Advanced Separation Techniques in the Analysis of Environmental Pollutants

by

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Author's declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Recent developments in chromatographic supports and instrumentation for liquid chromatography are enabling rapid and highly efficient separations. Various analytical strategies have been recently proposed, for example the use of monolithic supports, elevated mobile phase temperatures, columns packed with sub-2 μm fully porous particles for use in ultra-high-pressure liquid chromatography (UHPLC) and superficially porous particles (fused core). All these approaches could be used to enhance the efficiency and shorten the analysis time.

In the presented work, a high efficiency HPLC method was proposed based on coupling three columns packed with fully porous sub-2 μm particles and operating them at high temperature to reduce the solvent viscosity, thus reducing the column backpressure. The developed method could increase the number of theoretical plates compared to a single column at 30 ºC. The approach of column coupling was applicable for both isocratic and gradient mode of separation.

Also, fast analysis methods were proposed based on using either a column packed with fully porous sub-2 μm particles and operated at high temperature or a column packed with superficially porous particles as a tool to increase the analysis speed. An ultra-fast green method was also proposed by using a short narrow bore column packed with fully porous particles.

In addition, the chromatographic performance of columns packed with fused-core particles was investigated and compared to that of fully porous particles (sub-2 µm) at elevated temperature and extended column lengths. The study involved a comparison of chromatographic parameters such as retention, selectivity, resolution, efficiency and pressure drop. This study demonstrated that the fused-core particles can produce key advantages over the sub-2 µm particle columns in terms of separation speed, resolution and efficiency.

All the developed methods were validated and applied to the analysis of environmental pollutants in surface water and/or waste water. The fast and efficient methods developed could be used as an alternative to the traditional ones for the environmental analysis of many pollutants.

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Chapter 1

Introduction

Chromatography was invented by Russian botanist Michael Tswett (1872-1919) during his work on the separation of colored plant pigments into bands on a column packed with calcium carbonate. A century later, chromatography is being applied in many different ways.

Chromatography can be defined as a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary while the other moves in a definite direction.[1] The goal of this technique is to obtain quantitative or/and qualitative chemical information about mixtures of interest by separating and detecting their components. Liquid chromatography is a separation method in which a mixture is applied initially as a narrow zone to a stationary phase (typically a sorbent), and the components undergo differential migration by the flow of a liquid mobile phase.

High performance liquid chromatography (HPLC) is a well-established separation technique that can be used to solve numerous analytical problems. HPLC offers many features like robustness, ease of operation, well-understood separation principles, sensitivity and tunable selectivity. However, the number of components that can be separated in a single separation (peak capacity) is limited by the peak width, which in turn depends on the efficiency of the column. The limited efficiency in HPLC originates from the small diffusion coefficients of the analytes in the liquid phase leading to slow diffusion speed into the stationary phase.[2] HPLC has wide applications in the pharmaceutical, environmental, forensic, clinical, food

and fragrance sectors. HPLC is the most reliable method for analyzing non-volatile organic compounds.

High-throughput separations (fast analysis) are in great demand in many fields, such as clinical, forensics, toxicology, environmental and pharmaceutical analyses.[3] On the other hand, highly efficient separations are necessary for many applications, including metabolomics, proteomics and genomics. Very complex samples, such as biological materials, tryptic digests or natural plant extracts require highly efficient and fast analytical procedures to yield high resolution within an acceptable analysis time. The growing demand to enhance efficiency and reduce analysis speed directed many researchers to develop innovations in the traditional LC systems.

1.1 Recent trends in fast liquid chromatography

The reduction in LC method run time using fast analysis results in reduction in the overall solvent consumption and lowering costs. These improvements are advantageous for laboratories in terms of productivity enhancement and economic savings. Recently, manufacturers introduced new stationary phases, new column geometries and new instruments in order to reduce analysis times while maintaining high resolution and efficiency. Various analytical strategies have been proposed to increase the analysis speed. These strategies include making a single or multiple change (s) to the following parameters: reducing column length, increasing flow rate, decreasing column particle size, increasing pressure or increasing temperature. In fast LC method development, all factors are usually

not changed simultaneously, but step-by-step to optimize the separation to reduce the analysis time without losing resolution.

Gritti and Guiochon presented different possible approaches to achieving fast LC separations while maintaining high resolution and efficiency.[4] The first one is increasing the linear velocity of the mobile phase, which can be accomplished by operating the column under a higher inlet pressure or by decreasing the viscosity of the eluent. Solvent viscosity can be reduced by operating the column at elevated temperature, in which case the diffusion coefficients of the solutes increase due to reduction in viscosity, leading to faster mass transfer.[4] Another approach to increasing the mobile phase velocity is to increase column permeability, so that higher flow rates could be used with the same inlet pressure. This could be done by using monolithic columns. An alternative approach to speeding up the analysis without efficiency loss is to reduce the column length while decreasing the plate height. This can be accomplished by using smaller packing particles (e.g. sub-2 μm); however, in this case column permeability is decreased and very high pressures are needed. Finally, analysis time can be shortened by combining the increase in column permeability with an increase in efficiency, which could be accomplished by using sub-3 μm superficially porous particles.[4] These different approaches will be discussed in more details in the following section.

1.1.1 Monolithic supports

Monolithic supports consist of a single rod of porous material with several unique features in terms of permeability and efficiency. These columns can be used at high mobile phase velocities due to decreased flow resistance and improved mass transfer compared to

conventional fully porous particle-packed columns.[5, 6] The higher performance at high flow rates is due to the small size of the silica skeleton. The contribution of the stationary phase mass transfer term is consequently much lower compared with a particle-packed column. The high porosity and permeability, as well as small skeleton size of monolithic columns permit operation at high flow rates with low backpressure, which allows fast separations using conventional LC systems.[7] Unfortunately, the promise of this column technology has not been fulfilled thus far, mainly due to the fact that monolithic silica rods are not radially homogeneous, and their external porosity is smaller in the center than close to the wall, leading to poor efficiency.[8] In addition, the distribution of the injected sample at the column inlet and its collection at the column outlet may cause band broadening, leading to a loss of column efficiency.[8] Other drawbacks of this technology are the limited commercial availability of different stationary phases and often low retention.[7]

1.1.2 Ultra-high pressure liquid chromatography (UHPLC) and fully porous sub-2 µm particles

The second leap forward in column technology is the introduction of fully porous sub-2 µm particles. Columns packed with these small particles could improve the chromatographic performance and induce a simultaneous improvement in efficiency, optimal velocity and mass transfer.[9, 10]

These small particles can be used to reduce the analysis time since the optimal flow rate is inversely proportional to particle diameter according to the equation:[11]

$$\mu = \nu D_m / d_p \qquad (1)$$

(where ν is the reduced mobile phase velocity, μ is the optimal velocity and D_m the diffusion coefficient of the solute in the mobile phase). Smaller particles produce flatter van Deemter curves (see Section 1.1.3.2.2), as a result of which higher flow rates can be used without a significant loss in efficiency.

The main limitation of using sub-2 μm particles is the induction of high backpressure across the column. According to Darcy's law, the pressure is directly proportional to the column length and inversely proportional to the particle size at the optimum linear velocity:[12]

$$\Delta P = \mu L \eta \phi / d_p^2 \qquad (2)$$

Where ΔP is the pressure drop, η is the viscosity of the solvent and ϕ is the flow resistance.[13] Decreasing particle size results in reduced column permeability, hence the column backpressure increases.[4] The high backpressure produced by these particles hinders their usage with conventional LC systems. Consequently, improvements to the chromatographic systems have been made to overcome this problem. Recently introduced ultra-high pressure liquid chromatography (UHPLC) systems that can withstand pressures up to (and even exceeding) 1000 bar have been commercialized. The term UHPLC is used to define the use of columns packed with sub-2 μm particles at pressures exceeding 400 bar.[3] These systems provide many benefits by allowing the use of small particle size packings at high flow rates to speed up the analysis. Also, longer columns or coupled columns packed with small particles can be used to increase efficiency. The main drawback of UHPLC is the high cost related to the need for a dedicated system with high pressure pumps and injector, and high data acquisition rate of the detector.[3] In addition, frictional heating generated at the very high

pressure produces temperature gradients inside the column. This problem is pronounced for 4.6 mm I.D. columns when the pressure is close to or higher than 1,000 bar. It can be resolved by reducing the column I.D. to 2.1 or 1 mm.[3] In terms of analysis throughput, the benefits of the UHPLC approach are well documented in the literature.[14-16]

1.1.3 High-temperature liquid chromatography (HTLC)

An alternative option to overcome the high backpressure generated by sub-2-µm particles is the use of high mobile phase temperature. High-temperature liquid chromatography (HTLC) is considered a valuable technique in reversed phase liquid chromatography (RP-LC). Temperature can play a role in reducing analysis time, modifying retention and changing selectivity of the chromatographic separation.

HTLC is LC analysis performed at temperatures above ambient and below supercritical.[17] By working at higher temperatures, it is possible to improve the analysis conditions because some physical parameters playing an important role in HPLC, such as viscosity, mobile phase polarity or diffusivity depend strongly on temperature.[18]

1.1.3.1 Advantages of using high temperatures in LC

Recent interest in using temperature as a variable in LC is due to a number of significant advantages including:

(1) Increasing the analysis speed

Researchers are still very interested in finding more efficient ways to increase the analysis speed in order to improve the sample throughput for routine LC analysis. The use of ambient

operating temperature in conventional RP-LC systems with higher flow rates is a direct way to reduce analysis time. This approach is not recommended because the applicability of high flow rates is limited by the pressures that different parts of the chromatographic system, including pump, injector and column, can withstand. In addition, high flow rates in a conventional RP-LC system can cause a significant loss in resolution and shorten the column lifetime. Elevated column temperature can be used as a tool to overcome the flow rate problem associated with high back pressure, allowing the use of flow rates that otherwise could not be applied.[19] The possibility of increasing the analysis speed due to higher linear velocities is achieved as a result of the decrease in viscosity of the mobile phase with temperature. The relationship between the viscosity and temperature is shown in equation (3):[19]

$$ln\,\eta = a + b/T \qquad (3)$$

where a and b are empirically determined constants, η is the solvent viscosity and T is the absolute temperature (in K). Increasing mobile phase temperature leads to a decrease in solvent viscosity, which allows the use of higher flow rates to reduce the analysis time. As a result of lower viscosity, the backpressure is decreased according to Darcy's law. Decreasing the backpressure allows the use of smaller packing particles, such as sub-2 µm, without over-pressuring the pump. Reduction of mobile phase viscosity is also advantageous for increasing the column length through coupling to increase the number of theoretical plates, thus enhancing the efficiency.

Antia and Horvath showed that the effect of temperature on both viscosity and diffusivity allows the use of higher mobile phase linear velocities leading to faster analysis.[20] The relationship between the diffusion coefficient, the solvent viscosity and the absolute temperature can be expressed by the Wilke-Chang equation (4)[20]:

$$D_m = 7.4 \times 10^{-2} \frac{(\emptyset M)^{1/2} T}{\eta V_a{}^{0.6}} \quad (4)$$

where D_m is the solute diffusion coefficient, M is the solvent molecular weight, T is the absolute temperature (in K), η is the solvent viscosity, V_a is the solute molar volume and $\emptyset$ is the solvent association factor.

According to Wilke-Chang equation, the diffusion coefficient is directly proportional to temperature and inversely proportional to viscosity. Consequently, by increasing the temperature, the diffusion of analytes in the mobile and stationary phase will be increased. This effect is enhanced because viscosity is also a strong function of temperature. Thus, the reduction of the mobile phase viscosity with temperature leads to an increase in the diffusion coefficient of analytes in the solvent. Therefore, the minimum of plate height (H) in the van Deemter curve shifts to higher linear velocities allowing the use of high flow rates without compromising the efficiency.[17]

(2) Changing the amount or the type of modifier in the eluent making the separation greener

Temperature can be used to change the amount or type of the modifier in the eluent. Due to reduction in the backpressure at high temperature, acetonitrile and methanol can be replaced

with the more viscous and the less toxic ethanol. This can be used to optimize resolution and make the separation greener. Also, increasing the analysis speed at elevated temperature allows the decrease in the consumption of organic solvents. At high temperature, the eluotropic strength of water in RP- LC increases, so the amount of organic modifier used in HPLC can be greatly reduced or even the need for it can be completely eliminated. This helps reduce the analysis costs, especially in toxic waste disposal.[17]

(3) Using pure water as mobile phase in RP-LC

The use of pure water as an eluent has been recently studied by a number of researchers. This approach is known as "superheated water chromatography", "subcritical water chromatography" or "chromatography in very hot water".[20] At elevated temperatures, water becomes a stronger solvent in RP-LC because its dielectric constant decreases at 150 ºC to that of a mixture of 50:50 v/v methanol–water at ambient temperature, therefore increasing the solvent strength in RP-LC.[17] Elimination of organic modifier and using pure water as an eluent allow LC separations to be greener. In addition to its attractive advantage of non-toxicity, using pure water in LC offers the possibility of hyphenation with special detectors such as flame ionization detector (FID).[20] Also, UV detection can be carried out at very short wavelengths (~190 nm), allowing the detection of many species that do not absorb at longer wavelengths.[17]

(4) Compatibility with MS detectors

Heated effluents are more compatible with MS detectors and have shown to increase the ionization efficiency and signal to noise ratio (S/N) for some compounds.[17]

Due to the attractive advantages of elevated temperature, many researchers use high temperature as a variable in LC separations.[21-24]

1.1.3.2 Theoretical considerations of temperature in HTLC

1.1.3.2.1 Influence of temperature on retention

The effect of temperature on retention is given by the van't Hoff equation (5)[25]

$$ln\ k = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + ln\ \beta \qquad (5)$$

Where ΔH is the enthalpy of transfer of the solute from the mobile into the stationary phase, ΔS is the entropy of transfer of the solute from the mobile into the stationary phase, R is the gas constant and β is the volume phase ratio of the stationary and mobile phases. Theoretically, a plot of the natural logarithm of *k* against the inverse absolute temperature (*ln k* vs. 1/T in Kelvin) yields a straight line. The enthalpy of transfer may be estimated from the slope of the resulting line, whereas the intercept yields the entropy of transfer of the solute from the mobile to the stationary phase.[25] Changes in retention by varying temperature can be caused either by changes in enthalpy or in entropy.

Commonly, van't Hoff plots are linear and in most cases retention decreases with an increase in temperature.[17] For example, linear van't Hoff plots were observed for analytes such as alkylbenzenes in a temperature range from 40 to 100 °C[26], phenolic compounds in a temperature range of 100 to 150 °C using superheated water as the mobile phase[27], or substituted anilines on a C18 hybrid stationary phase in the temperature interval between 150 and 200 °C[28]. The data showed that linear van't Hoff plots can be obtained if non polar

analytes are involved and only one mechanism governs the retention, such as pure hydrophobic interactions.[25]

Deviations from the linear behavior of van't Hoff plots have recently been reviewed. Several researchers demonstrated that temperature has a marked effect on the pH of the mobile phase and pK_a of the analytes.[25] Castells and co-workers [29]showed that the retention of ionic compounds can be influenced by changing the temperature leading to a shift in the pH of the mobile phase. McCalley and co-workers demonstrated that for bases such as amitriptyline, benzylamine, nortryptiline and quinine, retention increased with increasing temperature. This could be due to temperature-dependent pK_a shifts of the solutes. Also, unusual retention behavior was observed for protryptiline, whose retention decreased at temperatures between 30 and 45 °C and then increased at higher temperatures. This finding could be explained by the change of the pH of the mobile phase, as well as the change of the effective degree of ionization of the analyte due to a shift of its pK_a.[25]

Other researchers demonstrated that proteins and polypeptides show highly different retention behaviors as a function of temperature compared to small solutes, and in some cases undergo an increase in retention as the temperature is elevated.[17] This could be explained by the changes in the three dimensional structures of the protein and the displacement of solvent molecules at its surface at elevated temperature, so both ΔH and ΔS may increase giving non-linear plots.[30]

Many researchers observed that a temperature increase of 4–5 °C had roughly the same effect on retention as a 1% increase in organic solvent concentration.[25] All these observations show

that deviations from the linear behavior of van't Hoff plots could be due to many factors, including different desorption kinetics of different functional groups, mixed retention mechanisms or changes related to the mobile phase or analyte properties that occur at elevated temperatures.[17]

1.1.3.2.2 Influence of temperature on efficiency

The influence of temperature on efficiency can be expressed by its effect on the height of a theoretical plate. According to the van Deemter equation, the height equivalent to a theoretical plate (HETP) depends on the linear velocity of the mobile phase (μ) and can be written as:

$$H = A + B/\mu + C\mu \quad (6)$$

Here, the HETP depends on three terms: band broadening due to eddy diffusion (A term), which is due to the non-uniformity of the column packing, longitudinal diffusion coefficient (B term), and the resistance to mass transfer coefficient between the mobile and stationary phases (C term). It is assumed that the A term does not depend on temperature. However Carr states that high mobile phase temperatures might improve the laminar flow and lateral mixing of molecules among different flow channels in a column[26]. The B and C terms are temperature dependent. The B term is directly proportional to the diffusion coefficient and increases with increasing temperatures. It becomes significant at low linear flow rates. The C term is inversely proportional to the diffusion coefficient and is reduced at higher temperatures. The C term predominates at high flow rates. According to the Wilke and Chang equation[20], the diffusion coefficient is directly proportional to temperature and

inversely proportional to viscosity. By increasing the temperature, the diffusion of analytes will be increased and viscosity will be decreased, so the overall result is enhancing the diffusion of analytes. The influence of temperature on the efficiency under different flow rates is shown in **Figure 1-1**. It is shown that the improvement in efficiency is pronounced at higher linear velocities where the C term is the dominant contribution to the plate height.[17] As elevated temperatures increase the mass transfer of solutes and decrease eluent viscosity, separation can be achieved at higher flow rates without significant losses in efficiency. Also, at high temperature the minimum plate height in the van Deemter curve shifts to higher linear velocities, and the increase in plate height at flow rates higher than optimum is not as pronounced as at ambient temperature. Consequently, increasing the temperature can improve the efficiency of the chromatographic systems when $u > u_{opt}$[25]

In routine analysis, one of the benefits of operating HPLC columns at higher temperatures is that strict reproducibility of the mobile phase flow rate is not as critical provided that the flow rate is higher than u_{opt}. Since liquid chromatographic methods are usually not run at the optimum linear velocity of the mobile phase and during method development a van Deemter curve is not plotted to find out the minimum plate height at different operating conditions, working at higher flow rates with elevated temperature is generally beneficial.[25]

However, some studies came to different conclusions. For example, Yang et al. noted that column efficiency was either improved or remained almost unchanged with increasing temperature in the temperature range between 60 and 120 °C, but decreased when temperatures between 120 and 160 °C were applied. On the other hand, these authors noted

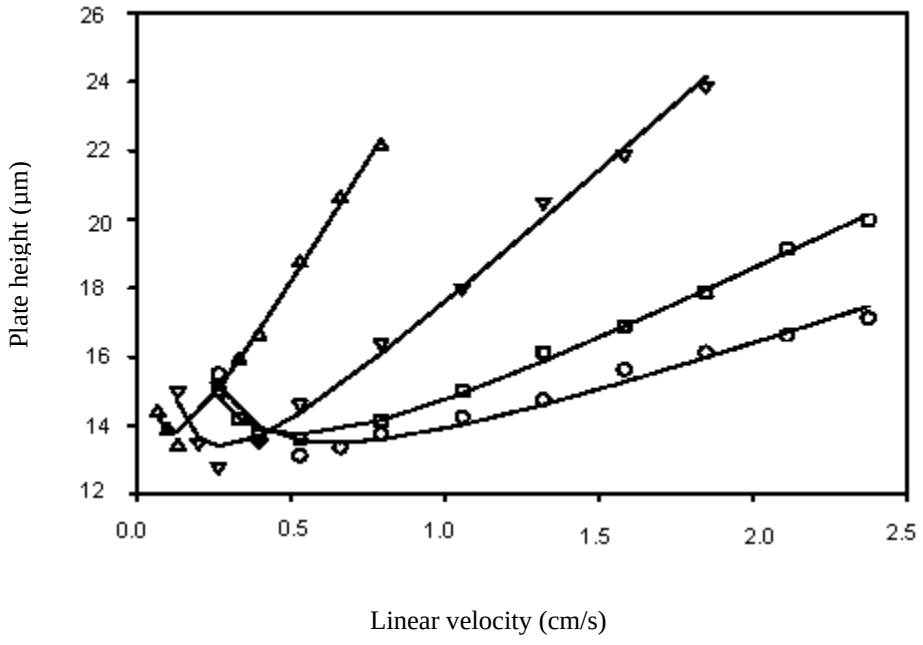


Figure 1-1: Plate height versus linear velocity at various temperatures for moderately retained solutes.[17] Experimental conditions: 3 µm ZirChrom-PS column (ZirChrom Separations), 5 cm × 4.6 mm id, 40% ACN/60% water, Δ = 25 ºC, octanophenone, k = 3.87, ▽ = 80 ºC decanophenone, k = 3.15, □ = 120 ºC, decanophenone, k = 5.70, ○ = 150 ºC, decanophenone, k = 1.65.

in another study that plate numbers decreased with higher temperatures except for one analyte when the temperature was increased from 100 to 140 °C.[25] This means that there are other factors which are responsible for band broadening, and thus a loss in efficiency when temperature is elevated. The formation of radial temperature gradients inside the column at elevated temperature, practical limitations of the heating system, as well as the connecting tubing, might all compromise efficiency.[25]

1.1.3.2.3 Influence of temperature on selectivity

Temperature can be used to control the selectivity of the separation. Differences in enthalpies of transfer for different solutes are the key driving force for changes in selectivity at elevated temperature.[25] Since a solute with a large ΔH will be more affected by changing the temperature than a solute with a small ΔH, the slopes of the van't Hoff plots for the selected analytes will differ. Thus, even if all analytes obey the van't Hoff equation and yield linear plots, a change in selectivity can often be obtained by changing the temperature.

A good example for changing selectivity as a function of temperature is given by Edge et al., where linear van't Hoff plots were observed for selected test probes between 40 and 180 °C using pure water as the mobile phase. Although linearity was obtained for all compounds, the elution order for caffeine and aminoantipyrine was reversed. The peaks were separated at low temperature, while at 113 °C they co-eluted. By increasing the temperature further they could again be separated. Consequently, temperature can be effectively used to optimize LC separation, especially when complex mixtures with polar and ionizable compounds are concerned.[25]

1.1.3.3 Practical aspects and limitations of high-temperature liquid chromatography

1.1.3.3.1 HTLC instrumentation

To successfully implement HTLC, special instrumentation including mobile phase preheating, column heating and post column effluent cooling system is required. Without a specially designed heating system, the benefits of high eluent temperatures cannot be obtained. Operating a column at a high linear velocity with poor instrumental design can result in a severe drop in efficiency. The instrumental system design used for HTLC should minimize both thermal mismatch and extra-column band broadening. Thermal mismatch results from insufficient mobile phase preheating and viscous heat dissipation across the column length, leading to longitudinal and radial thermal gradients. Radial thermal gradients decrease efficiency, whereas longitudinal thermal gradients affect solute retention factors.[20] Also, the system used should have low dead volume in order to minimize extra-column band broadening.

Mobile phase preheating

A critical element in a HTLC design is the preheater. Mobile phase preheating is necessary to prevent the formation of axial and radial temperature gradients within the column, which has been termed “thermal mismatch” by some authors.[25] This problem arises because silica or polystyrene column packings are relatively poor thermal conductors compared to stainless steel.[25] When the mobile phase enters the column at a temperature different from the column set temperature, a longitudinal thermal gradient occurs due to a difference in temperature between the column outlet and the column inlet, leading to a decrease in retention factor from

the cold column inlet to the warmer column outlet. Also, radial temperature gradients occur when the mobile phase molecules entering the wall region of the column reach the column set temperature before those entering the centre of the column. These gradients may cause the sample front to distort, leading to severe band broadening or even peak splitting.[25] Therefore, an efficient mobile phase preheater is essential to avoid temperature mismatch.[20] The acceptable difference in temperature between the incoming mobile phase and the column itself is recommended to be less than ±5 °C to keep good column performance.[20, 31, 32] The mobile phase preheating must be achieved after the injector valve because the maximum temperature of the valve rotor is usually 75 °C.

A major problem with HTLC analysis is the need for additional tubing length for preheating of the mobile phase, which results in additional extra-column dispersion. The required length of additional tubing can be critical for shorter columns. The tubing must be long enough to effectively preheat the mobile phase and avoid thermal mismatch broadening, but at the same time must be short enough to avoid excessive extra-column broadening. A good compromise is obtained by reducing tube length and tube internal diameter.[20] Commercially available column preheaters give fast response and have very low volumes.[17]

Column heating

The second component that must be incorporated into an HTLC design is a thermally controlled compartment for the column. The column temperature can be controlled by block heaters, forced circulating air ovens (e.g. converted GC ovens), as well as jackets with liquids circulating in them (water or oil).[20] Standard heaters such as water baths and block heaters

are less popular among the researchers. The majority of available column ovens use either still air or forced circulating air.[20] An air-bath oven is the most suitable solution because of its ability to rapidly change the temperature while allowing sufficient heat transfer from air to the column wall and through the column into the mobile phase. Block-heating ovens can effectively transfer heat because a tight contact is established between the column and the heating unit, but the drawback of block heaters is that they act as heat sinks with large thermal mass. This is not a problem when isothermal separation is used because the temperature is kept constant.[25] When temperature programming has to be used, though, ovens should rapidly come back to the initial temperature of the temperature program. In this case, heaters with large thermal mass such as water baths and block heaters should be avoided because high thermal mass will result in slow temperature changes. A forced circulating air oven is the most suitable for this purpose as heat transfer into the column is fast, which results in small thermal lag between the oven set-point and the column[19].

Post-column effluent cooling

In addition to mobile phase preheating, mobile phase cooling is often required prior to detection without causing a significant broadening of peaks eluted from the column. One approach for effluent cooling is the use of an efficient heat exchanger to reduce the mobile phase temperature below the upper limit of the detector (80 ºC for UV absorbance detectors).[17] Cooling the mobile phase is required when UV detector is used in order to avoid baseline noise and to maintain flow-cell longevity.[20] An alternative approach is to immerse a coil of tubing in a water bath or ice bath placed before the detector.[17]

Detector requirements

The temperature of the mobile phase entering the detector can have a negative or positive impact on detector sensitivity since its response could be reduced or increased due to high temperature.[25] Both effects have been reported in the literature. The effect of high eluent temperatures on UV, fluorescence and evaporative light scattering detection was studied. For a UV and fluorescence detector, a reduction in the peak area was observed after an increase in temperature; however, for the evaporative light scattering detector an increase in the detector response was noticed. Detection systems which are based on a conversion of the eluent from the liquid to the gaseous state, such as evaporative light scattering detector or a mass spectrometer, could thus benefit from high eluent temperatures. Consequently, it is not recommended to cool the mobile phase after it leaves the column when these detectors are used. Pereira et al.[33] pointed out that the use of high eluent temperatures has a positive impact in LC–MS methods. It could decrease the analysis time, improve the signal-to-noise ratios and enhance the sensitivity of the method.

Column stability

The column is considered the heart of the chromatographic system. Analytical column used for HTLC should be sufficiently stable over an extended period of time and must be able to withstand the aggressive conditions applied in HTLC. Not only the stationary phase has to be stable, but also the column hardware needs to be improved.[25] The use of elevated temperature might cause expansion of the column hardware and the stationary phase material leading to

the formation of voids and channels through the packed bed, which will result in additional band broadening.

Alkyl silane-bonded phases are the most popular in reversed phase HPLC (RP-LC). The major shortcoming of these phases is their thermal and chemical instability.[25] Conventional alkyl silane-bonded silica phases are unstable at temperatures above 50 °C since higher temperature accelerates the dissolution of silica in aqueous solution. At temperatures above 100 °C with water as the mobile phase, silica-based C18 phases are rapidly degraded.[30] In addition, these stationary phases could degrade at pH outside the range of 2.5-8. To overcome the problem of thermal and chemical instability of alkyl silane-bonded silica phases, different approaches have been developed such as grafting an organic protective layer on the silica surface, modifying silica with bonding and endcapping technologies and synthesizing hybrid particles resulting in organic groups incorporated into the silica matrix.[20] Also, alternative packing materials have been developed to withstand high temperatures, including metal oxide-based columns, porous graphitic carbon and organic polymers. However, these stationary phases are not a real alternative to replace silica-based stationary phases since they are less efficient, especially with polar and basic analytes.[20]

1.1.3.3.2 Analyte stability

One major concern for performing separations at elevated temperature is the analyte stability. The analytes should be stable and not susceptible to thermal degradation at the temperature of the analysis. The instability of some solutes at high temperature can limit the use of high temperatures in the pharmaceutical industry. The occurrence of on-column reactions depends

on both the solute residence time in the column and the reaction rate under the column conditions.[20] Antia and Horvath concluded that the on-column reactions could have insignificant effect if the increase in the reaction rate by increasing temperature was well compensated by the decrease in the residence time due to an increase in flow rate. The combination of using high mobile phase temperature with UHPLC and columns packed with small particles could be very useful for reducing the analysis time and improving the on-column stability of analytes.[20] Also, it should be pointed out that the reaction rate is also dependent on the mobile phase pH and expected to be lower in neutral than acidic or basic media. Therefore, neutral pH conditions are preferred at high temperature. Thompson and Carr[34] demonstrated that decreasing the residence time of thermally labile compounds in the column might decrease the extent of on-column reactions and eliminate analyte degradation. They examined criteria to check whether a given analyte can be analyzed at high temperature. The evidence for the absence of on-column degradation reactions could be achieved by observing the normalized peak area for each analyte at each temperature. A change in the normalized peak area (peak area × flow rate) with column residence time for a given solute would indicate on-column degradation. Other evidence is the observation of peak shape and the absence of additional peaks at elevated temperatures.[34] Thompson and Carr also demonstrated that thermally unstable pharmaceuticals could be separated at very high temperatures even though the compound was decomposed, provided that the decomposition peak was not separated from the parent peak.[20]

1.1.4 Superficially porous fused core particles

The recent introduction of fused core particles has provided an attractive alternative option for fast HPLC analysis using conventional instrumentation. The development of fused core particles was considered a breakthrough in column technology. It aims to reduce analysis times while maintaining column efficiencies and requiring relatively low pressures.[35, 36] The technology was originally developed by Kirkland in the 1990s. The major breakthrough in this technology occurred in 2006 when Kirkland et al. commercialized sub-3μm superficially porous particles. Currently superficially porous sub-2 μm particles are also available from an increasing number of manufacturers. Columns packed with superficially porous particles are offered under different brand names, such as Halo (Advanced Materials Technology, DE, USA), Ascentis (Supelco, Bellefonte, Pennsylvania, USA), Poroshell (Agilent, Little River, DE, USA) and Kinetex (Phenomenex, Torrance, CA, USA).[37] The columns are commercially available in different lengths, internal diameters and various bonding chemistries.

Unlike fully porous sub-2 μm column technology, which utilizes a completely porous particle, 2.6 μm fused-core Kinetex particles are comprised of 1.9 μm diameter solid silica core surrounded by 0.35 μm thick porous silica shell.[37] Fused core particles exhibit improvements in eddy diffusion term due to narrower particle size distribution, thus enhancing the efficiency. The thin porous shell on fused-core particles allows solutes to diffuse more quickly in and out of the porous structure for interaction with the stationary phase, which results in improved mass transfer. The resulting reduction in the resistance to mass transfer allows operation at higher flow rates without a significant loss in efficiency.[38] Also, the narrow particle size distribution of fused-core particles facilitates packing of

columns with great ruggedness.[7] Finally, fused-core particles produce lower back pressure compared to fully porous smaller particles, which makes it possible to use them with conventional HPLC systems.

The performance of fused-core particles was evaluated.[7, 36, 38-44] These columns show significant progress in performance compared to fully porous sub-2 µm particles. Columns packed with Kinetex® particles were shown to exhibit better efficiency than Halo-packed columns[37] and yield better efficiency than columns of the same length packed with sub-2 µm particles.[37] For these reasons, Kinetex® columns were chosen for the work presented.

