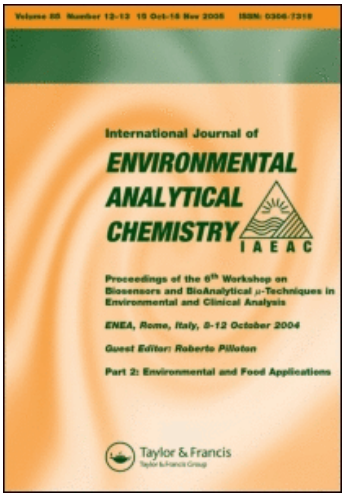


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# Dual gradient LC method for the determination of pharmaceutical residues in environmental samples using a monolithic silica reversed phase column

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Detailed below is a simple reversed-phase liquid chromatography (RP-LC) method for the simultaneous separation of up to 21 acidic, basic, and neutral pharmaceuticals using Merck Chromolith Performance RP-C18e monolithic columns with direct ultraviolet (UV) absorption detection. By simultaneously applying a solvent elution gradient program with a mobile phase flow gradient, both a decrease in the overall analysis time and a general increase in peak efficiencies were observed. Mobile phase pH and buffer concentration were optimised using the overall resolution product under applied gradient conditions. Under optimised conditions peak area reproducibility ($n = 6$) ranged between 0.4 and 9.3%, determined at the method LOQ level. For real sample analysis pharmaceutical residues were extracted using an optimised solid phase extraction (SPE) procedure, utilising Strata-X extraction cartridges, which overall provided the highest relative recovery data in comparison with four other commercially available SPE sorbents (17 out of 20 residues investigated had recoveries over 70%). Complete method precision, including all sample pre-treatment and LC analysis for six spiked river water samples at the 1 and 2 $\mu g\,L^{-1}$ level was between 10 and 29%. Using 1 L volumes of 1 $\mu g\,L^{-1}$ spiked estuarine water samples, the majority of detection limits were found to be in the 10–50 $ng\,L^{-1}$ range.

*Keywords:* Monolithic silica; Dual gradient; RP-LC; SPE; Pharmaceuticals

## 1. Introduction

In Ireland, according to the Irish Medicines Board, there are in excess of 3000 pharmaceutical compounds licensed for use in human and veterinary medicine, [1] and in 2003 alone there were more than 22.5 million prescriptions issued to the Irish public through the 'General Medical Services' [2]. Such diversity and volumes are relatively in-line with the majority of western European countries, resulting in a high

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probability that many of these compounds may persist through wastewater treatment processes and be present at ultra trace levels in the aquatic environment.

Pharmaceuticals can enter the environment by a variety of routes including disposal of manufacturing wastes and the improper disposal of expired or unused pharmaceuticals to landfill. However, the primary route is through the sewage system via human and animal excreta as either parent molecules or subsequent metabolites [3–5]. Albeit at ultra trace levels, pharmaceutical compounds have been detected in a variety of environmental matrices including sewage treatment plant effluents, river water, marine and estuarine waters and surface water [6–9]. In addition, under certain circumstances, some pharmaceutical compounds have been shown to survive potable water treatment processes and therefore be present in finished product drinking waters [10]. By design pharmaceuticals are bioactive, highly stable and resistant to degradation but concern has arisen due to the unknown toxicological effects on both humans and aquatic life continuously exposed to contaminated waters. Therefore there exists an increasing need for both qualitative and quantitative analytical techniques to monitor and identify this ever-increasing range of pharmaceuticals and their metabolites. Within the pharmaceutical industry a large proportion of quality control assays for product purity determination are performed using reversed-phase liquid chromatography (RP-LC) [11]. Liquid chromatography is suited to pharmaceutical analysis as the majority of pharmaceutical compounds are non-volatile, relatively polar and of moderately high molecular weight. Because of its inherent suitability, RP-LC has become the method of choice for the analysis of pharmaceuticals in environmental samples, with the majority of recent methods published using micro-bore RP-LC in conjunction with electrospray ionisation mass spectrometry (ESI-MS) [6–9].



Monolithic stationary phases are still a relatively new concept in LC. Unlike traditional packed columns, monoliths consist of a single continuous silica skeleton with a bimodal pore system; macropores ($\approx$2 μm diameter) that provide a relatively high flow through porosity ($\approx$80% porosity compared with $\approx$65% for traditional particle packed columns) and mesopores ($\approx$13 nm diameter) that provide a high surface area ($\approx$300 $m^2 g^{-1}$) for the chromatographic separation to take place [12]. The higher porosity of monolithic phases allows separations to be performed at higher flow rates than attainable with packed columns due to the lower back-pressure generated by the monolith. Monolithic stationary phases have therefore been used for the fast separation of a variety of analytes including pharmaceuticals, peptides, oligonucleotides, anions, cations etc. [13–18]. It has also been shown in the above studies that the efficiency of reversed-phase silica monoliths, at flow rates between 1 and 3 mL $min^{-1}$, is approximately equivalent to that seen with similar size columns containing 3 μm reversed-phase silica particles, with plate counts of >1,00,000 plates $m^{-1}$ having been readily achieved using monolithic columns [19].

In the method presented here, a combined solvent elution gradient and a continuous flow gradient was used for the separation of over 20 commonly prescribed acidic, basic, and neutral pharmaceuticals. Using the dual gradient approach, it was possible to use a more gradual solvent strength gradient to maintain desired resolution, whilst reducing overall run times through the simultaneous application of elevated flow rates. An additional benefit of this approach is an increase in column efficiency when compared with a single solvent gradient. When combined with an off-line preconcentration procedure (700–1000 fold preconcentration), the dual gradient method was suitable for the analysis of natural water samples.

## 2. Experimental

### 2.1. *Target pharmaceuticals*

Based upon those that showed prevalence in the environment as previously described in the literature the pharmaceuticals shown in table 1 were chosen for investigation.

### 2.2. *Chemicals and reagents*

Reagent water used throughout this study was obtained from a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA) and was 18.2 MΩ or greater. Methanol was received from Labscan (Dublin, Ireland), acetone and ethyl acetate were obtained from Aldrich (Gillingham, UK). All solvents used were HPLC grade. Dichlorodimethylsilane, ammonium formate and formic acid were also purchased from Aldrich. BDH Analar grade sulphuric acid was used for sample pH adjustment (Poole, UK). Metformin hydrochloride, acetaminophen, salicylic acid, *o*-toluic acid, propranolol hydrochloride, clofibric acid, ketoprofen, diclofenac sodium salt, clotrimazole and 2-naphthoic acid were obtained from Aldrich (Steinheim, Germany). Trimethoprim, caffeine, naproxen and triclosan were received from Fluka (Buchs, Switzerland). Bezafibrate, warfarin, flurbiprofen, indomethacin, ibuprofen sodium salt, meclofenamic acid sodium salt, mefenamic acid, gemfibrozil and ivermectin were all obtained from Sigma (Steinheim, Germany). All pharmaceuticals were of a purity >95%.

Stock 1000 mg $L^{-1}$ solutions of each pharmaceutical were prepared in methanol and were stored as described on the MSDS sheets for maximum stability, i.e. refrigerated if necessary and in the dark. In the case of salts, standards were prepared in terms of the parent analyte. These standards were periodically replaced. Working standards were prepared from these stock standards by appropriate dilution using methanol.



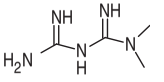
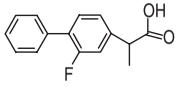
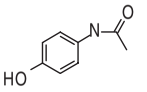
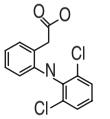
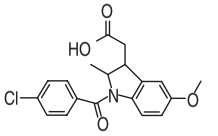
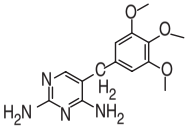
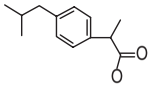
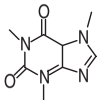
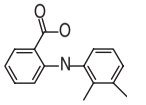
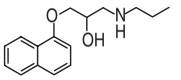
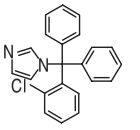
### 2.3. *Methods*

**2.3.1. Glassware preparation.** Prior to use all glassware was silanised by rinsing thoroughly with a 10% v/v solution of dichlorodimethylsilane in dichloromethane, followed by rinsing twice with dichloromethane and twice with methanol.

**2.3.2. Sample extraction.** A variety of sorbents were investigated as suitable stationary phases for sample extraction. These included Phenomenex Strata C18-E; Phenomenex Strata-X; (Phenomenex, Macclesfield, UK), Merck LiChrolut EN; (Merck, Darmstadt, Germany), Waters Oasis HLB; and Waters Oasis MAX; (Waters, Milford, MA, USA). Details of their sorbent mass and cartridge volumes are shown in table 2.

From initial investigations the Phenomenex Strata-X and Oasis HLB sorbents demonstrated high analyte recovery and were therefore chosen for further study. Prior to extraction 1 L water samples were filtered through Whatman GF/C glass microfibre filters to remove particulate matter (Whatman, Maidstone, UK). The filtrate was then spiked with surrogate standards (to yield an overall concentration of 2 μg $L^{-1}$ of both *o*-toluic acid and meclofenamic acid) to assess extraction performance and adjusted to pH 4.0 with concentrated sulphuric acid. The SPE cartridge was conditioned with 6 mL of methanol and 6 mL of water respectively.

Table 1. Pharmaceutical structures and product names [20].

| Target analyte | Product name | Structure | Target analyte | Product name | Structure |
|---|---|---|---|---|---|
| Metformin | Glucophage |  | Flurbiprofen | Strepsils |  |
| Acetaminophen | Paracetamol, e.g. Panadol |  | Diclofenac | Difene |  |
| Salicylic acid | Aspirin metabolite |  | Indomethacin | Indocin |  |
| Trimethoprim | Bactrim |  | Ibuprofen | Nurofen |  |
| Caffeine | Neutraceutical |  | Mefenamic acid | Ponstel |  |
| Propranolol | Inderal |  | Clotrimazole | Canesten |  |

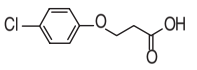
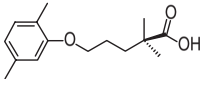
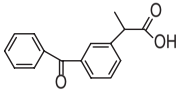
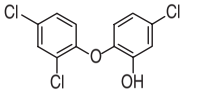
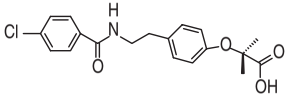
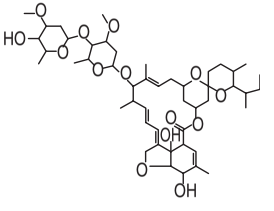
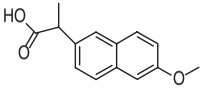
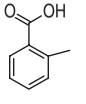
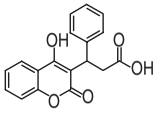
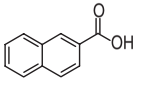
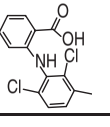
| | | | | | |
|---|---|---|---|---|---|
| Clofibric acid | Clofibrate metabolite |  | Gemfibrozil | Lopid |  |
| Ketoprofen | Oruvail |  | Triclosan | Personal care products |  |
| Bezafibrate | Bezalip |  | Ivermectin | Ivomec |  |
| Naproxen | Naprosyn |  | *o*-Toluic acid | Surrogate standard |  |
| Warfarin | Coumadin |  | 2-Naphthoic acid | Internal standard |  |
| | | | Meclofenamic acid | Surrogate standard |  |

Table 2. Mass and volume data for solid phase extraction cartridges.

| Product | Sorbent mass (mg) | Cartridge volume (mL) |
|---|---|---|
| Phenomenex Strata C18-E | 200 | 3 |
| Phenomenex Strata-X | 200 | 6 |
| Merck LiChrolut EN | 200 | 3 |
| Waters Oasis HLB | 200 | 6 |
| Waters Oasis MAX | 150 | 6 |

Samples were introduced by vacuum through Teflon tubing and extracted under an operating pressure of 20″ Hg on a vacuum manifold. After complete sample loading (without letting the cartridge run dry), the sorbent was washed with 1 mL of water. The sorbents were then dried by vacuum aspiration for a minimum of 30 min. Elution was then performed using 10 mL of a 50% v/v solution of ethyl acetate in acetone [21]. The eluate was collected and reduced to near dryness under a stream of $N_2$. The residue was reconstituted in 1 mL of internal standard solution (1 mg $L^{-1}$ 2-naphthoic acid in methanol) and transferred to an autosampler vial for HPLC analysis.



**2.3.3. Liquid chromatography.** A Hewlett Packard HP 1050 Series high performance liquid chromatograph was used throughout and consisted of a quaternary pump with online vacuum degasser, a variable wavelength detector model 79853C and an auto-sampler. Agilent ChemStation for LC systems version A.09.03 was used for system control and data analysis (Agilent Technologies, Palo Alto, CA, USA). The analytical column used consisted of two Chromolith Performance C18e; 100 × 4.6 mmID monolithic octadecyl silica columns combined in series using a Chromolith Column Coupler. A 10 × 4.6 mmID Chromolith RP-18e guard cartridge was also incorporated to prevent spoilage of the analytical columns (Merck, Darmstadt, Germany). A multi-step binary gradient, in conjunction with a flow gradient was employed for analyte elution using a mobile phase consisting of methanol and 1 mM ammonium formate/formic acid buffer at pH 4.5. The temperature was held at 25°C throughout. A 20 µL injection volume was employed for both samples and standards. The total analysis time per injection was 70 min followed by a 10 min re-equilibration period. Absorbance was monitored at 225 nm. At lower wavelengths the mobile phase itself showed significant absorbance and at higher wavelengths detector sensitivity for the majority of the pharmaceuticals was reduced.

## 3. Results and discussion

### 3.1. *Methanol gradient*

Using an initial mobile phase consisting of MeOH and water adjusted to pH 3.0 using formic acid, simple linear gradients were investigated. Initial experiments involved starting conditions of 40% MeOH that was held isocratically for 10 min and then raised to 70%. However, results showed that for polar analytes, such as trimethoprim, caffeine and paracetamol the initial percentage of MeOH in the mobile phase was too high, with subsequent poor retention. A number of peak pairs were also found to coelute, these included ketoprofen and clofibric acid, warfarin and bezafibrate and gemfibrozil and triclosan.

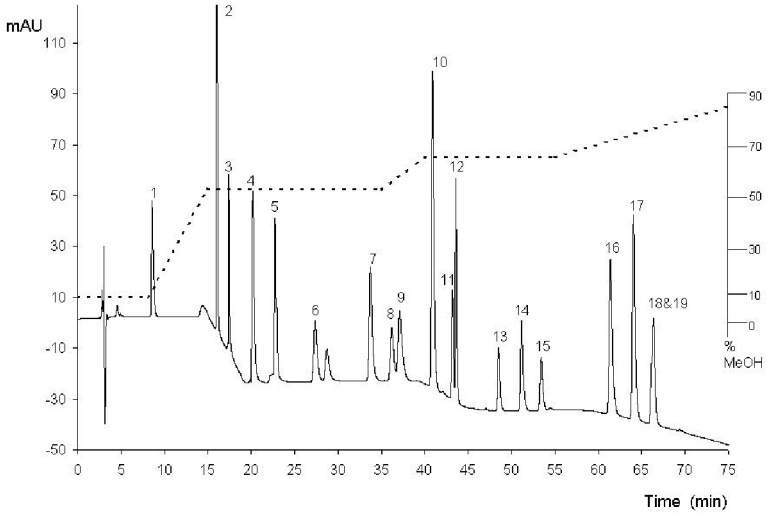



Figure 1. Optimised MeOH gradient separation of 19 pharmaceuticals on 20 cm reversed-phase silica monolithic column (pH 3.0 formic acid). Peak identification: 1. Paracetamol; 2. Trimethoprim; 3. Caffeine; 4. Salicylic acid; 5. *o*-Toluic acid; 6. Clotrimazole; 7. 2-Naphthoic acid (internal standard); 8. Ketoprofen; 9. Clofibric acid; 10. Naproxen; 11. Warfarin; 12. Bezafibrate; 13. Flurbiprofen; 14. Diclofenac; 15. Ibuprofen; 16. Meclofenamic acid; 17. Mefenamic acid; 18 and 19. Gemfibrozil and Triclosan.



Therefore, the starting percentage of methanol was reduced in order to promote retention of polar analytes. It was found that at 10% MeOH, polar analytes like paracetamol could be retained for ≈8 min. A series of individual linear gradients was then investigated to reduce the retention of all other less polar species and avoid the co-elution of the above mentioned peak pairs. To facilitate the complete elution and separation of all of the pharmaceuticals investigated, including ivermectin, the MeOH concentration had to be gradually increased over three individual linear gradients to >90% over a 75 min run.

Figure 1 depicts a 10 mg $L^{-1}$ standard chromatogram (excluding ivermectin) recorded under optimum conditions, a near complete separation of all analytes is observable with the exception of gemfibrozil and triclosan. The dashed line describes the percentage MeOH gradient used.

### 3.2. *pH and buffer strength*

Using the previously optimised organic modifier the pH and the buffer strength of the aqueous proportion of the mobile phase was systematically varied to evaluate selectivity effects.

Buffers considered included ammonium formate and ammonium acetate for compatibility with possible MS detection. Of the buffered phases ammonium acetate demonstrated considerable absorbance at 225 nm and caused baseline disruption over the course of the gradient. Ammonium formate demonstrated a considerably lower absorbance and although baseline disturbance was observed it was considerably less. Based on the above ammonium formate was chosen as the most suitable modifier.

For optimisation, an experimental space was designed, governed by the buffering activity of formate ($pK_a = 3.75$) and the desire to keep the ionic strength low enough

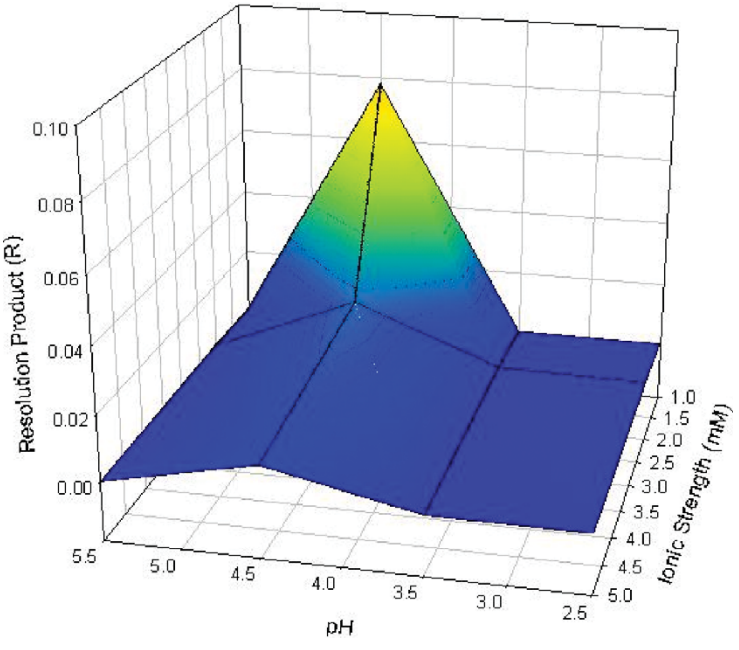



Figure 2. Resolution response surface for pH and buffer strength optimisation. Standard mixture as in figure 1. Other conditions as in figure 1.



to prevent salt precipitation at high proportions of organic solvent. Therefore, mobile phases containing ammonium formate concentrations of 1–5 mM, over the pH range of 2.5–5.5 were investigated. Twelve individual experiments were run within the above concentration and pH range. The resulting chromatograms were assessed using the resolution product criterion, $R$, calculated according to equation (1) [22].

$$R = \prod_{i=1}^{n-1} \left( \frac{R_s(i, i-1)}{(1/n-1)\sum R_s(i, i-1)} \right) \tag{1}$$

where, $n$ is the number of analytes present; $R_s(i, i-1)$ is the resolution between peaks $i$ and $i+1$. Calculated using,

$$R_s = \frac{2(Tr_{i+1} - Tr_i)}{W_{i+1} + W_i}$$

where, $Tr_{i+1}$ is the retention time of peak $i+1$; $Tr_i$ is the retention time of peak $i$; $W_{i+1}$ is the baseline width of peak $i+1$; $W_i$ is the baseline width of peak $i$; $R$ has values in the range of $0 < R < 1$. A value of 1 indicates that the resolution between all peaks is evenly distributed over the span of the separation and conversely a value of 0 indicates co-elution of two or more peaks somewhere within the chromatogram.

From the calculated $R$-values a response surface was constructed, which is shown in figure 2. The optimum combination of pH and buffer strength was determined from the apex of the response surface within the experimental space.

The response curve depicts a mobile phase condition where all analytes were optimally resolved, this being 1 mM ammonium formate at pH 4.5. Figure 3(a) depicts a standard chromatogram recorded under the optimised pH and buffer strength.

A complete separation of all chosen analytes can be observed in the optimised chromatogram, including the previously co-eluting peak pair. It is also notable that a change in the retention order of certain basic compounds is also observable, for example clotrimazole is now retained for ≈58 min as opposed to ≈28 min at pH 3.0, and increase in retention of some 30 min due to the higher pH of the mobile phase, attributable to the deprotonation of the nitrogen atoms of the imidazole group leading to a neutral moiety as opposed to the cationic moiety that exists at the lower pH.

### 3.3. *Combined solvent and flow gradients*

Although a complete separation of all pharmaceuticals in the optimisation standard is shown in figure 3(a), the overall analysis time is quite long at 90 min per injection (including re-equilibrium time). The high permeability of the porous monolithic columns is ideally suited for use at elevated flow rates. In this case operation under constant (isofluentic) elevated flow conditions were not suitable due to the desire to maintain resolution of early eluting peaks. Therefore, an approach first used by Paci *et al*. was investigated [23]. Paci *et al*. utilised a flow gradient combined with an elution gradient to reduce the retention of the anti-malarial agents chloroquine and proguanil on a $250 \times 4$ mmID particulate $C_{18}$ column. However due to the pressure constraints of the particulate packed column used in the study, the flow could only be increased by $0.3\,mL\,min^{-1}$. More recently, Paull *et al*. [24] reported more significant reductions in analysis times, together with increased efficiency, when a 'dual gradient' was applied to the separation of UV absorbing anions on short monolithic column coated with a zwitterionic reagent. In this case flow gradients from 1.0 to $6.0\,mL\,min^{-1}$ were applied.

A number of flow gradient programs were investigated in combination with the optimised mobile phase gradient. Comparison of resultant separations showed that a continual linear flow gradient from 1 to $3\,mL\,min^{-1}$ over the course of the run provided the best distribution of peaks across the entire chromatogram (with no detrimental effect upon resolution), combined with an approximate reduction in analysis time of 40–45%. Figure 3(b) depicts a standard chromatogram obtained by separation using the dual gradient. Although not shown in figure 3(b), the dual gradient approach also allowed for the inclusion of ivermectin to the test mixture, which eluted after 65 min. Ivermectin is moderately lipophilic but it was found that an extremely high quantity of methanol was required to elute it from the column under normal conditions.

### 3.4. *Detection limits and method validation*

Having developed and optimised the dual gradient separation the performance of the method was then investigated. The method was validated for linearity, reproducibility and repeatability and the instrumental limits of detection (LOD) and quantitation (LOQ) were calculated. The results for the validated parameters are presented in table 3.

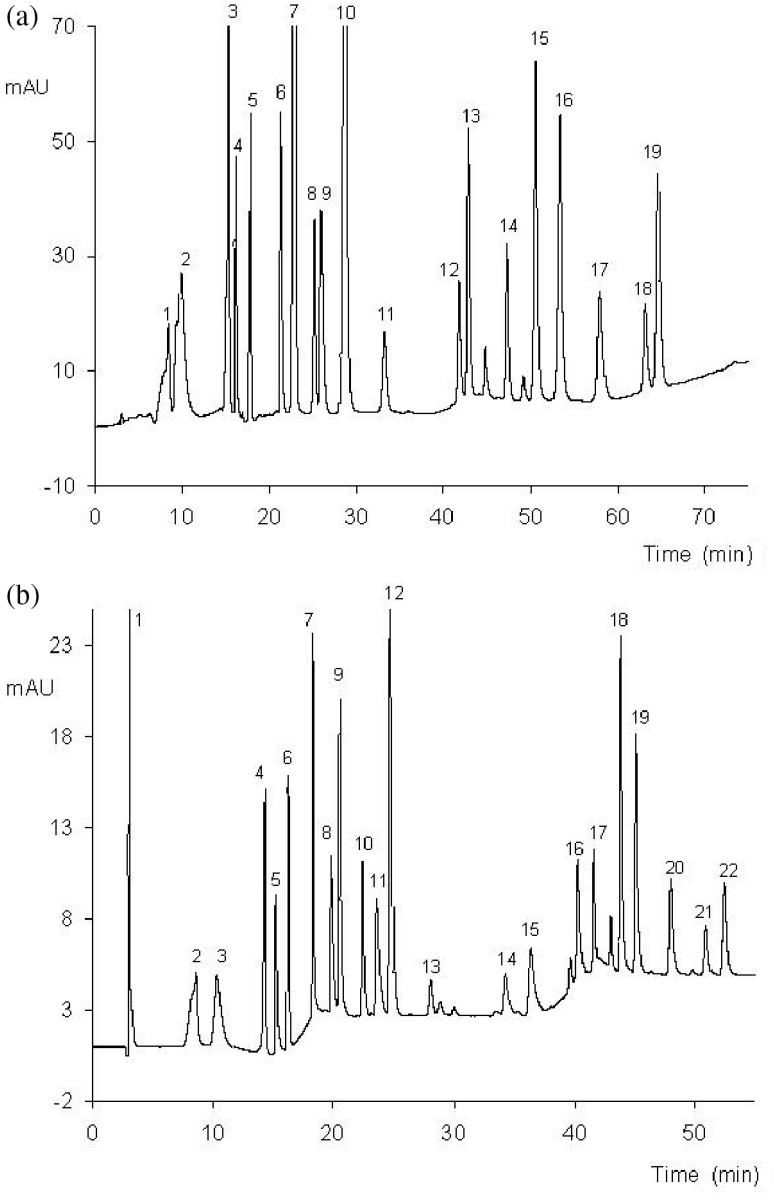



Figure 3. (a) Optimised MeOH gradient separation of 19 pharmaceuticals on 20 cm reversed-phase silica monolithic column (pH 4.5, 1 mM ammonium formate). Peak identification: 1. Paracetamol; 2. Salicylic acid; 3. Trimethoprim; 4. Caffeine; 5. *o*-Toluic acid; 6. Clofibric acid; 7. 2-Naphthoic acid (internal standard); 8. Ketoprofen; 9. Bezafibrate; 10. Naproxen; 11. Warfarin; 12. Flurbiprofen; 13. Diclofenac; 14. Ibuprofen; 15. Meclofenamic acid; 16. Mefenamic acid; 17. Clotrimazole; 18. Gemfibrozil; 19. Triclosan. (b) As in figure 3(a) with applied linear flow gradient from 1 mL $min^{-1}$ at $t = 0$ to 3 mL $min^{-1}$ at $t = 75$. Peak identification: 1. Metformin; 2. Paracetamol; 3. Salicylic acid; 4. Trimethoprim; 5. Caffeine; 6. *o*-Toluic acid; 7. Propranolol; 8. Clofibric acid; 9. 2-Naphthoic acid (internal standard); 10. Ketoprofen; 11. Bezafibrate; 12. Naproxen; 13. Warfarin; 14. Flurbiprofen; 15. Diclofenac; 16. Indomethacin; 17. Ibuprofen; 18. Meclofenamic acid; 19. Mefenamic acid; 20. Clotrimazole; 21. Gemfibrozil; 22. Triclosan.

Table 3. LC Method performance data.

| Analyte | Linearity ($R^2$) | LOD (mg $L^{-1}$) | LOQ (mg $L^{-1}$) | Reproducibility (% RSD) | Repeatability (% RSD) |
|---|---|---|---|---|---|
| Metformin | 0.9999 | 0.0 | 0.07 | 0.4 | 2.1 |
| Paracetamol | 0.9967 | 0.14 | 0.46 | 9.3 | 5.3 |
| Salicylic acid | 0.9999 | 0.22 | 0.72 | 2.4 | 7.8 |
| Trimethoprim | 0.9999 | 0.02 | 0.07 | 0.4 | 1.3 |
| Caffeine | 0.9982 | 0.09 | 0.29 | 1.9 | 3.4 |
| *o*-Toluic acid | 0.9999 | 0.06 | 0.19 | 0.8 | 1.0 |
| Propranolol | 0.9973 | 0.03 | 0.10 | 2.3 | 1.4 |
| Clofibric acid | 0.9955 | 0.02 | 0.06 | 3.3 | 3.6 |
| 2-Naphthoic acid (I.S.) | – | – | – | – | – |
| Ketoprofen | 0.9931 | 0.08 | 0.26 | 4.2 | 7.0 |
| Bezafibrate | 0.9954 | 0.06 | 0.21 | 1.1 | 7.2 |
| Naproxen | 0.9999 | 0.01 | 0.04 | 0.4 | 1.5 |
| Warfarin | 0.9999 | 0.24 | 0.81 | 7.5 | 10.2 |
| Flurbiprofen | 0.9999 | 0.24 | 0.81 | 0.9 | 11.1 |
| Diclofenac | 0.9998 | 0.15 | 0.49 | 9.7 | 15.1 |
| Indomethacin | 0.9997 | 0.10 | 0.33 | 2.0 | 9.3 |
| Ibuprofen | 0.9999 | 0.13 | 0.42 | 5.9 | 7.9 |
| Meclofenamic acid | 0.9999 | 0.04 | 0.13 | 0.6 | 2.5 |
| Mefenamic acid | 0.9999 | 0.05 | 0.15 | 2.1 | 3.7 |
| Clotrimazole | 0.9998 | 0.11 | 0.37 | 1.8 | 7.1 |
| Gemfibrozil | 0.9999 | 0.25 | 0.84 | 1.6 | 2.2 |
| Triclosan | 0.9999 | 0.12 | 0.40 | 2.7 | 3.7 |
| Ivermectin | 0.9996 | 0.12 | 0.04 | 4.2 | 3.5 |



For such a varied mixture of target analytes detector wavelength is obviously a key factor in relation to assessing method performance data. Absorbance spectra for all of the target species were taken and signal-to-noise data evaluated, taking into account the absorbance of the mobile phase itself, which was significant below 215 nm. Absorbance readings at 225 nm proved an acceptable compromise as most target species contained an aromatic moiety. A minority of target species showed slightly increased absorbance above 225 nm, namely flurbiprofen and bezafibrate. The use of wavelength programming was not seen as a viable approach to the above variation in absorbance spectra due to the large number of closely eluting target analytes and the significant baseline disturbances that result from using such an approach.

The use of a diode array detector to monitor all wavelengths simultaneously and obtain individual peak spectra was briefly investigated, but it was found that although some specific peak spectra could be identified in 1 μg $L^{-1}$ spiked samples, generally the detector was less sensitive and baseline disturbances more significant.

Linearity was determined at five individual concentration levels within the range of 0.1–20 mg $L^{-1}$. A linear response was observed for each analytes as demonstrated by the correlation coefficients that were all greater than $R^2 = 0.99$. The limit of detection was defined as a signal three times the standard deviation of the baseline noise and the limit of quantitation was defined as 10 times the standard deviation of the baseline noise. Using the ChemStation software the 'peak-to-peak' baseline noise was measured for a triplicate blank injection (HPLC grade methanol) over a window of 20 times the peak width either side of each peak. The peak heights of a combined 0.5 mg $L^{-1}$ standard were measured and from these measurements the LOD and LOQ for each analyte were determined. The reproducibility of the method was determined by performing six replicate injections of the same standard at the LOQ level using the same mobile phase.

Reproducibility is acceptably low for most analytes, generally lower than 5% RSD. Repeatability was determined by the injection of six individual standards at the LOQ level using six fresh preparations of mobile phase for the separation. The calculated repeatability values are also quite low, >10% RSD for all analytes with the exception of flurbiprofen and diclofenac.

Paci *et al.* [23] acknowledged that dual gradient separations suffer from lower precision due to the more complex dynamic processes occurring during the separation, however in this study the observed reproducibility and repeatability values were within acceptable limits.

### 3.5. *Solid phase extraction*

**3.5.1. Sorbent selection.** The chosen group of pharmaceuticals displayed a range of chemical properties, ranging from very hydrophilic to hydrophobic, and acidic to basic. Due to these distinct differences the selection of an extraction sorbent that showed good recoveries for all species was not trivial. Sorbents examined included those based upon standard octadecyl silica, hyper cross-linked styrene divinyl benzene, hydrophilic lipophilic balanced polymers and mixed functionality reversed-phase anion exchangers. Five commercial SPE phases were compared and evaluated using recovery performance data for a $5\,\mu g\,L^{-1}$ test mix from a 'clean' Milli-Q water matrix. The resultant data is presented in table 4.

From initial studies the hydrophilic lipophilic balanced sorbents, i.e. Waters Oasis HLB and Phenomenex Strata-X showed the greatest promise as relatively high recovery was observed across the range with these sorbents. The $C_{18}$ sorbent demonstrated poor ability to extract and retain the more polar analytes. Recovery was generally good using the Merck LiChrolut EN sorbent, however indomethacin was completely unretained on



Table 4. Sorbent selection performance data (calculated percentage recoveries from spiked Milli-Q water solution).

| Analyte | Phenomenex C18e | Phenomenex Strata-X | Merck LiChrolut EN | Waters Oasis HLB | Waters Oasis MAX |
|---|---|---|---|---|---|
| Paracetamol | – | 11 | 76 | 18 | 15 |
| Salicylic acid | – | 79 | 49 | 70 | 58 |
| Trimethoprim | 20 | 98 | 44 | 98 | 65 |
| Caffeine | 14 | 90 | 82 | 76 | 289 |
| *o*-Toluic acid (Surrogate 1) | 6 | 62 | 53 | 57 | 47 |
| Propranolol | 13 | 78 | 36 | 72 | 54 |
| Clofibric acid | 66 | 89 | 83 | 77 | 65 |
| 2-Naphthoic acid (I.S.) | – | – | – | – | – |
| Ketoprofen | 71 | 85 | 77 | 78 | 23 |
| Bezafibrate | 71 | 86 | 81 | 78 | 46 |
| Naproxen | 66 | 83 | 76 | 78 | 22 |
| Warfarin | 74 | 89 | 95 | 90 | 27 |
| Flurbiprofen | 83 | 91 | 85 | 90 | 45 |
| Diclofenac | 62 | 75 | 80 | 72 | 15 |
| Indomethacin | – | 61 | – | 56 | 238 |
| Ibuprofen | 69 | 88 | 69 | 86 | 38 |
| Meclofenamic acid (Surrogate 2) | 72 | 85 | 83 | 75 | 65 |
| Mefenamic acid | 45 | 78 | 74 | 76 | 63 |
| Clotrimazole | – | 73 | 51 | 60 | 31 |
| Gemfibrozil | 76 | 93 | 100 | 86 | 70 |
| Triclosan | 66 | 79 | 79 | 75 | 45 |

the styrene divinylbenzene resin, although it was retained on all of the other polymeric sorbents investigated. As the majority of the pharmaceuticals was acidic, a mixed functionality reversed-phase anion exchange sorbent, Oasis MAX was investigated. The extraction was performed at pH 6.0 so as to promote the dissociation of carboxyl functionalities. Recovery for acidic analytes was low, generally $<50\%$ and recovery of basic and neutral analytes was also lower than that calculated from the reversed-phase sorbents, suggesting an ion exclusion based upon electrostatic repulsion.

As the Oasis HLB and the Phenomenex Strata-X were chemically similar and share common retention chemistry, a *t*-test was performed using the calculated percentage recovery values for a $5\,\mu g\,L^{-1}$ spike standard to determine whether the two sorbents were significantly different. It was discovered that at the 95% confidence interval there was no significant difference between the two sorbents ($t$-experimental $=$ $0.31 < t$-critical $= 2.03$, two tailed assuming unequal variances).

**3.5.2. Extraction pH.** A subset of six analytes was chosen to optimise extraction pH. The selected analytes were salicylic acid, clofibric acid, ketoprofen, diclofenac, ibuprofen and mefenamic acid. These solutes were selected as they span across the chromatographic separation. Buffer solutions were prepared within the pH range of 2–8 and spiked with $5\,\mu g\,L^{-1}$ of each. Samples were extracted using the Oasis HLB cartridges and eluted with 10 mL methanol, reduced in volume, reconstituted and analysed using the HPLC method. It was observed that an extraction pH of 4.0 provided optimum analyte recovery for all compounds.



**3.5.3. Elution solvent.** Within the literature many SPE procedures recommend elution with methanol. However methanol is difficult to reduce under $N_2$ without significant sample loss and so alternative solvents were investigated. These included acetone, acetonitrile, ethyl acetate, isopropyl alcohol (IPA), 50% v/v ethyl acetate in acetone [20], and 10% v/v methanol in methyl *t*-butyl ether (MtBE) [25]. Using the Oasis HLB sorbent, a $5\,\mu g\,L^{-1}$ mixed standard was extracted and eluted using each of the above solvents and solvent mixtures. Recovery from cartridges eluted with ethyl acetate and acetonitrile were unacceptably low, $<50\%$ for the test solutes. Recovery was in the range of 70–90% with IPA and acetone. For the mixed solvents, recovery was $\approx 50\%$ or less for cartridges eluted with 10% v/v methanol in MtBE, however 50% v/v ethyl acetate in acetone provided near quantitative recovery for all analytes, $>90\%$. Of all the solvents examined the 50% v/v ethyl acetate in acetone mixture provided the highest recoveries (due to increased solvent strength) and it is also easily reduced under $N_2$ with minimal splashing and sample loss. The recovery of salicylic acid was low in all extraction experiments, however it is the most polar of the compounds examined ($pK_a \approx 3.0$). At this stage in the study it was noted that during the SPE procedural development there was significant batch-to-batch variability with the Oasis HLB sorbent, reflected in some cases by large reductions in solute recovery. Such batch variability was not apparent with the Phenomenex Strata-X phase and so these cartridges were preferred in further sample extractions.

**3.5.4. Elution volume.** The optimum volume of elution was determined by eluting an extracted $5\,\mu g\,L^{-1}$ standard with ten 2 mL portions of 50% v/v ethyl acetate in acetone.

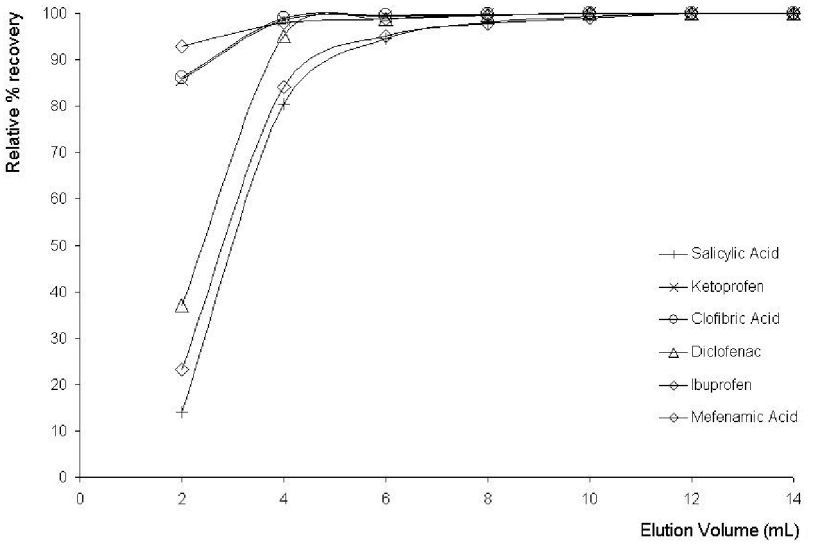



Figure 4. Plot of percentage relative recovery *vs*. SPE elution volume using Strata-X SPE cartridges and elution with 50% ethyl acetate in acetone.



Each 2 mL fraction was collected, reduced in volume and reconstituted in 100 µL of methanol. The relative recovery of each analyte was determined and plotted against the elution volume as in figure 4. It was observed that 10 mL of elution solvent was sufficient to completely elute all compounds from the sorbent bed. It was also observed that as expected the more polar the solute the less solvent was required for complete elution. The high recovery data shown for warfarin and diclofenac obviously reflect the higher than average recovery precision for these species, but still fall within the acceptable range of 70–130% normally adopted by the USEPA for methods requiring some form of analyte preconcentration.

**3.5.5. Surrogate performance.** Two surrogate standards were also included into the method at a concentration level of $2\,\mu g\,L^{-1}$ to assess the extraction procedure. The recovery of *o*-toluic acid is quite low and outside the acceptable limits at 56%, while the recovery of meclofenamic acid is more acceptable at 81%. *o*-Toluic acid is the more polar of the pair and although it is not in itself a pharmaceutical compound, it was chosen as it shares a common functionality with many of the more polar pharmaceuticals. Meclofenamic acid was previously used as a surrogate standard by Clara *et al*. [26].

Using the optimised parameters, a $1\,\mu g\,L^{-1}$ standard prepared in Milli-Q water was extracted using the Phenomenex Strata-X sorbents and recovery data is presented in table 5. The determined recovery data from the Strata-X sorbent was generally in the acceptable range of 70–130% and for many analytes is near quantitative. As expected from the LC method development, the water solubility of the analytes limits its retention on the polymeric sorbent, i.e. the more hydrophilic the analyte the less the retention. This fact is clearly demonstrated by metformin that shows little retention.

Table 5. SPE percentage recovery of mixed standard in Milli-Q water ($1\,\mu g\,L^{-1}$) using Strata-X (200 mg/6 mL/33 μm), $n = 3$.

| Analyte | Percentage recovery |
|---|---|
| Metformin | 18 ± 4 |
| Paracetamol | 48 ± 6 |
| Salicylic acid | 75 ± 3 |
| Trimethoprim | 92 ± 25 |
| Caffeine | 73 ± 5 |
| *o*-Toluic acid (Surrogate 1) | 56 ± 1 |
| Propranolol | 39 ±1 |
| Clofibric acid | 79 ± 5 |
| 2-Naphthoic acid (I.S.) | – |
| Ketoprofen | 93 ± 4 |
| Bezafibrate | 97 ± 1 |
| Naproxen | 98 ± 2 |
| Warfarin | 117 ± 19 |
| Flurbiprofen | 94 ± 12 |
| Diclofenac | 110 ± 18 |
| Indomethacin | 82 ± 7 |
| Ibuprofen | 81 ± 12 |
| Meclofenamic acid (Surrogate 2) | 81 ± 5 |
| Mefenamic acid | 102 ± 4 |
| Clotrimazole | 107 ± 11 |
| Gemfibrozil | 91 ± 3 |
| Triclosan | 105 ± 1 |
| Ivermectin | 46 ± 10 |



Theretention of basic analytes was generally lower than their acidic counterparts, which was due to the low pH at which the extraction was performed.

### 3.6. *Application to environmental samples*

Grab samples were collected in silanised amber Winchester bottles from areas in north Co. Dublin and Co. Meath, namely the Malahide Estuary (which receives treated effluent from sewage treatment plants in north Co. Dublin), in the vicinity of sewage sea outfalls near Howth and Portmarnock, and from the River Boyne downstream of the Navan Wastewater Treatment Centre in Co. Meath (>40,000 population equivalents). A sample of potable water from the laboratory was also analysed. Samples were filtered and extracted as previously described and analysed using the dual gradient method. A 1 L portion of each sample was also spiked with $1\,\mu g\,L^{-1}$ of each pharmaceutical and also extracted and analysed using the dual gradient method.

None of the chosen analytes were detected in any of the samples collected. However, the spiked samples showed very clear peaks for all of the pharmaceuticals added at the expected retention times. From analysis of the spiked chromatograms it was possible to recalculate LODs and LOQs based upon the use of the optimised extraction of 1 L of sample. This data is shown in table 6. As can be seen from the table shown, for the majority of target analytes the LODs ranged from between 10 and $50\,ng\,L^{-1}$ in the spiked samples, indicating the method is indeed practical for real sample screening purposes. Indeed, the percentage recovery data and LOD data compares favourably with recent studies on alternative pharmaceuticals analysed using SPE with LC [27–28], although in the work presented here, a significantly larger number of analytes can be monitored in a single run.

Table 6. Calculated LOD and LOQ values from spiked (1–2 μg $L^{-1}$) estuarine and river water samples (values in μg $L^{-1}$).

| | Estuarine/Sea water | | River water | |
|---|---|---|---|---|
| Analyte | LOD | LOQ | LOD | LOQ |
| Metformin | 0.35 | 1.17 | – | – |
| Paracetamol | 0.33 | 1.09 | – | – |
| Salicylic acid | 0.66 | 2.21 | 0.18 | 0.62 |
| Trimethoprim | 0.03 | 0.11 | – | – |
| Caffeine | 0.27 | 0.91 | – | – |
| *o*-Toluic acid | 0.13 | 0.44 | 0.17 | 0.58 |
| Propranolol | 0.04 | 0.13 | – | – |
| Clofibric acid | 0.12 | 0.41 | 0.07 | 0.23 |
| Ketoprofen | 0.16 | 0.53 | 0.11 | 0.38 |
| Bezafibrate | 0.13 | 0.45 | 0.08 | 0.28 |
| Naproxen | 0.02 | 0.08 | 0.02 | 0.06 |
| Warfarin | 0.73 | 2.42 | 0.55 | 1.85 |
| Flurbiprofen | 0.41 | 1.36 | 0.32 | 1.06 |
| Diclofenac | 0.29 | 0.96 | 0.18 | 0.62 |
| Indomethacin | 0.09 | 0.31 | 0.15 | 0.49 |
| Ibuprofen | 0.19 | 0.64 | 0.33 | 1.12 |
| Meclofenamic acid | 0.11 | 0.36 | 0.12 | 0.39 |
| Mefenamic acid | 0.11 | 0.38 | 0.12 | 0.39 |
| Clotrimazole | 0.24 | 0.80 | – | – |
| Gemfibrozil | 0.46 | 1.52 | 0.37 | 1.24 |
| Triclosan | 0.23 | 0.78 | 0.14 | 0.46 |
| Ivermectin | 0.32 | 1.08 | 0.43 | 1.42 |



Figure 5 depicts the resulting chromatogram of extracted spiked Malahide Estuary sample.

In addition to the above analysis, a large volume river water sample was collected and again spiked at the 1 μg $L^{-1}$ concentration. This sample was then divided into six sub-samples and each sub-sample subjected to the entire analytical method, including sample filtration, pH adjustment, extraction, elution, volume reduction, reconstitution and LC analysis. From the resultant six chromatograms, both recovery repeatability and overall method precision data were obtained for the river water sample. This data is shown in table 7.

## 4. Conclusions

A simple method has been developed that allows for the preconcentration and separation of >20 acidic and basic pharmaceuticals. Using monolithic columns a highly efficient separation was developed based upon a dual mobile phase and flow gradient approach. Solid phase extraction was applied to reduce detection limits to sub-μg $L^{-1}$ for the majority of analytes, making the method suitable for real sample screening. The method has been validated and applied to a variety of environmental waters. The method may become useful to those who wish to determine the selected pharmaceuticals in environmental samples but who do not have access to LC-MS instrumentation.

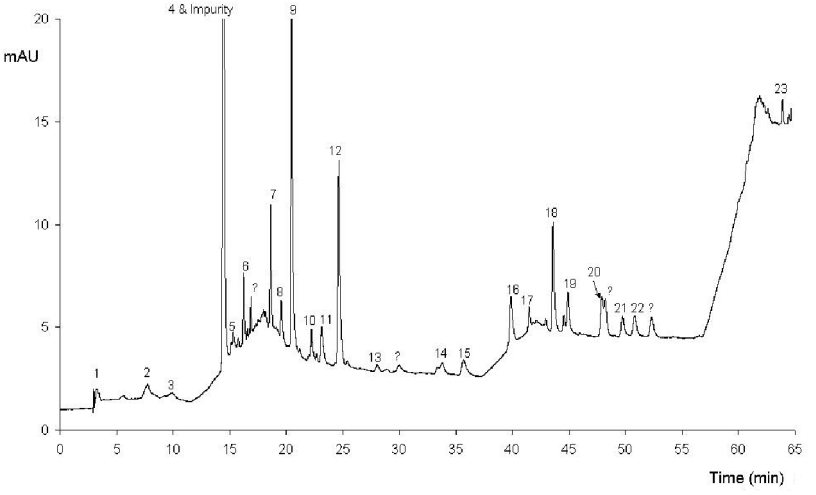



Figure 5. Chromatogram of the extracted $1\,\mu g\,L^{-1}$ spiked Malahide Estuary sample. Conditions are as in figure 3(b). Peak identification: 1. Metformin; 2. Paracetamol; 3. Salicylic acid; 4. Trimethoprim; 5. Caffeine; 6. *o*-Toluic acid; 7. Propranolol; 8. Clofibric acid; 9. 2-Naphthoic acid (internal standard); 10. Ketoprofen; 11. Bezafibrate; 12. Naproxen; 13. Warfarin; 14. Flurbiprofen; 15. Diclofenac; 16. Indomethacin*; 17. Ibuprofen; 18. Meclofenamic acid; 19. Mefenamic acid; 20. Clotrimazole*; 21. Gemfibrozil*; 22. Triclosan; 23. Ivermectin* (*Actual spiking level: $2\,\mu g\,L^{-1}$).



Table 7. Percentage RSD data for recovery repeatability and overall method precision for spiked river water samples (spiking level was $1\,\mu g\,L^{-1}$).

| Analyte | Recovery repeatability ($n=6$) | Method precision ($n=6$) |
|---|---|---|
| Metformin | – | – |
| Paracetamol | – | – |
| Salicylic Acid | $67 \pm 20$ | 29 |
| Trimethoprim | – | – |
| Caffeine | – | – |
| *o*-Toluic Acid | $45 \pm 12$ | 27 |
| Propranolol | – | – |
| Clofibric Acid | $77 \pm 18$ | 24 |
| Ketoprofen | $81 \pm 16$ | 19 |
| Bezafibrate | $67 \pm 13$ | 20 |
| Naproxen | $75 \pm 14$ | 18 |
| Warfarin | $97 \pm 18$ | 19 |
| Flurbiprofen | $77 \pm 16$ | 21 |
| Diclofenac | $73 \pm 15$ | 21 |
| Indomethacin* | $72 \pm 15$ | 21 |
| Ibuprofen | $92 \pm 17$ | 19 |
| Meclofenamic acid | $72 \pm 13$ | 18 |
| Mefenamic acid | $75 \pm 14$ | 18 |
| Clotrimazole* | – | – |
| Gemfibrozil* | $72 \pm 7$ | 10 |
| Triclosan | $104 \pm 24$ | 24 |
| Ivermectin* | $42 \pm 5$ | 13 |

Analytes marked with an asterisk were spiked at $2\,\mu g\,L^{-1}$.

## Acknowledgement

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