PERFORMANCE OF ZWITTERIONIC HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY FOR DETERMINATION OF IODINATED X-RAY CONTRAST AGENTS

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PERFORMANCE OF ZWITTERIONIC HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY ~~IN A METHOD~~ FOR ~~DETERMINING~~ DETERMINATION OF ~~A GROUP OF~~ IODINATED X-RAY CONTRAST ~~MEDIA~~ AGENTS

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Abbreviations: hydrophilic interaction liquid chromatography (HILIC); solid-phase extraction (SPE); stationary phase (SP); iodinated X-Ray contrast media (ICMs)

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Abstract

This study compares the separation performance of a group of iodinated X-Ray contrast media ~~(ICMs)~~ ~~in~~ on four different columns. The first three were two stationary phases ~~(SPs)~~ modified with C_{18} and a polar-embedded ~~SP~~ stationary phase (polar amide group bonded to an alkyl chain), all of which worked under reversed-phase liquid chromatography ~~(RPLC)~~. The fourth was a zwitterionic sulfoalkylbetaine ~~SP~~stationary phase, working under hydrophilic interaction liquid chromatography ~~(HILIC)~~. After the optimization of the different parameters, the zwitterionic column displayed the best separation, which also overcomes the problems encountered when these analytes were separated under RPLC. Moreover, when HILIC is coupled to a tandem mass spectrometry ~~(MS/MS)~~ detector, sensitivity is enhanced.

However, ~~the sensitivity of the method was affected~~ when sewage samples were then analysed by solid-phase extraction ~~(SPE)~~ followed by the optimal HILIC-MS/MS the sensitivity of the method was affected, due to the high matrix effect, which had to be solved by dilution of the extract. Finally, the method was validated with sewage and the figures of merit were comparable to those of the SPE~~-~~/RPLC-MS/MS.

1. Introduction

Iodinated X-Ray contrast media (ICMs) are used in clinical diagnosis to image soft tissues like organs or blood vessels. They are metabolically stable in the body, so they pass through the human body without interaction, and are rapidly eliminated via urine and faeces. Most of the chemical structures of ICMs are based on a benzene ring system containing three iodine atoms (to enhance X-ray absorption) and several hydrophilic moieties (to ensure high water solubility) [1, 2].

Their hydrophilic character and their (metabolic) stability are the main reasons that they are hardly eliminated at all during the sewage treatment process. In fact, it is widely known that compounds characterized by high solubility and low biodegradability are not eliminated in conventional sewage treatment plants [3]. Several studies have reported their occurrence in different aqueous samples, such as influent and effluent sewage, and surface waters, among others [2, 4-8]. Moreover, as a consequence of the high dosage administration and the lack of human metabolism, the concentrations of ICMs in the environment are at µg/L levels in sewage or ng/L in surface waters [4]. In view of this, different research groups have focused their attention on the development of analytical methods for determining ICMs in aqueous samples [1, 9-12].

Typically, the methods developed for the determination in environmental samples have included solid-phase extraction (SPE) followed by liquid chromatography (LC) with mass spectrometry (MS) or MS in tandem. For separation, several authors have used conventional RPLC [1, 2, 6-8, 10] or ion-pair chromatography, but this is not recommended if MS detection is required [10]. However, due to the strongly polar properties of ICMs, high water content mobile phase is necessary in order to achieve suitable separations. The higher the aqueous content in the mobile phase, the more difficult its desolvation in the ESI source, leading to a lack of sensitivity. Moreover, this presents an additional problem, as all of the sample extracts that have to be injected to the LC must also have a high aqueous content, which is not usually the case with solutions obtained from any extraction technique.

Recently, a new modality known as hydrophilic interaction liquid chromatography (HILIC) has regained popularity, and it is well suited for the separation of polar and/or ionizable compounds. First employed by Alpert [13], HILIC is a method in which a polar stationary phase (SP) (typically bare silica) is used in combination with a hydrophobic mobile phase (which contains a high percentage of organic solvent). The retention mechanism in HILIC is quite complex, involving a partition mechanism together with adsorption, ion-exchange, and even hydrophobic ~~reactions~~interactions. This has the effect of increasing retention as the polarity of

the analytes increases, providing alternate selectivity to RPLC [14, 15]. Moreover, the highly organic mobile phases used in HILIC provide enhanced sensitivity in MS detection, due to their efficient desolvation and low back pressures resulting from their low viscosity [15-17]. In recent years, this separation mode has been reported for the determination of different compounds, such as polar pharmaceuticals, polar pesticides and biomedical compounds, among others [18-23]. However, to the best of our knowledge, the separation of a group of compounds as highly polar as ICMs has never been reported using HILIC separation.