1.2 High efficiency liquid chromatography

High efficiency in LC separations is necessary for many applications such as metabolomics, proteomics and genomics. Efficiency in HPLC can be expressed by two related terms: plate height and plate count. These two terms are related by the equation

$$N = L/H \tag{7}$$

where L is the column length, H is the plate height and N is the number of theoretical plates[1]. The efficiency (plate count) can be increased by increasing the column length and/or decreasing the plate height.

The A, B and C terms of the Van Deemter equation (eqn. (6) in Section 1.1.3.2.2) are dependent on the particle size d_p and the diffusion coefficient of the solute in the mobile phase D_m in the following fashion:

$$A \propto d_p$$

$$B \propto D_m$$

$$C \propto d_p^2/D_m$$

It is clear from these relationships that the A and C terms can both be decreased by using smaller particle size columns, which leads to a decrease in the plate height and a corresponding increase in efficiency for a given column length. The use of sub-2 µm particles improves the chromatographic performance as these particles provide minimum plate heights nearly four times smaller than those observed with conventional columns packed with 5 µm particles or with monolithic columns.[45] However, the price for using such particles is the induction of high backpressure, as discussed in section 1.1.2. The number of theoretical plates can also be increased by increasing the column length, e.g. by coupling several columns in series. However, this approach also induces high backpressure.

Increasing the column length through coupling several analytical columns together is a simple way to achieve high efficiency in HPLC, but the challenge is how to implement this approach using commercially available instrumentation. Numerous attempts at increasing the efficiency in HPLC by coupling several columns in series have been reported, e.g. coupling conventional silica columns of 5 µm particle size.[46-49] The main drawback of this approach is the longer analysis time (e.g. Lestremau et al. generated 162,000 plates by coupling eight 25 cm × 4.6 mm × 5 µm ODS columns to separate seven compounds in ~ 100 min[46]). Coupling of monolithic columns has also been reported.[50-52] In this approach, a large number of

columns (up to fourteen) were required to achieve high numbers of theoretical plates. However, this approach is not promising due to the drawbacks of the monolithic columns discussed in section 1.1.1.

1.3 Analysis of environmental contaminants

The occurrence of pharmaceuticals as emerging contaminants in the environment has been a growing concern in the recent years. Pharmaceuticals are a group of biologically active compounds of different classes used mainly as human or veterinary medications. These compounds differ in the degree of hydrophilicity and hydrophobicity depending on their structure and chemical composition.[53] Pharmaceuticals can enter the aquatic environment in their intact form or as metabolites.[54] Generally, pharmaceuticals are metabolized extensively or partially to form more polar and water-soluble derivatives that are rapidly excreted.[53] These compounds can reach wastewater from households, hospitals and industrial units. Most of them are not completely eliminated in wastewater treatment plants because of their high polarity and stability. They could be detected in sewage-treatment plants, surface water, ground water, sediments, soils and drinking water at concentrations ranging from ng/L to µg/ L.[55]

The growing use of pharmaceutical products is becoming a new environmental problem. Continual input of pharmaceuticals into the environment might lead to long-term exposure resulting in adverse effects on humans and wildlife. Some of these effects include acute or chronic toxicity, endocrine disruption and inhibition of primary productivity.[56] The most important groups of pharmaceuticals which could be detected in environmental water

samples are non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics, nervous stimulants, estrogens and lipid regulators.[57] NSAIDs are used mainly for the treatment of pain and inflammation because they have antipyretic, anti-inflammatory and analgesic effects,[11, 58] while sulfonamides are used for the prevention and treatment of diseases and infections, as well as feed additives to promote growth in animals.[59] Although the use of these two groups of pharmaceuticals has positive effects on treating many diseases in animals and humans, their input to the environment can affect human health. For example, accumulation of analgesics such as diclofenac in water can cause harmful renal effects in humans, and accumulation of antibiotics can develop resistance to them among microorganisms.[59] Most worrying is the input of endocrine disrupting compounds (EDCs) into water. The serious effects of EDCs on fish include the feminization of male fetuses, reproductive dysfunctions, behavioral changes and developmental problems.[60] Humans may also experience adverse effects on reproductive and sexual development, such as decreased sperm quality, sex ratio changes, puberty disorders and development of hormonally sensitive carcinomas (female breast cancers, testicular and prostate cancers).[61] Consequently, monitoring of pharmaceuticals in aquatic environment is of great importance and has been the focus of an increasing number of recent studies.

Very sensitive and highly efficient methods are required for the analysis of pharmaceuticals in environmental samples, particularly wastewater. Several analytical methodologies based on GC–MS[62, 63], LC-MS[64-67] and LC-MS-MS[59, 68-74] have been developed to determine various pharmaceuticals in different environmental samples. Methods based on mass spectrometric detection are favored by many analysts because they offer significant

advantages, including high sensitivity and ability to provide compound confirmation. However, using GC–MS for the determination of many pharmaceuticals is not feasible due to the low volatility of these compounds. Derivatization could overcome this problem, but the main disadvantage of this process is the long sample preparation time, especially with a large number of samples. Also, some of the derivatizing agents (e.g. diazomethane) have high reactivity toward pharmaceuticals, high toxicity and carcinogenicity.[75]

As most pharmaceuticals are polar compounds, the technique of choice is HPLC, which remains the main tool for the analysis of pharmaceuticals in most commercial and research laboratories. While LC–MS is the preferred combination, its high costs make it not available to many laboratories.

The use of newly developed column technologies with UHPLC is a promising approach leading to improvements in the area of environmental analysis, especially when large number of samples and very complex matrices have to be analyzed. In this case, decreasing the analysis time, reducing solvent consumption and enhancing the efficiency are very beneficial.

1.4 Sample preparation

In addition to advanced separation techniques, a preconcentration step is required to reach the sensitivity necessary to detect the low concentrations normally present in environmental samples (in the ng/L to µg/L range). Sample preparation can be achieved by employing a wide range of techniques, such as liquid–liquid extraction (LLE), solid-phase extraction (SPE), solid-phase microextraction (SPME), liquid-phase microextraction (LPME) and

lyophilization.[76] The goal of all sample preparation methods is the removal of interferences, increasing the concentration of an analyte and in some cases converting an analyte into a more suitable form. The extraction method should be robust and reproducible.[77]

Solid phase extraction (SPE) is the most common technique applied to sample preparation and clean up in the analysis of pharmaceuticals. SPE provides many advantages including high recoveries, good selectivity and reproducibility, elimination of the formation of emulsions, low organic solvent consumption, short sample preparation time, easy operation and the possibility of automation.[77] SPE overcomes most of the disadvantages of liquid–liquid extraction (LLE)[78] and is considered the technique of choice for analyte enrichment in the analysis of pharmaceuticals.

SPE is a non-equilibrium exhaustive technique used for semi-volatile and non-volatile analytes.[79] The analytes are partitioned between a solid phase and a liquid phase, hence they must have greater affinity for the solid phase than for the sample matrix. The impurities in the sample are washed away, and the analytes retained on the solid phase can then be removed with an eluting solvent.[80] The experimental procedure of SPE consists of several stages including conditioning or activation of the sorbent by applying an appropriate solvent, application of the sample so the analytes will be retained on the solid phase, removal of interfering compounds with a weak solvent (washing step), and elution of the analytes from the sorbent with an appropriate strong solvent (elution step).[80] The procedure uses a vacuum manifold, so that several samples (usually 12 or 24) can be processed simultaneously, thus minimizing the time and effort required for sample preparation.[76] Many SPE materials are available, ranging from classical sorbents such as chemically-bonded silica with C8 or C18

groups and carbon or ion-exchange materials, to recent polymeric materials, immunosorbents, molecularly-imprinted polymers (MIPs) and restricted access materials (RAMs).[77]

Pharmaceuticals of adequate hydrophobicity can easily be preconcentrated using any reversed-phase material (e.g., C18, C8). In this case, adjustment of the pH is necessary to avoid deprotonation of acidic compounds and protonation of basic compounds to ensure sufficient hydrophobicity of the analytes. Acidic pharmaceuticals should therefore be preconcentrated under acidic conditions, as opposed to basic analytes. However, since most pharmaceuticals have polar properties, their enrichment on traditional reversed-phase materials is difficult. Recently, new polymeric sorbents have been introduced and are becoming increasingly popular.[81] These sorbents improve the retention of polar compounds and are suited for multi- class analysis of pharmaceuticals in water samples even without adjusting the pH of the sample. Polymeric sorbents have novel functional groups in their structure resulting in a hydrophilic–hydrophobic balance. One of the most widely used polymeric sorbents is a copolymer of divinylbenzene and vinylpyrrolidone, which has been commercialized under the trade name Oasis HLB by Waters.[81]

Recently, Oasis HLB became the prime sorbent for multi-residue methods for the determination of pharmaceuticals. Mixed mode sorbents based on Oasis HLB and containing ion-exchange groups (such as Oasis MCX and Oasis WCX containing strong and weak cation exchange groups, and Oasis MAX and WAX containing strong and weak anion exchange groups) have also been extensively used for the extraction of pharmaceuticals from water samples. For the extraction of real water samples, Oasis HLB cartridges were

selected because of their advantages such as improved wetting characteristics leading to better mass transfer, higher retention capabilities for extracting acidic analytes from water samples without acidification, and the ability to extract a large number of compounds simultaneously without the need for sample pH adjustment.[11] Performing the extraction on Oasis HLB cartridges without pH adjustment provides many advantages, such as simplified sample handling, especially for large volume samples, and no enhanced risk of acidic hydrolysis of other compounds in the sample.

In addition to Oasis HLB, other polymeric sorbents such as Strata-X manufactured by Phenomenex and Sampli Q OPT manufactured by Agilent are also used.[81] SampliQ OPT is a competitive material to Oasis HLB. It is a polymer with lipophilic divinylbenzene and hydrophilic N-vinylpyrrolidine groups. Compared to bonded silica sorbents which has limited operational pH range (pH 2-9), polymer sorbents have a very wide pH range and can tolerate solvents that would harm or destroy silica-based sorbents.[82]

Polymeric sorbents are spherical giving very homogeneous packed beds that display reproducible flow characteristics and minimal back pressure. In addition, the polymers have higher surface area leading to much higher sample capacity than silica, so the sorbent bed volumes can be smaller. Decreasing the sorbent volume has some advantages, such as decreasing the volume of the sample and the solvents needed for the extraction. Lower elution volumes result in higher analyte concentrations in the extracts and better overall method sensitivity. While silica-based bonded phases have silanol groups on the bonded surface which can be ionized and interact with the analytes affecting their retention and recovery, the absence of silanols on polymeric SPE sorbents prevents such interactions.[82]

Silica-based sorbents are not the sorbent of choice for tetracyclines because they have been found to bind irreversibly to the sorbent.[77] SPE of pharmaceuticals is generally done in an off-line mode prior to the chromatographic analysis step. However, SPE is well-suited to be coupled on-line with an HPLC system, which allows a high degree of automation. In this case, the preconcentrated analytes are directly eluted onto the analytical column. On-line SPE provides smaller sample volumes, smaller volumes of organic solvents for elution, shorter analysis time and lower costs.[81]

1.5 Aim of the research

The overall objective of this research was the development of advanced separation techniques for the analysis of environmental pollutants. Recently, the occurrence of pharmaceuticals in aquatic environment has been recognized as an environmental concern and these compounds are classified as emerging contaminants. Therefore, this research focused mainly on pharmaceuticals as environmental pollutants. The studied analytes covered a wide range of compounds including veterinary antibiotics, non-steroidal anti-inflammatory drugs, steroids and central nervous system (CNS) stimulants. These groups of pharmaceuticals were selected for the study because their occurrence in the aquatic environment leads to detrimental effects on wildlife and human health. Besides, based on the literature, these pharmaceuticals are very frequently detected in environmental water samples. In addition, other pollutants such as preservatives (personal care products) were chosen for the analysis in one of the presented studies.

The aim of the research was the development and validation of advanced LC separation techniques to replace the traditional methods used for the environmental analysis. The methods developed aim at cutting the analysis time, enhancing efficiency and minimizing the consumption of organic solvents making the analysis of environmental pollutants greener. This goal was accomplished by using the latest developments in liquid chromatography, including columns packed with the newly developed fully porous sub-2 µm and superficially porous sub-3 µm stationary phase particles, mobile phases at high temperature, ultra high pressure liquid chromatographic system (UHPLC) and reduced column dimensions.

The goals of the research have been accomplished through the following steps:

1. Development of highly efficient HPLC methods based on coupling of multiple columns packed with fully porous sub-2 µm particles at elevated temperature, thus reducing the backpressure.
2. Development of very fast HPLC methods based on using fully porous sub-2 µm or superficially porous sub-3 µm particles at elevated mobile phase temperatures.
3. Development of "green" HPLC method using short, small diameter columns operated at the maximum allowable pressure to be used as an alternative to the traditional methods used in environmental analysis
4. Comparing the performance of columns packed with superficially porous sub-3 µm particles with that of fully porous sub-2 µm particles at ambient and high temperature using single and coupled columns.

5. Separating different mixtures of pharmaceuticals on columns packed with superficially porous particles to illustrate the possibility of using fused-core particles as an alternative to sub-2 μm particles in terms of speed and efficiency in pharmaceutical analysis.
6. Validating the methods developed and applying them for the analysis of pharmaceuticals in samples of surface and/or wastewater.

Chapter 2

Experimental

2.1 Experimental

2.1.1 Materials and reagents

All standards (> 99% purity), solvents (acetonitrile, acetic acid and triethylamine are HPLC grade) and uracil were purchased from Sigma–Aldrich (Mississauga, ON, Canada). Ultrapure water purified using a Milli-Q water purification system from Millipore (Bedford, MA, USA) was used in all studies. All solvents for HPLC separation were filtered using 0.45 µm × 47 mm nylon membrane filters (Supelco, Bellefonte, PA, USA) and degassed by sonication for 20 min using a sonication bath (Crest, Ultrasonics, Bellefonte, PA, USA) before use. The pH of the mobile phase was adjusted with the help of a pH-meter (SevenEasy™ pH, Mettler Toledo, Switzerland).

2.1.2 Instrumentation

All separations were performed using an Agilent model 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, thermostated column compartment, binary pump and UV diode array detector. The column temperature was controlled by an external GC oven (model 5890 A gas chromatograph, Hewlett-Packard, Palo Alto, CA, USA). A preheater purchased from Restek (Catalog number 22484, Bellefonte, PA, USA) was used to preheat the mobile phase to avoid band broadening due to thermal

mismatch across the column. Cooling of the mobile phase was required prior to UV detection in order to avoid baseline noise and to maintain flow-cell longevity[83], so the temperature of the mobile phase effluent was set to 25 ºC using Agilent 1200 cooler in the column compartment. The maximum pressure produced by the system was 600 bar. Data were collected and analyzed using Chemstation software (Agilent Technologies, Waldbronn, Germany). A 4.6 mm I.D. in-line filter (0.2 µm) (Agilent Technologies, Waldbronn, Germany) was used to protect the analytical columns. In all studies, a stock solution of 1 mg/ mL of each studied analyte was prepared by dissolving 10 mg of each standard in 10 mL of methanol. The working standard solutions were prepared by serially diluting the stock solution in the mobile phase.

2.1.3 Sample collection

Water samples were collected from a small river, a lake and a municipal wastewater treatment plant in Southern Ontario, Canada. Raw influent wastewater samples were used in all studies. The sewage sample was pumped directly from the sewage pipe (4 m below the surface) straight into the sampling bottles. All samples were collected in pre-washed amber glass bottles with Teflon-lined caps. Bottles were completely filled with the sample, wrapped in hermetic plastic bags and transported to the laboratory in iceboxes. The samples were filtered to eliminate suspended matter through 0.45µm cellulose acetate filters. The samples were stored at 4 °C until the extraction, which was performed within 24 hours in order to avoid degradation of the analytes. The filtered samples were extracted and the extracts were dried and redissolved in the mobile phase.

2.1.4 Sample preparation and extraction

Solid phase extraction (SPE) was used for sample preparation. Visiprep solid phase extraction vacuum manifold system (Supelco, Bellefonte, PA, USA) was used for the extraction.

In all studies Oasis HLB and/or SampliQ OPT cartridges were used for the sample extraction. Oasis HLB with 60 mg packing material and 3 mL reservoir was purchased from Waters (Milford, MA, USA). SampliQ OPT polymer with 60 mg packing material and 3 mL reservoir was donated by Agilent Technologies.

During the extraction, the sorbent was never allowed to dry during either the conditioning period or sample loading procedures. A sample volume of 100 mL was applied to the cartridge at a flow rate not exceeding 4 mL/min. The cartridges were conditioned with 3 mL methanol, followed by 3 mL of 0.5 M HCL and 3 mL deionised water. After vacuum drying of the sorbent, the loaded cartridges were eluted with 5 mL of ammoniated methanol (ammonia:methanol, 1:19, v/v). Following the elution, the filtrates were evaporated to dryness under a gentle nitrogen stream. The dried extract was dissolved in l mL of the mobile phase to obtain a 100-fold preconcentration. Non-spiked water samples were also extracted in all experiments, using the same procedure, in order to detect any possible contribution of the water matrix to the analytes' signals.

2.2 Validation of the methods

The methods developed were validated according to International Conference on Harmonization (ICH) recommendations.[84] The performance of all methods developed was

evaluated through estimation of parameters such as selectivity, linearity, precision, accuracy and detection and quantitation methods.

2.2.1 Selectivity

Unlike LC-MS, HPLC with UV detection lacks the ability to provide compound identity confirmation. Therefore selectivity of the method was evaluated by comparison of the compound retention times obtained from the chromatogram of fortified deionised water to the chromatogram of unfortified deionised water (blank samples). The absence of false positive results for all sample blanks and good separation ensure that the signal measured is not influenced by other substances. These were considered acceptable for selectivity.

2.2.2 Linearity

The linearity of the method was evaluated using five concentrations of the analytes spiked in deionized water. Each concentration was analyzed in triplicate. Calibration curves were developed using aqueous standards for each compound studied by plotting the peak area versus the concentration. The results were analyzed by least-squares linear regression method.[84]

2.2.3 Precision

Precision was validated based on the evaluation of intraday and inter-day repeatability of the method. Intra-day and inter-day repeatabilities were determined by analyzing three replicates of deionized water samples spiked with the standards at two concentration levels (low and high). Intra-day and inter-day precision were expressed as relative standard deviations (RSD).

2.2.4 Accuracy

The recovery of the analytes from spiked water samples was evaluated by calculating the ratio of the peak area obtained from the extraction of spiked deionized water sample to the peak area of the corresponding standard solution at two concentration levels using Oasis HLB and/or SampliQ OPT cartridges.

2.2.5 Detection and Quantitation Limits

Limits of detection (LOD) and quantification (LOQ) were determined according to International Conference on Harmonization (ICH) recommendations.[84] LODs and LOQs were experimentally estimated from the injection of standard solutions serially diluted until the signal to noise ratio for any single analyte reached a value of ten for LOQ and three for LOD.

Chapter 3

High-Efficiency Liquid Chromatography Using Columns Packed With Sub-2 µm Particles at Elevated Temperature for the Analysis of Sulfonamides in Wastewater*

HPLC is the most widely used analytical separation technique, yet analyzing very complex matrices is still a very difficult problem. Enhancing the efficiency is very important in this context. Efficiency can be enhanced either by increasing the column length to gain high number of theoretical plates, or decreasing the plate height. Both approaches induce high backpressure across the column, especially when conventional HPLC systems are used. The direct way to extend the column length is to couple several columns in series; however, the challenge is how to implement this approach using commercially available instrumentation. Researchers have been trying to overcome the problem of high pressure drop by developing HPLC systems able to produce very high pressures (> 400 bar) to allow the use of small size packing particles and longer columns. Another approach to overcome the high backpressure is working at elevated temperature, hence reducing the viscosity of the mobile phase.

Attempts at increasing the column length through coupling are reported in the literature, e.g.[46, 50] These methods were based on coupling either conventional silica columns of 5 µm particle size, or monolithic columns. The drawbacks of these methods include longer analysis time and/or high number of columns that need to be coupled, as discussed before in Section 1.2. In addition, these methods consume significant amounts of the organic modifier, which

* This Chapter is based on a paper published in Chromatographia[85].

must be properly disposed of after the analysis. Thus, an alternative approach at column coupling allowing shorter analysis time and lower organic modifier consumption is highly desirable.

In this study, high plate count was obtained by coupling three columns packed with fully porous sub-2 μm particles. To overcome the high backpressure, the temperature of the mobile phase was elevated to 80 ºC to decrease the solvent viscosity and an HPLC system providing pressures up to 600 bar was used. A large number of theoretical plates (~ 84,000 for the last eluting compound) was generated. The plate number was increased on average by a factor of ~3.6 when three columns were coupled at 80 ºC compared with one column at 30 ºC.

The highly efficient method developed was then applied to the analysis of selected sulfonamides in wastewater, which is characterized by a very complex matrix, after sample preconcentration and clean-up on SPE cartridges and using isocratic mode of separation. Enhancing efficiency and increasing resolution offered by coupled columns allows the analytes to be separated from each other and from the interfering components in the matrix.

Sulfonamides were selected for this study because they are veterinary antibiotic commonly used in swine farms. Continual discharge of these compounds into the aquatic environment is considered a serious issue due to the high risk of the development of resistance in some bacterial strains.

The method developed offered high efficiency with shorter analysis time. It was also considered the first trial in using coupled columns for the analysis of pharmaceuticals in the very complex wastewater matrix.

3.1 Experimental

Analytes used in this study were sulfonamides including sulphanilamide (SNM), sulfacetamide (SAM), sulfapyridine (SPD), sulfamerazine (SMR), sulfamethazine (SMZ), sulfamethoxazole (SMX), sulfaisoxazole (SIX) and sulfadimethoxine (SDM). The detection wavelength was set at 272 nm. The columns used were Zorbax SB C18 (Agilent Technologies, Waldbronn, Germany) of 4.6 mm ID, 150 mm length and 1.8 micron particle size. These columns are stable up to 90 ºC. When the columns were coupled, stainless steel tubing of 10.5 cm length and 0.12 mm ID (Agilent Technologies, Waldbronn, Germany) was used to connect them. The injection volume was 5 µL. The standards were distributed into five concentrations ranging from 100 µg/L to 10 mg/L. The chromatographic separations were performed in isocratic mode using a mobile phase consisting of 75% of water (containing 0.5% acetic acid) and 25% acetonitrile.

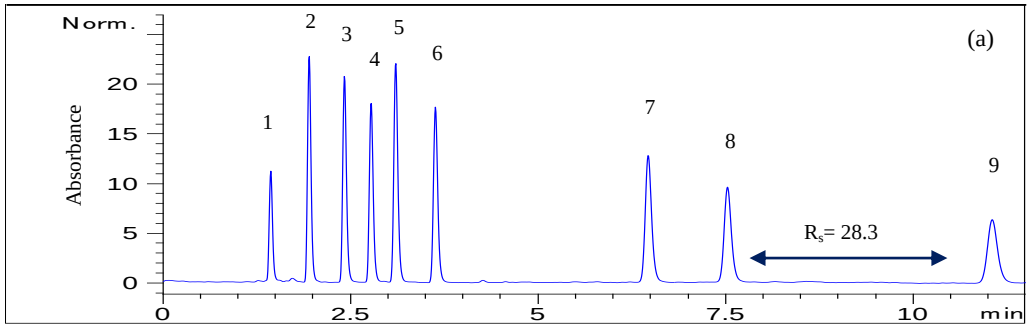
3.2 Results and discussion

3.2.1 Selection of the column

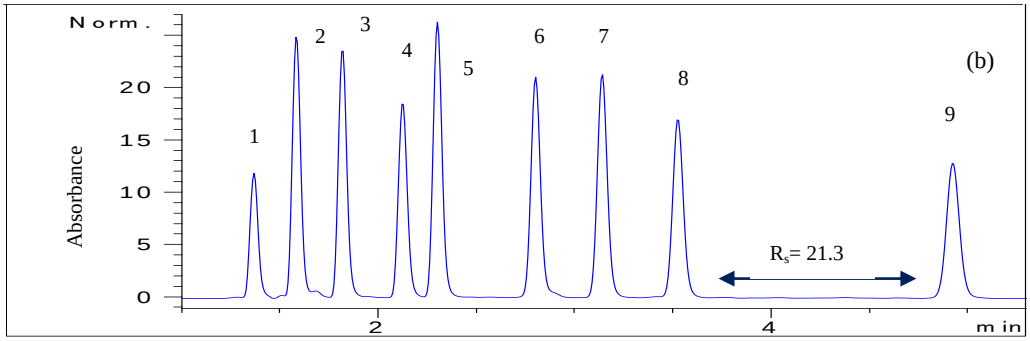
In this study, 4.6 mm I.D. columns were selected for the analysis. Such columns are still the most popular, although there is an on-going shift to narrower diameter columns. Using small diameter columns can save solvent and improve detection limits; however, a decrease in efficiency might be observed due to extracolumn effects, unless special precautions are taken. In addition, geometrical sidewall effects are more pronounced in narrow diameter columns[86], and packing of sub-2 µm particles into small diameter columns is practically difficult and leads to increased minimum plate heights.[2]

3.2.2 Effect of temperature on chromatographic performance

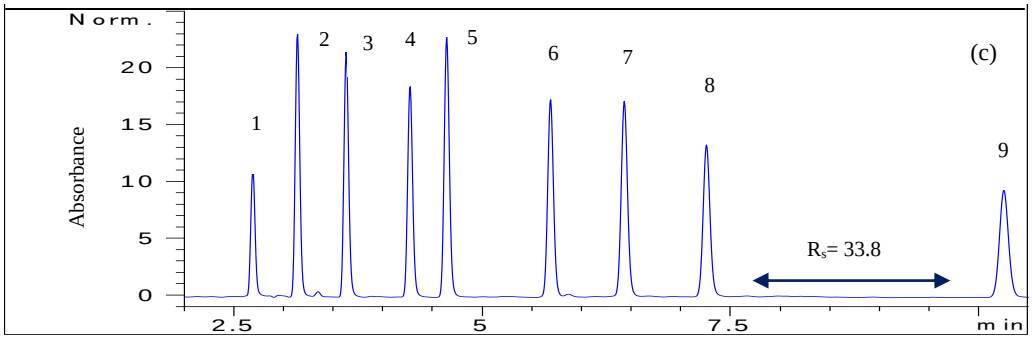
Efficiency and resolution are related to particle size and column length. The reduction in backpressure due to the decreased solvent viscosity at high temperature allows the use of longer columns to increase efficiency and resolution without significantly increasing the analysis time.[13] Separation of a mixture of eight sulfonamides was used to illustrate this approach. The mixture was separated at 30 ºC on a single column (150 mm long) packed with 1.8 µm particles under isocratic conditions and at 80 ºC on a 450 mm long column (three 150 mm columns coupled in series) at flow rate of 1 mL/min. The flow rate could not be set much higher because the system already operated close to the maximum pressure for the three coupled columns. At elevated temperature, the dielectric constant of water decreases. Thus, water can replace a large proportion of the organic solvent in the mobile phase. A temperature increase of 5 ºC has a similar effect on retention as a 1% increase in acetonitrile concentration.[13] In this study, the mobile phase was not buffered as crystallization of salt traces from the buffer in the column can reduce its separation capacity and shorten its lifetime. Acetic acid (0.5%) was added to the mobile phase instead to control the pH and enhance peak resolution. In the absence of the acid, coelution of SPD and SMR was observed. **Figure 3-1** shows the chromatograms of the test mixture separated at 30 ºC on a single column (150 mm) and at 80 ºC using one, two and three coupled columns at a flow rate of 1 mL/min under isocratic conditions. A comparison of the chromatograms obtained on a single column at 30 ºC (**Figure 3-1 a**) and at 80 ºC (**Figure 3-1 b**) demonstrated that the analysis time was reduced by ~56% at the same mobile phase composition with a concomitant loss in efficiency and resolution. This was due to the decrease in partition



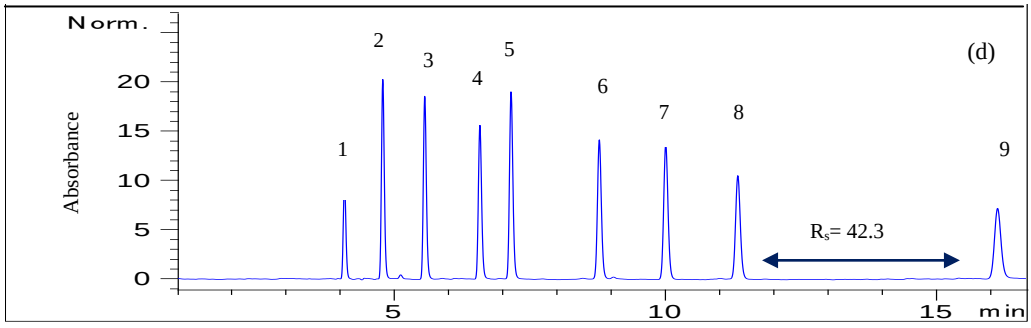
Retention time (min)



Retention time (min)



Retention time (min)



Retention time (min)

Figure 3-1: Chromatograms of the sulfonamides studied. a) One column at 30 ºC; b) one column at 80 ºC; c) two columns at 80 ºC; d) three columns at 80 ºC. Flow rate was 1 mL/min in all cases. Peak identification: 1- uracil, 2- SNM, 3- SAM, 4- SPD, 5- SMR, 6- SMZ, 7- SMX, 8- SIX, 9- SDM.

coefficients of the analytes at higher temperature (hence smaller capacity factors) and to the increase in longitudinal diffusion.[20, 25, 87] Also, the loss in resolution on a single column operated at 80 ºC was due to the fact that the analysis was not performed at the flow rate optimal for this temperature, which should be above 1 mL/min.

The change in selectivity for sulfamethazine (peak 6), whose relative retention changed quite dramatically at the elevated temperature, is worth noting. The reduced viscosity of the mobile phase at 80 ºC allowed the increase in the column length through the coupling of several columns in series. A maximum number of three columns could be coupled in this study due to instrumental limitations. **Figure 3-1 c** shows the separation of the test mixture obtained with two coupled columns (total length 300 mm). Compared with the single column at the same temperature (80 ºC) and the same flow rate (1 mL/ min), the analysis time was doubled, but the efficiency was increased. For example, resolution between the critical pair of sulfapyridine (peak 4) and sulfamerazine (peak 5) was increased from 3.5 using one column at 80 ºC to 6.6 by coupling two columns at elevated temperature. Also, the resolution between sulfaisoxazole (peak 8) and sulfadimethoxine (peak 9) increased to 33.8 compared with 21.3 for a single column (**Figure 3-1 b**). The analysis time in this case was nearly the same as for a single column at 30 ºC, but the separation efficiency was significantly higher, which is a definite advantage.

By increasing the column length to 450 mm (**Figure 3-1 d**, three coupled columns), the analysis time was increased by ~64% compared with two coupled columns (**Figure 3-1 c**). In this case, resolution between sulfapyridine (peak 4) and sulfamerazine (peak 5) was increased to 8.5, while the resolution between sulfaisoxazole (peak 8) and sulfadimethoxine

(peak 9) was increased to 42.3. While the resolution between the peaks could be considered excessive under all conditions, it should be kept in mind that in real samples the target analytes have to be separated both from each other and from the matrix components, which calls for the highest efficiency separation possible. The number of theoretical plates calculated for sulphanilamide (peak 2) was 6,158, 21,366 and 39,091 for one, two and three columns at 80 ºC, respectively. For sulfadimethoxine (peak 9), the respective numbers were 25,554, 57,122 and 84,433. The number of theoretical plates was increased by a factor of ~4.7 when three columns were coupled at 80 ºC compared with one column at 30 ºC for sulfanilamide (8,362 vs. 39,091) and more than doubled for sulfadimethoxine (84,000 vs. 32,000). On average, the number of theoretical plates increased by a factor of ~3.6 when three columns were used at 80 ºC compared with one column at 30 ºC, with greater increases for earlier eluting peaks.

The plate counts obtained for less retained compounds, especially for shorter column lengths, were comparatively low. This was most probably caused by band broadening due to the use of components such as the preheater and the cooler, whose significant volume led to extra-column band broadening. The more retained compounds were focused at the head of the column, which led to a higher number of theoretical plates. The effect of temperature on the chromatographic parameters (capacity factor, resolution, selectivity and efficiency) of the separated analytes is summarized in

Table 3-1.

Table 3-1: Chromatographic parameters (capacity factor, resolution, selectivity, efficiency) of the separated analytes

Analyte	One column at 30 °C				One column at 80 °C				Two columns at 80 °C				Three columns at 80 °C			
	k'[a]	R_s	α	N	k'[a]	R_s	α	N	k'[a]	R_s	α	N	k'[a]	R_s	α	N
Sulfanilamide	0.4	9.0	1.9	8362	0.2	5.0	2.1	6158	0.2	9.6	2.1	21366	0.17	13.1	2.1	39091
Sulfacetamide	0.7	6.4	1.4	10900	0.3	6.3	1.7	8128	0.4	12.0	1.7	27157	0.36	15.9	1.7	45256
Sulfapyridine	0.9	5.7	1.3	13608	0.6	3.5	1.2	9913	0.6	6.6	1.2	33007	0.61	8.5	1.2	54262
Sulfamerazine	1.2	8.8	1.3	15309	0.7	9.6	1.5	11704	0.7	17.3	1.5	37046	0.76	22.3	1.5	60056
Sulfamethazine	1.5	36.9	2.3	19731	1.0	6.2	1.2	14703	1.1	11.2	1.3	43928	1.15	15.0	1.3	70751
Sulfamethoxazole	3.5	10.7	1.2	26710	1.3	6.7	1.2	17658	1.4	11.4	1.2	48223	1.46	14.6	1.2	74404
Sulfaisoxazole	4.2	28.3	1.6	28581	1.6	21.3	1.65	19636	1.7	33.8	1.7	50572	1.78	42.3	1.7	76632
Sulfadimethoxine	6.7			32263	2.6			25554	2.8			57122	2.96			84433

[a] k': capacity factor; R_s: resolution; α: selectivity; N: number of theoretical plates.

3.2.3 Effect of temperature on backpressure

According to Darcy's law[12], the decrease in the mobile phase viscosity at high temperatures leads to a reduction in column backpressure, which allows the use of coupled columns packed with small particles (sub-2 µm). The system backpressure (ΔP) was measured as a function of temperature using single and coupled columns. At 30 ºC, the total pressure drop (ΔP) for a single column (150 mm) at 1 mL/min was 399 bar. By increasing the temperature to 80 ºC, the backpressure was reduced to 197 bar due to the reduction in the mobile phase viscosity. This also allowed the coupling of columns in series to extend the column length, thus enhancing the efficiency. At 80 ºC, the total pressure drop (ΔP) was 361 and 526 bar for two (300 mm) and three (450 mm) coupled columns, respectively. Due to instrumental limitations, a maximum number of three columns packed with 1.8 µm particles could be coupled at 80 °C because the system was operated close to the maximum allowable pressure.

3.2.4 Van Deemter Plots

The plots of plate height as a function of the mobile phase velocity using one column at 30 ºC, as well as two and three coupled columns at 80 ºC for the last eluting compound are shown in **Figure 3-2**. At 30 ºC, the optimum flow rate corresponding to the minimum plate height (H_{min}) was 0.6 mL/min. As the temperature increased to 80 ºC using two and three coupled columns, H_{min} moved to faster flow rates. At the same time, the plots were flatter at higher temperature, which allowed the analysis to be performed at flow rates higher than optimal without a significant loss in efficiency. Flow rates higher than 1mL/min could not be used when three columns were coupled due the increase in the backpressure. The plot for coupled columns at 80

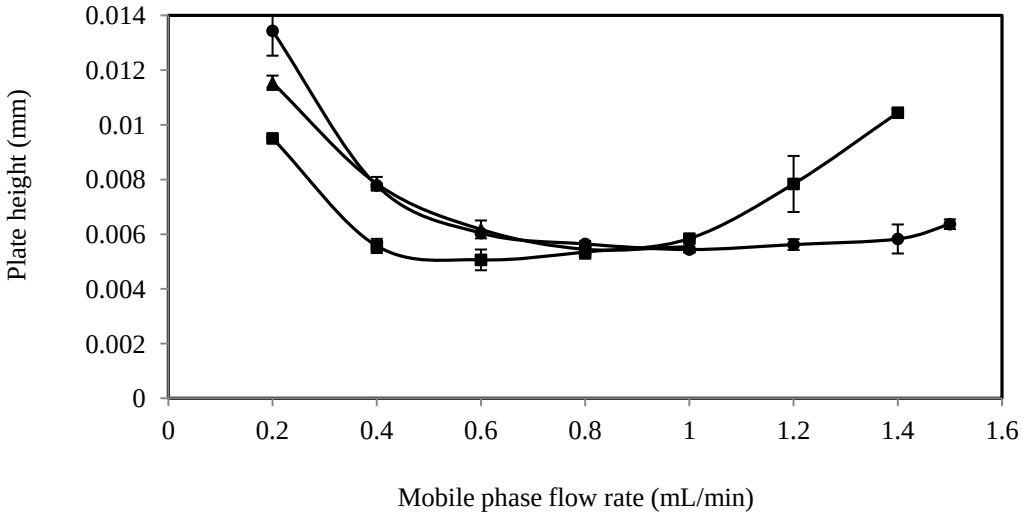


Figure 3-2: Plate height as a function of the mobile phase velocity for one column at 30 ºC (square), two columns at 80 ºC (circle) and and three columns at 80 ºC (triangle) for the last eluting compound.

ºC showed lower plate height values at flow rates higher than the optimum and thus higher efficiency compared with a single column at 30 ºC. For two and three coupled columns, the plate height values remained practically constant, which indicated that the tubing used for the connections had no significant effect on band broadening. At flow rates lower than the optimum, the coupled columns showed a marked increase in the plate height, which can be explained by increased band broadening due to longitudinal diffusion when the columns operated at flow rates lower than the optimum while the diffusion coefficients were significantly higher at 80 °C than at 30 °C. The analysis time on one column operated at 30 ºC at the optimum flow rate (0.6 mL/min) was slightly shorter than that on three columns at 80 ºC and 1 mL/min flow rate, but the

number of theoretical plates was significantly increased at the higher temperature. The higher number of theoretical plates obtained by increasing the column length at higher temperature resulted in better resolution between the peaks at a cost of marginally longer analysis time.

3.2.5 Application of the high-temperature, high-efficiency HPLC method to environmental samples

The method using three coupled columns packed with sub-2 µm particles at 80 ºC was used for the determination of sulfonamides in wastewater to illustrate the potential of high-temperature HPLC on long columns. The method was validated based on parameters such as linearity, precision, detection and quantification limits, and accuracy.

3.2.5.1 Sample preparation and extraction

Sulfonamides were extracted from 100 mL wastewater sample using Oasis HLB cartridges. Theses cartridges were preferred due to their chemical composition (the combination of the lipophilic divinylbenzene and the hydrophilic N-vinylpyrrolidone polymers) resulting in an excellent wettability and the ability to extract acidic, neutral and basic compounds at a wide range of pH values. Thus, a large number of compounds can be extracted simultaneously without the need for sample pH adjustment.

3.2.5.2 Validation of the method

3.2.5.2.1 Linearity

The linearity was evaluated using five concentrations ranging from 100 µg/L to 10 mg/L. The results were analyzed by least-squares linear regression method. The linearity of the calibration

curves was validated by the high values of the correlation coefficients, with the lowest one being R^2 = 0.9967 for sulfapyridine. The parameters of the regression equations and the calibration ranges for the studied analytes are summarized in **Table 3-2**.

3.2.5.2.2 Precision

Repeatability and intermediate precision were evaluated by analyzing three replicates of aqueous sulfonamide standards at two concentration levels (low and high). Good precision of the instrumental analysis was obtained, shown by the intra-day and inter-day RSDs at 100 µg/L (except for sulfanilamide at 500 µg/L) and 1 mg/L of 1.1–12.2% and 2.9–13.5%, respectively. The results are illustrated in **Table 3-3**. The results obtained confirmed good measurement repeatability of the SPE–HPLC method.

3.2.5.2.3 Detection and quantitation limits

The LOD of the method ranged from 0.6–2 µg/L, while LOQ were in the range 2–6.7 µg/L with UV detection. The detection and quantitation limits determined in the study are given in **Table 3-4**. The low limits of detection confirmed the applicability of the proposed method to real samples. Detection limits could be decreased even further by using mass spectrometry detection; however, one of the most recognized drawbacks of LC–MS analysis is the matrix effect (signal suppression or enhancement by the sample matrix that may occur in the ionization process). Co-eluting, undetected matrix components such as humic and fulvic acids in the aqueous matrix may reduce or enhance the ion intensity of the analytes and affect the reproducibility and accuracy of the assay.[88] This effect is particularly pronounced when very complex samples such as wastewater are analyzed.

Table 3-2: Characteristic parameters of the calibration curve equations

Compound	R^2	Range (mg/L)	Slope	Intercept
Sulfanilamide	0.9993	0.5 -10	4.33	1.53
Sulfacetamide	0.9995	0.1-10	29.88	4.81
Sulfapyridine	0.9967	0.1-10	11.76	8.78
Sulfamerazine	0.9992	0.1-10	15.26	4.02
Sulfamethazine	0.9973	0.1-10	12.55	4.97
Sulfamethoxazole	0.9981	0.1-10	11.39	5.58
Sulfaisoxazole	0.9996	0.1-10	11.51	1.01
Sulfadimethoxine	0.9992	0.1-10	11.63	2.62

Table 3-3: Intra-day and inter-day precision for the analysis of the sulfonamides studied

Compound	Intra-day precision				Inter-day precision			
	Conc. µg/L	RSD	Conc. mg/ L	RSD	Conc. µg/L	RSD	Conc. mg/L	RSD
Sulfanilamide	500	5.1	10	1.0	500	7.5	10	1.0
Sulfacetamide	100	1.1	10	2.8	100	3.4	10	1.4
Sulfapyridine	100	2.6	10	1.7	100	2.9	10	1.4
Sulfamerazine	100	3.0	10	3.9	100	3.7	10	4.3
Sulfamethazine	100	2.7	10	3.5	100	9.8	10	3.4
Sulfamethoxazole	100	3.0	10	3.5	100	5.2	10	4.0
Sulfaisoxazole	100	2.7	10	4.5	100	10.6	10	5.1
Sulfadimethoxine	100	12.2	10	5.2	100	13.5	10	5.7

RSD: relative standard deviation (%)

Table 3-4: Recovery, LOD and LOQ of the studied sulfonamides

Compound	Recovery (% ± SD)	LOD (µg/L)[a]	LOQ (µg/L)[b]
Sulfanilamide	26.4 ± 0.5	2	6.7
Sulfacetamide	99.0 ± 4.7	2	6.7
Sulfapyridine	84.6 ± 1.1	0.6	2
Sulfamerazine	71.7 ± 0.7	2	6.7
Sulfamethazine	83.0 ± 3.6	0.6	2
Sulfamethoxazole	85.0 ± 3.7	0.6	2
Sulfaisoxazole	90.0 ± 1.8	2	6.7
Sulfadimethoxine	85.9 ± 0.8	2	6.7

SD: standard deviation.

[a]S/N ≥ 3 and [b] S/N ≥ 10

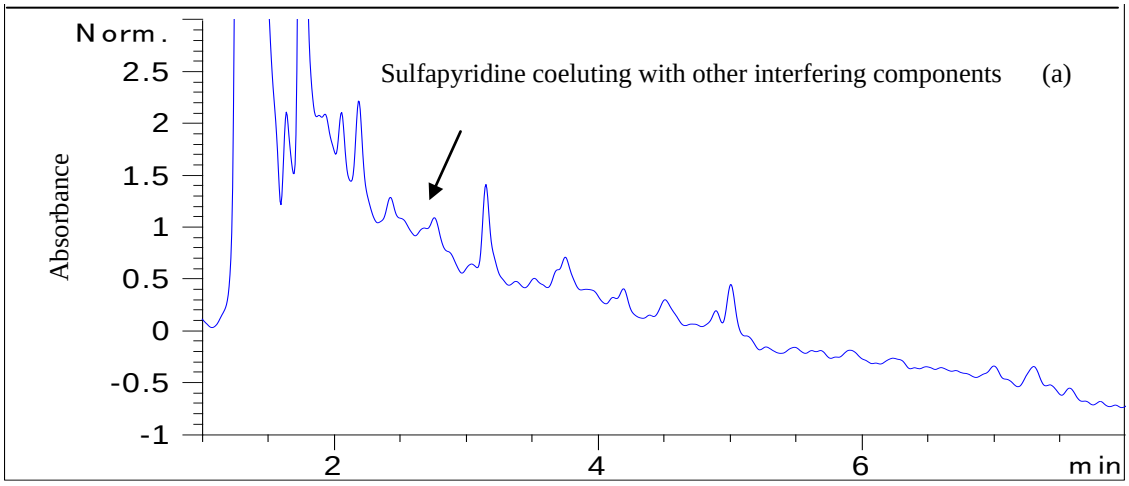
The limits of detection and quantitation could also be lowered by increasing the volume of the sample and/or reducing the volume of the solvent in which the dried extract was reconstituted. A reduction of the LOD and LOQ by at least an order of magnitude could be easily obtained by the combination of the two approaches if desired.

3.2.5.2.4 Accuracy

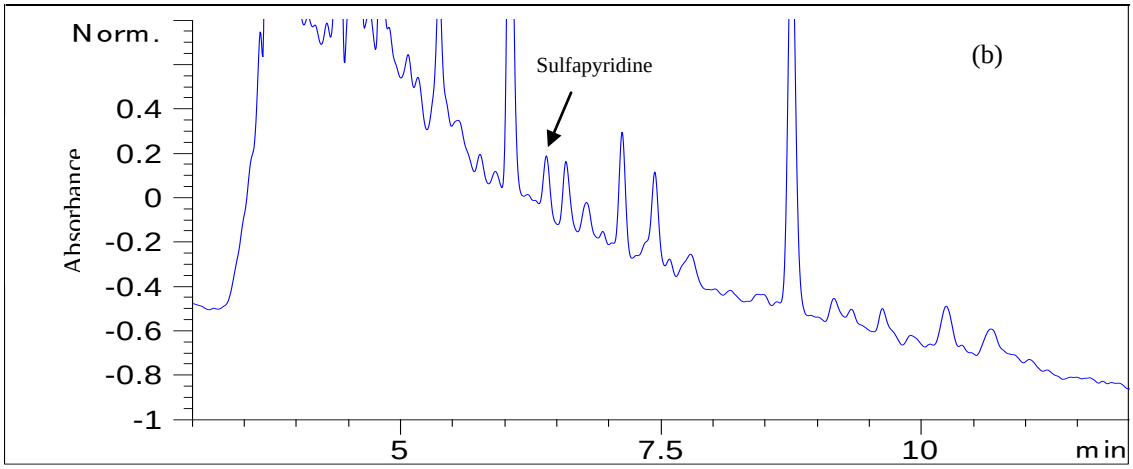
The recovery of the studied sulfonamides was evaluated by calculating the ratio of the peak area obtained from the extraction of spiked deionized water sample to the peak area of the corresponding standard solution at a concentration of 10 mg/L. The recoveries obtained are shown in **Table 3-4**. They fell within the range 71–99% for all analytes except sulfanilamide (26.5%). The recovery of sulfanilamide was low due to the fact that it has a high pK_a value of 10.43, hence should be extracted under alkaline conditions. However, if the extraction was performed under such conditions, the recoveries of other analytes would be reduced. The extraction was carried out in this study without adjusting the sample pH to accommodate all analytes studied. Low recoveries of sulphanilamide were documented in the literature under similar extraction conditions.[89]

3.2.5.3 Analysis of sulfonamides in wastewater

The method developed was successfully applied to the analysis of sulfonamides in real wastewater samples. The separation of the wastewater samples was initially carried out at 30 ºC using a single column at flow rate of 1 mL/min (**Figure 3-3 a**). The very complex wastewater sample was poorly resolved under such conditions, and the target analytes could not be detected. By coupling three columns at 80 ºC, both the efficiency and the resolution were enhanced, which allowed the detection of one of the target analytes (sulfapyridine) among the interfering



Retention time (min)



Retention time (min)

Figure 3-3: Chromatogram of wastewater sample showing (a) sulfapyridine (SPD) coeluting with other interfering components when using a single column at 30 ºC and (b) sulfapyridine (SPD) well separated from other interfering components when using three coupled columns at 80 ºC.

components (**Figure 3-3 b**). Its concentration was below the limit of quantitation. Other analytes were not detected. The presence of sulfapyridine was confirmed based on the retention time, UV spectrum and the increase in the response after spiking the sample with the standard solution of this analyte. While lower detection limits and better selectivity could likely be obtained by MS detection, matrix effect could adversely influence the analytical sensitivity and reliability of the method. In addition, this technique is more expensive and not available in many laboratories.

3.2.6 Conclusions

High-temperature HPLC is an attractive way to increase separation efficiency or reduce the analysis time. It offers many advantages such as decrease in the mobile phase viscosity and reduction in backpressure, allowing coupling of columns and the use of small size packing particles. Sub-2 µm particles offer many advantages, including enhanced efficiency, higher resolution and/or faster analysis. Efficiency can be significantly enhanced by coupling columns at elevated temperature and using conventional HPLC systems. The method is applicable to the analysis of environmental samples.

Chapter 4

High Temperature-High Efficiency Liquid Chromatography Using Coupled Columns Packed With Sub-2 µm Particles for The Analysis of Selected Non-steroidal Anti-inflammatory Drugs and Veterinary Antibiotics in Environmental Samples*

In the previous study, column coupling was successfully implemented for the analysis of eight sulfonamides in wastewater using isocratic mode of separation. The aim of this study was to illustrate the possibility of using coupled columns with gradient elution mode for enhancing the efficiency, as well as increasing the peak capacity in LC separation.

In this work, three columns packed with fully porous sub-2 µm particles were coupled at 80 °C and operated with gradient elution. The number of the analytes studied was extended to 24 pharmaceuticals, including sulfonamides and non-steroidal anti-inflammatory drugs. NSAIDs were selected because they are often detected in environmental samples. These drugs are available over-the-counter and can be used without prescriptions, resulting in their extensive consumption. Continual input of NSAIDs into the environment is harmful to the humans and wildlife.

The increase in peak capacity and enhancement in efficiency achieved by the developed method allowed 24 pharmaceuticals to be separated in the shortest possible analysis time (~28 min) on three coupled columns compared to a single column operated at 30 ºC. The studied analytes

* This chapter is based on a paper published in Analytica Chimica Acta[90]

could be separated from each other and from interferences in the complex wastewater samples when the method was applied to real samples.

4.1 Experimental

Analytes used in this study were non-steroidal anti-inflammatory drugs included acetaminophen, acetyl salicylic acid, ketoprofen, diflunisal, fenoprofen, oxaprozin, flurbiprofen, diclofenac, indomethacin, ibuprofen, phenyl butazone, mefenamic acid and meclofenamic acid. The sulfonamides studied included sulfaisomidine, sulfathiazole, sulfadiazine, sulfapyridine, sulfamerazine, sulfamethoxypyridazine, sulfamonomethoxine, sulfamethaxazole, sulfaisoxazole, sulfadimethoxine and sulfaquinoxaline. The chemical structures of the pharmaceuticals studied are given in **Figure 4-1**. The same columns were used as in the previous Chapter. A diode array detector (DAD) was used to detect each analyte at its maximum absorption wavelength. The standards were distributed into five concentrations ranging from 7 µg/L to 1000 µg/L. Analytes were separated by gradient elution using a mobile phase consisting of acetonitrile (solvent B) and ultrapure water containing 0.5% acetic acid (solvent A) at a flow-rate of 1.1 mL/min in gradient mode.

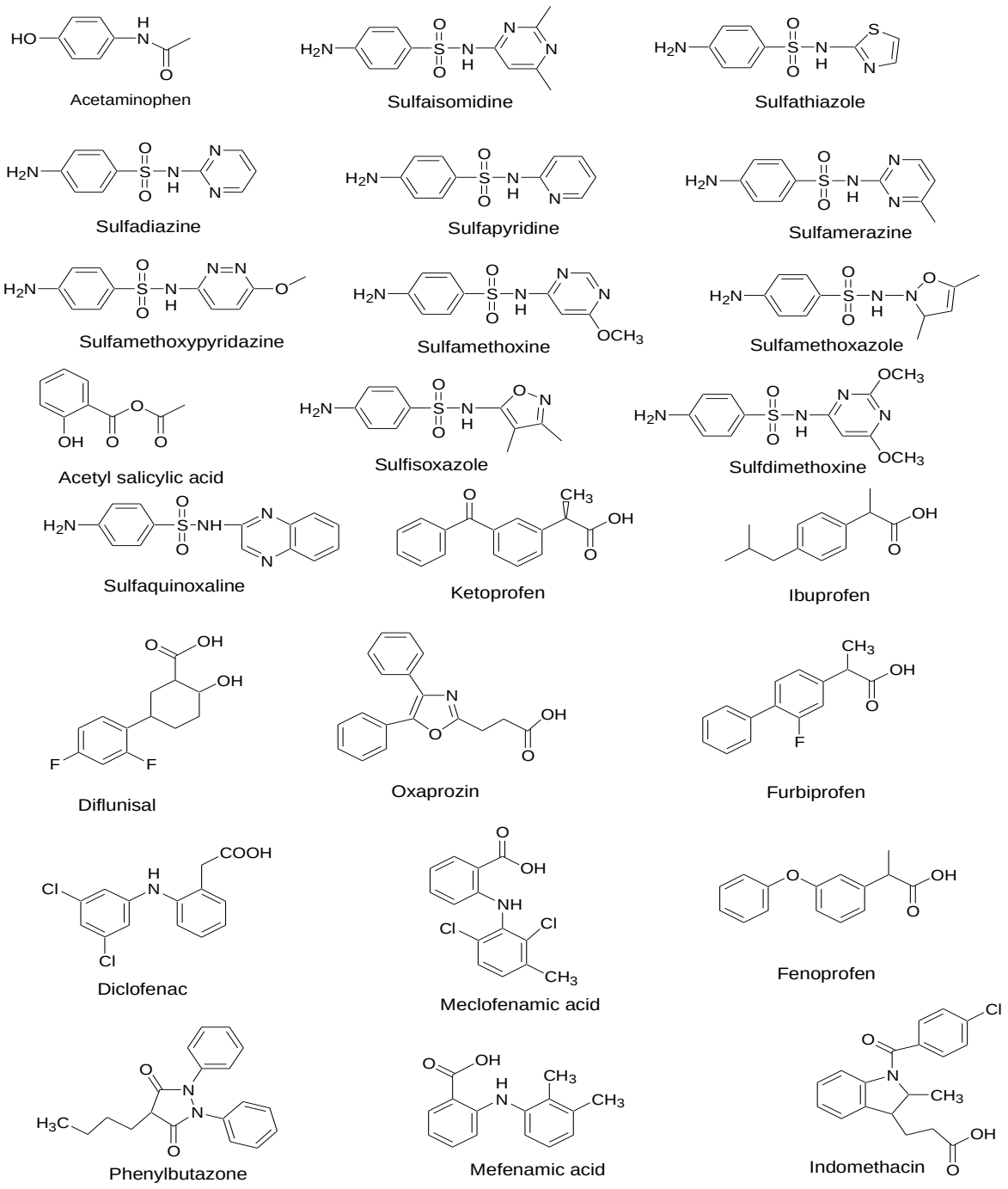


Figure 4-1: Chemical structures of the pharmaceuticals studied

4.2 Results and discussion

4.2.1 HPLC method

The columns used in this study had the same dimensions as in the previous Chapter. Standard size columns (4.6 mm I.D.) were selected to maintain high efficiency in addition to the previously discussed advantages of such columns. The mobile phase used in the analysis was not buffered, as crystallization of salt traces from the buffer in the column can reduce its separation capacity and shorten its lifetime. Acetic acid (0.5%) was added to the mobile phase instead to control the pH and enhance peak resolution. For higher sensitivity, each analyte was detected using DAD at its wavelength of maximum absorption. **Table 4-1** shows the wavelengths used for the detection of each analyte. An investigation to select the adequate composition of the mobile phase was performed. Gradient elution was used to obtain the best separation of the 24 analytes in the shortest possible time. In spite of numerous attempts, the whole suite of analytes could not be completely separated under any conditions when using a single column at 30 ºC. The optimal gradient used for the separation of the pharmaceuticals studied on three coupled columns (450 mm) at 80 ºC was 0 min, 22.5% acetonitrile; 4.1 min, 22.5% acetonitrile then increasing to 75% acetonitrile at 27.3 min. An equivalent gradient was then used for comparison with the single column at 30 ºC. The equilibration time was 10 min in all cases.

Table 4-1: Target analytes and relevant parameters for separation at 80 ºC

Analyte	**λ (nm)**	**R_t at 80** °C	**pKa**
Acetaminophen	247	4.8	9.5
Sulfisomidine	272	5.1	7.4
Sulfthiazole	272	5.4	7.2
Sulfadiazine	272	5.7	6.4
Sulfapyridine	272	6.4	8.4
Sulfamerazine	272	7.0	6.9
Sulfamethoxypyridazine	272	7.6	7.2
Sulfamonomethoxine	272	8.8	6.0
Sulfamethaxazole	272	10.1	5.9
Acetyl salicylic acid	230	10.7	2.97
Sulfisoxazole	272	11.2	4.6
Sulfadimethoxine	272	13.8	6.2
Sulfaquinoxaline	272	14.0	5.5
Ketoprofen	254	21.3	5.94
Diflunisal	254	21.9	3.3
Fenoprofen	272	24.1	4.5
Oxaprozin	279	24.5	4.3
Furbiprofen	247	24.7	4.2
Diclofenac	272	25.6	4.0
Indomethacin	272	25.8	4.5
Ibuprofen	225	26.6	4.4
Phenylbutazone	237	27.9	4.5
Mefenamic acid	279	28.1	4.2
Meclofenamic acid	279	28.4	3.7

4.2.2 Effect of temperature on chromatographic performance

At elevated temperature, the diffusivity and mass transfer of the solutes are enhanced as previously discussed. Alternatively, the low viscosity of the eluent allows higher flow rates with reasonable backpressure to be used to achieve fast analysis without a significant loss in resolution. In addition, the dielectric constant of the mobile phase in RP-LC also decreases with increasing temperature. This allows the reduction of the amount of organic modifier used in LC, making the analysis greener and saving solvent and disposal costs.[91] In this study, temperature was used as a variable parameter to enhance separation in HPLC. **Figure 4-2** shows the chromatograms obtained during both experiments for the test mixture under gradient conditions at a flow rate of 1.1 mL/min. At 30 ºC using a single column (**Figure 4-2 a**), coelutions of some analytes were observed. Complete coelution was observed between sulfthiazole and sulfadiazine; sulfadimethoxine and sulfaquinoxaline; diclofenac and indomethacin, as well as mefenamic acid and meclofenamic acid. These pairs of analytes eluted at retention times of 2.5, 6.5, 10.3 and 11.3 min, respectively. Also, partial coelutions were observed between acetaminophen and sulfisomidine, which eluted at 1.86 and 1.95 min, as well as oxoprozin and fenoprofen, which eluted at 9.65 and 9.74 min, respectively. Complete separation of all analytes could not be achieved on a single column at 30 ºC.

Elevated temperature had a great effect on selectivity and resolution. **Figure 4-2 b** shows the separation of the analytes at 80 ºC using three coupled columns. Under these conditions, complete separation of the critical pairs of acetaminophen and sulfisomidine; sulfathiazole and sulfadiazine; sulfadimethoxine and sulfaquinoxaline; diclofenac and indomethacin; mefenamic acid and meclofenamic acid, as well as oxoprozin and fenoprofen was accomplished with

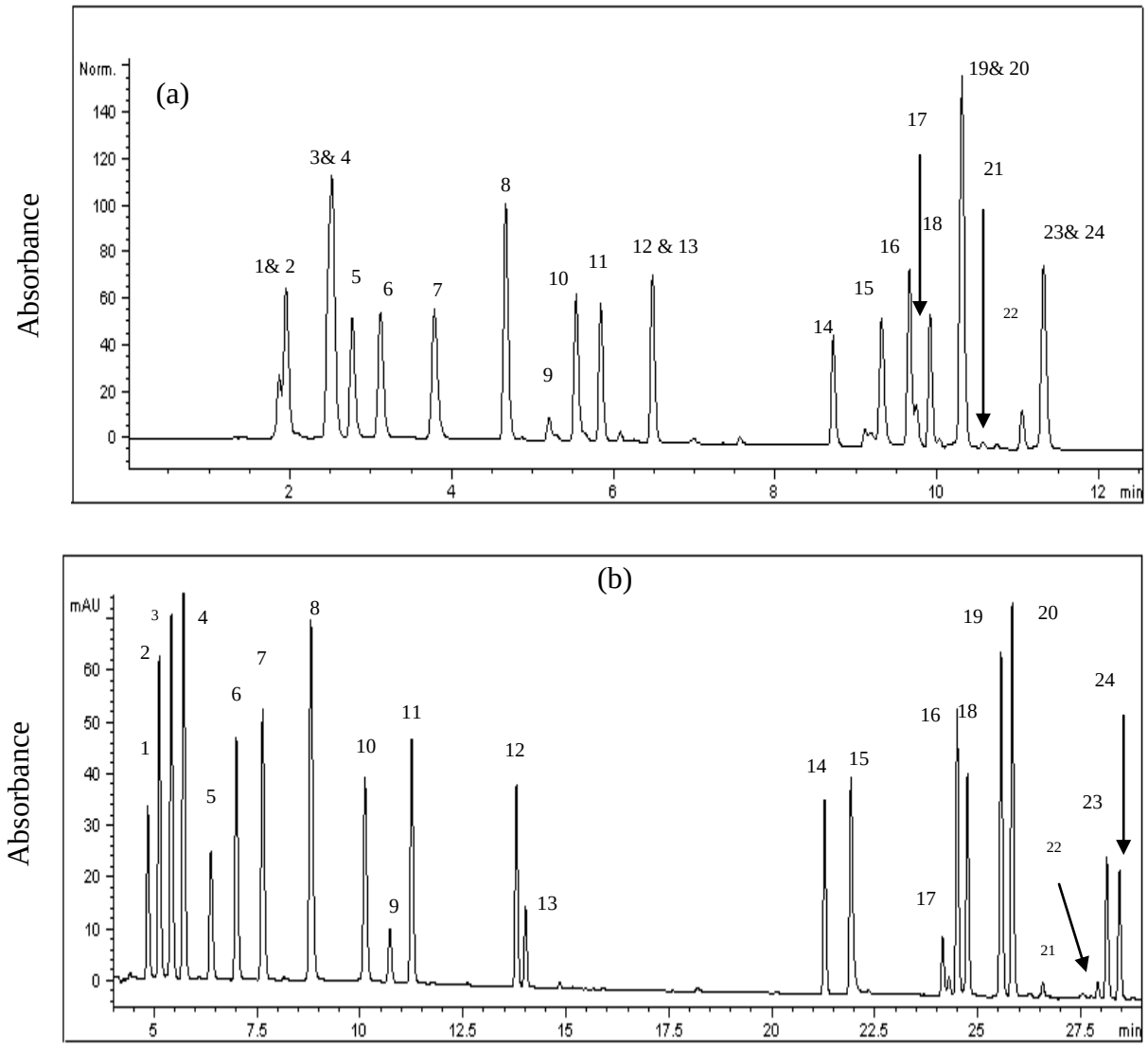


Figure 4-2: Chromatograms of test analytes at a detection wavelength of 272 nm and a flow rate of 1.1 mL/min. (A) One column at 30 ºC; (B) Three columns at 80 ºC. Peak identification: 1 - acetaminophen, 2 - sulfisomidine, 3 - sulfthiazole, 4 - sulfadiazine, 5 - sulfapyridine, 6 - sulfamerazine, 7 - sulfamethoxypyridazine, 8 - sulfamonomethoxine, 9 - acetyl salicylic acid, 10 - sulfamethaxazole, 11 - sulfaisoxazole, 12 - sulfadimethoxine, 13 - sulfaquinoxaline, 14 - ketoprofen, 15 - diflunisal, 16 - oxaprozin, 17 - fenoprofen, 18 - flurbiprofen, 19 - diclofenac, 20 - indomethacin, 21 - ibuprofen, 22 - phenyl butazone, 23 - mefenamic acid, 24 - meclofenamic acid.

resolution of 4.1, 4.1, 3.3, 3.9, 4.3 and 4.9, respectively. All the analytes studied (24 pharmaceuticals) were separated in 28.4 min. The retention times of the analytes recorded at 80 ºC on three coupled columns at 1.1 mL/min mobile phase flow rate are listed in **Table 4-1**. The effect of elevated temperature on selectivity manifested itself particularly strongly through the change in elution order observed between acetyl salicylic acid (peak 9) and sulfamethaxazole (peak 10), as well as oxaprozin (peak 16) and fenoprofen (peak 17) at 80 ºC. Extending the column length at high temperature had also a significant effect on peak capacity. The peak capacities at 30 ºC on a single column (150 mm) and at 80 ºC on three coupled columns (450 mm) were calculated using equation $Pc = 1 + \frac{t_g}{\bar{w}}$ (8)[15] where t_g is the gradient time and $\bar{w}$ is the average peak width at the base in time units. The peak capacity increased from 77 on the single column (150 mm) at 30 °C to 158 on the three coupled columns (450 mm), an increase by a factor of ~2. On the other hand, the rate at which the peak capacity was generated (total peak capacity divided by the separation time) decreased marginally when using three coupled columns (5.6 vs. 6.4 at 30 ºC).

Elevated temperature had a great effect on column backpressure. The initial backpressure at 30 ºC using a single column at a flow rate of 1.1 mL/min was 445 bar, while at 80 ºC and the same column length and flow rate it was only 192 bar, which allowed up to 3 columns to be coupled at this temperature. The initial backpressure across three columns packed with sub-2 µm particles operated at 80 ºC and 1.1 mL/min was 575 bar. A maximum number of three columns could be

coupled in this study due to instrumental limitations, as the system operated close to its maximum allowable pressure of 600 bar.

4.2.3 Validation of the method

The SPE–HPLC method was validated based on parameters such as linearity, precision, detection and quantification limits, selectivity and accuracy. The linearity of the method was evaluated using deionized water spiked with the analytes in the concentration range of 7–1000 µg/L. The characteristic parameters of the regression equations for the analytes studied are given in **Table 4-2**. Precision was validated based on the evaluation of intra- and inter-day repeatability of the method. Satisfactory results were achieved for all analytes. The inter-day repeatability RSDs ranged from 1.1% to 7.4%, and the intra-day repeatability ranged from 1.3% to 10.5%, except acetyl salicylic acid (which had very low recovery). The results of the intraday and inter-day repeatability experiments expressed as relative standard deviations are summarized in **Table 4-3**.

The detection and quantitation limits determined in the study are given in **Table 4-4**. LODs of the method ranged from 2 to 16 µg/L, while LOQs were in the range of 7 to 54 µg/L with UV detection. The low limits of detection confirmed the applicability of the proposed method to the analysis of real samples. The detection range of single µg/L has often been reported in studies based on using UV detection for the determination of pharmaceuticals in environmental samples.[92-98] Mass spectrometry detection could allow decreasing these limits even further; however, matrix effects (signal suppression or enhancement by the sample matrix that may occur

Table 4-2: Characteristic parameters of the calibration curve equations of the analytes studied

Analyte	R^2	Slope	Intercept
Acetaminophen	0.998	11.0	25.4
Sulfisomidine	0.994	2.1	11.9
Sulfthiazole	0.998	8.5	12.0
Sulfadiazine	0.999	3.6	5.4
Sulfapyridine	0.991	5.5	11.8
Sulfamerazine	0.998	3.2	26.9
Sulfamethoxypyridazine	0.998	3.0	16.1
Sulfamonomethoxine	0.992	2.8	17.2
Sulfamethaxazole	0.995	2.5	20.4
Sulfisoxazole	0.998	3.4	7.5
Sulfadimethoxine	0.996	1.9	12.2
Sulfaquinoxaline	0.995	1.0	7.6
Ketoprofen	0.997	3.1	12.1
Diflunisal	0.995	1.1	4.5
Fenoprofen	0.999	1.4	12.9
Oxaprozin	0.999	2.1	10.5
Flurbiprofen	0.999	1.3	8.2
Diclofenac	0.998	1.7	10.7
Indomethacin	0.997	2.7	7.4
Ibuprofen	0.996	1.4	7.0
Phenyl butazone	0.995	0.9	6.1
Mefenamic acid	0.994	1.7	21.9
Meclofenamic acid	0.996	1.1	6.5

Table 4-3: Intra-day precision and inter-day precision of the studied analytes

Analyte	**Intra-day precision (RSD)**		**Inter-day precision (RSD)**	
	100 µg/L	**1000 µg/L**	**100 µg/L**	**1000 µg/L**
Acetaminophen	4.5	4.8	6.3	5.5
Sulfisomidine	3.8	2.7	7.2	4.1
Sulfthiazole	4.6	1.1	8.3	1.4
Sulfadiazine	4.0	1.6	6.5	1.9
Sulfapyridine	4.4	1.3	3.8	2.2
Sulfamerazine	2.2	2.1	7.7	2.4
Sulfamethoxypyridazine	3.7	1.5	7.1	1.6
Sulfamonomethoxine	3.5	2.6	6.3	3.2
Sulfamethaxazole	7.4	2.0	10.5	2.1
Acetyl salicylic acid	11.7	7.1	15.3	8.9
Sulfisoxazole	3.8	1.5	6.7	1.9
Sulfadimethoxine	3.8	1.0	4.9	1.3
Sulfaquinoxaline	2.8	1.4	7.9	1.6
Ketoprofen	6.2	1.7	6.8	2.1
Diflunisal	4.4	2.6	5.0	2.8
Fenoprofen	4.6	3.4	7.7	4.8
Oxaprozin	2.4	2.8	4.1	5.6
Flurbiprofen	2.8	2.6	8.6	6.0
Diclofenac	4.0	1.7	9.3	2.1
Indomethacin	3.8	2.1	7.2	5.5
Ibuprofen	4.6	3.9	6.3	4.9
Phenyl butazone	2.8	2.9	3.8	6.4
Mefenamic acid	4.4	6.4	4.9	7.1
Meclofenamic acid	3.8	3.4	7.2	4.8

*RSD: relative standard deviation (%)

Table 4-4: Recovery, LOD and LOQ of the studied analytes

Compound	Recovery (% ± SD)		LOD (µg/L)[a]	LOQ (µg/L)[b]
	100 µg/L	1000 µg/L		
Acetaminophen	30.7 ± 1.2	31.3 ± 1.3	9	30
Sulfisomidine	95.4 ± 4.0	95.6 ± 2.3	3	10
Sulfthiazole	92.8 ± 1.7	93.4 ± 1.3	9	30
Sulfadiazine	88.2 ± 2.3	94.2 ± 3.1	7	23
Sulfapyridine	92.3 ± 5.3	95.0 ± 2.8	3	10
Sulfamerazine	83.6 ± 2.9	89.9 ± 2.4	2	7
Sulfamethoxypyridazine	89.8 ± 3.1	93.6 ± 2.7	7	23
Sulfamonomethoxine	86.7± 2.1	90.4 ± 4.0	7	23
Sulfamethaxazole	95.0 ± 4.5	96.0 ± 2.2	4	13
Acetyl salicylic acid	9.7 ± 4.5	10.1 ± 4.1	16	54
Sulfisoxazole	95.3 ± 2.6	95.9 ± 2.4	7	23
Sulfadimethoxine	97.0 ± 4.0	98.2 ± 2.1	4	13
Sulfaquinoxaline	88.9 ± 5.2	87.4 ± 2.8	3	10
Ketoprofen	88.7 ± 4.9	90.2 ± 5.5	8	26
Diflunisal	75.8 ± 5.2	79.1 ± 1.5	8	26
Fenoprofen	90.9 ± 6.1	90.7 ± 5.6	7	24
Oxaprozin	73.9 ± 4.7	74.7 ± 3.7	3	10
Flurbiprofen	92.7 ± 3.9	93.9 ± 1.6	3	11
Diclofenac	89.9 ± 3.2	93.0 ± 2.4	12	39
Indomethacin	72.9 ± 3.9	72.7 ± 4.2	11	37
Ibuprofen	91.4 ± 2.5	92.6 ± 2.9	9	30
Phenyl butazone	76.7 ± 4.6	77.5 ± 4.3	16	54
Mefenamic acid	75.0 ± 5.0	75.2 ± 3.5	14	46
Meclofenamic acid	73.2 ± 2.8	74.3 ± 2.8	15	50

SD: standard deviation.
[a] $S/N \geq 3$
[b] $S/N \geq 10$

in the ionization process) might be a serious issue. Also, matrix components such as humic and fulvic acids might affect the reproducibility and accuracy of the assay.[59] These effects are particularly pronounced when very complex samples such as wastewater are analyzed. As was mentioned before, the detection and quantitation limits could also be lowered by increasing the volume of the sample and/or reducing the volume of the solvent in which the dried extract was reconstituted. The recoveries of sulfonamides were evaluated at two concentration levels using Oasis HLB cartridges.

The recoveries were within the range of 72.7% to 98.2% with SDs not higher than 6%, except for acetaminophen and acetyl salicylic acid. The recoveries of the analytes using Oasis HLB cartridges without adjusting the pH are listed in **Table 4-4**. The recovery of acetaminophen was low due to the fact that it has a pKa value of 9.5, thus it should be processed under alkaline conditions. However, if the extraction was performed under such conditions, the recoveries of the remaining analytes would be reduced. The low recovery found for acetaminophen was also reported in other studies carried out with Oasis HLB cartridges.[99, 100] On the other hand, acetyl salicylic acid has a pK_a value of 2.97, which requires strongly acidic conditions. However, adjusting the pH to a low value could again result in reduced recoveries of the remaining analytes. Carrying out the extraction without adjusting the sample pH was a compromise solution to accommodate as many analytes as possible. The low recovery of acetyl salicylic acid (~10%) introduced high uncertainty in its quantitation at very low concentrations, but detection was still possible.

4.2.4 Analysis of real wastewater samples

The applicability of the proposed method for the determination of the most commonly used non-steroidal anti-inflammatory drugs and sulfonamides in real samples was tested by analyzing raw influent wastewater from a treatment plant. **Figure 4-3** shows the chromatograms obtained on three coupled columns at 80 ºC. Three of the studied analytes could be detected. Acetaminophen was detected at its maximum absorption wavelength of 247 nm (**Figure 4-3 a**), while sulphapyridine and sulphamethoxazole were detected at 272 nm (**Figure 4-3 b**). Acetaminophen was found at a concentration of 30 µg/L, while the concentrations of sulphapyridine and sulphamethoxazole were below their LOQs. The identities of these pharmaceuticals were confirmed as in the previous chapter. Other analytes were not detected. While lower detection limits and better selectivity could likely be obtained by MS detection, matrix effect could adversely influence the analytical sensitivity and reliability of the method. In addition, this technique is not available in many laboratories due to its high cost.

4.3 Conclusions

Temperature is an important parameter in HPLC. Elevated temperature can be used to decrease the analysis time, change the selectivity of the system and reduce the viscosity of the mobile phase allowing coupling of multiple columns packed with small particles. Extending the column length at elevated temperatures allows the generation of a large number of theoretical plates, thus enhancing the efficiency in HPLC. The approach of coupling many columns at high temperature is applicable to both isocratic and gradient modes of separation. Enhanced efficiency at elevated

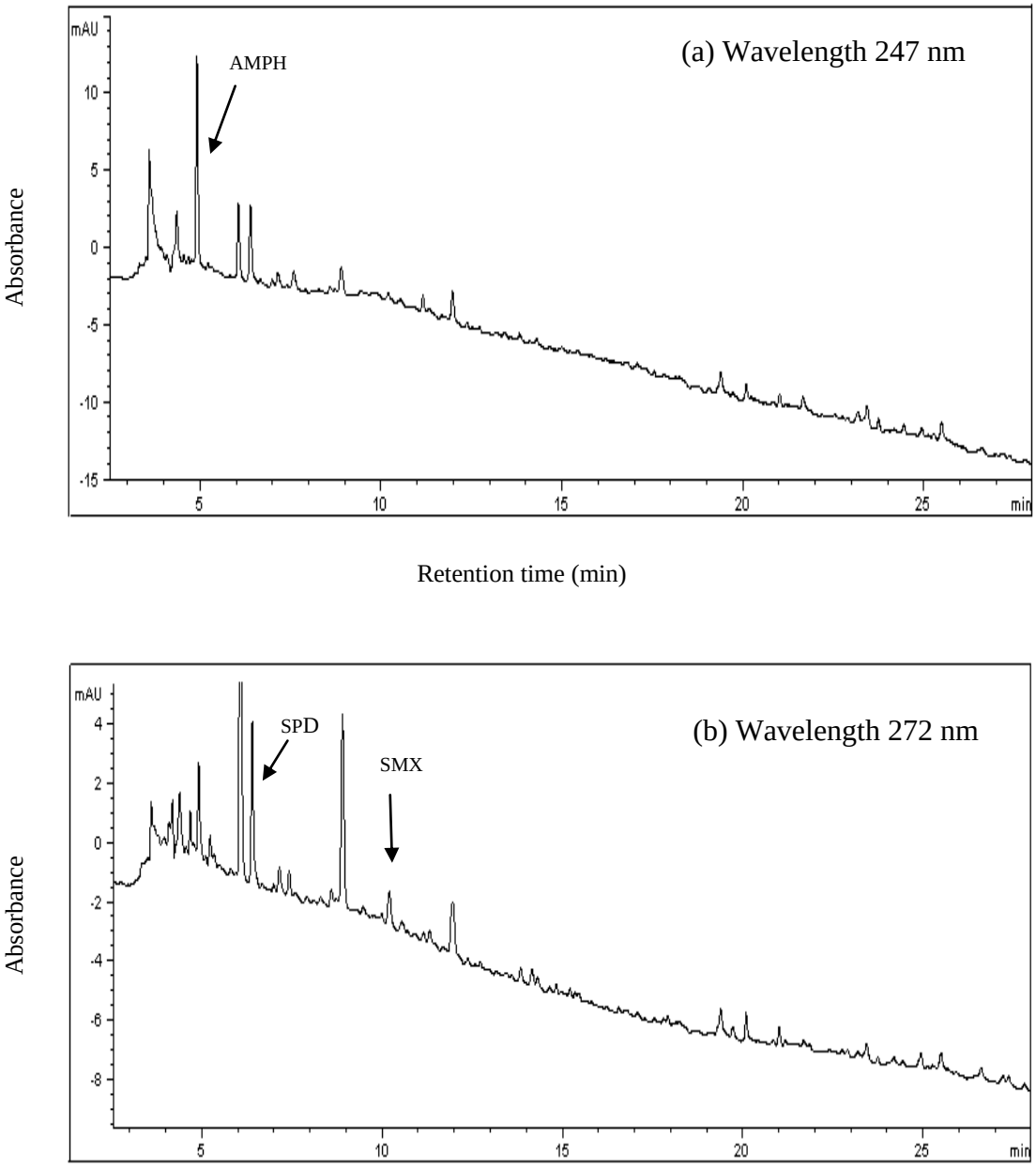


Figure 4-3: Chromatograms of wastewater sample extract analyzed at 80 ºC using three coupled columns showing (A) acetaminophen (AMPH) at a detection wavelength of 247 nm, and (B) sulphapyridine (SPD) and sulphamethoxazole (SMX) at 272 nm.

temperature facilitates the analysis of non-steroidal anti-inflammatory drugs and veterinary antibiotics in a very complex wastewater matrix. The proposed method was capable of determining 24 pharmaceuticals simultaneously in wastewater in a reasonable analysis time.

Chapter 5

Optimization and Validation of a Fast, Ultrahigh Pressure Liquid Chromatographic Method for Simultaneous Determination of Selected Sulfonamides in Water samples Using a Column Packed With Fully Porous Sub-2 µm Particles at Elevated Temperature*

In the two previous studies, LC methods based on coupling columns at elevated temperature operated in both isocratic and gradient modes were developed to enhance the separation efficiency. In addition to the importance of developing highly efficient methods, high speed analytical methods are also needed. Cost effective methodologies with reduced analysis time are required in many laboratories operating in various areas such as environmental, pharmaceuticals, toxicology, forensics and food analysis.

Most methods used for the analysis of pharmaceuticals in water rely on HPLC with standard columns packed with 5 µm particles for analyte separation. Analysis times of tens of minutes are not unusual. This leads to the consumption of significant amounts of the organic modifier, which must be properly disposed of after the analysis. Thus, alternative approaches allowing the reduction of the amount of the organic modifier without the loss in chromatographic performance are highly desirable.

Reducing the analysis time can be achieved by different approaches, such as using monolithic columns, columns packed with fully porous sub-2 µm particles, columns packed with superficially porous particles and high mobile phase temperature. These approaches gain a wide

*This chapter is based on a paper published in Journal of Separation Science[101]

interest among researchers, especially with the availability of very high pressure UPLC systems. However, these approaches are not extensively used in the area of environmental analysis. Despite the advantage of using temperature as a parameter to optimize LC separations, its use in environmental analysis is very limited.

The aim of this work was to develop a fast LC method to separate sulfonamides in surface water and wastewater in a very short analysis time while maintaining high efficiency. The method was based on using fully porous sub-2 µm particles in combination with high mobile phase temperature and operating the system at high flow rates. In this study, the effects of changing the solvent temperature on the system backpressure and chromatographic performance were studied, as well as the effect of changing the organic modifier concentration and the flow rate. The developed method was applied for the analysis of sulfonamides in surface water and wastewater after extraction using SPE method.

This method is the first application of fully porous sub-2 µm particles in combination with elevated temperature in the environmental analysis of pharmaceuticals. It illustrates the advantages of using temperature as a tool to optimize the separation through its effect on solvent viscosity, allowing the use of high flow rates while maintaining the efficiency. Achieving the separation in less than 10 min is considered fast analysis[45] The developed method offers many advantages such as shorter analysis time (3 min) compared with any other published HPLC-UV methods used to determine sulfonamides in environmental samples (e.g.[85, 89, 90]), lower solvent consumption making the analysis greener, and lower labor cost. The method developed could be used as an alternative to other HPLC-UV methods for the analysis of sulfonamides.

5.1 Experimental

Analytes used in this study were sulfonamides including sulfanilamide, sulfisomidine, sulfacetamide, sulfadiazine, sulfapyridine, sulfamerazine, sulfamethoxypyridazine, sulfamethazine and sulfamonomethoxine. The chemical structures and pK_a values of the sulfonamides studied are given in **Table 5-1**.[89, 90] The columns used were the same as in Chapter 3. The detection wavelength was set at 272 nm. Stock solutions of the analytes were prepared by dissolving 10 mg of each standard in 10 ml methanol. The standards were distributed into five concentrations ranging from 5 µg/L to 30 mg/L. The chromatographic separations were performed in isocratic mode using a mobile phase consisting of 80 % water containing 0.5% acetic acid and 20 % acetonitrile, at a flow rate of 1.5 mL/min.

5.1.1 Sample preparation and extraction

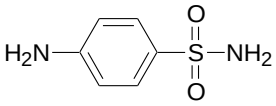
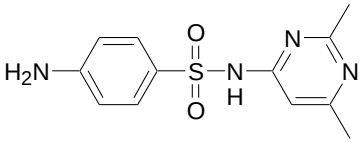
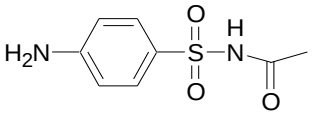
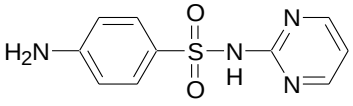
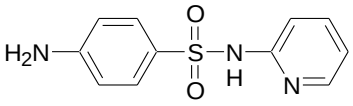
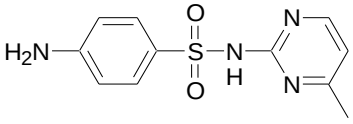
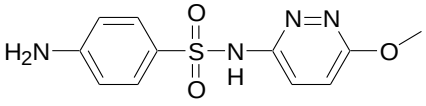
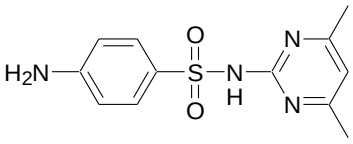
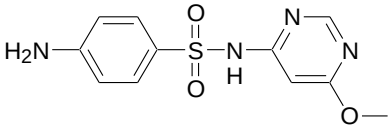
In this study, both Oasis HLB and SampliQ OPT cartridges were used for sample extraction. For extraction of real water samples, 500 mL of river and lake water were extracted and 150 mL of wastewater sample was used in order to avoid blocking of the SPE sorbents. The extracted samples were evaporated and dissolved in 0.5 mL of the mobile phase to increase the sensitivity.

5.2 Results and discussion

5.2.1 Optimization of the chromatographic conditions

The most important aspect of method development in liquid chromatography is the achievement of sufficient resolution ($R \geq 1.5$) of all the studied analytes, short analysis time and high sensitivity with low solvent consumption. These goals can be achieved by adjusting different chromatographic factors to give the desired response. The main analytical parameters to be

Table 5-1: Characteristics of the sulfonamides studied.

Analyte	Abbreviation	Chemical structures	pKa
Sulfanilamide	SNM		10.4
Sulfisomidine	SIM		7.5
Sulfacetamide	SAM		5.4
Sulfadiazine	SDZ		6.5
Sulfapyridine	SPD		8.4
Sulfamerazine	SMR		7.1
Sulfamethoxypyridazine	SMX		7.2
Sulfamethazine	SMZ		7.4
Sulfamonomethoxine	SMM		6.0

optimized are the stationary phase and mobile phase composition, flow rate and column temperature.

5.2.1.1 Selection of the analytical column

In this study, columns packed with sub-2 µm particles were selected for the chromatographic separation of the sulfonamides studied because they allow one to achieve higher analysis speeds compared to standard 3-5 µm particles without loss of resolution.[7] Smaller particles result in flatter van Deemter curves, allowing higher flow rates to be used while maintaining good efficiency. A 150 mm long column was selected for this study to maximize the efficiency. Although there is an on-going shift to narrower diameter columns, 4.6 mm I.D. columns were used. The relative merits of small vs. standard diameter columns were discussed in the previous chapter.

One of the problems that have to be dealt with when operating columns at very high pressures is frictional heating. This effect is more pronounced when wide bore columns (e.g. 4.6 mm ID) are operated at elevated pressures, leading to potential efficiency loss due to temperature gradients inside the column. In this study, the column was operated at pressures limited to the maximum system pressure (600 bar), at which frictional heating is not considered to be significant. Consequently, thermal gradients inside the column were not expected to affect the efficiency significantly.

5.2.1.2 Mobile phase composition and effect of organic modifier percentage on retention time

The mobile phase was not buffered in the study, as crystallization of salt traces from the buffer in the column can reduce its separation capacity and shorten its lifetime. Acetic acid

(0.5%) was added to the mobile phase to enhance peak resolution. In the absence of the acid, lower resolution between SIM and SAM was observed. This was first established in an earlier study.[102] An investigation to select the adequate composition of the mobile phase was performed and different mobile phase compositions were tested to obtain the best separation of the studied sulfonamides in the shortest possible time. **Figure 5-1** shows the effect of acetonitrile content in the mobile phase on the retention of the compounds studied. All analytes could be separated in 2.2 min with the mobile phase containing acetonitrile /water (25:75% v/v); however, poor resolution between SNM and SIM peaks was observed. Mobile phase composition of 20% acetonitrile allowed all analytes to be baseline separated with good resolution in 3 min. The method developed has the highest performance in terms of analytical speed compared to other published HPLC-UV methods for the determination of sulfonamides.[103-105] The isocratic mode of separation eliminated the need for re-equilibration, minimizing the turn-around time**.** However, in cases when not all matrix components elute from the column during the time required for the separation of the target analytes, gradient elution might be a better alternative.

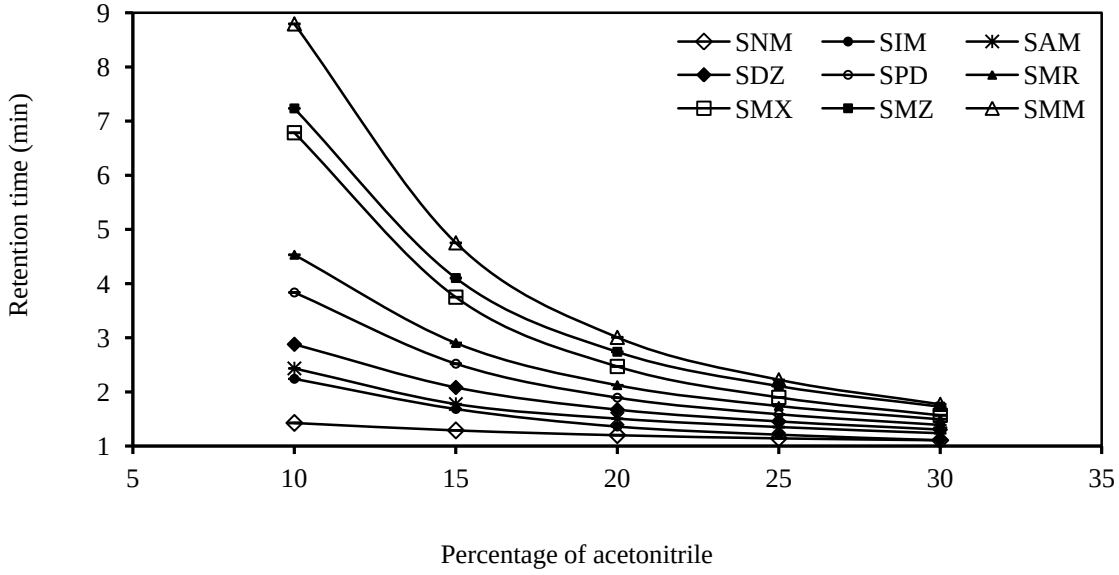


Figure 5-1: Effect of acetonitrile percentage on the retention times of the sulfonamides studied at a temperature of 60 ºC and a flow rate of 1.5 mL/min. Error bars indicate one standard deviation.

5.2.1.3 Effect of temperature on retention time

The effect of temperature on retention times of the sulfonamides was investigated using a temperature range of 30-80 ºC under a mobile phase composition of acetonitrile and water containing 0.5% acetic acid (20:80 v/v) at flow rate of 1.5 mL/min. As shown in **Figure 5-2 a,** retention times decreased with an increase in temperature. The changes in retention followed the expected trends, as illustrated by the van't Hoff plots shown in **Figure 5-2 b**. The effect of temperature on the retention time is discussed in more detail in the literature.[3, 20]

Temperature can also be used to tune selectivity during the development of the analytical method because the enthalpic and entropic contributions of the interaction between the analyte and the stationary phase might have different magnitudes for different analytes, hence the rate of change of the magnitude of the partition coefficients with temperature might also be different. This can be clearly seen in **Figure 5-2 a**, where sulfamethazine is well separated from sulfamonomethoxine but nearly coelutes with sulfamethoxypyridazine at 30 °C, while at 80 °C the opposite is true. Complete separation of the three analytes can be easily achieved at intermediate temperatures, with the optimum around 60 °C. The retention time, capacity factor, resolution and selectivity of the studied sulfonamides under the optimized conditions at 60 °C are presented in **Table 5-2**. At this temperature, complete separation of all analytes was achieved in 3 minutes.

One major concern for performing separations at elevated temperature is analyte stability. Thompson and Carr[106] demonstrated that decreasing the residence time of thermally labile compounds in the column might decrease the extent of on-column reactions and eliminate analyte degradation. The evidence for the absence of on-column degradation reactions was obtained by observing the normalized peak area for each analyte at each temperature. A change in the normalized peak area (peak area × flow rate) with column residence time for a given solute would indicate on-column degradation. In this study, no significant changes in the normalized peak area were observed for all solutes. Evidence for on-column reactions can also be obtained by observing peak shape; again, the peak shapes did not indicate the occurrence of degradation. In addition, no additional peaks were observed at elevated temperatures. Overall, the shortest analysis time could be obtained at a temperature of 70 ºC, at which the separation

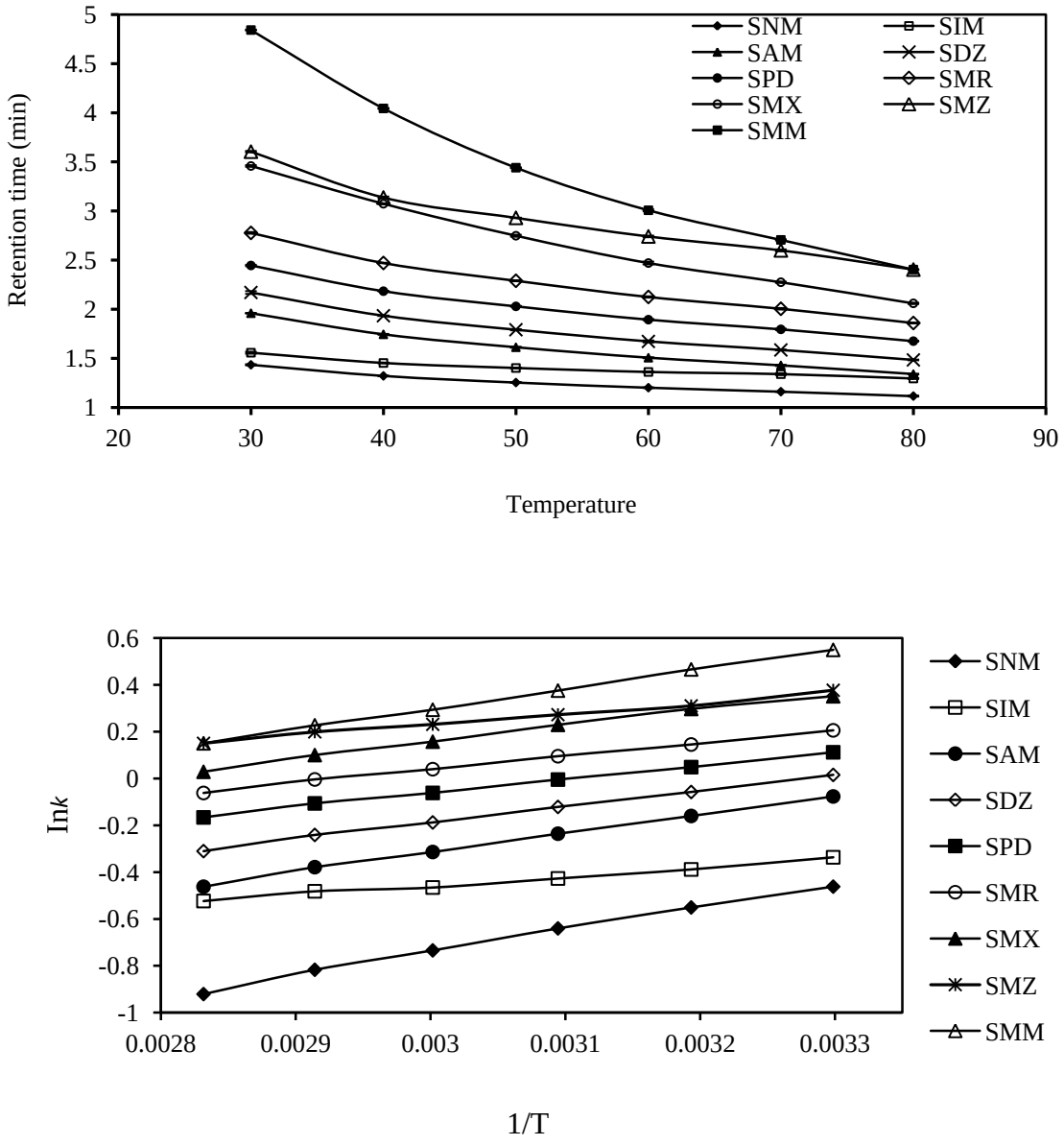


Figure 5-2: Effect of temperature on the retention times of the analytes: (a) retention times as a function of temperature; (b) van't Hoff plots. T is absolute temperature (in Kelvin) and *k* is capacity factor. Error bars indicate one standard deviation.

Table 5-2: Chromatographic parameters (retention time, capacity factor, resolution and selectivity) for the optimized method.

Analyte	**t_R ± S.D.**	k'	R_s	A
SNM	1.20 ± 0.005	0.26	5.5	1.67
SIM	1.37 ± 0.010	0.43	4.5	1.35
SAM	1.51 ± 0.009	0.58	5.1	1.30
SDZ	1.68 ± 0.012	0.76	6.5	1.31
SPD	1.90 ± 0.015	0.99	6.5	1.25
SMR	2.14 ± 0.017	1.24	9.3	1.30
SMX	2.49 ± 0.023	1.61	6.8	1.18
SMZ	2.76 ± 0.025	1.89	6.4	1.15
SMM	3.03 ± 0.029	2.17		

t_R: retention time; S.D: standard deviation; k': capacity factor; R_s: resolution; α: selectivity

was completed in 2.2 min. However, 60 ºC was considered as the optimum and selected for the separation of the sulfonamides studied to ensure complete resolution of all analytes from each other and from the matrix components when the method is applied to real water samples.

5.2.1.4 Effect of temperature on the system backpressure

The system backpressure (ΔP) was measured as a function of temperature (ranging from 30 to 80 ºC) using a mobile phase consisting of acetonitrile and water containing 0.5% acetic acid (20:80 v/v) at a flow rate of 1.5 mL/min. As shown in **Figure 5-3**, by increasing the temperature from 30 ºC to 80 ºC, the system backpressure was decreased from 575 bar to 306 bar. At 60ºC the backpressure was 386 bar, which allows the analysis to be performed with conventional

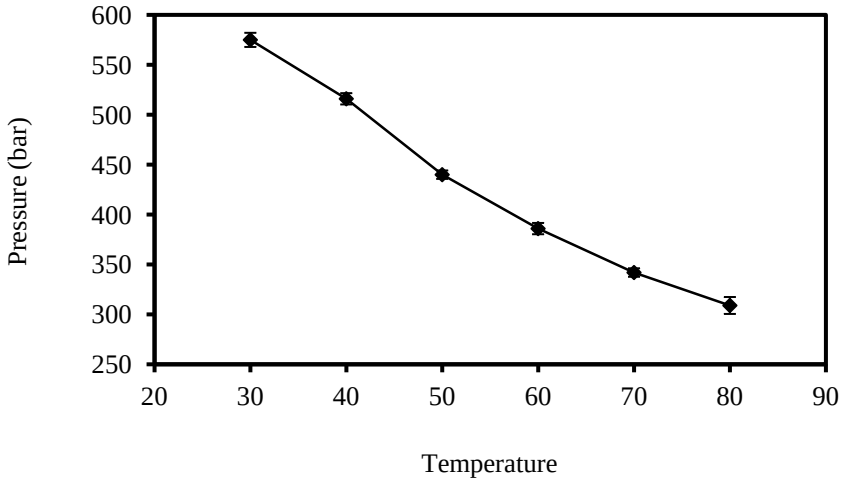


Figure 5-3: Effect of temperature on system backpressure using a mobile phase consisting of 20:80 (v/v) acetonitrile:water containing 0.5% acetic acid at a flow rate of 1.5 mL/min. Error bars indicate one standard deviation.

HPLC instrumentation. The reduction in the backpressure at elevated temperature was due to the decrease in the mobile phase viscosity according to Darcy's law.[12] The higher mobile phase flow rates made possible by the reduction in the backpressure decrease the contribution of longitudinal diffusion to band broadening since the analyte spends less time in the column. This, in turn, allows the separation to be performed faster without compromising the efficiency.[13]

5.2.1.5 Effect of the flow rate on retention time

The effect of mobile phase flow rate on the retention times of the analytes was studied at 60 ºC using the optimized mobile phase composition. By increasing the flow rate, the retention times were decreased equally for all analytes, as shown in **Figure 5-4**. All the analytes could be completely separated with high resolution (> 1.5) at a flow rate of 1.5 mL/min, at which the analysis time was 3 min. The analysis time could be decreased even further by using

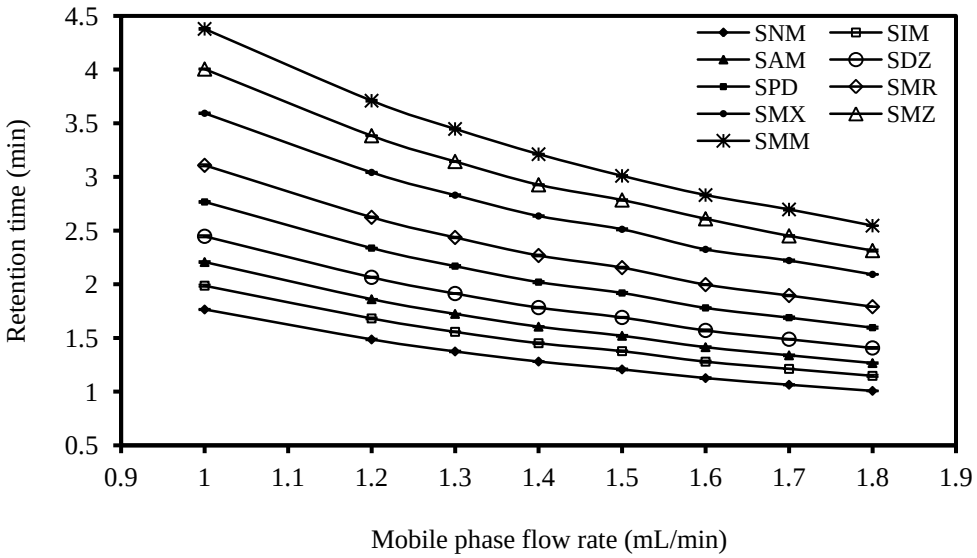


Figure 5-4: Effect of mobile phase flow rate on the retention time of the sulfonamides studied at 60 ºC. Error bars indicate one standard deviation.

higher flow rates, such as 1.7 mL/min, at which the total analysis time was 2.5 min. A flow rate of 1.5 mL/min was selected for the analysis to ensure higher resolution of all analyte from each other and from the interfering components when the method is applied to real water samples. The final optimum conditions were obtained with the mobile phase consisting of acetonitrile and water containing 0.5% acetic acid in a ratio of 20:80 (v/v) at a flow rate of 1.5 mL/min at 60 ºC. Under these conditions, all the analytes were separated in 3 minutes, as shown in **Figure 5-5**.

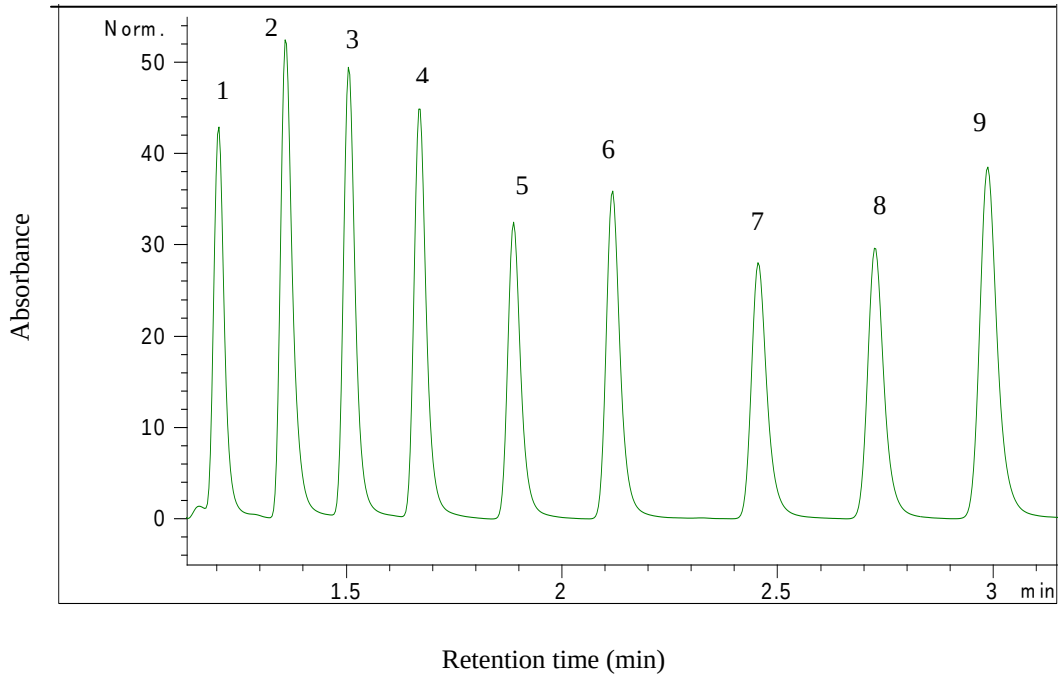


Figure 5-5: Chromatogram of the sulfonamides studied. Chromatographic conditions: acetonitrile:water containing 0.5% acetic acid (20:80 v/v) at 60 ºC and a flow rate of 1.5 mL/min with UV detection at 272 nm. Peak identification:1- SNM, 2- SIM, 3- SAM, 4- SDZ, 5- SPD, 6- SMR, 7- SMX, 8- SMZ, 9- SMM.

5.2.2 SPE optimization

In this study both Oasis HLB and SampliQ OPT cartridges were evaluated. From the comparison of two tested SPE cartridges, the highest average recovery was achieved with Oasis HLB cartridges. With this sorbent, the recovery ranged from 70.6 % to 96 % for all analytes except SNM, which was characterized by low recovery (31%). Compared to Oasis HLB, SampliQ OPT cartridge revealed higher recovery for SNM (59 %); SMZ and SMX were also more retained on this sorbent compared to Oasis HLB. On the other hand, SampliQ OPT produced very low recoveries for SIM and SDZ (38.5% and 26.8%, respectively). **Figure 5-6** shows the recovery of

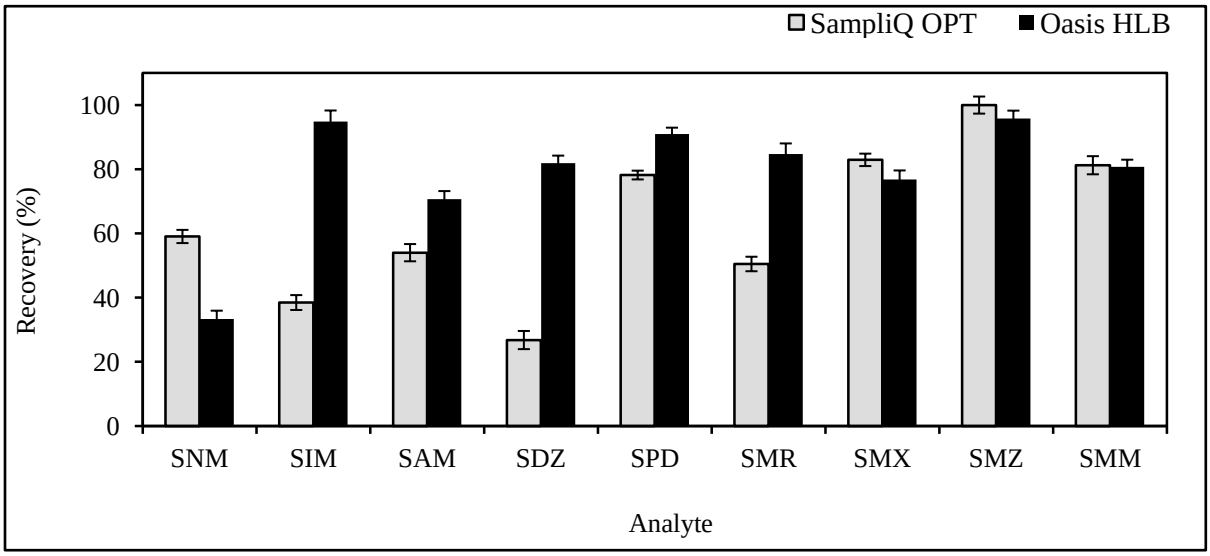


Figure 5-6: Recovery of the studied sulfonamides on Oasis HLB and SampliQ OPT SPE cartridges. Error bars indicate one standard deviation.

the analytes studied obtained with both sorbents. Based on the comparison, Oasis HLB was selected as the optimal sorbent, as it produced higher recoveries on average.

5.2.3 Validation of the method

Unlike LC–MS, HPLC with UV detection lacks the ability to provide compound identity confirmation. Therefore selectivity of the method was evaluated by comparison of the compound retention times obtained from the chromatogram of fortified deionised water to the chromatogram of unfortified deionised water (blank samples). The absence of false positive results for all sample blanks and good separation ensure that the signal measured is not influenced by other substances. These were considered acceptable for selectivity.

The linearity of the method was evaluated using five concentrations of the analytes spiked in deionized water in the range of 5 µg/L to 30 mg/L. The parameters of the regression equations and the calibration ranges for the studied analytes are presented in **Table 5-3**.

Precision was validated based on the evaluation of intra-day and inter-day repeatability of the method. The results are summarized in **Table 5-4**. Satisfactory results were achieved for all analytes. The intra- day repeatability ranged from 1.5 to 5.2 % RSD, while the inter-day precision ranged from 1.6 to 6.6 % RSD. The results obtained confirmed good measurement repeatability of the SPE–HPLC method.

The recovery ranged from 70.6 % to 96 % for all analytes with standard deviations not higher than 4.7%, except for SNM. The recoveries of the analytes using Oasis HLB cartridges are listed in **Table 5-5**.

The differences in the pK_a values of the sulfonamides studied made sample extraction difficult. While the recoveries for the majority of the analytes were the highest under acidic pH, the SNM recovery was markedly reduced under such conditions due to the fact that it has a high pK_a value of 10.43, thus it should be processed under alkaline conditions. However, if the extraction was performed under such conditions, the recoveries of other analytes would be reduced. The extraction was carried out in this study without adjusting the sample pH to accommodate all analytes studied. The low recovery found for SNM was also reported in other studies carried out with Oasis HLB cartridges.[89]

Table 5-3: Characteristic parameters of the calibration curve equations.

Analyte	R^2	Range (mg/L)	Slope	Intercept
SNM	0.9991	0.04 - 30	1.22	0.94
SIM	0.9989	0.15 - 3	33.26	-1.16
SAM	0.9987	0.025 - 10	2.90	0.14
SDZ	0.9989	0.025 - 10	2.77	-0.46
SPD	0.9991	0.025 - 10	6.69	0.18
SMR	0.9980	0.025 - 10	7.46	-1.57
SMX	0.9990	0.025 - 10	4.81	0.08
SMZ	0.9993	0.005 - 2	38.29	0.24
SMM	0.9985	0.025 - 10	6.19	-1.04

Table 5-4: Intra-day and inter-day precision of the determination of the analytes studied

Analyte	Intra-day precision (RSD)		Inter-day precision (RSD)	
	Low conc.[a]	High conc.[b]	Low conc. [a]	High conc. [b]
SNM	4.4	3.9	4.5	4.0
SIM	2.5	2.6	2.8	4.8
SAM	3.3	2.8	3.0	3.5
SDZ	5.2	5.0	6.6	4.0
SPD	3.0	2.2	3.1	4.7
SMR	3.7	1.9	3.4	1.6
SMX	4.3	2.1	4.1	4.8
SMZ	4.0	1.5	4.2	3.9
SMM	3.7	1.8	4.0	2.5

[a] the concentration for all analytes was 25 µg/L except sulfanilamide (40 µg/L).

[b] the concentration for all analytes was 10 mg/L except sulfanilamide (30 mg/L), sulfisomidine (3 mg/L) and sulfamethazine (2 mg/L).

RSD: relative standard deviation (%).

Table 5-5: Recovery of the analytes, limits of detection (LOD) and limits of quantitation (LOQ) on Oasis HLB SPE cartridges.

Analytes	**Recovery (% ± SD)**		**LOD (µg/L)**[c]	**LOQ (µg/L)**[d]
	Low conc.[a]	**High conc.**[b]		
SNM	30.0 ± 2.6	31.4 ± 4.7	10	33
SIM	92.3 ± 3.6	94.4 ± 2.2	4	13
SAM	71.0 ± 3.1	70.6 ± 2.5	6	20
SDZ	81.6 ± 2.6	81.7 ± 1.8	7.5	25
SPD	90.9 ± 2.7	90.4 ± 2.0	6	20
SMR	82.6 ± 4.3	84.3 ± 3.0	7.5	25
SMX	73.5 ± 4.0	76.0 ± 4.7	7.5	25
SMZ	95.9 ± 4.5	96.0 ± 3.7	1	3.3
SMM	81.7 ± 3.2	81.9 ± 4.3	7.5	25

SD: standard deviation.

The detection and quantitation limits determined in the study are given in **Table 5-5**. The LODs ranged from 1 to10 µg/L, while the LOQs ranged from 3.3 to 33 µg/L. The low limits of detection confirmed the applicability of the proposed method for the analysis of real water samples.

5.2.4 Analysis of real water samples

The applicability of the method to the analysis of real samples was tested by the determination of the selected sulfonamides in river, lake and wastewater samples. None of the target analytes were found in the former two samples. In the raw wastewater samples, only sulfapyridine was detected at a concentration of 8 µg/L. The presence of sulfapyridine was confirmed as previously discussed. **Figure 5-7** presents the chromatogram of the analyzed raw wastewater sample.

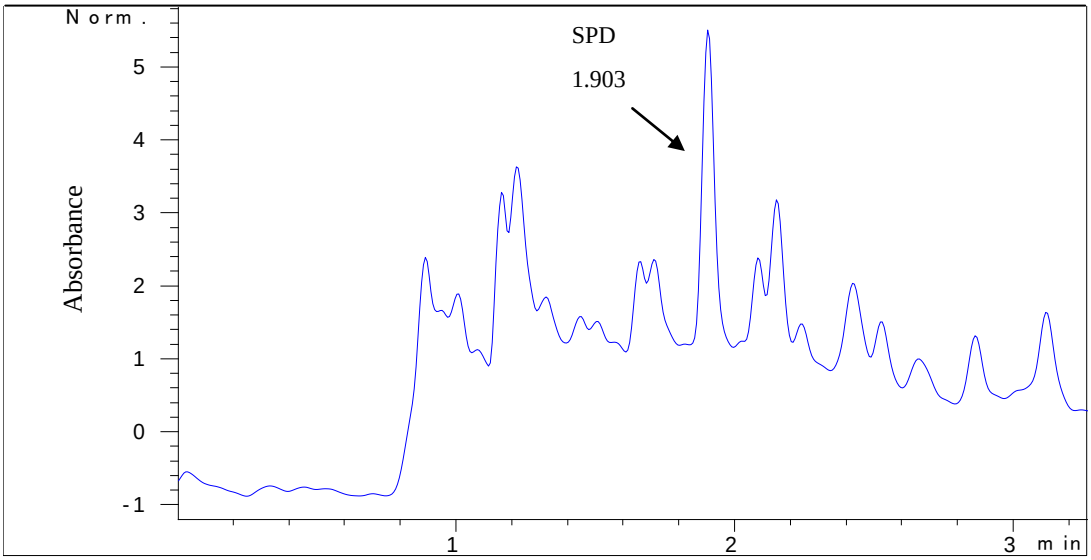


Figure 5-7: HPLC-UV chromatogram of wastewater sample extract showing detection of SPD.

5.3 Conclusions

Temperature is an important parameter in HPLC. At elevated temperature, the viscosity of the mobile phase can be reduced and the mass transfer improves, allowing higher flow rates to be used without a significant loss in efficiency. Consequently, analysis time can be shortened. In

this study, nine sulfonamides could be separated in 3 min using HPLC-UV at elevated temperature. The method is applicable to the analysis of sulfonamides in different environmental water samples such as river, lake and wastewater. The developed method has the highest performance in terms of analytical speed compared to other published HPLC-UV methods for the determination of sulfonamides. It is also compatible with MS detection when unambiguous analyte identification is required. The fast method introduced in this study should be an attractive proposition for many users.

Chapter 6

Green Ultra-Fast High Performance Liquid Chromatographic Method Using a Short Narrow Bore Column Packed With Fully Porous Sub-2 µm Particles for Simultaneous Determination of Selected Pharmaceuticals as Wastewater and Surface Water Pollutants*

In the previous chapter, a fast LC method was developed and applied to the analysis of sulfonamides in environmental samples. As an extension to developing high-throughput analytical methods to be used as an alternative to the traditional ones and with the growing awareness about the environment, hereby the aim of this work was to develop an ultra-fast green LC method for the analysis of pharmaceuticals in environmental samples.

Fast separations have become a necessity in laboratories that analyze hundreds of samples per day or those needing short turn-around times. Using high throughput HPLC analysis, results for a sample batch can be reported in a few hours rather than a few days.

Traditional HPLC methods using conventional stationary phases and standard column dimensions require large amounts of organic solvents and generate large volumes of waste. For example, traditional HPLC instruments operated with a column of 4.6 mm internal diameter and 25 cm length at a mobile phase flow rate of about 1–1.5 mL/min generate over 1 L of effluent per day, and this waste has to be disposed of.[108] Typical solvents used in HPLC are volatile and harmful to the environment. With this in mind, the development of green technologies has received increased attention aiming at minimizing or eliminating the enormous amounts of hazardous organic solvents consumed everyday worldwide.[109]

* This chapter is based on a paper submitted to "Journal of Separation Science" [107]

Finding alternative approaches allowing fast analysis and lower solvent consumption without the loss in chromatographic performance are highly desirable.

The approaches to speed up the analysis have been described in the previous sections. They include the use of monolithic columns, fully porous sub-2 µm particles with very high pressure systems, superficially porous particles and high mobile phase temperatures. However, not all fast chromatographic techniques are sparing in solvent use. For example, monolithic columns have a wide application in speeding LC analysis. These columns can be operated at elevated flow rates without generating excessive back-pressure. High speed analysis using this approach is very common; however, the amount of waste generated is the same as or even greater than with conventional HPLC columns of comparable dimensions.[108]

Smaller particle size packing (sub-2 µm) is well known to speed up the analysis time. These particles allow much higher flow rates to be used to achieve faster separation. However, when these particles are used with short columns of standard diameter (4.6 mm), no waste reduction can be observed. This could be explained by the fact that solvent savings through the reduction in the analysis time are compensated for by the solvent consumption increase through the use of a higher volumetric flow rate,[108] therefore the reduction in column diameter could be a better option.[110, 111]

Narrow bore columns packed with sub-2 µm particles can be used to achieve fast analysis, to reduce the solvent consumption and to minimize the frictional heating effects.[108] Despite the advantages of reducing the column diameter in solvent savings, this approach is not widely used in the environmental field.

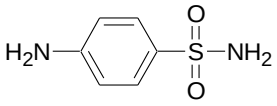
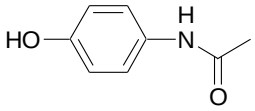
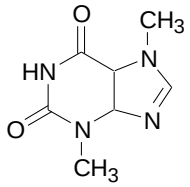
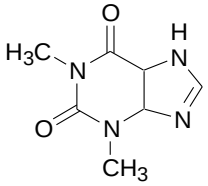
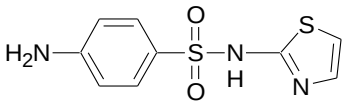
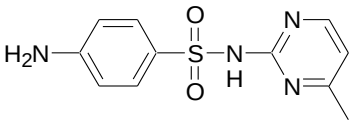
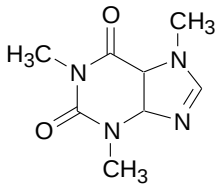
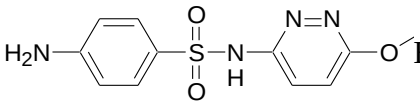
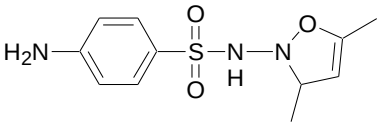
In this study, a short column (3 cm) packed with fully porous sub-2 µm particles operated at the maximum allowable pressure was used to separate nine analytes in only one minute. In addition to sulfonamides and NSAIDs, CNS stimulants were selected for this study. The selected analytes are widely detected in the aquatic environment.[54]

Achieving the LC separation in less than 1 min is widely known as ultrafast analysis.[45] The method developed using a short, small diameter column and small packing particles could decrease the analysis time and lower the solvent consumption while maintaining efficiency. The method could be used as an alternative to traditional approaches, making the analysis of pharmaceuticals greener.

6.1 Experimental

The pharmaceuticals studied were sulfanilamide, acetaminophen, theobromine, theophylline, sulfathiazole, sulfamerazine, caffeine, sulfamethoxypyridazine, and sulfamethoxazole. These compounds are frequently detected in the aquatic media.[112, 113] The chemical structures and pK_a values of the analytes studied are given in **Table 6-1**. The column used was Zorbax SB-AQ column (Agilent Technologies, Waldbronn, Germany) of 2.1 mm ID, 30 mm length and 1.8 µm particle size. The detection wavelength was set at 272 and 248 nm. Stock solutions of the analytes were prepared by dissolving 10 mg of each standard in 10 mL methanol. The standards were distributed into five concentrations ranging from 10 µg/L to 1000 µg/L. The chromatographic separations were performed in isocratic mode using a mobile phase consisting of 15 % water containing 0.5% acetic acid and 85 % acetonitrile, at a flow rate of 1 mL/min.

Table 6-1: Characteristics of the studied analytes

Analyte	Chemical structures	Therapeutic use	pKa	Abb.
Sulfanilamide		Bacteriostatic	10.4	SNM
Acetaminophen		Analgesic	9.5	AMPH
Theobromine		CNS stimulant	10	TBM
Theophylline		CNS stimulant	8.8	TPH
Sulfathiazole		Bacteriostatic	7.2	SZL
Sulfamerazine		Bacteriostatic	7.1	SMZ
Caffeine		CNS stimulant	14	CAF
Sulfamethoxypyridazine		Bacteriostatic	7.2	SPZ
Sulfamethoxazole		Bacteriostatic	5.7	SMX

6.1.1 Sample preparation and extraction

In this study, two SPE cartridges were investigated: Oasis HLB and SampliQ OPT. For extraction of real water samples, 500 mL of river and lake water were extracted and 150 mL of wastewater sample was used in order to avoid blocking of the SPE sorbents. The extracted samples were evaporated and dissolved in 0.5 mL of the mobile phase.

6.2 Results and discussion

6.2.1 Optimization of the chromatographic conditions

6.2.1.1 Selection of the analytical column

Reducing column length is one of the simplest approaches to decrease analysis time.[45] To compensate for the loss in efficiency associated with shorter column lengths, a simultaneous reduction in particle size is mandatory. In this study, a 3 cm column packed with fully porous sub-2 µm particles was employed. The use of short columns in combination with small particle size packing allows higher flow rates to be used to decrease the analysis time without a loss in efficiency. However, using short columns packed with small particles in a standard column diameter (4.6 mm I.D) is not the optimum solution for saving solvent because the use of higher volumetric flow rates to speed up the analysis results in increasing the solvent consumption. Hereby, the direct way to minimize solvent consumption was to reduce the column volume. This could be achieved by shortening the column length with a decrease in the column diameter.[110, 111] In order to obtain the maximum plate number at the optimal velocity (u_{opt}) when the internal diameter of the column is reduced, the flow rate has to be reduced by a factor (F) according to the following equation:[109]

$$F = (I.D.conventional/I.D.downscaled)^2 \quad (9)$$

Upon switching from a standard 4.6 mm I.D. column to narrow bore 2.1 mm I.D. column, F equals 4.8. When a 4.6 mm column is operated at 1 mL/min, the 2.1 mm column should be operated at 0.21 mL/min. The major advantage of reducing the flow rate is the reduction in solvent consumption without compromising the separation.

The volume of the mobile phase consumed in a given separation can be expressed by the following equation:[108]

$$V = F \times t \quad (10)$$

where F is the volumetric flow rate and t is the analysis time. In this study, at a flow rate of 1 mL/min, all the analytes were separated in 1 min. Therefore, the volume of the mobile phase consumed in each run was 1 mL. Thus, in this method a significant reduction in solvent consumption could be achieved.

In addition to solvent savings offered by small diameter columns, these columns provide increased sensitivity due to the reduced dilution of the solutes in the mobile phase and the appearance of more concentrated bands at the detector.[109] Furthermore, narrow bore columns are highly preferred when very high pressure is used due to faster heat dissipation than standard columns.[45] However, these columns also have significant limitations in terms of efficiency, as discussed in the preceding Chapters.

Because extra-column band broadening can significantly affect narrow bore column performance, extra-column volumes must be minimized. In this study, all connections used

between the injector and the detector were 0.12 mm ID of the shortest possible length to minimize the extra-column band broadening.

A critical aspect with the use of ultra-high pressure is the frictional heating which causes temperature gradients inside the column. The radial temperature gradient, due to the heat dissipation at the column wall, can cause a significant loss in efficiency. In this study, the column was operated at maximum system pressure (600 bar), at which frictional heating is not very significant, hence thermal gradients inside the column were not expected to affect the efficiency.

6.2.1.2 Mobile phase composition and the effect of organic modifier percentage on retention time

Acetonitrile is the most commonly used solvent in HPLC because of its unique characteristics such as ability to dissolve a wide range of solutes, low acidity, minimal chemical reactivity, low UV cut-off and low viscosity.[109] The unique properties of acetonitrile make it the solvent of choice in separations of pharmaceuticals.[110]

In this study, acetonitrile was used as an organic modifier. Acetic acid (0.5%) was added to the mobile phase to enhance peak resolution. In the absence of the acid, lower resolution between sulfathiazole and sulfamerazine was observed. An investigation to select the adequate composition of the mobile phase was performed and different mobile phase compositions were tested to obtain the best separation of the studied pharmaceuticals in the shortest possible time. Mobile-phase composition of 85% acetonitrile allowed all analytes to be baseline separated with good resolution in only 1 min. At higher acetonitrile content (> 85 %), poor resolution between sulfathiazole and sulfamerazine peaks was observed. Reducing the overall consumption of

organic solvents is an attractive option for green separation.[109] In this study, separating all analytes in only one minute allows the overall acetonitrile consumption to be minimized, making the analysis greener. The isocratic mode of separation eliminated the need for re-equilibration, minimizing the turn-around time. However, gradient elution might be a better alternative when not all matrix components elute from the column during the time required for the separation of the target analytes. **Figure 6-1** shows the effect of acetonitrile content in the mobile phase on the retention of the studied compounds.

The final optimum conditions were obtained with the mobile phase consisting of acetonitrile and water containing 0.5% acetic acid in a ratio of 85:15 v/v at a flow rate of 1 mL/min at 30 ºC. Under these conditions, all the analytes were separated in 1 min as shown in **Figure 6-2**. Complete separation of all analytes in short analysis time with high resolution (not less than 2.1) ensures good separation of the studied analytes from each other and from the interfering

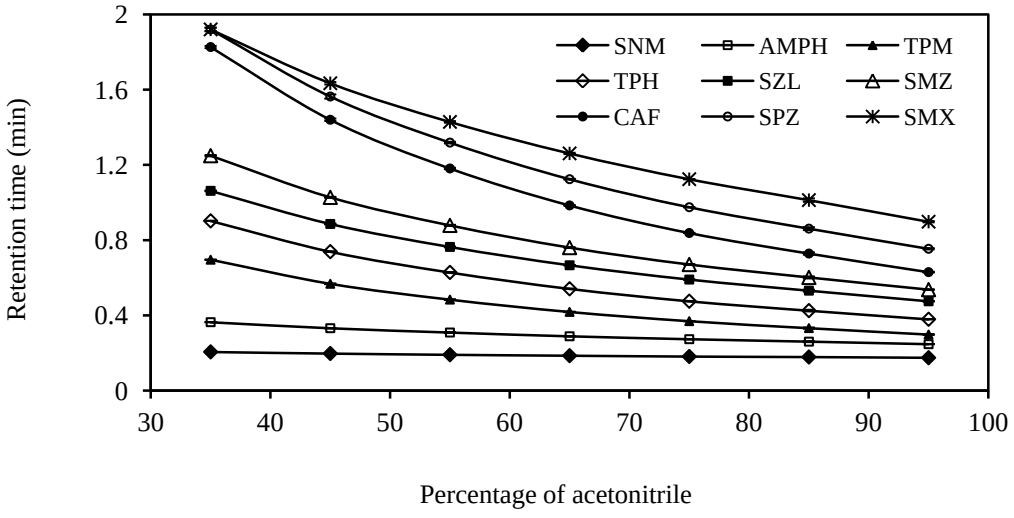


Figure 6-1: Effect of acetonitrile percentage on the retention time of the pharmaceuticals studied.

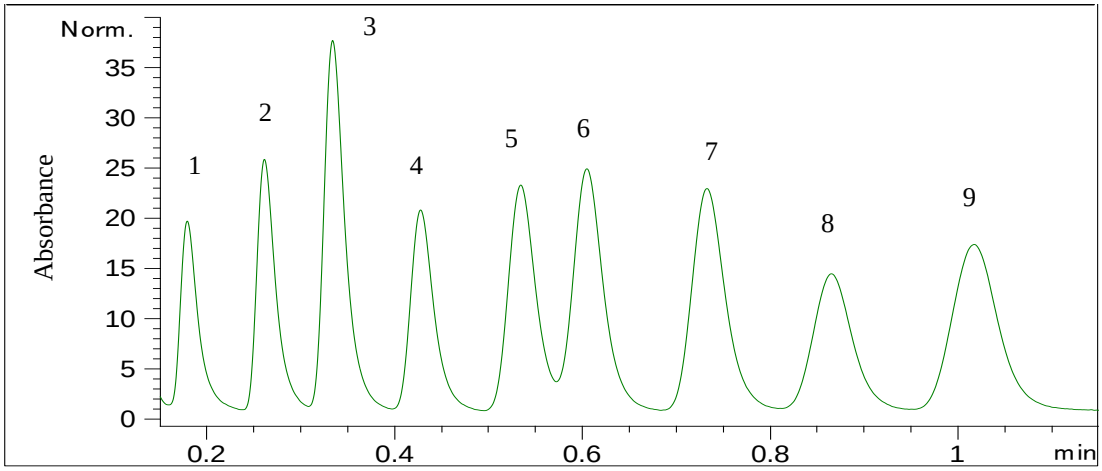


Figure 6-2: Chromatogram of the pharmaceuticals studied. Chromatographic conditions: acetonitrile:water containing 0.5 % acetic acid (85:15 v/v) at 30 ºC and a flow rate of 1 mL/min with UV detection at 272 nm. Peak identification: 1 - sulphanilamide, 2 - acetaminophen, 3 - theobromine, 4 - theophylline, 5 - sulfathiazole, 6 - sulfamerazine, 7 - caffeine, 8 - sulfamethoxypyridazine and 9 - sulfamethoxazole.

components when the method is applied to real wastewater samples. The chromatographic parameters such as retention time, capacity factor, resolution and selectivity of the studied analytes are presented in **Table 6-2**.

Table 6-2: Chromatographic parameters (retention time, capacity factor, resolution and selectivity.

Analyte	**t_R ± R.S.D.**	$k^{\backslash}$	R_s	α
Sulfanilamide	0.18 ± 1.17	0.34	3.7	2.80
Acetaminophen	0.26 ± 1.02	0.95	3.1	1.57
Theobromine	0.33 ± 1.04	1.50	3.5	1.47
Theophylline	0.43 ± 1.03	2.20	3.6	1.36
Sulfathiazole	0.53 ± 0.98	2.99	2.1	1.18
Sulfamerazine	0.60 ± 0.92	3.52	3.4	1.27
Caffeine	0.73 ± 0.87	4.48	3.1	1.22
Sulfamethoxypyridazine	0.86 ± 0.87	5.47	3.0	1.21
Sulfamethoxazole	1.01 ± 0.89	6.61		

t_R: retention time; R.S.D: relative standard deviation; k': capacity factor; R_s:resolution; α: selectivity

6.2.2 SPE optimization

From the comparison of the two tested SPE cartridges (Oasis HLB and SampliQ OPT), higher average recoveries were achieved with Oasis HLB. With this sorbent, the recovery ranged from 70.9 % to 92.5 % for all analytes except SNM, which was characterized by low recovery (32.1%). Compared to Oasis HLB, SampliQ OPT cartridge revealed higher recovery for SNM (54.8 %); also, AMPH was more retained on this sorbent compared to Oasis HLB. On the other hand, SampliQ OPT produced very low recovery for the other analytes as shown in **Figure 6-3**. Based on the comparison, Oasis HLB was selected as the optimal sorbent, as it produced higher recoveries on average.

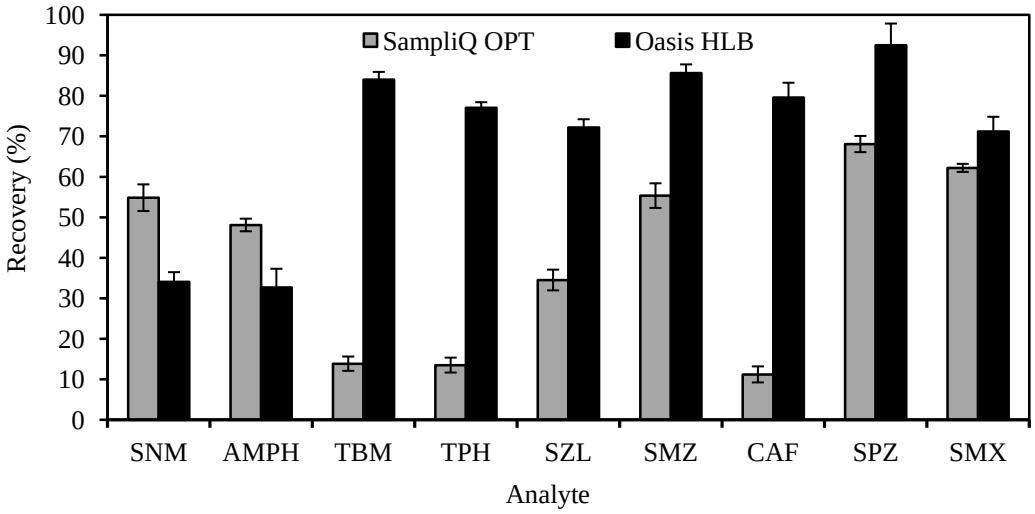


Figure 6-3: Recovery of the studied pharmaceuticals on Oasis HLB and SampliQ OPT cartridges

6.3 Validation of the method

The linearity of the method was evaluated using five concentrations of the analytes spiked in deionized water in the range of 10 µg/L to 1000 µg/L. The parameters of the regression equations for the studied analytes are presented in **Table 6-3**. Precision was validated based on the evaluation of intra-day and inter-day repeatability of the method and the results are summarized in **Table 6-4**. Satisfactory results were achieved for all analytes. The intra-day repeatability ranged from 1.4 to 5.3 % RSD, while the inter-day precision ranged from 1.3 % to 5.5 % RSD. The results obtained confirmed good measurement repeatability of the SPE–HPLC method.

The recovery ranged from 70.9 % to 92.5 % for all analytes with standard deviations not higher than 5.4%, except for SNM and AMPH. The recoveries of the analytes using Oasis HLB cartridges are listed in **Table 6-5**. The recovery of SNM and AMPH was low due to the fact that SNM and AMPH have a high pK_a value of 10.4 and 9.5 respectively, thus they should be processed under alkaline conditions. However, if the extraction was performed under such conditions, the recoveries of other analytes would be reduced. The extraction was carried out in this study without adjusting the sample pH to accommodate all analytes studied. The low recovery found for those compounds carried out with Oasis HLB cartridges was reported in other studies for SNM[85, 89, 101] and AMPH.[90]

Table 6-3: Characteristic parameters of the calibration curve equations

Analyte	**R^2**	**Slope**	**Intercept**
Sulfanilamide	0.9953	2.7	6.7
Acetaminophen	0.9979	11.4	2.2
Theobromine	0.9981	3.4	6.8
Theophylline	0.9981	2.9	6.9
Sulfathiazole	0.9972	7.7	2.2
Sulfamerazine	0.9992	3.3	4.6
Caffeine	0.9999	1.2	3.4
Sulfamethoxypyridazine	0.9991	3.1	2.9
Sulfamethoxazole	0.9990	3.1	2.7

Table 6-4: Intra-day precision and inter-day precision of the studied analytes

Analyte	**Intra-day precision (RSD)**		**Inter-day precision (RSD)**	
	100 µg/L	1000 µg/L	100 µg/L	1000 µg/L
Sulfanilamide	4.4	5.1	4.6	2.4
Acetaminophen	2.6	2.9	2.8	3.4
Theobromine	3.3	2.2	3.1	1.3
Theophylline	5.3	3.6	5.5	1.6
Sulfathiazole	2.8	1.4	3.5	2.5
Sulfamerazine	3.1	2.6	2.9	2.0
Caffeine	4.2	5.3	4.3	4.0
Sulfamethoxypyridazine	4.0	2.2	4.2	3.9
Sulfamethoxazole	4.6	2.0	4.0	2.3

RSD: relative standard deviation (%).

Table 6-5: Recovery on Oasis HLB, LODs and LOQs of the analytes studied

Analyte	**Recovery (% ± SD)**		**LOD (µg/L)**[a]	**LOQ (µg/L)**[b]
	100 µg/L	1000 µg/L		
Sulfanilamide	32.1 ± 4.7	34.1 ± 2.4	10	33
Acetaminophen	30.8 ± 4.2	32.7 ± 4.6	9	30
Theobromine	82.7 ± 4.0	84.0 ± 1.9	7	23
Theophylline	75.0 ± 2.2	77.0 ± 1.4	10	33
Sulfathiazole	71.5 ± 2.9	72.2 ± 2.0	9	30
Sulfamerazine	84.5 ± 2.4	85.6 ± 2.1	3	10
Caffeine	79.3 ± 3.5	79.6 ± 3.6	12	40
Sulfamethoxypyridazine	92.0 ± 2.7	92.5 ± 5.4	7	23
Sulfamethoxazole	70.9 ± 2.8	71.2 ± 3.6	8	26

SD: standard deviation.

[a] $S/N \geq 3$ and [b] $S/N \geq 10$

The detection and quantitation limits determined in the study are given in **Table 6-5**. The LODs ranged from 3 to 12 µg/L, while LOQs ranged from 10 to 40 µg/L. The limits of detection and quantitation could be decreased by mass spectrometry detection.

6.4 Analysis of real water samples

The applicability of the method was tested by the determination of the selected pharmaceuticals in river, lake and wastewater samples. None of the target analytes were found in the surface water; however, in the raw wastewater samples acetaminophen could be detected at a concentration of 21.6 µg/L. The presence of acetaminophen was confirmed as explained before. **Figure 6-4** shows the chromatogram of the analyzed raw wastewater sample.

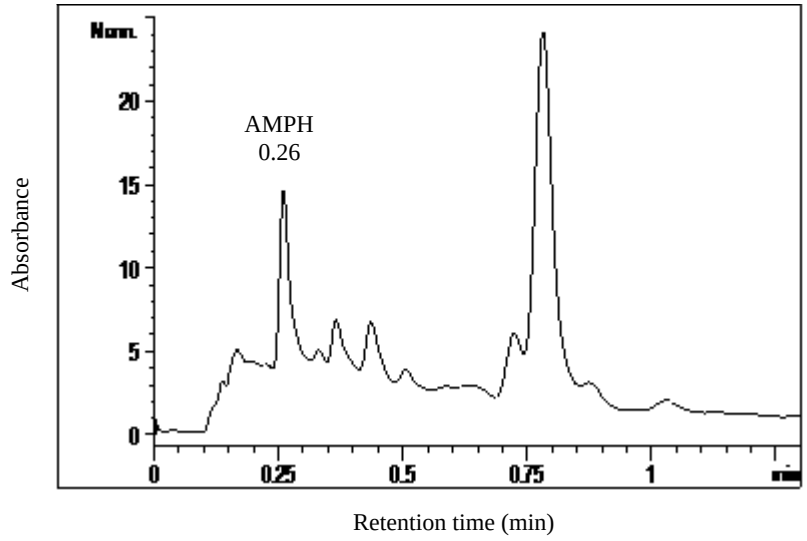


Figure 6-4: HPLC-UV chromatogram of wastewater sample extract showing detection of AMPH

Chapter 7

Chromatographic Performance of Superficially Porous Sub-3 µm and Fully Porous Sub-2 µm Particles at Elevated Temperature and Extended Column Length: Application to Pharmaceutical Analysis*

The use of fully porous sub-2 µm particles leads to significant improvements in terms of efficiency and analysis speed in HPLC separations. In the preceding studies, small particle size packings could be effectively used to enhance efficiency and reduce analysis time in the analysis of pharmaceuticals in aquatic environment. Despite the advantages offered by fully porous sub-2 µm particles, the induction of high backpressure still limits their extensive use in many laboratories where conventional HPLC systems are still used.

Recent advances in HPLC column technology have focused on manufacturing new packing materials aiming at further reduction in the analysis time while maintaining column efficiencies and requiring relatively low back pressures compatible with conventional HPLC systems.[17, 35] The development of columns packed with superficially porous silica particles (fused-core particles) was considered a breakthrough in column technology. Therefore, studying the chromatographic performance of these newly developed particles and comparing it to that of fully porous sub-2 µm particles is highly needed. The aim of this study was to compare the performance of columns packed with fused-core particles to that of fully porous particles (sub-2 µm) at elevated temperatures and extended column lengths, and to investigate the possibility of using these particles as an alternative to fully porous sub-2 µm particles for the pharmaceutical

* This chapter is based on a paper submitted to "Analytical Methods" [114]

analysis. The study involved a comparison of chromatographic parameters such as retention, selectivity, resolution, efficiency and pressure drop on single and coupled columns.

At the time when this study was performed, it was the first one aiming to compare the performance of the two different stationary phases (fully porous and superficially porous) at elevated temperatures on single and coupled columns. Also, at that time only superficially porous sub-3 µm particles were commercially available. Among many commercially available fused core particles such as Halo and Ascentis, Kinetex® columns packed with 2.6 µm particles were selected for this study because they were shown to exhibit better efficiency than Halo-packed columns[37, 115] and yield better efficiency than columns of the same length packed with sub-2 µm particles.[116] With the improvements in the manufacturing of fused-core particles, new column dimensions and smaller particles sizes are now commercially available under different brand names, and today columns packed with fused-core sub-2 µm particles are also commercially available. Due to the increasing use of fused core particles in HPLC separations, many studies have focused recently on the evaluation of their performance, e.g.[7, 36, 39, 41-44]

In this study, several columns packed with fused core particles could be coupled in series at high temperature to increase the number of theoretical plates, hence enhancing the efficiency. Lower backpressure across the fused core particles allowed two columns to be coupled at 30 ºC to generate ~ 66,000 theoretical plates, and three columns could be coupled at 60 ºC generating ~129,000 plates. On the other hand, only two columns packed with sub-2 µm particles could be coupled at 60 ºC generating ~ 49,000 plates at the optimum flow rate. This column set could be used to separate a mixture of tricyclic anti-depressant drugs with high efficiency in a short time. Due to lower backpressure across the fused core particles, higher flow rate could be used to

separate a pharmaceutical mixture of 14 compounds in less than 4 minutes without exceeding the pressure limits of the system.

7.1 Experimental

7.1.1 Materials and reagents

The test analytes used for the construction of the van Demter curves and for the backpressure study were sulfanilamide, sulfacetamide, sulfapyridine, sulfamerazine and sulfamethazine. The mixture of tricyclic antidepressant drugs separated on coupled columns operated under isocratic conditions was composed of desipramine, nortriptyline, doxepin, imipramine, amitriptyline and clomipramine. The mixture used for gradient elution contained sulphanilamide, acetaminophen, sulfisomidine, sulfacetamide, sulfadiazine, sulfapyridine, sulfamerazine, sulfamethazin, sulfamonomethoxine, sulfamethaxazole, sulfaisoxazole, sulfadimethoxine, piroxicam and naproxen.

7.1.2 Instrumentation and chromatographic conditions

The columns used in this study were Kinetex® C18 columns of 4.6 ID, 150 mm length packed with 2.6 µm superficially porous particles (Phenomenex, USA) and Zorbax StableBond C18 columns packed with sub-2 µm particles (Agilent Technologies, Waldbronn, Germany) of 4.6 ID, 150 mm length and 1.8 µm particles. The columns packed with fused-core particles and sub-2 µm particles are stable up to 60 °C and 90 °C, respectively, according to their manufacturers. Stainless steel tubing with an internal diameter of 0.12 mm and 10.5 cm length (Agilent Technologies) was used for column coupling. Connections could not be made with shorter tubing due to the physical layout of system; hence some additional extra-column band broadening was practically unavoidable.

7.1.3 Column efficiency and van Deemter curve determination

Van Deemter curves were generated using a mixture composed of five sulfonamides (see above) separated under isocratic conditions. The mobile phase was water containing 0.5 % acetic acid–acetonitrile (75/25, v/v). No attempt was made to adjust the mobile phase composition to ensure constant *k* for all analytes, because this would introduce additional variability in terms of viscosity and analyte diffusion coefficients, which would outweigh the minor effects of slightly variable retention.

In literature, the column efficiency is often reported using non-retained compounds. However, in practical HPLC analysis of pharmaceutical products, compounds are always well retained. Therefore, the efficiency for retained compounds is more relevant and meaningful for practitioners. In this study, the test mixture contained uracil as the void marker. Among the five tested compounds, sulfamethazine was selected as the model analyte because it had the highest retention factor. The mobile phase flow rate was increased (in 0.2 mL/min increments) from 0.2 mL/min until the maximum backpressure accepted by the column and the chromatographic system was reached, depending on the temperature. Detection was set at 272 nm. Experimental plate height (H) and mobile phase linear velocity (u_0) values were obtained using single and coupled columns of different lengths for both fused-core and sub-2 µm particles at column temperatures of 30 °C and 60 °C. The linear velocity u_0 was calculated by dividing the column length (L, mm) by the void time (t_0, s) at each flow rate. The void time was determined by using uracil as unretained/void volume marker. Plate height values were calculated from the equation $H= L/N^1$ where N is the number of theoretical plates and L is the column length. The number of theoretical plates (N) for each test mixture analyte was calculated using the moments method (as reported by the Chemstation software).

7.1.4 System backpressure

The test mixture of five sulfonamides separated under isocratic conditions was used to compare the backpressures generated by the fused-core and the sub-2 µm particles at different flow rates at temperatures of 30 °C and 60 °C. The system backpressure was plotted as a function of the flow rate on each column set.

7.1.5 Pharmaceutical applications

As an application to pharmaceutical analysis, two mixtures of pharmaceuticals from different classes were separated to prove the possibility of using fused-core particles as an alternative to sub-2 µm particles in terms of efficiency and analysis speed.

7.1.5.1 Enhanced efficiency

A mixture composed of six tricyclic antidepressant drugs was separated on both types of stationary phases under isocratic conditions. The separation was performed on a single column packed with sub-2 µm particles (150 mm at 30 °C) and on columns packed with fused-core particles of different lengths (two columns coupled at a temperature of 30 °C, two and three columns coupled at a temperature 60 °C). The mobile phase used for the analysis contained 80 % acetonitrile and 20 % ultra-pure water containing 0.1 % triethylamine at pH 9 (adjusted with acetic acid). The mobile phase flow rate was 1.8 mL/min. The analytes were detected at 254 nm.

7.1.5.2 Reduced analysis time

Gradient mode of separation was employed to analyze a mixture composed of 14 pharmaceuticals (11 sulfonamides and 3 non-steroidal anti-inflammatory drugs) using a column packed with fused-core particles (Kinetex 2.6 µm single column, 150 mm) at a temperature of 30 °C and mobile phase flow rate of 2.1 mL/min. The gradient used for the analysis was 0 min, 10

% acetonitrile; 1.6 min, 38 % acetonitrile then increasing to 95% acetonitrile at 3.5 min. The equilibrium time was set to five minutes. The detection wavelength was 272 nm.

7.2 Results and discussion

7.2.1 Comparison of chromatographic parameters

7.2.1.1 Retention and selectivity

The retention characteristics of the fused-core particles were compared to those of the sub-2 µm particles. **Figure 7-1** shows the chromatograms obtained from the separation of the test mixture of five sulfonamides under isocratic conditions on single columns (150 mm) using sub-2 µm and fused-core particles at 30 °C and 60 °C.

At 30 °C, the retention of the studied analytes on the column packed with fused-core particles (Kinetex®, 150 mm) was lower than on column packed with sub-2 µm particles (Zorbax SB 150 mm) (**Figure 7-1 a and c**, respectively). The lower retention factors on the fused-core particles could be due to lower accessible surface area (lower carbon loading) compared to Zorbax column packed with sub-2 µm particles ,[7] smaller partition constant and/or smaller volume of the stationary phase. Also, the short diffusion paths of the thin outer shell lead to smaller t_0, so the unretained solutes eluted faster on such columns compared to totally porous particles. Similar observations have previously been reported for fused-core particles and Zorbax Eclipse Plus column packed with sub-2 µm particles,[7] as well as columns packed with fused-core particles and Acquity columns packed with 1.7 µm particles.[36]

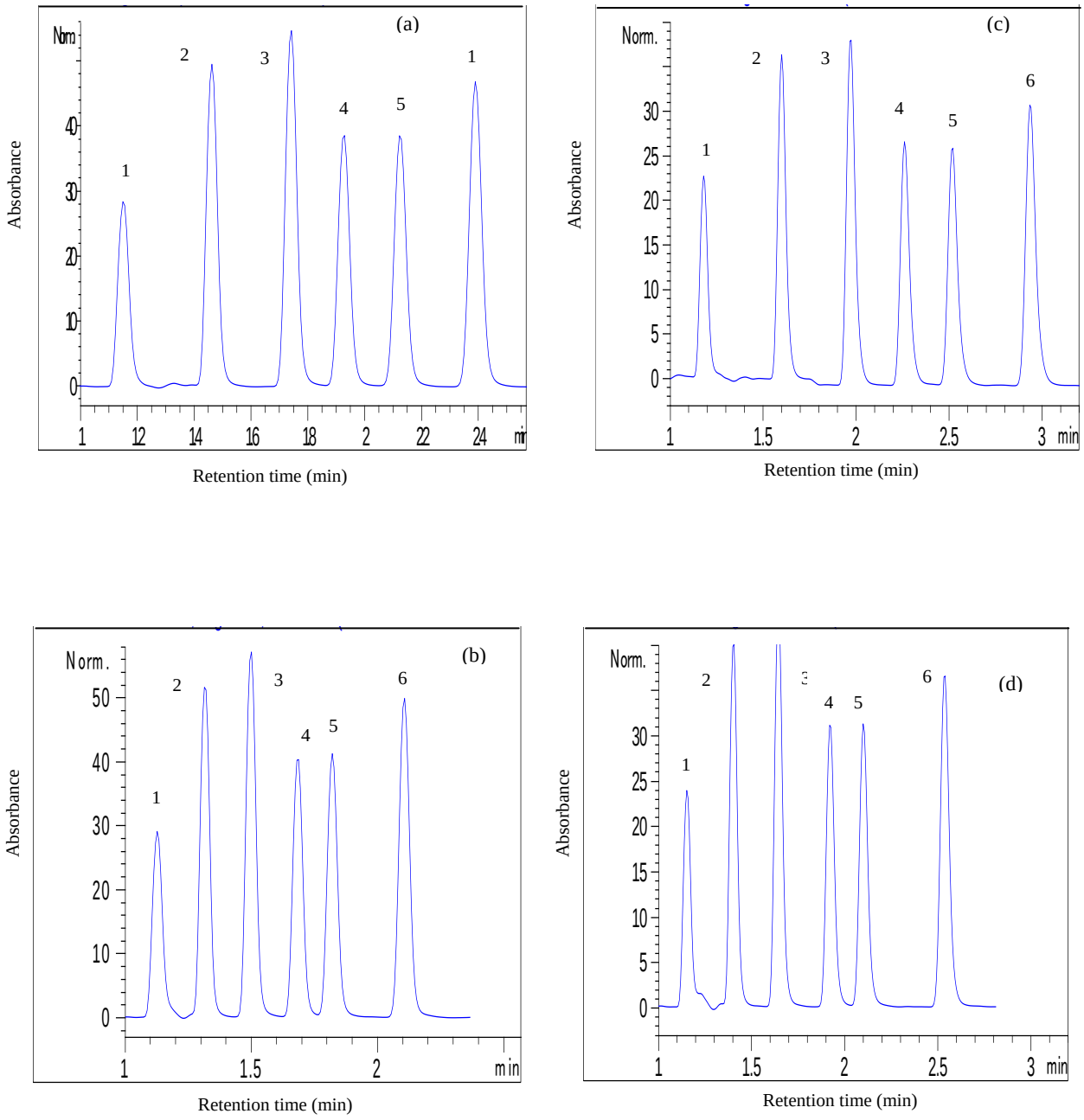


Figure 7-1: Chromatograms of sulfonamides separated on single columns (150 mm) at a flow rate of 1.2 ml/min; (A) a column packed with fused-core particles at 30 °C, (B) a column packed with fused-core particles at 60 °C, (C) a column packed with sub-2 µm particles at 30 °C and (D) a column packed with sub-2 µm particles at 60 °C. Detection: UV at 254 nm. Peak identification: 1 - uracil, 2 - sulfanilamide, 3 - sulfacetamide, 4 - sulfapyridine, 5 - sulfamerazine and 6 - sulfamethazine.

At a temperature of 60 °C, the retention significantly decreased on both kinds of packing compared to separation performed at 30 °C (**Figure 7-1 b, d**). The retention was decreased at high temperature due to the reduction of the partition coefficient between the stationary phase and the mobile phase at higher temperature. Another benefit of operation at high temperature was the reduction of column backpressure (due to the decrease in solvent viscosity). This allowed coupling of several columns in series. At 60 °C, up to three columns packed with fused-core particles could be coupled to give a total length of 450 mm, while only two columns packed with sub-2 µm particles (300 mm) could be coupled due to pressure limitations of the instrumentation. Analyte retention times on two coupled columns packed with fused-core particles were shorter than on two coupled columns packed with sub-2 µm particles of the same length (8.3 vs. 9.8 min at the optimum flow rate, and 2.8 compared to 5.1 min at the maximum flow rate). Retention times for the analytes separated on single and coupled columns on both kinds of stationary phases are given in **Table 7-1**.

Additionally, the selectivity characteristics of the columns packed with fused-core particles and sub-2 µm particles were compared. The relative retention of the last eluting compound was calculated for separations carried out 30 °C and 60 °C on single and coupled columns. The results are shown in **Figure 7-1**. At the optimum flow rate and a temperature of 30 °C, the relative retention on single columns was 1.31 and 1.29 for column packed with sub-2 µm particles and fused-core particles, respectively. At 60 °C, it increased to 1.46 and 1.44 on sub-2 µm and fused-core particles, respectively, on both single and coupled columns. The data

Table 7-1: Fundamental parameters of 2.6 µm fused-core and 1.8 µm fully porous particles for different column lengths at 30 °C and 60 °C

Column set	Flow rate		N	α	R_s	ΔP	R_t
Single column packed with sub-2 µm particles at 30°C	Optimum	0.4	25003	1.31	9.5	160	8.6
	Maximum	1.45	12406	1.29	5.3	575	2.0
Single column packed with fused-core particles at 30°C	Optimum	0.4	26523	1.29	8.7	110	7.2
	Maximum	2.1	5231	1.30	3.7	557	1.4
Single column packed with sub-2 µm particles at 60°C	Optimum	0.6	22647	1.46	11.2	98	5.0
	Maximum	2.2	5122	1.46	4.7	584	1.4
Single column packed with fused-core particles at 60°C	Optimum	0.4	23328	1.44	10.1	70	6.3
	Maximum	3.1	2131	1.43	3.1	554	0.9
Two columns packed with fused-core particles at 30°C	Optimum	0.4	66743	1.29	13.3	299	14.3
	Maximum	1.1	44443	1.29	11.1	546	5.6
Two columns packed with sub-2 µm particles at 60°C	Optimum	0.6	49897	1.46	17.3	280	9.8
	Maximum	1.2	37020	1.46	15.1	559	5.1
Two columns packed with fused-core particles at 60°C	Optimum	0.6	53341	1.44	15.3	184	8.3
	Maximum	1.8	22021	1.44	9.0	552	2.8
Three columns packed with fused-core particles at 60°C	Optimum	0.6	129576	1.44	21.4	256	12.5
	Maximum	1.3	78547	1.43	17.5	570	6.1

Data calculated for the last eluting peak (flow rate in mL/min). N: number of theoretical plates; α: selectivity; R_s: resolution; ΔP: system backpressure (bar); R_t: retention time (min).

indicates similarity in selectivity for both columns packed with fused-core particles and sub-2 µm particles.

7.2.1.2 System backpressure

The system backpressure was measured at various flow rates for different column lengths (single and coupled) of columns packed with fused-core particles and sub-2 µm particles at 30 °C and 60 °C (**Figure 7-2**). According to Darcy's law, the pressure is directly proportional to the column length and inversely proportional to the square of the particle size.[117] Consequently, columns with the smallest particle diameter (such as sub-2 µm) produce the highest backpressure. As a result of the larger particle size of the fused-core particles, they can be operated at much lower backpressures than sub-2-µm particles, as illustrated in **Figure 7-2.** A single column packed with fused-core particles (150 mm) at 30 °C produced lower backpressure (by ~32 % on average) compared to a single column of the same length packed with 1.8 µm porous particles at the same mobile phase flow rate. This results in the possibility of using columns packed with fused-core particles at higher flow rates to achieve faster separations on conventional HPLC instruments (limited to 400 bar). Due to the lower pressure drop on fused-core particles, two columns packed with fused-core particles (300 mm) could be coupled at 30 °C operated at the maximum flow rate of 1.1 ml/min to stay within instrument pressure specifications, while columns packed with sub-2 µm particles could not be coupled at 30 °C due to high backpressure across the column.

By increasing the temperature to 60 °C, the mobile phase viscosity was reduced leading to a reduction in backpressure. The column packed with fused-core particles produced lower backpressure (by ~36 % on average) compared to 30 °C at the same flow rate. Reduced

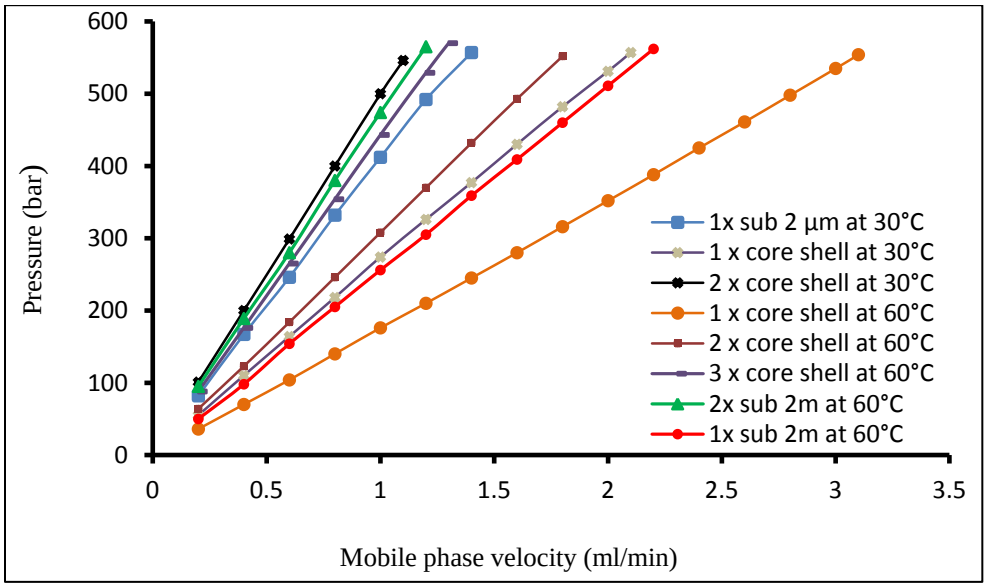


Figure 7-2: Pressure drop as a function of mobile phase velocity observed for the fused-core and sub-2µm particles on columns of different lengths at 30 °C and 60 °C.

backpressure at high temperature allowed coupling of several columns in series. Up to three columns packed with fused-core particles could be coupled at 60 °C (450 mm total length and maximum flow rate of 1.3 ml/min; for two columns of a total length of 300 mm the maximum flow rate was 1.8 ml/min), while only two sub-2 µm could be coupled at that temperature and operated at a maximum flow rate of 1.2 ml/min due to the instrumental limitations. Interestingly, the backpressure of two columns packed with fused-core particles coupled at 60 °C was lower (by ~23%) than that for a single column packed with sub-2 µm particles operated at the same flow rate at 30 °C. Similarly, three coupled fused-core at 60 °C produced lower backpressure (~5% on average) than two columns packed with sub-2 µm particles coupled at the same temperature. Also, columns packed with fused-core particles could be operated at higher flow

rates to reduce the analysis time on both conventional HPLC and UHPLC instruments. These features demonstrate the advantages of using columns packed with fused-core particles as an alternative to columns packed with sub-2 µm particles.

7.2.1.3 Column efficiency/van Deemter curves

To evaluate the chromatographic performance of the two different stationary phases, van Deemter curves were plotted for columns packed with fused-core and sub-2 µm particles of different lengths at temperatures of 30 °C and 60 °C. Plate height was plotted as a function of mobile phase linear velocity (mm/sec) for sulfamethazine, which was used as a model analyte. **Figure 7-3 a and b** show the curves plotted for a single column packed with fused-core particles and a single column packed with sub-2 µm particles at 30 °C and 60 °C, respectively. The curves show that there was no significant difference between the performance of a single column packed with fused-core particles and a single column packed with sub-2 µm particles at and below the optimum linear velocity at 30 °C (u_{opt} ~ 0.73 mm/s) and at 60 °C (u_{opt} ~ 1.1 mm/s).

To compare the performance of the columns independently of the particle size, the reduced plate height (h) was used. Reduced plate height is a dimensionless parameter that allows direct comparison of the efficiency of two or more columns packed with different particle size packing materials.

$$h = \frac{H}{d_p} \qquad (11)^{118}$$

Where H is the height equivalent to a theoretical plate (µm), and d_p is particle size (µm). The reduced plate height for the Kinetex column was found to be ~ 2.5. In another study,

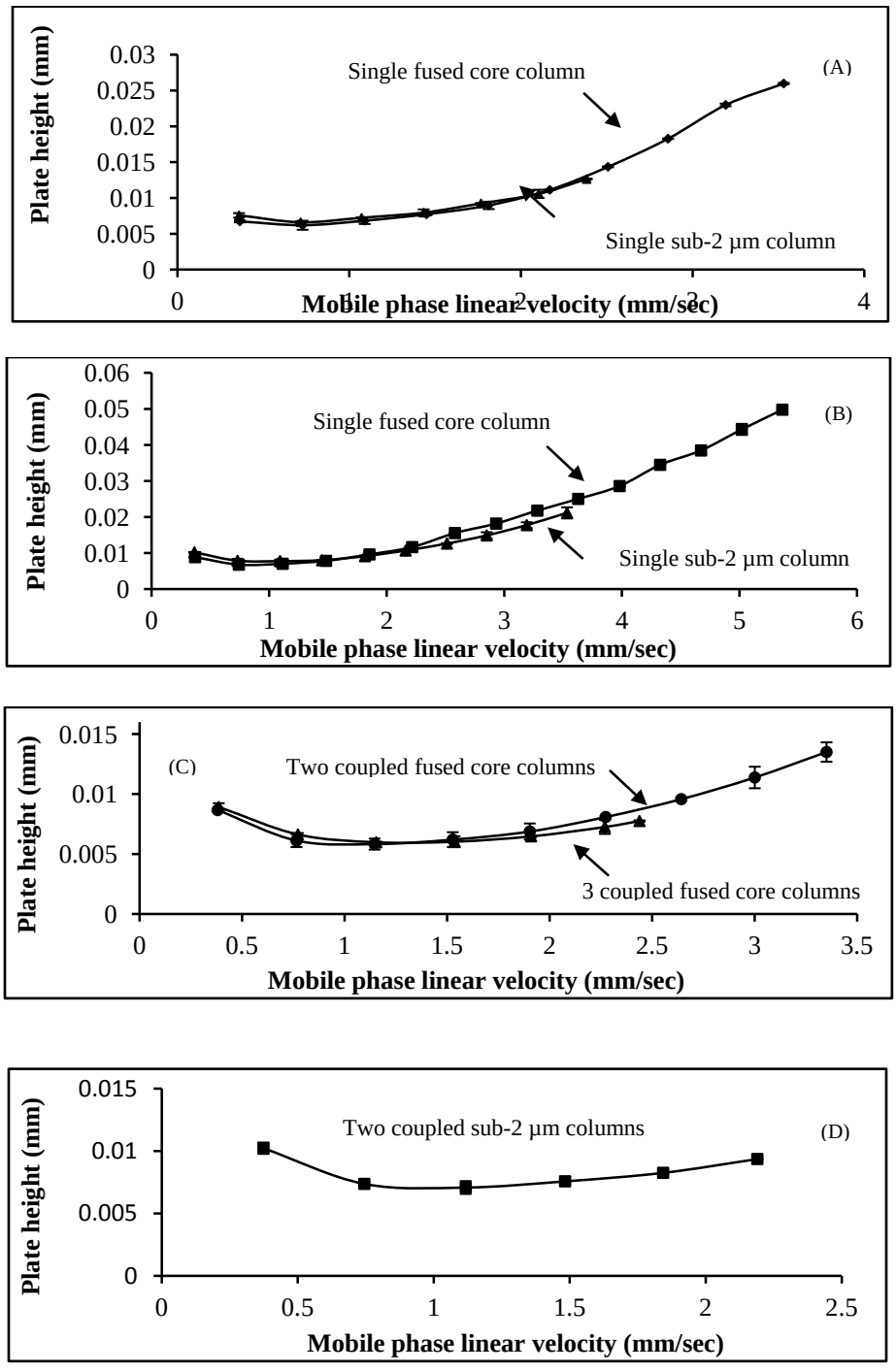


Figure 7-3: Van Demeeter plots constructed for (A) single column packed with fused-core particles and single column packed with fully porous sub-2 µm particles at 30 °C (B) single column packed with fused-core particles and single column packed with fully porous sub-2 µm particles at 60 °C (C) two and three coupled columns packed with fused-core particles at 60 °C (D) two columns packed with fully porous sub-2 µm particles at 60 °C.

the halo column had the lowest reduced plate height (h_{min} < 2) and the Ascentis Express column also displayed a low reduced plate height of h_{min} = 2; on the other hand, the Acquity and the Zorbax columns had h_{min} ~ 3, and the Thermo columns had h_{min} = 4.[36] A different study reported that the $HETP_{min}$ values obtained with the new Kinetex columns were higher than the $HETP_{min}$ values of the Ascentis Express columns and Waters Acquity BEH columns.[41] The larger reduced plate heights of the column packed with 1.8-µm particles (~ 3.5), compared to ~ 2.5 for column packed with 2.6-µm fused-core particles, suggest that there might be difficulties in obtaining homogeneous packed beds of these very small particles. On the other hand, the small reduced plate height of the column packed with shell particles can be explained by the shorter diffusion paths and by the very narrow particle size distribution. Similar observations were also reported in other studies.[39, 119] In general, the column packed with fused-core particles showed similar efficiency to columns packed with fully porous sub-2 µm particles at and below the optimum linear velocity, but with lower backpressure. **Figure 7-3 c** shows the curves plotted for coupled columns packed with fused-core particles at 60 °C (two columns (300 mm), and three columns packed with fused-core particles (450 mm), while **Figure 7-3 d** shows the curves plotted for two columns packed with fully porous sub-2 µm particles (300 mm) coupled at 60 °C. Due to lower backpressure on fused core particles, three columns could be coupled in series, while only two columns packed with fully porous sub-2 µm particles could be coupled at this temperature. There was no significant difference between the performance of two coupled columns packed with fused-core particles and sub-2 µm particles at and below the optimum linear velocity (u_{opt} ~ 1.1 mm/s). Again, the reduced plate height for coupled columns packed with fused-core particles (~2.3) was lower than that of coupled columns packed with fully porous sub-2 µm particles (~3.7), hence the performance of columns packed with the larger superficially porous particles

was significantly better. It should be emphasized that when using efficient columns with the physical layout of the system used (external column oven, pre-heater and cooler), the extra-column variance is not negligible. Coupling two columns packed with fully porous sub-2 µm particles at 60 °C leads to higher minimum plate height of 3.7 compared to 3.5 when single column is used due to the small incremental increases in extra-column dead volume associated with column coupling. Nevertheless, the results of the comparison between the sub-2 µm and superficially porous particles remain valid, as both have been tested under identical experimental conditions.

The maximum linear mobile phase velocity that could be reached for three coupled columns packed with fused-core particles was 2.4 mm/s (corresponding to the volume flow rate of 1.3 ml/min) due to the instrumental limitations, as the system was operated at 573 bar, close to the maximum allowable pressure (600 bar). Two columns packed with fused-core particles coupled at 60°C could generate ~53,000 theoretical plates at the optimum flow rate compared to ~49,000 plates achieved on two columns packed with fully porous sub-2 µm particles coupled at the same temperature, while the analysis time was shorter (8.3 min vs 9.8 min). In addition, three columns packed with fused-core particles coupled at 60°C could generate ~129,000 plates at the optimum flow rate and ~78,000 plates at the maximum flow rate, with a slight increase in the analysis time compared to two columns packed with fully porous sub-2 µm particles coupled at 60 º (6.1 vs. 5.1 min).

The results demonstrate the ability of the Kinetex® 2.6 µm fused-core technology to achieve chromatographic efficiencies comparable to those of columns packed with fully porous sub-2 µm particles at substantially lower system backpressures. The lower pressures generated by columns packed with Kinetex® 2.6 µm particles allow them to be used with conventional LC instruments

(400 bar), whereas fully porous sub-2 µm particles require UPLC for optimum use. Additionally, these results show the potential of coupling columns packed with fused-core particles at elevated temperature to enhance the efficiency while keeping lower backpressure.

7.2.2 Applications

Two mixtures of pharmaceuticals were separated to illustrate the possibility of using columns packed with fused-core particles as an alternative to columns packed with fully porous sub-2 µm particles in terms of efficiency and analysis speed.

7.2.2.1 Enhancing the efficiency

Figure 7-4 shows the chromatograms obtained for the separation of a mixture composed of six tricyclic antidepressant drugs as an example of separation of basic analytes. Initially, the separation was performed on a single column packed with sub-2 µm particles (150 mm) at 30 °C with a flow rate of 1.8 mL/min and all the analytes were completely resolved (**Figure 7-4 a**). Then the separation was performed on columns packed with fused-core particles : two columns (300 mm) coupled at 30 °C (**Figure 7-4 b**), two columns coupled at 60 °C (300 mm) (**Figure 7-4 c**) and three columns at 60 °C (450 mm) (**Figure 7-4 d**) using the same mobile phase composition and flow rate. The separation on two columns packed with fused-core particles at 30 °C resulted in the number of theoretical plates for the last eluting peak increasing by ~46 %, while the backpressure was only increased by ~17 % (**Figure 7-4 b**). At 60 °C, two coupled columns packed with fused-core particles generated a higher number of theoretical plates (increase by ~64 %) and the retention time was reduced by ~44 % compared to

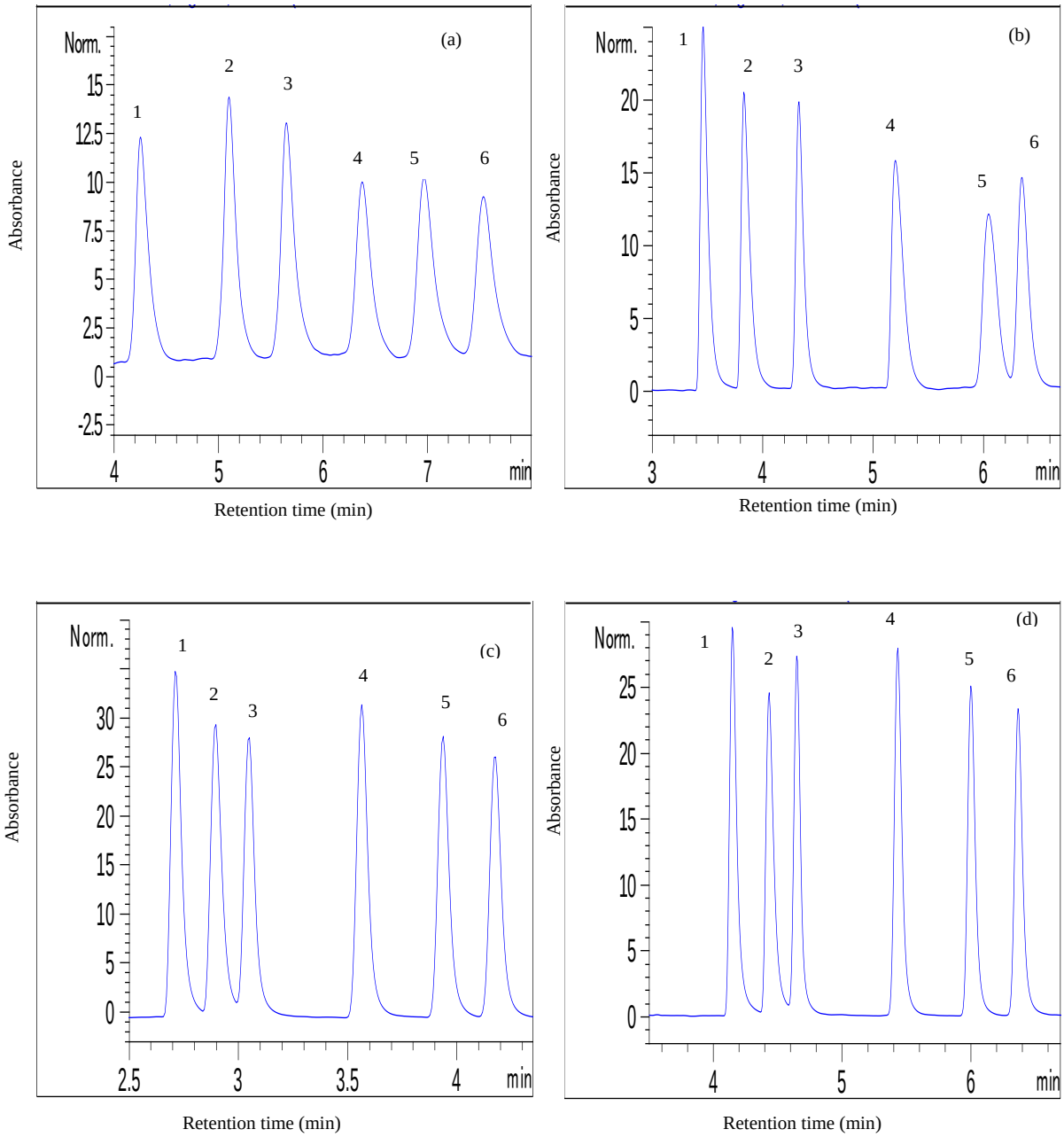


Figure 7-4: Chromatograms of tricyclic antidepressant drugs separated at a flow rate of 1.8 ml/min using (a) a single column packed with fully porous sub-2 µm particles at 30 °C (150 mm), (b) two columns packed with fused-core particles coupled at 30 °C (300 mm), (c) two columns packed with fused-core particles coupled at 60 °C (300 mm) and (d) three columns packed with fused-core particles coupled at 60 °C (450 mm). Detection: UV at 254 nm. Peak identification: 1 - desipramine, 2 - nortriptyline, 3 - doxepin, 4 - imipramine, 5 - amitriptyline and 6 - clomipramine.

separation obtained from a single column packed with fully porous sub-2 µm particles at 30 °C (**Figure 7-4 c**), while the system backpressure was only 375 bar, which indicates the possibility of using columns packed with fused-core particles at higher flow rates to achieve faster separations on conventional HPLC instruments (providing up to 400 bar). To obtain a higher number of theoretical plates, column length was extended from two (300 mm) to three columns packed with fused-core particles (450 mm) at 60 °C. On three columns packed with fused-core particles, the number of theoretical plates was increased by a factor of ~5 compared to one column packed with fully porous sub-2 µm particles at 30 °C, while the analysis time was shorter (6.4 vs 7.5 min) (**Figure 7-4 d**). Additionally, the resolution of the critical pair of peaks (5 and 6) increased from 3.3 on the single column packed with fully porous sub-2 µm particles (150 mm) at 30 °C to 4.1 and 5.7 on two and three coupled columns packed with fused-core particles at 60 °C, respectively. **Table 7-2** shows the chromatographic parameters obtained for the separation of tricyclic antidepressant drugs on a single column packed with 1.8 µm fully porous particles and coupled columns packed with fused-core particles at 30 °C and 60 °C. This example illustrates the possibility of coupling columns packed with fused-core particles at elevated temperature to increase the number of theoretical plates in a shorter analysis time compared to columns packed with fully porous sub-2 µm particles.

7.2.2.2 Reducing the analysis time

The second application involved the analysis of a mixture composed of 14 pharmaceuticals on a single column packed with fused-core particles operated at the maximum allowable flow rate (2.1 ml/min) at 30 °C. The mixture was separated in less than 4 min using gradient mode of

Table 7-2: Fundamental parameters of columns packed with 2.6 µm fused-core and 1.8 µm fully porous particles for columns of different lengths at 30 °C and 60 °C in the analysis of tricyclic antidepressant drugs*

Column set	**N**	**R_t**	**α**	**R_s**	**Δ P**
One column packed with fully porous sub-2 µm particles at 30 °C	10031	7.5	1.21	3.3	427
Two columns packed with fused-core particles at 30 °C	18738	6.3	1.12	2.4	512
Two columns packed with fused-core particles at 60 °C	27985	4.2	1.19	4.1	375
Three columns packed with fused-core particles at 60 °C	53303	6.4	1.20	5.7	544

*Calculated for the last eluting compound (clomipramine).
N: number of theoretical plates; R_t: retention time in minutes; α: selectivity; R_s: resolution; Δ P: system backpressure (bar).

separation (conditions described in section 2.5.2). The capability of a column packed with fused-core particles to carry out very fast separation is illustrated in **Figure 7-5**. The low backpressure generated by the column packed with fused-core particles allows flow rates higher than the optimum to be used to achieve faster analysis without significant loss in efficiency or resolution.

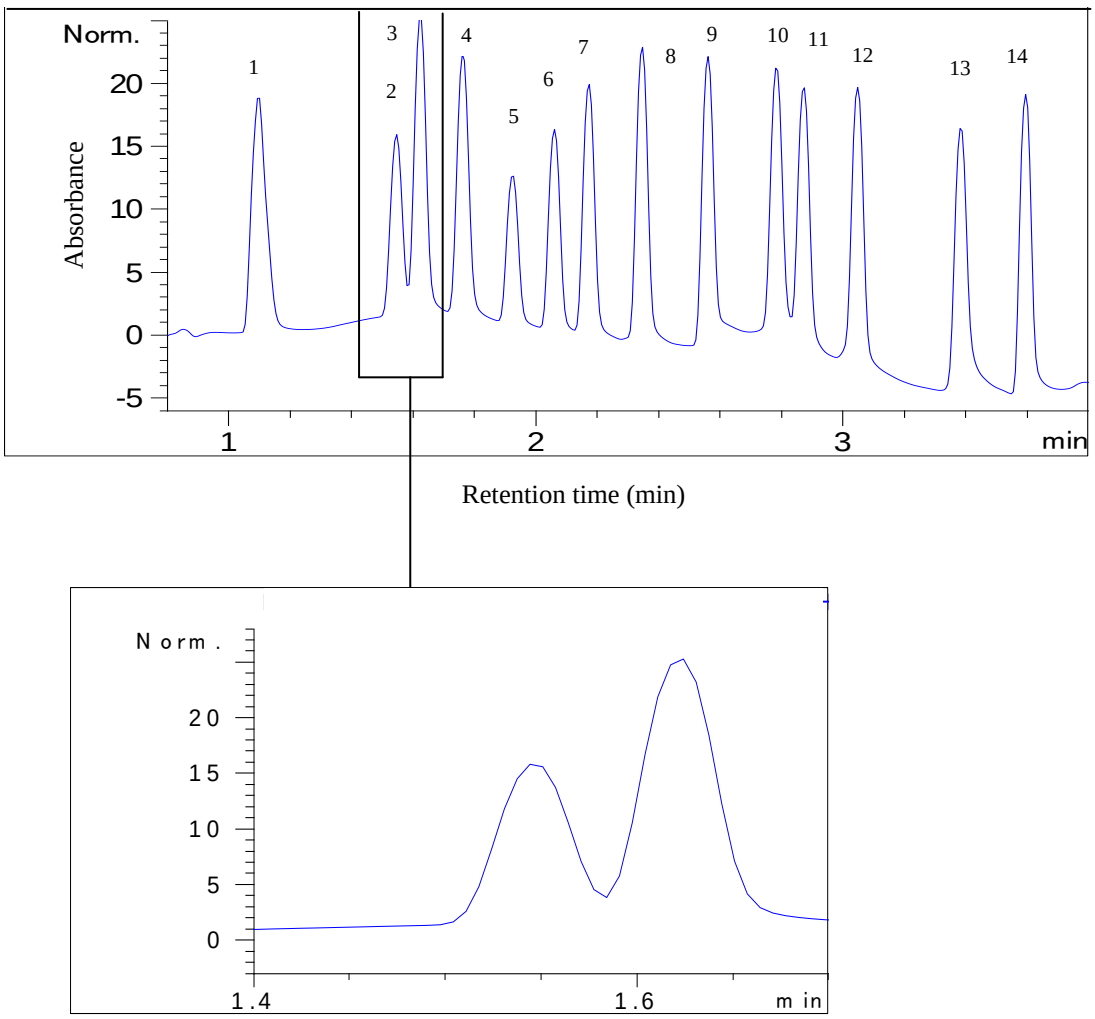


Figure 7-5: Rapid gradient separation of 14 pharmaceuticals. Column: 150 × 4.6 mm C18 fused-core particles. Detection: UV at 272 nm. Peak identification: 1 -sulphanilamide, 2 - acetaminophen, 3 - sulfisomidine, 4 - sulfacetamide, 5 - sulfadiazine, 6 - sulfapyridine, 7 - sulfamerazine, 8 - sulfamethazin, 9 -sulfamonomethoxine, 10 - sulfamethaxazole, 11 - sulfaisoxazole, 12 -sulfadimethoxine, 13 - piroxicam and 14 - naproxen. The inset shows complete baseline separation of acetaminophen and sulfisomidine with a resolution of 1.9.

7.3 Conclusions

Fused-core particles are a very good alternative to sub-2 µm particles. Columns packed with these particles are capable of yielding fast and efficient separations at conventional pressure limits. Columns packed with fused-core particles produce lower back pressure, which allows much higher flow rates to be used than with fully porous sub-2 µm particles. This allows fast LC applications, or extending the column length (through coupling) to improve separation efficiency without exceeding the allowable pressure limits of standard HPLC systems.

Chapter 8

Fast Ultrahigh Performance Liquid Chromatographic Method for The Simultaneous Determination of 25 Emerging Contaminants in Surface Water and Wastewater Samples Using Superficially-Porous Sub-3 µm Particles as an Alternative to Fully porous Sub-2 µm Particles*

Comparing the chromatographic performance of superficially porous sub-3 µm particles and fully porous sub-2 µm particles showed that superficially porous particles can be used as an alternative to fully porous sub-2 µm particles in achieving fast analysis at lower backpressure. However, since the use of superficially porous particles is a relatively recent trend in HPLC separations, using these particles for the analysis of pharmaceuticals is limited to food analysis (e.g. [121, 122]) or biological fluids (e.g. [123, 124]). Thus far the superficially porous particles have not found widespread use in environmental analysis. The aim of this work was to illustrate the advantages of using columns packed with fused core particles as an alternative to fully porous sub-2 µm particles in the analysis of environmental pollutants for achieving fast HPLC separations.

At the time this work was performed, it was the first trial of using fused core particles in environmental analysis. However, several papers describing the use of these columns in environmental analysis have been published since 2011 (e.g.[125, 126]). These studies were limited to a few compound classes. The objective of this work was to develop and validate a high-throughput, economical method using fused-core C18 silica column to determine 25 emerging

* This chapter is based on a paper submitted to "Talanta"[120]

contaminants from different classes in river, lake and wastewater in the shortest possible time in a single run.

In this study, the selection of the analytes was extended to cover a wide range of classes including veterinary antibiotics, CNS stimulants, non-steroidal anti-inflammatory drugs, steroids and preservatives from personal care products. These groups are the most commonly detected groups in the aquatic environment.[54]

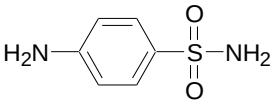
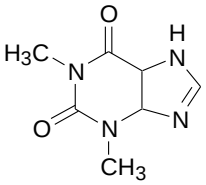
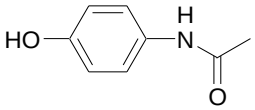
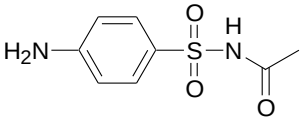
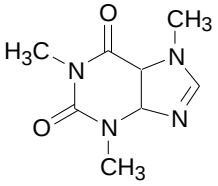
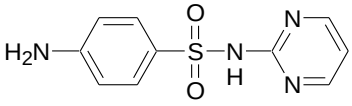
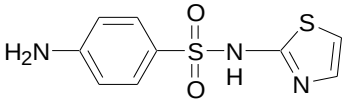
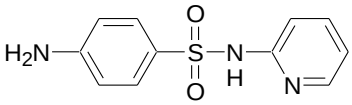
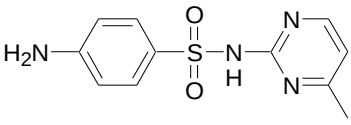
8.1 Experimental

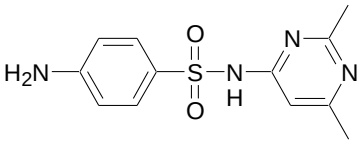
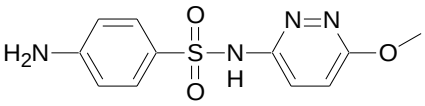
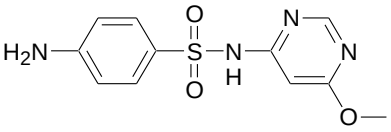
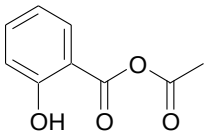
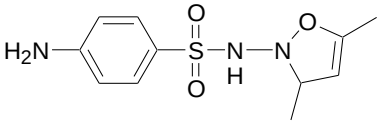
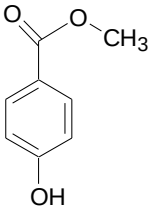
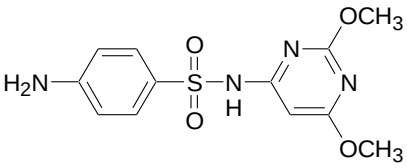
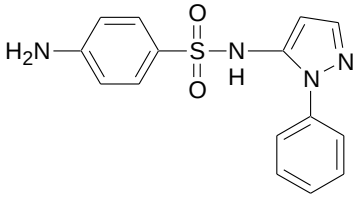
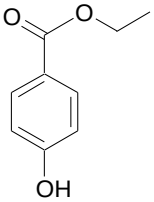
The studied analytes were sulphanilamide, sulfacetamide, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamethazine, sulfamethoxypyridazine, sulfamonomethoxine, sulfamethoxazole, sulfadimethoxine, sulfaphenazole, acetaminophen, acetyl salicylic acid, ketoprofen, fenoprofen, flurbiprofen, diclofenac, theophylline, caffeine, methylparaben, ethylparaben, propylparaben, 17 α-ethinyl estradiol and estrone. The chemical structures and pK_a values of the studied analytes are given in **Table 8-1**.

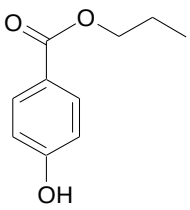
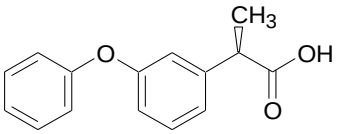
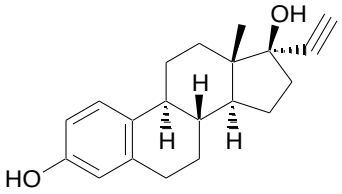
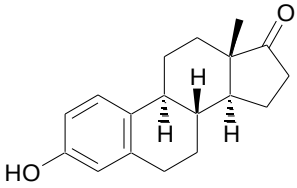
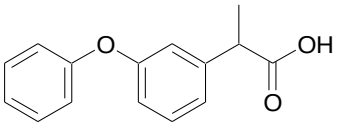
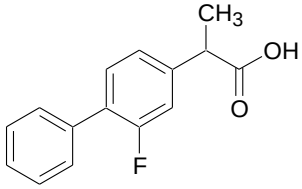
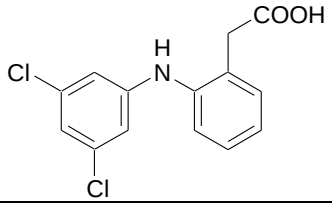
8.1.1 Instrumentation and chromatographic conditions

The columns used in this study were Kinetex® C18 columns of 4.6 mm ID, 150 mm length packed with 2.6 µm fused-core particles (Phenomenex, USA) and Zorbax Stable Bond C18 columns (Agilent Technologies, Waldbronn, Germany) of 4.6 mm ID, 150 mm length and 1.8 µm particles. Analytes were separated by gradient elution at 30 ºC using a mobile phase consisting of acetonitrile (solvent B) and ultra-pure water containing 0.5% acetic acid (solvent A). The gradient used for the analysis was 0 min, 15 % acetonitrile and flow rate 1 mL/min; 3

Table 8-1: Characteristics of the studied analytes

Analyte	Abb.	Chemical structures	pKa	Λ	Therapeutic use
Sulfanilamide	SNM		10.4	272	Bacteriostatic
Theophylline	THP		8.8	272	CNS stimulant
Acetaminophen	APH		9.5	247	Analgesic
Sulfacetamide	SAM		5.4	272	Bacteriostatic
Caffeine	CFN		14	272	CNS stimulant
Sulfadiazine	SDZ		6.5	272	Bacteriostatic
Sulfathiazole	STZ		7.2	272	Bacteriostatic
Sulfapyridine	SPD		8.4	272	Bacteriostatic
Sulfamerazine	SRZ		7.1	272	Bacteriostatic

Sulfamethazine	SMZ		7.4	272	Bacteriostatic
Sulfamethoxypyridazine	SPZ		7.2	272	Bacteriostatic
Sulfamonomethoxine	SMX		6.0	272	Bacteriostatic
Acetyl salicylic acid	ASA		2.97	230	Anti-inflammatory
Sulfamethoxazole	SXZ		5.7	272	Bacteriostatic
Methylparaben	MPN		8.5	254	Preservative & antifungal
Sulfadimethoxine	SDM		6.1	272	Bacteriostatic
Sulfaphenazole	SPZ		6.5	272	Bacteriostatic
Ethylparaben	EPB		8.4	254	Preservative & antifungal

Propylparaben	PPN		8.4	254	Preservative
Ketoprofen	KFN		5.9	254	Anti-inflammatory
17 α-ethinyl estradiol	ESD		10.0	280	Steroid
Estrone	ETN		10.4	280	Steroid
Fenoprofen	FEN		4.5	272	Anti-inflammatory
Flurbiprofen	FLP		4.2	247	Anti-inflammatory
Diclofenac	DCF		4.0	272	Anti-inflammatory

min, 15 % acetonitrile and flow rate 1.3 mL/min then increasing to 82 % acetonitrile at 10 min with flow rate 1.3 mL/min. The equilibration time was set to five minutes. For higher sensitivity, each analyte was detected at its wavelength of maximum absorption. **Table 8-1** shows the wavelengths used for the detection of each analyte.

8.1.2 Sample preparation

In this study, two SPE cartridges were investigated, Oasis HLB and SampliQ OPT.

8.2 Results and discussion

8.2.1 HPLC method

In this study, 4.6 mm I.D. columns were selected for the analysis because of their previously discussed advantages. A 150 mm long column was selected to maximize the efficiency. Columns of the same length and internal diameter packed with 2.6 µm superficially porous C18 silica particles and fully porous sub-2 µm particles were used to perform the analysis.

Acetic acid (0.5%) was added to the mobile phase to enhance peak resolution. An investigation to select the adequate composition of the mobile phase was performed and different mobile phase compositions were tested to obtain the best separation of the studied analytes in the shortest possible time on the column packed with fused-core particles without sacrificing peak shape. Solvent gradient and flow rate gradient were used to obtain the best separation for the studied analytes. The gradient elution program described in section 7.1.1 was the best option in terms of analysis time and peak shape. Under these conditions, all the analytes studied were fully separated on the column packed with fused-core particles in a short time (10 min), as shown in **Figure 8-1**. After the optimization of the separation conditions on the column packed with fused-core particles, the same gradient was applied to the column packed with fully porous sub-2 µm particles for comparison. The chromatogram of the analytes studied is presented in **Figure 8-2**. **Figure 8-1** presents the chromatogram obtained using the fused-core particles, while **Figure 8-2**

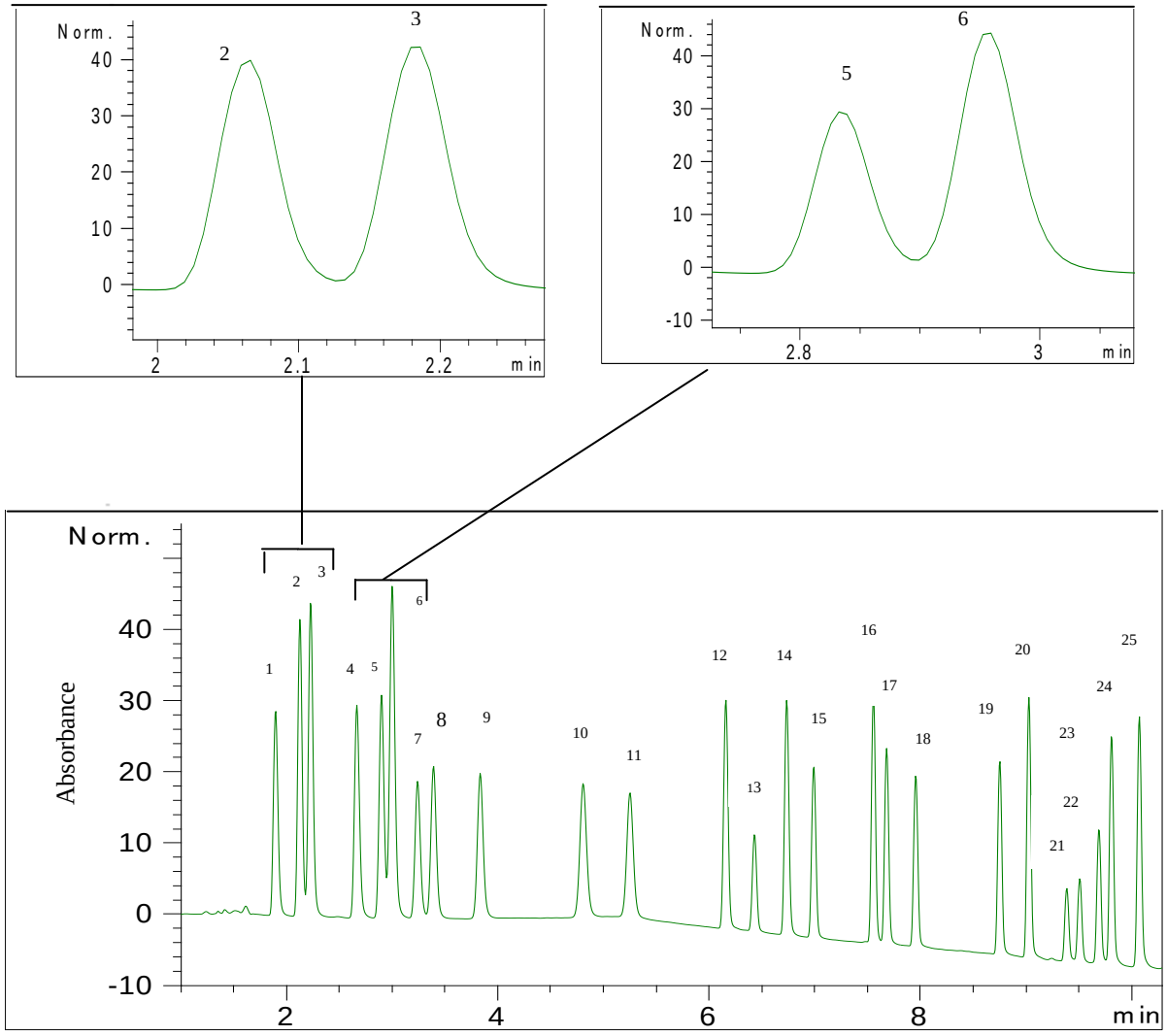
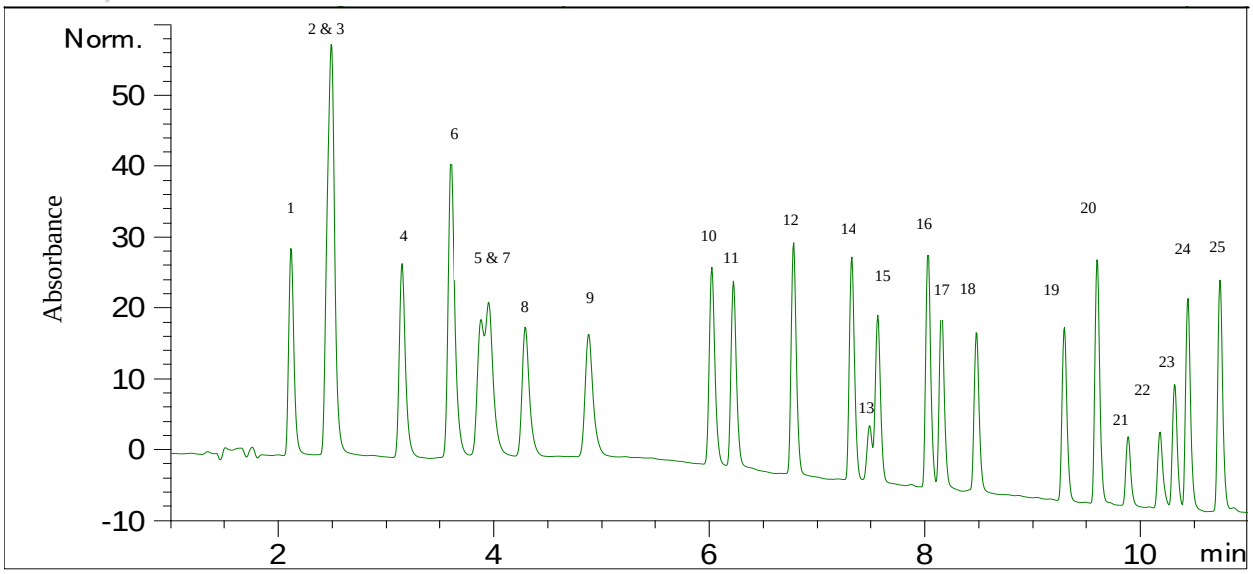


Figure 8-1: Chromatogram of the analytes studied separated on the column packed with fused-core particles. Peak identification: 1 - sulphanilamide, 2 - theophylline, 3 - acetaminophen, 4 - sulfacetamide, 5 - caffeine, 6 - sulfadiazine, 7 - sulfathiazole, 8 - sulfapyridine, 9 - sulfamerazine, 10 - sulfamethazine, 11 - sulfamethoxypyridazine, 12 - sulfamonomethoxine, 13 - acetyl salicylic acid, 14 - sulfamethoxazole, 15 - methylparaben, 16 - sulfadimethoxine, 17 - sulfaphenazole, 18 - ethylparaben, 19 - propylparaben, 20 - ketoprofen, 21 - 17 α -ethinyl estradiol, 22 - estrone, 23 - fenoprofen, 24 - flurbiprofen and 25 - diclofenac. The inset shows complete baseline separation of peaks 2, 3 and 5, 6.



Retention time (min)

Figure 8-2: Chromatogram of the analytes studied separated on the column packed with fully porous sub-2 µm particles . Peak identification: 1 - sulphanilamide, 2 & 3 theophylline and acetaminophen, 4 - sulfacetamide, 5 - sulfadiazine, 6 - caffeine, 7 - sulfathiazole, 8 - sulfapyridine, 9 - sulfamerazine, 10 - sulfamethazine, 11 - sulfamethoxypyridazine, 12 - sulfamonomethoxine, 13 - sulfamethoxazole, 14 - acetyl salicylic acid, 15 - methylparaben, 16 - sulfadimethoxine, 17 - sulfaphenazole, 18 - ethylparaben, 19 - propylparaben, 20 - ketoprofen, 21 - 17 α -ethinyl estradiol, 22 - estrone, 23 - fenoprofen, 24 - flurbiprofen and 25 - diclofenac.

shows the chromatogram obtained using fully porous sub-2 µm particles under the same conditions. A change in selectivity manifested through the change in elution order was observed between some analytes such as caffeine (peak 5) and sulfadiazine (peak 6), as well as acetyl salicylic acid (peak 13) and sulfamethaxazole (peak 14) on fully porous sub-2 µm column. The change in selectivity could be due to differences in column chemistry of both stationary phases.

While all analytes were fully resolved on the column packed with fused-core particles (resolution between 2.09 and 18.06, above the critical value of 1.5 in all cases), coelutions of many analytes were observed for the column packed with fully porous particles. For example, the critical pair of theophylline and acetaminophen (peaks 2 and 3) which were fully separated on the column packed with fused-core particles with a resolution of 2.25, coeluted completely on the fully porous sub-2 µm column. Also, partial coelutions were observed between caffeine and sulfathiazole, as well as acetyl salicylic acid and methyl paraben with this column. Another observation is that all the analytes could be fully separated on the column packed with fused-core particles in only 10 min, while the analysis time on column packed with fully porous sub-2 µm particles was greater than 10 min without complete separation for all analytes. Complete simultaneous separation of all analytes could not be achieved on the column packed with fully porous sub-2 µm particles under this operating condition. Consequently, gradient conditions would have to be optimized in order to separate all the analytes on the column packed with fully porous sub-2 µm particles at a cost of a longer analysis time. Finally, the system pressure observed when the separation was performed on the column packed with fused-core particles was 355 bar compared to 520 bar when column packed with fully porous sub-2 µm particles was used under the same mobile phase composition and flow rate. The lower pressure for the column packed with fused-core particles is advantageous when conventional HPLC systems (400 bar) are used, while ultrahigh pressure instrumentation (≥ 600 bar) is needed when the separation has to be performed on fully porous sub-2 µm columns.

Based on this comparison, the column packed with fused-core particles was chosen for the analysis as it allows reducing the analysis times at relatively low back pressures compared to

fully porous sub-2 µm particles. The column packed with fused-core particles was then used for the determination of the 25 emerging contaminants in real water samples. The chromatographic parameters (retention time, capacity factor, resolution and selectivity) of the analytes on the column packed with fused-core particles are listed in **Table 8-2**.

8.2.2 SPE optimization

To meet the objectives for the monitoring of the studied analytes in surface water and wastewater, a preliminary preconcentration step is necessary. In this study, two polymeric sorbents (Oasis HLB and SampliQ OPT) were evaluated to quantitatively extract the investigated analytes and to eliminate the influence of matrix components. From the comparison of the two tested SPE cartridges, the highest average recovery was achieved with Oasis HLB cartridges. With this sorbent, the recovery ranged from 67.5 % to 97 % for all analytes except SND, APH and ASA which were characterized by low recovery (33.9 %, 32.2 % and 15% respectively). Compared to Oasis HLB, the SampliQ OPT cartridge revealed higher recovery for SND, APH, SMZ, ASA and SDM (54.8%, 48%, 100%, 24.4% and 98% respectively). On the other hand, SampliQ OPT produced very low recoveries for many analytes, such as THP, CFN, SDZ, STZ and FPB (12.3%, 11.7%, 35.5%, 35% and 31% respectively). **Figure 8-3** shows the comparison of the recoveries of the analytes studied obtained with both sorbents. Based on this comparison, Oasis HLB was chosen as the optimal sorbent, as it produced higher recoveries on average.

Table 8-2: Chromatographic parameters (retention time, capacity factor, resolution and selectivity) of the analytes separated on the Kinetex® C18 column of 4.6 mm ID, 150 mm length and packed with 2.6 µm fused-core particles

Analyte	**t_R ± R.S.D**	k'	R_s	A
Sulfanilamide	1.88 ± 1.02	0.36	4.48	1.43
Theophylline	2.09 ± 2.03	0.51	2.25	1.15
Acetaminophen	2.20 ± 1.38	0.59	8.58	1.54
Sulfacetamide	2.64 ± 0.96	0.91	4.23	1.18
Caffeine	2.87 ± 1.53	1.07	2.09	1.08
Sulfadiazine	2.98 ± 0.97	1.15	4.21	1.15
Sulfathiazole	3.21 ± 1.15	1.32	2.73	1.08
Sulfapyridine	3.36 ± 0.99	1.43	7.62	1.22
Sulfamerazine	3.81 ± 0.82	1.75	15.01	1.40
Sulfamethazine	4.78 ± 0.71	2.46	6.32	1.13
Sulfamethoxypyridazine	5.22 ± 0.75	2.77	14.45	1.24
Sulfamonomethoxine	6.14 ± 0.23	3.44	5.24	1.06
Acetyl salicylic acid	6.42 ± 0.18	3.64	6.62	1.06
Sulfamethoxazole	6.72 ± 0.13	3.86	5.62	1.05
Methylparaben	6.98 ± 0.11	4.05	12.81	1.10
Sulfadimethoxine	7.55 ± 0.07	4.46	2.84	1.02
Sulfaphenazole	7.67 ± 0.06	4.55	6.27	1.04
Ethylparaben	7.95 ± 0.06	4.75	18.06	1.12
Propylparaben	8.74 ± 0.04	5.32	6.26	1.04
Ketoprofen	9.02 ± 0.04	5.52	8.13	1.05
17 α-ethinyl estradiol	9.38 ± 0.04	5.78	2.75	1.02
Estrone	9.50 ± 0.03	5.87	4.02	1.02
Fenoprofen	9.68 ± 0.04	6.00	2.68	1.01
Flurbiprofen	9.80 ± 0.03	6.09	5.87	1.03
Diclofenac	10.07 ± 0.03	6.28		

t_R: retention time; R.S.D: relative standard deviation; k': capacity factor; R_s: resolution; α: selectivity

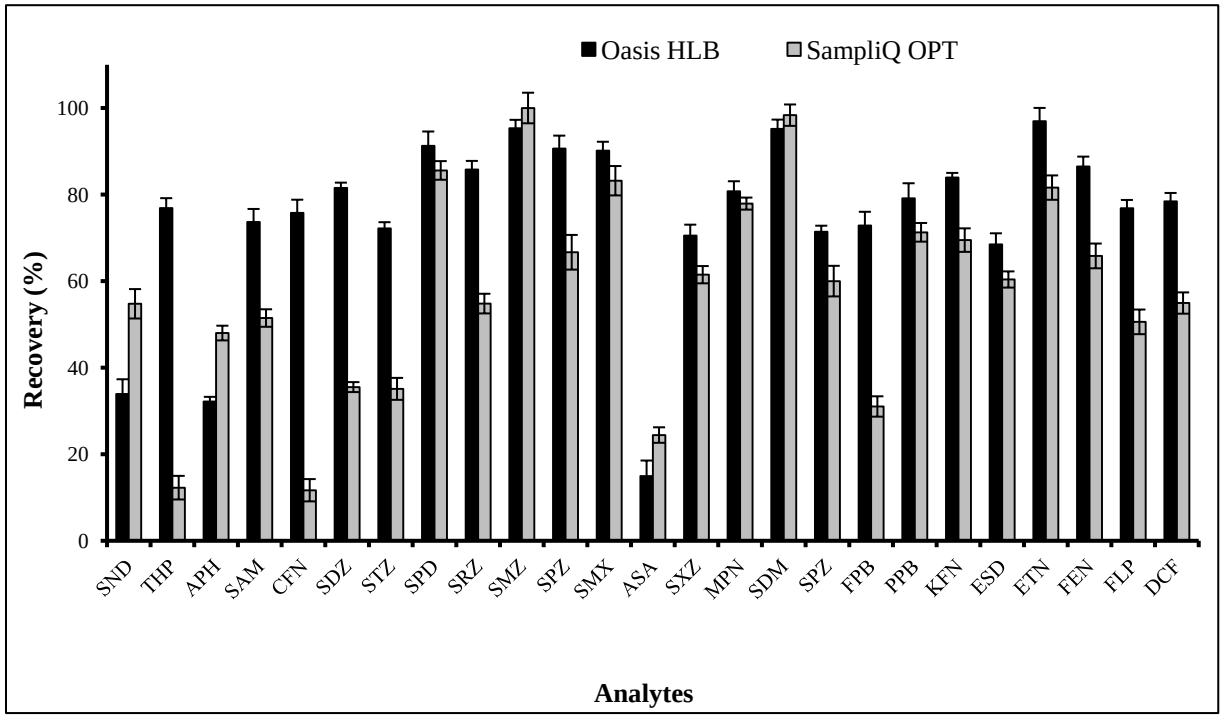


Figure 8-3: Recovery of the analytes on the SPE Oasis HLB and SampliQ OPT cartridges

8.2.3 Validation of the method

The linearity of the method was evaluated using deionized water spiked with the analytes in the concentration range from 5 µg/L to 1000 µg/L. The characteristic parameters of the regression equations for the analytes studied are given in **Table 8-3**.

Precision was validated based on the evaluation of intra- and inter-day repeatability of the method. Satisfactory results were achieved for all analytes. The intra-day repeatability RSDs

Table 8-3: Characteristic parameters of the calibration curve equations of the analytes studied

Analyte	R^2	Slope	Intercept
Sulfanilamide	0.9961	2.7	2.2
Theophylline	0.9973	2.7	4.1
Acetaminophen	0.9989	10.9	5.7
Sulfacetamide	0.9990	3.4	6.9
Caffeine	0.9989	1.7	6.4
Sulfadiazine	0.9992	3.6	4.4
Sulfathiazole	0.9989	8.5	5.8
Sulfapyridine	0.9950	5.6	3.0
Sulfamerazine	0.9998	3.2	4.2
Sulfamethazine	0.9986	1.0	4.7
Sulfamethoxypyridazine	0.9993	3.0	2.4
Sulfamonomethoxine	0.9974	2.8	1.3
Sulfamethoxazole	0.9979	2.5	1.5
Methylparaben	0.9980	3.4	4.7
Sulfadimethoxine	0.9967	2.0	4.8
Sulfaphenazole	0.9974	2.7	4.8
Ethylparaben	0.9979	1.4	4.8
Propylparaben	0.9990	1.1	1.3
Ketoprofen	0.9989	3.1	6.0
17 α-ethinyl estradiol	0.9956	1.1	6.5
Estrone	0.9956	0.9	7.5
Fenoprofen	0.9992	1.4	4.6
Flurbiprofen	0.9998	1.3	4.6
Diclofenac	0.9990	1.8	5.1

ranged from 1.1% to 5.4% and the inter-day repeatability ranged from 1.4% to 5.4%, except for acetyl salicylic acid (which had a very low recovery). The results of the intra-day and inter-day repeatability experiments expressed as relative standard deviations are summarized in **Table 8-4**. The detection and quantitation limits determined in the study are given in **Table 8-5**. The LODs of the method ranged from 1.5 to 15 µg/L, while limits of quantification were in the range from 5 to 50 µg/L with UV detection.

The recovery of the studied analytes was within the range from 67.5 % to 97.0 % with standard deviations not higher than 5.7 %, except for SND, APH and ASA, for which low recoveries were obtained. The recoveries of the analytes using Oasis HLB cartridges are listed in **Table 8-5**. The recoveries of SND and APH were low due to the fact that the pK_a values for these compounds are 9.5 and 10.4, respectively, which means that the extraction should be performed under alkaline conditions. However, if the extraction was performed under such conditions, the recoveries of the remaining analytes would be reduced. The low recovery found for these compounds were also reported in other studies carried out with Oasis HLB cartridge for APH[99, 100] and for SND[38, 98]. On the other hand, ASA has a pK_a value of 2.97, which requires strongly acidic conditions. However, adjusting the pH to a low value could again result in reduced recoveries of the remaining analytes. Carrying out the extraction without adjusting the sample pH was a compromise solution to accommodate as many analytes as possible. The low recovery of ASA (< 15%) introduced high uncertainty in its quantitation at very low concentrations, but detection was still possible. The low recovery of ASA was reported in the literature under similar extraction conditions.[89]

Table 8-4: Intra-day precision and inter-day precision of the determination.

Analyte	**Intra-day precision (RSD)**		**Intra-day precision (RSD)**	
	100 µg/L	1000 µg/L	100 µg/L	1000 µg/L
Sulfanilamide	3.1	3.0	4.5	3.9
Theophylline	2.7	1.6	2.8	2.7
Acetaminophen	2.2	3.4	5.1	4.2
Sulfacetamide	2.9	2.5	2.6	2.2
Caffeine	5.4	3.5	3.5	5.2
Sulfadiazine	2.7	2.1	5.2	2.8
Sulfathiazole	2.2	2.1	5.2	1.4
Sulfapyridine	3.8	1.1	5.4	2.1
Sulfamerazine	5.0	1.9	4.3	2.8
Sulfamethazine	5.0	1.8	5.4	3.5
Sulfamethoxypyridazine	3.4	3.5	4.9	1.9
Sulfamonomethoxine	4.6	3.0	3.6	4.2
Acetyl salicylic acid	8.7	7.6	11.3	10.2
Sulfamethoxazole	2.8	2.4	5.2	2.1
Methylparaben	3.4	3.4	4.8	4.1
Sulfadimethoxine	4.5	3.1	4.0	4.3
Sulfaphenazole	3.3	4.0	5.2	3.0
Ethylparaben	2.9	3.2	4.3	4.0
Propylparaben	3.4	4.4	3.3	3.2
Ketoprofen	3.4	4.7	3.0	2.8
17 α-ethinyl estradiol	4.8	1.6	4.8	4.2
Estrone	4.4	1.9	2.5	4.0
Fenoprofen	5.4	2.1	4.5	4.2
Flurbiprofen	3.3	2.3	2.7	3.7
Diclofenac	5.2	2.3	4.5	5.2

Table 8-5: Recovery on Oasis HLB cartridges, LODs and LOQs of the analytes

Analyte	Recovery (% ± SD)		LOD (µg/L)[a]	LOQ (µg/L)[b]
	100 µg/L	1000 µg/L		
Sulfanilamide	32.2 ± 4.8	33.9 ± 2.6	10	33
Theophylline	74.8 ± 2.5	76.9 ± 1.3	12	40
Acetaminophen	31.6 ± 2.4	32.2 ± 2.9	9	30
Sulfacetamide	71.5 ± 2.6	73.7 ± 2.3	6	20
Caffeine	75.6 ± 1.7	75.8 ± 2.1	12	40
Sulfadiazine	81.3 ± 2.1	81.5 ± 2.5	7.5	25
Sulfathiazole	71.7 ± 2.7	72.2 ± 2.0	9	30
Sulfapyridine	90.6 ± 1.7	90.1 ± 1.6	3	10
Sulfamerazine	84.6 ± 2.5	85.8 ± 1.9	3	10
Sulfamethazine	95.1 ± 4.8	95.3 ± 3.5	1.5	5
Sulfamethoxypyridazine	91.6 ± 2.1	90.6 ± 2.7	7	23
Sulfamonomethoxine	89.9 ± 2.2	91.2 ± 5.7	7	23
Acetyl salicylic acid	12.7 ± 3.6	14.9 ± 2.6	15	50
Sulfamethoxazole	71.5 ± 2.8	70.5 ± 2.6	7.5	25
Methylparaben	81.4 ± 2.3	80.8 ± 2.5	7.5	25
Sulfadimethoxine	92.8 ± 3.9	95.2 ± 1.9	6	20
Sulfaphenazole	70.5 ± 5.3	71.4 ± 4.1	12	40
Ethylparaben	69.4 ± 4.1	72.8 ± 1.9	7.5	25
Propylparaben	77.8 ± 2.5	79.2 ± 2.2	7.5	25
Ketoprofen	82.9 ± 3.8	84.0 ± 1.9	8	26
17 α-ethinyl estradiol	67.5 ± 3.1	68.5 ± 2.3	15	50
Estrone	95.6 ± 3.0	97.0 ± 2.3	15	50
Fenoprofen	85.4 ± 4.6	86.5 ± 4.2	7	24
Flurbiprofen	76.3 ± 3.5	76.9 ± 4.2	3	10
Diclofenac	77.4 ± 3.8	78.4 ± 4.0	12	40

SD: standard deviation.[a] $S/N \geq 3$ and [b] $S/N \geq 10$

8.2.4 Analysis of real water samples

The method developed was successfully applied to the analysis of different pharmaceuticals and preservatives in real water samples. None of the target analytes were found in the analyzed river and lake water samples; however, in raw wastewater samples two of the studied analytes, sulfapyridine and sulfaphenazole, could be detected at their maximum absorption wavelength of 272 nm. Other analytes were below their LODs. **Figure 8-4** shows the chromatogram of the analyzed wastewater sample. The identities of these pharmaceuticals were confirmed as previously described.

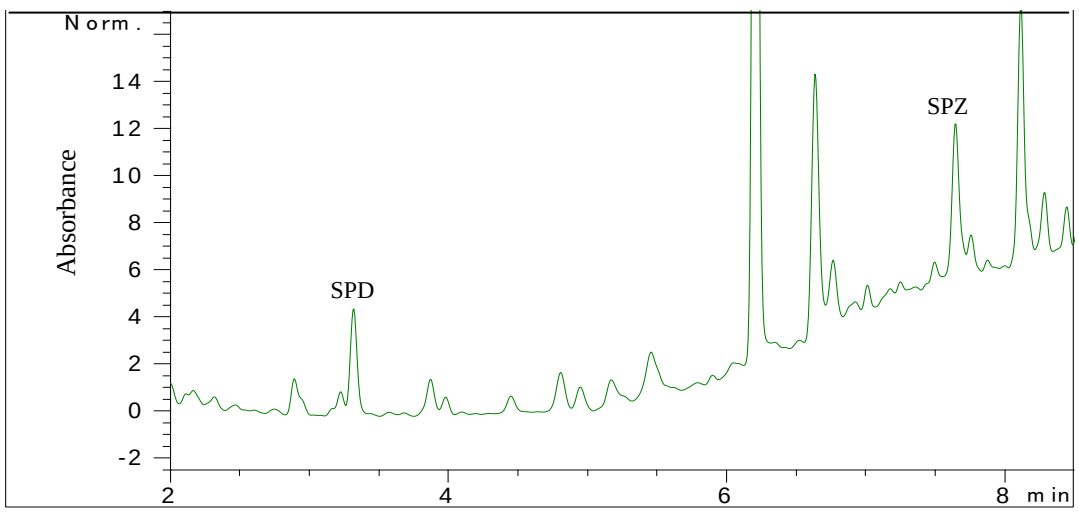


Figure 8-4: HPLC-UV chromatogram of wastewater sample extract (Oasis HLB cartridges) separated on the column packed with fused-core particles, showing sulfapyridine and sulfaphenazole at a detection wavelength of 272 nm.

8.3 Conclusions

A fast HPLC method with diode-array absorbance detector for the analysis of 25 emerging contaminants in different types of water samples was developed using a C18 reversed-phase column packed with fused-core particles. Separation of all compounds was achieved in 10 min with acceptable reproducibility, resolution and selectivity. Columns packed with fused-core particles are capable of running fast and efficient separations at conventional pressure limits. These particles produce lower back pressure, which allows much higher flow rates to be used. Fused-core particles can be used as an alternative to fully porous sub-2 μm particles for fast separations in environmental analysis. The method developed using the column packed with fused-core particles was used for the determination of selected analytes in river, lake and wastewater samples. Two of the studied analytes were detected in the wastewater sample.

Final conclusions and future work

Using temperature as a parameter in LC separations is an attractive way to speed up the analysis and/or increase separation efficiency. Increasing the mobile phase temperature could decrease the solvent viscosity, hence reducing the backpressure allowing the use of small particle size packing and the use of longer columns.

To enhance efficiency in LC, different approaches can be employed including reducing the packing particle size and/or increasing the column length through coupling. In the presented studies, both approaches were used. Coupling three columns packed with fully porous sub-2 µm particles and operating them at elevated temperature (80 ºC) resulted in a large number of theoretical plates compared to a single column at (30 ºC). The approach of column coupling was successfully applied to the analysis of pharmaceuticals in aquatic environment using both isocratic and gradient modes of LC separations. The method developed, based on column coupling, was the first implementation of this approach in environmental analysis and could be used as an alternative to the traditional methods for enhancing the efficiency.

In addition to the need for developing efficient methods, high speed analysis is also required for routine work. Most of the traditional methods used for the analysis of pharmaceuticals require a long time resulting in large solvent consumption. Therefore alternative fast analytical methodologies are highly needed. Based on this idea, very fast LC methods were developed in this presented work either by using fully porous sub-2 µm columns operated at 80 ºC to reduce the analysis time (3 min), or by using superficially porous sub-3 µm columns using solvent and

flow rate gradient to separate 25 emerging contaminants in only 10 minutes. These methods could decrease the analysis time while high efficiency was maintained.

Comparing the chromatographic performance of columns packed with fully porous sub-2 µm and superficially porous sub-3 µm particles showed that the use of columns packed with fully porous sub-2 µm particles might require special instrumentation because of the induction of high backpressure, but this drawback can be eliminated by the use of superficially porous particles. Columns packed with superficially porous particles can be used with conventional HPLC or UHPLC instruments while attaining similar efficiency as fully porous sub-2 µm particles. From this point of view, columns packed with superficially porous particles seem to be a more advantageous approach to easily achieve high speed LC separations even with conventional LC instrumentation. Using superficially porous particles is a promising approach for achieving fast separation in environmental analysis.

By increasing the awareness about the environment, green analytical methods are highly desired aiming to minimize or eliminate the consumption of toxic organic solvents. Based on this idea, an ultra-fast green method using a short narrow bore column packed with fully porous sub-2 µm particles was developed and applied for environmental analysis of some selected pharmaceuticals in an aquatic environment. All the analytes could be separated in one minute. The developed method using short narrow bore column could lower the solvent consumption making the analysis greener.

In conclusion, the proposed strategies presented in this work offer many advantages in terms of speed and efficiency, and can be used as an alternative to replace the traditional methods for environmental analysis.

The availability of new stationary phases, new column geometries and very high pressure systems opens the way for many researches to develop new effective analytical methods for the analysis of pharmaceuticals in different complex matrices such as food, environmental and biological fluids. While the most convenient approach to achieve fast, efficient and economic HPLC separations is the use of UHPLC technology with fully porous sub-2 µm or superficially porous particles, its application in environmental analysis is very limited. Although using high temperature in HPLC is a good alternative to improve separation efficiency and reduce analysis time, this approach is not yet routinely used in environmental analysis. The use of elevated temperature in environmental analysis has been typically limited to increases in temperature up to 60–80 ºC with conventional solvents. At even higher temperatures, the possibility of using pure water as an eluent in HPLC separations opens up, making the analysis completely green. The scarcity of stable high temperature packing materials and the perceived temperature instability of some compounds analyzed in environmental applications hinder the use of high temperature HPLC separations in the environmental area. Thus, the development of more stable packing materials is required in the near future to enable exploring the advantages of high temperature HPLC in the environmental field. More attention also should be given to replacing the traditional HPLC methods with green ones. This aim can be achieved either by replacing

toxic solvents with green alternatives, such as ethanol, carbon dioxide or pure water as an eluent, or by reducing the overall scale of the analytical instrument.

In addition to the need for improvements in one-dimensional HPLC separations, two-dimensional liquid chromatography (2D-LC) should also receive more attention. Although this technique has been considered a more powerful alternative for the analysis of complex samples compared with one-dimensional LC, it found limited applications in environmental analysis.

The speed of the second dimension separation is the main limitation of the total speed of 2D-LC. