction and the enrichment of small molecules, limiting the access of macromolecules to the interaction sites of a stationary phase bonded to their inner surface. Only small molecules are able to penetrate into the pores and interact with the solid sorbent on the inner surface, while large molecules are eluted with the clean-up solvent. RAMs were initially designed to remove proteins in the analysis of drugs in biological matrices [12–14], although they have also found applications in environmental analysis [15]. However, only a few studies have been reported regarding the applications of RAMs to sample treatment steps in food analysis [16].

The present study was aimed at developing a simple, fast, precise, accurate, and highly automated analytical approach for the identification and quantification of several EDCs in honey. The analytes assayed were: bisphenol A (BPA), bisphenol F (BPF), 4-*tert*-butylbenzoic acid (tBBA), the chlorophenols 2,4-dichlorophenol (DCPL), 2,4,5-trichlorophenol (TCPL) and pentachlorophenol (PCPL), 4-*tert*-butylphenol (tBPL), 4-*tert*-octylphenol (tOPL) and 4-*n*-octylphenol (nOPL). These compounds are used in industry or are produced as intermediates in the production of epoxy resins and polycarbonate plastics. These plastics are used in many food- and drink-packaging applications, whilst the resins are commonly used as lacquers to coat metal products such as food cans, bottle tops, and water supply pipes. These compounds have been widely analyzed in environmental matrices such as water, sludge, and sediments [17, 18]. Food may be another important route of exposure to endocrine-disrupting compounds. Some EDCs could enter the food chain at several stages of its production and also via plastic packaging material, stretch films used for food packaging, and many foodstuff containers for oven or microwave cooking [19]. The presence of phenolic EDCs in different foods [20–24], among which honey is included [25, 26], has been reported by several authors, highlighting a possible risk for consumers.

The method proposed here is based on the use of a RAM coupled on-line to an LC-MS/MS system for the identification and quantification of nine EDCs in honey. The use of RAM material enables fast on-line clean-up of honey samples, efficiently eliminating matrix components and providing appropriate selectivity and sensitivity for the determination of these compounds at trace levels. The on-line configuration developed involves minimum sample treatment (a simple dilution and filtration step) and affords a shorter analysis time. The method was validated as a quantitative confirmatory method according to the EU Decision 2002/657/EC: linearity, precision (repeatability and intra-laboratory reproducibility), recovery, decision limits, detection capabilities, specificity, and ruggedness were evaluated for the target compounds.

Experimental

Chemicals

Analytical standards of *bisphenol-F* (BPF), bis-(4-hydroxyphenyl)methane, CAS RN [620-92-8]; *bisphenol-A* (BPA), 2,2-bis(4-hydroxyphenyl)propane, CAS RN [80-05-7]; *tert-butylphenol* (tBPL), 4-(1,1-dimethylethyl)phenol, CAS RN [98-54-4]; *dichlorophenol* (DCPL), 2,4-dichlorophenol, CAS RN [120-83-2]; *trichlorophenol* (TCPL), 2,4,5-trichlorophenol, CAS RN [95-95-4] and *pentachlorophenol* (PCPL), 2,3,4,5,6-pentachlorophenol, CAS RN [87-86-5] were purchased from Dr. Ehrenstorfer (Augsburg, Germany). *tert-butylbenzoic acid* (tBBA), 4-(1,1-dimethylethyl)benzoic acid, CAS RN [98-73-7]; *n-octylphenol* (nOPL), 4-octylphenol CAS RN [1806-26-4] and *tert-octylphenol* (tOPL), 4-(1,1,3,3-tetramethylbutyl)-phenol, CAS RN [140-66-9] were obtained from Sigma-Aldrich (Steinheim, Germany).

The organic solvents acetonitrile (ACN) and methanol (MeOH) were of HPLC grade (Merck, Darmstadt, Germany) and were used as received. Ultra-high quality (UHQ) water was obtained with a Wasserlab (Noain, Navarra, Spain) Ultramatic water purification system.

All chemicals used for the preparation of the buffer and all other chemicals were of analytical reagent grade.

Instrumentation

HPLC analyses were performed on a HP 1100 Series chromatograph from Agilent (Waldbronn, Germany) equipped with a binary pump, an additional isocratic pump, a membrane degasser, an autosampler (equipped with a 1,500-μL capillary seat), a six-port valve, and a diode-array detector (DAD). The system was controlled by a HP ChemStation, which also performed data collection from the detectors and quantitative measurements. The RAMs used were LiChroCART 25-4LiChrospher alkyl-diol-silica (ADS, 25 μm, 25 mm×4 mm) RP18, RP8, and RP4 from Merck (Darmstadt, Germany). The analytical column was a 150×4.60 mm Luna PFP(2) packed with 3-μm particles (Phenomenex, Torrance, CA, USA).

The clean-up mobile phase, impelled by the isocratic pump, consisted of a 2.5 mmol L^{-1} formic acid/ammonium formate buffer (pH: 2.9) with 10% ACN. The separation mobile phase, impelled by the binary pump, consisted of a 1 mmol L^{-1} formic acid/ammonium formate buffer, pH: 3.4, (solvent A) and methanol (solvent B) gradient from

80% to 100% of B. The analytical column was thermostatted at 20 °C.

Mass spectrometry

The LC/MSD Trap XCT ion-trap mass spectrometer (Agilent, Waldbronn, Germany) was equipped with an electrospray (ESI) source with a nebulizer spacer. The ESI settings were a capillary voltage of −3,500 V; a drying gas flow of 10 L min^{-1} at a temperature of 350 °C, and a nebulizer pressure of 50 psi. Optimization of the ionization and fragmentation parameters was achieved manually while injecting standard solutions of each analyte (5 μg mL^{-1}) with a syringe pump at a flow rate of 1 mL h^{-1}; these solutions were mixed with the mobile phase at 0.3 mL min^{-1} by means of a T piece. The trap parameters were set at a smart target of 50,000 to 100,000 and a maximum accumulation time of 200 ms at an *m/z* range from 60 to 400 u. A narrow isolation width of 4 u was selected. The optimized parameters and retention times for each analyte are listed in Table 1.

Honey samples

Samples of polyfloral honey commercially available from different commercial brands on the retail market were used in this work. All honey samples were first analyzed by the proposed method in order to check the natural occurrence of these compounds. No signals corresponding to the target analytes were found, and hence fortified honey samples were used.

Calibrant preparation and calibration procedure

Stock solutions of analytical calibrants were prepared by dissolving 12.5 mg of each analyte in 25 mL of acetonitrile (500 μg mL^{-1}). These stock solutions were stored at 4 °C in brown glass bottles. Matrix-matched calibrants, in the 10–200 ng g^{-1} range, were prepared daily by adding the appropriate amount of each stock solution to the honey samples. The fortified honey samples were capped and stored in the dark at room temperature for approximately 12 h to permit the interaction between the analytes and the matrix compounds. Finally, the fortified honey samples were diluted 1:5 (m/V) with UHQ water, shaken vigorously and filtered with a 5.0 μm nylon filter before analysis. Calibration curves were obtained by plotting the peak areas of the analytes versus concentration using matrix-matched standards.

Procedure for on-line sample preparation and separation

The experimental setup for RAM-LC-MS/MS was an on-line column switching configuration [27]. First, a predetermined volume of diluted honey was injected with the autosampler and the isocratic pump was immediately started to pump the clean-up mobile phase at 1 mL min^{-1} for 3 min with the valve in the "sample enrichment" position. The target compounds were withheld in the RAM, while the matrix components of the honey were washed to waste. At 3 min, the system setup was changed to the "sample elution" position and the separation gradient (binary pump) eluted the analytes at a flow rate of 0.65 mL min^{-1} in backflush mode to the analytical column, where they were separated and finally detected by the mass spectrometer. During this time, the isocratic pump changed the flow rate to 0.3 mL min^{-1} in order to save solvent until next injection. At 23 min, the separation ended and the gradient was returned to the initial conditions. The flow rate of the isocratic pump was changed to 1 mL min^{-1}. At 26 min, the valve was switched to the "sample enrichment"

Table 1 Ion-trap tandem mass spectrometer parameters optimized for the studied EDCs

Analyte[a]	t_R (min)	Identification transition	Confirmation transition	Fragmentation amplitude (V)	IPs[b]	Window
BPF	14.2	199→93	199→123	1.10	4	12–17.3 min
BPA	14.6	227→212	227→133	0.95	4	12–17.3 min
tBPL	15.7	149→133	–[c]	0.67	2.5	12–17.3 min
DCPL	15.9	161→125	163→125	0.68	3.5	12–17.3 min
tBBA	16.4	177	–[c]	–[c]	1	12–17.3 min
TCPL	17.6	195→159	197→161	0.95	5	17.3–25 min
tOPL	18.6	205→133	205→190	0.87	4	17.3–25 min
nOPL	20.1	205→106	–[c]	0.86	2.5	17.3–25 min
PCPL	21.4	265	267, 263[d]	–[c]	3	17.3–25 min

[a] *BPF* bisphenol-F, *BPA* bisphenol-A, *tBPL* 4-*tert*-butylphenol, *DCPL* 2,4-dichlorophenol, *tBBA* 4-*tert*-butylbenzoic acid, *TCPL* 2,4,5-trichlorophenol, *tOPL* 4-*tert*-octylphenol, *nOPL* 4-*n*-octylphenol, *PCPL* pentachlorophenol

[b] Identification Points (IPs) according to 2002/657/EC

[c] No satisfactory transition was found

[d] Chloride isotopes ions

mode and a 4-min post-run program was started, keeping the system under the initial conditions in order to equilibrate the analytical column for the next analysis.

Method validation

The method was validated, according to the pertinent legislation [28], by evaluating the following parameters:

- Specificity: by calculating ion suppressions and comparing patterns with matrix-matched samples.
- Determination of the statistical parameters and the quality of the linear regression using the confirmation transition. The matrix-matched calibrations were obtained in the 10–150 ng g^{-1} range. The decision limit (CC_α) and the detection capability (CC_β) were also calculated.
- Repeatability and within-laboratory reproducibility were determined as intraday and interday precisions by analyzing honey samples at two concentrations levels (20 and 100 ng g^{-1}).
- Recoveries were determined at two concentration levels (12.5 and 25 ng g^{-1}).
- Stability: by controlling the storage conditions of the analytes and stock solutions (see "Calibrant preparation and calibrant procedure" section), keeping them in the range in which stability is guaranteed by the manufacturer.

Results and discussion

The chromatographic separation of the analytes was optimized by searching for maximum compatibility with the detection performed with mass spectrometry. Thus, water/methanol mixtures with a high content in organic component were used as the mobile phase, facilitating the electrospray ionization process. Likewise, the presence of salts was reduced as much as possible, this aiming and increasing the ionizing efficiency of the analytes in the source [29]. Following these criteria, optimum chromatographic separation was observed in 19 min for the nine EDCs studied, applying a gradient of 80% to 100% of methanol, and using a 1.0 mmol L^{-1} formic acid/ammonium formate buffer, pH 3.4, as the aqueous component of the mobile phase, with a mobile phase flow rate of 0.65 mL min^{-1}.

Restricted-access materials: optimization of sample treatment using RAM-LC-DAD

The analysis of complex matrices using chromatographic methods usually requires one or several clean-up steps in order to remove the greatest amount possible of the compounds present in the matrix that could hinder analysis and shorten the useful lifetime of the chromatographic column.

RAMs have frequently been used in the clean-up steps of biological samples, but their use in the clean-up of food samples has not received much attention. In previous studies carried out by us, we observed that these materials may be highly suitable for the clean-up of samples with high contents of sugars. Accordingly, here we studied the possibilities offered by a restricted-access material of the ADS type as a sample treatment step for the analysis of phenolic EDCs in honey samples. For this study, an ADS-RAM coupled on-line to a LC-DAD system was used. Figure 1 shows the chromatograms for an unfortified honey sample injected directly into the LC-DAD system (Fig. 1a), together with those obtained upon injecting the same sample volume through the RAM-LC-DAD system: using ADS-RAMs of the C18 and C4 types (Fig. 1b–c). Regarding the direct injection, it is possible to observe a large number of very intense chromatographic peaks, corresponding to the matrix. The number and intensity of these peaks decreased considerably when the samples were injected through the RAM, which acted as an extraction and on-line clean-up step. The results obtained reveal the efficiency of the RAM in cleaning the components of the matrix in the honey, which is an important aspect in order to preserve the instrumental system and lengthen the life of the chromatographic column. The chromatograms obtained upon using the ADS-RAM of the C18 (Fig. 1b) and C4 (Fig. 1c) types for the same unfortified honey sample indicate that the level of clean-up is adequate in both cases, although the most favorable one was obtained with the ADS-RAM with the C4 stationary phase. It was thus decided to use the C4 RAM for later studies.

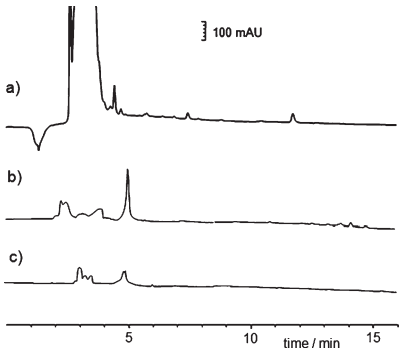

Fig. 1 LC-DAD chromatograms obtained for an unfortified honey sample injected **a** directly into the chromatographic column, **b** through the on-line RAM-LC-DAD using a C18 RAM, and **c** the same as in **b** using a C4 RAM. Injected volume, 100 μL, signal 214 nm

The next step consisted of optimizing the RAM washing solvent, i.e., the mobile phase impelled by the isocratic pump that acted as a washing solvent and at the same time transported the sample through the RAM. Study of the composition of this mobile phase (washing solvent) aimed at maximizing sample clean-up without negatively affecting the analyte recovery. There are two main factors that jointly affect these parameters: the percentage of organic phase in the washing solvent and the time that the washing step lasts. Increases in the organic component afforded a better cleaning of the interferents, although an excessive increase may lead to the elution of the analytes of interest, low recoveries being obtained. We therefore studied percentages of acetonitrile in the washing solvent between 0% and 25%, keeping a clean-up time of 3 min throughout the study. In each case, the recoveries of each analyte were determined by comparing the concentration obtained, by means of a calibration, with the real concentration at which the sample was originally fortified. The recoveries remained above 90% when a washing solvent with up to 10% acetonitrile was used. An increase in this percentage up to 25% led to a dramatic decrease in the recoveries for all the analytes studied. In light of the results, it was decided to use as a washing solvent, corresponding to the mobile phase impelled by the isocratic pump, 2.5 mmol L^{-1} formic acid/ammonium formate buffer (pH 2.9) with 10% of acetonitrile because this was the highest percentage of organic phase with which satisfactory recoveries were obtained.

With this composition for the washing solvent, a study was made of the effect of the RAM washing time; that is, the time during which it was circulating through the RAM once the sample had been injected. For washing times between 3 and 15 min, an increase in the washing time did not afford a significantly better clean-up but did elicit the corresponding increase in the analysis time. Accordingly, a time of 3 min was chosen as optimum since it afforded an adequate washing of the undesired components of the matrix.

Optimization of the on-line coupling RAM-LC-MS/MS

In the literature, it has been reported that RAMs admit the injection of large sample volumes [30]. Thus, the possibility of injecting large sample volumes into the RAM-LC-MS/MS system was studied in order to improve the sensitivity of the method. The sample volumes injected were in the 20–1,500-μL range. Figure 2 shows the results obtained for a honey sample fortified at 4 μg g^{-1}. For high injection volumes it is possible to observe detector saturation. Accordingly, an injection volume of 500 μL was chosen, considering this value as the highest volume that could be injected without saturating the MS/MS system.

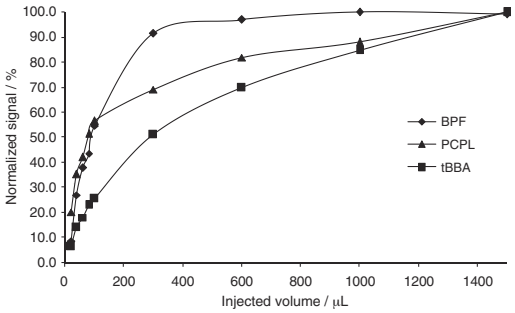

Fig. 2 Normalized (to the highest area) signals as a function of the volume of fortified honey injected according to the RAM-LC-MS/MS method developed. Fortified level: 4 μg g^{-1} for each analyte. *BPF* bisphenol-F, *PCPL* pentachlorophenol, *tBBA* 4-*tert*-butylbenzoic acid

Study of ion suppression

When ESI-MS is used as a method of detection, it is known that this ionization source (ESI) may undergo losses of sensitivity owing to the presence of other compounds that co-elute with the analytes of interest, a phenomenon known as ion suppression. Moreover, this effect may be more pronounced when large sample volumes are injected.

Here, we studied the possible effect of ion suppression on the signals of the analytes when 500 μL of honey sample was injected into the RAM-LC-MS/MS system. To accomplish this, the total ion chromatograms (TIC) were recorded (Fig. 3) for a standard aqueous sample of the analytes studied, fortified at 100 ng g^{-1} in UHQ water (Fig. 3a) and for a honey sample fortified at 100 ng g^{-1} injected using the proposed RAM-LC-MS/MS methodology (Fig. 3b). This honey sample was also directly injected into the LC-MS/MS system (Fig. 3c). It may be observed that the total signal of the analytes in the standard in UHQ water and in the honey injected through the RAM was similar both in shape and in intensity. However, when fortified honey was injected directly into the LC-MS/MS system, the signal of the analytes underwent a strong decrease owing to ion suppression. This points to the efficiency of the RAM in the cleaning of the matrix components.

Although upon injecting 500 μL of honey sample the RAM-LC-MS/MS configuration strongly reduced the ion suppression of the analytes, such suppression persisted. Thus, this matrix effect was calculated as ion suppression according to the method suggested by Feitosa-Felizzola et al. [31]. Table 3 shows the ion suppression (expressed as a percentage with respect to the signal obtained in UHQ water) for each of the analytes separately. The values of ion suppression varied between 4.6% for tBBA and 24% for tBPL.

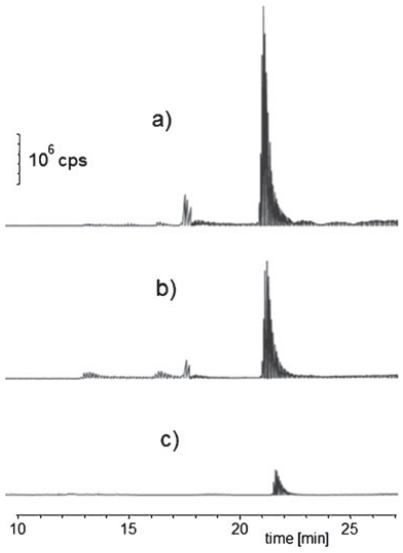

Fig. 3 Total ion chromatograms (*TICs*) obtained by the analysis of: **a**) a UHQ standard, **b**) a fortified honey sample injected according to the RAM-LC-MS/MS method developed, and **c**) a fortified honey sample injected directly into the LC-MS/MS system. Injected volume, 500 μL, fortified level, 100 ng g^{-1} for each analyte

The level of ion suppression undergone by the analytes did not seem to be related to their retention times. However, we did observe a correlation between ion suppression and the acidity constant (pK_a) of the analytes, i.e., with their tendency to lose a proton. The formation of ions in the electrospray is one of the most critical factors in liquid chromatography coupled to mass spectrometry. The efficiency of this step depends on the previous presence of the ions studied in liquid phase or on their later generation, through highly complex mechanisms, in the gas phase [29]. Accordingly, the pK_a of the compounds of interest is one of the most relevant factors affecting the efficiency of the formation of such ions. Table 2 shows the pK_a value for each of the EDCs studied, together with its corresponding ion suppression, calculated experimentally. The correlation between ion suppressions and pK_a (r^2=0.954) is coherent with the fact that all the compounds of interest were detected as anions arising from the loss of a proton: $[M-H]^-$. Since the effect of ion suppression leads to a loss of analytical signal, and hence sensitivity, this correlation could be extended to different analytical characteristics, such as the limit of detection.

Analytical characteristics of the RAM-LC-MS/MS method

Once the parameters of the RAM-LC-MS/MS method had been optimized, their analytical characteristics were studied. The MS/MS detection system was configured in multiple reaction monitoring (MRM) mode, analyzing the transition of identification for each of the analytes. The limits of detection (LODs), calculated as three times the signal–noise ratio, varied between 0.6 ng g^{-1} for PCPL and 7.2 ng g^{-1} for BPF. The limits of quantification (LOQs), calculated as ten times the signal–noise ratio, ranged between 2.0 ng g^{-1} for PCPL and 22.6 ng g^{-1} for BPA. Figure 4 shows the MS/MS chromatograms and total ion chromatogram (TIC) of the analytes studied, at a concentration level of 10 ng g^{-1}, in MRM mode, analyzing the transition of identification.

Validation of the RAM-LC-MS/MS method according to 2002/657/EC

In recent years, the Commission Decision of the 12 August 2002 implementing Council Directive 96/23/EC establishes criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results generated by official laboratories. This decision establishes common criteria for the interpretation of test results and introduces a procedure to progressively establish minimum required performance limits (MRPL) for analytical methods employed to detect substances for which no permitted limit (maximum limit) has been established. This, especially for substances whose use is not authorized or is specifically prohibited in the EU. Thus, through the 2002/657/EC Decision [28] the EU has established the characteristics that must be fulfilled by an analytical method for it to be required as a confirmatory quantitative method. The parameters to be evaluated are: the detection capacity (CC_β), the limit of decision (CC_α), recovery, precision, selectivity, and ruggedness.

Additionally, for confirmatory methods based on MS/MS detection, the 2002/657/EC Decision establishes a minimum of three points of identification (IPs) that must be presented for each analyte based on the number of mass and mass/mass signals found. The IPs found for the nine EDCs studied are shown in Table 1. Unfortunately, it was not possible to achieve the minimum number of identification points required by the EU [28] in the case of tBPL, tBBA, and nOPL; even when MS^3 experiments were performed for tBPL and nOPL, no additional fragment ions were observed. This is due to the poor fragmentation that these analytes present by collision-inducted dissociation (CID) in the ion trap. Accordingly, these analytes were removed from the validation process. For the other six target analytes (BPF, BPA, DCPL, TCPL, tOPL, and PCPL) IP values between 3 and 5 were observed. For PCPL, the three IPs corresponded to the three m/z ratios of the chloride isotope ions. A higher number of IPs can be obtained when it is possible to analyze two transitions for each analyte: a transition of identification

Table 2 Retention times, acidic constants and ion suppressions for the EDCs studied obtained by analyzing fortified honey samples

Analyte[a]	BPF	BPA	tBPL	DCPL	tBBA	TCPL	tOPL	nOPL	PCPL
t_r	14.2	14.6	15.7	15.9	16.4	17.6	18.6	20.1	20.4
pK_a	9.7	9.8	10.3	7.7	4.4	6.7	10.2	10.2	4.9
Ion supp. (%)	20	21	24	15	4.6	16	22	22	6.3

[a] *BPF* bisphenol-F, *BPA* bisphenol-A, *tBPL* 4-*tert*-butylphenol, *DCPL* 2,4-dichlorophenol, *tBBA* 4-*tert*-butylbenzoic acid, *TCPL* 2,4,5-trichlorophenol, *tOPL* 4-*tert*-octylphenol, *nOPL* 4-*n*-octylphenol, *PCPL* pentachlorophenol

and the other one for confirmation. To validate the method, the MS/MS detection system was configured in the Product Ion Scan mode. With this configuration, a complete spectrum of product ions was observed, with which it was possible to check the existence of both transitions and the correspondence of the ratios. For each analyte, the analytical signal was obtained by collecting an extracted ion chromatogram (EIC) of the product ion corresponding to the confirmation transition.

Table 3 shows the calibration characteristics for each analyte (intercept, slope, and r^2) corresponding to the confirmation transition and the validation parameters

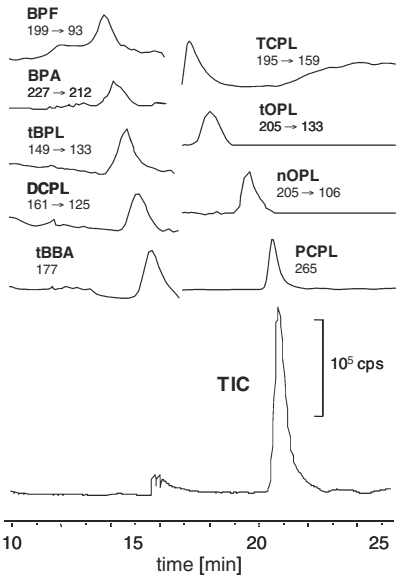

Fig. 4 MS/MS chromatograms and total ion chromatogram (*TIC*) of a fortified honey sample obtained using the developed RAM-LC-MS/MS method. Fortified level, 10 ng g^{-1} for each analyte. *BPF* bisphenol-F, *BPA* bisphenol-A, *tBPL* 4-*tert*-butylphenol, *DCPL* 2,4-dichlorophenol, *tBBA* 4-*tert*-butylbenzoic acid, *TCPL* 2,4,5-trichlorophenol, *tOPL* 4-*tert*-octylphenol, *nOPL* 4-*n*-octylphenol, *PCPL* pentachlorophenol

established by the 2002/657/EC Commission Decision. The limit of decision (CC_α) varied between 1.7 ng g^{-1} for PCPL and 12.6 ng g^{-1} for BPA and the values of the detection capabilities (CC_β) ranged between 2.8 ng g^{-1} for PCPL and 21.6 ng g^{-1} for BPA. The correlations between CC_α and CC_β versus the pK_a had r^2 values of 0.904 and 0.908, respectively.

Since no reference materials are available, recovery studies were carried out in order to check the accuracy of the proposed RAM-LC-MS/MS method. Polyfloral honey samples from different commercial brands that had not been used previously in the method were analyzed, none of the analytes studied being found in them at concentrations higher than the CC_α of the method. These samples were fortified at two levels, 12.5 and 25 ng g^{-1}. The signal obtained for each of the analytes was introduced into the corresponding calibration line. In the case of honey fortified at the lowest level of concentration, the recoveries varied between 88±16% for tOPL and 120±24% for BPA.

Repeatability and within-laboratory reproducibility were assessed as intraday and interday precision, respectively for the two concentration levels; 20 and 100 ng g^{-1}. The highest values of the relative standard deviation (RSD) varied in the 5.5–11% range for interday analysis at a level of 20 ng g^{-1}.

Finally, the specificity and ruggedness of the method were determined by the high level of selectivity characteristic of tandem mass spectrometry and by the different checks and calibrations to which the system had been subjected, respectively. At all times along the work, the pressure of the system was controlled; both that of the isocratic pump connected to the RAM column and that of the binary pump connected to the analytical column. The pressure control of both pumps, together with the background noise and the shape of the peaks obtained, allowed the proper functioning of the system to be checked. Moreover, a standard solution was injected each week with a view to controlling the intensity of the signals obtained in the MS/MS detector.

Along this work, only one RAM column was employed, supporting about 150 injections. This implies a total sample

Table 3 Statistic and performance characteristics, according to 2002/657/EC, of the proposed RAM-LC-MS/MS method obtained by analyzing fortified honey samples

Analyte[a]		BPF	BPA	DCPL	TCPL	tOPL	PCPL
Intercept (area units; au)		$(0.4\pm5.4)\times10^2$	$(-0.5\pm1.8)\times10^2$	$(0.0\pm3.0)\times10^2$	$(0.5\pm1.0)\times10^3$	$(1.7\pm2.2)\times10^3$	$(-0.6\pm6.0)\times10^5$
Slope (au; ng g^{-1})		4.70±0.04	11±0.2	$(2.8\pm0.2)\times10^2$	$(1.3\pm0.1)\times10^2$	$(0.7\pm0.1)\times10^2$	$(2.4\pm0.1)\times10^5$
r^2		0.998	0.997	0.999	0.999	0.998	1.000
CC_α[b] (ng g^{-1})		9.6	12.6	7.6	6.8	10.8	1.7
CC_β[c] (ng g^{-1})		16.6	21.6	12.9	11.5	18.4	2.8
Recovery[d] (%)	12.5 ng g^{-1}	112±16	120±24	104±16	112±16	88±16	96±8
	25.0 ng g^{-1}	100±12	96±8	108±8	104±8	98±8	104±4
RSD[e,f] Intraday (%)	20 ng g^{-1}	6.7	4.9	5.9	7.4	7.1	3.9
	100 ng g^{-1}	2.5	2.1	2.3	3.2	4.5	1.6
RSD[e,g] Interday (%)	20 ng g^{-1}	11	11	7.5	9.5	9.5	5.5
	100 ng g^{-1}	7.6	9.9	4.3	5.8	7.7	3.0

[a] Concentration range from 10 to 150 ng g^{-1} (five calibration points). *BPF* bisphenol-F, *BPA* bisphenol-A, *DCPL* 2,4-dichlorophenol, *TCPL* 2,4,5-trichlorophenol, *tOPL* 4-*tert*-octylphenol, *PCPL* pentachlorophenol

[b] Decision limit (CC_α) calculated from the confirmation transition in product ion scan mode. r^2 (CC_α vs. pK_a): 0.904

[c] Detection capability (CC_β) calculated from the confirmation transition in product ion scan mode. r^2 (CC_β vs. pK_a): 0.908

[d] Confidence intervals at 95% significance level

[e] *RSD* relative standard deviation

[f] Intraday precision (repeatability) was determined by eight injections

[g] Interday precision (within-laboratory reproducibility) was determined in three consecutive days (eight injections each day)

volume of approximately 80 mL (equivalent to 16 g of honey). It is unquestionable that the half-life of RAMs will depend on their use (number of injections). As an indication, replacement of the RAM column should be carried out when the pressure of the system is excessively high (>20%) with respect to the usual values or when the intensity of the signal and the shape of the peaks so indicate.

Conclusions

An automated method for the analysis of endocrine disruptors in honey samples, by using a configuration that incorporates a RAM material, has been developed. The results obtained showed that the use of restricted-access materials in the clean-up of honey samples with different origins is acceptable. The proposed on-line configuration allowed prior sample treatment to be minimized, this being reduced to a dilution and filtration step. Additionally, the RAM used (ADS-C4) proved to have a fairly long half-life (150 injections) and allowed the injection of at least 16 g of honey.

The method described, RAM-LC-MS/MS, is a sensitive, selective, and precise automated tool for the determination and confirmation of several different EDCs in a complex matrix such as honey. The ESI-MS/MS system showed acceptable sensitivity, which is related to the pK_a value of each of the analytes studied. In any case, it's acceptable sensitivity allowed limits of detection at the low nanogram per gram level to be obtained. For six of the nine analytes studied (BPF, BPA, DCPL, TCPL, tOPL, and PCPL) between three and five points of identification were found, such that for these compounds the proposed method has been validated as a quantitative confirmatory method in accordance with the 2002/657/EC Commission Decision.

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References

1. Anklam E (1998) Food Chem 63:549–562
2. Fernández M, Picó Y, Mañes J (2002) J Food Prot 65:1502–1511
3. Tahboub YR, Zaater MF, Barri TA (2006) Anal Chim Acta 558:62–68
4. Pang GF, Cao YZ, Fan CL, Zhang JJ, Li XM, Li ZY, Jia GQ (2003) Anal Bioanal Chem 376:534–541
5. Nozal MJ, Bernal JL, Martín MT, Jiménez JJ, Bernal J, Higes M (2006) J Chromatogr A 1116:102–108
6. Herrero-Hernández E, Carabias-Martínez R, Rodríguez-Gonzalo E (2009) Anal Chim Acta 650:195–201
7. Campillo N, Peñalver R, Aguinaga N, Hernández-Córdoba M (2006) Anal Chim Acta 562:9–15
8. Bogialli S, Di Corchia A (2007) J Biochem Biophys Methods 70:163–179
9. Rissato SR, Galhiane MS, Knoll FRN, Apon BM (2004) J Chromatogr A 1048:153–159
10. Tadeo JL, Sánchez-Brunete C, Albero B, García-Valcárcel AI (2009) J Chromatogr A. doi:10.1016/j.chroma.2009.11.066
11. Korta E, Bakkali A, Berrueta LA, Gallo B, Vicente F (2002) J Food Prot 65:161–166
12. Desilets CP, Rounds MA, Regnier FE (1991) J Chromatogr 544:25–39
13. Souverain S, Rudaz S, Veuthey JL (2004) J Chromatogr B 801:141–156
14. Sadílek P, Satínsky D, Solich P (2007) Trends Anal Chem 26:375–384
15. Lopez de Alda MJ, Díaz-Cruz S, Petrovic M, Barceló D (2003) J Chromatogr A 1000:503–526
16. Chico J, Meca S, Companyó R, Prat MD, Granados M (2008) J Chromatogr A 1181:1–8
17. Li D, Oh JR, Park J (2003) J Chromatogr A 1012:207–214
18. Basheer C, Lee HK (2004) J Chromatogr A 1057:163–169
19. Muncke J (2009) Sci Total Environ 407:4549–4559
20. Kuo HW, Ding WH (2003) J Chromatogr A 1027:67–74
21. Ballesteros-Gómez A, Rubio S, Pérez-Bendito D (2009) J Chromatogr A 1216:449–469
22. Nerín C, Philo MR, Salafranca J, Castle L (2002) J Chromatogr A 963:375–380
23. Shao B, Han H, Li D, Ma Y, Tu X, Wu Y (2007) Food Chem 105:1236–1241
24. Shao B, Han H, Tu X, Huang L (2007) J Chromatogr B 850:412–416
25. Campillo N, Peñalver R, Hernández-Córdoba M (2006) J Chromatogr A 1125:31–37
26. Inoue K, Murayama S, Takeba K, Yoshimura Y, Nakazawa H (2003) J Food Compos Anal 16:497–506
27. Rodríguez-Gonzalo E, García-Gómez D, Carabias-Martínez R (2009) J Chromatogr A 1217:40–48
28. 2002/657/EC: Commission Decision. Official Journal of the European Union L 221, 17.8.2002
29. Kostiainen R, Kauppila TJ (2009) J Chromatogr A 1126:685–699
30. Liu M, Yan W, Lin JM, Hashi Y, Liu LB, Wei Y (2008) J Chromatogr A 1198–9:87–94
31. Feitosa-Felizzola J, Temine B, Chiron S (2007) J Chromatogr A 1164:95–104

RESEARCH ARTICLE II

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Research Article

Determination of endocrine disruptors in honey by CZE-MS using restricted access materials for matrix cleanup

An analytical method based on CZE coupled to ESI-MS is proposed for the identification and simultaneous quantification of several endocrine-disrupting chemicals in honey. The target compounds were the chlorophenols: 2,4-dichlorophenol, 2,4,5-trichlorophenol and pentachlorophenol, and bisphenol-A, 4-*tert*-butylphenol, and 4-*tert*-butylbenzoic acid. A two-step optimization of the ESI-MS detection was carried out. First, the organic solvent present in the sheath liquid was selected and its effect on the analytical signal was studied. The best results in terms of the intensity of the MS signals were obtained with methanol. Thus, an experimental design technique (Doehlert type) was used for the optimization of the other parameters: the NH_3 concentration in the sheath liquid, the flow of the sheath liquid, the nebulizer pressure in ESI, and the drying gas temperature and flow. Here, we developed a new sample treatment based on the combined use of a restricted access material and a polymeric sorbent for SPE. The LOD achieved were in the range of 5–31 ng/g. The intraday precision of the proposed method was determined from replicate analyses ($n = 4$) at a concentration level of 50 ng/g, with RSD values in the range of 15–23%. The results revealed that the proposed method is suitable for the reliable quantification of endocrine-disrupting chemicals in honey at nanograms *per* gram levels.

Keywords:
CE-ESI-MS / Endocrine-disrupting chemicals / Honey / Restricted access material / SPE
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1 Introduction

There is increasing concern about a series of substances used in industrial, agricultural and consumer goods, because some chemicals may cause disruption of the endocrine system and may affect the hormonal control of development in aquatic organisms, wildlife and even humans. These chemicals have often been described as endocrine-disrupting chemicals (EDCs) and include different groups of chemicals such as pesticides, alkylphenols, dioxins, furans, certain polychlorinated biphenyls, synthetic steroids, *etc*. A report from the European Commission [1] has proposed a candidate list of 553 substances for further evaluation of their role in endocrine disruption. The analytes studied in the present work are included among these substances: bisphenol A (BPA), 4-*tert*-butylbenzoic acid (*t*-BBA), 4-*tert*-butylphenol (*t*-BP), and the chlorophenols 2,4-dichlorophenol (DCP), 2,4,5-trichlorophenol (TCP), and pentachlorophenol (PCP).

These compounds are used in industry or are produced as intermediates in certain industrial processes. Alkylphenols are mainly used to make alkylphenol ethoxylate surfactants (detergents) and can themselves be used as plasticisers in plastics. BPA is used in the production of epoxy resins and polycarbonate plastics. These plastics are used in many food and drinks packaging applications, while the resins are commonly used as lacquers to coat metal products such as food cans, bottle tops, and water supply pipes. Chlorophenols are released as byproducts in a number of chemical processes involving chlorine. PCP is still used in some countries as a heavy-duty wood preservative, and 4-*t*-BBA can be used as an intermediate in pharmaceuticals.

Although these compounds have been widely analyzed in water, sludge and sediments, food may be another important route of exposure to the EDCs. Some EDCs could enter food at several stages of food production and *via* the

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Abbreviations: AcOET, ethyl acetate; **BPA,** bisphenol A; **DCP,** dichlorophenol; **EDC,** endocrine-disrupting chemical; **GA,** genetic algorithm; **IS,** internal standard; **PCP,** pentachlorophenol; **RAM,** restricted access material; ***t*-BBA,** *tert*-butylbenzoic acid; ***t*-BP,** *tert*-butylphenol; **TCP,** trichlorophenol

plastic packaging material, stretch films used for food packaging, or many foodstuff containers for oven or microwave cooking. Therefore, it is important to consider food packaging [2] as an important route of exposure of humans to EDCs by leaching from packaging the food. The presence of phenolic EDCs in different foods [3–5] among which is honey [6, 7] has been reported by several authors, showing the potential risk for consumers. Some of the analytes studied here (BPA, PCP – banned according to EC Directive (91/173/EEC) – and *t*-BP) appear in the list compiled by the Scientific Committee for Food of the European Commission concerning monomers and additives used in the manufacture of plastic materials intended to come into contact with foodstuffs. The globally increased concern about these compounds underscores the need to develop highly specific and sensitive analytical methods for their analysis in food samples.

Honey is a natural and wholesome product that is consumed worldwide. The nutritional and quality aspects of honey are important since they determine consumer acceptance. The safety of honey is also critical because it affects human health. According to European Union (EU) regulations, honey as a natural product must be free of chemicals (Directive 74/409/EEC).

In this sense, CE is a good alternative to the more traditional analytical separation techniques such as LC or GC. Moreover, the coupling of CE-MS has some very attractive features, since it combines the highly separative capacity of CE with the good selectivity and high-power identification of MS. Nevertheless, CE-MS currently presents a limited concentration sensitivity that may prevent its use in the analysis of residues of organic contaminants in foods. This is why the development and application of preconcentration techniques before CE-MS is of special importance in this field. The application of capillary electromigration methods for the analysis of foods and food components has been the subject of different reviews [8, 9] as have the main applications of CE-MS for the analysis of organic contaminants in foods [10–13]. Several CE and CE-MS methods for the determination of chlorophenols and other phenolic compounds have also been reported [14–16].

Often, the most critical point in analysis is the need for efficient sample preparation, which may involve analyte isolation, trace enrichment, and further cleanup to remove the matrix interferents. These pretreatment steps often take up most of the total analysis time, contributing strongly to the total cost of analysis and greatly influencing the reliability and accuracy of the analysis. Liquid–liquid extraction, SPE, and solid-phase microextraction are the sample treatment techniques most frequently employed for the extraction of these kinds of EDCs in food. In the particular case of the analysis of honey samples, SPE has been widely used for the extraction and enrichment of different organic contaminants [4, 17–19].

Restricted access materials (RAMs) are porous chromatographic supports specifically designed to allow solid–liquid extraction and the enrichment of small molecules, thereby limiting the access of macromolecules to the interaction sites of a stationary phase bonded on their inner surface. Only small molecules are able to penetrate into the pores and interact with the solid sorbent on the inner surface, while large molecules are eluted with the cleanup solvent. RAMs were initially designed to remove proteins in the analysis of drugs in biological matrices [20–22]. The literature contains reports concerning naphthols [23], bisphenol A, and octylphenol [24]. However, applications to food analysis are still scarce [25, 26].

Here, a procedure based on the combined use of RAMs and polymeric SPE sorbents was developed for the extraction–preconcentration–cleanup of several EDCs in honey. After this sample treatment, the analytes were separated and analyzed by CE-ESI-MS. The optimum conditions for the electrophoretic separation and detection by MS were optimized. The whole method developed, RAM+SPE before CE-ESI-MS, was applied to commercial honey samples. To our knowledge, this work describes for the first time the use of a RAM in sample treatment steps before the analysis of microcontaminants in food by CE-MS.

2 Materials and methods

2.1 Chemicals

The compounds studied were as follows: 4-*t*-BP, CAS RN [98-54-4]; BPA, CAS RN [80-05-7]; 2,4-DCP, CAS RN [120-83-2]; 2,4,5-TCP, CAS RN [95-95-4];PCP and CAS RN [87-86-5] obtained from Dr. Ehrenstorfer (Augsburg, Germany); and 4-*t*-BBA, CAS RN [98-73-7] obtained from Aldrich. Stock solutions of each compound were prepared in ACN at 500 mg/L. Table 1 summarizes some of the physicochemical properties of these compounds.

The organic solvents – ACN, methanol, and ethyl acetate (AcOET) – were of HPLC grade (Merck, Darmstadt, Germany) and were used as received. All the chemicals used for the preparation of the buffer and all other chemical were of analytical reagent grade.

The RAM used was LiChroCART 25-4LiChrospher RP4 ADS (25 μm, 25 × 4 mm) from Merck. The polymeric sorbent used for the SPE step was Oasis HLB 60 mg, a copolymer of poly(divinylbenzene-co-*N*-vinylpyrrolidone) from Waters (Milford, Massachussetts, USA).

2.2 Instrumentation

All the experiments were carried out with a Hewlett-Packard HP^{3D} CE instrument (Agilent Tecnologies, Waldbron, Germany) equipped with a UV-visible DAD device working at 214 nm with a bandwidth of 16 nm. MS was performed using an Agilent LC/MSD SL mass spectrometer equipped with a single quadrupole analyzer. Both, the UV-visible DAD detector and the MS device were controlled by Agilent HP ChemStation software, version B.02.01 SR1.

Table 1. Physichochemical properties of the target compounds[a)]

Compound	Log k_{ow}	pK_a	Solubility[b)] (mg/L)	Vapor pressure[b)] (mmHg)	Ions monitorized (*m/z*)
4-*t*-BP	3.31	10.3	580	3.8×10^{-2}	149
4-*t*-BBA	3.85	4.4	28	6.4×10^{-4}	177
BPA	3.32	9.8	120	3.9×10^{-7}	227
PCP	5.12	4.9	14	1.1×10^{-4}	265, 267, 263
2,4,5-TCP	3.72	6.7	1200	7.5×10^{-3}	195, 197, 199
2,4-DCP	3.06	7.7	4500	9.0×10^{-2}	161

a) Data from Interactive PhysProp Database Demo, Syracuse Research Corporation, USA (http://esc.syrres.com/interkow/physdemo.htm).
b) Water, 25°C.

Fused silica capillaries (75 μm id) were from Polymicro Technologies (Phoenix, AZ, USA), supplied by Composite Metal Services (West Yorkshire, UK), and were used throughout the work.

Sample treatments using the RAM were performed on an HP 1100 Series instrument from Agilent equipped with two high pressure pumps, two six-port valves, and a 4-mL homemade injection loop. The system was controlled with an HP ChemStation.

For SPE, Oasis HLB sorbents were used. The conditioning of these sorbents and sample passage were accomplished using a syringe. The cartridge was dried in a vacuum pump (Afa, Barcelona, Spain) coupled to a 20-place manifold for sample preparation (Varian, Harbor City, USA)

2.3 Sample preparation

In this work, samples of polyfloral honey available commercially in the retail market were used. All the honey samples were first analyzed with the proposed method to check the natural occurrence of the target EDCs. No signals corresponding to the target analytes were found, and hence spiked honey samples were used. An amount of 2.5 g of honey sample was weighed in a small beaker and spiked with all six compounds. The sample was kept covered for approximately 12 h before the extraction to permit the interaction between the analytes and the matrix compounds. Quantitative results were obtained by using matrix-matched standards (blank honey samples spiked with standard analyte solutions). *t*-BBA was used as internal standard (IS). Calibration curves were established by considering the relative peak areas (as the ratio of analyte peak to IS peak). All the honey samples were diluted with UHQ water at a ratio 1:5 w/v.

2.3.1 Treatment of honey samples

The experimental setup for RAM sample treatment is shown in Fig. 1. The solution impelled by pump A consisted of a 2.5 mM ammonium formate (pH 2.9) solution with 10% ACN. This solution was used to transfer the sample bolus to the RAM column and in turn to clean the RAM. Pump B impelled the elution solvent (methanol) to elute the analyte retained on the RAM.

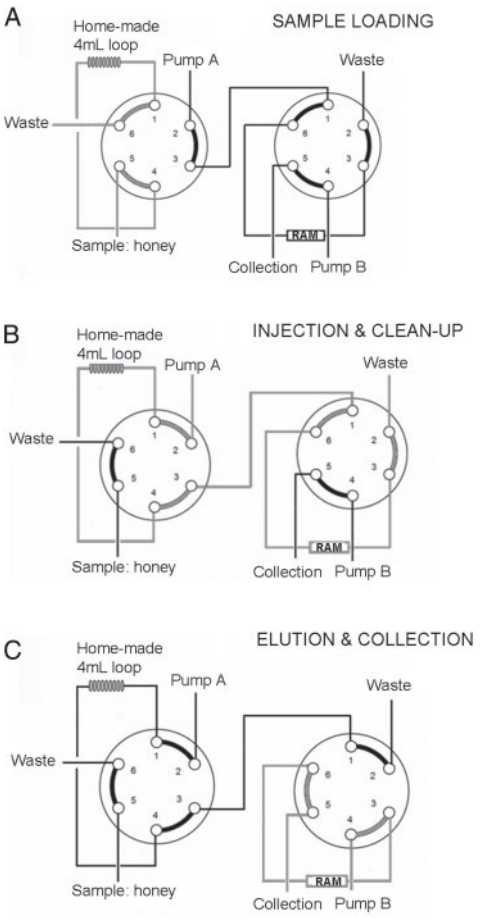

Figure 1. Experimental setup for RAM sample treatment.

First, a predetermined volume of honey was loaded manually into the loop with a syringe, with the system in the ''sample loading'' position. Immediately thereafter, the system was changed to the ''injection and cleanup position'' (Fig. 1B) and pump A was started to pump the cleanup solution at 1 mL/min for 10 min. While the matrix components of the honey were washed to waste, the target compounds studied were withheld in the RAM. At 10 min, the system setup was changed to the ''elution and collection'' position (Fig. 1C) and methanol, from pump B, was used to elute the analytes at a flow rate of 1 mL/min in backflush mode to the collection exit, where they were collected manually into a vial. Controlling the elution time, 167 μL were collected. After the collection had been completed, the system was kept for 3 min in the ''elution and collection position'' to wash the RAM completely. Then, the system was changed to the initial positions. Three minutes were allowed to elapse in order for the system to become reequilibrated. The syringe and the loop were cleaned manually with methanol before each analysis. In the proposed method (RAM+SPE before CE-MS), initially 4.0 mL of honey was injected and the fraction eluted was collected, this process being repeated four times. Thus, the total amount of honey preconcentrated was 16 mL.

The sum of the fractions eluted from the RAM (4×167 μL) was later preconcentrated by SPE, using an Oasis HLB sorbent. Dilution of methanolic RAM extracts with UHQ water to a methanol content of about 20% was performed to prevent elution of the compounds from the SPE sorbent during the sample-loading step. Since preconcentration must be carried out under acidic conditions to avoid ionization of the compounds in the sample, 10 mM of ammonium formate was also added.

The SPE method, using Oasis HLB sorbents, has been optimized elsewhere [27] and involves the conditioning of the cartridges with 5 mL of AcOET, then 5 mL of ACN and, finally, 5 mL of UHQ water. Sample passage (~3.5 mL) was carried out by gravity. Once the retention step had been completed, the cartridges were dried for 15 min under a vacuum of 15 mmHg (1 mmHg = 133.322 Pa). The compounds retained were eluted with 0.5 mL of ACN and 3 mL of AcOET. The organic phase obtained was carefully evaporated to dryness under a gentle stream of nitrogen at 30–35°C, and the residue was reconstituted with the appropriate injection medium (60/40 v/v methanol/water).

2.4 CE separation conditions

Optimization of the electrophoretic separation was carried out with a 57-cm total-length capillary, with 50 cm to the UV detector. The rest of the experiments were carried out with an 87.5-cm total-length capillary, with 21 cm to the UV detector.

Injections were made at the anodic end, using a pressure of 50 mbar for 17 s. Electrophoretic separation was achieved with a voltage of 22 kV (normal mode), with an initial ramp of 7 s. The running buffer was an aqueous solution of 15 mM ammonium acetate adjusted to pH 11.0 with a concentrated aqueous solution of ammonium hydroxide. The temperature of the capillary was kept constant at 25°C. Before the first use, the capillary was conditioned by flushing with the BGE for 10 min at 4 bar. This was also implemented as a daily startup procedure. After each run, the capillary was prewashed at 4 bar for 1 min with fresh running buffer.

2.5 ESI interface

An Agilent coaxial sheath liquid sprayer was used for CZE-MS coupling. The fused silica capillary was mounted in such a way that the tip just protruded from the surrounding steel needle; ~1/2 of the capillary od. The sheath liquid consisted of methanol containing 3% v/v of aqueous ammonium hydroxide concentrated solution and was delivered at a flow rate of 0.78 mL/min by a Agilent 1100 series pump, equipped with a 1:100 flow splitter. The ESI voltage was set at –4000 V. Other electrospray parameters at optimum conditions were: nebulizer pressure, 2 psi; drying gas flow rate, 2 L/min; drying gas temperature, 100°C.

2.6 MS conditions

The mass spectrometer was operated in the negative ion mode. Analyte quantification was carried out under SIM acquisition mode using deprotonated molecules. The ions monitored simultaneously were as follows: 149 *m/z* for 4-*t*-BP; 177 *m/z* for 4-*t*-BBA; 227 *m/z* for BPA; 265, 267, and 263 *m/z* for PCP; 195, 197, and 199 *m/z* for 2,4,5-TCP; and 161 for 2,4-DCP (Table 1). The number of identification points was 1 for all the analytes, except for PCP and 2,4,5-TCP for which it was 3. The optimized fragmentor voltage was 60 V for all the analytes.

3 Results and discussion

3.1 Electrophoretic separation

Before the application of MS detection, previous studies were carried out to optimize the electrophoretic separation of the selected compounds using a UV-Vis DAD device. Thus, the pH and ionic strength of the BGE, the separation voltage, and the temperature of the capillary during separation were evaluated. A volatile buffer of low conductivity (*i.e.*, an electric current below 50 μA) must be used to consider the compatibility of the CE method with MS. An ammonium acetate buffer was employed. The optimized buffer concentration was 15 mM, since this was found to provide a good compromise among peak shape, electrical current intensity, and analysis time.

The pH of the CE separation buffer must be set at a high value to achieve good separation and to improve the presence of deprotonated molecules in the electrospray device (Table 1). The optimized value was 11.0 (range studied 9.7–11.6). The separation voltage and the temperature of the capillary were also optimized, and values of 22 kV and 25°C were selected. Methanol was employed as an additive in the BGE; the range studied was 0–30% v/v and the optimum value proved to be 0%.

3.2 CE-ESI-MS optimization

Starting from the optimum values for separation, the optimization of the parameters affecting the CE-ESI-MS method was performed. Initially, different sheath liquids were tested, all of them containing 1% v/v of aqueous ammonium hydroxide concentrated solution to improve the presence of the deprotonated molecules in the electrospray. Water, methanol, isopropanol, and ACN, as well as all their binary mixtures (ratio 50:50 v/v) were tested. Each analyte dissolved in the CE buffer was introduced by direct infusion through the capillary into the spray chamber and the scan (100–400 *m/z*) was monitored over 5 min. The ESI and MS parameters were set at typical values. As shown in Fig. 2, the best results in terms of intensity of the MS signal for the endocrine disruptors studied were obtained using methanol containing 1% v/v of aqueous ammonium hydroxide concentrated solution. The main ion obtained was the deprotonated molecular ion, except for ACN as sheath liquid, where different adducts with BGE anions were obtained for 2,4-DCP, BPA, and 4-*t*-BP. In addition, for 4-*t*-BP another main adduct, $[2M\text{-}H]^-$, was obtained for the isopropanol/methanol, isopropanol/ACN, and methanol/ACN mixtures.

Using methanol as sheath liquid, all the parameters related to electrospray formation were studied to obtain the highest abundance signal for the molecular ions for all the analytes. The factors studied and their corresponding ranges were as follows: percentage of the aqueous ammonium hydroxide concentrated solution in the sheath liquid, 0–3% v/v; nebulizer pressure, 2–20 psi; sheath liquid flow, 0.05–2 mL/min (before 1:100 split); drying gas flow, 2–20 L/min; and drying gas temperature 100–200°C. The intervals studied for these parameters were imposed by different constraints such as initial proofs, the stability of the spray, and the instrumental and capillary limitations. The use of the experimental design techniques for the

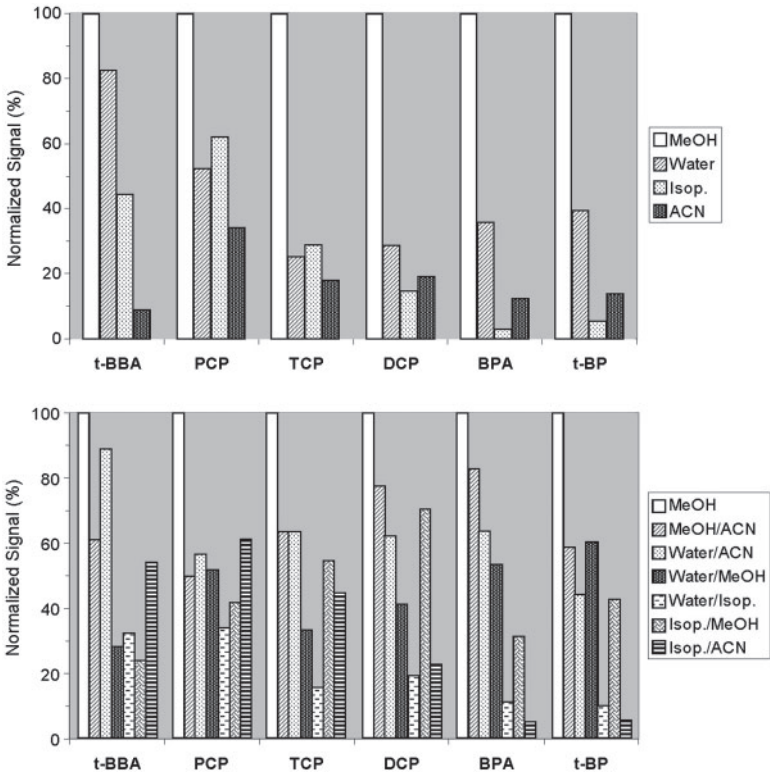

Figure 2. Number of counts for each analyte normalized to the largest value (%) employing different sheath liquids containing 1% v/v aqueous ammonium hydroxide concentrated solution.

optimization of ESI parameters is suitable [28–30] for reducing the redundancies in the experiments and searching for optimal conditions simultaneously instead of using a step-by-step method. In our case, the optimization of these five factors was carried out by means of a Doehlert design [31], plus three central points to evaluate the lack of fit. The levels for the factors were 5, 7, 7, 7, and 3, respectively. This kind of design is not much used in analytical chemistry in spite of its advantages, such as its high efficiency in comparison with other designs such as the Box–Behnken or central composite designs, and because of the possibility of studying a higher number of levels for the most significant variables, taking into account the observed effects from screening designs or initial experiments.

The analytical response to be optimized was the geometric mean for peak heights working in SIM mode. The analyses were performed electrophoretically under the optimized CE separation conditions, each time injecting an aqueous sample containing all the analytes at the 10 mg/L concentration level. The MS capillary voltage was set to its maximum allowed value (−4000 V).

The experimental analytical responses obtained were fitted to a model containing the linear terms and the double interactions among the five factors without containing the quadratic interactions. The model-fitting characteristics, coefficients and their variance inflation factors are shown in Table 2. The experimental response values were significantly fitted to the model (<5%). Also, the experimental responses did not show significantly lack of fit to the model (>5%).

The search for the maximum value of the analytical response was carried out using a genetic algorithm (GA). GAs differ substantially from more traditional search and optimization methods since they use probabilistic, and not deterministic, transition rules, and they search for a population of points in parallel and not merely a single point; hence, GAs are potentially useful for identifying all alternative or similar solutions simultaneously.

The factor values giving the maximum of the analytical responses were located at: percentage of aqueous ammonium hydroxide concentrated solution in the sheath liquid, 3% v/v; nebulizer pressure, 2 psi; sheath liquid flow, 0.78 mL/min; drying gas flow, 2 L/min; and drying gas temperature 100°C.

Under the optimized ESI conditions, the MS fragmentation curves were studied for all the analytes. Typical fragmentation curves were obtained and hence a voltage of 60 V was selected for all the analytes. The amount of sample injected in the CE capillary was also studied, obtaining the maximum of the SIM peak height for all the analytes at 17 s when the hydrodynamic injection pressure was set at 50 mbar.

3.3 Characteristics of the method

The whole optimized CE-ESI-MS method was initially characterized for standard aqueous solutions of the analytes with a residual content of methanol lower than 14% v/v. Linear calibration curves were obtained for all compounds across the range tested (0.2–42 μg/mL, depending on the analyte), employing 4-*t*-BBA as IS. Calibration curves were prepared at six levels and each calibration level was replicated four times. The LOD (S/N of 3) ranged from 0.04, for PCP, to 0.80 μg/mL for 4-*t*-BP. The RSD values

Table 2. Coefficients, statistical parameters, and model fitting[a)] for ESI optimization

	Related term[b)]	Coefficient × 10^3	VI F[c)]	Confidence interval[d)]	Significance
b_0	–	3.7711	1.1316	3.4 to 4.1	Yes
b_1	C_{NH3}	−0.0458	1.0925	−0.7 to 0.6	No
b_2	**P_{Neb}**	−2.1256	1.1987	−2.9 to −1.4	**Yes**
b_3	**Q_{Sheat}**	1.1180	1.1179	0.3 to 1.9	**Yes**
b_4	Q_{N2}	−0.1148	1.1219	−0.9 to 0.7	No
b_5	T_{N2}	0.5697	1.0314	−0.1 to 1.3	No
b_{12}	$C_{NH3} \cdot P_{Neb}$	0.3991	1.7302	−1.9 to 2.7	No
b_{13}	$C_{NH3} \cdot Q_{Sheat}$	−1.3194	1.4843	−4.0 to 1.4	No
b_{14}	$C_{NH3} \cdot Q_{N2}$	0.7053	1.3809	−2.1 to 3.5	No
b_{15}	$C_{NH3} \cdot T_{N2}$	−1.9089	1.3128	−4.4 to 0.6	No
b_{23}	**$P_{Neb} \cdot Q_{Sheat}$**	2.4621	1.2678	0.2 to 4.7	**Yes**
b_{24}	**$P_{Neb} \cdot Q_{N2}$**	3.9699	1.2440	1.6 to 6.4	**Yes**
b_{25}	$P_{Neb} \cdot T_{N2}$	0.8471	1.3623	−1.1 to 2.8	No
b_{34}	$Q_{Sheat} \cdot Q_{N2}$	1.9982	1.0982	−0.2 to 4.2	No
b_{35}	$Q_{Sheat} \cdot T_{N2}$	0.0998	1.0569	−2.1 to 2.3	No
b_{45}	**$Q_{N2} \cdot T_{N2}$**	2.6836	1.0079	0.5 to 4.9	**Yes**

a) Model significance lower than 5% (0.03%); lack of fit significance higher than 5% (6.0%); correlation coefficient 0.899; $S_{y/x}$ 0.78 × 10^3.
b) C_{NH3}, concentration (% volume) of aqueous ammonium hydroxide concentrated solution in the sheath liquid; P_{Neb}, nebulizer pressure (psi); Q_{Sheat}, sheat liquid flow (mL/min); Q_{N2}, drying nitrogen flow (L/min); T_{N2}, drying nitrogen temperature (°C).
c) Variance inflation factors (<4 acceptable values, 1 optimal value).
d) Confidence intervals for each coefficient at 95% significance level.

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(intraday, $n = 7$) for the analytical signal ranged from 11.0, for TCP, to 16.7%, for 4-*t*-BP at a concentration level of 0.4–1.6 mg/L, depending on the compound.

3.4 Sample treatment

The analysis of complex matrices using MS requires previous sample treatment since quantitative determinations may be affected by the ion suppression that occurs in electrospray, mainly due the coelution of matrix interferents with the analytes. It is well known that the matrix effects due to coextracted and coeluting matrix substances can seriously affect analyte signals.

To apply the optimized CE-ESI-MS method to the analysis of EDCs in honey, it was necessary to develop a suitable sample treatment. Initially, a commercial Oasis HLB polymeric cartridge was used. The SPE procedure for Oasis HLB is described in Section 2.3. With these materials, it was not possible to pass sample volumes (honey dissolved at a proportion of 1:5 w/v) above 5 mL through the cartridge, since the sorbent became blocked. For this 5-mL sample volume, working with honey samples spiked at 250 ng/g, only PCP was detected.

3.4.1 Use of the RAM before CE-MS

The use of RAMs is very effective in the analysis of small molecules in biological fluids. They act by limiting the access of macromolecules to the sorbent by means of a porous membrane, thereby achieving the cleanup of the biological matrix. Here, we checked the possibility of using a RAM column as an efficient step for the cleaning of honey samples, which have a high content of sugars. Accordingly, a study was made of the variables involved in the process.

Initially, 500 μL of sample were injected using the configuration shown in Fig. 1A. The solvent that transfers the sample bolus to the RAM material (Fig. 1B) also acted as a washing solvent. The composition of this solvent was optimized. By means of pump A different solutions containing 2.5 mM ammonium formate (pH 2.9) with different percentages of ACN were passed through the system. The results showed that the use of aqueous solutions afforded not very clean extracts; however, upon increasing the percentage of ACN above 25% analyte elution occurred. The use of solutions containing 2.5 mM ammonium formate (pH 2.9) with 10% ACN for 10 min eliminated the matrix interferents and did not elute the analytes retained in the RAM.

The initial studies were carried out eluting the RAM with methanol and collecting 1 mL of the eluted fraction (Fig. 1C, pump B). To increase the sensitivity of the method, the methanolic solution was brought to dryness under a gentle stream of N_2 and the dry residue was reconstituted with a solution containing 60/40 v/v of methanol/water. These experiments indicated that the evaporation of methanolic solutions involves the loss of DCP, TCP, and *t*-BP, which are the most volatile analytes (Table 1). In light of this, and with a view to eliminating the evaporation step, we determined the fraction of eluate that contained the highest percentage of analyte. Figure 3 shows the variations in the signals as a function of the fraction eluted and analyzed. It may be seen that upon using methanol all the analytes eluted in the same fraction. The fraction richest in analytes was the second one, whereas in the third and fourth fractions the amount of analyte eluted was lower than 15%. In later studies, the second fraction, a volume of 167 μL, was collected. This fraction was diluted with UHQ water until a solution with 60/40 v/v of methanol/water was obtained.

With a view to enhancing sensitivity, the possibility of injecting volumes of up to 6000 μL was assayed. For the injected volumes greater than 4000 μL, a decrease in the signal was obtained (Fig. 4). This kind of behavior reveals that the injection of large volumes of sample into the RAM column decreases the cleaning efficiency and hence greater ion suppression is obtained.

The analytical characteristics of the method (RAM before CE-MS) were determined by injecting a volume of 4000 μL of honey into the RAM. The estimated LOD were in the range 20–120 ng/g.

Since EDCs are still being evaluated [1], no legislation limits for these compounds in foodstuffs are available. Thus,

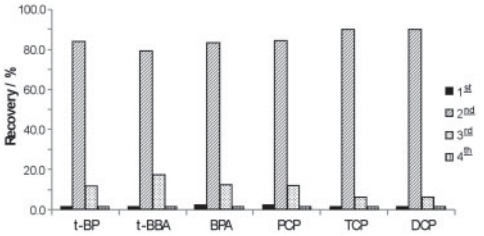

Figure 3. Elution profiles for each compound from the RAM system using methanol as eluent. Volume of each fraction is 167 μL.

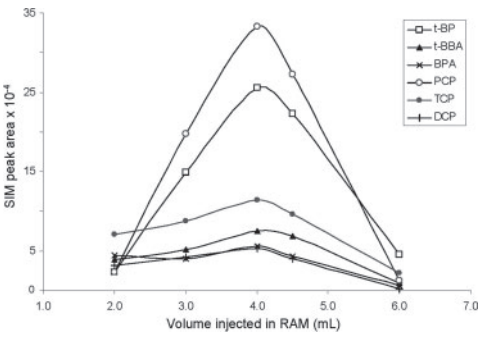

Figure 4. Effect on the analytical signal of the RAM injected volume of 1:5 w/v diluted honey sample.

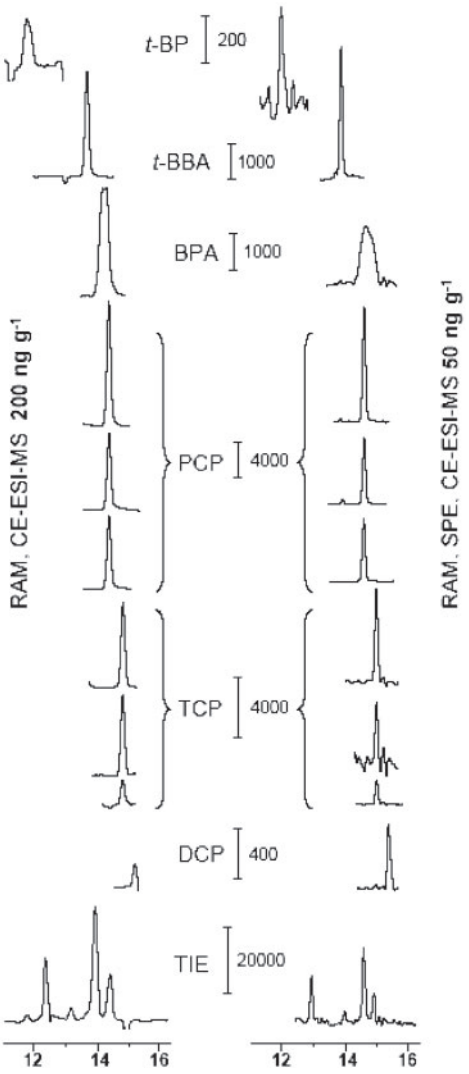

Figure 5. Electropherograms for two different honey samples spiked with the EDCs and analyzed by RAM and RAM+SPE before CE-ESI-MS. The concentration levels of each analyte in the honey samples are 200 and 50 ng/g, respectively. CE conditions: BGE, 15 mM ammonium acetate buffer (pH 11.0) with ammonium hydroxide; uncoated fused silica capillary, 75 μm id × 87.5 cm; injection by pressure at 50 mbar for 17 s; applied voltage, 22 kV; temperature 25°C. ESI conditions: negative ion mode; spray voltage −4.0 kV; sheath liquid, methanol with 3% v/v of aqueous ammonium hydroxide concentrated solution at 0.78 mL/min (1:100 flow splitter); drying gas flow, 2 L/min; drying gas temperature, 100°C; nebulizer pressure, 2 psi. MS conditions: SIM acquisition mode of deprotonated molecules, PCP, and TCP with three isotopic identification points; fragmentation amplitude, 60 V. TIE, total ion electropherogram.

the development of sensitive methods to identify and assess EDCs is a basic requirement for comprehensive legislative action aimed at protecting people and the environment from the potential dangers posed by these chemicals. In view of the lack of legislation, it has been considered that the method should have LOD of 10 ng/g (values proposed by default in the legislation as maximum residue limits for food contaminants).

3.4.2 Use of a RAM and SPE step before CE-MS

To obtain a more sensitive method for the quantification of the target compounds in honey, a combination of the cleanup performed with the RAM followed by another new step with a polymeric SPE sorbent was studied. The experimental procedure is described in Section 2.3. In Fig. 5, the electropherograms for two differently spiked honey samples analyzed by RAM and by RAM+SPE before CE-MS are depicted. The combined RAM+SPE procedure appeared to be appropriate for the cleanup and enrichment of target compounds from honey.

Figure 6 shows the normalized signals for a honey sample spiked at 400 ng/g and subsequently treated following the RAM+SPE before CE-ESI-MS procedure and for a honey blank treated using the same procedure and spiked shortly before injection. From a comparison of both signals, it may be observed that the recovery values of the proposed method lie between 71 and 113%. The same figure shows the signal generated by a standard solution of the same injected concentration as the previous ones. The difference between the signal of the standard and that of the spiked honey samples reflects an important degree of ion suppression. Accordingly, quantification should be carried out using the matrix-matched method.

3.5 Performance of the analytical method for the determination EDCs in honey

The optimized method, based on RAM+SPE before CE-MS, was validated with respect to linearity, precision, recoveries, and detection limits. Calibration was accomplished in the spiked honey samples using *t*-BBA as IS; the IS was added before starting sample treatment (Section 2.3). Calibration curves were prepared at seven levels and each calibration level was injected in quadruplicate. The range of concentrations and LODs ($S/N = 3$) are summarized in Table 3.

The repeatability (intraday precision) of the analytical signal was determined. To do so, four honey samples spiked at two different concentration levels were used, 50 and 200 ng/g (Table 3) were used. Intraday RSD values ranged between 8 and 23%. Interday precision (within laboratory reproducibility) was determined in three consecutive days (four injections each day). The RSD values did not differ significantly from these percentages, ranging between 20

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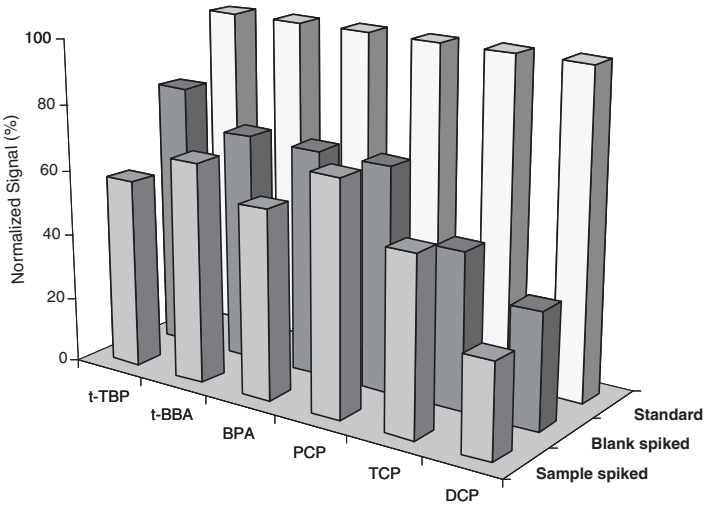

Figure 6. Effect on the analytical signal normalized to the highest value (%) for a standard; a honey blank (blank spiked) treated following the RAM+SPE before CE-ESI-MS procedure and spiked shortly before injection; and a honey sample spiked and subsequently treated following the same procedure. Concentration level in honey, 400 ng/g. Conditions as in Fig. 5.

Table 3. Analytical characteristics of the RAM, SPE, CE-ESI-MS methods applied to the analysis of EDCs in spiked honey samples

	t-BP	*t*-BPA	PCP	TCP	DCP
Intercept[a)]	−2.5 (2)	11.3 (13)	9.9 (25)	18.1 (14)	0.2 (2)
Slope[a)]	0.225 (0.005)	1.363 (0.025)	1.919 (0.044)	1.040 (0.031)	0.167 (0.003)
R^2	0.991	0.993	0.992	0.989	0.996
LOD (ng/g)[b)]	31	7.5	5.3	9.5	23
Range studied (ng/g)	50–1000	50–1000	50–1000	50–1000	50–1000
Recoveries (%)					
Per concentration level 100 ng/g	114 (13)	103 (14)	96 (20)	110 (23)	96 (17)
Per concentration level 200 ng/g	94 (7)	96 (7)	86 (10)	96 (11)	108 (9)
RSD (%) signal[c)]					
Per concentration level 50 ng/g	22.0	22.5	14.9	13.4	23.0
Per concentration level 200 ng/g	15.0	21.2	8.3	15.1	21.0

a) Normalized peak areas in SIM mode against IS (*t*-BBA). Standard deviations in brackets.
b) LOD ($S/N = 3$). Simultaneous detection in SIM mode.
c) Intraday ($n = 4$).

and 24%. This result is expected due to the normalization brought about by the use of *t*-BBA as IS.

In view of the lack of certified samples of EDCs in honey, the model was validated by determining the recovery values at two concentration levels in honey samples from a different commercial brand. To accomplish this, we analyzed honey samples that had not been used previously in the development of the RAM+SPE before CE-MS method; no EDCs were found at concentrations above the LOD of the method in them. These samples were spiked at two levels: 100 and 200 ng/g. The signal obtained for each of the analytes was introduced into the corresponding calibration line. The recovery values obtained and their precision are shown in Table 3. In all cases, satisfactory recovery values were obtained.

4 Concluding remarks

In this paper, a CE-ESI-MS method has been developed and validated for the quantification of EDCs in honey.

The use of an experimental design method for optimizing the variables affecting the ESI allows a reduction in the experimentation necessary to achieve maximum efficiency in the ionization of these compounds by means of the negative ionization in mode ESI.

A sample pretreatment procedure is proposed based on the combined use of a RAM and a SPE step with a polymeric sorbent. The use of the RAM column before the SPE sorbent allows volumes up to 16 mL to be preconcentrated, while in the absence of the RAM the SPE cartridge is blocked at sample volumes greater than 5 mL. This shows that the use of a RAM

before SPE efficiently removes sugars from the honey samples, which enables the preconcentration of a greater volume in the SPE sorbent. Also, the use of the RAM exerts a positive effect on the decrease in the ion suppression in the ESI-MS detector.

The procedure is simple and effective for the cleaning–extraction–preconcentration of the target compounds, including volatile compounds from honey samples, allowing detection limits in the range 5–31 ng/g.

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The authors have declared no conflict of interest.

5 References

[1] Communication from the Commission to the Council and the European Parliament. *Implementation of the Community Strategy for Endocrine Disrupters: a range of substances suspected of interfering with the hormone systems of humans and wildlife*. COM (1999) 706. Brussels, 14-06-2001, COM (2001) 262.

[2] Muncke, J., *Sci. Total Environ.* 2009, *407*, 4549–4559.

[3] Kuo, H.-W., Ding, W.-H., *J. Chromatogr. A* 2003, *1027*, 67–74.

[4] Ballesteros-Gómez, A., Rubio, S., Pérez-Bendito, D., *J. Chromatogr. A* 2009, *1216*, 449–469.

[5] Nerín, C., Philo, M. R., Salafranca, J., Castle, L., *J. Chromatogr. A* 2002, *963*, 375–380.

[6] Campillo, N., Peñalver, R., Hernández-Córdoba, M., *J. Chromatogr. A* 2006, *1125*, 31–37.

[7] Inoue, K., Murayama, S., Takeba, K., Yoshimura, Y., Nakazawa, H., *J. Food Comp. Anal.* 2003, *16*, 497–506.

[8] García-Cañas, V., Cifuentes, A., *Electrophoresis* 2008, *29*, 2309–2904.

[9] Óbice, M. C., *Electrophoresis* 2007, *28*, 4046–4062.

[10] Herrero, M., García-Cañas, V., Simó, C., Cifuentes, A., *Electrophoresis* 2010, *31*, 205–228.

[11] Font, G., Ruiz, M. J., Fernández, M., Picó, Y., *Electrophoresis* 2008, *29*, 2059–2078.

[12] Mũller, L., Barták, P., Bednář, P., Fryšová, I., Ševčík, J., Lemr, K., *Electrophoresis* 2008, *29*, 2088–2093.

[13] Hernández-Borges, J., Borges-Miquel, T. M., Rodríguez-Delgado, M. A., Cifuentes, A., *J. Chromatogr. A* 2007, *1153*, 214–226.

[14] Arráez-Román, D., Gómez-Caravaca, A. M., Gómez-Romero, M., Segura-Carretero, A., Fernández-Gutiérrez, A., *J. Pharm. Biomed. Anal.* 2006, *41*, 1648–1656.

[15] Jáuregui, O., Moyano, E., Galceran, M. T., *J. Chromatogr. A* 2000, *896*, 125–133.

[16] Jáuregui, O., Puignou, L., Galceran, M. T., *Electrophoresis* 2000, *21*, 611–618.

[17] Martel, A. C., Zeggane, S., *J. Chromatogr. A* 2002, *954*, 173–180.

[18] Rissato, S. R., Galhiane, M. S., Almeida, M. V., Gerenutti, M., Apon, B. M., *Food Chem.* 2007, *101*, 1719–1726.

[19] Michalkiewicz, A., Biesaga, M., Pyrzynska, K., *J. Chromatogr. A* 2008, 18–26.

[20] Sadílek, P., Satínský, D., Solich, P., *Trends in Anal. Chem.* 2007, *26*, 375–384.

[21] Schafer, C., Lubda, D., *J. Chromatogr. A* 2001, *909*, 73–78.

[22] Souverain, S., Rudaz, S., Veuthey, J. L., *J. Chromatogr. B* 2004, *801*, 141–156.

[23] Preuss, R., Angerer, J., *J. Chromatogr. B* 2004, *801*, 307–316.

[24] Liu, M., Hashi, Y., Pan, F., Yao, J., Song, G., Lin, J. M., *J. Chromatogr. A* 2006, *1133*, 142–148.

[25] Chico, J., Meca, S., Compayó, R., Prat, M. D., Granados, M., *J. Chromatogr. A* 2008, *1181*, 1–8.

[26] Marazuela, M. D., Bogialli, S., *Anal. Chim. Acta* 2009, *645*, 5–17.

[27] Carabias-Martínez, R., Rodríguez-Gonzalo, E., Revilla-Ruiz, P., *J. Chromatogr. A* 2004, *1056*, 131–138.

[28] Lara, F. J., García-Campaña, A. M., Alés-Barrero, F., Bosque-Sendra, J. M., García-Ayuso, L. E., *Anal. Chem.* 2006, *78*, 7665–7673.

[29] Hernández-Borges, J., Rodríguez-Delgado, M. A., García-Montelongo, F. J., Cifuentes, A., *Electrophoresis* 2004, *25*, 2065–2076.

[30] Hernández-Borges, J., Rodríguez-Delgado, M. A., García-Montelongo, F. J., Cifuentes, A., *J. Sep. Sci.* 2005, *28*, 948–956.

[31] Doehlert, D. H., *Appl. Stat.* 1970, *19*, 231–239.

2.B. DETERMINATION OF ANTHELMINTIC BENZIMIDAZOLES IN MILK

RESEARCH ARTICLE III

Analytical and Bioanalytical Chemistry 404 (2012) 2909

Anal Bioanal Chem (2012) 404:2909–2914
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TECHNICAL NOTE

A fast and reliable method for the quantitative determination of benzimidazoles and metabolites in milk by LC-MS/MS with on-line sample treatment

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Abstract This work reports the development of a simple, reliable and automated method based on LC-MS/MS for the quantitative determination of benzimidazole residues and some of their metabolites in milk. The method involves the use of an extraction cartridge coupled on-line to the chromatographic system for the clean-up of the milk samples, efficiently eliminating matrix macromolecules and providing appropriate selectivity for the determination of such compounds. In the online method developed here, only a reduced manual sample manipulation was required (protein precipitation and filtration) prior to injection into the chromatographic system. The limits of detection of the target anthelmintics ranged from 0.1 to 0.8 ngmL^{-1} in milk samples, these values being below the maximum residue limit established for these compounds. The whole method developed was validated in real samples according to the requirements set by the Commission Decision 2002/657/EC. The optimized method was successfully applied to commercial and raw milk samples of different origin demonstrating that the proposed method may find application in routine laboratory analyses of food control safety.

Keywords Benzimidazole drugs · Milk · Restricted access material · On-line sample treatment · Liquid chromatography–tandem mass spectrometry · Validation according to Commission Decision 2002/657/EC

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Introduction

Benzimidazole derivatives are drugs used in veterinary practice as broad-use anthelmintic agents for the treatment of parasite infestations in animals destined for human consumption [1]. The extensive use of these anthelmintic agents increases the likelihood of the appearance of residues in foods of animal origin [2]. Thus, the control of these residues from veterinary drugs in foods of animal origin is especially important. Some other benzimidazoles used as fungicides, like carbendazim, may become accessible to organisms feeding on leaf litter too, because they are stable for several weeks on plant material.

The determination of benzimidazole residues in milk continues to be problematic because despite the similarities in their chemical structure and mode of action, their chemical properties, such as lipophilicity and acid–base behavior, are very different [3]. In addition, maximum residue limits of pharmacologically active substances in milk are usually lower than in other foodstuffs of animal origin.

The literature describes several methods for the determination of benzimidazoles in milk [4].

In recent years, detection by mass spectrometry (MS) has been the main method used for both benzimidazole residues and for other veterinary drugs in biological matrices. LC-MS [5] or LC-MS/MS coupling [2, 6] allow the development of highly sensitive and selective methods.

Most of the above-mentioned methods require relatively expensive instruments and/or need time-consuming sample treatment procedures prior to analysis. Liquid–liquid extraction [5], solid-phase extraction [6] and QuEChERS [2] have been described for the extraction of benzimidazoles from milk.

In the present work, we focus on the development of an automated analytical method for the sample treatment, separation, identification, and quantification at trace level of a broad set of benzimidazole drugs in milk samples with minimum sample pretreatment that could be applied in routine laboratory work in order to guarantee food safety and quality.

To accomplish this, we developed a procedure for on-line sample treatment based on the use of Restricted Access Materials [7] that allows the low food residue levels currently established in the legislation to be achieved. To our knowledge, this is the first time that this configuration has been proposed to determine benzimidazole drugs in milk. As a final goal, we validated the method developed as a quantitative confirmatory method, according to the criteria set forth by European legislation (Commission Decision 2002/657/EC) for toxic residues in foods [8].

Experimental

Chemicals

Analytical standards of *Albendazole* (ABZ), *Albendazole-sulfoxide* (ABZ-SO), *Albendazole-sulfone* (ABZ-SO_2), *Albendazole-2-aminosulfone* (ABZ-NH_2-SO_2), and *Carbendazim* (MBC) were purchased from Dr. Ehrenstorfer (Augsburg, Germany).

Fenbendazole (FBZ), *Oxfendazole* (OFZ), *Fenbendazole-sulfone* (FBZ-SO_2), *Oxibendazole* (OXI), *Flubendazole* (FLU), *Flubendazole-2-amine* (FLU-HMET), and *Febantel* (FBT) were obtained from Sigma-Aldrich (Steinheim, Germany).

Standard preparation and calibration procedure

Stock solutions of analytical standards were prepared by dissolving each analyte in dimethylsulfoxide (100–500 μg mL^{-1}). Working solutions were prepared daily by diluting adequate amounts of stock solutions in McIlvane buffer at pH 7.2 plus 10 % MeOH (*v*/*v*).

Instrumentation

HPLC analyses were performed on a HP 1200 Series chromatograph from Agilent (Waldbronn, Germany). The restricted access material used was a LiChroCART 25-4LiChrospher RP4 alkyl-diol-silica (ADS) (25 μm, 25× 4 mm) from Merck (Darmstadt, Germany). The analytical column was a 150×4.60 mm Zorbax Eclipse XDB-C18 packed with 3 μm particles (Agilent, Waldbronn, Germany). The 6410 Triple Quad mass spectrometer (Agilent, Waldbronn, Germany) was equipped with an electrospray ionization source (ESI). The ESI settings were a capillary voltage of +3,500 V; a drying gas flow of 12 $L min^{-1}$ at a temperature of 350 °C, and a nebulizer pressure of 35 psi.

Sample collection and pretreatment

Six samples of cow's milk of different types were analyzed: whole milk (milk 1), skimmed milk (milk 2), and milk enriched in fiber (milk 3) calcium (milk 4), soya isoflavones (milk 5), and vitamin B (milk 6). They were purchased at local supermarkets. Two samples of raw non-commercial goat's milk (milk 7), and cow's milk (milk 8), collected from organic farms, were also analyzed.

Prior to their analysis, the milk samples were pretreated according to the following procedure: 2.5 mL of milk were mixed with 5 mL of methanol, 2.5 mL of McIlvaine buffer (pH 2.5) and 0.37 g of EDTA. The resulting whey, whose pH was adjusted to 7.2 with NaOH, was separated by centrifugation for 15 min.

On-line sample preparation and HPLC separation

The experimental setup and the schedule for clean-up and chromatographic separation was similar to that reported previously [9]. Briefly, 50 μL of pretreated milk (with a content of 50 %*v*/*v* of MeOH) were first injected with the autosampler and the isocratic pump was started immediately to pump the mobile phase (unbuffered water) at 0.5 mL min^{-1} for 3 min with the system in the "sample loading" position. After a prefixed clean-up time, the system setup was changed to the "sample elution" position and the separation gradient from 25 to 90 % of ACN (binary pump), eluted the analytes to the chromatographic column at a flow rate of 0.5 $mL min^{-1}$ in backflush mode.

Results and discussion

Sample treatment

Optimization of sample pretreatment

Initially, a McIlvane buffer (pH 3.5) with 0.1 M EDTA was tested as precipitation solution, following a procedure described in the literature for tetracyclines [10]. However, this procedure is not valid for benzimidazoles owing to their low solubility in aqueous medium. With a view to solubilizing the analytes, the pH was changed to 2.5 and a portion of organic solvent was added. For the organic solvent, ACN and MeOH were assayed at proportions of 40–50 %. It was found that ACN precipitation was poor and a turbid whey was obtained that was much harder to filter than that obtained with MeOH.

In addition, a study to check whether spiking the sample before or after protein precipitation might have some effect on recovery was carried out. When the milk samples were spiked before protein precipitation, recoveries ranged between 82 and 117 %, and very similar values were obtained when it was the supernatant obtained in the separation that was spiked.

Optimization of sample treatment using on-line extraction

The instrumental configuration used here for coupling was that of the so-called coupled columns and has been described previously [11].

Different cartridges from the ADS family with stationary phases made of alkyl chains of different lengths (C4, C8, and C18) were studied. The best results were obtained with the C4 since in the C8 and C18 cartridges the analytes were retained too strongly, later providing broader and less well resolved peaks. The next step consisted of optimizing the washing time in order to maximize sample clean-up without negatively affecting analyte recovery, which could be hindered by the fact that the pretreated sample was injected in a high content of organic solvent (40–50 %*v*/*v*). A washing time of 3 min was selected.

It was noticed that upon injecting the sample of unspiked milk, peaks belonging to unidentified compounds in the milk were obtained. One of these unidentified peaks coeluted with the more retained analyte (FBT) and hence, with the DAD detector only 11 of the 12 benzimidazoles studied could be determined.

Development of the online extraction-LC-MS/MS method

In order to achieve the levels demanded by current legislation for the determination of veterinary drugs in milk it was necessary to use MS/MS with a QqQ analyzer since with the previous method—DAD detection—it was not possible to attain the values of maximum residue limit (MRL) demanded by the Commission Regulation 37/2010/EC [12]. It should be noted that for all the analytes at least two transitions were obtained; the most abundant one was that used in quantification and the following ones for confirmation. FBZ-SO_2 was an exception, since it only showed one satisfactory transition.

The incorporation of prior clean-up steps of the sample that eliminates the possible interferents responsible for possible matrix effects is one of the strategies that have been proposed [13] to minimize ion suppression in ESI. In this work, the on-line extraction cartridge device behaved as an efficient clean-up step for the milk samples, reducing ion suppression.

The matrix effect was evaluated by comparison of the response of the target compounds in spiked milk samples

Table 1 Validation parameters of the developed method according to Commission Decision 2002/657/EC

2002/657/EC VALIDATION		ABZ-NH_2-SO_2	ABZ-SO	MBC	ABZ-SO_2	OFZ	FLU-HMET	OXI	FLU	ABZ	FBZ	FBT
Calibration parameters	Intercept/ua	−5±10	−4±10	−30±60	4±30	4±40	−30±30	−20±60	40±70	20±100	−3±80	90±400
	Slope/ua $mL·ng^{-1}$	30±1	35±1	347±6	71±3	113±3	110±3	449±5	352±6	450±10	341±7	741±25
	CC_α/$ngmL^{-1}$	0.8	0.9	0.4	1.0	0.8	0.7	0.3	0.5	0.6	0.6	1.1
	CC_β/$ngmL^{-1}$	1.3	1.6	0.6	1.6	1.4	1.2	0.5	0.8	1.0	1.0	1.9
Repeatability[a] and (reproducibility) as RSD/%	5 $ngmL^{-1}$	3.3 (6.9)	2.4 (13)	1.4 (9.0)	3.0 (8.1)	2.2 (9.3)	2.9 (5.2)	16 (20)	7.9 (16)	12 (16)	9.2 (20)	3.2 (9.4)
	10 $ngmL^{-1}$	5.6 (4.8)	5.2 (9.7)	6.8 (5.2)	4.8 (7.3)	9.7 (7.6)	2.6 (4.9)	4.6 (9.4)	7.1 (4.7)	9.4 (7.1)	4.9 (8.0)	4.7 (5.6)
	15 $ngmL^{-1}$	2.0 (4.5)	3.5 (8.7)	1.3 (3.9)	2.1 (5.7)	0.8 (6.7)	2.1 (4.6)	1.6 (12)	1.9 (6.7)	1.8 (7.8)	2.3 (6.6)	1.4 (5.6)
Recovery[b]/%	5 $ngmL^{-1}$	108±8	110±8	108±3	103±9	114±7	105±7	100±3	108±4	104±7	101±12	87±9
	10 $ngmL^{-1}$	102±4	95±4	105±2	93±4	101±4	100±4	88±2	92±2	91±3	90±9	106±12
	15 $ngmL^{-1}$	99±3	93±3	102±1	95±3	99±2	93±3	82±1	87±2	115±2	93±7	93±7

[a] Repeatability calculated as intraday precisions (eight injections per level). Reproducibility calculated as interday precisions (three consecutive days, eight injections per level each day)

[b] Recoveries (process efficiency) calculated as the average of six injections per concentration level

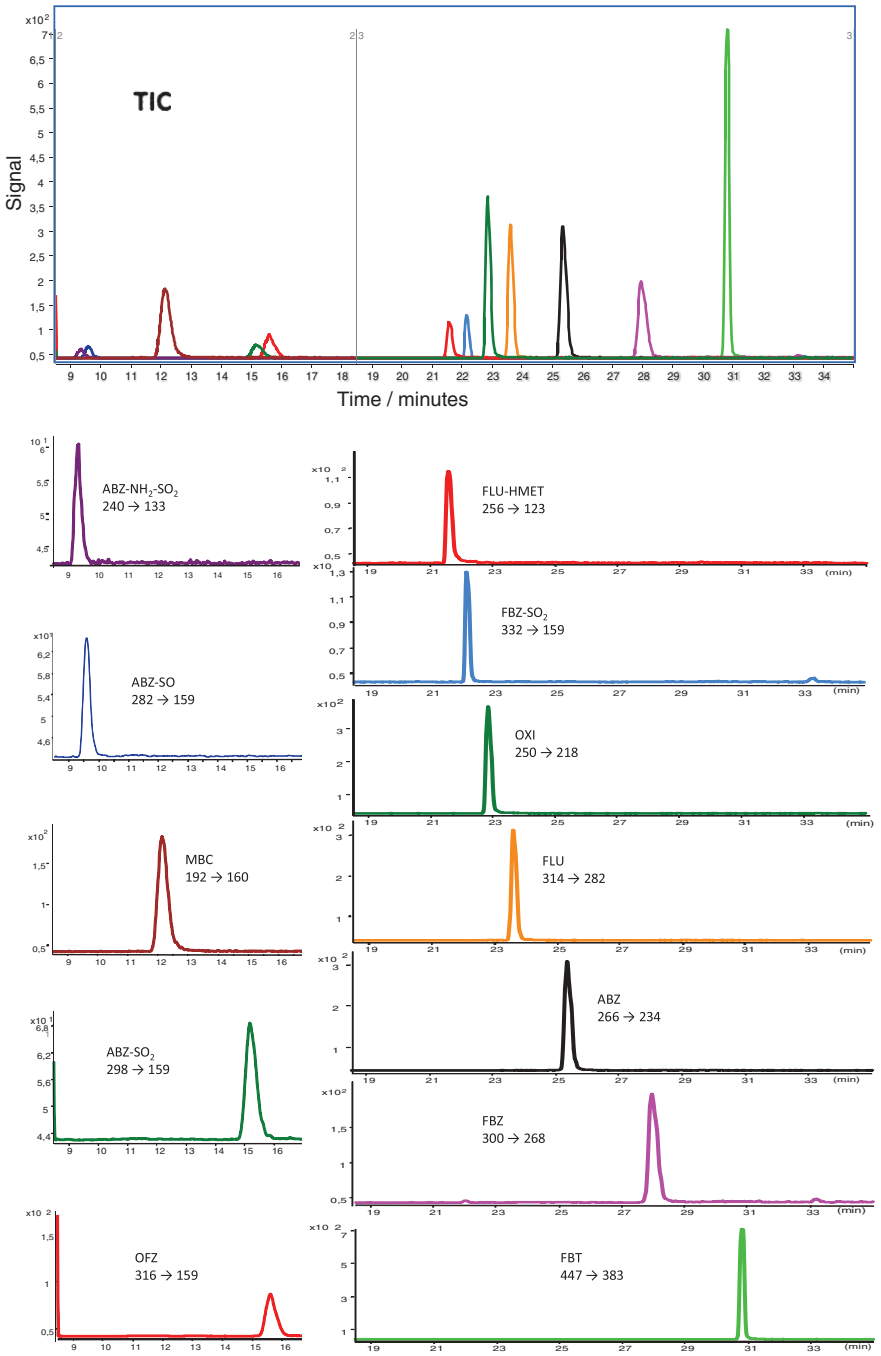

Fig. 1 Total ion chromatogram (TIC) and extracted ion chromatograms (XIC) obtained with the whole proposed method for a milk sample spiked at 10 ngmL^{-1}

and in aqueous standards. A two-tailed *t* test was applied to compare the slopes. It may be seen that for most of the analytes probability values (*p*) lower than 0.05 were obtained. These results confirm that for 8 of the 12 compounds there was a matrix effect. Accordingly, quantification in milk samples with the proposed method requires the use matrix-matched calibration. A calibration in milk samples in the 5–200 $ng mL^{-1}$ concentration range was performed, this being linear for most of the benzimidazoles studied (Table 1). Figure 1 shows the MS/MS extracted ion chromatograms (XIC) obtained in the analysis of a milk sample.

Validation of method according to Commission Decision 2002/657

Owing to the low stability of benzimidazoles in water, the stability of the stock solutions was studied in three different media: water, McIlvane buffer at pH 7.2, and McIlvane buffer at pH 7.2 plus 10 % MeOH (*v*/*v*). The results revealed that the analytes were more stable in McIlvane buffer at pH 7.2 plus 10 % MeOH, with stable signals for at least 72 h, such that in later studies stock solutions of this medium were used.

Validation of the proposed method was performed following the Commission Decision 2002/657/EC which establishes the validation criteria of analytical methods for the determination of organic residues or contaminants in foods of animal origin.

Two analytical limits are recommended: the decision limit (CC_α) and the detection capability (CC_β). These parameters were determined in whole milk and verified by respective experiments, estimated using the calculations described by Verdon et al. [14] (Table 1). According to current legislation [12], the MRL for the albendazole family (ABZ, ABZ-NH_2-SO_2, ABZ-SO and ABZ-SO_2) is 100 μg kg^{-1}. Calculated values for the CC_α and the CC_β for this family were 3.2 and 5.4 $\mu g kg^{-1}$, respectively. Both values are much lower than those established by the legislation as the MRL for this family.

In the case of the febantel family (FBT, FBZ, OFZ, and FBZ-SO_2), CC_α and CC_β were 3.3 and 5.7 $\mu g kg^{-1}$, which are again much lower than those established.

In order to determine the intralaboratory repeatability and reproducibility of the proposed method, milk samples were spiked with all the analytes at three concentration levels: 5, 10, and 15 $ng mL^{-1}$. The relative standard deviations (RSD) for the three concentrations studied are shown in Table 1. The values found were lower than 20 %, even in the most unfavorable of cases.

Validation of the method requires estimation of its accuracy. The recoveries of milk samples spiked at 5, 10, and 15 $ng mL^{-1}$ were determined. The results obtained, as percentages of analyte recovered, are shown in Table 1. It may be seen that for the three concentrations studied the recoveries were in the accepted range of the Codex Guidelines (CAC/GL 71–2009): 70–120 %, with relative standard deviations less than 15 % (Table 1).

In order to validate the ruggedness of the method, seven different milk samples were analyzed. None of the samples showed benzimidazole compounds at concentrations above the LOD and hence they were spiked at 10 $ng mL^{-1}$. The signal obtained for each of the analytes was introduced into the corresponding calibration line. Table 2 shows the recovery values obtained. In all cases, satisfactory recoveries were obtained.

One of the disadvantages of the use of on-line preconcentration with the same sorbent for different samples is possible contamination among them. It was shown that a memory effect, or contamination among samples, existed at elevated concentrations (500 $ng mL^{-1}$), although the magnitude of the effect was very small since it did not reach 1 % in any of the cases. However, it is recommended that in the analysis of samples with high concentrations of analyte, an

Table 2 Recoveries found for the analyses, using the developed method, of milk samples from different sources and of different nature spiked at 10 $ng mL^{-1}$. Average of six injections

Analyte	Milk 2	Milk 3	Milk 4	Milk 5	Milk 6	Milk 7	Milk 8
ABZ-NH_2-SO_2	109±4	113±4	108±4	112±5	99±5	107±5	98±5
ABZ-SO	99±4	101±4	99±4	100±5	98±5	99±5	93±5
MBC	98±2	92±2	92±2	106±3	106±3	106±3	106±3
ABZ-SO_2	109±4	108±4	105±4	98±4	99±4	96±4	94±4
OFZ	110±4	97±3	110±3	105±3	109±4	108±4	105±4
FLU-HMET	102±3	101±4	104±3	99±4	97±4	100±4	114±4
OXI	109±4	110±4	111±4	103±4	105±4	98±4	108±4
FLU	110±4	113±4	108±4	104±4	107±4	100±4	111±4
ABZ	115±7	115±6	108±7	104±6	109±6	104±6	107±7
FBZ	95±2	85±2	78±2	106±1	109±5	93±2	107±3
FBT	109±10	100±10	85±10	13±17	128±17	114±19	125±17

injection of water should be made between samples with a view to minimizing this effect and the possibility of obtaining "false positives".

Conclusions

In this work, a rapid and efficient method for the determination of benzimidazoles in milk samples was developed, at the levels required by current legislation. The method is user-friendly at control laboratories since it involves a minimum of sample manipulation. The results obtained show that the use of a restricted access material as a prior clean-up step in milk samples is very efficient in reducing the matrix effect, being useful for samples containing up to 50 % of MeOH.

Additionally, the C4 ADS-cartridge studied shows good ruggedness, cost and half-life.

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References

1. Townsend LB, Wise DS (1990) Parasitol Today 6:107–112
2. Whelan M, Kinsella B, Furey A, Moloney M, Cantwell H, Lehotay SJ, Danaher M (2010) J Chromatogr A 1217:4612–4622
3. Danaher M, Ruyck H, Crooks SRH, Dowling G, O'Keeffe M (2007) J Chromatogr B 845:1–37
4. Keegan J, Whelan M, Danaher M, Crooks S, Sayers R, Anastasio A, Elliott C, Brandon D, Furey A, O'Kennedy R (2009) Anal Chim Acta 654:111–119
5. Takeba K, Fujinuma K, Sakamoto M, Miyazaki T, Oka H, Itoh Y, Nakazawa H (2000) J Chromatogr A 882:99–107
6. Ruyck H, Daeseleire E, Ridder H, Renterghem R (2002) J Chromatogr A 976:181–194
7. Souverain S, Rudaz S, Veuthey JL (2004) J Chromatogr B 801:141–156
8. Commission Decision 657/2002/EC (2002) Off J Eur Commun L 221/8
9. Rodríguez-Gonzalo E, García-Gómez D, Carabias-Martínez R (2010) J Chromatogr A 1217:40–48
10. Chico J, Meca S, Companyó R, Prat MD, Granados M (2008) J Chromatogr A 1181:1–8
11. Rodríguez-Gonzalo E, García-Gómez D, Herrero-Hernández E, Carabias-Martínez R (2010) J Sep Sci 33:2240–2249
12. Commission Regulation 37/2010/EC (2010) Off J Eur Union. L 15/1
13. Eeckhaut A, Lanckmans K, Sarre S, Smolders I, Michotte Y (2009) J Chromatogr B 877:2198–2207
14. Verdon E, Hurtaud-Pessel D, Sanders P (2006) Accred Qual Assur 11:58–62

3 DETERMINATION OF BIOMARKERS IN BIOLOGICAL SAMPLES

3.A. DETERMINATION OF BIOMARKERS OF EXPOSURE TO XENOBIOTIC COMPOUNDS

RESEARCH ARTICLE IV

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Research Article

Automated sample treatment with the injection of large sample volumes for the determination of contaminants and metabolites in urine

This work reports the development of a simple and automated method for the quantitative determination of several contaminants (triazine, phenylurea, and phenoxyacid herbicides; carbamate insecticides and industrial chemicals) and their metabolites in human urine with a simplified sample treatment. The method is based on the online coupling of an extraction column with RP LC separation–UV detection; this coupling enabled fast online cleanup of the urine samples, efficiently eliminating matrix components and providing appropriate selectivity for the determination of such compounds. The variables affecting the automated method were optimized: sorbent type, washing solvent and time, and the sample volume injected. The optimized sample treatment reported here allowed the direct injection of large volumes of urine (1500 μL) into the online system as a way to improve the sensitivity of the method; limits of detection in the 1–10 ng/mL range were achieved for an injected volume of 1500 μL of urine, precision being 10% or better at a concentration level of 20 ng/mL. The online configuration proposed has advantages such as automation (all the steps involved in the analysis – injection of the urine, sample cleanup, analyte enrichment, separation and detection – are carried out automatically) with high precision and sensitivity, reducing manual sample manipulation to freezing and sample filtration.

Keywords: Contaminants and metabolites / Human urine / Large volume injection / LC / Online sample treatment
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1 Introduction

Analytical methods for the analysis of contaminants are mainly based on chromatographic techniques. Although many methods have been reported for measuring pesticides and industrial chemicals in human matrices, most of them refer to a limited number of compounds or analytes belonging to the same chemical group [1]. There is a continual need for methods that assess exposure to emerging pesticides, as well as for the multiresidue determination of contaminants of different chemical groups for application in occupational and environmental toxicology.

One of the main problems in multiresidue analysis of contaminants in real samples is the tediousness and complexity of the procedures required for the extraction, cleanup, and preconcentration of the matrix analytes [2]. This highlights the importance of, and indeed current interest in, the search for new procedures that will allow sample treatment to be simplified, with the collection of reliable results and also allowing automation. Most methods described in the literature for the determination of contaminants employ a combination of some extraction methods with organic solvents with one or several washing steps aimed at removing co-extractants before the samples are injected into the chromatographic system [3, 4]. Moreover, a preconcentration/evaporation step is usually carried out to increase the concentration and/or replace the solvent with one that is more compatible with the chromatographic system.

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Abbreviations: ADS, alkyl-diol-silica; **Atz,** atrazine; **BPF,** bisphenol-F; **ClAnil,** chloroaniline; **Clt,** chlortoluron; **Cbl,** carbaryl; **CMPU,** 3-chloro-4-methylphenylurea; **DiClAnil,** dichloroaniline; **Din,** diuron; **LC-DAD,** LC-diode array detector; **1NPL,** naphthol; **2,4,5-T,** 2,4,5-trichlorophenoxyacetic acid; **2,4,5-TP,** 2,4,5-trichlorophenoxypropionic acid; **TCPL,** trichlorophenol

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Some of these steps can be combined and automated through coupling to the chromatographic system. Advances in the automation of pesticide analyses in biological samples focus on the cleanup procedure, which involves the separation of target analytes from a large number of interferents present in the matrix [5].

Sample treatment steps based on the use of restricted access materials were initially designed to remove proteins in the analysis of drugs in biological matrices; they allow online solid–liquid extraction and the enrichment of small molecules contained in the biofluid in question, limiting the access of macromolecules to the interaction sites by means of a porous membrane [6–8]. Souverain *et al.* [9] have reviewed a large number of applications for the purification of pharmaceutical and endogenous compounds in biological matrices such as serum, urine, saliva, milk, and tissues. The literature contains reports concerning phthalates [10], triazines [11], naphthols [12], organophosphorus compounds [13], bisphenol and octylphenol [14], together with many articles addressing drugs. However, to our knowledge no authors have applied these materials to the multiresidue analysis of compounds of very different chemical nature.

The determination of contaminants in biological matrices involves the measurement and assessment of compounds or their metabolites in fluids, tissues, secretions, exhaled air, or a combination of these as a method for evaluating exposure and health risks [15]. The determination of contaminants and their metabolites is often linked to the concentration of the internal dose and can be useful for the estimation of the integrated exposure by all routes [16, 17]. Choice of the biological matrix is determined by the nature of the contaminant of interest and its pharmacokinetics and also by the population characteristics. When attempting to analyse low-molecular weight compounds with possible toxic activity, urine is the most favorable candidate [18]; usually, metabolites are excreted in urine since they have half-lives of hours or days and are not bioaccumulated in tissues. Furthermore, their concentration in urine is much higher than in other matrices, and they can be detected over longer periods of time. All the foregoing, together with the fact that urine is very easy to obtain, means that it is the fluid of choice in this type of analysis [19].

In light of the above, here we were prompted to develop a method for the determination of contaminants and metabolites of known toxic activity and from different families and provenances. The aim of this work was to develop a simple, robust, and automated method for the determination of contaminants at trace levels in human urine, with minimum sample treatment and avoiding the use of sophisticated and/or expensive instrumentation. With this in mind, the work described here focuses on (i) the study and optimization of a sample treatment step coupled online to an LC-diode array detector (DAD) system, and (ii) the development of a procedure based on the injection of large sample volumes that can be applied directly to biological samples such as urine.

Thus, here we propose an online configuration that allows the direct injection of volumes of urine up to 1500 μL into the in-line system, with no losses of resolution or deterioration of the chromatographic devices. In this way, a considerable improvement in sensitivity can be achieved. The contaminants and their respective metabolites used in the present study were as follows: (i) Pesticides, such as the 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 2,4,5-trichlorophenoxypropionic acid (2,4,5-TP) phenoxyacids, which are the herbicides globally most used domestically [20]; the carbamate carbaryl (Cbl), one of the most popular domestic insecticides throughout the world, and its metabolite 1-naphthol (1NPL) [21]; the phenylureas chlortoluron (Clt) and diuron (Din), designated as "priority hazardous substances" by the EU [22] and their metabolites 3-chloro-4-methylphenylurea (CMPU) [23], chloroaniline (ClAnil) and dichloroaniline (DiClAnil) [4] and the triazine atrazine (Atz), the second most widely used herbicide [20]. (ii) Contaminants of industrial origin, such as bisphenol F (BPF), which is widely used in the preparation of epoxy resins and polycarbonates [24], and trichlorophenol (TCPL), associated with the always dangerous dioxins [25], and the already mentioned 1NPL, a metabolite of polycyclic aromatic hydrocarbons [26].

2 Materials and methods

2.1 Chemicals

Analytical standards were obtained from two different suppliers; seven contaminants were from Dr. Ehrenstorfer (Augsburg, Germany): Atz, 6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine, CAS RN [1912-24-9]; Cbl, 1-naphthyl-*N*-methylcarbamate, CAS RN [63-25-2]; DiClAnil, 3,4-DiClAnil, CAS RN [95-76-1]; Clt, 3-(3-chloro-4-methyl)-1,1-dimethylurea, CAS RN [15545-48-9]; CMPU, *N*-(3-chloro-4-methylphenyl)urea, CAS RN [13142-64-8]; Din, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, CAS RN [330-54-1] and 1NPL, 1-hydroxynaphthalene, CAS RN [90-15-3]. Another five contaminants were obtained from Sigma-Aldrich (Steinheim, Germany): ClAnil, 4-ClAnil, CAS RN [106-47-8]; BPF, bis-(4-hydroxyphenyl)methane, CAS RN [620-92-8]; TCPL, 2,4,5-TCPL, CAS RN [95-95-4]; 2,4,5-T, CAS RN [93-76-5]; and 2,4,5-TP, CAS RN [93-72-1].

The organic solvents – ACN and methanol – were of HPLC grade from Merck (Darmstadt, Germany) and were used as received. Ultra high-quality water was obtained with an Elgastat UHQ water purification system.

All the chemicals used for the preparation of the buffer and all the other chemicals were of analytical reagent grade.

2.2 Sample collection

Urine samples collected from four healthy volunteers were used for developing the method and the preparation of the calibration standards. Urine samples were collected in 250-mL brown glass bottles and frozen immediately until analysis. Before use, the urine samples were thawed at

room temperature and spiked daily with diluted standard solutions. Samples were filtered through 0.45 μm CAMEO filters to remove the precipitated proteins. Creatinine was determined to normalize the results for concentration and rule out overdiluted or overconcentrated samples; values from 877 to 1268 mg/L were obtained. All urine samples were first analyzed with the proposed method to check the natural occurrence of the target compounds. No signal corresponding to the target analytes was found, and hence spiked urine samples were used.

2.3 Instrumentation

LC-DAD was performed on a HP 1100 Series chromatograph from Hewlett Packard (Waldbronn, Germany) equipped with a binary pump, an additional isocratic pump, a membrane degasser, an autosampler (equipped with a 1500-μL capillary seat), a six-port valve and a DAD. The system was controlled by a HP ChemStation, which also performed data collection from the DAD and quantitative measurements. The extraction columns used were alkyl-diol-silica (ADS), restricted access materials LiChroCART 25-4LiChrospher RP4 ADS, RP8 ADS, and RP18 ADS (25 μm, 25 mm × 4 mm) from Merck. The separation column used was a 150 × 4.60 mm Luna PFP(2) packed with 3-μm particles (Phenomenex, Torrance, CA, USA). The DAD was set at 214 and 244 nm. The spectra were recorded in the 190–400 nm range. Full-scan UV detection fulfills the performance criteria set by 2002/657/EC. These criteria are based on the comparison of the sample and calibration spectra. The absorption maxima in the spectrum of the analyte will be at the same wavelengths as those of the calibration standard within a ±2-nm margin. The spectrum of the analyte above 220 nm will, for those parts of the two spectra with a relative absorbance ≥10%, not be visibly different from the spectrum of the calibration standard.

The mobile phase of the isocratic pump consisted of 2.5 mM ammonium formate buffer (pH 2.9) with 10% ACN. The mobile phase of the binary pump consisted of a 2.5 mM ammonium formate buffer, pH 3.5 (solvent A), and ACN (solvent B) gradient. The analytical column was thermostated at 25°C.

2.4 Online sample preparation and LC separation

The experimental setup for online configuration is shown in Fig. 1 and the schedule for cleanup, enrichment, and analysis is shown in Table 1. First, after the injection of the

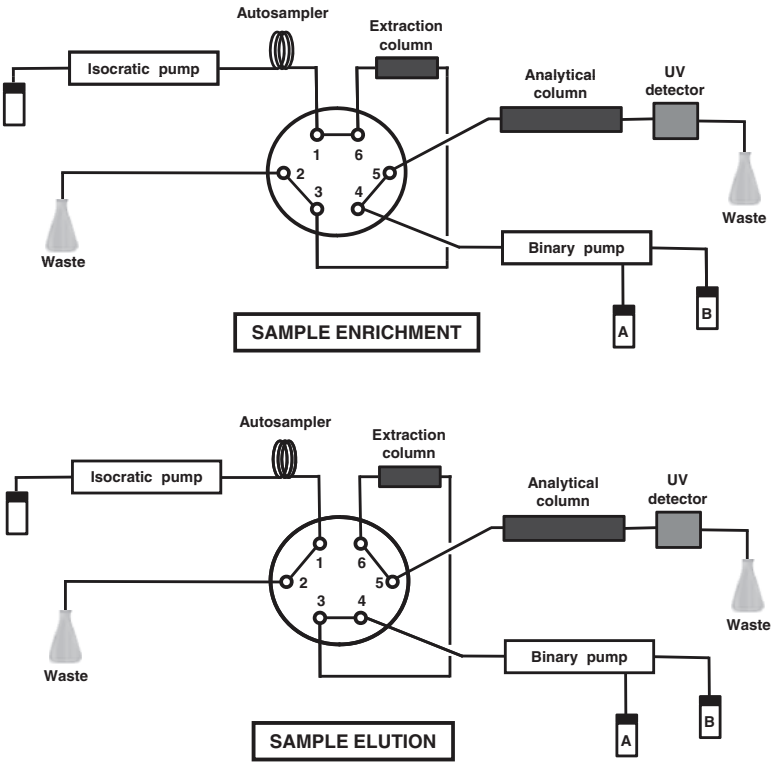

Figure 1. Schematic diagrams of the proposed online method.

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Table 1. Chromatographic conditions of online proposed method

Time (min)	Isocratic pump[a)] flow (mL/min)	Binary pump[b,c)] (% A)	Six-port valve position	Event
0–10	1	85	Enrichment	Sample charging
10–11	1→0.3	85	Elution	Analyte transfer
11–12	0.3	85→65	Elution	Analyte separation
12–19	0.3	65	Elution	Analyte separation
19–30	0.3	65→45	Elution	Analyte separation
30–35	0.3	45→0	Elution	Analyte separation
35–37	0.3	0	Elution	Analyte separation
37–39	0.3→1	0→85	Elution	Equilibrating
39–42	1	85	Enrichment	Equilibrating

a) Mobile phase: ammonium formate buffer 2.5 mM (pH = 2.9) with 10% ACN.
b) Flow: 1 mL/min.
c) Mobile phase: (solvent A) ammonium formate buffer 2.5 mM (pH = 3.4), (solvent B) ACN.

urine (up to 1.5 mL) by the autosampler, the isocratic pump was run 10 min at 1 mL/min with the valve in ''enrichment'' mode. While the matrix components of the urine were being washed to the waste, the analytes studied were withheld in the extraction column. At 10 min, the valve was changed to ''elution'' mode and a gradient (binary pump), shown in Table 1, with a flow rate of 1 mL/min eluted the analytes in backflush mode to the column, where they were separated and finally detected by the DAD. The isocratic pump changed the flow rate to 0.3 mL/min to avoid wasting solvent. At 37 min, the separation was terminated and the gradient was returned to the initial conditions. The flow rate of the isocratic pump was changed to 1 mL/min. At 39 min, the valve was switched to ''enrichment'' mode and equilibrated for 3 min. Then, at 42 min, the system was ready for the next injection.

For offline SPE experiments, commercial OASIS HLB 60 mg cartridges from Waters System (Milford, MA, USA) were conditioned with 3 mL of ACN and then with 3 mL of UHQ water. Following this, 400 μL of urine was loaded, including a cleanup step with 10 mL of water containing 5% of ACN, once the sample had been passed. The Oasis HLB cartridges were easily fouled, admitting the passage of 400 μL but not much more, which to a large extent limits the preconcentration of the analytes. After loading the sample a slight vacuum was applied. Analytes were eluted with 3 mL of ACN. The extracts were evaporated to complete dryness in a nitrogen stream and reconstituted in 400 μL of UHQ water for further analysis.

2.5 Standard preparation and calibration procedure

The initial stock solutions for the standards were prepared by dissolving 12.5 mg of each analyte in 25 mL of ACN (500 μg/mL). These stock solutions were stored at 4°C in brown glass bottles. For a 100-μL injection, calibration standards were prepared in a range from 250 to 500 ng/mL. In the case of 1500-μL injection, standards between 10 and 200 ng/mL were prepared. Before analysis, the standards were filtered with a Cameo (0.45 μm Nylon) filter. Calibration curves were obtained by plotting the peak areas of the analytes *versus* the concentration using matrix-matched samples.

The stability of these stock solutions was ensured by controlling the storage conditions (temperature, humidity, absence of light) of the analytes, keeping them in the range in which stability is guaranteed by the manufacturer. Moreover, a standard solution was injected each week with a view to controlling the intensity of the signals obtained.

Spiked urine standards were prepared daily by spiking urine with the appropriate amounts of each analyte.

2.6 Stability of the online procedure

Throughout the work, the pressure of the LC system was controlled, both that of the isocratic pump connected to the extraction column and that of the binary pump connected to the analytical column. Control of the pressure of both pumps, together with the background noise and the shape of the peaks obtained, allowed the proper functioning of the system to be checked.

Along this work, two extraction columns were employed, of which the one that lasted the shortest time supported 110 injections of urine, corresponding to a total volume of 92.2 mL. As an indication, replacement of the extraction column should be carried out when the pressure of the LC system is excessively high with respect to the usual values or when the intensity of the signal and the shape of the peaks so indicate.

3 Results and discussion

The analysis of urine samples with methods based on LC that include an extraction–preconcentration step normally requires a cleanup step to eliminate as many of the compounds as possible present in the urine that would hinder the analysis and would shorten the useful life of the chromatographic columns. Figure 2 shows the chromatograms corresponding

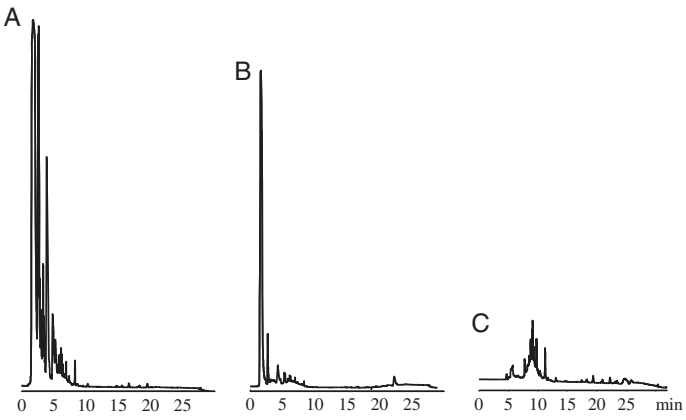

Figure 2. Comparison of different sample cleanup procedures for an unspiked urine sample: (A) injected directly into the LC-DAD system, (B) after offline cleanup with Oasis HLB cartridges, and (C) after online cleanup with a C4-ADS extraction column. Sample volume injected: 100 μL. *Y*-axis at the same scale.

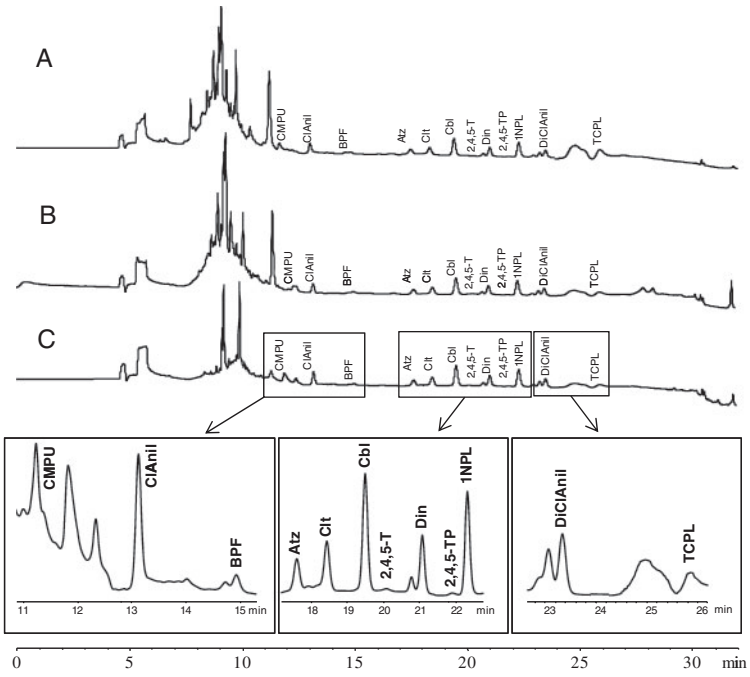

Figure 3. Chromatograms of a urine sample spiked with 400 ng/mL using sorbents with alkyl chains of different lengths: (A) C18, (B) C8, (C) C4. Sample volume injected: 100 μL. (A–C) *Y*-axis at the same scale.

to urine samples injected directly into the LC-DAD system (Fig. 2A) or subjected to extraction and cleanup through a conventional cartridge Oasis HLB (Fig. 2B), or through a column containing a restricted access material (C4 ADS type) with a time of 3 min for washing it (Fig. 2C). As may be seen, in the urine sample injected directly into the LC-DAD system many peaks were observed, corresponding to impurities present in the urine. These peaks decreased in number when an extraction and cleanup step through Oasis HLB was introduced, and even more so when the cleanup was performed through the extraction column with the restricted access material. Peaks before 10 min, although not relevant for the determination of analytes, are relevant for the analytical method. Thus, the use of Oasis HLB would elicit the entry of a large front of interferents into the column, which would strongly affect its life.

It should also be taken into account that it is necessary to use an Oasis HLB cartridge for each sample since they

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collapse rapidly (Section 2.4), whereas restricted access materials allow the injection of a large number of samples [27]. Accordingly, the use of the restricted access materials seems to be more favorable than that employing offline Oasis HLB cartridges.

3.1 Selection of the material used as sorbent in the extraction column

A study was made of the effect of the type of ADS restricted access material used, for which materials with different lengths of the alkyl chain were assayed: C4, C8, and C18. Figure 3 shows the chromatograms corresponding to the injection of 100-μL urine samples spiked with 400 ng/mL of each of the analytes studied. The isocratic pump was used for loading and washing the extraction column, with a 2.5 mM ammonium formate buffer, pH 2.9, with 5% of ACN as the mobile phase and a washing time of 3 min. As may be observed, cleaner chromatograms were obtained as the chain length of the sorbent decreased, with no appreciable decrease in the peaks corresponding to the analytes studied. It was decided to use the C4 sorbent for later studies.

3.2 Online sample cleanup and preconcentration of urine samples (small volume, 100 μL)

Once the most suitable sorbent for the target analytes had been selected, we studied different factors that might improve the cleaning of the sample, eliminating possible interferents and thus enabling the identification of the different analytes, since an efficient washing step is also important for preserving the instrumental system and lengthening the column life.

3.2.1 Optimization of the washing step. Evaluation of matrix effects

The process of washing the extraction column is affected by two main factors: the solvent used and the washing time. The mobile phase impelled by the isocratic pump acts as a washing solvent and at the same time transports the sample through the extraction column. The composition of the mobile phase is therefore a decisive factor for achieving an efficient washing of the sample matrix with no elimination of the compounds of interest. As the proportion of organic component in this solution increases, the washing effect also increases, although so does the possibility of removing the target compounds. To optimize the composition of the mobile phase, ammonium formate buffer (pH 2.9)–ACN mixtures were assayed and the content in ACN was modified from 5 to 25%. The results (Fig. 4) revealed that the more polar analytes (CMPU, BPF, Atz) were not retained in the extraction column, or only sparingly so, when the ACN content surpassed 10%. For the rest of the analytes no losses were observed and percentages of ACN of 15% could be used, maintaining recoveries above 80%. In the later studies, for the washing solution it was decided to use one with a composition of 2.5 mM ammonium formate buffer (pH 2.9)–10% ACN, since this was the one offering the best cleaning without producing appreciable losses of the more polar analytes.

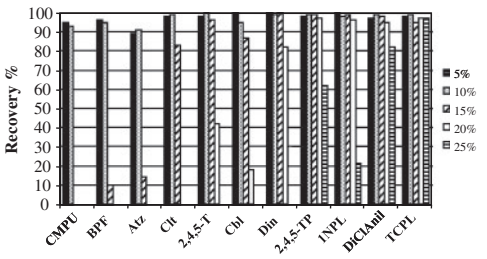

Figure 4. Recoveries as a function of the percentage of ACN in the mobile phase used as washing solvent (isocratic pump).

Another parameter to be controlled was the washing time that is the time during which the previously optimized washing solvent is circulating through the extraction column. This period comprises the time that elapses since sample introduction to the change in position of the injection valve. Four analyses were made modifying the washing time between 3 and 15 min. There were no large differences in the chromatograms obtained, apart from the consequent increase in the analysis time such that it was decided to use a 3 min washing time.

Once the parameters that might affect the sample treatment step had been established, a study was made of the influence of the concentration of the target compounds. The calibration curves obtained for all the analytes exhibited linearity throughout the range studied.

The matrix effect was studied by comparing the slopes, using linear regression analysis, obtained from aqueous

Table 2. Recoveries and precisions of online cleanup step injecting 100 μL of a urine sample spiked with 400 ng/mL

	LOD[a] (ng/mL)	Recovery (%)	RSD[b] (%)
CMPU	76	100	8
ClAnil	20	85	2
BPF	80	91	3
Atz	34	93	6
Clt	31	99	4
Cbl	51	97	5
2,4,5-T	23	91	7
Din	15	97	7
2,4,5-TP	44	93	9
1NPL	53	89	4
DiClAnil	31	97	7
TCPL	48	86	9

a) Limit of detection for a signal-to-noise ratio of 3.
b) RSD: $n = 6$.

standards and matrix-matched calibration graphs obtained by using spiked urine samples. A paired *t*-test was applied and the *p* values (probability that the slopes in the aqueous standards were not significantly higher than slopes in spiked urine) obtained were less than 0.05 in all cases, which points at the existence of a matrix effect. As a consequence, the matrix-matched calibration procedure is recommended for quantification purposes.

The limits of detection (Table 2), determined for a signal-to-noise ratio of 3, varied between 15 ng/mL for Din, and 80 ng/mL for BPF. Recovery values obtained by injecting 100 μL of urine are also shown in Table 2. The recoveries for each analyte were calculated by comparing the concentration obtained from the matrix-matched calibration lines and the real value of the concentration used to spike the sample. Precision was studied by analyzing 8 replicates of a urine sample spiked with 400 ng/mL, in all cases RSD values lower than 10% were obtained (Table 2).

3.3 Influence of the volume of sample injected

The studies performed up to this point involved the injection of a sample volume of 100 μL. A study was made of the possibility of injecting larger volumes, which would allow the sensitivity to be increased, thereby improving the limits of detection. Accordingly, the system was modified by installing a capillary seat that would allow sample volumes of up to 1500 μL to be injected. Figure 5 shows the variation in peak areas for three of the analytes studied upon injecting volumes of 400, 750, 1000, and 1500 μL. It may be seen that in all cases there was a linear relationship between the signal and the volume injected. Additionally, upon comparing the chromatographic areas obtained on injecting samples of 100 μL spiked with 1500 ng/mL with those obtained upon injecting samples of 1500 μL spiked with 100 ng/mL (*i.e.* the amount of sample injected was the same), no significant differences were observed in the signals of the different analytes (Table 3). It may also be seen that the signal corresponding to the urine samples was slightly lower than that achieved with the aqueous samples due to matrix effect, this effect being smaller for medium polarity analytes. This points to the need to perform quantification using matrix-matched samples.

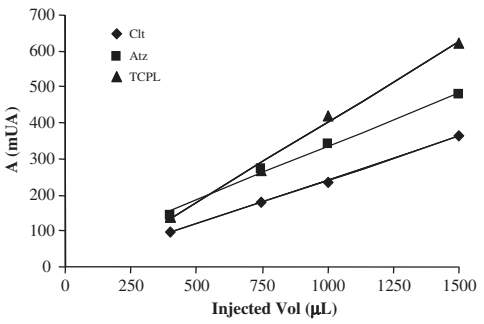

Figure 5. Influence of the volume of urine injected into the online system.

3.4 Online sample cleanup and preconcentration for urine sample volumes of 1500 μL

In view of the linear relationship between the signal and volume injected, it was decided to inject the maximum volume accepted by the device (1500 μL) to improve the sensitivity of the method.

First, it was ascertained whether for these volumes of urine the efficiency of the previously optimized washing step of the extraction column would be maintained. Since previously it had been observed that for proportions of ACN higher than 10% no retention of the more polar analytes occurred, it was decided to use this same solvent: ammonium formate buffer (pH 2.9), plus 10% ACN. However, the washing times required another optimization owing to the important increase in the amount of urine injected. Figure 6 shows the effect of applying washing times of 3 min (Fig. 6A), 5 min (Fig. 6B) and 10 min (Fig. 6C), injecting a volume of unspiked urine of 1500 μL. Large differences can be seen in the efficiency of the washing process, which was notably improved with the increase in time (Fig. 6C), although it also increased the analysis time. To work with these sample volumes (1500 μL), a washing time of 10 min was chosen because correct washing allows the useful life of the column to be prolonged and facilitates the identification of the different analytes in question.

Table 3. Comparison of areas, in UHQ and urine matrix, of a sample spiked with 100 ng/mL injecting 1500 μL and a sample spiked with 1500 ng/mL injecting 100 μL

Analyte	UHQ		Urine	
	1500 μL of 100 ng/mL	100 μL of 1500 ng/mL	1500 μL of 100 ng/mL	100 μL of 1500 ng/mL
CMPU	361.4	350.9	–[a)]	267.6
ClAnil	585.8	592.5	292.2	287.5
BPF	286.8	290.7	158.1	157.2
Atz	467.1	434.5	391.7	433.3
Clt	323.4	318.4	291.3	235.5
Cbl	1186	1158	915.1	910.0
2,4,5-T	550.8	566.6	391.9	355.4
Din	334.1	330.6	277.3	257.0
2,4,5-TP	601.5	591.6	517.7	518.4
1NPL	1136	1109	600.5	580.2
DiClAnil	348.1	347.8	234.2	287.5
TCPL	59.8	52.2	47.5	48.3

a) Concentration below limit of quantification.

3.5 Analytical characteristics of the proposed method injecting 1500 μL of urine

Once the different parameters of the method developed had been optimized and selected – choice of sorbent (an ADS restricted access material, C4-type), injection volume (1500 μL), washing solvent (10% ACN), and washing time (10 min) – we evaluated the spiking of the samples because this is performed after freezing and must be compared with the samples spiked before freezing, in the sense that this latter situation would correspond to a real contaminated sample.

Two urine samples spiked at 100 ng/mL were prepared and subjected to freezing before and after spiking, respectively. The signal of each of the analytes was analyzed, and it was concluded that there were no significant differences in any case, such that it can be affirmed that spiking before or after freezing does not affect the results of the method.

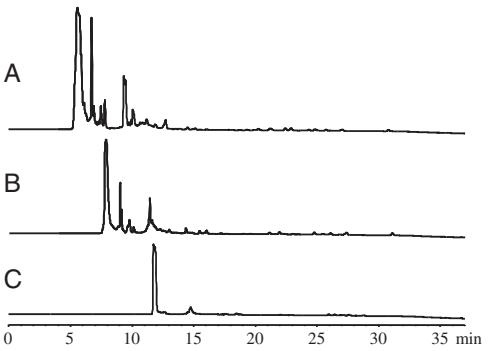

Figure 6. Influence of the washing time in the chromatograms of an unspiked urine sample: (A) 3 min, (B) 5 min, and (C) 10 min. Sample volume injected: 1500 μL. *Y*-axis at the same scale.

We also studied the relationship between the analytical signal and the concentration of each of the compounds studied. For all the analytes assayed, linear relationships were observed between the peak areas and the concentrations within the range studied: 10–200 ng/mL. Table 4 shows the analytical characteristics of the method applied. The limits of detection, determined for a signal-to-noise ratio of 3, varied between 1 ng/mL for Din and DiClAnil, and 10 ng/mL for BPF, Cbl and TCPL improving one order of magnitude from the 100-μL injection results. These limits can be compared with those obtained by LC-MS/MS methods [28]. In both cases, limits in the low ng/mL level have been achieved. For CMPU, the most polar compound, the limit of detection determined was higher (50 ng/mL) owing to the strong matrix signal obtained from 12 to 19 min, as can be seen in Fig. 7. For this reason the method developed would not be appropriate for the determination of CMPU and other more polar analytes. The limits of quantification, determined for a signal-to-noise ratio of 10, ranged from 3 ng/mL for Din and DiClAnil to 20 ng/mL for Cbl and TCPL.

The RSD was determined by analyzing low (20 ng/mL) and medium concentrations (100 ng/mL) of spiked urine samples ($n = 6$). Intraday precision for low concentration was in the range 2.6–8.9% for BPF and Atz, respectively (Table 4).

Figure 7 shows a chromatogram corresponding to the injection of 1500 μL of urine spiked at a concentration level of 20 ng/mL. The interday precision, at this level, was also determined by analyzing injections (8 each day) carried out

Table 4. Analytical characteristics of online proposed method obtained by the analysis of spiked urine samples. Injection volume: 1500 μL

Analyte	λ (nm)	Intercept (mAU)	Slope (mAU/ng/mL)	r^2	LOD[a] (ng/mL)	LOQ[b] (ng/mL)	RSD[c] (%)	
							20 ng/mL	100 ng/mL
ClAnil[d]	214	−2 ± 12	2.9 ± 0.1	0.998	3	10	5.4	3.2
BPF[e]	214	0 ± 30	1.8 ± 0.3	0.995	10	19	2.6	3.9
Atz[d]	214	−3 ± 22	3.9 ± 0.2	0.996	4	13	8.9	7.6
Clt[d]	244	−11 ± 20	2.7 ± 0.2	0.997	7	19	7.1	7.1
Cbl[e]	214	−32 ± 120	11 ± 1	0.996	10	20	7.7	2.7
2,4,5-T[e]	214	21 ± 36	2.2 ± 0.3	0.995	9	19	6.1	6.1
Din[d]	244	7 ± 10	2.7 ± 0.1	0.998	1	3	3.4	2.5
2,4,5-TP[d]	214	−9 ± 33	5.0 ± 0.3	0.996	3	11	4.8	3.0
1NPL[d]	214	−46 ± 20	6.3 ± 0.2	0.999	4	13	3.6	4.5
DiClAnil[d]	244	−6 ± 13	2.3 ± 0.1	0.997	1	3	4.5	9.6
TCPL[e]	214	−25 ± 46	4.5 ± 0.4	0.995	10	20	5.5	8.5

a) Limit of detection for a signal-to-noise ratio of 3.
b) Limit of quantification for a signal-to-noise ratio of 10.
c) RSD (intraday, $n = 6$).
d) Concentration range: 10–200 ng/mL.
e) Concentration range: 40–200 ng/mL.

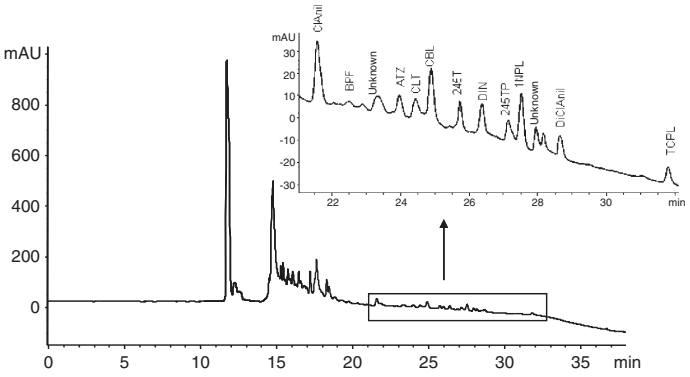

Figure 7. Chromatogram obtained in the analysis of a urine sample spiked at 20 ng/mL. Sample volume injected: 1500 μL.

over three consecutive days which was in the range from 7.2% for BPF to 14.3% for TCPL.

As no reference materials are available, recovery studies were carried out to validate the accuracy of the proposed method [29]. Urine samples that had not been used previously in the method were analyzed. These samples were spiked at two levels: 20 and 100 ng/mL. The signal obtained for each of the analytes was introduced into the corresponding matrix-matched calibration line. In the case of urine spiked with 100 ng/mL, the recoveries varied between 90 ± 5% for TCPL and 104 ± 7% for Cbl. For the urine spiked with 20 ng/mL, they varied between 87 ± 10% for ClAnil and 107 ± 12% for Clt. In all cases, satisfactory recoveries were obtained.

4 Concluding remarks

The analytical methodology proposed here allows the detection and quantification of contaminants with different chemical structures and provenances (different pesticides, metabolites, and industrial products) in urine. The online configuration developed means that manual sample manipulation is minimum (freezing and filtration); this has a positive effect on precision and affords a shorter analysis time. Additionally, the development of a procedure based on the injection of large sample volumes (up to 1500 μL) allowed limits of detection at the low ng/mL level to be achieved.

The method described here is a suitable (rapid, reliable, and accessible) tool for the determination of these compounds in a short time, with good sensitivity. According to 2002/657/EC Decision, this LC-full scan DAD methodology can be used as a suitable confirmatory method for organic residues or contaminants. The method is therefore appropriate for its application within the context of the biological monitoring of contaminants.

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The authors have declared no conflict of interest.

5 References

[1] Aprea, C., Colosio, C., Mammone, T., Monoia, C., Maroni, M., *J. Chromatogr. B* 2002, *769*, 191–219.

[2] Norrgran, J., Bravo, R., Bishop, A. M., Restrepo, P., Whitehead, R. D., Needham, L. L., Barr, D. B., *J. Chromatogr. B* 2006, *830*, 185–195.

[3] Zao, R. S., Wang, X., Yuan, J. P., Wang, X. D., *J. Sep. Sci.* 2009, *32*, 630–636.

[4] Baker, S. E., Olsson, O., Needham, L. L., Barr, D. B., *Anal. Bioanal. Chem.* 2005, *383*, 963–976.

[5] Chalányová, M., Hutta, M., Pagác, M., *J. Sep. Sci.* 2010, *33*, 133–142.

[6] Kanda, T., Kutsuna, H., Ohtsu, Y., Yamaguchi, M., *J. Chromatogr. A* 1994, *672*, 51–57.

[7] Sadílek, P., Satinsky, D., Solich, P., *Trends Anal. Chem.* 2007, *26*, 375–384.

[8] Machtejevas, E., Marko-Varga, G., Lindberg, C., Lubda, D., Hendriks, R., Unger, K. K., *J. Sep. Sci.* 2009, *32*, 2223–2232.

[9] Souverain, S., Rudaz, S., Veuthey, J. L., *J. Chromatogr. B* 2004, *801*, 141–156.

[10] Preuss, R., Koch, H. M., Angerer, J., *J. Chromatogr. B* 2005, *816*, 269–280.

[11] Sambe, H., Hoshina, K., Haginaka, J., *J. Chromatogr. A* 2007, *1152*, 130–137.

[12] Preuss, R., Koch, H. M., Angerer, J., *J. Chromatogr. B* 2004, *801*, 307–316.

[13] Amini, N., Crescenzi, C., *J. Chromatogr. B* 2003, *795*, 245–256.

[14] Liu, M., Hashi, Y., Pan, F., Yao, J., Song, G., Lin, J. M., *J. Chromatogr. A* 2006, *1133*, 142–148.

[15] Zielhuis, R. L., *Am. J. Ind. Med.* 1985, *8*, 515–516.

[16] WHO, *Environmental Health Criteria 222*, World Health Organization 2001, Geneva.

[17] Angerer, J., Ewers, U., Wilhelm, M., *Int. J. Hyg. Environ. Health* 2007, *210*, 201–228.

[18] Petersen, G. O., Leite, C. E., Chatkin, J. M., Thiesen, F. V., *J. Sep. Sci.* 2010, *33*, 516–521.

[19] Barr, D. B., Barr, J. R., Maggio, V. L., Whitehead Jr, R. D., Sadowski, M. A., Whyatt, R. M., Needham, L. L., *J. Chromatogr. B* 2002, *778*, 99–111.

[20] Kiely, T., Donaldson, D., Grube, A., *Pesticide Industry Sales and Usage 2000 and 2001 Market Estimates,* US Enviromental Protection Agency, 2004.

[21] WHO, *Environmental Health Criteria 153 Carbaryl*, World Health Organization 1994, Geneva.

[22] European Commision, *Decision 2455/2001/CE*, European Union 2001 Brussels.

[23] Carabias-Martínez, R., Rodríguez-Gonzalo, E., Revilla-Ruiz, P., Domínguez-Álvarez, J., *J. Chromatogr. A* 2003, *990*, 291–302.

[24] Jana, S. K., Okamoto, T., Kugita, T., Namba, S., *Appl. Catal. A* 2005, *288*, 80–85.

[25] Collins, J. J., Bodner, K., Haidar, S., Wilken, M., Burns, C. J., Lamparski, L. L., Budinsky, R. A., Martin, G. D., Carson, M. L., *Chemosphere* 2008, *73*, S284–S289.

[26] Barr, D. B., Needham, L. L., *J. Chromatogr. B* 2002, *778*, 5–29.

[27] Baeyens, W. R. G., Van Der Weken, G., Haustraete, J., Aboul-Enein, H. Y., Corveleyn, J. P., Remon, J. P., García-Campaña, A. M., Deprez, P., *J. Chromatogr. A* 2000, *871*, 153–161.

[28] Rodríguez-Gonzalo, E., García-Gómez, D., Carabias-Martínez, R., *J. Chromatogr. A* 2010, *1217*, 40–48.

[29] European Commission, Decision 657/2002/EC, *Off. J. Eur. Commun.* L 221/8 (2002).

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Development and validation of a method for the detection and confirmation of biomarkers of exposure in human urine by means of restricted access material-liquid chromatography–tandem mass spectrometry

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ABSTRACT

The present article describes the development and validation of a LC–MS/MS method for the determination and confirmation of biomarkers of exposure to different types of xenobiotics in human urine. The method combines the use of a restricted access material (RAM) coupled on-line to a LC–IT-MS system; in this way, a rapid and efficient matrix cleanup was achieved, reducing manual sample preparation to freezing and sample filtration. The ion trap (IT) mass spectrometry detector provided the selectivity, sensitivity and ruggedness needed for confirmatory purposes. The on-line RAM-LC–MS/MS method developed here has been validated as a quantitative confirmatory method according to the European Union (EU) Decision 2002/657/EC. The validation steps included the verification of linearity, repeatability, specificity, trueness/recovery, reproducibility, stability and ruggedness in fortified urine samples. Repeatability and within-laboratory reproducibility, measured as intraday and interday precisions, were evaluated at two concentration levels, being 12.7% or below at the concentration corresponding to the quantification limits. Matrix effects and non-targeted qualitative analyses were also evaluated in fortified urine samples. Decision limits (CC_α) and detection capabilities (CC_β) were in the range of 3.6–16.5 and 6.0–28.1 ng mL^{-1} respectively. The results of the validation process revealed that the proposed method is suitable for reliable quantification and confirmation of biomarkers of exposure to xenobiotics in human urine at low ng mL^{-1} levels. In addition, working in Data-Dependent Scan mode the proposed method can be used for the screening of these compounds in urine samples.

1. Introduction

Human health is affected by all the activities of an individual, who is subject to a continuum of chemical exposures in the external environment, including air, water, soil and food. Biological monitoring involves the measurement and evaluation of chemical compounds or their metabolites (biomarkers of exposure) in biological fluids as a method for assessing the risk to health deriving from exposure to a toxic agent [1]. Biomonitoring may be used to assess the exposure (amount absorbed or internal dose) and the effects of chemicals and the susceptibility of individuals, and it may be applied regardless of whether such exposure has been from dietary, environmental or occupational sources. The data gleaned from biological monitoring studies can be used in conjugation with other data in clinical diagnosis, health risk assessment, and for risk management programs. Although many methods for measuring pesticides and industrial chemicals in human matrices have been reported, most of them refer to a limited number of compounds or analytes belonging to the same chemical group. There is an important need for validated methods that will allow the unequivocal identification and quantification of an important variety of xenobiotics of different chemical natures for application in the context of biomonitoring [2].

The analytical methodology used in the biological monitoring of exposure to pesticides and other chemicals is mainly based on chromatographic techniques. The application of liquid chromatography–mass spectrometry (LC–MS) in occupational and environmental toxicology has proved to be a very useful tool in the determination of biomarkers of exposure as well as in metabolism studies aimed at investigating new biomarkers [3,4].

In this context, tandem mass spectrometry (MS/MS) seems to be the most efficient technique for quantification and identification. The MS/MS detector most widely used is the triple quadrupole (QqQ), used in multiple reaction monitoring (MRM) mode [5–7]. However, in MRM mode the qualitative information necessary for full confirmation of analytes is lost at low concentration levels owing to the low sensitivity of the confirmation transition. Since confirmation of identification is insufficient with a single MS/MS

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transition, it is necessary to perform a second injection to confirm the positive samples [8,9]. Use of an ion trap (IT) has great potential as a confirmatory method because it allows a complete spectrum of the product ions to be obtained. Nevertheless, there are few applications in this field [10,11] owing to the low sensitivity of ITs when the number of analytes to be determined is high and to their poor reliability in complex matrices [12]. One good approach for addressing these limitations is by coupling the chromatographic system to an on-line preconcentration step [13]. This coupling also automates prior sample treatment, which in many cases is the limiting step in the analysis time.

Restricted access materials (RAMs) can be satisfactorily used for the on-line pretreatment of biological fluids in the analysis of low-molecular weight substances since they permit liquid–solid extraction and the concentration of small molecules. These materials act by limiting the access of macromolecules to the sorbent by means of a porous membrane [14], thereby achieving a highly efficient cleanup of the biological matrix. Souverain et al. [15] have reviewed a large number of applications in which endogenous and pharmaceutical compounds were purified in different biological matrices, such as plasma, urine, saliva and milk.

The aim of the present work was to develop and validate a sensitive and specific multiresidue method based on LC–IT-MS for the detection and confirmation of a broad variety of biomarkers of exposure to xenobiotics in human urine. The xenobiotics and their respective biomarkers of exposure used were selected on the basis of their importance in environmental studies: several pesticides, such as Carbaryl, one of the most popular domestic insecticides [16] and its metabolite, 1-naphthol; 2,4,5-T and 2,4,5-TP phenoxyacids, which are the herbicides most used domestically [17]; chlortoluron and diuron, which are phenylureas and are designated "priority hazardous substances" by the EU [18] and one of their metabolites, 3-chloro-4-methylphenylurea [19]. Several xenobiotics of industrial origin were also included, such as bisphenol-A and bisphenol-F, which are widely used in the preparation of epoxy resins and polycarbonates [20], trichlorophenol, associated with the always dangerous dioxins [21], and the already mentioned 1-naphthol, a metabolite of polycyclic aromatic hydrocarbons (PAHs) [3]. All these analytes and their metabolites are well known to be excreted in urine after environmental exposure [22–26].

To accomplish this, here we propose a combination of RAMs for a fast on-line sample treatment of the urine samples with the high selectivity of IT-MS, thus affording a rapid, automatic and sensitive multi-residue method that will permit the determination of a broad range of biomarkers of exposure to pesticides and industrial compounds, all fulfilling the requisites concerning full validation and identification legislated by the European Union [27].

In addition, a Data-Dependent Scan procedure for non-targeted qualitative analysis was developed. To our knowledge, this methodology has not been applied to the multiresidue analysis of biomarkers of exposure of very different chemical natures.

2. Experimental

2.1. Chemicals

Analytical standards of *chlortoluron* (CLT), 3-(3-chloro-4-methyl)-1,1-dimethylurea, CAS RN [15545-48-9]; *CMPU* (CMPU), *1*-(3-chloro-4-methylphenyl)urea, CAS RN [13142-64-8]; *diuron* (DIN), 3-(3,4-dichlorophenyl)-1,1-dimethylurea, CAS RN [330-54-1] and *1-naphthol* (1NPL), 1-hydroxynaphthalene, CAS RN [90-15-3] were purchased from Dr. Ehrenstorfer (Augsburg, Germany). *Bisphenol-A* (BPA), 2,2-bis(4-hydroxyphenyl)propane, CAS RN [80-05-7]; *bisphenol-F* (BPF), bis-(4-hydroxyphenyl)methane, CAS RN [620-92-8]; *trichlorophenol* (TCPL), 2,4,5-trichlorophenol, CAS RN [95-95-4]; *2*,4,5-T (245T), 2,4,5-trichlorophenoxyacetic acid, CAS RN [93-76-5] and 2,4,5-TP (245TP), 2-(2,4,5-trichlorophenoxy)propionic acid, CAS RN [93-72-1] were obtained from Sigma–Aldrich (Steinheim, Germany).

The organic solvents—acetonitrile (ACN) and methanol (MeOH)—were of HPLC grade (Merck, Darmstadt, Germany) and were used as received. Ultra-high quality (UHQ) water was obtained with an Elgastat UHQ water purification system.

All chemicals used for the preparation of the buffer and all other chemicals were of analytical reagent grade.

2.2. Instrumentation

HPLC analyses were performed on a HP 1100 Series chromatograph from Agilent (Waldbronn, Germany) equipped with a binary pump, an additional isocratic pump, a membrane degasser, an autosampler (equipped with a 1500-μL capillary seat), a six-port valve, and a diode-array detector (DAD). The system was controlled by a HP ChemStation which also performed data collection from the mass spectrometer and quantitative measurements. The restricted access material (RAM) used was a LiChroCART 25-4LiChrospher RP4 ADS (25 μm, 25 mm × 4 mm) from Merck (Darmstadt, Germany). The analytical column was a 150 × 4.60 mm Luna PFP(2) packed with 3 μm particles (Phenomenex, Torrance, CA, USA).

The clean-up isocratic mobile phase, impelled by the isocratic pump, consisted of a 2.5 mM ammonium formate buffer (pH 2.9) with 10% ACN. The separation mobile phase, impelled by the binary pump, consisted of an unbuffered UHQ water (solvent A) and methanol (Solvent B) gradient from 70% to 0% of A. The analytical column was thermostated at 25 °C.

2.2.1. Mass spectrometry

The LC/MSD Trap XCT ion trap mass spectrometer (Agilent, Waldbronn, Germany) was equipped with an electrospray (ESI) source with a nebulizer spacer. The ESI settings were a capillary voltage of 3500 V; a drying gas flow of 10 L min^{-1} at a temperature of 350 °C, and a nebulizer pressure of 50 psi. Optimization of the ionization and fragmentation parameters was achieved manually while injecting standard solutions of each analyte (5 μg mL^{-1}) with a syringe pump at a flow rate of 1 mL/h; these solutions were mixed with the mobile phase at 0.3 mL/min by means of a T piece. The trap parameters were set at a smart target of 50,000–100,000 and a maximum accumulation time of 200 ms at an m/z range from 60 to 400 u. A narrow isolation width of 4u was selected. The optimized parameters and retention times for each analyte are listed in Table 1.

2.3. Sample collection

Urine samples collected from two healthy volunteers were used for method development and the preparation of calibration standards. Urine samples were collected in 250-mL brown glass bottles and frozen immediately until analysis. Before use, the samples were thawed at room temperature. An appropriate amount of urine was spiked with dilute analytical standards daily. Samples were filtered through 0.45 μm filters to remove precipitated proteins. Creatinine was determined in order to normalize the results with respect to the concentration and to rule out overdiluted or overconcentrated samples, the concentration values proving to be 87.7 and 126.8 mg dL^{-1}.

2.4. On-line sample preparation and HPLC separation

The experimental setup for RAM-LC–MS/MS is shown in Fig. 1, and the schedule for clean-up and chromatographic separation is shown in Table 2. First, a predetermined volume of urine was

Table 1
Ion trap tandem mass spectrometer parameters optimized for the studied biomarkers of exposure.

Analyte	t_R (min)	Quantification transition	Confirmation transition	Fragmentation amplitude (V)	ESI mode	Window
245T	19.8	253 ⇒ 195	255 ⇒ 197	0.70	–	18–25 min
245TP	23.4	267 ⇒ 195	269 ⇒ 197	0.64	–	18–25 min
CMPU	26.4	185 ⇒ 142	185 ⇒ 168	0.98	+	25–27 min
BPF	27.9	199 ⇒ 93	199 ⇒ 123	1.10	–	27–29.5 min
CLT	30.7	211 ⇒ 166	211 ⇒ 140	0.95	–	29.5–33 min
BPA	31.3	227 ⇒ 212	227 ⇒ 133	0.95	–	29.5–33 min
1NPL	32.3	143[a]	–[a]	–[a]	–	29.5–33 min
DIN	33.8	233 ⇒ 72	–[b]	0.70	+	33–34 min
TCPL	36.1	195 ⇒ 159	197 ⇒ 161	0.95	–	34–39 min

[a] No satisfactory fragmentation was found for 1NPL.
[b] Only one satisfactory fragmentation was found for DIN.

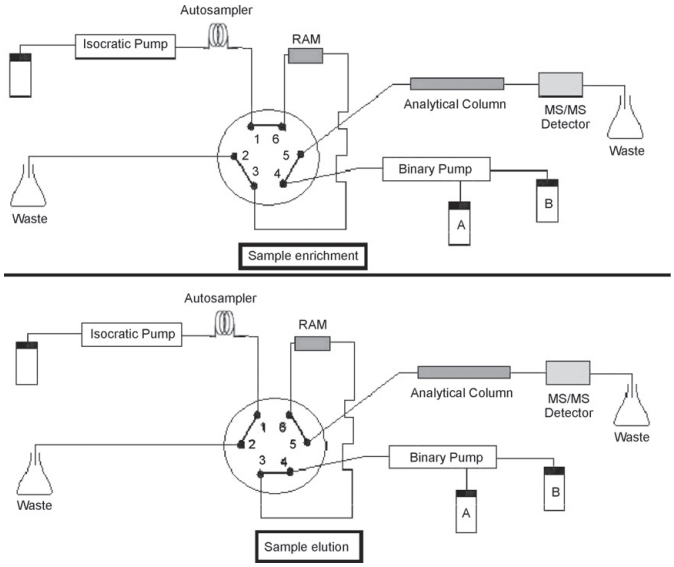

Fig. 1. RAM-LC–MS/MS instrumental setup. Top: switching valve in "sample enrichment" position. Bottom: valve in "sample elution" position (backflush mode).

injected with the autosampler and the isocratic pump was immediately started to pump the clean-up mobile phase at 1 mL min^{-1} for 10 min with the system in the "sample enrichment" position. While the matrix components of the urine were washed to waste, the xenobiotic compounds studied were withheld in the RAM. At 10 min, the system setup was changed to "sample elution" position and the separation gradient (binary pump), shown in Table 2, eluted the analytes at a flow rate of 0.8 mL min^{-1} in backflush mode to the analytical column, where they were separated and finally detected by the mass spectrometer. During this time, the isocratic pump changed the flow rate to 0.3 mL min^{-1} in order to save solvent until the next injection. At 37 min, the separation ended and the gradient was returned to the initial conditions. The flow rate of the isocratic pump was changed to 1 mL min^{-1}. At 39 min, the system was switched to "sample enrichment" mode and a 3-min post-run program was started, keeping the system in the initial conditions, in order to equilibrate the analytical column for the next analysis.

Table 2
Time procedure and valve events of RAM-LC–MS/MS method.

Time (min)	Isocractic pump[a] flow (mL min^{-1})	Binary pump[b,c] % A	Six-port valve position	Event
0–10	1	70	Enrichment	RAM charging and cleanup
10–11	1 ⇒ 0.3	70	Elution	Analyte transfer
11–12	0.3	70 ⇒ 50	Elution	Analyte separation
12–19	0.3	50	Elution	Analyte separation
19–30	0.3	50 ⇒ 30	Elution	Analyte separation
30–35	0.3	30 ⇒ 0	Elution	Analyte separation
35–37	0.3	0	Elution	Analyte separation
37–39	0.3 ⇒ 1	0 ⇒ 70	Elution	Re-equilibrating
39–42	1	70	Enrichment	Equilibrating

[a] Mobile phase: ammonium formate buffer 2.5 mM (pH 2.9) with 10% ACN.
[b] Flow rate: 0.8 mL min^{-1}.
[c] Mobile phase: A: UHQ water B: MeOH.

2.5. Standard preparation and calibration procedure

Individual stock solutions of the analytical standards, at 500 μg mL^{-1}, were prepared by dissolving 12.5 mg of each analyte in 25 mL of acetonitrile. These stock solutions were stored at 4 °C in brown glass bottles. Matrix-matched standards were prepared by adding the appropriate amount of each stock solution to urine. Calibration standards for quantification were prepared in the 10–200 ng mL^{-1} range. The standards were filtered before analysis with a Cameo (0.45 μm Nylon) filter. Calibration curves were obtained by plotting the peak areas of the analytes *versus* concentration using matrix-matched standards.

2.6. Method validation

The method was validated, according to the pertinent legislation [27], by evaluating the following parameters:

- Specificity: by calculating ion suppressions, comparing patterns with matrix-matched samples, and developing a non-targeted analysis.
- Determination of the statistical parameters and the quality of the linear regression, using the transition confirmation. The matrix-matched calibrations (five calibration points) were obtained in the 30–150 ng mL^{-1} range. The decision limit (CC_α) and the detection capability (CC_β) were also calculated.
- Repeatability and within-laboratory reproducibility were determined as intraday and interday precisions by analyzing urine samples at two concentrations levels (10 ng g^{-1} and at the respective limits of quantification).
- The recoveries and RSD% were determined at two concentration levels (25 and 12.5 ng mL^{-1}).
- Ruggedness: evaluating sample preparation by analyzing spiked urine samples before and after freezing and the possible memory effect of the system by analyzing blanks after high-concentration samples.
- Stability: by controlling the storage conditions of the analytes and samples, keeping them in the range in which stability is guaranteed by the manufacturer.

3. Results and discussion

Chromatographic separation was optimized, with the achievement of a satisfactory separation in 27 min on using a water/MeOH gradient. This mobile phase was chosen for the proposed method (Table 2) because it showed greater compatibility with the mass detector [28] than water/ACN mixtures. Additionally, no buffer was employed, since the presence of salts negatively affects the ionization of some analytes in the ESI [29].

3.1. Study of the behavior of the RAM coupled on-line to LC–MS/MS

Liquid chromatography analysis of complex matrices (urine, plasma, etc.) usually requires a previous sample treatment step to eliminate large amounts of interferents from the matrices, which positively affects selectivity and sensitivity and at the same time prolongs columns life and preserves the integrity of the instrumental setup. This step is especially important when mass spectrometry with electrospray (ESI) is used as detector since this source of ionization undergoes severe losses of sensitivity (ion suppression) owing to the presence of interferents. Among the different strategies described to minimize such suppression [30] the following are important: modification of the ionization conditions; the use of a suitable internal standard, usually a stable isotope-labeled ana-

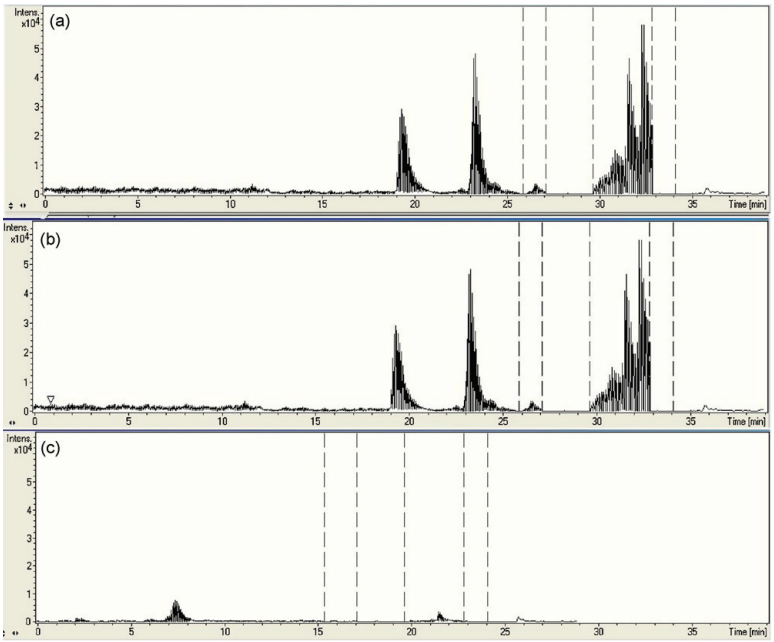

Fig. 2. Total ion chromatograms (TICs) of UHQ standard and urine samples spiked with 100 ng mL^{-1}. (a) UHQ standard, (b) urine injected through the RAM, (c) urine injected directly in the LC–MS/MS system. Dashed lines represent the windows for IT-MS/MS detection.

logue; strong dilution of the sample, or a clean-up step. In our case, we propose a clean-up step using a RAM. Attending to the different chemical natures of the target compounds, the optimization of the behaviour of the RAM column is an important step in the development of the method. The efficiency of the RAM as a clean-up step is mainly affected by the solvent used to eliminate the matrix interferences. The mobile phase impelled by the isocratic pump acts as a washing solvent and at the same time transports the sample through the RAM. The composition of the mobile phase is thus a decisive factor for achieving an efficient washing of the sample matrix with no elimination of the compounds of interest. Different washing solutions based on ammonium formate buffer—ACN mixtures were assayed. It was decided to use one with a composition of 2.5 mM ammonium formate buffer (pH 2.9) −10% ACN, since this was the one offering the best cleaning without producing appreciable losses of the target compounds.

Fig. 2 shows the total ion chromatograms (TICs), the result of summing the signals corresponding to all the analytes, for a standard of 100 ng mL^{-1} in UHQ (Fig. 2a.) and for samples of urine spiked with 100 ng mL^{-1}, injected through the RAM according to the proposed methodology (Fig. 2b) or directly into the analytical columns (Fig. 2c). It may be seen that the signals of the standard in UHQ and the urine sample injected through the RAM are very similar both in shape and in signal intensity. However, when direct injection of urine sample was carried out the signals of all the analytes underwent a strong decrease owing to interferences from the matrix. These observations point to the effectiveness of the proposed clean-up step.

The use of the RAM coupled on-line with the chromatographic system serves not only as a clean-up method but has also been described to be a valid preconcentration step [31]. Accordingly, in order to increase sensitivity, a study was made of the possibility of injecting volumes up to 1500 μL. Fig. 3 shows the normalized signals obtained for three of the analytes (245TP, BPA and TCPL) upon injecting increasing volumes of samples spiked at different concentrations (400, 100 and 10 ng mL^{-1}). It may be seen that the signal becomes saturated upon increasing the volume injected, this effect being especially pronounced in the samples with higher concentrations. Therefore, an injection volume of 100 μL was selected since this was the greatest volume for which there was no signal saturation in the concentration range studied (3–200 ng mL^{-1}). So, to study the possible preconcentration achieved for each analyte upon injecting 100 μL, UHQ water samples spiked with 100 and 20 ng mL^{-1} were injected through the RAM and also injected directly into the LC–MS/MS system. At both levels, the peaks areas of the analytes obtained through RAM and directly were not significantly different, indicating that no analyte preconcentration was occurring in the RAM. This was probably due to the low sample volume injected (100 μL).

3.2. Evaluation of matrix effects

Quantitative determinations using ESI-MS/MS for detection may be affected by the ion suppression that occurs in the electrospray device, mainly due to the co-elution of matrix interferents with the analytes. This suppression can be calculated via equation [13]: ion suppression (%) = $A_s - (A_{su} - A_{usu})/A_s$, where A_s is the analyte peak area in spiked UHQ water; A_{su} is the analyte peak area in urine, and A_{usu} is the analyte peak area, if present, in unspiked urine. Ion suppression was calculated for each analyte, both in direct injection mode and with the proposed instrumental configuration, RAM-LC–MS/MS (Table 3), obtaining values from 77.7% to 99.6% for direct injection and between 13% and 89% for the proposed method. Very high degrees of ion suppression were observed in the case of direct injection of urine samples, which to a large extent was corrected with the use of the RAM. The trend shown

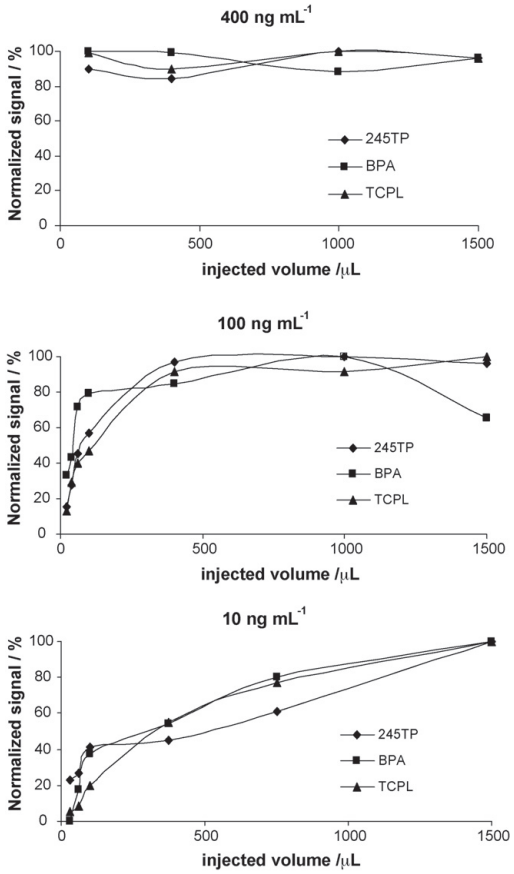

Fig. 3. Influence of the injected urine on the RAM-LC–MS/MS. Analyte response expressed as the signal normalized to the largest peak (%). Urine samples spiked at 400, 100 and 10 ng mL^{-1}, from top to bottom. Analyte identification as seen from Section 2.1.

by these values does not seem to be related to the retention times and hence to the polarity of the analytes; this relationship has been described for other matrices [13]. The use of the RAM-LC–MS/MS configuration afforded a marked decrease in ion suppression but it still persisted, rendering quantification via an external standard inadequate. Therefore, calibration was performed with the matrix-matched method. Fig. 4 compares the calibrations carried out with an external standard in UHQ water and in matrix-matched mode. It may be seen that in fortified urine, sensitivity was decreased, but that this did not affect linearity.

3.3. Limits of detection (LODs) and quantification (LOQs) of the RAM-LC–MS/MS method

Starting out from the optimized parameters, a study was made of the analytical characteristics of the method. Linear relationships were established between the peak areas and the concentration of the analytes for the range studied (10–200 ng mL^{-1}). Table 4 shows the analytical characteristics of the proposed method.

The limits of detection were determined working with the ion trap (IT) in Multiple Reaction Monitoring (MRM) mode, isolat-

Table 3
Ion suppressions for the biomarkers studied obtained by analyzing spiked urine samples.

	Analyte	245T	245TP	CMPU	BPF	CLT	BPA	1NPL	DIN	TCPL
Ion suppression (%)	Directly in LC–MS/MS	88.5	99.6	97.7	77.7	82.7	97.8	–[a]	90.1	72.2
	RAM-LC–MS/MS	40.5	27.3	89.4	43.3	16.4	64.3	36.3	59.1	12.7

[a] No signal was found for 1NPL when urine was directly injected.

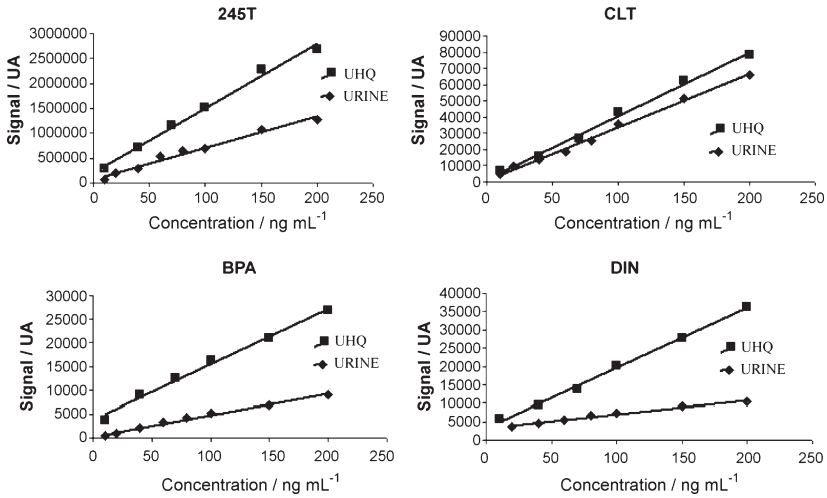

Fig. 4. Comparison of calibration curves for UHQ standards and fortified urine samples. Analyte identification as seen from Section 2.1.

ing the most abundant fragmented ion (quantification transition) and setting six different windows, as shown in Table 1. The chromatograms of the urine samples spiked with 10 ng mL^{-1} and the noise generated by an unspiked urine sample are shown in Fig. 5. The limits of detection were calculated as the concentration for which a signal-to-noise ratio of 3 would be obtained in a real sample. The values thus obtained for all the analytes are shown in Table 4, and they range between 0.2 ng mL^{-1} for 245T and 245TP, and 3.2 ng mL^{-1} for BPF, with the exception of DIN, which had a limit of detection of 15.5 ng mL^{-1}, probably due to the important difference in the mass/charge ratio between the precursor ion and the product ion (233 ⇒ 72). Likewise, the limits of quantification were calculated, in real samples, as the concentration for which a signal-to-noise ratio of 10 would be obtained (Table 4). In this case, values between 3 and 10 ng mL^{-1} were obtained, again with the exception of DIN which had a limit of 50 ng mL^{-1}.

3.4. Method validation

As a confirmatory criterion, the EU Decision suggests the use of at least three points of identification [27]. One of the greatest advantages of tandem mass spectrometry is that it is possible to record a complete spectrum of product ions, thus being able to analyze both the quantification transition and that of confirmation from a single injection, which ensures the presence of at least one precursor and two product ions. With the proposed methodology, four identification points were achieved (one precursor and two products) for CMPU, BPF, CLT and BPA, and five (two precursors and two products) for 245T, 245TP and TCPL. However, for DIN and 1NPL it was not possible to find more than 2.5 (one precursor and one product) and 1 points of identification (one precursor), respectively, such that they were removed from the validation step. This is due to the poor fragmentation that these analytes present by collision-inducted dissociation (CID) in the ion trap.

Table 4
Analytical characteristics of RAM-LC–MS/MS method calculated by the analysis of fortified urine samples.

Analyte	Intercept (area units (au))	Slope (au ng^{-1} mL)	LOD[c] (ng mL^{-1})	LOQ[d] (ng mL^{-1})
245T[a]	$(8.1 \pm 8.5) \times 10^4$	$(6.3 \pm 0.8) \times 10^3$	0.2	3
245TP[a]	$(0.2 \pm 1.3) \times 10^5$	$(1.2 \pm 0.1) \times 10^4$	0.2	3
CMPU[a]	$(2.6 \pm 2.1) \times 10^2$	(28 ± 2)	3.0	10
BPF[a]	$(-0.8 \pm 1.4) \times 10^2$	(19 ± 1)	3.2	10
CLT[a]	$(1.2 \pm 2.6) \times 10^3$	$(3.3 \pm 0.3) \times 10^2$	2.0	10
BPA[a]	$(3.6 \pm 3.9) \times 10^2$	(44 ± 4)	1.4	5
1NPL[a]	$(1.1 \pm 1.0) \times 10^5$	$(8.6 \pm 1.0) \times 10^3$	0.7	5
DIN[b]	$(3.4 \pm 0.5) \times 10^3$	(37 ± 5)	15.5	50
TCPL[a]	$(2.4 \pm 2.9) \times 10^3$	$(4.1 \pm 0.5) \times 10^2$	2.6	10

[a] Concentration range from 10 to 200 ng mL^{-1}.
[b] Concentration range from 20 to 200 ng mL^{-1}.
[c] Limit of detection (LOD) for a signal-to-noise ratio of 3 from quantification transition in MRM mode.
[d] Limit of quantification (LOQ) for a signal-to-noise ratio of 10 from quantification transition in MRM mode.

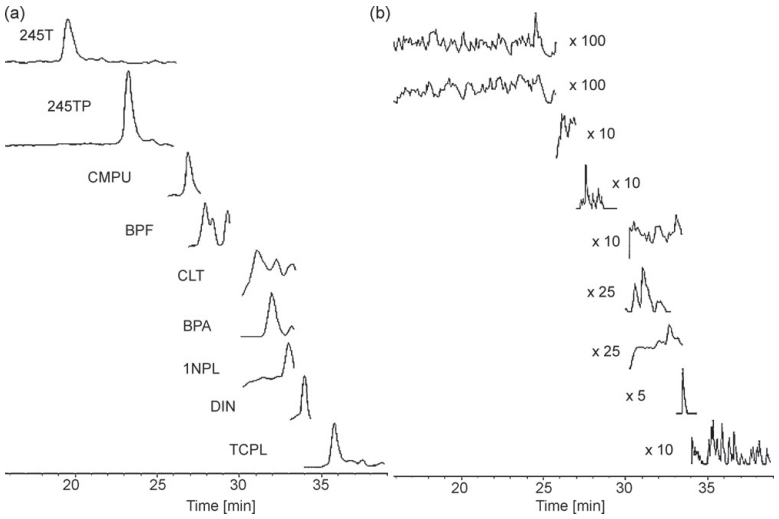

Fig. 5. LC–MS/MS chromatograms of: (a) a urine sample spiked at 10 ng mL^{-1} (except from DIN at 20 ng mL^{-1}) and (b) a blank urine sample (magnified). Analyte identification as seen from Section 2.1.

3.4.1. Calibration curves, decision limits and detection capabilities

Two analytical limits are recommended in the European Decision 657/2002/EC: the decision limit (CC_α), which is defined as "the lowest concentration level of the analyte that can be detected in a sample with a chance of 1% of a false positive decision", and the detection capability (CC_β), which is "the smallest content of the analyte that can be detected in a sample with a chance of 5% of a false negative decision" [27].

To determine these limits, urine samples were studied in MRM mode, recording the full spectrum of product ions. Then, after verifying the existence of both transitions (quantification and confirmation transitions) and ratios according to the EU criteria, an extracted ion chromatogram (EIC) was made of the ion that was the product of the confirmation transition. Thus a calibration of five points ranging between 30 and 150 ng mL^{-1} was built; its characteristics are shown in Table 6. CC_α and CC_β were estimated using the calculations described by Verdon et al. [32]. The values calculated are shown in Table 6. It may be seen that the CC_α values varied between 3.6 ng mL^{-1} for 245T and 16.5 ng mL^{-1} for BPA, and the CC_β values between 6.0 ng mL^{-1} for 245T and 28.1 ng mL^{-1} for BPA.

3.4.2. Repeatability and reproducibility

Keeping the same instrumental configuration as that used to calculate the LODs, the repeatability and reproducibility of the proposed method were evaluated. To accomplish this, urine samples spiked with two different concentrations were analyzed: first at 10 ng mL^{-1} of each analyte, and then with each analyte spiked at its limit of quantification (LOQs, Table 4). Repeatability, as intraday precision, was assessed with eight injections performed on the same day and reproducibility, as interday precision, was determined by analyzing injections (eight each day) carried out over three consecutive days. The values of the different precisions are shown in Table 5. The lowest values were obtained for the sample spiked with 10 ng mL^{-1} in the intraday assay, with precisions between 1.0% for 245T and 5.1% for CLT. The highest values were found upon evaluating the interday precision in samples spiked at the limit of quantification, values of between 7.1% for CMPU and 11.5% for BPA being recorded.

3.4.3. Recoveries

As an additional analysis to validate the method, urine samples that had not been used previously in the method were analyzed. These samples were spiked at two levels: 12.5 and 25 ng mL^{-1}. The signal obtained for each of the analytes in MRM mode by measuring the quantification transition was introduced into the corresponding calibration. For both samples, the results shown in Table 6 were obtained. In the case of urine spiked with 25 ng mL^{-1}, the precisions varied between 7.7% for CLT and 12.5% for 245TP. For the urine spiked with 12.5 ng mL^{-1} they varied between 8.3% for 245T and 18.2% for CLT. In all cases, satisfactory recoveries were obtained.

3.4.4. Ruggedness

Only sample pretreatment was investigated because the LC–MS device was regularly subjected to checks and maintenance, ensuring its ruggedness [33]. Additionally, the method developed is characterized by involving minimum sample pretreatment, such that it is only necessary to evaluate the spiking of the samples

Table 5
Repeatability, evaluated as intraday precision, and within-laboratory reproducibility, evaluated as interday precision, of developed RAM-LC–MS/MS method obtained by analyzing spiking urine samples.

Analyte	RSD[a] (%)			
	Intraday[b] at 10 ng mL^{-1}	Interday[c] at 10 ng mL^{-1}	Intraday[b] at LOQ	Interday[c] at LOQ
245T	1.0	3.1	5.2	9.6
245TP	1.1	4.2	6.3	8.8
CMPU	2.5	7.1	2.5	7.1
BPF	4.9	11.3	4.9	11.3
CLT	5.1	10.4	5.1	10.4
BPA	4.0	8.4	5.3	11.5
TCPL	3.9	10.1	3.9	10.1

[a] RSD, relative standard deviation.
[b] Intraday precision (repeatability) was determined by eight injections.
[c] Interday precision (reproducibility) was determined in three consecutive days (eight injections each day).

Table 6
Statistic and performance characteristics of the proposed RAM-LC–MS/MS method obtained by analyzing fortified urine samples[a].

Analyte	Intercept (au)	Slope (au ng^{-1} mL)	r^2	CC_α (ng mL^{-1})	CC_β (ng mL^{-1})	Recoveries	
						At 25 ng mL^{-1}	At 12.5 ng mL^{-1}
245T	$(-0.09 \pm 3.02) \times 10^4$	$(4.61 \pm 0.03) \times 10^3$	0.999	3.6	6.0	25 ± 3	12 ± 1
245TP	$(-0.2 \pm 1.1) \times 10^5$	$(1.1 \pm 0.1) \times 10^4$	0.999	5.0	8.6	24 ± 3	11 ± 2
CMPU	$(1.8 \pm 2.2) \times 10^2$	(8.3 ± 2.3)	0.996	14.4	24.5	23 ± 2	12 ± 2
BPF	$(-0.7 \pm 1.6) \times 10^2$	(8.6 ± 1.7)	0.998	10.3	17.5	27 ± 3	12 ± 2
CLT	$(5.7 \pm 4.4) \times 10^3$	$(1.6 \pm 0.5) \times 10^2$	0.996	15.2	25.9	26 ± 2	11 ± 2
BPA	$(-0.4 \pm 5.2) \times 10^2$	(17.1 ± 5.4)	0.995	16.5	28.1	25 ± 3	14 ± 2
TCPL	$(-0.3 \pm 4.5) \times 10^3$	$(2.7 \pm 0.5) \times 10^2$	0.998	9.0	15.4	24 ± 3	13 ± 2

[a]Concentration range from 30 to 150 ng mL^{-1} (five calibration points).

because this is performed after freezing and must be compared with samples spiked prior to freezing, in the sense that this latter situation would correspond to a real contaminated sample.

Two urine samples spiked at 100 ng mL^{-1} were prepared and subjected to freezing before and after spiking, respectively. The signal of each of the analytes was analyzed, and it was concluded that there were no significant differences in any case, such that it can be affirmed that spiking before or after freezing does not affect the results of the method.

One of the disadvantages of the use of on-line preconcentration with the same sorbent for different samples is possible contamination among them. This may be especially relevant when RAMs are used, because they have a long half-life. In order to study such a possibility, a high (1500 μL) volume of a high-concentration (200 ng mL^{-1}) sample of urine was analyzed. Following this, a blank of UHQ water was injected and no signal was observed for any of the analytes. Accordingly, the washing to which the RAM had been subjected was effective, since even under the most unfavourable conditions the system did not display carryover.

3.5. Non-targeted screening analysis

All the studies performed using the proposed method led to the presetting of the most suitable parameters for the quantification of each analyte (Table 1). In order to check that the method can be used for screening purposes, it is necessary to check its ability to detect any other type of analyte that has not been preset in the chromatographic program. To accomplish this, the Data-Dependent Scan mode (called "Auto Ms(n)" in the software used [34]) was implemented. This mode does not require the presetting of any type of parameter characteristics of the analytes (transitions, windows, etc.). The detector performs a mass scan from 60 to 400 u and, when the signal surpasses a given threshold, it starts the fragmentation, recording the whole MS/MS spectrum. The optimum fragmentation amplitude was determined empirically in an automatic way as the voltage, between 0.3 and 2.0 V, that reduced the signal of the precursor ion to 10%. Working in this mode, with a signal threshold of 150,000 ua, samples were analyzed in UHQ water—from 10 to 200 ng mL^{-1}—and in real matrices using urine spiked at 0–1000 ng mL^{-1}. In all cases we analyzed whether each of the analytes was detected, taking as a criterion: (i) whether fragmentation would begin or not and (ii) whether the characteristic MS/MS spectrum of the analyte would match the home-made library reference, (iii) observing a signal/noise ratio greater than 3. In that conditions a minimum of four identification points was assured. Fig. 6 shows the chromatogram and the spectrum obtained for a urine sample spiked with 100 ng mL^{-1} of 245T (Fig. 6b) and the corresponding chromatogram and spectrum of the standard (Fig. 6a). All the results thus obtained are shown in Table 7. It may be seen that there are no false negatives and that the identification was correct for all the analytes up to a concentration level of 40 ng mL^{-1} in UHQ and 60 ng mL^{-1} in urine; these limits were improved in the case of 245T and 245TP up to levels of 10 and 20 ng mL^{-1} respectively. Such results suggest the use of Data-Dependent Scan as a general screening method for the detection of low-molecular weight xenobiotics in urine.

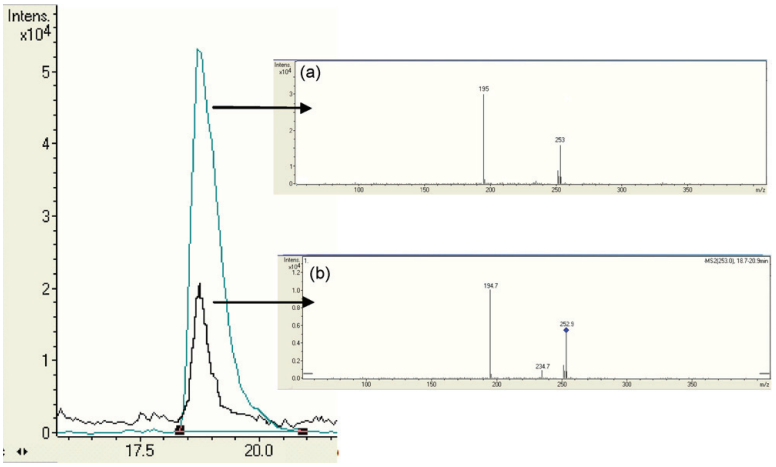

Fig. 6. Non-targeted qualitative analysis: 245T MS/MS chromatograms and spectra obtained from (a) an UHQ standard used as home-made library and (b) a urine sample spiked at 100 ng mL^{-1}.

Table 7
Performance of proposed non-targeted screening analysis for fortified UHQ water and urine samples[a].

Analyte	245T	245TP	CMPU	BPF	CLT	BPA	1NPL	DIN	TCPL
Precursor ion	253	267	185	199	211	227	143	233	195
Product ion	195	195	142	93	166	212		72	159
UHQ ($ng\,mL^{-1}$)									
10	♦	♦	▽	▽	▽	▽	▽	▽	▽
40	♦	♦	♦	♦	♦	♦	▽	▽	♦
100	♦	♦	♦	♦	♦	♦	▽	♦	♦
200	♦	♦	♦	♦	♦	♦	▽	♦	♦
Urine ($ng\,mL^{-1}$)									
Blank	▽	▽	▽	▽	▽	▽	▽	▽	▽
10	▽	▽	▽	▽	▽	▽	▽	▽	▽
20	♦	♦	▽	▽	▽	▽	▽	▽	▽
40	♦	♦	▽	♦	♦	▽	▽	▽	♦
60	♦	♦	♦	♦	♦	♦	▽	♦	♦
80	♦	♦	♦	♦	♦	♦	▽	♦	♦
100	♦	♦	♦	♦	♦	♦	▽	♦	♦
150	♦	♦	♦	♦	♦	♦	▽	♦	♦
200	♦	♦	♦	♦	♦	♦	▽	♦	♦
1000	♦	♦	♦	♦	♦	♦	♦	♦	♦

[a] ♦: Identified analyte; ▽: unidentified analyte.

4. Conclusions

The on-line configuration developed, incorporating a RAM, means that sample treatment is minimum (freezing and filtration); this has a positive effect on precision and affords a shorter analysis time. The configuration has advantages such as automation, high sensitivity and an important reduction in ion suppression. Another advantage is cost, since the half-life of the RAM allowed the injection of at least 190 samples of urine, involving a total volume 20.7 mL of urine. The method described, RAM-LC–MS/MS, is a sensitive, selective, and precise automated tool for the determination and confirmation of several different biomarkers of exposure to xenobiotic compounds in a complex matrix such as human urine. The method has been validated as a quantitative confirmatory method in accordance with the Commission Decision 2002/657/EC. Additionally, IT-MS/MS detection provided high sensitivity and selectivity, allowing limits of detection to be achieved at the low $ng\,mL^{-1}$ level. The method is therefore appropriate for application within the context of the biological monitoring of these biomarkers and as a general screening method.

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References

[1] R.L. Zielhuis, Am. J. Ind. Med. 8 (1985) 515.
[2] L.L. Needham, A.M. Calafat, D.B. Barr, Int. J. Hyg. Environ. Health 210 (2007) 229.
[3] D.B. Barr, L.L. Needham, J. Chromatogr. B 778 (2002) 5.
[4] C. Aprea, C. Colosio, T. Mammone, C. Minoia, M. Maroni, J. Chromatogr. B 769 (2002) 191.
[5] X. Ye, Z. Kukenyik, L.L. Needham, A.M. Calafat, Anal. Chem. 77 (2005) 5407.
[6] M. Kellert, K. Scholz, S. Wagner, W. Dekant, W. Völkel, J. Chromatogr. A 1131 (2006) 58.
[7] R. Tuyttem, F. Lemière, W.V. Dongen, E. Witters, E.L. Esmans, R.P. Newton, E. Dudley, Anal. Chem. 80 (2008) 1263.
[8] F. Hernandez, M. Ibañez, J.V. Sancho, O.J. Pozo, Anal. Chem. 76 (2004) 4349.
[9] A.A. Stolker, W. Niesing, E.A. Hogendoorn, J.F. Versteegh, R.U.A. Fuchs, Th. Brinkman, Anal. Bioanal. Chem. 378 (2004) 955.
[10] R.L. Fitzgerald, J.D. Rivera, D.A. Herold, Clin. Chem. 45 (1999) 1224.
[11] F. Hernandez, J.V. Sancho, M. Ibañez, C. Guerrero, Trends Anal. Chem. 26 (2007) 466.
[12] V. Andreu, Y. Pico, Anal. Chem. 76 (2004) 2878.
[13] J. Feitosa-Felizzola, B. Temine, S. Chiron, J. Chromatogr. A 1164 (2007) 95.
[14] P. Sadílek, D. Satinsky, P. Solich, Trends Anal. Chem. 26 (2007) 375.
[15] S. Souverain, S. Rudaz, J.L. Veuthey, J. Chromatogr. B 801 (2004) 141.
[16] WHO, 1994 WHO, Environmental Health Criteria 153. Carbaryl, World Heatlh Organization, Geneva (1994).
[17] T. Kiely, D. Donaldson, A. Grube, US Enviromental Protection Agency Pesticide Industry Sales and Usage 2000 and 2001 Market Estimates, 2004.
[18] European Commision Decision 2455/2001/EC. Off. J. Eur. Commun. L 331/1 (2001).
[19] R. Carabias-Martínez, E. Rodríguez-Gonzalo, P. Revilla-Ruiz, J. Domínguez-Álvarez, J. Chromatogr. A 990 (2003) 291.
[20] S.K. Jana, T. Okamoto, T. Kugita, S. Namba, Appl. Catal. A 288 (2005) 80.
[21] J.J. Collins, K. Bodner, S. Haidar, M. Wilken, C.J. Burns, L.L. Lamparski, R.A. Budinsky, G.D. Martin, M.L. Carson, Chemosphere 73 (2008) 284.
[22] R. Bravo, L.M. Caltabiano, C. Fernández, K.D. Smith, M. Gallegos, R.D. Whitehead Jr., G. Weerasekera, P. Restrepo, A.M. Bishop, J.J. Perez, L.L. Needham, D.B. Barr, J. Chromatogr. B 820 (2005) 229.
[23] D.B. Barr, J. Chem. Health Saf. 15 (2008) 20.
[24] P. Panuwet, T. Prapamontol, S. Chantara, D.B. Barr, Int. J. Hyg. Environ. Health 212 (2009) 288.
[25] W. Völkel, T. Colnot, G.A. Csanady, J.G. Filser, W. Dekant, Chem. Res. Toxicol. 15 (2002) 1281.
[26] F. Onyemauwa, S.M. Rappaport, J.R. Sobus, D. Gajdosová, R. Wu, S. Waidyanatha, J. Chromatogr. B 877 (2009) 1117.
[27] European Commission Decision 657/2002/EC. Off. J. Eur. Commun. L 221/8 (2002).
[28] R.F. Straub, R.D. Voyksner, J. Am. Soc. Mass Spectrom. 4 (1993) 578.
[29] R. Kostiainen, T.J. Kauppila, J. Chromatogr. A 1216 (2009) 685.
[30] S. Wang, M. Cyronak, E. Yang, J. Pharm. Biomed. Anal. 43 (2007) 701.
[31] K.S. Boos, C.T. Fleischer, Fresen. J. Anal. Chem. 371 (2001) 16.
[32] E. Verdon, D. Hurtaud-Pessel, P. Sanders, Accredit. Qual. Assur. 11 (2006) 58.
[33] R. Zeleny, S. Harbeck, H. Schimmel, J. Chromatogr. A 1216 (2009) 249.
[34] LC/MSD Trap software (6.0). Agilent Technologies Inc.

3.B. DETERMINATION OF BIOMARKERS OF EFFECT BY ZWITTERIONIC HYDROPHILIC LIQUID CHROMATOGRAPHY

RESEARCH ARTICLE VI

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Study of retention behaviour and mass spectrometry compatibility in zwitterionic hydrophilic interaction chromatography for the separation of modified nucleosides and nucleobases

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ABSTRACT

A study has been made of the chromatographic behaviour of modified nucleosides and nucleobases using different stationary phases with functional groups of polar nature, all of them compatible with aquoorganic mobile phases. The stationary phases assayed were a pentafluorophenylpropyl (PFP) column for reverse phase separation, and another two for hydrophilic interaction chromatography (HILIC) separation. Six modified nucleosides and nucleobases (hydroxylated and methylated derivatives) were chosen as the target analytes. In the study, chromatographic resolution as well as the sensitivity in detection by mass spectrometry were taken into account. The results obtained showed that the zwitterionic (ZIC-HILIC) column was the most suitable one for the separation of these analytes. From the study of the different parameters affecting separation it may be concluded that in the ZIC-HILIC column separation is based on a mechanism of partition and interaction through weak electrostatic forces.

1. Introduction

Oxidative stress is defined as cellular damage caused by reactive oxygen species (ROS). These species are continually being produced as a result of the cell's metabolism and their production may be increased as a result of exposure to xenobiotics or to radiation [1]. ROS interact with and modify several biomolecules, especially DNA, lipids and proteins [1]. DNA lesions are of special importance because they can alter the sequence of nucleotides. The natural mechanisms for DNA repair involve the excision of the damaged bases or nucleosides [2]. The products thus released cannot be reused and circulate freely through the blood until they are eliminated in the urine [3]. Additionally, the action of hydrolytic enzymes such as ribonucleases and phosphatases releases normal and modified nucleosides during RNA turnover [4], a process that is enhanced by the presence of different types of tumours [5]. Thus, modified nucleosides and nucleobases (MNN) arise mainly from the post-transcriptional modification of the nucleosides forming transfer RNA (tRNA) [6]. Around 100 MNN related to these metabolic processes have been detected to date [7].

Studies have addressed the potential of MNN as markers of several diseases [8,9], and, mainly, as early markers of different types of cancer [10–14]. Other studies have related the urinary concentration of MNN to different life styles [15].

Among the most widely studied MNN are the methylation or hydroxylation products of guanosine; this is because they are the most abundant [16–18]. Simultaneous determination of both groups of MNN (hydroxylated and methylated) is of great interest since the mechanisms of mutagenesis and carcinogenesis brought about by the oxidation and methylation processes are different [19].

The analytical techniques most widely employed for the simultaneous determination include capillary electrophoresis [20], immunoassay [21] and, mainly, liquid chromatography (HPLC) [22]. UV detection [23] has been replaced by mass spectrometry (MS) [13,24], which allows a surer identification and confirmation as well as affording structural information about the compounds being assayed.

The analysis of highly polar compounds, such as MNN, involves important difficulties in reverse phase liquid chromatography (RPLC) owing to the poor retention of these analytes in the stationary phase. Normal phase liquid chromatography (NPLC) would afford a better retention but is not desirable owing to the difficulty involved in coupling this chromatographic technique with mass spectrometry. Hydrophilic interaction chromatography (HILIC) is a viable alternative to RPLC for the separation of polar compounds. HILIC, a term coined by Alpert [25], is based on the use of polar stationary phases combined with mobile phases with a high organic content and a small amount of water. In addition, HILIC has the

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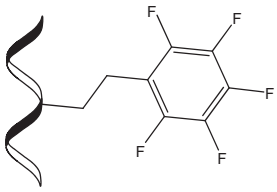
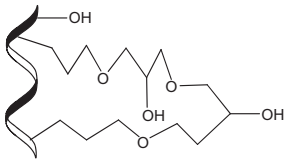
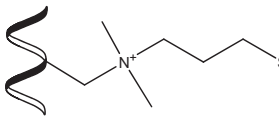
Table 1
Characteristics of the chromatographic columns used for the analysis of the modified nucleosides and nucleobases.

Chromatographic column	Dimensions (mm)	Pore size (Å)	Particle size (μm)	Stationary phase
Luna PFP(2)	150 × 4.6	100	3	 Pentafluorophenylpropyl
Luna HILIC	150 × 4.6	200	3	 Cross-linked diol
ZIC-HILIC	150 × 4.6	100	3.5	 Sulfobetaine

advantage of enhanced detection sensitivity when used in conjunction with MS due to the high organic content of the mobile phase, which allows efficient spraying and desolvation in electrospray ionization (ESI). The applications of HILIC include the analysis of different polar molecules, especially those of biological nature [26]. In recent years, new possibilities have emerged with the development of stationary phases specific to HILIC, such as zwitterionic hydrophilic interaction chromatography (ZIC-HILIC) columns [27].

The aim of the present work was to study the chromatographic behaviour shown by different MNN, compounds of similar and very high polarity, using a pentafluorophenylpropyl (PFP) reverse phase column and two polar columns based on cross-linked diol (Luna-HILIC) and sulfobetaine (ZIC-HILIC). The MNN selected were 8-hydroxy-guanine, 8-hydroxy-guanosine, 8-hydroxy-2′-deoxyguanosine, 1-methyl-guanine, 7-methyl-guanine and 9-methyl-guanine. The ZIC-HILIC column showed the highest selectivity. The optimum conditions were chosen bearing in mind resolution and sensitivity in later detection by means of mass spectrometry. Using this column, a detailed study was performed to elucidate the mechanisms involved in the retention of the analytes.

In sum, this study explores the benefits and limitations of using HILIC for the analysis of MNN. To our knowledge, no other work has attempted to study the mechanisms of retention, for the separation of MNN using ZIC-HILIC.

2. Experimental

2.1. Chemicals

Analytical standards of *8-hydroxy-guanine* (8OHGua), CAS RN [5614-64-2] and *8-hydroxy-guanosine* (8OHG), CAS RN [3868-31-3] were purchased from Cayman Chemical (Michigan, USA). *8-hydroxy-2′-deoxyguanosine* (8OH2dG), CAS RN [88847-89-6]; *9-methyl-guanine* (9mGua), CAS RN [5502-78-3]; *1-methyl-guanine* (1mGua), CAS RN [938-85-2] and *7-methyl-guanine* (7mGua), CAS RN [578-76-7] were obtained from Sigma–Aldrich (Steinheim, Germany).

The organic solvents – acetonitrile (ACN) and methanol (MeOH) – were of HPLC grade (Merck, Darmstadt, Germany) and were used as received. Ultra-high quality (UHQ) water was obtained with a Wasserlab (Noain, Spain) Ultramatic water purification system. All other chemicals were of analytical reagent grade.

2.2. Instrumentation

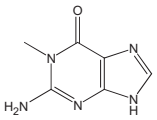
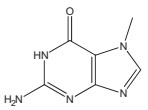
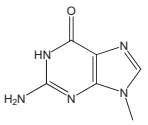
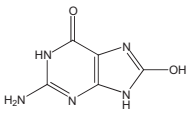
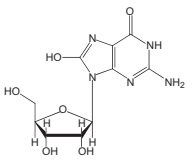
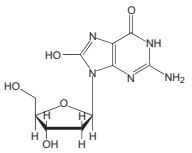
HPLC analyses were performed on a HP 1100 Series chromatograph from Agilent (Waldbronn, Germany). The Diode Array Detector (DAD) recorded the spectra in the 190–400 nm range. The Agilent single quadrupole LC/MSD SL mass spectrometer was equipped with an electrospray (ESI) source. The analytical columns were a reverse phase Luna PFP(2) packed with 3 μm particles and a Luna HILIC packed with 3 μm particles from Phenomenex (Torrance, CA, USA) and a ZIC-HILIC packed with 3.5 μm particles from Merck (Darmstadt, Germany). The characteristics of the chromatographic columns used are shown in Table 1. $_{w}^{w}$pH values were determined with a 691 pH Meter from Metrohm (Herisau, Switzerland).

2.3. Preparation of standards

The initial stock solutions for the 1mGua, 7mGua, 9mGua (500 μg mL^{-1}) and for the 8OH2dG and 8OHG standards (150 μg mL^{-1}) were prepared in acidified UHQ water. The stock solution for 8OHGua (150 μg mL^{-1}) was prepared in 0.1 M sodium hydroxide. These stock solutions were stored at 4 °C in brown glass bottles. Working solutions were prepared daily at 5 μg mL^{-1} for 1mGua, 7mGua and 9mGua, and 3 μg mL^{-1} for 8OH2dG, 8OHG and 8OHGua.

The mobile phase was a mixture of organic solvent and an aqueous medium at different pH values and with different concentrations of salts. To measure $_{w}^{w}$pH values, the pH meter was calibrated using aqueous buffers and pH measurements were performed before the addition of organic solvent. The apparent $_{w}^{s}$pH, after adding the organic solvent, was not measured.

Table 2
Physical properties of the modified nucleosides and nucleobases analyzed.

Modified nucleosides and nucleobases	Mw	$\log D^{a}_{pH\,3}$	Structure
1-Methyl-guanine (1mGua)	165.07	−1.4	
7-Methyl-guanine (7mGua)	165.07	−1.8	
9-Methyl-guanine (9mGua)	165.07	−1.4	
8-Hydroxy-guanine (8OHGua)	167.04	−1.5	
8-Hydroxy-guanosine (8OHG)	299.09	−1.9	
8-Hydroxy-2′-deoxy-guanosine (8OH2dG)	283.09	−1.3	

[a] Calculated using ChemBioDraw Ultra 12.0 from CambridgeSoft by Crippen's and Viswanadhan's fragmentations and Advanced Chemistry Development (ACD/Labs) software.

3. Results and discussion

The high number of known MNN (approx. 100) means that some of them must be selected as a model to study their behaviour in HILIC. In the present case, we chose hydroxylated and methylated forms because these are the commonest modifications. As the base structure, we chose guanine, the most studied and abundant base as regard a possible relationship between the modified nucleosides and different diseases. Some of the physical and chemical properties of the six MNN studied are shown in Table 2. The pK_a values reported in the literature for these compounds lie within a broad interval. This is because the nucleobases and their derivatives present multiple tautomers with different site-specific microscopic pK_a values. Thus, values of 3.1–3.3 for guanine and values of −0.1 to 0.2 for hydroxylated-MNN were found [28,29]. In addition, pK_a values were experimentally calculated by UV spectrophotometry [30], measuring absorbance at different $_w{}^w$pH values in a 80% ACN: 20% H_2O medium; values of 3.2, 3.3 and 3.9 for 7mGua, 9mGua and 1mGua, respectively, were found. However, it was not possible to determine the pK_a values for the hydroxylated-MNN.

3.1. Reversed phase chromatography and HILIC for the separation of modified nucleosides and nucleobases

Using the three chromatographic columns indicated in Section 2, a study was made of the chromatographic behaviour of the six analytes selected (Fig. 1).

A mobile phase with an aqueous phase of 90% in RPLC (Fig. 1a$_1$) did not afford a satisfactory separation of the analytes, retention being almost completely absent ($k < 0.8$) for all the MNN.

It was necessary to increase the aqueous phase up to 95% (Fig. 1a$_2$) to obtain significant retentions, and even under these conditions the coelution of two sparingly retained analytes occurred ($0.2 < k < 2.2$).

When a Luna HILIC column was used employing 20% formic acid 2.6 mM ($_w{}^w$pH = 3.1): 80% ACN, the analytes exhibited greater retention than in RPLC ($k < 1.2$), but the resolution was not satisfactory (Fig. 1b$_1$). A decrease in the proportion of aqueous solvent to 10% (Fig. 1b$_2$) allowed appropriate retentions to be obtained ($1.6 < k < 3.0$), although two pairs of MNN coeluted.

The ZIC-HILIC column with a mobile phase containing 20% of aqueous phase (Fig. 1c) afforded suitable retentions ($1.6 < k < 3.2$).

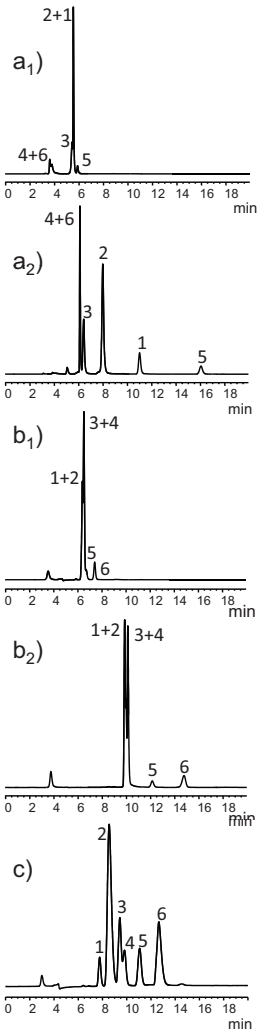


Fig. 1. UV chromatograms obtained by the analysis of the modified nucleosides and nucleobases using isocratic elution. By RPLC using 90% formic acid 2.6 mM $_w^w$pH = 3.1: 10% ACN (a_1) and 95% formic acid 2.6 mM $_w^w$pH = 3.1: 5% ACN (a_2). By Luna HILIC using 20% formic acid 2.6 mM $_w^w$pH = 3.1: 80% ACN (b_1) and 10% formic acid 2.6 mM $_w^w$pH = 3.1: 90% ACN (b_2). By ZIC-HILIC using 20% formic acid 2.6 mM $_w^w$pH = 3.1: 80% ACN (c). Analyte identification: (1) 8OH2dG, (2) 9mGua, (3) 1mGua, (4) 8OHGua, (5) 8OHG and (6) 7mGua. Column temperature: 20 °C. Chromatographic flow rate: 0.5 mL min^{-1}. Detection: DAD 254 nm.

The high selectivity of the ZIC-HILIC column should be noted, since it resolved the six MNN in a total analysis time of less than 14 min.

For all six compounds studied, the order of elution in RPLC was different from that observed in Luna HILIC and ZIC-HILIC columns. In the latter two, hydrophilic interactions play an important role in the separation. No correlation was observed between the number of hydroxyl groups of the molecule and the retention observed, a relationship, however, that has been found for other molecules [31].

For the analytes studied, the best conditions as regard separation were obtained with RPLC (separation of five MNN) and with ZIC-HILIC (separation of all six MNN). However, with RPLC it was necessary to use a high water content in the mobile phase, usually above 90% (Fig. 1a_2). This has a negative effect in mass spectrometry when it is used as the method of detection because solvents with a high water content produce a less efficient ionization in the electrospray ionization (ESI). Table 3 shows the signal ratio obtained, using mass spectrometry with a single quadrupole, when the chromatographic eluent has a water content of 20% (ZIC-HILIC separation) or of 95% (RPLC separation). The relationship between both signals varied by one order of magnitude, between 10- and 23-fold (ratio I/II). Likewise, the signal/noise ratios observed (data not shown) were between 8- and 26-fold higher with ZIC-HILIC than with RPLC.

3.2. Separation of modified nucleosides and nucleobases in ZIC-HILIC

In light of the results obtained, the ZIC-HILIC column was selected for all the later studies since it seems to offer the optimum possibilities for analysis of the MNN. Alpert proposed a partition mechanism between aqueous layer associated with the stationary phase, and the organic phase to explain separation in HILIC [25]. However, later studies have shown that the mechanism of retention involves more complex equilibria [32]. Moreover, the presence of charged sites in the stationary phase in ZIC-HILIC would propitiate the appearance of other retention mechanisms. All these factors prompted us to study the different factors that could affect the separation of the MNN.

3.2.1. Effect of the nature and content of the organic phase

A study was made of analyte separation in a typical protic polar solvent, in this case MeOH, and an aprotic polar solvent, ACN. MeOH can act as a donor and an acceptor of hydrogen bonds, whereas ACN can act as an acceptor of hydrogen bonds and furthermore provides stronger dipole–dipole interactions. It has been suggested that protic polar solvents are able to compete for the polar sites of the stationary phase disturbing the formation of the aqueous layer required for the partition mechanism [33]. This leads to a more hydrophobic aqueous stationary phase, whose consequence is a poor retention of analytes with a high capacity to form hydrogen bonds. The results obtained, upon varying composition of the mobile phase for ACN or MeOH/2.6 mM formic acid (pH: 3.1) mixtures, show that for all the analytes MeOH acted as a stronger solvent than ACN. Thus the retention factors were always lower with MeOH, although the order of elution persisted in both cases. Optimum separation was achieved with a mobile phase made up of 80% ACN: 20% formic acid 2.6 mM ($_w^w$pH = 3.1). For higher percentages of ACN (>80%), very strong retentions occurred and the analysis time being more than 40 min for a percentage of 85% of ACN and 75 min for a percentage of 90%.

3.2.2. Effect of buffer pH

The nature of the stationary phase in ZIC-HILIC restricts the range that can be used to pH 3–8. We assessed its performance from $_w^w$pH = 3.1 (2.6 mM formic acid) to 6.7 (2.5 mM ammonium formate) with intermediate points at 3.8, 4.7 and 5.7 (2.5 mM formic acid/ammonium formate buffer, setting ammonium concentration to 2.5 mM with ammonium perchlorate). Fig. 2 shows the chromatograms obtained at $_w^w$pH: 3.1, 4.8 and 6.7. In these experiments, confirmation of the identity of the analytes was achieved with ESI-MS. Comparison of the chromatograms at $_w^w$pH = 3.1 (Fig. 2a) and 4.8–6.7 (Fig. 2b and c) allowed us to conclude that hydroxylated-MNN were not charged in the range of pH from 3.1 to 6.7. However, at $_w^w$pH = 3.1, methylated-MNN were positively charged as can be deduced from the variation in their retention times. This theory is supported by the pK_a values found in the literature [28,29], and by those pK_a values obtained experimentally by us. The retention of these MNN is greater in the positively

Table 3
Comparison between mass spectrometric signals of the modified nucleosides and nucleobases studied using different elution solvents in ZIC-HILIC and RPLC separation.

Modified nucleosides and nucleobases	MS signal[a]			Ratio I/II	Ratio I/III
	ZIC-HILIC acid[b] (I)	RPLC acid[c] (II)	ZIC-HILIC neutral[d] (III)		
8OH2dG	4.6×10^7	2.0×10^6	4.6×10^6	23	10
9mGua	3.0×10^9	3.0×10^8	4.1×10^8	10	7.3
1mGua	7.2×10^8	3.2×10^7	5.1×10^7	22	14
8OHGua	1.2×10^8	8.5×10^6	1.3×10^7	14	9.4
8OHG	2.5×10^8	1.1×10^7	3.0×10^7	23	8.3
7mGua	4.0×10^8	2.6×10^7	3.3×10^7	15	12

[a] ESI settings: capillary voltage: +3500 V; drying gas flow: 10 L min^{-1}; temperature: 350 °C; nebulizer pressure: 50 psi. Signals as chromatographic areas in Selected Ion Monitoring (SIM) mode for: 284, 166, 168 and 300 *m/z*.
[b] Mobile phase: 20% 2.6 mM formic acid ($_w{}^w$pH = 3.1): 80% ACN.
[c] Mobile phase: 95% 2.6 mM formic acid ($_w{}^w$pH = 3.1): 5% ACN.
[d] Mobile phase: 20% 2.6 mM ammonium formate ($_w{}^w$pH = 6.7): 80% ACN.

charged form (Fig. 2a) than in their neutral form (Fig. 2b and c). This behaviour is consistent with a mechanism based on partition, since the protonated forms would be more soluble in the aqueous phase retained on the stationary phase. Likewise, the electrostatic interactions with the residue of sulfobetaine of the stationary phase would be greater in the case of positively charged molecules. The electrostatic interactions would thus occur mainly with the terminal sulfonic group and not with the interior ammonium group, which is clearly sterically hindered.

Regarding the separation of the MNN, it can be accomplished at any $_w{}^w$pH values from 3.1 to 6.7, but better resolution was obtained at more acidic pHs. Moreover, the electrospray ionization is much more favourable if the analytes arrive at the ionization system at a $_w{}^w$pH where they are easily charged. Thus, it is seen that a change in $_w{}^w$pHs from 6.7 to 3.1, maintaining the same concentration of salts, elicited an increase in the signal varying between 7.3- and 14-fold (Table 3, ratio I/III).

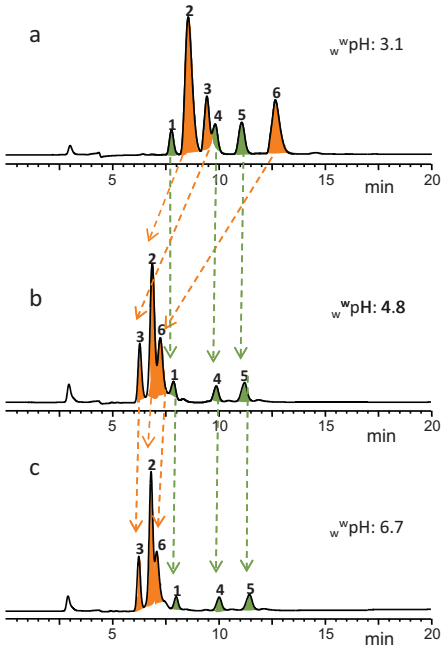


Fig. 2. UV chromatograms, normalized to the highest peak, obtained using isocratic elution 20% formic acid: 80% ACN at $_w{}^w$pH = 3.1 (a); 20% formic acid/ammonium formate buffer 2.5 mM: 80% ACN at $_w{}^w$pH = 4.8 (b); and 20% ammonium formate 2.5 mM: 80% ACN at $_w{}^w$pH = 6.7 (c). Analyte identification as in Fig. 1. Column temperature: 20 °C. Chromatographic flow rate: 0.5 mL min^{-1}. Detection: DAD 254 nm. Peak identification confirmed by ESI-MS.

3.2.3. *Effect of the salt concentration*

Owing to the high organic content of the mobile phase in ZIC-HILIC, the number of salts available is limited to those showing an acceptable solubility in organic medium, like perchlorate or organic salts.

In HILIC columns, the increase in salt concentration usually leads to an increase in retention [34]. Additionally, other effects may appear in ZIC-HILIC [35,36], since when working at low salt concentrations a displacement equilibrium is established between charged analytes and the cations of the mobile phase in the stationary phase (ion exchange).

These effects were assessed varying the salt concentration from 0.25 to 200 mM for ammonium perchlorate and from 0.25 to 5.0 mM for potassium perchlorate (because of its poor solubility) at $_w{}^w$pHs of 3.1 and 6.7.

At high salt concentrations, above 50 mM (Fig. 3a), an increase in retention occurs for all MNN at any $_w{}^w$pHs, in agreement with the characteristics of a partition mechanism. The high organic content of the mobile phase led the salts to be located mainly in the aqueous phase bound to the stationary phase of the column. Thus, high concentrations could have produced an increase in the volume or hydrophilicity of the aqueous layer, attracting the solvated MNN and causing a greater retention of the analytes.

At low salt concentrations, methylated-MNN at a $_w{}^w$pH where they are not charged (Fig. 3a: 7mGua, $_w{}^w$pH = 6.7) and hydroxylated-MNN at any $_w{}^w$pH (Fig. 3a and b: 8OH2dG) keep relatively constant retention values. However, for positively charged methylated-MNN (Fig. 3a and b: 7mGua, $_w{}^w$pH = 3.1), an increase in retention occurs, indicating that if analytes are in its protonated form, a electrostatic interaction occurs. Starting out from equilibrium (1), whose equilibrium constant is *K*, where BH^+ are the charged analytes, whose acid–base ionization constant is K_a, and M^+ represents the cations of the salts present:

$$BH^+ + RSO_3{}^-M^+ \leftrightarrow RSO_3{}^-BH^+ + M^+ \quad (1)$$

It has been reported [32] that electrostatic interactions fit Eq. (2):

$$k_{el} = A\frac{1}{[M^+]} \quad (2)$$

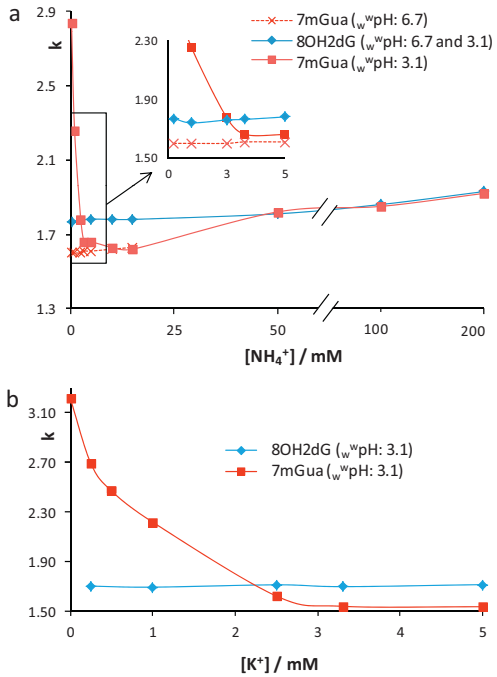


Fig. 3. Retention factors for 7mGua and 8OH2dG, plotted versus salt concentration, obtained using 20% ammonium perchlorate: 80% ACN (a) and 20% potassium perchlorate: 80% ACN (b). Column temperature: 20 °C. Chromatographic flow rate: 0.5 mL min^{-1}.

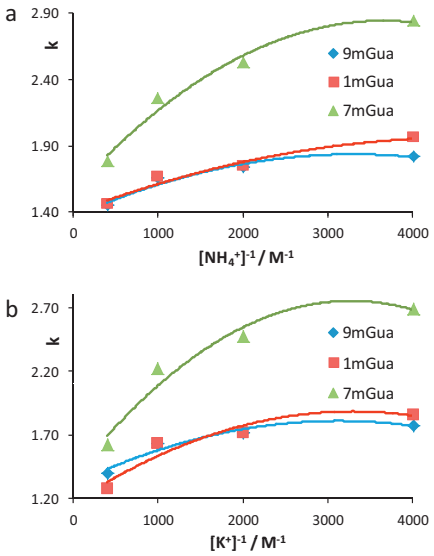


Fig. 4. Retention factors for methylated-MNN, plotted versus the inverse of salt concentration, obtained using 20% ammonium perchlorate (with formic acid, 2.6 mM at $_w$wpH = 3.1): 80% ACN (a), and 20% potassium perchlorate (with formic acid 2.6 mM at $_w$wpH = 3.1): 80% ACN (b). Column temperature: 20 °C. Chromatographic flow rate: 0.5 mL min^{-1}.

where A is a constant similar to that described by McCalley [32]:

$$A \propto \frac{K[RSO_3^-M^+]}{1 + Ka/[H^+]} \quad (3)$$

Accordingly, a plot of the experimental retention factors (k_{exp}) against the inverse of the salt concentration ($[M^+]^{-1}$) should be a straight line, proportional to A, with the ordinate at the origin being zero if electrostatic interactions are the only retention mechanism (Eq. (2)). Experimentally, the values found for ammonium and potassium salt concentrations of 0.25–2.5 mM (corresponding to $[M^+]^{-1}$ values ranging from 4000 to 400 M^{-1}) fitted a second-order polynomial equation (Fig. 4). The presence of other non-electrostatic retention mechanisms (e.g. partition mechanism) was indicated by the fact that values for the ordinate at the origin are different from zero and a straight line was not obtained.

The results obtained show that the presence of salt and the increase in its concentration do not elicit any significant improvement in the separation of the MNN. If it is also considered that the presence of salts can increase ion suppressions in the possible detection by mass spectrometry, sensitivity thus decreasing, it is possible to set 2.6 mM formic acid in the absence of salts as the most appropriate medium for these analytes.

3.2.4. *Effect of temperature*

The Van't Hoff equation has been proposed as a way to relate the retention factor and temperature. Likewise, it has been suggested that if the retention mechanism in HILIC is a mechanism of partition, then the Van't Hoff equation must be fulfilled [37]. Table 4 shows the results obtained upon modifying the column temperature between 20 °C and 60 °C (5 calibration points). A good correlation was obtained for the nucleobases modified by methylation, negative enthalpy values being observed for the three analytes. Accordingly, the retention process is exothermic, and its values seem to be compatible with a partition mechanism. However, the nucleosides and the nucleobases modified by hydroxylation do not fit the Van't Hoff equation. This can be related to the presence of hydroxyl groups and their ability to form hydrogen bonds. Thus, the presence of a greater number of hydroxyl groups (one in 8OHGua, three in 8OH2dG and four in 8OHG) produces a larger deviation from the ideal behaviour of a partition mechanism that could be described by the Van't Hoff equation.

Regarding separation, variations in temperature did not seem to be critical. Neither the efficiency nor the selectivity of the separation was significantly affected. Thus, a temperature of 20 °C can be considered optimum for performing the separation of the MNN studied.

Table 4
Effect of temperature on ZIC-HILIC separation of the modified nucleosides and nucleobases analyzed. Thermodynamic parameters calculated from the Van't Hoff equation.[a]

Modified nucleosides and nucleobases	r^2	Slope (K)	ΔH° (kJ mol^{-1})
8OH2dG	0.430	–	–
9mGua	0.987	480	−4.0
1mGua	0.900	325	−2.7
8OHGua	0.739	–	–
8OHG	0.288	–	–
7mGua	0.970	440	−3.7

[a] Isocratic elution: 80% ACN: 20% formic acid 2.6 mM ($_w$wpH = 3.1). Temperature: variable from 20 to 60 °C. Flow rate: 0.5 mL min^{-1}. Injection: 50 μL in ACN.

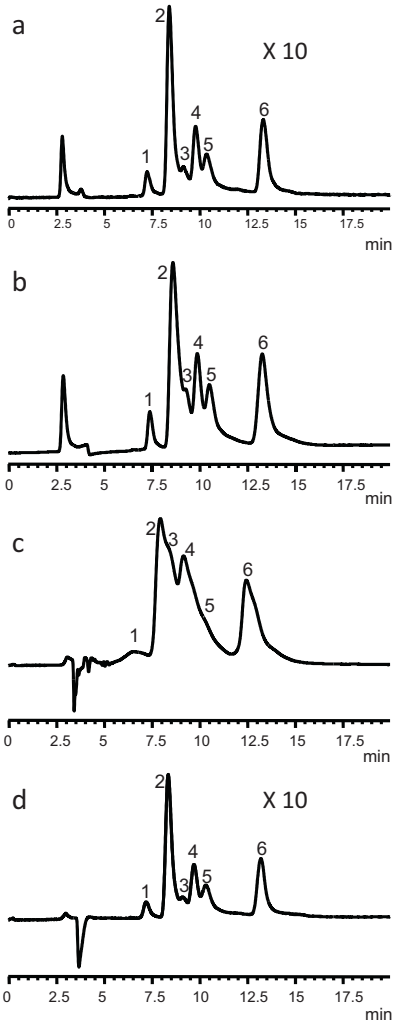


Fig. 5. UV chromatograms obtained using isocratic elution 20% formic acid 2.6 mM $_{w}^{w}$pH = 3.1: 80% ACN for a working standard solution at 5 μg mL^{-1} for methylated-MNN and 3 μg mL^{-1} for hydroxylated-MNN. Column temperature: 20 °C. Chromatographic flow rate: 0.5 mL min^{-1}. Detection: DAD 254 nm. Injection of 5 μL in ACN (a); 50 μL in ACN (b); 50 μL in 50% ACN: 50% water (c); and 5 μL in water (d). Analyte identification as in Fig. 1.

3.2.5. Effect of chromatographic flow rate

The flow rate directly affects the efficiency of the column, as may be deduced from the equation of Van Deemter. For two of the analytes (9mGua and 8OHG), a study was made of the effect of the chromatographic flow rate, analyzing 22 flow rates ranging from 0.1 mL min^{-1} to 2 mL min^{-1}. The correlation coefficients (r^2) for the theoretical curve (HETP = A + B/u + C·u) were 0.947 for 9mGua and 0.955 for 8OHG. For both analytes, the minimum experimental value for the HETP was seen at 0.5 mL min^{-1}. As a side effect, the increase in flow produced a corresponding increase in column pressure. Thus, for a flow rate of 0.5 mL min^{-1} the pressure was 55 bar; for 1.0 mL min^{-1} it was 121 bar, and for 2.0 mL min^{-1} it was 204 bar. These observations allowed a flow rate of 0.5 mL min^{-1} to be selected as optimum for separation. With this flow rate value, HETP of around 20 μm were obtained (equivalent to 50,000 N/m) with a total analysis time of 15 min.

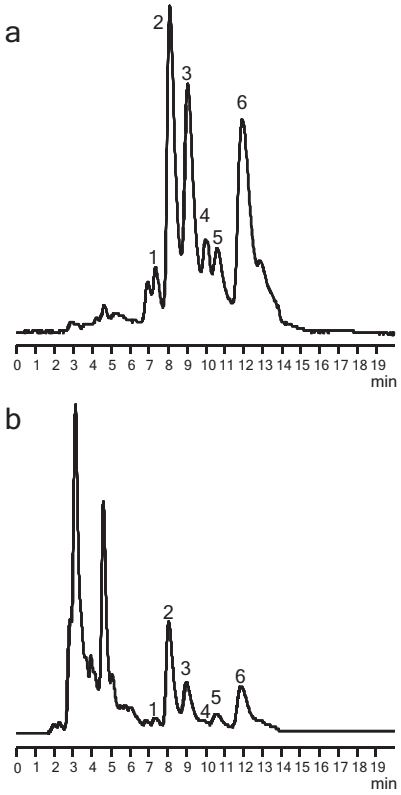


Fig. 6. (a) Total Ion Chromatogram (TIC) and (b) UV chromatogram obtained from a urine sample fortified at 5 μg mL^{-1} for methylated-MNN and 3 μg mL^{-1} for hydroxylated-MNN using isocratic elution 20% formic acid 2.6 mM $_{w}^{w}$pH = 3.1: 80% ACN. Column temperature: 20 °C. Chromatographic flow rate: 0.5 mL min^{-1}. Analyte identification as in Fig. 1.

3.3. Injection in ZIC-HILIC

Another important advantage of HILIC as compared with RPLC is the possibility of injecting samples with a high content of organic solvent, without this affecting the separation and the symmetry of the chromatographic peaks. The usual procedures of extraction, preconcentration and sample cleaning, which are essential for the analysis of trace amounts of compounds in complex samples, as is the case of the analysis of MNN [38], generate a solution that contains the analytes dissolved in predominantly organic media, which means that it is necessary to incorporate an evaporation/redissolution step. This step can be sidestepped by injecting the organic extract directly in HILIC. As organic solvent, ACN has been suggested as the best choice although ACN/isopropanol mixtures could be used in case of solubility issues. In addition, proportion of water greater than 10% in the sample diluent should be avoided to maintain sharp peaks [39].

The separation and symmetry of peaks obtained were satisfactory when injections of 5 μL and 50 μL were performed in a 100% organic medium (Fig. 5a and b) thus showing the injection

volume capacity of the column. The correlation coefficients (r^2) between the volume injected and the peak area obtained (6 calibration points) for the analytes studied varied between 0.986 and 0.997.

Injections of 50 μL with different water contents showed a deterioration in the separation and symmetry of the peaks as expected (Fig. 5c). However, when 5 μL were injected in a 100% aqueous medium (Fig. 5d), the separation and symmetry of the peaks were satisfactory and very similar to those obtained in totally organic medium. Nevertheless, injection of 5 μL in ACN afforded a better separation and peak shape.

Fig. 6 shows the injection of 5 μL of a real urine sample purified following the procedure proposed by Li et al. [40], eluting the SPE cartridge with mobile phase (80% ACN: 20% formic acid 2.6 mM) and sidestepping the evaporation/redissolution step.

3.4. Reproducibility in ZIC-HILIC

One of the best-known disadvantages of NPLC is its lack of reproducibility [34]. To determine the precision of the separation achieved with the ZIC-HILIC column, three samples (prepared daily) were analyzed along three consecutive days, performing 7 injections per day (21 injection in all). The relative standard deviations (RSD) were lower than 2.5% for the retention times and lower than 3.5% for the adjusted retention times. Regarding the analytical signal obtained, measured as chromatographic peak areas, the corresponding RSD remained below 4%.

4. Conclusions

It has been showed that ZIC-HILIC is a suitable alternative for the chromatographic analysis of MNN. A mobile phase containing 80% ACN and 20% of formic acid (2.6 mM) allowed appropriate resolution to be achieved. Additionally, the characteristics of ZIC-HILIC mean that the compatibility between chromatographic separation and detection by mass spectrometry is very high, allowing the injection of volumes up to 50 μL in a completely organic medium, leading to high sensitivity and compatibility with the usual methods of extraction/preconcentration used for the analysis of MNN. The retention of these compounds in ZIC-HILIC occurs through a mechanism of partition between the aqueous phase bound to stationary phase and the organic component of the mobile phase; however, the mechanism is complex, and it presumably involves other processes such as retention of the MNN with hydroxyl groups by hydrogen bonding or electrostatic interactions, for positively charged analytes.

Acknowledgements

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References

[1] S.M. Cooke, R. Olinski, S. Loft, Cancer Epidemiol. Biomarkers Prev. 17 (2008) 3.
[2] M. Harri, H. Kasai, T. Mori, J. Tornaeus, K. Savela, K. Peltonen, J. Chromatogr. B 853 (2007) 242.
[3] H.M. Liebich, S. Müller-Hagedorn, F. Klaus, K. Meziane, K.R. Kim, A. Frickenschmidt, B. Kammerer, J. Chromatogr. A 1071 (2005) 271.
[4] H. Topp, R. Duden, G. Schöch, Clin. Chim. Acta 218 (1993) 73.
[5] E. Borek, B.S. Baliga, C.W. Gehrke, C.W. Kuo, S. Belman, W. Troll, T.P. Waalkes, Cancer Res. 37 (1977) 3362.
[6] D. Jégourel, R. Delépée, F. Breton, A. Rolland, R. Vidal, L.A. Agrofoglio, Bioorg. Med. Chem. 16 (2008) 8932.
[7] B. Kammerer, A. Frickenschmidt, C.E. Müller, S. Laufer, C.H. Gleiter, H. Liebich, Anal. Bioanal. Chem. 382 (2005) 1017.
[8] S.H. Lee, I. Kim, B.C. Chung, Clin. Biochem. 40 (2007) 936.
[9] G. la Marca, S. Malvagia, B. Casetta, E. Pasquini, I. Pela, M. Hirano, M.A. Donati, E. Zammarchi, J. Mass Spectrom. 41 (2006) 586.
[10] D. Bullinger, H. Fröhlich, F. Klaus, H. Neubauer, A. Frickenschmidt, C. Henneges, A. Zell, S. Laufer, C.H. Gleiter, H. Liebich, B. Kammerer, Anal. Chim. Acta 618 (2008) 29.
[11] W.Y. Hsu, W.T. Chen, W.D. Lin, F.J. Tsai, C.T. Lin, W.Y. Lo, L.B. Jeng, C.C. Lai, Clin. Chim. Acta 402 (2009) 31.
[12] K. Roszkowski, D. Gackowski, R. Rozalski, T. Dziaman, A. Siomek, J. Guz, A. Szpila, M. Foksinski, R. Olinski, Int. J. Cancer 123 (2008) 1964.
[13] H.Y. Li, S.M. Wang, H.M. Liu, S.S. Bu, J. Li, D. Han, M.Z. Zhang, G.Y. Wu, J. Mass Spectrom. 44 (2009) 641.
[14] L.B. Jeng, W.Y. Lo, W.Y. Hsu, W.D. Lin, C.T. Lin, C.C. Lai, F.J. Tasi, Rapid Commun. Mass Spectrom. 23 (2009) 1543.
[15] K. Tamae, K. Kawai, S. Yamasaki, K. Kawanami, M. Ikeda, K. Takahashi, T. Miyamoto, N. Kato, H. Kasai, Cancer Sci. 100 (2009) 715.
[16] R. Mateos, L. Bravo, J. Sep. Sci. 30 (2007) 175.
[17] I.H. Lin, M.R. Chao, C.W. Hu, M.L. Ho, J.Y. Huang, H.S. Lee, C.C. Chen, R.H. Wong, Toxicology 260 (2009) 1.
[18] M.R. Chao, C.J. Wang, H.H. Yang, L.W. Chang, C.W. Hu, Rapid Commun. Mass Spectrom. 19 (2005) 2427.
[19] A. Seidel, S. Brunner, P. Seidel, G.I. Fritz, O. Herbarth, Br. J. Cancer 94 (2006) 1726.
[20] Y. Jiang, Y. Ma, Anal. Chem. 81 (2009) 6474.
[21] A.J. Sasco, F. Rey, C. Reynaud, J.Y. Bobin, M. Clavel, A. Niveleau, Cancer Lett. 108 (1996) 157.
[22] R. Tuytten, F. Lemière, E.L. Esmans, W.A. Herrebout, B.J. van der Veken, B.U.W. Maes, E. Witters, R.P. Newton, E. Dudley, Anal. Chem. 79 (2007) 6662.
[23] D.L. Walters, D.L. Jacobs, J.E. Tomaszewski, S.J. Graves, J. Pharm. Biomed. Anal. 19 (1999) 955.
[24] R. Tuytten, F. Lemière, W. Van Dongen, E. Witters, E.L. Esmans, R.P. Newton, E. Dudley, Anal. Chem. 80 (2008) 1263.
[25] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[26] F. Qin, Y.Y. Zhao, M.B. Sawyer, X.F. Li, Anal. Chem. 80 (2008) 3404.
[27] S. Mysling, G. Palmisando, P. Højrup, M. Thaysen-Andersen, Anal. Chem. 82 (2010) 5598.
[28] Y.H. Jang, W.A. Goddard III, K.T. Noyes, L.C. Sowers, S. Hwang, D.S. Chung, Chem. Res. Toxicol. 15 (2002) 1023.
[29] V. Verdolino, R. Cammi, B.H. Munk, H.B. Schlegel, J. Phys. Chem. B 112 (2008) 16860.
[30] A. Albert, E.P. Serjeant, The Determination of Ionization Constants: A Laboratory Manual, 3rd edition, Chapman & Hall, London, 1984.
[31] A. Yanagida, H. Murao, M. Ohnishi-Kameyama, Y. Yamakawa, A. Shoji, M. Tagashira, T. Kanda, H. Shindo, Y. Shibusawa, J. Chromatogr. A 1143 (2007) 153.
[32] D.V. McCalley, J. Chromatogr. A 1217 (2010) 3408.
[33] M. Liu, J. Ostovic, E.X. Chen, N. Cauchon, J. Chromatogr. A 1216 (2009) 2362.
[34] Y. Guo, S. Gaiki, J. Chromatogr. A 1074 (2005) 71.
[35] J.A. Alpert, Anal. Chem. 80 (2008) 62.
[36] G. Marrubini, B.E.C. Mendoza, G. Massolini, J. Sep. Sci. 33 (2010) 803.
[37] Z. Hao, B. Xiao, N. Weng, J. Sep. Sci. 31 (2008) 1449.
[38] E. Dudley, S. El-Shakawi, D.E. Games, R.P. Newton, Nucleosides Nucleotides Nucleic Acids 19 (2000) 545.
[39] J. Ruta, S. Rudaz, D.V. McCalley, J.L. Veuthey, D. Guillarme, J. Chromatogr. A 1217 (2010) 8230.
[40] H.Y. Li, S.M. Wang, H.M. Liu, J. Li, D. Han, S.S. Bu., M.Z. Zhang, Rapid Commun. Mass Spectrom. 22 (2008) 3161.

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Research Article

Evaluation of the Electrostatic Contribution to the Retention of Modified Nucleosides and Nucleobases by Zwitterionic Hydrophilic Interaction Chromatography

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This work explores the benefits and limitations, on a quantitative basis, of using zwitterionic hydrophilic interaction chromatography (ZIC-HILIC) for the separation of several modified nucleosides and nucleobases of clinical interest. The target compounds were hydroxylated and methylated derivatives: 8-hydroxy-guanine, 8-hydroxy-guanosine, 8-hydroxy-2′-deoxyguanosine, 1-methyl-guanine, 7-methyl-guanine, and 9-methyl-guanine. A quantitative evaluation of the electrostatic interaction based on a systematic study of the nature and concentration of the salts in the mobile phase has been carried out. From the obtained results, it may be concluded that separation is based on a mechanism of partition and interaction through weak electrostatic forces: the contribution of the electrostatic interaction to the retention of the charged analytes reaching values between 25 and 52% at low salt concentration. However, the electrostatic contribution decreased progressively as the salt concentration rose.

1. Introduction

Hydrophilic interaction chromatography (HILIC) is a mode of chromatography in which polar compounds are retained using hydrophilic stationary phases combined with mobile phases with a high concentration of organic solvent and a small amount of water [1]. A partition mechanism between the aqueous layer associated with the stationary phase and the organic component of the mobile phase has been proposed to explain the type of separation that occurs in HILIC [1]. However, later studies have shown that the mechanism of retention involves more complex equilibria [2]. Moreover, the presence of charged sites in the stationary phase in zwitterionic hydrophilic chromatography (ZIC-HILIC) would propitiate the appearance of other retention mechanisms [3, 4].

Despite the many applications described in the literature, the retention mechanism in ZIC-HILIC is still under debate [2, 4–14].

The chromatographic behaviour of several modified nucleosides and nucleobases (MNNs) with different stationary phases in HILIC mode has been described previously [15], the zwitterionic stationary phase being the most suitable one for their separation. The MNNs selected were hydroxylated and methylated derivatives, used as biological markers of several diseases: 8-hydroxy-guanine (8OHGua), 8-hydroxy-guanosine (8OHG), 8-hydroxy-2′-deoxyguanosine (8OH2dG), 1-methyl-guanine (1mGua), 7-methyl-guanine (7mGua), and 9-methyl-guanine (9mGua). The results obtained from the study of the different parameters affecting separation—content of organic solvent in the mobile phase, pH, salt concentration, and temperature inside the column—showed that separation in the ZIC-HILIC column was based on a mechanism of partition between the aqueous phase bound to stationary phase and the organic compound of mobile phase. However, the mechanism is complex, and it presumably involves other processes such as retention of the MNN with hydroxyl groups by hydrogen bonding or interaction through weak electrostatic forces for charged analytes.

In this work, we describe a quantitative evaluation of these electrostatic interactions in ZIC-HILIC in order to elucidate the contribution of the interactions to the retention

of the target analytes. The evaluation was based on a detailed study of the influence of the type and concentration of the salt used.

Thus, the aim of the present work was to contribute to a better understanding of the retention mechanism of these compounds in ZIC-HILIC mode.

2. Experimental

2.1. Chemicals. Analytical standards of *8-hydroxy-guanine* (8OHGua), CAS RN [5614-64-2]; and *8-hydroxy-guanosine* (8OHG), CAS RN [3868-31-3] were purchased from Cayman Chemical (Michigan, USA). *8-hydroxy-2′-deoxyguanosine* (8OH2dG), CAS RN [88847-89-6]; *9-methyl-guanine* (9mGua), CAS RN [5502-78-3]; *1-methyl-guanine* (1mGua), CAS RN [938-85-2]; and *7-methyl-guanine* (7mGua), CAS RN [578-76-7] were obtained from Sigma-Aldrich (Steinheim, Germany).

Working solutions were daily prepared at 5 μg mL^{-1} for 1mGua, 7mGua, and 9mGua, and at 3 μg mL^{-1} for 8OH2dG, 8OHG, and 8OHGua.

Acetonitrile (ACN) was of HPLC grade (Merck, Darmstadt, Germany). Ultrahigh-quality (UHQ) water was obtained with a Wasserlab (Noain, Spain) Ultramatic water purification system. All other chemicals were of analytical reagent grade.

2.2. Instrumentation. HPLC analyses were performed on a HP 1100 Series chromatograph from Agilent (Waldbronn, Germany). The Diode Array Detector (DAD) recorded the spectra in the 190–400 nm range. The analytical column was a ZIC-HILIC packed with 3.5 μm particles from Merck (Darmstadt, Germany). Analyses were performed in isocratic elution: 80% ACN: 20% formic acid (2.6 mM, $_{w}{}^{w}$pH = 3.1) with variable concentration of ammonium or potassium perchlorate. Column temperature was set to 20°C, flow rate at 0.5 mL min^{-1}, and an injection volume of 50 μL in ACN was selected.

3. Results and Discussion

Application of the ZIC-HILIC mode to the separation of MNN has been described previously [15]. A mobile phase containing 80% ACN and 2.5 mM formic acid ($_{w}{}^{w}$pH 3.1) allowed appropriate resolution to be achieved. At that pH value, only methylated MNNs were in the protonated form, and hence electrostatic interactions with the sulfobetaine groups in the stationary phase could be expected for these analytes. Hydroxylated MNNs were not charged at $_{w}{}^{w}$pH 3.1, and therefore different behavior that for methylated MNN would be expected.

3.1. Effect of the Nature and Salt Concentration in the Mobile Phase. Owing to the high organic content of the mobile phase in ZIC-HILIC, the number of salts available is limited to those showing acceptable solubility in organic medium, such as perchlorate or organic salts.

In HILIC mode, increases in salt concentrations usually lead to an increase in analyte retention [16]. Additionally, other effects may appear in ZIC-HILIC: for eluents with low salt concentration, ion exchange equilibrium should be established between the cations of the mobile phase in the stationary phase and the charged analytes.

We have previously reported the behavior of the target compounds in ZIC-HILIC mode for salt concentrations in the 0.25–200 mM range for ammonium perchlorate and 0.25–5.0 mM for potassium perchlorate (because of its poor solubility) [15]. Briefly, two zones showing different behaviour were observed: at low salt concentrations (0–2.5 mM), a strong decrease occurred in the retention of all the charged MNNs studied (methylated MNN at $_{w}{}^{w}$pH 3.1), indicating that a decrease in electrostatic retention had taken place (Figure 1(a)). The electrostatic nature of this decrease was confirmed by the behaviour of hydroxylated MNNs, which were not charged at $_{w}{}^{w}$pH: 3.1 (Figure 1(b)), and by carrying out the same experiment at $_{w}{}^{w}$pH: 6.7, where both the methylated and hydroxylated MNN were uncharged; both experiments resulted in the disappearance of this zone, confirming the electrostatic nature of the decrease. The other zone appeared at high salt concentrations (15–200 mM), pointing to an increase in retention and in agreement with the characteristics observed for a partition mechanism.

3.1.1. Quantitative Evaluation of the Electrostatic Contribution to Retention. The retention of a charged analyte (BH^+) on the sulfonic group ($R\text{-}SO_3^-$) of the stationary phase can be described according to

$$BH^+ + RSO_3{}^-M^+ \longleftrightarrow RSO_3{}^-BH^+ + M^+, \quad (1)$$

where M^+ represents the cation of the salt present in the eluent.

It has been reported [2, 4] that electrostatic interactions fit (2):

$$k_{EI} = A\frac{1}{[M^+]}, \quad (2)$$

where A is a constant similar to that described by McCalley [2, 4]:

$$A \propto \frac{K[RSO_3{}^-M^+]}{1 + K_a/[H^+]}, \quad (3)$$

K being the equilibrium constant of (1), and K_a, the acid-base ionization constant of the analyte (BH^+).

Accordingly, a plot of the experimental retention factors (k_{exp}) against the inverse of the salt concentration ($[M^+]^{-1}$) should be a straight line, proportional to A, with the ordinate at the origin being zero if electrostatic interactions are the only retention mechanism (2). Experimentally, the values found for salt concentrations in the 0.25–2.5 mM range (values of $[M^+]^{-1}$ ranging from 4000 to 400 M^{-1}) fit a second-order polynomial equation, with r^2 values between 0.945 and 0.989 for $NH_4{}^+$ and between 0.913 and 0.957 for K^+, indicating the existence of a minor additional effect (Table 1). The presence of other nonelectrostatic retention mechanisms

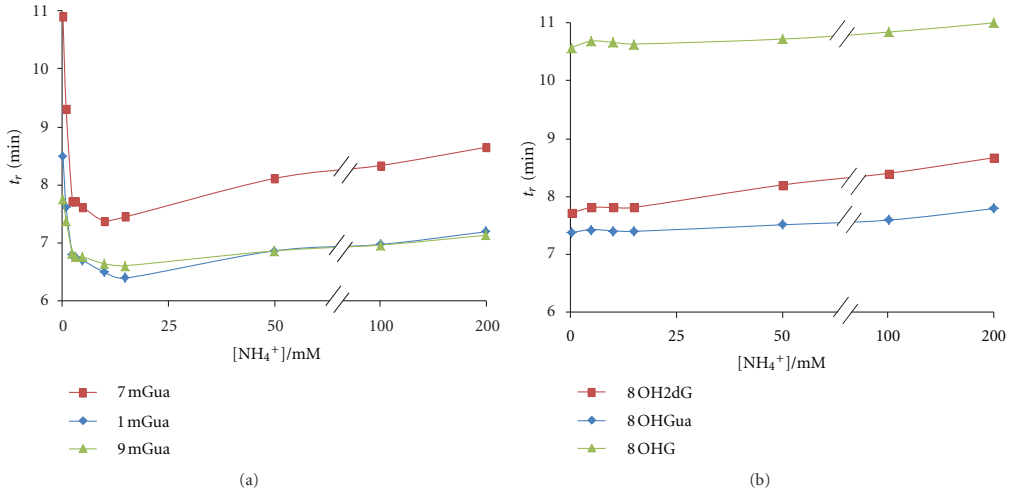

Figure 1: Plots of retention time (t_r) versus ammonium perchlorate concentration for methylated MNNs (a) and hydroxylated MNNs (b).

Table 1: Parameters of the equation for the second-order polynomial fitting of the experimental retention factors (k_{exp}) versus the inverse of the salt concentration ($([M^+]^{-1})$[(a)].

Modified nucleosides and nucleobases		$k_{exp} = a/[M^+]^2 + b/[M^+] + c$		
		M^+: Ammonium perchlorate		
	R^2	a	b	c
9mGua	0.9538	$-4E-5$	0.3	1.3667
1mGua	0.9681	$-3E-5$	0.2	1.3969
7mGua	0.9782	$-1E-4$	0.7	1.5624
		M^+: Potassium perchlorate		
	R^2	a	b	c
9mGua	0.9392	$-5E-5$	0.3	1.3109
1mGua	0.9127	$-7E-5$	0.4	1.1628
7mGua	0.9574	$-1E-4$	0.8	1.3724

(a) Isocratic elution: 80% ACN: 20% formic acid (2.6 mM, $_w{}^w$pH = 3.1) with variable concentrations of ammonium or potassium perchlorate. Temperature: 20°C. Flow: 0.5 mL min^{-1}. Injection: 50 μL in CAN.

Table 2: Contribution of electrostatic interactions to the ZIC-HILIC retention of the positively charged MNNs, at different salt concentrations [(a)].

Charged MNN	% of electrostatic interaction in retention				
	Ammonium perchlorate				
	0.25 mM	0.50 mM	1.00 mM	2.50 mM	$(k_{EI})_{0.25\,mM}$
9mGua	25	21	17	6	0.45
1mGua	28	20	16	4	0.75
7mGua	52	46	39	23	1.66
	Potassium perchlorate				
	0.25 mM	0.50 mM	1.00 mM	2.50 mM	$(k_{EI})_{0.25\,mM}$
9mGua	26	24	20	6	0.54
1mGua	38	32	29	9	0.99
7mGua	49	44	38	16	1.85

(a) Isocratic elution: 80% ACN: 20% formic acid (2.6 mM, $_w{}^w$pH = 3.1) with variable concentrations of ammonium or potassium perchlorate. Temperature: 20°C. Flow: 0.5 mL min^{-1}. Injection: 50 μL in ACN.

(e.g., partition mechanism) is indicated by the fact that values for the ordinate at the origin are different from zero (Table 1) and a straight line was not obtained.

Therefore, bearing in mind that the experimental retention factors (k_{exp}) must be the sum of the contribution of the electrostatic (k_{EI}) and nonelectrostatic (k_{NEI}) interactions:

$$k_{exp} = k_{EI} + k_{NEI}, \quad (4)$$

and substituting (2) in (4), we have

$$k_{exp} = A\frac{1}{[M^+]} + k_{NEI}. \quad (5)$$

According to (4) and (5), extrapolation of the second-order polynomial fittings to the ordinate at the origin ($[M^+]^{-1} \rightarrow 0$) allows the retention factor due to any mechanism other than electrostatic interactions (k_{NEI}) to be calculated, since when $[M^+]^{-1} \rightarrow 0$, then $k_{EI} = 0$. For a hypothetical infinite concentration of salts (when $[M^+]^{-1} \rightarrow 0$ then $[M^+] \rightarrow \infty$), there would be "*infinite competition*" (of the salts against the target analytes) for the electrostatically active sites of the stationary phase; this would completely eliminate the electrostatic contribution to the retention mechanism of the analytes.

From these calculated k_{NEI} values, the percentage of contribution of the electrostatic interaction (%EI) to the overall retention was calculated, according to (6), for each analyte and for the different of salt concentrations studied (Table 2):

$$\%EI = \frac{k_{EI}}{k_{exp}} \cdot 100. \quad (6)$$

For the lowest salt concentration, 0.25 mM, the contribution of the electrostatic interactions to the retention mechanism varied between 25% and 52% for NH_4^+, and between 26% and 49% for K^+. These decreased progressively as the salt concentration rose, observing, for a salt concentration of 2.5 mM, contributions between 6% and 23% for NH_4^+ and between 6% and 16% for K^+. These results are in good agreement with those found by McCalley working with four basic compounds [2] and by Kumar et al. for catecholamines [4].

The retention factors due to electrostatic interactions at a salt concentration of 0.25 mM ($k_{EI})_{0.25\,mM}$ were also calculated (Table 2). These values can be determined for (4) as the difference between the experimental retention factors at 0.25 mM (k_{exp}) and the retention factors for an infinite concentration of salts (k_{NEI}). Here, it should be recalled that these k_{NEI} values were estimated by extrapolation to zero of the experimental data (Table 1). Accordingly, the percentage values (Table 2) should be seen as a quantitative approximation to the electrostatic contribution to the overall retention.

4. Conclusions

The results obtained in the present work show that for charged MNNs separation in the ZIC-HILIC column is based on a shared mechanism of partition and interaction through weak electrostatic forces. The electrostatic contribution to retention was about 25–50% at low salt concentration in the eluent, although it should be noted that this contribution decreased significantly as the salt concentration rose. In order to exploit these interactions with a view to enhancing selectivity and resolution in ZIC-HILIC separations, the use of mobile phases at low salt concentrations should be considered.

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References

[1] A. J. Alpert, "Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds," *Journal of Chromatography*, vol. 499, pp. 177–196, 1990.

[2] D. V. McCalley, "Study of the selectivity, retention mechanisms and performance of alternative silica-based stationary phases for separation of ionised solutes in hydrophilic interaction chromatography," *Journal of Chromatography A*, vol. 1217, no. 20, pp. 3408–3417, 2010.

[3] S. Mysling, G. Palmisano, P. Hojrup, and M. Thaysen-Andersen, "Utilizing ion-pairing hydrophilic interaction chromatography solid phase extraction for efficient glycopeptide enrichment in glycoproteomics," *Analytical Chemistry*, vol. 82, no. 13, pp. 5598–5609, 2010.

[4] A. Kumar, J. P. Hart, and D. V. McCalley, "Determination of catecholamines in urine using hydrophilic interaction chromatography with electrochemical detection," *Journal of Chromatography A*, vol. 1218, no. 25, pp. 3854–3861, 2011.

[5] A. J. Alpert, "Electrostatic repulsion hydrophilic interaction chromatography for isocratic separation of charged solutes and selective isolation of phosphopeptides," *Analytical Chemistry*, vol. 80, no. 1, pp. 62–76, 2008.

[6] D. V. McCalley, "Is hydrophilic interaction chromatography with silica columns a viable alternative to reversed-phase liquid chromatography for the analysis of ionisable compounds?" *Journal of Chromatography A*, vol. 1171, no. 1-2, pp. 46–55, 2007.

[7] J. Ruta, S. Rudaz, D. V. McCalley, J. L. Veuthey, and D. Guillarme, "A systematic investigation of the effect of sample diluent on peak shape in hydrophilic interaction liquid chromatography," *Journal of Chromatography A*, vol. 1217, no. 52, pp. 8230–8240, 2010.

[8] Y. Guo and S. Gaiki, "Retention and selectivity of stationary phases for hydrophilic interaction chromatography," *Journal of Chromatography A*, vol. 1218, no. 35, pp. 5920–5938, 2011.

[9] P. Jandera, "Stationary and mobile phases in hydrophilic interaction chromatography: a review," *Analytica Chimica Acta*, vol. 692, no. 1-2, pp. 1–25, 2011.

[10] Z. Hao, B. Xiao, and N. Weng, "Impact of column temperature and mobile phase components on selectivity of hydrophilic interaction chromatography (HILIC)," *Journal of Separation Science*, vol. 31, no. 9, pp. 1449–1464, 2008.

[11] A. E. Karatapanis, Y. C. Fiamegos, and C. D. Stalikas, "A revisit to the retention mechanism of hydrophilic interaction liquid

chromatography using model organic compounds," *Journal of Chromatography A*, vol. 1218, no. 20, pp. 2871–2879, 2011.

[12] R.-I. Chirita, C. West, S. Zubrzycki, A. -L. Finaru, and C. Elfakir, "Investigations on the chromatographic behaviour of zwitterionic stationary phases used in hydrophilic interaction chromatography," *Journal of Chromatography A*, vol. 1218, no. 35, pp. 5939–5963, 2011.

[13] B. Buszewski and S. Noga, "Hydrophilic interaction liquid chromatography (HILIC)—a powerful separation technique ," *Analytical and Bioanalytical Chemistry*, vol. 402, no. 1, pp. 231–247, 2011.

[14] K. Jinno, N. S. Quiming, N. L. Denola, and Y. Saito, "Modeling of retention of adrenoreceptor agonists and antagonists on polar stationary phases in hydrophilic interaction chromatography: a review," *Analytical and Bioanalytical Chemistry*, vol. 393, no. 1, pp. 137–153, 2009.

[15] E. Rodríguez-Gonzalo, D. García-Gómez, and R. Carabias-Martínez, "Study of retention behaviour and mass spectrometry compatibility in zwitterionic hydrophilic interaction chromatography for the separation of modified nucleosides and nucleobases," *Journal of Chromatography A*, vol. 1218, no. 26, pp. 3994–4001, 2011.

[16] Y. Guo and S. Gaiki, "Retention behavior of small polar compounds on polar stationary phases in hydrophilic interaction chromatography," *Journal of Chromatography A*, vol. 1074, no. 1-2, pp. 71–80, 2005.

RESEARCH ARTICLE VIII

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Development and validation of a hydrophilic interaction chromatography–tandem mass spectrometry method with on-line polar extraction for the analysis of urinary nucleosides. Potential application in clinical diagnosis

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ABSTRACT

The present paper describes the development, validation and application of a quantitative method for the determination of endogenous nucleosides and nucleobases in urine based on the on-line coupling of a solid-phase extraction step with hydrophilic interaction chromatography–tandem mass spectrometry. The method combines the use of a highly polar restricted-access material (RAM), based on an N-vinylacetamide copolymer, for efficient analyte extraction and matrix removal, with separation by zwitterionic hydrophilic interaction chromatography (ZIC-HILIC), that revealed a satisfactory retention of the polar analytes studied. Detection using a triple quadrupole analyser allowed reliable identification and high-sensitivity quantitation of the target compounds. The on-line configuration developed, RAM-ZIC-HILIC–MS/MS, provides a convenient approach to automate the application to urine analysis, with minimum sample manipulation. The whole method was validated according to European Legislation for bioanalytical methods. The validation steps included the verification of matrix effects, calibration curve, precision, accuracy, selectivity, stability and carry-over in real samples. The results of the validation process revealed that the proposed method is suitable for the reliable determination of nucleosides and nucleobases in human urine, showing limits of detection from 0.1 to 1.3 ng mL^{-1}. The application to clinical samples was also checked; the results obtained in analyses of urine samples from healthy volunteers and cancer patients using Principal Component Analysis, Hierarchical Cluster Analysis and Soft Independent Modeling of Class Analogy are also shown.

1. Introduction

The degradation products of nucleic acids form a particular class of compounds present in human urine [1,2]. Modified nucleosides are widely known as the metabolites of ribonucleic acids (RNA), in particular transfer-RNA (tRNA). Simple structural modifications such as base or ribose methylation, base isomerization, reduction, thiolation or deamination are the most frequent [3]. This primary source of modified nucleosides is enhanced by the presence of different types of tumours [3]. These modified nucleosides cannot be reutilized or further degraded but are excreted in the urine as intact molecules. Therefore, any malignant disease, such as cancer or metabolic imbalances affecting RNA breakdown or turnover, alters the urinary levels of excreted modified nucleosides. More than 93 modified nucleosides have been reported in urine for all forms of RNA [3]. In recent decades, modified nucleosides excreted in human urine have been studied to examine their biomedical significance as possible biomarkers of cancer and other degenerative diseases [4,5].

Another type of important biomarker for cancers is damaged deoxyribonucleic acid (DNA) and its products/metabolites, especially 8-hydroxy-2′-deoxyguanosine (8OH2dG). Among all the different types of DNA damage, oxidative damage by reactive oxygen species is considered to be one of the most important contributors to diseases such as cancer, aging, heart disease and other age-related diseases. Of more than 20 known products of oxidative DNA damage [6] 8OH2dG has been identified as a biomarker for a few types of cancers, including breast, lung and liver cancer [7]. Because 8OH2dG is excreted in urine without any further metabolism [8], the determination of urinary 8OH2dG levels has been considered as a non-invasive method for the diagnosis of cancer.

Different analytical techniques have been reported for the determination of modified nucleosides, immunoassay [9], capillary electrophoresis [10] and, mainly, liquid chromatography [11] being the most widely employed. However, most of the proposed

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methods are relatively complicated and/or need time-consuming procedures for analyte extraction and clean-up prior to analysis. The analysis of highly polar compounds such as nucleosides involves important difficulties in reverse-phase liquid chromatography (RPLC) owing to the poor retention of these analytes in the stationary phase. Hydrophilic interaction chromatography (HILIC) has been shown to be a viable alternative to RPLC for the separation of such polar compounds [12–14].

Here we developed a procedure for the extraction and clean-up of nucleosides from urine based on solid-phase extraction (SPE) with a restricted-access material (RAM) [15,16] of polar nature (N-vinylacetamide copolymer) that can be applied to a broad range of nucleosides. In most methods reported, nucleosides were isolated from urine by SPE in affinity mode using an immobilized phenylboronic acid group [11,17], which specifically binds cis-diols. However, this is not applicable to non-cis-diol nucleosides (other nucleosides that do not have vicinal hydroxyl groups in their structure) or nucleobases, as in the case of 8-hydroxy-2′-deoxyguanosine, 7-methyl-guanine, and guanine [18]. Nevertheless, on-line coupling of polar restricted-access material and the ZIC-HILIC column is the main problem to be solved; this is because of the requirement that the solvent used in the elution step must be compatible with the chromatographic separation media used for later separation. It should be noted that in most on-line SPE-LC systems proposed in the literature, LC in reverse-phase was used for the separation of analytes eluted from SPE sorbents [3,4,18,19]; other modes of LC, such as HILIC, have been little used because the solvents required for sorbent elution may not be compatible with the mobile phase employed in HILIC. To our knowledge, this is the first time that an on-line coupling of a RAM of polar nature to a HILIC column has been proposed.

In light of the above, the main aim of this work was to develop a fast and reliable method for the separation, identification, and quantification of modified nucleosides and nucleobases in human urine. This method could easily be used for clinical purposes such as the early diagnosis of cancer and other diseases, and also for monitoring the progression of disease and the response to therapy. This target was addressed in two ways: (i) through the development of a simple on-line treatment for analyte isolation and matrix removal based on a solid-phase extraction step with a RAM device of polar nature (MSpak) coupled on-line to the HILIC column; and (ii) validation of the automated method, RAM-ZIC-HILIC–MS/MS, according to current legislation for bioanalytical methods [20].

Here the potential application of the proposed approach in clinical areas was tested by analysing real samples from cancer patients. Several endogenous modified nucleosides and nucleobases were detected and quantified in the urine samples and the urinary levels were related to the presence of malignant disease.

2. Experimental

2.1. Chemicals

The analytical standard of *8-hydroxy-guanosine* (8OHG), CAS RN [3868-31-3] was purchased from Cayman Chemical (Michigan, USA). *Uridine* (URN), CAS RN [58-96-8]; *8-hydroxy-2′-deoxyguanosine* (8OH2dG), CAS RN [88847-89-6]; *inosine* (INN), CAS RN [58-63-9]; *adenosine* (ADN), CAS RN [58-61-7]; *guanine* (Gua), CAS RN [73-40-5]; *7-methyl-guanine* (7mGua), CAS RN [578-76-7]; *1-methyl-adenosine* (1mA), CAS RN [15763-06-1] and *7-methyl-guanosine* (7mG), CAS RN [20244-86-4] were obtained from Sigma–Aldrich (Steinheim, Germany). Isotopically Labeled Internal Standards (ILIS)-*8-13C-guanine* (^{13}C-Gua), 98% and *ribose-2-13C-adenosine* (^{13}C-ADN), 99% – were purchased from Cambridge Isotope Laboratories, Inc. (Massachusetts, USA).

Acetonitrile (ACN) was of HPLC grade (Merck, Darmstadt, Germany) and was used as received. Ultra-high quality (UHQ) water was obtained with a Wasserlab (Noain, Spain) Ultramatic water purification system. All other chemicals were of analytical grade.

2.2. Instrumentation

The liquid chromatography–tandem mass spectrometry (LC–MS/MS) system consisted of an Agilent Technologies (Waldbronn, Germany) 1200 series HPLC system with a binary pump, an additional isocratic pump, a membrane degasser, an autosampler, a six-port valve, a diode-array detector (DAD) and a 6410 LC/MS triple quadrupole (QqQ) mass spectrometer. The DAD recorded the spectra in the 190–400 nm range. The triple quadrupole mass spectrometer was equipped with an electrospray (ESI) source. The nebulizer pressure and voltage were set at 35 psi and +3500 V, respectively. Nitrogen was used as the drying (12 L min^{-1}, 350 °C) and collision gas. The separation of analytes was performed on a ZIC-HILIC column (150 mm × 4.6 mm) packed with 3.5 μm particles from Merck (Darmstadt, Germany). For on-line sample treatment, two restricted-access materials (RAM) were used as on-line extraction columns: a LiChroCART^{TM} 25-4LiChrospher Alkyl-Diol-Silica (ADS, 25 μm, 25 mm × 4.0 mm) RP4 from Merck (Darmstadt, Germany) and a MSpak^{TM} PK-4A (N-vinylacetamide copolymer, 10 mm × 4.0 mm) from Shodex (Kawasaki, Japan). Other on-line sorbents used were a 25 mm × 4.0 mm home-made Isolute^{TM} ENV+ packed with 100 μm particles from International Sorbent Technology (Cambridge, UK) and a 20 mm × 4.6 mm Oasis^{TM} HLB packed with 25 μm particles from Waters (Massachusetts, USA).

2.3. Preparation of standards

The initial stock solutions for the ADN, 7mGua, 1mA (500 μg mL^{-1}), Gua, ^{13}C-Gua and ^{13}C-ADN (250 μg mL^{-1}), and 8OHG (100 μg mL^{-1}) standards were prepared in acidified UHQ water (HCl 0.1 M). The stock solutions for the URN, INN, 7mG (500 μg mL^{-1}) and 8OH2dG (100 μg mL^{-1}) standards were prepared in UHQ water. These stock solutions were stored at 4 °C in brown glass bottles. Working solutions were prepared daily.

2.4. Sample collection

Urine samples collected from six healthy volunteers (numbered from H1 to H6) were used for the development and validation of the method. The samples were collected in 250-mL brown glass bottles and frozen immediately until analysis. Before use, the samples were thawed at room temperature. Samples were filtered through 0.22 μm filters.

Urine samples collected from six hospitalized cancer patients (numbered from C1 to C6) were kindly supplied by the Teaching Hospital of Salamanca and were used in the application of the method to clinical samples.

To compensate for variations in urine concentrations, all analyte concentrations were normalized against creatinine and expressed as nmol analyte/μmol creatinine. Urinary creatinine levels were determined with the Jaffe method [21], based on the reaction between creatinine and picric acid, using photometric detection.

2.5. On-line sample preparation and LC–MS/MS separation

A similar experimental setup for on-line extraction-LC–MS/MS has been reported previously [22]. Briefly, 10 μL of urine were injected and the isocratic pump was started immediately to pump the clean-up mobile phase (UHQ water) at 0.5 mL min^{-1} for 1 min. While the matrix components of the urine were washed to waste, the compounds studied were withheld in the extraction column. At

1 min, the six-port valve position was changed and the separation gradient (binary pump, A: ACN, B: 2.5 mM formic acid/ammonium formate buffer – pH 3.4; 1–10 min 30% B, 11–30 min 50% B) eluted the analytes at a flow rate of 0.5 mL min^{-1} in backflush mode to the analytical column, where they were separated and finally detected by the DAD and MS/MS. At 30 min, the separation ended and the gradient and the six-port valve were returned to the initial conditions. A 12-min post-run program was started, keeping the system in the initial conditions in order to equilibrate the analytical column for the next analysis. The system dead volume associated with the extraction column was minimized in order to avoid the disturbance to the chromatographic separation produced by the water from the on-line sample preparation. To accomplish this, the ends of the RAM were connected to the six-port valve using two polyether ether ketone (PEEK) tubes 100 mm × 0.12 mm (approximately 1.2 μL each).

2.6. Method validation

The method was validated according to "*Draft guideline on validation of bioanalytical methods*" published by the European Medicines Agency (EMEA) [20]. The guideline defines the main characteristics of a bioanalytical method that are essential to ensure the acceptability of the performance and the reliability of analytical results. These are: selectivity, lower limit of quantitation (LLOQ), the response function (calibration curve performance), accuracy, precision, matrix effects and stability of the analytes and of any internal standard in the biological matrix, and the stock and working solutions under the entire period of storage and processing conditions. Matrix effects were calculated as matrix factors (MF) according to the following experiments: (A) peak areas obtained in a standard solution injected directly into the ZIC-HILIC–MS/MS system; (B) peak areas obtained for a spiked urine sample injected directly into the ZIC-HILIC–MS/MS system, and (C) peak areas obtained for a spiked urine sample injected according to the RAM-ZIC-HILIC–MS/MS method developed. Urine sample were spiked at a level at which endogenous concentrations can be considered negligible. Thus, three different parameters were calculated: the matrix effects for the ZIC-HILIC–MS/MS system ((B/A) × 100), the matrix effects using the RAM-ZIC-HILIC–MS/MS developed method ((C/A) × 100), and normalized matrix effects calculated using ILIS ($(C/C_{ILIS})/(A/A_{ILIS}) \times 100$).

2.7. Graphic and statistical analyses for pattern recognition

Graphical analysis was carried out as proposed by La et al. [23] using Microsoft™ Excel 14.0 from Microsoft™ Office Professional Plus 2010. Briefly, each creatinine-corrected concentration of the nucleosides measured for the cancer patients and healthy volunteers were plotted to produce scatter plots and a star plot. Statistical analyses were performed using The Unscrambler™ X 10.1 from CAMO™ software. The pattern recognition techniques used were Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) as unsupervised methods, and Soft Independent Modeling of Class Analogy (SIMCA) as a supervised method. In these techniques, the data were standardized to a common scale so that no variable would dominate over the others.

3. Results and discussion

3.1. Method development: on-line sample extraction

Liquid chromatography analysis of a matrix as complex as urine usually requires a previous sample-treatment step to eliminate large amounts of interferents from the matrix, which positively affects selectivity and sensitivity and at the same time prolongs column life. This step is especially important when mass spectrometry with electrospray interface (ESI) is used for detection since this source of ionization undergoes severe losses of sensitivity (ion suppression) owing to the presence of matrix compounds that co-elute with the analytes. Several authors have proposed different strategies to extract nucleosides from urine. Affinity chromatography with phenylboronic acid gel (Affigel 601™, Bio-Rad), showing selectivity for cis-diol compounds, has been tested in off-line [24] and on-line configurations [25]. Oasis™ HLB [18] and MCX [26], more general sorbents capable of extracting cis-diol and non-cis-diol nucleosides, have been tested off-line. MSpak, a highly polar restricted-access material based on an N-vinylacetamide copolymer, has shown good results coupled on-line with RPLC columns [19].

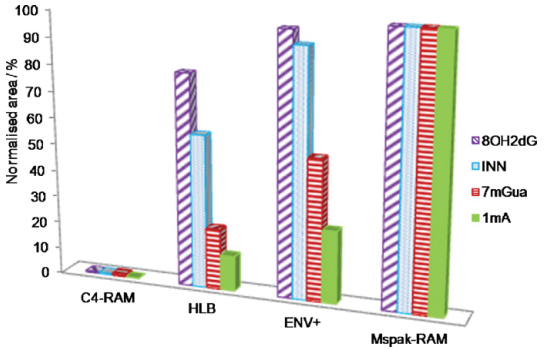

Fig. 1. MS/MS signals, normalized to the highest peak, obtained for 8OH2dG, INN, 7mGua and 1mA, working with different on-line extraction columns: C4-RAM, HLB, ENV+ and MSpak-RAM. Washing conditions: 25 mM ammonium formate – pH 6.0, 0.5 mL min^{-1}, 2 min.

3.1.1. Selection of on-line extraction column

The high polarity of the nucleosides studied hinders their retention in extraction columns where retention is achieved by hydrophobic interactions. Four different extraction columns were selected: an Oasis HLB divinylbenzene-N-vinylpyrrolidone copolymer; an Isolute™ ENV+ hydroxylated styrene-divinylbenzene copolymer and two restricted-access materials: an ADS-C4 (butyl-diol-silica) and a MSpak (N-vinylacetamide copolymer). Four different nucleosides were chosen for the study, covering the expected range of polarity: 8OH2dG, INN, 7mGua and 1mA ($\log D_{pH\,3}$: −1.3, −1.4, −1.8 and −2.1, respectively, calculated using ChemBioDraw Ultra 12.0 from CambridgeSoft™). Analyses were carried out by directly coupling the extraction columns with the mass spectrometer. Fig. 1 shows the results obtained. As may be observed, C4-RAM provided a poor retention of the four analytes studied. The HLB polymeric column afforded better results, especially with the less polar nucleosides. The ENV+ sorbent resulted in higher areas for all nucleosides, reaching almost the maximum area for the least polar of them, 8OH2dG, although the most polar one, 1mA, only reached 27% of its maximum area. Finally, MSpak-RAM provided the highest individual areas obtained for the four extraction columns tested. In light of these results, it was decided to use the MSpak-RAM extraction column for later studies.

3.1.2. Optimization of extraction parameters

Once the most suitable extraction column for the target analytes had been selected, different factors that might improve the cleaning of the sample by eliminating possible interferents were studied. The washing process is affected by two main factors: the solvent used and the washing time, both being decisive factors for achieving an

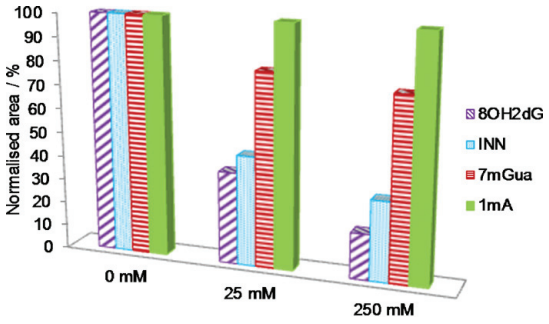

Fig. 2. MS/MS signals, normalized to the highest peak, obtained for 8OH2dG, INN, 7mGua and 1mA, working with different concentrations of ammonium formate: 0, 25 and 250 mM. Washing conditions: MSpak-RAM, 0.5 mL min^{-1}, 1 min.

efficient washing of the sample matrix with no elimination of the compounds of interest. The solvent parameters studied were the proportion of organic component, the pH, and the salt concentration. Regarding the proportion of organic component, it should be noted that when the proportion of organic solvent is high the effect of the washing of the interferents in the RAM is favoured, but the possibility of the removal of the target compounds also increases. To optimize the composition of the washing solvent, UHQ water: ACN mixtures were assayed and the content in ACN was modified from 0 to 10%. It was observed that increasing amounts of ACN produced a loss of the nucleosides, so a washing solvent without organic solvent was selected since this was the choice offering the best cleaning results without producing appreciable losses of the more polar analytes. pH was assayed from 3.0 to 9.0. The results showed that pH has no influence on the analyte washing process. Finally, the concentration of salt, in the form of ammonium formate, was studied from 0 to 250 mM. It was found that increasing amounts of salt in the washing solvent resulted in the elution of the less polar analytes (Fig. 2). As may be observed, while the 1mA signal remained constant, the signals of 8OH2dG and INN were reduced to less than 33% for 250 mM. Thus, with these results in mind, a washing solvent of UHQ water with no organic solvent or salts was selected as optimum.

The other main factor in the extraction process, the washing time, was assayed from 0.5 to 10 min. The analyses revealed a maximum for the analyte signals when the washing solvent was pumped through the extraction column for 1 min. When longer times were assayed, a loss of the more polar nucleosides was observed. Thus, for a clean-up time of 10 min the signal obtained for 8OH2dG was 60% of that found for a time of 1 min; this loss of signal was even more marked for 1mA, whose signal for 10 min of clean-up was reduced to 1% of that obtained with 1 min of clean-up.

Another important parameter in on-line extraction methods is the solvent used for elution and transfer of the analytes from the extraction column to the analytical column. The solvent must both elute the analytes and be compatible with the chromatographic separation. Furthermore, in HILIC mode ACN acts as weak chromatographic solvent, water being the strong one. Accordingly, it was important to reduce the aqueous dead volume associated with the coupling of the extraction column (MSpak-RAM) to the six-port valve in order to minimize the water that would reach the ZIC-HILIC column, disturbing the separation. In addition, for an adequate analysis the elution process should result in a narrow elution band and should be compatible with the gradient applied for subsequent chromatographic separation. As elution solvent, different mixtures of A: ACN and B: 2.5 mM formic acid/ammonium formate buffer – pH 3.4 – were assayed. Fig. 3a shows the elution profiles of

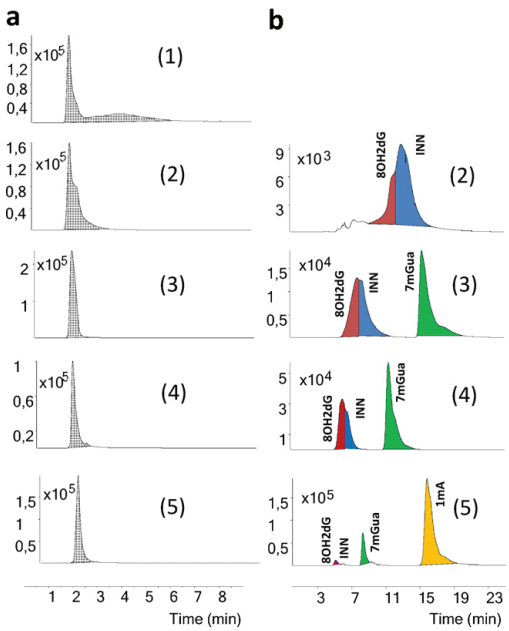

Fig. 3. MS/MS Total Ion Chromatogram (TIC) obtained with RAM–MS/MS (a, elution profiles) or with the RAM-ZIC-HILIC–MS/MS coupling developed (b) for 8OH2dG, INN, 7mGua and 1mA. MSpak-RAM on-line extraction column eluted with mixtures of 95:5 (1), 90:10 (2), 85:15 (3), 80:20 (4) and 70:30 (5) of ACN: 2.5 mM formic acid/ammonium formate buffer – pH 3.4. Washing conditions: UHQ water, 0.5 mL min^{-1}, 1 min.

mixtures ranging from 95% to 70% of ACN (obtained by bypassing the chromatographic column) and Fig. 3b shows the corresponding chromatograms (which were collected by maintaining the elution solvent constant for 5 min and then applying the separation gradient). As may be observed, high percentages of ACN produced a very broad elution peak (Fig. 3a.1 and a.2, w_b: 5.5 min), which resulted in an inadequate transfer to the ZIC-HILIC column, yielding a very poorly defined chromatogram (Fig. 3b.2). The increase in the UHQ water proportion afforded narrower elution peaks (Fig. 3a.3 and a.4), resulting in better transfer and chromatographic separation (Fig. 3b.3 and b.4). A mixture of 70% ACN: 30% 2.5 mM formic acid/ammonium formate buffer – pH 3.4 – was selected since this mixture afforded a minimum of elution peak width (Fig. 3a.5, w_b: 1.0 min). Furthermore, this mixture allowed adequate transfer to the chromatographic column and later separation (Fig. 3b.5). In addition, this mixture with 30% of aqueous phase was the mixture with the highest aqueous proportion assayed. Therefore, it resulted in a high compatibility with the residual UHQ water that reached the ZIC-HILIC column from the dead volume of the extraction column.

3.1.3. Optimization of chromatographic separation

Chromatographic separation of the nucleosides and nucleobases studied was optimized in the ZIC-HILIC column with (A) ACN and (B) 2.5 mM formic acid/ammonium formate buffer – pH 3.4 – as solvents. A low-concentration buffer was employed, since the presence of salts negatively affects the ionization of some analytes in the ESI. Furthermore, the use of acid pH results in the analytes being positively charged, thereby increasing the mass spectrometer signals and the column retentions [14]. A gradient was set, starting with the optimized conditions for the extraction column

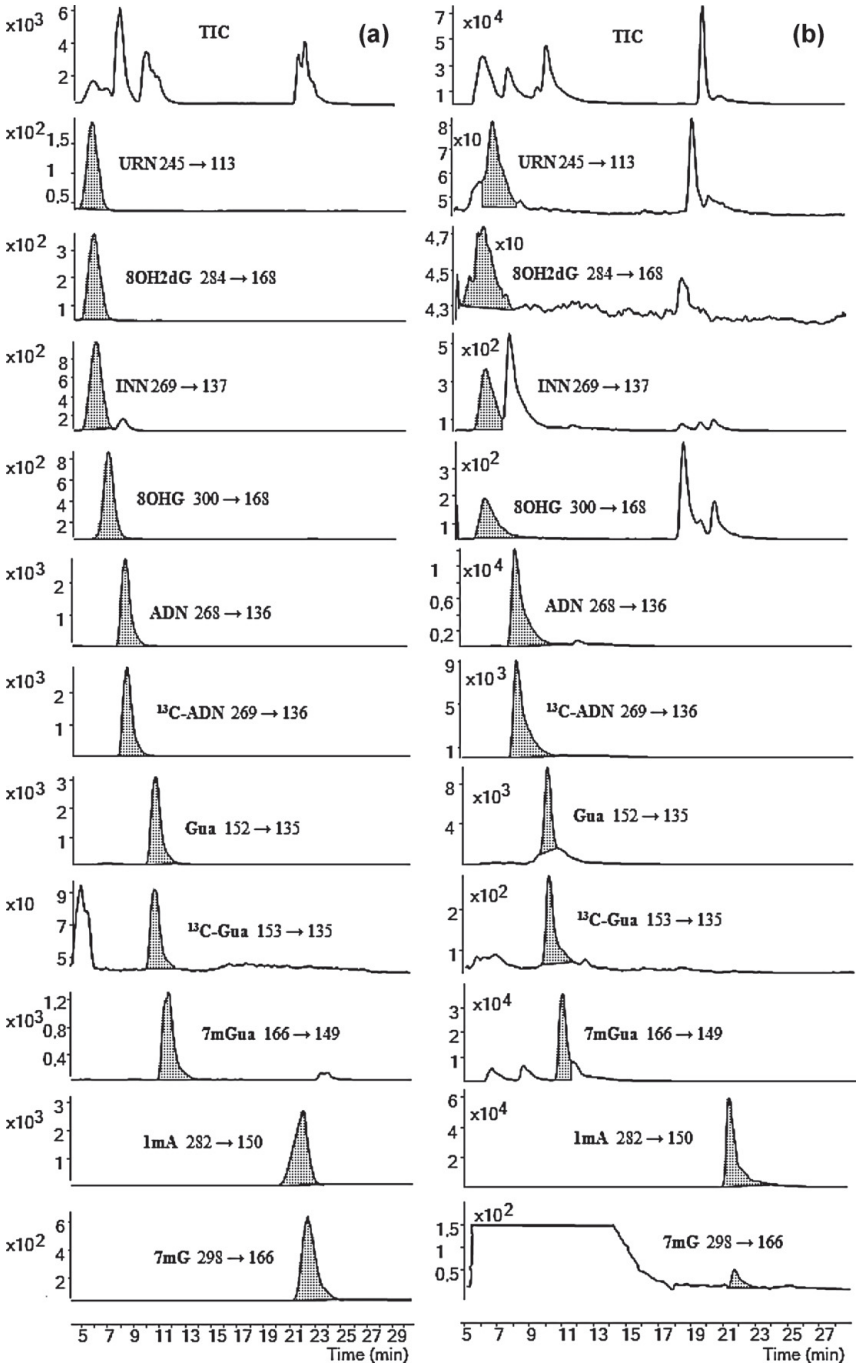

Fig. 4. LC–MS/MS Total Ion Chromatogram (TIC) and Selective Reaction Monitoring (SRM) transitions obtained by the analysis with the proposed RAM-ZIC-HILIC–MS/MS method of (a) a standard sample (2–4 μg mL^{-1}) and (b) unspiked urine H1.

Table 1
HPLC–MS/MS and calibration curve parameters for the developed RAM-ZIC-HILIC–MS/MS method.

	URN	8OH2dG	INN	8OHG	ADN	Gua	7mGua	1mA	7mG
HPLC–MS/MS[a]									
t_R/min	5.9	6.0	6.2	7.2	8.3	10.4	11.3	22.9	23.6
Fragmentor/V	80	80	80	80	80	120	120	80	80
Quantification transition	245 → 113 (8)	284 → 168 (8)	269 → 137 (4)	300 → 168 (12)	268 → 136 (16)	152 → 135 (16)	166 → 149 (16)	282 → 150 (16)	298 → 166 (8)
Confirmation transition	245 → 96 (40)	284 → 140 (20)	269 → 119 (40)	300 → 140 (28)	268 → 119 (40)	152 → 110 (20)	166 → 124 (20)	282 → 133 (40)	298 → 149 (40)
Calibration curve[b]									
Slope × 10^2/mL ng^{-1}	0.4 ± 0.02	3.0 ± 0.1	3.5 ± 0.1	6.7 ± 0.1	6.5 ± 0.1	8.9 ± 0.4	5.7 ± 0.1	19.0 ± 1.9	3.3 ± 0.2
Intercept	0.0 ± 0.1	0.3 ± 0.6	0.2 ± 0.9	1 ± 3	1 ± 4	1 ± 3	1 ± 1	-3 ± 6	0.9 ± 1.6
R^2	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.998	0.999
S/N at LLOQ	81	642	343	1560	1335	708	409	1298	297
LOD/ng mL^{-1}	1.3	0.2	0.3	0.1	0.2	0.2	0.4	0.1	0.9
ILIS	^{13}C-ADN	^{13}C-ADN	^{13}C-ADN	^{13}C-ADN	^{13}C-ADN	^{13}C-Gua	^{13}C-Gua	^{13}C-ADN	^{13}C-ADN

[a] ZIC-HILIC column. A: ACN, B: 2.5 mM formic acid/ammonium formate buffer (pH 3.4). Gradient from 30 to 50% B in 30 min. Flow rate: 0.5 mL min^{-1}. Column temperature: 20 °C. Detection: triple quadrupole in Selective Reaction Monitoring (SRM) mode; collision energy in brackets. ESI nebulization in positive mode with N_2 as drying gas at 12 L min^{-1} (350 °C). Nebulizer voltage: +3500 V. Nebulizer pressure: 35 psi. Collision gas: N_2.
[b] Six points calibration curve in UHQ water with isotopically labeled internal standards (ILIS). S/N at LLOQ, signal to noise ratio at lower limit of quantitation (30 ng mL^{-1}); LOD, limits of detection calculated for a signal to noise ratio of 3.

(70% A: 30% B) and ending with 50% A: 50% B; with this, a satisfactory separation was achieved in less than 25 min. Quantitation was carried out using a Selective Reaction Monitoring (SRM) transition for each analyte and two isotopically labeled compounds as Internal Standards (ILIS). The HPLC–MS/MS parameters are listed in Table 1. Fig. 4a shows the Total Ion Chromatogram (TIC) and the nine SRMs obtained for the analysis of the nucleosides and nucleobases studied in a standard mixture (2–4 μg mL^{-1}). In Fig. 4b, the TIC and SRMs of a real unspiked sample are shown.

3.2. Method validation

The RAM-ZIC-HILIC–MS/MS method developed was validated according to the "*Draft guideline on validation of bioanalytical methods*" [22]. This guideline provides the requirements for the validation of bioanalytical methods, the main objective being "to demonstrate the reliability of a particular method for the determination of an analyte concentration in a specific biological matrix, such as blood, plasma, urine, saliva or tissue".

The characteristics explored were: matrix effects, the response function (calibration curve performance), the lowest limit of quantitation (LLOQ), the precision, accuracy, selectivity and stability of the analytes and any internal standard in the biological matrix, and the stock and working solutions along the entire period of the storage and processing conditions.

3.2.1. Matrix effect

Quantitative determinations, using ESI as an ion source, may be affected by the ion suppression that mainly occurs due to the co-elution of matrix compounds with the analytes. These matrix effects were calculated as matrix factors (MF), as stated in Section 2.6. Fig. 5 shows the MF obtained for the ZIC-HILIC–MS/MS system (a), for the RAM-ZIC-HILIC–MS/MS method developed (b) and normalized MF, calculated using ILIS (c). As may be observed, MF below 10% were obtained when the urine samples were injected directly into the ZIC-HILIC column (Fig. 5a). On-line coupling of the separation column with the MSpak-RAM improved the MF up to 60–80% (Fig. 5b); in other words, the matrix effects were reduced almost 10-fold. From the normalized MF values (Fig. 5c) it is clear that 8OHG, 7mG, 8OH2dG, 1mA, INN, ADN and URN should be normalized with ^{13}C-AND, and that 7mGua and Gua should be normalized with ^{13}C-Gua, because of the pairing of MF values. This pairing was correlated with the molecular structure of the analytes; on one hand, 8OHG, 7mG, 8OH2dG, 1mA, INN, ADN and URN are nucleosides like ^{13}C-ADN, and, on the other hand, 7mGua and Gua are nucleobases, like ^{13}C-Gua. It was also found that the normalized MF values were not significantly different for 100% (differences above 100% can be correlated with the endogenous occurrence of nucleosides). This points to the notion that ILIS could be used to correct matrix effects in the method developed. Thus, quantification via standards prepared in UHQ water would be adequate if the signals were corrected with an appropriate ILIS.

3.2.2. Calibration curve

An ILIS calibration curve was built from six standards prepared in UHQ water, covering the expected range from 30 ng mL^{-1} to 10 μg mL^{-1},spiking with ILIS at 500 ng mL^{-1}. The ILIS calibration curve parameters for each analyte are shown in Table 1. The lowest limit of quantitation (LLOQ) was assessed using water standards at 30 ng mL^{-1}, the lowest concentration in the calibration curve. Precisions for the LLOQ ranged from 4.1% to 6.7% with signal-to-noise ratios ranging from 81 to 1560. The back-calculated concentrations of the calibration standards were in all cases within ±15% of the nominal value. The limits of detection (LOD), calculated as three times the signal-to-noise ratio, were in the 0.1–1.0 ng mL^{-1} range.

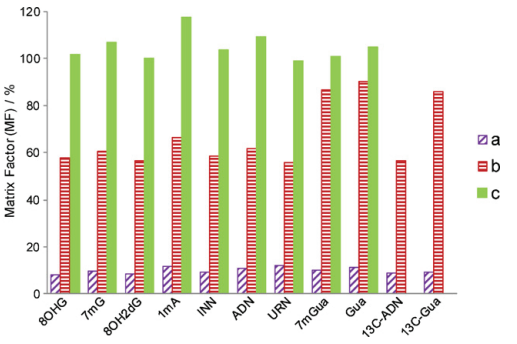

Fig. 5. Matrix effects, calculated as matrix factors (MF) for: (a) ratio between peak areas obtained for a spiked urine sample injected directly into the ZIC-HILIC–MS/MS system and peak areas obtained in standard solutions, (b) ratio between peak areas obtained for a spiked urine sample injected according to the RAM-ZIC-HILIC–MS/MS method developed and peak areas obtained in standard solutions, and (c) ratio between peak areas obtained for a spiked urine sample injected according to the RAM-ZIC-HILIC–MS/MS method developed normalized to their corresponding ILIS and peak areas obtained in standard solutions normalized to their corresponding ILIS.

Table 2
Signal precisions obtained by the analysis of an unspiked urine sample (H1) by the developed RAM-ZIC-HILIC–MS/MS method.

Analytes	Coefficient of variation (CV)[a]/%				[Analyte][d]/ng mL^{-1}
	Within-run[b]		Between-run[c]		
	a	*b*	*a*	*b*	
URN	2.3	2.6	4.6	4.1	660 ± 30
8OH2dG	1.5	1.6	5.9	4.8	230 ± 10
INN	1.5	1.6	5.8	4.6	1390 ± 30
8OHG	2.3	1.7	7.5	5.4	160 ± 20
ADN	1.8	1.7	9.6	7.8	1800 ± 30
Gua	2.1	2.2	4.9	4.4	1480 ± 30
7mGua	1.8	1.9	7.9	5.1	4870 ± 40
1mA	2.6	2.2	15	13	2890 ± 80
7mG	4.5	4.4	11	10	280 ± 20

[a] Precision, calculated using analysis of variance (ANOVA), expressed as coefficient of variation (CV).
[b] Within-run precision: 8 consecutive injections of unspiked urine H1 without (*a*) and with (*b*) ILIS correction.
[c] Between-run precision: 8 consecutive injections, in 3 different days, of unspiked urine H1 without (*a*) and with (*b*) ILIS correction.
[d] Concentration of analytes in unspiked urine H1 obtained by calibration in UHQ water with ILIS.

3.2.3. Precision

Signal precision was evaluated by analyzing an unspiked urine sample (H1). For each analyte, the within-run and between-run precisions were calculated, always with respect to the ILIS used (Table 2). The values were obtained from eight consecutive injections on three different days of unspiked urine H1. As endogenous compounds, nucleosides were present in unspiked urine at low (8OHG, 7mG and 8OH2dG), medium (URN, INN and Gua) and high (1mA, ADN and 7mGua) levels, and hence the analysis of precision in an unspiked urine sample allowed the performance of the system to be checked over a broad concentration range. The concentrations calculated, expressed in ng mL^{-1}, are shown in Table 2. Within-run values varied in the 1–5% range and between-run values were in the 4–15% range (Table 2). Correction with ILIS did not afford a significant improvement in the precision of the signals obtained.

3.2.4. Accuracy

Accuracy, assessed in samples spiked with known amounts of the analyte (quality control samples, QC), must be determined by replicate analysis using a minimum of 5 determinations at a minimum of 4 concentration levels that cover the calibration curve range. However, this approach cannot be used in the case of endogenous compounds, since quality control samples cannot be prepared owing to the lack of blank matrix samples. A well-known approach to circumvent this problem and one that has been widely applied over many years is application of the standard addition method [27]. Thus, the accuracy was checked by analyzing six different matrices (H1–H6) by standard additions (quality control samples, QC) and comparing the results with those obtained by the RAM-ZIC-HILIC–MS/MS method developed. As in the case of precision, the analysis of unspiked urine samples demonstrated accuracy over a broad range of concentrations. The accuracy results are shown in Table 3; as may be observed, values within 13% of the nominal values for the QC samples were found.

Furthermore, the slopes of the standard addition and proposed methods were compared for each nucleoside in the six samples through a two-tailed paired *t*-test. In all cases p values > 0.05 were found, suggesting that there were no significant differences between the two methods.

3.2.5. Selectivity, stability, and carry-over

One of the main advantages of tandem mass spectrometry is its good ability to differentiate the analytes of interest and ILIS from endogenous components in the matrix, when working in SRM mode. Selectivity was checked by using six different matrix samples (H1–H6), which were analyzed and evaluated individually for interference. Selectivity was also confirmed by reanalyzing three of the nucleosides studied with secondary SRM transitions (1mA: 282 → 133, ADN: 268 → 119 and 7mGua: 166 → 124). The concentrations found were not significantly different from those obtained with the primary SRM transitions, confirming that no interferences were present in the transitions used for quantification.

The stabilities of the standards and stock solutions were guaranteed by the manufacturer under the appropriate storage conditions. Working solutions were prepared daily. The stability of the samples was tested as regards freeze and thaw stability. A urine sample was analyzed just after collection and after 5 cycles of freezing–thawing. No significant differences were found for the analytical signals obtained.

One of the disadvantages of the use of an on-line extraction column for different samples is possible carry-over among them. This may be especially relevant when RAMs are used, because they have a long half-life. Carry-over was assessed by injecting a blank sample after twelve urine samples, the last one being a high-concentration sample (urine spiked at 2 μg mL^{-1}). Signals, when found, were under 5% of the LLOQ.

3.3. Method application: pattern recognition

The analysis of biological samples usually affords very complex data that require computer-aided graphic analysis and statistical methods, such as pattern-recognition techniques, to discriminate among samples. Here, scatter and star plots [23] and PCA, HCA and SIMCA are suggested as adequate tools for discriminating between urine samples from healthy volunteers and cancer patients.

3.3.1. Scatter and star plots

Fig. 6 shows the scatter and star plots. The scatter plots (Fig. 6a) represent the creatinine-corrected concentrations from healthy volunteers (samples H1–H6, represented with filled diamonds) and cancer patients (samples from C1 to C6, represented with open diamonds) obtained by the proposed RAM-ZIC-HILIC–MS/MS method. As may be seen, the level of several nucleosides was higher for cancer patients. In the star plot (Fig. 6b) the concentrations were converted to a percentage of the corresponding concentration in healthy volunteers. The percent relative concentration of each nucleoside was plotted as a line radiating from a common central point and the lines were joined together to produce the star plot for each group average. Star plots represent the increase in level in a better way, since normalization allows both the minor and major nucleosides to have the same weight. Thus, URN (medium concentration) and 8OHG and 7mG (low concentration) can be considered as the main nucleosides to discriminate between groups.

Table 3
Accuracy for the developed RAM-ZIC-HILIC–MS/MS method calculated with addition standard as reference. Analysis of unspiked urine samples (H1–H6).

	H1			H2			H3			H4			H5			H6		
	A[a]	B[b]	Acc[c]/%	A[a]	B[b]	Acc[c]/%	A[a]	B[b]	Acc[c]/%	A[a]	B[b]	Acc[c]/%	A[1]	B[2]	Acc[3]/%	A[a]	B[b]	Acc[c]/%
URN	0.19	0.206	106	0.17	0.149	89	0.30	0.294	97	0.42	0.459	110	0.29	0.271	93	0.10	0.113	107
8OH2dG	0.06	0.062	100	0.01	0.013	100	0.15	0.154	102	0.03	0.027	92	0.01	0.011	87	0.03	0.030	88
INN	0.40	0.395	100	0.59	0.590	100	0.29	0.277	97	0.42	0.350	89	1.07	1.016	88	0.58	0.565	95
8OHG	0.05	0.041	89	0.07	0.070	100	0.15	0.147	96	0.12	0.106	88	0.10	0.089	90	0.27	0.257	96
ADN	0.51	0.514	100	1.10	1.064	97	0.99	0.982	99	1.10	1.091	99	1.06	1.118	106	1.59	1.670	103
Gua	0.73	0.750	102	1.31	1.310	100	0.34	0.320	95	0.27	0.234	86	0.18	0.170	93	0.16	0.150	93
7mGua	2.30	2.253	99	3.92	3.951	101	1.41	1.515	106	3.50	3.617	104	2.12	2.085	102	2.41	2.482	104
1mA	0.81	0.781	97	0.92	1.015	107	0.72	0.746	101	0.68	0.716	106	1.04	1.053	101	0.79	0.785	99
7mG	0.07	0.072	104	0.08	0.071	88	0.06	0.059	100	0.12	0.110	94	0.24	0.223	92	0.16	0.145	93

[a] A: concentration of analytes in unspiked urines, expressed as nmol μmol^{-1} of creatinine, obtained by standard addition with the proposed method.
[b] B: concentration of analytes in unspiked urines, expressed as nmol μmol^{-1} of creatinine, obtained by calibration in UHQ water with ILIS.
[c] Accuracy calculated using standard addition as quality control.

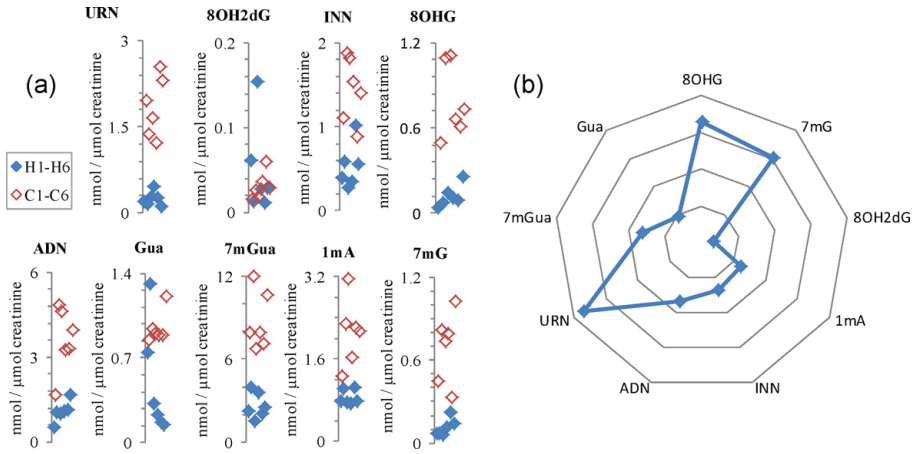

Fig. 6. Scatter plots (a) and star plot (b) obtained by analysis of urine samples from H1 to H6 (healthy volunteers) and C1 to C6 (cancer patients) with the proposed RAM-ZIC-HILIC–MS/MS method.

3.3.2. *Pattern recognition*

Standardized and creatinine-corrected concentrations were used for statistical pattern recognition. First, unsupervised methods, PCA and HCA, were applied. PCA showed that the total variance explained by the first PC was 80% whereas the second PC explained 90% of it. The scores of the samples in these two dimensions (PC-1 and PC-2) are plotted in Fig. 7a. The healthy volunteer samples grouped close on the left of the graph while the cancer patient samples formed a looser cluster on the right. In any case, taking into account only the two first PCs, the samples were clearly separated in two groups. The main loadings for these two PCs were 8OHG (0.7), URN (0.5) and 7mG (0.4) for PC-1 and URN (0.8) and 8OH2dG (0.2) for PC-2. HCA was applied using complete linkage and squared Euclidean distance. The dendogram generated (Fig. 7b)

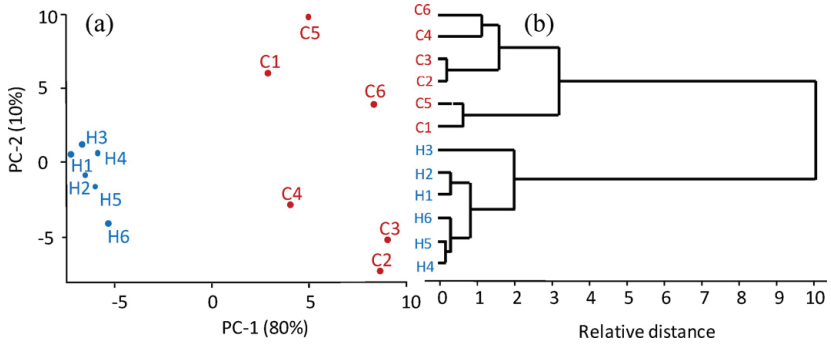

Fig. 7. Principal Component Analysis (PCA) scores of the two first PCs (PC-1 and PC-2) (a) and complete linkage, squared Euclidean distance dendogram obtained by Hierarchical Cluster Analysis (HCA) (b). H1–H6: healthy volunteer urine samples. C1–C6: cancer patient urine samples.

showed an adequate separation, forming two groups at a relative distance lower than 4. Not a single wrong linkage was observed.

Finally, SIMCA, a supervised pattern-recognition method, was applied to the data. Two models were constructed: one for the healthy volunteer samples – from H1 to H5 – using two PCs, and another one for the cancer patient samples – from C1 to C5 – using one PC. The H6 and C6 samples, not included in the model, were subsequently used for prediction. Coomans' diagram revealed the two sets to be separated, each in its own box. The H6 and C6 samples were correctly predicted. The nucleosides with the highest loadings in each model were 8OH2dG (0.9585 for H1–H5) and 8OHG (0.7709 for C1–C5). The analytes with the highest discriminant power were as follows: 8OHG (9.9682), URN (8.0543), 7mG (6.0277) and ADN (5.2008). These results are in good agreement with those obtained with the star plot.

4. Conclusions

This work proposes the coupling of a restricted-access material (RAM), based on N-vinylacetamide copolymer, with a ZIC-HILIC column. This novel coupling allows the on-line extraction of urine and subsequent separation, detection and confirmation of urinary nucleosides and nucleobases up to the low-ng mL^{-1} level. This configuration means that sample treatment is minimum, affording shorter analysis times, more automation, and lower costs than off-line methods.

The method was successfully applied to the analysis of urine samples from cancer patients. These samples had increased levels of nucleosides and nucleobases in comparison with the control urine samples. The application of pattern-recognition methods, such as PCA, HCA and SIMCA, allowed correct grouping of the samples studied, 8OHG, URN and 7mG the being the nucleosides that showed the highest discriminatory power. These results show that the method developed could potentially be used as a control method for clinical purposes such as early diagnosis and therapeutic response monitoring.

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References

[1] M. Harri, H. Kasai, T. Mori, J. Tornaeus, K. Savela, K. Peltonen, J. Chromatogr. B 853 (2007) 242.
[2] H.M. Liebich, S. Müller-Hagedorn, F. Klaus, K. Meziane, K.R. Kim, A. Frickenschmidt, B. Kammerer, J. Chromatogr. A 1071 (2005) 271.
[3] B. Kammerer, A. Frickenschmidt, C.E. Müller, S. Laufer, C.H. Gleiter, H. Liebich, Anal. Bioanal. Chem. 382 (2005) 1017.
[4] D. Bullinger, H. Fröhlich, F. Klaus, H. Neubauer, A. Frickenschmidt, C. Henneges, A. Zell, S. Laufer, C.H. Gleiter, H. Liebich, B. Kammerer, Anal. Chim. Acta 618 (2008) 29.
[5] L.B. Jeng, W.Y. Lo, W.Y. Hsu, W.D. Lin, C.T. Lin, C.C. Lai, F.J. Tasi, Rapid Commun. Mass Spectrom. 23 (2009) 1543.
[6] C. Richter, J.W. Park, B.N. Ames, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 6465.
[7] K. Otsuka, S. Terabe, J. Chromatogr. A 875 (2000) 163.
[8] S. Schmalzing, W. Nashabeh, Electrophoresis 18 (1997) 2184.
[9] A.J. Sasco, F. Rey, C. Reynaud, J.Y. Bobin, M. Clavel, A. Nivelau, Cancer Lett. 108 (1996) 157.
[10] Y. Jiang, Y. Ma, Anal. Chem. 81 (2009) 6474.
[11] R. Tuytten, F. Lemière, E.L. Esmans, W.A. Herrebout, B.J. van der Veken, B.U.W. Maes, E. Witters, R.P. Newton, E. Dudley, Anal. Chem. 79 (2007) 6662.
[12] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[13] F. Qin, Y.Y. Zhao, M.B. Sawyer, X.F. Li, Anal. Chem. 80 (2008) 3404.
[14] E. Rodríguez-Gonzalo, D. García-Gómez, R. Carabias-Martínez, J. Chromatogr. A 1218 (2011) 3994.
[15] P. Sadílek, D. Satinsky, P. Solich, Trends Anal. Chem. 26 (2007) 375.
[16] N.M. Cassiano, V.V. Lima, R.V. Oliveira, A.C. de Pietro, Q.B. Cass, Anal. Bioanal. Chem. 384 (2006) 1462.
[17] E. Dudley, S. El-Shakawi, D.E. Games, R.P. Newton, Nucleosides Nucleotides Nucleic Acids 19 (2000) 545.
[18] H.Y. Li, S.M. Wang, H.M. Liu, S.S. Bu, J. Li, D. Han, M.Z. Zhang, G.Y. Wu, J. Mass Spectrom. 44 (2009) 641.
[19] S. Cho, B.H. Jung, S.H. Lee, W. Lee, G. Kong, B.C. Chung, Biomed. Chromatogr. 20 (2006) 1229.
[20] European Medicines Agency (EMEA), Guideline on validation of bioanalytical methods, CHMP/EWP/192217/2009.
[21] M. Jaffe, Z. Physiol. Chem. 10 (1886) 391.
[22] E. Rodríguez-Gonzalo, D. García-Gómez, R. Carabias-Martínez, J. Chromatogr. A 1217 (2010) 40.
[23] S. La, J. Cho, J. Kim, K. Kim, Anal. Chim. Acta 486 (2003) 171.
[24] X. Zhao, W. Wang, J. Wang, J. Yang, G. Xu, J. Sep. Sci. 29 (2006) 2444.
[25] R. Tuytten, F. Lemière, W. Van Dongen, E. Witters, E.L. Esmans, R.P. Newton, E. Dudley, Anal. Chem. 80 (2008) 1263.
[26] W. Hsu, W.T. Chen, W. Lin, F. Tsai, Y. Tsai, C. Lin, W. Lo, L. Jeng, C. Lai, Clin. Chim. Acta 402 (2009) 31.
[27] N.C. van de Merbel, Trends Anal. Chem. 27 (2008) 924.

REVIEW ARTICLE
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Stationary phases for separation of nucleosides and nucleotides by hydrophilic interaction liquid chromatography

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This review describes recent trends in novel stationary phases for hydrophilic interaction liquid chromatography (HILIC), focusing on separation of nucleosides and nucleotides. The performance of these novel HILIC stationary-phases is discussed in terms of resolution, separation, improved sensitivity and increased speed in order to achieve more efficient, faster chromatographic separations.

Keywords: Column technology; Hydrophilic interaction liquid chromatography (HILIC); Monolith; Nucleobase; Nucleoside; Nucleotide; Resolution; Sensitivity; Separation; Stationary phase

Abbreviations: AAc-co-BA, Acrylic acid-co-n-butylacrylate ester; AAPBA, 3-acrylamidophenylboronic acid; ACN, Acetonitrile; APS, Aminopropylsilica; BACM, 4-aminocyclohexyl)methyl]cyclohexylamine; BVPE, 1,2-bis(p-vinylphenyl)ethane; CMCH, Carboxymethyl chitosan; CNP, Carbon nanoparticle; CTSAP, Click triazole-amino stationary phase; DBN, 1,5-diazabicyclo[4.3.0] non-5-ene; EDMA, Ethylene dimethacrylate; ERLIC, Electrostatic repulsion liquid chromatography; EtOH, Ethanol; $Et(OH)_2$, 1,2-ethanediol; GNP, Gold nanoparticle; HA, Humic acid; HILIC, Hydrophilic interaction liquid chromatography; HMMAA, N-(hydroxymethyl) methacrylamide; HTMA, 2-hydroxyl-3-[4-(hydroxymethyl)-1H-1,2,3-triazol-1-yl]propyl 2-methylacrylate; MAA, Methacrylic acid; ME, 2-mercaptoethanol; MEO, Oxidized 2-mercaptoethanol; MeOH, Methanol; MS, Monolith silica; MSA, [2-(methacryloyloxy)-ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide; NAHAM, N-acryloyl-tris(hydroxymethyl)-aminomethane; PA, Polyacrylamide; PAA, Polyacrylic acid; PEI, Polyethyleneimine; PETA, Pentaerythritoltriacrylate; PHA, Polyhydroxylethylaspartamide; pNA-SIL, Poly[N-acryoyl-tris(hydroxymethyl)-aminomethane]-coated silica; PSA, Polysulfoethyl; TG, 1-thioglycerol; RAM, Restricted access material; RF, Retention factor; SPE, N,N-dimethyl-N-methacryloxyethyl-N-3(sulfopropyl)-ammonium betaine; TCM, Traditional chinese medicine; TEPIC, Tris(2,3-epoxypropyl) isocyanurate; TGO, Oxidized 1-thioglycerol; ZIC, Zwitterionic interaction chromatography; 6TG, 6-thioguanine

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1. Introduction

In recent years, hydrophilic interaction liquid chromatography (HILIC) has been established as the appropriate chromatographic mode for the retention and the separation of polar and hydrophilic compounds. The combination of the retention of polar compounds and the high organic content in the mobile phase makes this mode of chromatography suited to high-sensitivity, quantitative analysis based on applying liquid chromatography coupled to mass spectrometry (LC-MS).

It has been accepted that partitioning between the aqueous layer associated with the stationary phase and the organic component of the mobile phase is the main retention mechanism. However, the participation of secondary interactions (e.g., dipole-dipole, hydrogen bonding and ion-exchange) can play an important role in separation, leading to changes in selectivity.

As the HILIC mode is becoming more popular for the chromatographic analysis of polar and ionic analytes, a wider range of HILIC stationary phases is available on the market. Novel materials with different types of polar functional groups (including amide, amino, diol, cyano, zwitterionic and other charged and non-charged groups) are continually being developed and assessed as stationary phases under HILIC conditions.

The increasing interest in this chromatographic mode as a viable alternative to reversed-phase LC (RPLC) can be seen

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in recent reviews that have appeared in the literature. In recent years, 26 review articles have been published on the topic, some of them general [1–6]. The applications of HILIC include the analysis of different polar molecules, such as toxic residues – antibiotics [7] and other pharmaceuticals [8] in foods and pesticides in environmental matrices [9]. HILIC also seems to be a promising alternative to more traditional LC methods in bioanalysis. In particular, the analysis of highly polar metabolites, such as nucleosides and nucleobases, involves important difficulties in RPLC, due to the poor retention of these analytes in the stationary phase. Normal-phase LC (NPLC) would afford a better retention but is not desirable, due to the difficulty involved in coupling this chromatographic technique with MS.

Nucleobases, nucleosides and nucleotides are the basic components of all cells, forming the different nucleic acids. Their clinical interest in bioanalysis has expanded considerably in recent decades, so nucleosides are investigated as potential biomarkers of oncological processes, being excreted with or without modifications through the urinary tract [10–13]. Apart from in field of biology, nucleobases, nucleosides and nucleotides are also important in other avenues of enquiry. In foods, monophosphate nucleotides are nutrients of special importance during periods of rapid growth or after injury, hence the importance of the supplementary contribution of such nutrients in neonatal feeding [14–16].

The aim of this article is to discuss current trends and advances in the development and the evaluation of the different approaches recently proposed for new HILIC stationary phases, which may allow selectivity and/or sensitivity to be enhanced, or faster separations to be achieved. It summarizes the information published in more than 75 papers in the literature, mostly in the period 2008–12, with the main focus on recent advances and trends in the HILIC approach for nucleoside and nucleotide analysis.

Also, we consider new trends in column technology (e.g., reduction in particle size down to 1.7 μm, the use of superficially-porous particles, and the use of monolithic columns of different types) as applied to the separation of nucleotides, nucleosides and their bases.

2. Commercial columns

2.1. Unmodified silica

The simplest stationary phase, the first to be used in HILIC, is that formed by unmodified silica (bare silica). The separation mechanism proposed for this stationary phase is based on a partition equilibrium between the aquo-organic phase (usually mixtures of water as the minor component and acetonitrile (ACN) as the major component), and a phase – mainly aqueous – bound to the stationary phase.

The different separations proposed for the compounds studied here have a high percentage of organic components in the mobile phase, which is usual in the stationary phases of bare silica (Table 1). Thus, it has been possible to separate four nucleobases and four nucleosides on Atlantis HILIC Silica, formed by bare silica, in 14 min [17].

The low retention factors (RFs) usually found for these compounds in bare silica columns can be improved by varying the nature of the composition of the mobile phase. Working with an isocratic regime with 90% of ACN, the replacement of the water by $Et(OH)_2$, MeOH and EtOH leads the RFs to increase in the following order: $H_2O < Et(OH)_2 <$ MeOH < EtOH. Thus, an order of elutropic strength of a protic modifier of EtOH < MeOH < $Et(OH)_2 < H_2O$ could be established [18]. This can be explained in terms of the fact that the replacement of water molecules by organic solvent may modify the formation of the semi-stationary layer.

2.2. Hydroxylated stationary phases

The low selectivity and retentions shown by HILIC columns made of bare silica can be improved by introducing functional polar groups. One of the simplest modifications consists of introducing alkyl chains with hydroxyl functional groups. The separation mechanism is based, as in the case of bare silica columns, on partition equilibrium, although the presence of hydroxyl residues allows the appearance of interactions via hydrogen bridges, leading to an increase in selectivity.

Within the columns with hydroxylated stationary phases, Luna-HILIC, formed by a cross-linked diol phase (Fig. 1a), is the one most used for the separation of the compounds of interest, offering a neutral stationary phase that allows a water-rich layer to form readily on the surface [19,20]. For this Luna HILIC column, plots of log k_0 versus log of the percentage of water in the mobile phase resulted in a linear fit in the range 45–55% water in the mobile phase, consistent with an adsorptive mechanism. At around 45% water composition in the mobile phase, a slight curvature of the line was observed, suggesting that a partitioning mechanism was taking hold as the column became saturated. Those authors [19,20] suggested that, during gradient elution at higher amounts of water, the adsorptive mechanism may be more prevalent and the separation will fall under conditions more common in RPLC.

In this phase, the replacement of water by $Et(OH)_2$, MeOH and EtOH has also been studied [18]. The gain in retention upon reducing the polarity of the protic modifier was stronger for nucleobases, although the general trend of increasing retention upon reducing the polarity of the modifier was not so evident for all solutes.

Other columns modified by hydroxylation tested for separation were polyhydroxylethylaspartamide (PHA, Fig. 1b) and Pronto-SIL-DIOL (Fig. 1c). Using PHA, the authors concluded that, in comparison with amine-bonded phases, the functionalization of stationary phases with hydroxy groups resulted in less retentive

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Table 1. Commercial HILIC columns proposed for the analysis of nucleosides and nucleotides

Analytes	HILIC column	Column dimensions/mm	Separation Time/min	Mobile phase	Flow/ mL/min	Detection	Ref.
Four nucleobases and four nucleosides	Atlantis HILIC Silica (Bare)	150 × 4.6 (5 µm)	14	90% ACN 10% AF:FA 200 mM	1.0	DAD: 260 nm	[17]
Four nucleobases and nine nucleosides	DAISOGEL (Bare silica)	150 × 4 (5 µm)	30/15/8/10	90% ACN AA 5 mM 10% EtOH/MeOH/Et(OH)$_2$/H$_2$O	1.0	DAD: 254 nm	[18]
Five nucleobases and nine nucleosides	Luna-HILIC	150 × 4.6 (5 µm)	7	90% ACN 10% AA 10 mM (pH: 5)	1.0	UV:254 nm	[19]
Four polynucleotides	Luna-HILIC	150 × 2.0 (3 µm)	25	A: ACN B: AA 5 mM (pH: 5.8) 0-2.5 min: 85%-70% A 2.5-17.5 min: 70%-40% A	0.1	ICP-MS: $^{31}P^{16}O^{+}$	[20]
Four nucleobases and nine nucleosides	Luna-HILIC	150 × 4.6 (5 µm)	30/20/10/7	90% ACN AA 5 mM 10% EtOH / MeOH / Et(OH)$_2$ / H$_2$O	1.0	DAD: 254 nm	[18]
Five nucleobases and ten nucleosides	PHA	100 × 2.1 (3 µm)	25	90% ACN 10% AA 20 mM (pH: 4.7)	0.1	UV: 254 nm	[21]
Four nucleobases and nine nucleosides	Pronto-SIL-DIOL	150 × 4 (5 µm)	35/20/10/7	90% ACN AA 5 mM 10% EtOH / MeOH / Et(OH)$_2$ / H$_2$O	1.0	DAD: 254 nm	[18]
Five polynucleotides	TSK-Gel-NH$_2$ 100	150 × 2.0 (3 µm)	30	A: MeOH B: AF:FA 30 mM (pH: 2.5) 0-10 min: 15% A 10.1-30 min: 2% A	0.2	MS/MS (QqQ)	[22]
Six nucleobases and thirteen nucleosides	TSK-Gel Amide 80	150 × 2.0 (3 µm)	110	95% ACN 5% B: AA 10 mM (pH: 6.8)	0.2	DAD: 260 nm	[23]
Four nucleosides	TSK-Gel Amide 80	150 × 4.6 (3 µm)	50	90% ACN 10% SA:AAc 20 mM (pH: 4.4)	0.6	UV: 262 nm	[24]
Five nucleobases and ten nucleosides	TSK-Gel Amide-80	150 × 4.6 (5 µm)	40	90% ACN 10% AA 20 mM (pH: 4.7)	0.5	UV: 254 nm	[21]
Six nucleobases and six nucleosides	TSK-Gel Amide 80	150 × 2.1 (5 µm)/(3 µm)	80	A: ACN B: AF 10 mM (pH: 4) 0-20 min: 95% A 20-40 min: 95%-90% A 40-80 min: 90%-85% A	0.15/0.1	DAD: 260 nm	[25]
Four polynucleotides	TSK-Gel Amide 80	150 × 2.0 (3 µm)	25	A: ACN B: AA 5 mM (pH: 5.8) 0-2.5 min: 85%-70% A 2.5-17.5 min: 70%-40% A	0.1	ICP-MS: $^{31}P^{16}O^{+}$	[20]
Four nucleosides and four nucleotides	XBridge Amide	150 × 4.6 (3.5 µm)	60	85% ACN 15% AP 5 mM	1.0	UV: 262 nm	[26]
Five nucleobases and nine nucleosides	XBridge Amide	150 × 3	16	90% ACN 10% AA 10 mM (pH: 5)	0.6	UV: 254 nm	[19]
Four nucleobases and four nucleosides	VENUSIL HILIC	150 × 4.6 (5 µm)	23	90% ACN 10% AF:FA 200 mM	1.0	DAD: 260 nm	[17]

continued on next page

Table 1. *continued*

Analytes	HILIC column	Column dimensions/mm	Separation Time/min	Mobile phase	Flow/ mL/min	Detection	Ref.
Seven nucleobases and nine nucleosides	VENUSIL HILIC	250 × 4.6 (5 μm)	75	A: ACN B: FA 0.2%, AF 20 mM 0-15 min: 95%A 15-20 min: 95%-90% A 20-30 min: 90%A 30-50 min: 90%-80% A 50-60 min: 80%-60% A 60-65 min: 60%-50% A 67-75 min: 50% A	0.8	MS (TOF)	[27]
Five nucleobases and ten nucleosides	COSMOSIL HILIC	150 × 4.6 (5 μm)	30	90% ACN 10% AA 20 mM (pH: 4.7)	0.5	UV: 254 nm	[21]
Two nucleobases	ZIC-HILIC	50 × 4.6 (5 μm)	2.5 1.0 0.5	80% ACN 20% AF 2.5 mM FA 25 mM	0.5 2.0 5.0	DAD: 254 nm	[32,33]
Five nucleobases	ZIC-HILIC	150 × 2.1 (5 μm)	15	80% ACN 20% AF 2.5 mM FA 25 mM	0.1	DAD: 254 nm	[32,33]
Four nucleobases and two nucleosides	ZIC-HILIC	150 × 4.6 (3.5 μm)	13	80% ACN 20% AF:FA 2.5 mM (pH: 3)	0.5	DAD: 254 nm MS (Q)	[29,34]
Five nucleobases and ten nucleosides	ZIC-HILIC	150 × 4.6 (5 μm)	30	90% ACN 10% AA 20 mM (pH: 4.7)	0.5	UV: 254 nm	[21]
Six nucleobases and six nucleosides	ZIC-HILIC	150 × 2.1 (5 μm)	60	A: ACN B: AF 10 mM (pH: 4) 0-10 min: 95%-85% A 10-60 min: 85% A	0.15	DAD: 260 nm	[25]
Two nucleobases and six nucleosides	ZIC-HILIC	150 × 4.6 (3.5 μm)	25	A: ACN B: AF:FA 2.5 mM (pH: 3.1) 0-10 min: 70% A 10-30 min: 50% A	0.5	MS/MS (QqQ)	[35]
Five nucleobases and eleven nucleosides	ZIC-HILIC	100 × 4.6 (3.5 μm)	30	A: ACN B: AP 15 mM (pH: 6) 0-18 min: 97%-95% A 18-19 min: 95%-90% A 19-30 min: 90%-80% A	1.0	DAD: 254 nm	[36]
Twelve nucleotides	PSA	200 × 4.6 (5 μm)	35	84% ACN 16% 80 mM TEAP pH: 3.0	1.0	UV: 260 nm	[31]

ACN, Acetonitrile; AF, Ammonium formate; FA, Formic acid; AA, Ammonium acetate; SA, Sodium acetate; AP, Ammonium phosphate; AC, Ammonium carbonate; TEAP, Triethylammonium phosphate. Other abbreviations: see Fig. 1.

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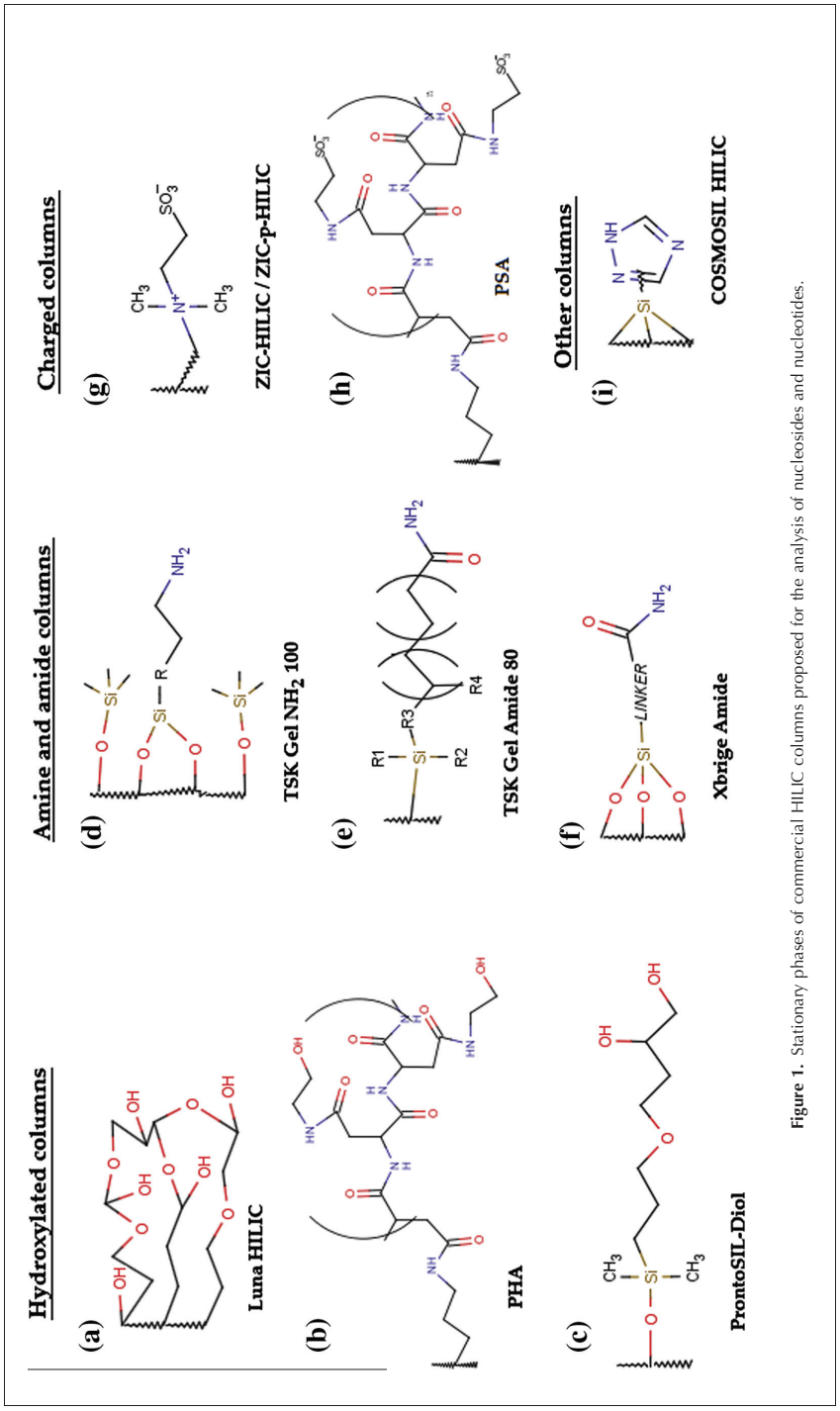

Figure 1. Stationary phases of commercial HILIC columns proposed for the analysis of nucleosides and nucleotides.

and less selective HILIC phases [21]. Using a Pronto-SIL-DIOL column, the separation of nucleosides and nucleobases with mixtures of ACN and H_2O, $Et(OH)_2$, MeOH and EtOH, respectively, has been studied [18]. The trends in changes of selectivity for adenosine/uridine, guanosine/adenosine, as well as cytidine/adenosine as a function of the type of modifier were in good agreement for the two commercial diol-type columns studied by those authors (Luna-HILIC and Pronto-SIL-DIOL).

2.3. Amine and amide stationary phases

Another usual modification used to increase selectivity is derivatization with amine and amide terminal groups. As in the case of columns modified by hydroxylation, the separation mechanism involves a partition equilibrium in which the appearance of hydrophilic interactions leads to an increase in selectivity. These stationary phases allow interactions by hydrogen bonding as donor and acceptor. It may be seen that, depending on the pH of the mobile phase, the amine stationary phase could be positively charged and, therefore, as well as partition phenomena, additional ionic interaction mechanisms may take place.

Inoue et al. [22] studied the retention of nucleotides in HILIC columns with amine residues. The separation of five nucleotides revealed strong retention in a TSK Gel NH_2-100 column (Fig. 1d). In comparison, it was difficult to determine the separation of adenosine monophosphate and uridine monophosphate in a TSK-gel Amide-80 column.

Among the best studied stationary phases in HILIC for the separation of nucleobases, nucleosides and nucleotides are those based on amide residues. Unlike amine-based columns, amide columns are not susceptible to Schiff-base formation, thus improving accuracy of quantitation. The results showed that Amide-HILIC was superior to RPLC for the separation of nucleosides and nucleobases [20,21,23–25]. It was also observed that TSK Gel Amide-80 columns (Fig. 1e) have good chemical and mechanical stability, although the separation of adenine and hypoxanthine could not be achieved.

XBridge Amide columns (Fig. 1f) have been used by different authors [19,26]. It was observed that on using this column a partition mechanism would be involved during oligonucleotide separation.

It has been reported [17] that, in a VENUSIL HILIC column packed with acrylamide-bonded silica, the elution order of peaks differed from that in other HILIC columns [27].

Finally, the separation of the compounds studied on a COSMOSIL HILIC (Fig. 1i) was tested. In this, the silica was modified with triazole groups [21]. This phase can be categorized as weakly hydrophilic, exhibiting less retention than amide columns.

2.4. Charged stationary phases

The search for new stationary phases with a view to increasing selectivity in HILIC has led to the development of charged stationary phases. In these, the silica is modified with different alkyl chains containing residues with positive and/or negative charged groups. The retention mechanism in this case is mixed. On one hand, the partition equilibrium typical of HILIC is maintained, but, on the other, weak electrostatic interactions occur between those charged analytes and the stationary phase. The contribution to retention of this mechanism has been studied in depth [28,29] and, to a large extent, it depends on the nature of the analytes and on the concentration of salts present in the mobile phase. This type of stationary phase is of special interest as regards the separation of nucleobases and nucleotides, which are found as cations at pH values lower than 3, and for nucleotides, present in anionic form across a broad pH range.

The most widely studied charged stationary phase in the separation of the compounds studied is ZIC-HILIC, whose stationary phase is zwitterionic, showing at the same time a quaternary ammonium group and a sulfonic group (Fig. 1g). However, several authors have shown that, due to steric hindrance, the interaction with the quaternary ammonium residue is negligible [30], giving rise to an electrostatic interaction with the analytes through the negatively-charged sulfonic group. Thus, the positively-charged analytes (nucleobases and nucleosides) undergo electrostatic attraction while the negatively-charged ones (nucleotides) undergo electrostatic repulsion-hydrophilic interactions (ERLIC) [31]. Thus, ERLIC is defined for HILIC separations where the surface chemistry of the ionic column is used to repel a common ionic polar group on an analyte or within a set of analytes, to facilitate separation by the remaining polar groups.

The first application of charged stationary phases dates back to 2004 [32,33]. In later references [29,34], the authors confirmed that the retention of these compounds in ZIC-HILIC occurs through a mechanism of partition between the aqueous phase bound to the stationary phase and the organic component of the mobile phase. However, the mechanism is complex, and it presumably involves other processes (e.g., the retention of the nucleosides and nucleobases with hydroxyl groups by hydrogen bonding or electrostatic interactions, the latter in the case of positively-charged analytes). The electrostatic contribution to retention was about 25–50% at low salt concentrations in the eluent, although it should be noted that this contribution decreased significantly as the salt concentration rose. In order to exploit these interactions with a view to enhancing selectivity and resolution in ZIC-HILIC separations, the use of mobile phases at low salt concentrations should be considered. It has also been suggested that the flow rate of the mobile phase should be raised to 5.0 mL/min, [32,33], still with acceptable back-pressure levels, which demonstrates its suitability for high-throughput separations. The ZIC-HILIC phase [21] exhibited greater selectivity than the amide-bonded phases, the latter giving peak fronting under the separation conditions. ZIC-HILIC

Table 2. New trends in commercial HILIC columns proposed for the analysis of nucleosides and nucleotides

Analytes	HILIC column	Column dimensions/mm	Separation Time/min	Mobile phase	Flow/mL/min	Detection	Ref
Eight nucleosides	KINETEX HILIC core-shell (bare silica)	50 × 4.6 (2.6 μm)	4	94% ACN 6% H_2O	2	DAD	[37]
Four nucleobases and four nucleosides	BEH HILIC (Bare silica)	100 × 2.1 (1.7 μm)	4	90% ACN 10% AF:FA 200 mM	0.2	DAD: 260 nm	[17]
One nucleobase	BEH HILIC (Bare silica)	50 × 2.1 (1.7 μm)	3	95% ACN 5% AF 10 mM, FA 160 mM	0.3	MS/MS (QqQ)	[38]
Eleven nucleotides	Bare titanium	150 × 4.6 (3 μm)	40	A: ACN B: 10 mM PB (pH: 6.0) 0-6 min: 75% A 6-40 min: 65% A	1.0	UV: 254 nm	[39]
Five nucleosides	PHA	150 × 0.3	50	A: ACN B: 0.2% FA, 10 mM AF 95%-50% A	0.005	MS/MS (QTrap)	[40]
One nucleobase and seven nucleosides	ZIC-HILIC	250 × 0.1	35	95% ACN 5% AF:FA 25 mM (pH: 3.8)	0.0008	DAD: 254 nm	[41]
Two nucleotides	ZIC-pHILIC	150 × 4.6 (5 μm)	4	A: ACN B: 10 mM AC (pH: 4.4) 0-1 min: 63%-20% A 1-2 min: 20% A	0.5	MS/MS (IT)	[42]
Eight nucleotides	ZIC-pHILIC	150 × 2.1 (5 μm)	20	70% ACN 30% AC 100 mM (pH: 8.9)	0.2	UV: 254 nm	[43]
Eight nucleotides	2 coupled in series ZIC-pHILIC	150 × 2.1 (5 μm)	35/70	70% ACN 30% AC 100 mM (pH: 8.9)	0.2/0.1	UV: 254 nm	[43]
Five nucleobases	Zorbax Rx-SIL (Bare silica)	250 × 4.6 (5 μm)	8	95% EtOH 5% AF:FA 20 mM (pH:3) 1.5 mL/min CO_2	1.0	DAD: 254 nm	[44]
Four nucleosides	TSK-Gel Amide 80	150 × 4.6 (3 μm)	16	90% MeOH 10% SA:AAc 20 mM (pH: 4.4) 0.2 mole fraction CO_2	0.4	UV: 262 nm	[24]
Four nucleosides and four nucleotides	XBridge Amide	150 × 4.6 (3.5 μm)	8	90% MeOH 10% AP 75 mM + DBN 5mM + NaCl 200 mM + 0.15 mole fraction CO_2	1.0	UV: 262 nm	[26]

ACN, Acetonitrile; AF, Ammonium formate; FA, Formic acid; PB, Phosphate buffer; AC, Ammonium carbonate; TEAP, Triethylammonium phosphate; DBN, 1,5-Diazabicyclo(4.3.0)non-5-ene. Other abbreviations: see Fig. 1.

provided the best selectivity for those analytes for all the stationary phases studied.

Also, gradient separation was found to be more versatile [25,35,36], highlighting the notion that only the ZIC-HILIC column provided full resolution of the selected compounds and that, although the 5-μm and 3-μm TSK Gel Amide-80 columns had demonstrated great chemical and mechanical stability, they were unable to separate all the analytes under the conditions applied. In one of those investigations, the throughput of the assay was improved by the use of 96-well and 384-well plates.

A column with polysulfoethyl groups (PSA, Fig. 1h) has been used for the separation of nucleotides [31]. Under these conditions, all nucleotides are retained through hydrophilic interaction, despite being repelled by the stationary phase to some extent (ERLIC). With mixtures of solutes that differ greatly in charge, repulsion effects can be exploited to antagonize selectively the retention of the solutes that would normally be retained most. This permits the isocratic resolution of mixtures that normally require gradients, including peptides, amino acids, and nucleotides.

2.5. New trends in commercial columns

In the present decade, new types of HILIC stationary phases are continually being released, and other ''classic'' stationary phases [e.g., bare silica and materials with amide, poly(succinimide)-derived, and sulfoalkylbetaine functional groups] have been improved in their separation efficiency thanks to down-sizing the particles and the internal diameter, apart from changes in the composition of mobile phases in the search for greener HILICs (Table 2).

One way of increasing the efficiency of separation in bare silica columns is to use stationary phases with particle diameter less than 3 μm. In this way, it is possible to achieve the separation of up to eight nucleosides in a KINETEX HILIC core-shell (2.6 μm) column in 4 min [37]. However, the results showed that, with the increase in the percentage of organic solvent, the peaks become more asymmetric. The RFs are greater with the increase of ACN in the mobile phase, and consequently the polar interactions occurring in HILIC mode are also more significant; this explains why the peaks become asymmetric.

A greater reduction in particle size down to 2 μm requires the separations to be adapted to the UHPLC technique. A BEH HILIC column, with a particle size of 1.7 μm, separated eight analytes in 4 min [17,38]. The elution order was the same with an Atlantis HILIC and a BEH HILIC column. The retention times of the nucleobases and nucleosides in the BEH HILIC column were shorter, but good separation was achieved due to the use of the 1.7-μm packings.

Another new avenue of enquiry consists of replacing the silica by titania as the stationary phase. Titania is attracting increasing interest as a chromatographic packing material. It has greater mechanical and pH stability (pH 1–14) than silica. It acts as an anion exchanger at low pH and a cation exchanger at high pH. More importantly, the unsaturated titanium(IV) ions are strong Lewis-acid sites, which have an affinity for compounds donating electron pairs. In contrast, silica behaves as a cation exchanger only and does not have any ligand-exchange properties [39]. The results showed that the separation was governed by a HILIC mechanism of partitioning, in which the main retention mechanism was not ion exchange.

As in other chromatographic modes, one of the new fields now being developed is the use of capillary chromatography. With capillary columns (PHA-HILIC), good resolution is obtained for nucleoside isomers {e.g., 1-methyl-guanosine and 2-methyl-guanosine are baseline separated, permitting their unambiguous identification, since their ESI product-ion spectra are not discriminatory [40]}.

Similarly, the use of a commercial ZIC-HILIC capillary monolithic silica (MS) column has been proposed. This column had zwitterionic functionality and measured 0.1 mm i.d., with a mobile-phase flow rate of 0.8 μL/min [41]. Also, separation could be improved by applying a temperature pulse of about 15 s in duration (40°C to 120°C) in the capillary columns, demonstrating that temperature pulsing provides a useful tool for the optimization of resolution in capillary HILIC, since the chromatographic retention and selectivity in discrete sections of the chromatograms can be manipulated to yield improvements in resolution.

The use of polymer-based columns is also gaining ground. Thus, for the separation of nucleotides [42,43], a polymer-based column has been proposed (ZIC-pHILIC); this allows the use of mobile phases with more extreme pH values than the common range for silica-based columns. A study has also been made of the coupling in series of two ZIC-pHILIC columns [43], since solvent optimization did not result in complete separation of the eight nucleotide triphosphates in a single column. Two identical ZIC-pHILIC columns were coupled in series to increase the number of plates from 1941 to 4252 (N/m = 12,927 and 14,159, respectively). It was observed that ZIC-pHILIC columns provided better separations than silica-based ZIC-HILIC columns, confirming the differences in performance and selectivity. In addition, the behavior of nucleotide retention when temperature is increased can be explained in terms of the dissociation of analyte/water clusters.

Other authors have focused their attention on modifying the composition of the mobile phase. In this sense, the replacement of ACN by EtOH has been proposed in a Zorbax Rx-SIL column [44]. The addition of CO_2 to a mobile phase composed of ethanol/buffer allowed ACN/buffer to be replaced in HILIC separation. The disposal of CO_2 not only converts HILIC into a green chromatographic method, but also offers the opportunity to work in the enhanced-fluidity mode for all chromatographic modes and to perform supercritical or

sub-critical fluid chromatography. The same approach has been tested in both TSK-Gel Amide 80 and Xbridge Amide columns, with the observation that the replacement of ACN by MeOH and CO_2 significantly reduces the separation times [24,26]. These studies show that the difference in the solvent strength of ACN compared to that of methanol leads to differences in the order of elution, selectivity and RFs, expanding the elution window when CO_2 is used in the mobile phase.

The use of 1,5-diazabicyclo[4,3,0] non-5-ene (DBN) proved to be valuable for improving peak shape. Methanol/H_2O mobile phases are not commonly used in HILIC, due to increased viscosity and lower selectivity in comparison with ACN/H_2O; however, the addition of salt is a valuable asset to the formation of the water-enriched layer, allowing for selective partitioning of the analytes. Coupled to the addition of CO_2, which lowers the viscosity and increases the change in entropy of many analytes, selectivity was greatly improved. These studies showed that a CO_2/methanol/water mixture provides an interesting alternative to ACN/water as a mobile phase in HILIC and that such a mixture is better suited than ACN/water to the separation of compounds with large differences in polarity.

3. Non-commercial columns

The rapid development of HILIC over the past decade has been accompanied by the appearance of different commercial stationary phases. However, these continue to be scarce, their variety clearly being less than that available for RPLC. Furthermore, knowledge of the mechanisms of retention involved in HILIC is still limited, with reports of significant differences among the different stationary phases studied. Accordingly, several groups have focused their attention on the development of new stationary phases that can be applied in HILIC. These special separation materials for HILIC demonstrate good selectivity and reproducibility for the separation of polar compounds. Furthermore, the preparation procedure is usually complicated and difficult, due to the complex structures and active groups on the bonded stationary phases (Table 3). In general, synthesis is based on derivatization, in one or more steps, introducing different types of polar residues, although the use of silica or polymeric monolithic columns has also been proposed.

3.1.1. Non-charged hydroxylated stationary phases

One of the most common modifications in non-commercial columns is that based on the introduction of hydroxylated residues into the stationary phase, since these provide important added selectivity upon interacting with the analytes through hydrogen bridges. In its simplest configuration, it comprises an alkyl chain bound to the silica, showing a diol group at the opposite end. Three such columns have been tested for the separation of the compounds of interest [45].

The simplest one comprised an 11-carbon-atom chain (C1, Fig. 2a) although an ether-bridged column (C2, Fig. 2b) and a stable cross-linked (ethylene-bridged) diol phase have also been proposed. The authors reported that the retention and the selectivity profiles of polar packings containing structurally quite related ligands, being tested under similar elution conditions, could vary considerably.

Another option in the synthesis of hydroxylated stationary phases is the introduction of a linker in an intermediate step of the synthesis. The nature of the linker is usually polar, providing an added selectivity factor. Thus, studies have been conducted using columns with a thioether linker [45] and its sulfoxide oxidation equivalent [18] with terminal hydroxyl groups (ME and MEO, Fig. 2c) and diol (TG and TGO, Fig. 2d). As may be seen, a differential introduction of phase polarity, by converting embedded sulfide groups to sulfoxides and/or increasing the amount of hydroxyl groups on the chromatographic ligand, effectively changes the HILIC characteristics. Each of these functionalities may add increments in specific retention and selectivity and hence the presence of different non-ionic polar motifs in a single ligand may lead to quasi-mixed-mode HILIC.

The study of TG and TGO was broadened by replacing the aqueous phase by $Et(OH)_2$ and MeOH [18]. The authors believed that, in particular, the type of protic modifier is an attractive yet scarcely explored mobile-phase parameter that can be used conveniently to adjust chromatographic selectivity for polar probes in a dedicated manner and suggested that, in analogy to RP chromatography, a plethora of adsorptive and partitioning mechanisms seem to contribute to the global retention process of HILIC.

Another widely-used linker is based on a triazole organic group. In this group, there are single OH columns (Fig. 2e), multi-OH columns (Fig. 2f) [17] with the elution order of the analytes being similar, and Poly(HTMA) columns (Fig. 2g). The Poly(HTMA) column, including a terminal molecule of triazole and a hydroxylated residue, was synthesized by an epoxide-azide-Cu(I)-catalyzed cycloaddition reaction of glycidyl methacrylate and propargyl alcohol to provide a β-alcohol in a one-pot process. The retention of the solutes in this column was mainly controlled by hydrogen bonding under HILIC conditions [46].

Another hydroxylated column that also has a complex structure is pNA-SIL (poly[N-acryoyltris(hydroxymethyl)aminomethane]-coated silica (Fig. 2h) [47], which has amide bonds and terminal amine and cyano groups. This novel hydrophilic polymer-coated silica sorbent was prepared using the radical ''grafting from'' polymerization method through surface-bound azo [47]. In this column, the retention of nucleosides was mainly based on the HILIC mechanism, exhibiting good reproducibility and stability.

3.1.2. β-cyclodextrin stationary phases

Among the various hydroxylated columns, one particular group comprises residues of β-cyclodextrins (Fig. 2i)

Table 3. Non-commercial HILIC columns proposed for the analysis of nucleosides and nucleotides

Analytes	HILIC column	Column dimensions/mm	Separation Time /min	Mobile phase	Flow/ mL/min	Detection	Ref.
Two nucleobases and five nucleosides	C1/C2/C3	150×4	5/10/9	90% ACN 10% AA 150 mM	1.0	DAD: 254 nm	[45]
Two nucleobases and five nucleosides	ME/MEO	150×4	4/7	90% ACN 10% AA 150 mM	1.0	DAD: 254 nm	[45]
Two nucleobases and five nucleosides	TG/TGO	150×4	6/9	90% ACN 10% AA 150 mM	1.0	DAD: 254 nm	[45]
Five nucleobases and nine nucleosides	TG	150×4	20/15/10/7	90% ACN 10% EtOH/MeOH/Et(OH)$_2$/H$_2$O	1.0	DAD: 254 nm	[18]
Five nucleobases and nine nucleosides	TGO	150×4	45/15/10	90% ACN AA 5 mM 10% MeOH/Et(OH)$_2$/H$_2$O	1.0	DAD: 254 nm	[18]
Four nucleobases and four nucleosides	Single OH	150×4.6 (5 µm)	6	90% ACN 10% AF:FA 200 mM	1.0	DAD: 260 nm	[17]
Four nucleobases and four nucleosides	Multi OH	150×4.6 (5 µm)	10	90% ACN 10% AF:FA 200 mM	1.0	DAD: 260 nm	[17]
Two nucleobases and three nucleosides	Poly(HTMA)	100×4.6	70	A: ACN B: AF 20 mM 0-30 min: 95% A 30-60 min: 95-70% A 60-80 min: 70% A	1.0	UV: 254 nm	[46]
Five nucleosides	pNA-sil	150×4.6	12	85% ACN 15% AF 20 mM (pH: 6.1)	1.0	UV: 254 nm	[47]
Four nucleobases and four nucleosides	β-Cyclodextrin	150×4.6 (5 µm)	12	90% ACN 10% AF:FA 200 mM	1.0	DAD: 260 nm	[17]
Nine nucleosides	β-Cyclodextrin	150×4.6	15	A: ACN B: AA 10 mM 0-8 min: 85%-80% A 8-15 min: 80%-57% A	1.0	DAD: 260 nm	[3]
Two nucleobases and three nucleosides	Amino	150×4.6	7	83% ACN 17% AF 5 mM	1.0	UV: 248 nm	[48]
Five nucleobases and nine nucleosides	APS 5µ-1	150×4	15	90% ACN 10% AA 10 mM (pH: 5)	1.0	UV:254 nm	[19]
Three nucleobases and three nucleotides	CTSAP	100×4.6	10	90% ACN 10% AF 10 mM (pH: 3.15)	1.0	UV: 254 nm	[49]
Four nucleosides	Poly(AAc-co-BA)	150×4.6	14	95% ACN 5% SA 10 mM	1.0	DAD: 254 nm	[50]
Three nucleobases and three nucleosides	Tetrazole	150×4.6	9	90% ACN 10% AF 100 mM (pH: 7)	1.0	UV: 254 nm	[51]
Five nucleobases and five nucleosides	EC-HA-APS	100×4.6	40	90% ACN 10% H$_2$O	0.5	DAD: 254 nm	[52]
Three nucleobases and three nucleosides	Glycosylphenyl glycine	100×2.1	20	90% ACN 10% H$_2$O	0.2	UV: 254 nm	[53]
Five nucleobases and nine nucleosides	C-Choc	150×4	15	90% ACN 10% AA 10 mM (pH: 5)	1.0	UV:254 nm	[19]
Two nucleobases and three nucleosides	CNPs	150×4.6	10	83% ACN 17% AF 5 mM	1.0	UV: 248 nm	[48]

ACN, Acetonitrile; AF, Ammonium formate; FA, Formic acid; AA, Ammonium acetate; SA, Sodium acetate. Other abbreviations: see Figs. 2 and 3.

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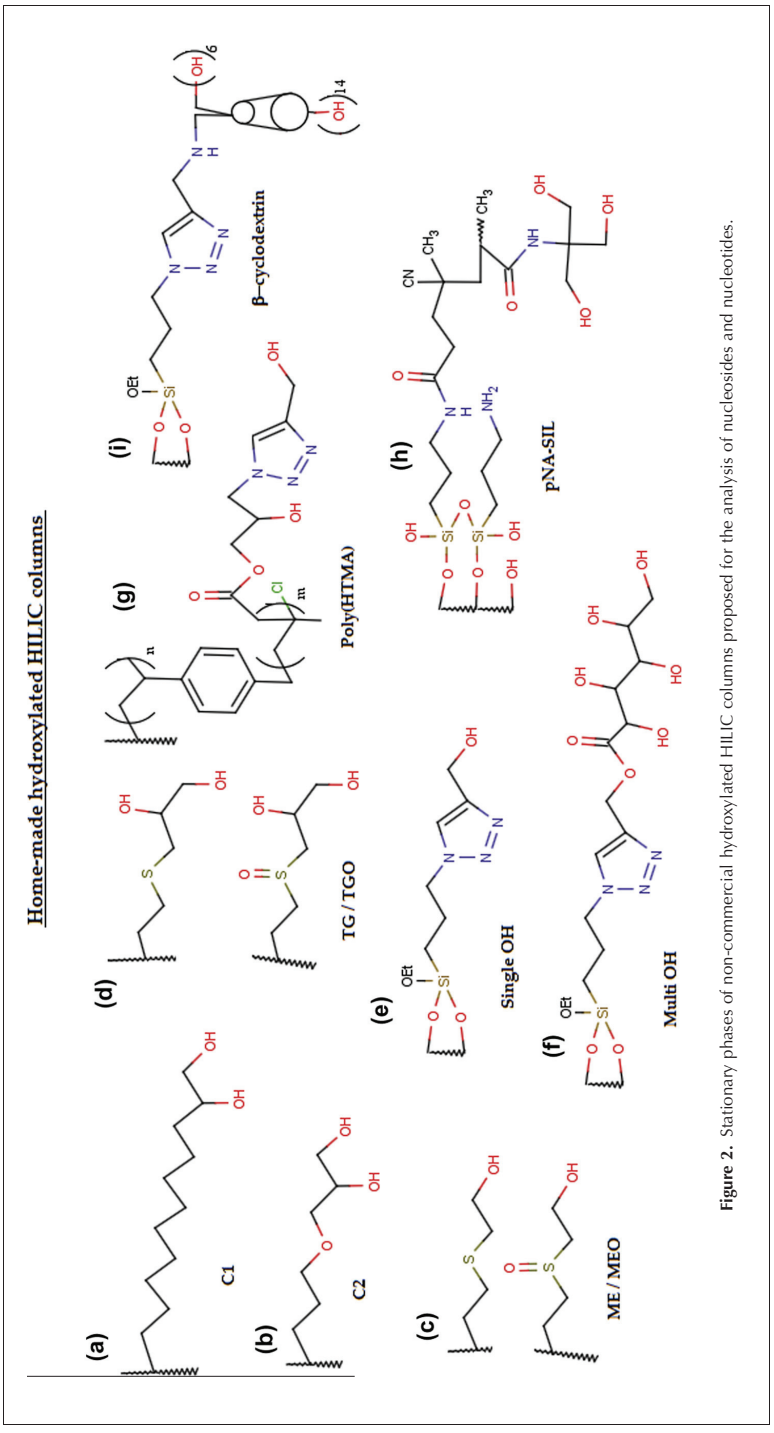

Figure 2. Stationary phases of non-commercial hydroxylated HILIC columns proposed for the analysis of nucleosides and nucleotides.

The presence of cyclodextrins may elicit an additional degree of selectivity due to the different interaction of the analytes (even enantiomers) with the internal cavity of the β-cyclodextrin column [17].

Another column proposed, formed by β-cyclodextrins as the stationary phase, is included in what is known as click chemistry [3]. The stationary phase was prepared by covalently bonding β-cyclodextrins on silica particles via a dipolar cycloaddition between the organic azide and the terminal alkyne. The resulting click β-cyclodextrin material exhibited the typical chromatographic behavior of HILIC, mainly providing a partitioning mechanism and some other interactions (e.g., ion exchange and electrostatic repulsion effects).

3.2. Amine stationary phases

As in the case of commercial columns, columns with terminal amino groups have been prepared. The simplest are those formed by amino [48] and aminopropyl silica (APS, Fig. 3a) [19]. This APS column was prepared by the reaction of bare silica with 1.7 mmol 3-aminopropyltrimethoxysilane per gram of silica. These columns were used at slightly acidic pH values, which could be responsible for electrostatic or ion-exchange interactions.

A CTSAP (Fig. 3b) column has also been assayed, prepared by the immobilization of propargylamine on azide-silica via click chemistry [49]. The retention mechanism was found to match the typical HILIC retention well. The retention of all analytes increased with the increase in the salt concentration, due to the enhancement of the polarity of the water-rich layer resulting from high salt concentration. Nevertheless, at low salt concentrations, the retention decreased with the increase of salt concentration, which could be explained by the ion-exchange mechanism dominating the separation process.

3.3. Other non-charged stationary phases

Although columns prepared with hydroxyl and amine residues are the most common, other types of column have been developed. In these, the goal is to use another class of polar residues {e.g., a mixed column of acid and ester groups (polyacrylic acid-co-n-butylacrylate ester, Fig. 3c) [50]}. Poly(Aac-co-BA) was covalently bonded onto the surface of silica by the ''grafting from'' polymerization method. The surface characteristics of the

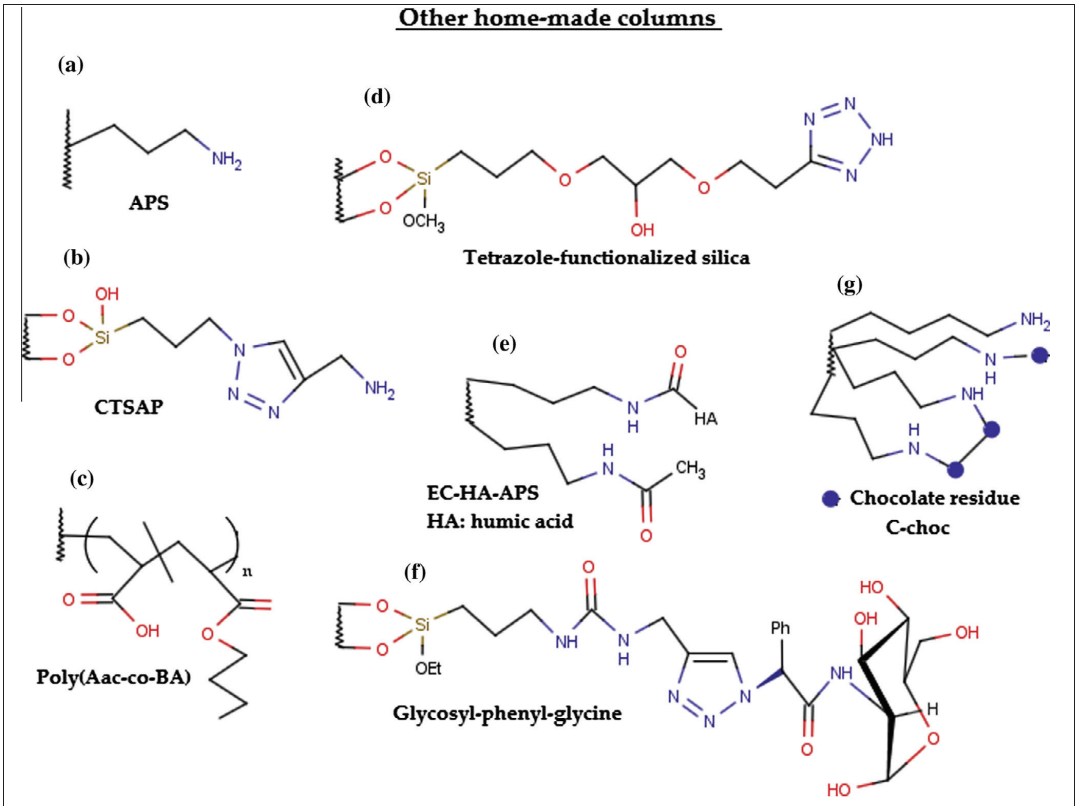

Figure 3. Stationary phases of other non-commercial HILIC columns proposed for the analysis of nucleosides and nucleotides.

polymer-grafted silica were strongly related to the environmental pH. The hydrophobic surface of the polymer-grafted silica can be obtained at lower pH, while the hydrophilic surface is obtained at higher pH.

Dai et al. [51] prepared a tetrazole-functionalized column (Fig. 3d). The effect of the organic modifier, buffer pH and ionic strength further supported the viewpoint that the retention of the solutes was mainly controlled by hydrogen bonding between the solutes and the tetrazole group on the surface of the stationary phase under HILIC conditions.

It is also possible to find columns in which the formation and the nature of the polar residues are more complex. Thus, there are columns with humic acids (HAs) [52], glycosyl groups [53] and groups arising from the Maillard reaction [19] as polar residues. In the column formed by HAs (Fig. 3e), the authors concluded that this stationary phase, which was obtained by immobilization of HA to APS via chemical bond formation and a subsequent end-capping process, exhibited mixed-mode RPLC/HILIC behavior towards some nucleosides and nucleobases [52].

The column formed by glycosyl groups (Fig. 3f) was prepared using click chemistry. This material showed typical HILIC characteristics in the separation of nucleosides and nucleobases, and no buffer was required, making it much easier to apply this kind of stationary phase to LC-MS. Based on its hydrophilic characteristics and special interaction, enhanced by structures similar to glycopeptides, it has great potential to enrich glycosylated peptides, and other applications in the separation of highly-polar compounds, showing longer retention times and better separation ability than the commercial Atlantis HILIC column [53].

Finally, the C-choc column (Fig. 3g) is prepared via the Maillard reaction of cellobiose derivatives. Of all the sugars investigated, cellobiose was found to be the most promising primer, resulting in the most efficient packing material. The reaction cascade using cellobiose resulted in an efficient chromatographic material, which further served as a Chocolate HILIC column (chocolate refers to the fact that these phases are brownish). Cellobiose is composed of two glucose sub-units that are clearly superior to other reducing sugars in the course of Maillard-product formation on a surface. The Chocolate HILIC column even outperformed the efficiency of the commercial Luna HILIC column [19].

Columns formed of carbon nanoparticles (CNPs) obtained by refluxing corn-stalk soot in nitric acid are of a different nature [48]. In this refluxing treatment process, –OH and $–CO_2H$ groups are generated on the surface of the CNPs, conferring a hydrophilic nature. All the components were well resolved and the nucleosides displayed stronger retention on the new column than on an amino column. It was reasonable to assume that the surface carboxylic groups on the new phase might interact with the basic adenine, thus leading to stronger retention and greater tailing for adenine.

3.4. Monolithic stationary phases

One approach for developing high-throughput HPLC methods is to use monolithic columns. In contrast to conventional HPLC columns, monolithic columns are formed from a single piece, affording them greater porosity and permeability than conventional particle columns. Based upon the nature of their construction materials, monolithic columns can be classified as organic polymer-based or silica-based columns. Both types have been addressed in depth [54–57], as has their general application in HILIC [58,59].

3.4.1. Silica monolithic columns. Silica monolithic columns, which appeared before polymeric columns, are prepared via a sol-gel process that begins with the generation of $Si(OR)_4$ to form Si-O-Si polymers. Once formed, the silica monolith can be functionalized chemically to obtain the desired stationary phase. These silica-based monolithic columns were first developed by the team of Nakanishi and Tanaka in the 1990s. However, they were not applied in HILIC until much later, when the chemical functionalization of silica allowed the introduction of polar groups, typical of hydrophilic stationary phases (Table 4).

Only four proposals for the use of monolithic columns based on silica functionalized for the separation of nucleobases, nucleosides and nucleotides have been put forward, two of them from the team of Nakanishi and Tanaka.

Ikegami et al. [60] polymerized acrylamide on an MS capillary column to obtain the polyacrylamide-coated MS capillary column that they designated PAMS(100), in which the value in brackets refers to the i.d. of the column in μm. It was found to be useful for HILIC-mode separation due to the high column efficiency and higher permeability in comparison with a particle-packed HILIC column (TSK Gel Amide 80) for the separation of several nucleosides.

The same authors [61] also prepared a poly(acrylic acid)-coated MS column that they call MS-200T-PAA. This column showed much higher separation efficiency than a commercially-available HILIC-type particle-packed column. Based on the high permeability and small-sized skeletons, the MS column was able to provide high efficiency even with very fast elutions.

Lü et al. [62] have proposed a novel cationic hydrophilic interaction monolithic stationary phase based on the chemical modification of carboxymethyl chitosan (CMCH) to the MS skeleton using carbodiimide as an activation reagent. The performance of the chitosan-functionalized monolithic column was superior to that of the non-modified native silica column. However, compared with the reported hydrophilic interaction monolithic columns, the chitosan-functionalized monolithic column showed relatively low column efficiency.

More recently, the preparation of organic-silica hybrid capillary monolithic columns was proposed by Lin et al. [63]. These monoliths have the advantage of being prepared in a single step, using a simple ''one-pot'' thermal

Table 4. Monolithic columns (silica and polymer based) proposed for the analysis of nucleosides and nucleotides

Analytes	Functional group	Column dimensions/mm	Mobile phase	Separation Time/min	Comments	Ref.
Five nucleobases and four nucleosides	Acrylamide	380 × 0.1	90% ACN 10% H_2O	17	Silica modified with N-(3-trimethoxy-silylpropyl) methacrylamide	[60]
Three nucleosides	Acrylic acid	200 × 0.2	90% ACN 10% FA 0.2%	17/1.4	Silica modified with 3-methacrylamidopropyl silyl as anchor	[61]
Four nucleosides	Carboxymethyl-chitosan	400 × 0.2	90% / 70% ACN 10% / 30% 10 mM PB (pH: 5.6)	8/15	Carbodiimide as an activation reagent	[62]
Four nucleobases and three nucleosides	Zwitterionic (MSA)	486 × 0.075	80% ACN 20% 10 mM AA	13	"one-pot" approach by replacing the cross-linker silane with γ-MAPS	[63]
Four nucleobases and four nucleosides	Epoxy (TEPIC)	364 × 0.1	90% ACN 10% H_2O	10	A chiral column could be prepared using a chiral TEPIC	[64]
Four nucleobases	Zwitterionic (SPE)	285 × 0.1	95% ACN 5% 5 mM AF (pH:3.0)	13	In order to provide anchoring sites for the polymer, the capillaries were treated with γ-MAPS	[65]
Five nucleobases	Zwitterionic (MPC)	290 × 0.1	92% ACN 8% 5 mM AF (pH: 6.4)	9	Methanol/THF mixture proved to be a good porogen	[66]
Three oligonucleotides	Acrylamide	180 × 0.32	94% ACN Decreasing 0.12% /min 6% TEAA 100 mM	35	Porogenic system composed of propanol and butane-1,4-diol	[67]
Five nucleosides	Hydroxy (NAHAM)	300 × 0.1	95% ACN 5% FA (pH: 3.5)	10	In situ polymerization. Polyethylene glycol (PEG) in dimethyl sulfoxide (DMSO) as the porogen	[68]
Five nucleosides	Methacrylic acid	300 × 0.1	86% ACN 14% H_2O	15	One-step polymerization. PEG in DMSO as the porogen	[69]
Four nucleosides	Zwitterionic (SPE)	109 × 0.1	90% ACN 10% 20 mM AF (pH: 3.2)	8	Thiolene click chemistry	[70]
One nucleobase and four nucleosides	Cysteine	165 × 0.1	90% ACN 10% 25 mM AF (pH: 3.2)	18	10 nm GNP dual-layer as intermediate ligand	[71]
Six nucleobases	Zwitterionic (SPE)	275 × 0.2	95% to 50% ACN	10	Enlarged flow-channels and the presence of a higher proportion of mesopores provided a larger surface area	[72]

ACN, Acetonitrile; FA, Formic acid; PB, Phosphate buffer; AF, Ammonium formate; AA, Ammonium acetate; TEAA, Triethylammonium acetate. Other abbreviations: see text.

treatment. A zwitterionic organic-silica monolithic column was successfully synthesized by using [2-(methacryloyloxy)-ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (MSA). The optimized MSA monolith could not only develop hydrophilic interactions with polar and charged analytes but also offered the possibility of weak electrostatic interactions with charged analytes. Four purines and pyrimidines and three nucleosides were baseline-separated under the optimized chromatographic conditions.

3.4.2. Polymeric monolithic columns. The use of polymeric monolithic columns is one of the main new trends in LC. Nevertheless, despite their broad use in RPLC, polymeric monolithic columns for HILIC remain scarce as the result of several factors, since polar monomers are not very soluble in the usual porogenic solvents, and few polar monomers are available commercially. Further, the developments in this field have focused on the separation of biomacromolecules rather than on small analytes, such as those studied here. Thus, there have been few proposals for the separation of nucleobases, nucleosides and nucleotides using polymeric monolithic columns, and many of these were only published very recently (Table 4).

Hosoya et al. [64], used an epoxy resin-based polymer, called TEPIC-BACM, which is obtained by reacting the monomer tris(2,3-epoxypropyl) isocyanurate (TEPIC) with 4-aminocyclohexyl)methyl]cyclohexylamine (BACM) in the presence of polyethylene glycol. This column afforded really high performance, based on it containing hydroxyl and amine groups as functional groups and that its homogeneous co-continuous structure has a μm-sized skeleton and sub-μm-sized pores. In the separation of nucleosides, this column showed up to 60,000 plates.

Jiang et al. [65] prepared a hydrophilic zwitterionic monolith by thermoinitated copolymerization of N,N-dimethyl-N-methacryloxyethyl-N-3(sulfopropyl)ammonium betaine (SPE) and ethylene dimethacrylate (EDMA) to generate the poly(SPE-co-EDMA) monolith. Typical HILIC retention was observed at high organic-solvent contents (ACN > 60%). The polar zwitterionic monolith provided an environment not only capable of hydrophilic interaction with polar and charged analytes but also offering the possibility of weak electrostatic interaction with analytes bearing positive or negative charges.

This same research team [66] has proposed another hydrophilic zwitterionic monolith prepared by polymerization of zwitterionic monomer 2-(methacryloyloxy)-ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (MSA) and 3-methacryloxypropyltrimethoxysilane (γ-MAPS). Like SPE, this MSA monolith could not only have hydrophilic interactions with polar and charged analytes but also offer the possibility of weak electrostatic interactions with charged analytes.

Using N-(hydroxymethyl) methacrylamide as a monomer, an HMMAA monolithic column has been designed [67]. The HMMAA monolithic stationary phases are advantageous due to their easy preparation, since silanization of the inner wall of capillaries is unnecessary and the porogenic system is simplified from a ternary mixture to a binary one.

Chen et al. [68] have proposed the preparation of a polymeric column starting with N-acryloyltris(hydroxymethyl)aminomethane (NAHAM) and pentaerythritol-triacrylate (PETA). This novel poly(NAHAM-co-PETA) column was applied in capillary HILIC (cHILIC).

The same authors also proposed [69] another polymeric column based on methacrylic acid (MAA) and ethylene glycol dimethacrylate (EDMA). This poly(MAA-co-EDMA) monolith was used in HILIC mode and five nucleosides were baseline separated.

Lv et al. have proposed [70] the use of a thiolene click reaction to prepare a monolith with zwitterionic functionalities using SPE as monomer, and they demonstrated the application of this functionalized monolith in the chromatographic separation of nucleotides in HILIC mode. This monolithic column did not offer very high efficiency, because the surface area of the monolith was one order of magnitude less than that of silica-based monolithic columns.

One emerging area is the incorporation of nanostructures into the polymeric base for application in RP, ion-exchange and affinity chromatography. Recently, Svec et al. [71] combined the hyper-cross-linking of hydrophobic poly(4-methylstyrene-co-vinylbenzyl chloride-co-divinylbenzene) monoliths with the formation of a hydrophilic layered structure, including gold nanoparticles (GNPs) embedded in a polyethyleneimine (PEI) layer, and functionalized with cysteine, enabling the preparation of a very efficient monolithic stationary phase for the separation of small molecules in HILIC mode. The technique was more complex than single-step reactions. However, the results further demonstrate the versatility of functionalization approaches for monoliths relying on GNPs, which serve as a ''universal'' intermediate ligand that several research groups are currently developing.

Recently, Foo et al. [72] proposed a novel HILIC stationary phase prepared by the co-polymerization of SPE and cross-linker 1,2-bis(p-vinylphenyl)ethane (BVPE) in the presence of porogens toluene and methanol. This highly hydrophilic porous monolith, poly(SPE-co-BVPE), was successfully applied to the rapid, high-resolution separation of pyrimidine and purine nucleobases.

4. Applications

Despite the large number of authors addressing the separation of nucleobases, nucleosides and nucleotides in HILIC, most have used these analytes as a model for polar compounds without applying the separation in real analyses. However, the determination of nucleobases,

Table 5. Applications of HILIC columns in the analysis of nucleobases, nucleosides and nucleotides in real samples

Application	Column	Ref.
8 NS in urine and serum	KINETEX HILIC core shell	[37]
5 NS in urine samples	PHA	[40]
2 NB and 6 NS in urine of healthy and cancer patients	ZIC-HILIC	[35]
5 NS in urine	Home-made Poly(NAHAM-co-PETA)	[68]
2 NT in vitro (cells)	ZIC-pHILIC	[42]
8 NT in *Escherichia coli* cell samples	ZIC-pHILIC	[43]
6-Thioguanine in DNA	HILIC BEH	[38]
9 NS in traditional Chinese medicines	Home-made β-cyclodextrin	[3]
4 NB and 4 NS in safflower (*C. tintorius)*	Home-made β-cyclodextrin	[17]
2 NB and 3 NS in safflower (*C. tintorius)*	Home-made CNPs	[48]
7 NB and 9 NS in marine organisms	VENUSIL HILIC	[27]
6 NB and 13 NS in aqueous extracts of Geosaurus and Leech	TSK-Gel-Amide 80	[23]
5 NT in infant formula	TSK-Gel-NH_2-100	[22]
5 NB and 11 NS to evaluate the quality of *Ganoderma* (mushroom)	ZIC-HILIC	[36]

NS, Nucleosides; NB, Nucleobases; NT, Nucleotides. Other abbreviations: see text and figures.

nucleosides and nucleotides has found applications in biology, the food industry and medicine (Table 5).

In biology, the role of these compounds is crucial, since they form part of RNA and DNA. Thus, their determination in both those macromolecules and biological fluids (e.g., urine or serum) is of special importance in the study of nucleic acids. It should be noted that the measurement of certain modified nucleobases and nucleosides in urine has been proposed as a diagnostic and treatment-assessment method in different types of cancer [73–76].

In the food industry, the determination of nucleobases, nucleosides and nucleotides is important, because they are the primary constituents of nucleic acids. Nucleotide monophosphates are essential in infant foods, to which they are added to address the concentrations of these analytes in cow's milk being lower than they are in maternal milk [77–79].

4.1. Determination of nucleobases and nucleosides in blood and urine

A proposal has been made to use a bare silica column (KINETEX core-shell) in UHPLC mode for the determination of eight nucleosides in synthetic urine and blood samples, with spectrophotometric determination [37]. The results of nucleoside identification in serum were successful, with low LOQ values, so the method developed is suitable for routine analysis of these compounds in serum. In contrast, the results obtained in the case of urine were not as promising as those for serum.

Tuytten et al. [40] have proposed an on-line system formed by coupling aprotic boronic-acid chromatography with a hydroxylated HILIC column for the determination of five nucleosides in urine samples. The method developed represents the first workable on-line method with SPE-LC-ESI-MS-(/MS) for urinary nucleosides and has potential as a basis for a high-throughput automated means to profile urinary nucleosides.

We [35] have proposed the use of a highly polar restricted-access material (RAM), based on an N-vinylacetamide copolymer, for efficient analyte extraction and matrix removal, with separation by zwitterionic HILIC (ZIC-HILIC) for the determination of two nucleobases and six nucleosides. The application to clinical samples was also checked, observing that the method developed could be used as a control method for clinical purposes (e.g., early diagnosis and therapeutic-response monitoring).

Finally, an on-line system, in this case capillary, involving the combination of the poly(NAHAM-co-PETA) home-made monolith with a poly(AAPBA-co-EDMA) extraction monolith has also been proposed. This on-line SPME-cLC system was successfully applied for the rapid, sensitive determination of four nucleosides from urine samples, with spectrophotometric detection. This method also offers a potential application for clinical diagnoses [68].

4.2. Determination of nucleotides in cells

In 2010, Goutier et al. [42] presented for the first time the successful development, validation and application of a novel, robust, sensitive HILIC-MS/MS method for the simultaneous determination of two nucleotides, adenosine triphosphate (ATP) and cyclic adenosine monophosphate (cAMP), in biological samples to study receptor activity using a ZIC-pHILIC column. The method does not require extensive sample preparation, making it suitable for routine analyses, and it is amenable to 96-well technology.

Later, Johnsen et al. [43] proposed the separation of eight nucleotides applied to *Escherichia coli* cell samples. In these complex cell culture samples, NTP was separated selectively (also from other matrix compounds) in 70 min using two 15-cm ZIC-pHILIC columns coupled in series.

Also in this scenario, in 2012 Jacobsen et al. [38] proposed the determination of the pharmacological end-point of thiopurines [e.g., 6-thioguanine (6TG)] in chromosomal DNA using a BEH HILIC column with a particle size of

1.7 μm. Purine nucleobases were released from DNA, etheno-derivatized with chloroacetaldehyde, separated by HILIC, and quantified by tandem MS using endogenous chromosomal guanine as the internal standard. The method was easily applicable, and provided a fast, precise analysis to measure 6TG contents in genomic DNA without the need for enzymes or multiple incubations.

4.3. Determination of nucleobases and nucleosides in traditional Chinese medicine

For thousands of years, traditional Chinese medicine (TCM) has used different plant preparations (phytotherapy). The high polarity of these preparations means that HILIC is ideal for their analysis, especially in the case of the determination of the compounds addressed here.

In 2009, Guo et al. [3] analyzed nine nucleosides in a TCM compound called toad skin using a column made of β-cyclodextrins. A similar column was applied to the determination of four nucleobases and four nucleosides in Safflower (*C. tinctorius* L.) collected from Ta County, China [17]. Safflower has been widely used in China for thousands of years and is a popular TCM for the treatment of cardiovascular and hypertensive disease because of its reputation for facilitating blood circulation and dispersing blood stasis. More recently, with the same matrix, two nucleobases and three nucleosides were determined using a column formed by CNPs [48]. On a C18 column, the components in the safflower injection were not retained due to their hydrophilic character. A dozen compounds were well resolved and showed good retention in the new phase and in a commercial amine column.

Zhao et al. [27] have proposed the determination of seven nucleobases and nine nucleosides in medicinal marine organisms using a commercial Venusil HILIC column. The method was suitable for the separation of highly-polar nucleosides and nucleobases. The coupling of HILIC separation with the accurate-mass measurement of TOF provided a candidate tool for identification and determination of low concentrations of highly-polar nucleosides and nucleobases in marine organisms. In particular, they can be used for further identification of unknown non-target compounds.

Also, Chen et al. [23] determined six nucleobases and 15 nucleosides in two TCMs, the annelids Geosaurus and Leech. The results clearly suggested that the HILIC method could be employed as a useful tool for the quality assessment of Geosaurus and Leech using certain nucleosides and nucleobases as markers. Setting a minimum limit to the amount of these compounds would be helpful for the quality control of Geosaurus and Leech preparations.

4.4. Determination of nucleobases, nucleosides and nucleotides in foods

In the food industry, nucleotides have been identified as essential nutrients.

In 2010, Inoue et al. [22] determined five nucleotides in infant formulas using a TSK-Gel-NH_2-100 column. This screening assay allowed the discovery of interesting results in infant formulas, the concentrations of total nucleotides being considered reasonable and appropriate in comparison with referenced human-milk levels.

Also in food, Chen et al. [36] proposed the determination of five nucleobases and 11 nucleosides to assess the quality of *Ganoderma lucidum*, a nutritional mushroom, using a ZIC-HILIC column. With this method, for the first time, nucleosides and bases in *G. atrum* were quantified and discriminated from another two species of *Ganoderma* (*G. lucidum* and *G. sinense*). This HILIC method is simple, rapid and sensitive, and it offers an alternative to the older non-specific techniques, which are laborious and time consuming, and which cannot detect all those nucleosides and nucleobases simultaneously.

5. Conclusions and perspectives

HILIC has been shown to have great potential applications for the separation of polar biomolecules (e.g., nucleobases, nucleosides and nucleotides), so explaining its increasing importance in research fields (e.g., proteomics and bioanalysis). In order to enhance separation selectivity, a wide range of HILIC stationary phases were recently proposed to exploit the retention of these hydrophilic compounds effectively. The developments in stationary phases cover the introduction of new chemistries and new column geometries in order to obtain high resolution and selectivity, at the same time reducing the analysis time. In the years to come, we expected that investigations into new HILIC stationary phases will continue.

Zwitterionic, amide and amine stationary phases occupy a special position among the sorbents designed for HILIC separation of nucleotides, nucleosides and their bases. The presence of these groups enables the existence of the mixed-mode retention mechanisms in such a way that the order of elution can be altered to a certain extent by variations in the composition and the pH of the mobile phase. This affords greater selectivity. In all cases, particle-packed columns are available commercially and also zwitterionic monolithic capillary columns have been described.

Monoliths are seen to be a more convenient approach for efficient, economic and fast analysis, but their application remains limited, due to the restricted availability of stationary-phase chemistries. Monolithic columns are broadly accepted in other types of LC and future efforts should be directed to develop commercially-available monolithic phases for HILIC applications.

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References

[1] P. Hemstroem, K. Irgum, J. Sep. Sci. 29 (2006) 1784.
[2] P. Jandera, J. Sep. Sci. 31 (2008) 1421.
[3] Z. Guo, Y. Jin, T. Liang, Y. Liu, Q. Xu, X. Liang, et al., J. Chromatogr., A 1216 (2009) 257.
[4] P. Jandera, Anal. Chim. Acta 692 (2011) 1.
[5] B. Buszewski, S. Noga, Anal. Bioanal. Chem. 402 (2012) 231.
[6] M.R. Gama, R.G. da Costa Silva, C.H. Collins, C.B.G. Bottoli, Trends Anal. Chem. 37 (2012) 48.
[7] L. Mora, A. Hernández-Cázares, M.C. Aristoy, F. Toldrá, M. Reig, Food Anal. Methods 4 (2011) 121.
[8] B. Dejaegher, Y. Vander Heyden, J. Sep. Sci. 33 (2010) 698.
[9] T. Hayama, H. Yoshida, K. Todoroki, H. Nohta, M. Yamaguchi, Rapid Commun. Mass Spectrom. 22 (2008) 2203.
[10] A. Frickenschmidt, H. Fröhlich, D. Bullinger, A. Zell, S. Laufer, C.H. Gleiter, et al., Biomarkers 13 (2008) 435.
[11] C.M. Galmarini, J.R. Mackey, C. Dumontet, Lancet Oncol. 3 (2002) 415.
[12] A. Seidel, S. Brunner, P. Seidel, G.I. Fritz, O. Herbarth, Br. J. Cancer 94 (2006) 1726.
[13] W. Struck, M. Waszczuk-Jankowska, R. Kaliszan, M. Markuszewski, Anal. Bioanal. Chem. 401 (2011) 2039.
[14] J.D. Carver, Am. J. Clin. Nutr. 77 (2003) 1550S.
[15] E. Schlimme, D. Martin, H. Meisel, Br. J. Nutr. 84 (2000) S59.
[16] M. Sugawara, N. Sato, T. Nakano, T. Idota, I. Nakajima, J. Nutr. Sci. Vitaminol. 41 (1995) 409.
[17] G. Jin, Z. Guo, F. Zhang, X. Xue, Y. Jin, X. Liang, Talanta 76 (2008) 522.
[18] W. Bicker, J. Wu, M. Lämmerhofer, W. Lindner, J. Sep. Sci. 31 (2008) 2971.
[19] G. Schuster, W. Lindner, Anal. Bioanal. Chem. 400 (2011) 2539.
[20] R.N. Easter, K.K. Kroning, J.A. Caruso, P.A. Limbach, Analyst (Cambridge, UK) 135 (2010) 2560.
[21] Y. Kawachi, T. Ikegami, H. Takubo, Y. Ikegami, M. Miyamoto, N. Tanaka, J. Chromatogr., A 1218 (2011) 5903.
[22] K. Inoue, R. Obara, T. Hino, H. Oka, J. Agric. Food Chem. 58 (2010) 9918.
[23] P. Chen, W. Li, Q. Li, Y. Wang, Z. Li, Y. Ni, et al., Talanta 85 (2011) 1634.
[24] J.W. Treadway, G.S. Philibert, S.V. Olesik, J. Chromatogr., A 1218 (2011) 5897.
[25] G. Marrubini, B.E.C. Mendoza, G. Massolini, J. Sep. Sci. 33 (2010) 803.
[26] G.S. Philibert, S.V. Olesik, J. Chromatogr., A 1218 (2011) 8222.
[27] H. Zhao, J. Chen, Q. Shi, X. Li, W. Zhou, D. Zhang, et al., J. Sep. Sci. 34 (2011) 2594.
[28] D.V. McCalley, J. Chromatogr., A 1217 (2010) 3408.
[29] D. García-Gómez, E. Rodríguez-Gonzalo, R. Carabias-Martínez, ISRN Anal. Chem. (2012) 308062.
[30] S. Di Palma, P.J. Boersema, A.J.R. Heck, S. Mohammed, Anal. Chem. 83 (2011) 3440.
[31] A.J. Alpert, Anal. Chem. 80 (2007) 62.
[32] T. Jonsson, P. Appelblad, LC-GC Eur. (2004) 40.
[33] T. Jonsson, P. Appelblad, LCGC North Am. (2004) 72.
[34] E. Rodríguez-Gonzalo, D. García-Gómez, R. Carabias-Martínez, J. Chromatogr., A 1218 (2011) 3994.
[35] E. Rodríguez-Gonzalo, D. García-Gómez, R. Carabias-Martínez, J. Chromatogr., A 1218 (2011) 9055.
[36] Y. Chen, W. Bicker, J. Wu, M. Xie, W. Lindner, J. Agric. Food Chem. 60 (2012) 4243.
[37] S. Studzińska, B. Buszewski, J. Chromatogr., B 887–888 (2012) 93.
[38] J.H. Jacobsen, K. Schmiegelow, J. Nersting, J. Chromatogr., B 881–882 (2012) 115.
[39] T. Zhou, C.A. Lucy, J. Chromatogr., A 1187 (2008) 87.
[40] R. Tuytten, F. Lemiere, W. Van Dongen, E. Witters, E.L. Esmans, R.P. Newton, et al., Anal. Chem. 80 (2008) 1263.
[41] T.J. Causon, H.J. Cortes, R.A. Shellie, E.F. Hilder, Anal. Chem. 84 (2012) 3362.
[42] W. Goutier, P.A. Spaans, M.A.W. van der Neut, A.C. McCreary, J.H. Reinders, J. Neurosci. Meth. 188 (2010) 24.
[43] E. Johnsen, S.R. Wilson, I. Odsbu, A. Krapp, H. Malerod, K. Skarstad, et al., J. Chromatogr., A 1218 (2011) 5981.
[44] A. dos Santos Pereira, A. Jiménez Girón, E. Admasu, P. Sandra, J. Sep. Sci. 33 (2010) 834.
[45] J. Wu, W. Bicker, W. Lindner, J. Sep. Sci. 31 (2008) 1492.
[46] X. Dai, Y. He, Y. Wei, B. Gong, J. Sep. Sci. 34 (2011) 3115.
[47] X.-T. Peng, B.-F. Yuan, Y.-Q. Feng, J. Sep. Sci. 34 (2011) 3123.
[48] Y. Li, L. Xu, T. Chen, X. Liu, Z. Xu, H. Zhang, Anal. Chim. Acta 726 (2012) 102.
[49] Y. Liu, Q. Du, B. Yang, F. Zhang, C. Chu, X. Liang, Analyst (Cambridge, UK) 137 (2012) 1624.
[50] Q. Ma, M. Chen, H.-R. Yin, Z.-G. Shi, Y.-Q. Feng, J. Chromatogr., A 1212 (2008) 61.
[51] X. Dai, X. Qian, B. Gong, Y. Wei, Chromatographia 73 (2011) 865.
[52] O. Gezici, H. Kara, Talanta 85 (2011) 1472.
[53] H. Huang, H. Guo, M. Xue, Y. Liu, J. Yang, X. Liang, et al., Talanta 85 (2011) 1642.
[54] G. Guiochon, J. Chromatogr., A 1168 (2007) 101.
[55] O. Nuñez, K. Nakanishi, N. Tanaka, J. Chromatogr., A 1191 (2008) 231.
[56] K. Štulík, V. Pacáková, J. Suchánková, P. Coufal, J. Chromatogr., B 841 (2006) 79.
[57] F. Svec, J. Chromatogr., A 1228 (2012) 250.
[58] T. Ikegami, K. Tomomatsu, H. Takubo, K. Horie, N. Tanaka, J. Chromatogr., A 1184 (2008) 474.
[59] Z. Jiang, N.W. Smith, Z. Liu, J. Chromatogr., A 1218 (2011) 2350.
[60] T. Ikegami, H. Fujita, K. Horie, K. Hosoya, N. Tanaka, Anal. Bioanal. Chem. 386 (2006) 578.
[61] K. Horie, T. Ikegami, K. Hosoya, N. Saad, O. Fiehn, N. Tanaka, J. Chromatogr., A 1164 (2007) 198.
[62] Z. Lü, P. Zhang, L. Jia, J. Chromatogr., A 1217 (2010) 4958.
[63] H. Lin, J. Ou, Z. Zhang, J. Dong, M. Wu, H. Zou, Anal. Chem. 84 (2012) 2721.
[64] K. Hosoya, N. Hira, K. Yamamoto, M. Nishimura, N. Tanaka, Anal. Chem. 78 (2006) 5729.
[65] Z. Jiang, N.W. Smith, P.D. Ferguson, M.R. Taylor, Anal. Chem. 79 (2006) 1243.
[66] Z. Jiang, J. Reilly, B. Everatt, N.W. Smith, J. Chromatogr., A 1216 (2009) 2439.
[67] P. Holdšvendová, J. Suchánková, M. Bunček, V. Bačkovská, P. Coufal, J. Biochem. Biophys. Methods 70 (2007) 23.
[68] M.L. Chen, S.-S. Wei, B.-F. Yuan, Y.-Q. Feng, J. Chromatogr., A 1228 (2012) 183.
[69] M.L. Chen, L.-M. Li, B.-F. Yuan, Q. Ma, Y.-Q. Feng, J. Chromatogr., A 1230 (2012) 54.
[70] Y. Lv, Z. Lin, F. Svec, Analyst (Cambridge, UK) 137 (2012) 4114.
[71] Y. Lv, Z. Lin, F. Svec, Anal. Chem. 84 (2012) 8457.
[72] H.C. Foo, J. Heaton, N.W. Smith, S. Stanley, Talanta 100 (2012) 344.
[73] A.J. Sasco, F. Rey, C. Reynaud, J.-Y. Bobin, M. Clavel, A. Niveleau, Cancer Lett. 108 (1996) 157.
[74] K.H. Schram, Mass Spectrom. Rev. 17 (1998) 131.
[75] K. Nakano, T. Nakao, K.H. Schram, W.M. Hammargren, T.D. McClure, M. Katz, et al., Clin. Chim. Acta 218 (1993) 169.
[76] B.O. Feng, M.-H. Zheng, Y.-F. Zheng, A.-G. Lu, J.-W. Li, M.-L. Wang, et al., Hepatology 20 (2005) 1913.
[77] L. Thorell, L.-B. Sjoberg, O. Hernell, Pediatr. Res. 40 (1996) 845.
[78] L.K. Pickering, D.M. Granoff, J.R. Erickson, M.L. Masor, C.T. Cordle, J.P. Schaller, et al., Pediatrics 101 (1998) 242.
[79] B.D. Gill, H.E. Indyk, Int. Dairy J. 17 (2007) 596.

4 CONCLUSIONS

It may be concluded that the main aim of the thesis has been achieved, namely that there has been a satisfactory development of new analytical methodologies based on coupling of mass spectrometry and high-efficiency separation techniques, which have allowed problems of special interest in the field of food safety and environmental health to be overcome. With these new methodologies, it has been possible to simplify the analytical procedures involved in the sample treatment step and to quantify and perform a correct, unequivocal identification of the analytes studied.

The on-line coupling of restricted access materials (RAM) and liquid chromatography have allowed the author to automate the sample treatment step. This coupling is highly versatile, permitting a suitable clean-up in matrices as complex as foods and biological samples. It has satisfactory recovery values for analytes of very different natures. Additionally, it should be noted that the useful life of RAMs is long, with no memory effects. All these factors mean that the coupling of RAMs to liquid chromatography or capillary electrophoresis is a promising alternative in sample treatment steps.

Hydrophilic interaction liquid chromatography (HILIC) is seen to have good potential as a technique for the separation of high-polarity compounds. A detailed study of the retention mechanism in ZIC-HILIC shows that the separation of the analytes studied, nucleosides and nucleobases, occurs via a multiple mechanism in which electrostatic interactions play an important role under certain experimental conditions.

Additionally, although in a preliminary stage, the author highlights the capacity and analytical possibilities of the coupling of HILIC and RPLC (two-dimensional chromatography) in the analysis of endogenous and exogenous biomarkers in biological samples. The use of this methodology broadens the possibilities of the analysis of analytes of very different types by performing only a single chromatographic injection.

Tandem mass spectrometry, in its different modes, has allowed the development of highly sensitive methods, working with both an ion trap and a triple quadrupole. A further positive point is that study of the fragmentation patterns of the compounds of interest allows the follow-

up of different transitions, ensuring the unequivocal identification of the analytes at the level demanded by the corresponding legislations.

Overall, it may be concluded that the methods developed are rapid, sensitive and selective, both in the analysis of toxic residues in foods and biomarkers in biological samples. All the methods have proved to be suitable as quantitative confirmatory methods, meeting the different validation steps demanded by the pertinent European laws. With respect to more concrete conclusions, the work carried out along the development of this Thesis shows that in the determination of food pollutants highly efficient clean-ups can be obtained using C4 alkyl-chain RAMs for the analysis of both honey and milk samples. The author also demonstrates the possibilities of these materials (RAMs) in the field of food safety, an area in which they have rarely been used previously. In the determination of exogenous biomarkers in biological samples, the coupling of RAM materials, with an apolar nature, and chromatographic separation allows on-line sample treatment and automated quantitative determination. Of special importance is the development of a screening method that allows the development of a general follow-up of exposure to pesticides and toxic products of industrial origin. In the determination of endogenous biomarkers it is necessary to use a polar polymeric RAM. The method developed, applied to the analysis of control samples and samples from oncologic patients, has revealed the potential of the analysis of endogenous biomarkers as a method for diagnosis and follow-up in oncologic processes.

To conclude, the author has achieved the aims set, in particular those associated with studies using HILIC and the automation of the sample treatment steps, solving current problems linked to food safety and environmental toxicology, and showing the applicability of the methods developed and validated.