In view of this, the aim of this work is to develop an analytical method based on SPE coupled to HILIC-MS/MS for the determination of a group of ICMs in complex sewage, and to evaluate whether the proposed method addresses the issues with the early elution time of ICMs, with all of the related problems that this involves.

2. Materials and methods

2.1 Reagents and standards

The ICMs studied were: iopamidol, diatrizoic acid, iohexol, iomeprol and iopromide. All were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Standard individual solutions of 1,000 mg/L in methanol were prepared from the salt and stored at -20°C. A working solution of the mixture of each of compounds was prepared at 100 mg/L in 70:30 acetonitrile:pure water (v/v), and then daily solutions were prepared from the intermediate one in the same solution (70:30 acetonitrile:pure water (v/v)). These solutions were stored at 4°C.

The organic solvents methanol and acetonitrile (ACN) of HPLC grade were purchased from SDS (Pepin, France). Analytical grade ammonium acetate, acetic acid, ammonium formate, formic acid and ammonium hydroxide (25%), which were used to prepare the mobile phase, were supplied by Aldrich (St. Louis, MO, USA). Ultrapure water was obtained from a water purification system (Veolia, Sant Cugat del Vallès, Spain). The nitrogen (N_2) gas (99%) was obtained from Carburos Metálicos (Tarragona, Spain). The nylon filters of 0.22 µm pore size were purchased from Scharlab (Barcelona, Spain).

2.2 Chromatographic equipment and conditions

The chromatographic system was an Agilent 1200 Liquid Chromatograph-triple quadrupole tandem mass spectrometer-UHPLC-MS/MS - (Agilent Technologies, Waldbronn, Germany). It was equipped with an electrospray ionization interface (ESI), an automatic injector, a degasser, a binary pump and an oven for the column. The injected volume was 50 µL. For the evaluation of the separation, the LC instrument was connected to a UV detector (1200 series, Agilent) at 240 nm.

The LC separation was carried out using four different columns, three of them as reversed-phase (RP) SPs: Ascentis Express C_{18} (50 mm x 4.6 mm i.d., 2.7 µm fused core particle size), Ascentis Express RP-amide (100 mm x 2.1 mm i.d., 2.7 µm fused core particle size), both from Supelco (Bellafonte, PA, USA), and Zorbax Eclipse XDB-C_{18} (50 mm x 4.6 mm i.d., 1.8 µm particle size) from Agilent. The fourth was an HILIC SP: ZIC-HILIC column (150 mm x 4.6 mm i.d, 5 µm particle size from Merck (Darmstadt, Germany).

The three RP columns were tested under the same ~~optimized~~ conditions. The mobile phase was a mixture of solvent A, ultrapure water with 1% formic acid ~~adjusted to~~ (pH 2.6) ~~with formic acid~~, and solvent B, acetonitrile. The gradient profile started at 5% solvent B which was held for 4 min, and increased to 25% solvent B in 3 min (and held for 7 min), after which the

mobile phase was returned to the initial conditions (5% solvent B) in 3 min. The mobile phase flow-rate was 0.2 mL/min and the temperature of the column was 25°C.

The ZIC-HILIC column was used under the following optimized conditions. The mobile phase was a mixture of solvent A, 2mM $HCOONH_4$/HCOOH aqueous buffer at pH 3.5, and solvent B, ACN. The gradient profile was 90% solvent B which was reduced to 81% in 12 min, and to 50% in 3 min (and held for 5 min), after which the mobile phase was returned to the initial conditions (90% solvent B) in 2 min (and held for 5 min to equilibrate the column for the following analysis). The mobile phase flow-rate was 1 mL/min and the temperature of the column was 65°C.

The ionization and fragmentation were optimized by ~~direct injection~~infusion of each of the ICMs. Analyses were performed in the Multiple Reaction Monitoring (MRM) mode, using electrospray ionization (ESI) in the positive mode. Optimized MS/MS parameters were as follows: a N_2 flow-rate of 12 L/min, a capillary voltage of 3,500 V, a nebulizer pressure of 45 psi (N_2) and a source temperature of 350°C. The cone voltage and collision energies were optimized for each compound. The cone voltage was fixed at 140 V and collision energies were between 10 and 50 V for all compounds. The retention time and two MRM transitions were compared to confirm the presence of the analytes. The most abundant MRM transition was used to quantify. In the case of RP separation, two time windows were used: 0-9 minutes (iopamidol, diatrizoic acid, iohexol and iomeprol) and 9-16 minutes (iopromide). In the HILIC separation, just a single time window was used. Table 1 details the optimal MS/MS conditions.

2.3 Solid-phase extraction

Influent and effluent sewage water samples from ~~areas surrounding~~ treatment plants were previously mixed from different batches, and then ~~firstly~~ filtered through 0.45 µm nylon membranes (Scharlab) before the SPE step was performed to eliminate the particulate matter, after which they were adjusted to pH 2.5 with formic acid.

The final SPE protocol was as follows: Oasis HLB 500 mg (Waters, Milford, MA. USA) were placed in an SPE manifold (Teknokroma, Barcelona, Spain) and connected to a vacuum pump. The sorbent was conditioned with 5 mL of MeOH and 5 mL of water adjusted to pH 2.5. The samples (100 mL of influent and 250 mL of effluent) were loaded through the cartridge. A clean-up step was then performed with 2 x 5 mL of water acidified to pH 2.5 with formic acid. The retained analytes were then eluted with 5 mL of MeOH. Elution extracts were evaporated to dryness under a gentle flow of nitrogen. Before MS/MS injection, the elution fractions were reconstituted to 5 mL with the same solution consisting of 85:15 ACN:ultrapure water (pH 7) (v/v), which are the same as the initial mobile phase elution conditions.

3. Results and discussion

3.1 Development of the chromatographic separation

Four analytical columns were tested for the chromatographic separation. Initially, an Ascentis Express C_{18} (50 mm x 4.6 mm i.d., 2.7 µm fused core particle size) and a Zorbax Eclipse XDB-C_{18} (50 mm x 4.6 mm i.d., 1.8 µm particle size) column were tested. These two columns are typically RP SPs, with the latter being specially recommended for the separation of highly polar compounds since it uses the eXtra Dense Bonding (XDB) technology of organo-silane ligands and double end-capping to protect the ultrapure silica supports. Both columns were tested using the mobile phase as described in Section 2.2, and using a flow-rate of 0.2 mL/min.

Table 2 shows the optimal conditions and the analyte retention times for all of the columns tested. Under these pre-optimized conditions [9], this group of ICMs eluted with limited retention of these polar compounds at flow-rate of 0.2 mL/min and using initial mobile phase conditions, such as 95% water. The early elution of the ICMs might be a disadvantage if matrix components in complex samples (e.g. sewage) co-elute with the analytes of interest. Such co-elution might result in ion suppression/enhancement effects that are not easy to control. Another problem that arose with the optimal RP conditions is the high water content (from 95% to 75% during the compound elution). Even at the low flow-rate (i.e. 0.2 mL/min), the ESI interface was unable to convert the liquid mobile phase completely into an ionized vapour. All of this resulted in a ~~poorer~~ worse ICM response. Moreover, the high water content in the mobile phase is an added problem since all of the solution directly injected to the LC must be of high aqueous content, which is not usually the case with the usual solutions obtained from any extraction technique [24]. To date, different studies that have determined ICMs used similar LC conditions, which also involved related problems [1, 2, 10].

The next column tested was the Ascentis Express RP-amide, which is a new generation of polar-embedded SP that contains a polar amide group on the bonded alkyl chain. The polar amide moieties can interact with the residual silanol groups in the SP through electrostatic and/or hydrogen bonding interactions and, as a result, minimize silanophilic interactions [25] and also add retention increments ~~with~~ for compounds as polar as ICMs ~~[25]~~. Under the ~~optimum~~ selected conditions (see section 2.2), the analyte retention time was similar or even lower compared to the retention times achieved under C_{18} separation (see Table 2). The RP-amide phase provided different selectivity with respect to iohexol and diatrizoic acid. Moreover, the RP-amide phase was able to separate the two diastereoisomers of iohexol and iopromide, as each of these compounds contains two chiral carbon atoms [26], while both C_{18} modified phases were just able to separate the diastereoisomer of iopromide. In any case, this

feature did not improve the early elution of the ICMs studied. In fact, Busetti [26] also tested a RP polar-embedded type column for a similar group of ICMs. However, they ruled out this column as some of the ICMs presented fronting and/or tailing.

Next, a ZIC-HILIC column based on a zwitterionic sulfoalkylbetaine SP was evaluated. ZIC-HILIC consists of a wide-pore silica gel that contains both strongly acidic sulphonic acid groups and strongly basic quaternary ammonium groups separated by a short alkyl spacer. Thus, simultaneous separation of anionic and cationic compounds are possible. This type of column was selected as the five ICMs to separate present differences in terms of acidity (e.g. diatrizoic acid possesses a negative charge) and basicity. Moreover, the sulfoalkylbetaine bonded phases strongly adsorb water by hydrogen bonding and the bulk layer of water, which forms part of the SP, and therefore largely controls the retention mechanism. Polar (hydrogen-bonding and dipole-dipole) interactions in the SP are of primary importance. [17, 27, 28].

The mobile phase composition was evaluated testing different variables such as pH, ionic strength and organic modifier. Since HILIC is then directly coupled to MS/MS detector, volatile buffers should be considered. In fact, buffers for HILIC are typically acetic acid, formic acid and their ammonium salts because they are all volatile and soluble with high percentages of organic phase. These different aqueous mobile phases were tested: ultrapure water adjusted to pH 3.5 and 7 with formic acid and ammonium hydroxide, respectively; and the buffers ammonium formate and ammonium acetate both at pH 3.5 and 7, and both at different salt concentrations (2 mM, 10 mM and 50 mM). All of these mobile phases were combined with ACN as the organic mobile phase. The first observation was that buffered mobile phases worked better than ultrapure water adjusted with acidic or basic modifier. It has been extensively reported that the presence of buffering salts in the mobile phase can decrease electrostatic interactions through disruption [28]. Among the different concentration of salt tested, in general, no significant differences were observed in the separation of the analytes as well as the peak shape itself. The only difference was in the case of diatrizoic acid, which experienced a shift in the retention time when increasing the concentration of salt, and finally was overlapped with iomeprol. This behaviour is in line with the observation that increasing salt concentration suppresses both electrostatic attraction and repulsion, causing increasing retention of acidic analytes [15]. Therefore, as no improvements were achieved and in order to facilitate the buffer volatilization once in the ESI interfase, as well as avoiding problems of salt precipitation, it was decided to work at a salt concentration of 2mM. When comparing formate and acetate buffers, it was noticed that the formate buffer yielded shorter analysis time separation, presumably due to different eluting strength of the competing ions (formate versus acetate) in the ion-exchange interaction [19]. Therefore, the former was selected for

further analysis. Adjusting the pH of the $HCOONH_4$/HCOOH mobile phase to 3.5 or 7 provided differences in the retention time of diatrizoic acid ~~(~~because it is the only analyte that modifies its charge~~ability~~ depending on the pH~~. In other words, when the mobile phase is adjusted to pH 3.5, (~~i.e. it is in its neutral state at pH 3.5, and, it is in its anionic form ~~when it is adjusted to~~at pH 7 ~~is in its anionic form)~~. When the mobile phase was adjusted to pH 3.5, all five analytes were better separated and the resolution between them was better than at pH 7. Moreover, this pH favours the positive ionization of the analytes in the following ESI interface. Therefore, 2 mM $HCOONH_4$/HCOOH mobile phase at pH 3.5 was selected as the optimal mobile phase for this separation.

The type of organic mobile phase was also evaluated by comparing ACN and MeOH. However, MeOH did not provide any improvements in the separation of the ICMs, showing that the contributions of hydrogen bonding is not so determinant. In view of this, ACN was used as organic phase from then on.

Under these conditions, the flow-rate (0.6, 0.8 and 1.0 mL/min) and the temperature (25°C, 35°C, 60°C and 65°C) were also tested. With all of the flow-rates tested, similar separation was achieved, but the higher the flow-rate, the faster the separation. Therefore, 1.0 mL/min was selected as it provided the separation with the lowest analysis time. Moreover, this flow-rate is suitable for the inner diameter column dimensions (4.6 mm), and it is also compatible with ESI as it is mostly composed by ACN. With respect to temperature, the retention time of the analytes was randomly different when the temperature of the separation was modified. This feature can be observed in Figure 1, where the four chromatographic profiles are shown for the four temperatures tested. This behaviour may be due to the advantage of the partitioning or electrostatic retention mechanism with the SP of the analytes depending on the temperature, since higher temperatures would favour partitioning mechanisms, while not favouring electrostatic interaction mechanisms [29]. In addition, the phase temperature ~~can~~ might affect the separation selectivity of a zwitterionic SP, as the flexibility of the intercharge spacer arm increases with increased temperatures [29]. This behaviour also helped in the separation of iopamidol and iomeprol at 65°C, because, at the other temperatures tested, these two analytes appeared overlapped.

Once the mobile phase (2 mM $HCOONH_4$/HCOOH mobile phase at pH 3.5 combined with ACN), temperature (65°C) and flow-rate (1 mL/min) had been fixed, different gradients were tested in order to further increase the separation of the analytes. The optimum gradient as described in section 2.2., which takes into account that the higher the organic phase content in the HILIC separation, the higher the retention of the polar compounds. And also, that a minimum percentage of water (at least 3%) is necessary for sufficient hydration of the SP particles [30,

31]. Table 2 summarizes the analyte retention times, where the first analyte (i.e. iopromide) appeared at 4 minutes, circumventing the problems related with the early elution of the analytes that occurred in the case of RP separation.

In addition, the signal obtained in the subsequent MS/MS detector when using HILIC separation is enhanced (two-fold signal in the case of most of the analytes) compared to the response obtained under RPLC separation. This is attributed to the enhanced desolvation process in the MS interface, as the mobile phase that surrounds the analytes in HILIC conditions is mainly organic phase [32].

3.2. Sample preparation and matrix effect

Oasis HLB (500 mg) was the sorbent of choice for the SPE protocol since it had provided satisfactory results in previous studies [9]. Initially, 250 mL of ultrapure water adjusted to pH 2.5 and spiked at 5 µg/L of each ICM were loaded into the cartridge and the SPE protocol as described in section 2.3 was followed. Under these conditions, all of the studied analytes showed recoveries ranging from 67% to 91%, with the exception of iopamidol (%R 36%).

The recoveries were then studied in more complex environmental waters, such as influent and effluent sewage. Firstly, a blank sample was analysed in order to subtract the possible signal of existing analytes that appeared in all instances at low levels of concentration. When these type of matrices were used, the matrix effect was evaluated first, which was calculated by comparing the signal obtained for the analytes when spiked ~~over the~~to the blank extract of the SPE to the signal obtained for these analytes in the injection solution.

The matrix effect of the proposed method was very high with values of ion-suppression ranging between 75% and 90% for all analytes. To circumvent this high ion-suppression effect, the eluted SPE extract was diluted to 5 mL instead of 1 mL and, with this strategy, the ion-suppression decreased to values up to 50% as most. This high matrix effect had already been reported when HILIC was used, since this type of SP may become overloaded more easily and the sensitivity may be seriously compromised in real samples [22, 33, 34]. Therefore, the signal improvement achieved in ultrapure water using HILIC was lost in complex samples. One strategy for diminishing the complexity of the matrix is washing these interferences out of the SPE cartridge before eluting them together with the analytes of interest. For this reason, different washing solvents were tested: pure MeOH, ACN, water at various percentages (25%, 50% and 75%). However, all of them failed because the recoveries dropped to between 5% and 10% for all of the analytes. Washing with aqueous solvent was not strong enough to remove all of these interferences but it did not affect the recovery of the analytes. Thus, washing was carried out with 2 x 5 mL of water acidified to pH 2.5 with formic acid. Another strategy often

adopted to correct the matrix effect is the inclusion of isotopically labelled internal standards. As isotopically labelled ICMs were not commercially available, w~~W~~e tried some different deuterated drugs that we had available in the laboratory. However, none of them helped to reduce this effect. We then used a matrix matched calibration curve to correct the ion suppression and recovery of the analytes. After completion, the recovery values obtained (including the matrix effect) after loading 250 mL and 100 mL of effluent and influent sewage, respectively, are detailed in Table 3. As can be seen, the values are slightly low, but acceptable since they are in the line of those already reported in other studies that analyse similar complex samples, if one takes the matrix effect into account.

3.3 Method validation and analysis of samples

The overall analytical method was then validated for effluent and influent sewage (Table 3) considering: linear range, limits of quantification (LOQs), limits of detection (LODs), intra-day and inter-day repeatability.

The linear range was studied with a six-point calibration curve (details for the concentration range in Table 3), and in all of the cases, determination coefficients (r^2) were higher than 0.99.

The LOQs for each compound were taken as the lowest concentration level of the calibration curve, which was also checked as signal-to-noise ratio (S/N) of 10. The LODs, calculated as the ~~signal-to-noise ratio (S/N)~~ S/N of 3, were from 0.01 to 0.05 µg/L for effluent and from 0.03 to 0.5 µg/L for influent. However, when the compounds were present in real samples the LODs and LOQs were estimated as three and ten times the standard deviation of the analyte signal in the blank (n=3), respectively. The LODs for the rest of the compounds were similar to those reported in methods involving SPE-/RPLC-MS/MS [2, 35].

The intra-day and inter-day repeatability were determined by spiking three replicates at 50 µg/L of each type of sample and also at 2 µg/L for effluent and 5 µg/L for influent, and the results obtained, expressed as a % of relative standard deviation (%RSD), were less than 7% and 15%, respectively.

Finally, the developed SPE/HILIC-MS/MS method was applied for the determination of ICMs in influent and effluent samples, in triplicate, from two different urban sewage treatment plants (STP) with samples taken on different days. The presence of the analytes found were confirmed according to the Commission Decision 2002/657/EC [36]. As expected, iopromide and iohexol appeared in all of the samples analysed with concentration levels ranging from 6.5 to 9.2 µg/L and 0.1 to 1.9 µg/L, respectively for influent; and from 4.1 to 6.9 µg/L and 0.4 to 1.2 µg/L, respectively, for effluent sewage. The rest of ICMs appeared in both types of sample in a more random manner: diatrizoic acid (<LOQs to 3.5 µg/L for influent and <LOQs for effluent),

iomeprol (<LOQ – 4.1 µg/L for influent and <LOQ – 2.0 µg/L for effluent) and iopamidol (<LOQ – 1.2 µg/L for influent and <LOQ for effluent). These values are in line with those reported at similar STPs [2, 3, 9, 12]. It should be mentioned that some ICMs are present in effluent but not in influent. This might be due to the fact that the sampling of the influent and effluent sewage was not performed in the same time period, but also due to a possible conversion of their conjugated metabolite to the original substance after the treatment processes. As an example, Figure 2 shows a representative MRM chromatogram of an influent sample, where all of the studied ICMs were found.

4. Concluding remarks

For the first time, we present a new separation for a group of ICMs based on HILIC using a zwitterionic type column. After optimizing the parameters that affect the separation, the HILIC separation displayed good performance for all of the compounds, and improved the problems related to early elution associated with RPLC.

The instrumental method (HILIC-MS/MS) presented enhanced sensitivity compared to the conventional methods based on RPLC-MS/MS. However, the sensitivity of the overall analytical procedure (SPE/HILIC-MS/MS) used to analyse complex samples was affected by the high matrix effect encountered.

Therefore, further work focusing on the clean-up of the sample during the pretreatment process is needed in order to avoid the matrix effect and, thus, achieve even higher sensitivity.

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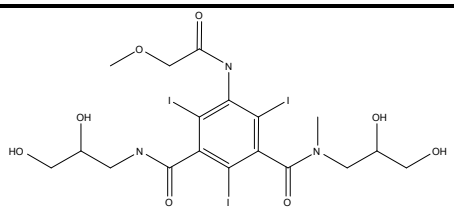
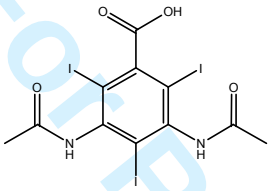
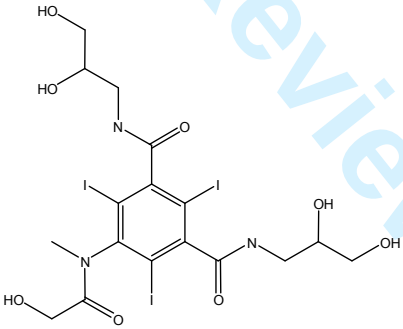
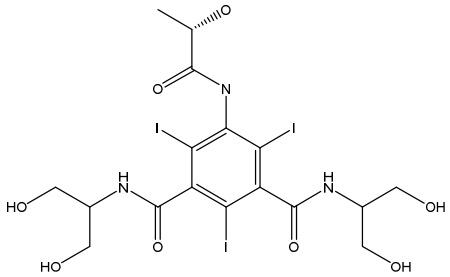
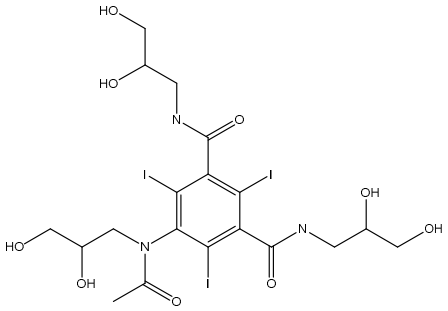
420

421 **Table 1.** Structures and MRM conditions for the ICMs studied.

Analyte	Structure	Cone voltage (V)	~~Parent~~ Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)
Iopromide		140	792	**559** 445	30 40
Diatrizoic acid		140	615	**361** 233	10 25
Iomeprol		140	778	**405** 532	35 25
Iopamidol		140	778	**559** 387	15 50
Iohexol		140	822	**804** 603	15 30



In bold the ion selected for quantification.

Table 2. Retention time and separation conditions for the four columns tested. See the text for the rest of conditions.

TYPE	RP		Polar embedded RP	HILIC
COLUMN	Ascentis Express Fused Core C_{18}	Zorbax Eclipse XDB C_{18}	Ascentis Express Fused Core RP-amide	ZIC-HILIC
~~CONDICIONS~~CONDITIONS	Optimised RP conditions; Flow-rate 0.2 ml/min			Optimised HILIC cond. Flow-rate 1 ml/min
	Retention time (minutes)			
IOPAMIDOL	1.9	2.~~15~~2	2.7	7.9
DIATRIZOIC ACID	2.7	2.6	4.5	6.2
IOHEXOL	3.5	3.9	3.4 + 4.1	9.5
IOMEPROL	3.9	4.3	4.4	7.7
IOPROMIDE	6.2 + 6.5	7.2 + 7.4	6.6 + 7.3	4.2

Multiple retention times for iohexol and iopromide correspond to the retention time of the respective enantiomers of each compound.

Table 3. % Extraction r~~R~~ecovery values and validation parameters for effluent and influent sewage.

	% ~~Recovery~~Extraction recovery		Linear range (µg/L)		LODs (µg/L)		Repeati~~i~~bili~~i~~ty (%RSD)			
							Intra-day[c]		Inter-day[c]	
	Effluent[a]	Influent[b]	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent
Iopromide	29	21	0.1 -100	0.5 -100	0.05	0.1	3	6	5	10
Diatrizoic acid	23	25	0.1 -100	1 -100	0.02	0.5	1	3	3	5
Iomeprol	55	52	0.1 -100	0.1 -100	0.01	0.03	2	5	3	10
Iopamidol	28	20	0.1 -100	0.5 -100	0.01	0.1	3	8	4	12
Iohexol	21	19	0.1 -100	0.1 -100	0.01	0.05	1	4	6	8

[a] 250 ml spiked at 2 µg/L.
[b] 100 ml spiked at 5 µg/L.
[c] n=3, 2 µg/L for effluent and 5 µg/L for influent.
LOQs set at the lowest point into the linear range.

Figure captions.

Figure 1. Effect of the temperature: (A) 25ºC; (B) 35ºC; (C) 60ºC; (D) 65ºC; in the separation of the compounds using the ZIC-HILIC column. For experimental conditions see the text. Peak assignment: (1) iopromide; (2)diatrizoic acid; (3) iomeprol; (4) iopamidol; (5) iohexol.

Figure 2. MRM chromatogram for an influent sewage. For experimental conditions see the text.

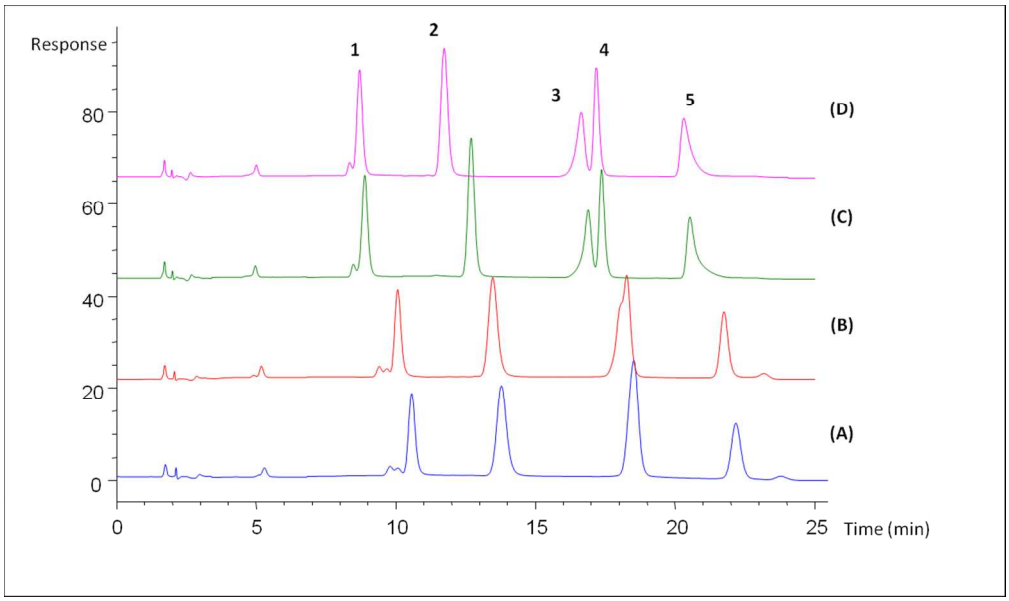


Figure 1. Effect of the temperature: (A) 25ºC; (B) 35ºC; (C) 60ºC; (D) 65ºC; in the separation of the compounds using the ZIC-HILIC column. For experimental conditions see the text. Peak assignment: (1) iopromide; (2)diatrizoic acid; (3) iomeprol; (4) iopamidol; (5) iohexol.
215x127mm (150 x 150 DPI)

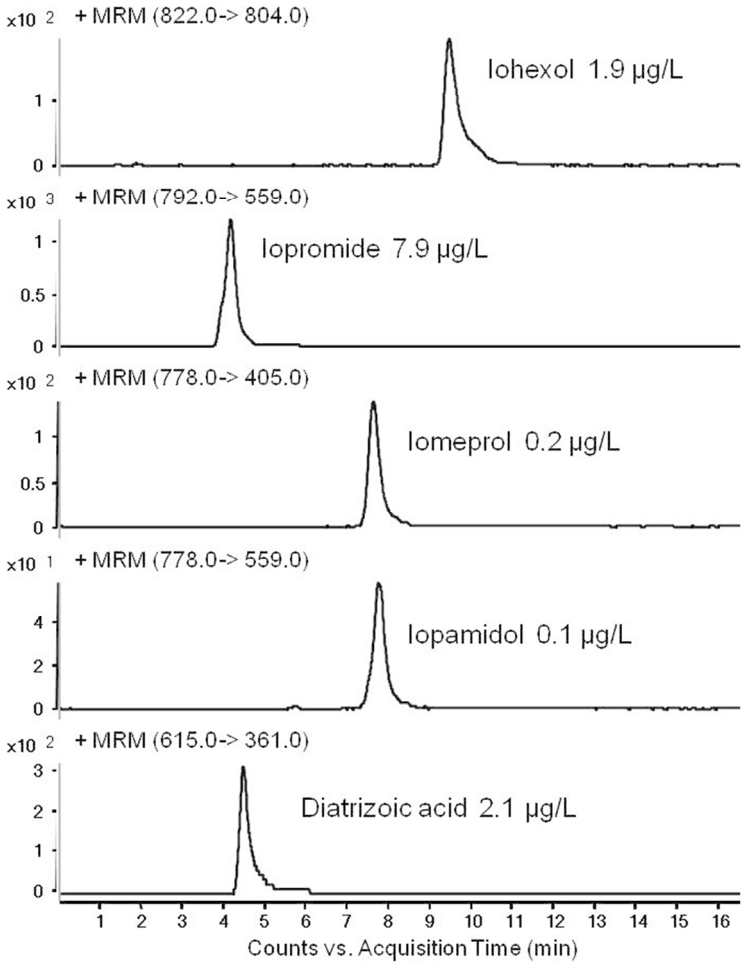


Figure 2

Figure 2. MRM chromatogram for an influent sewage. For experimental conditions see the text.
159x165mm (150 x 150 DPI)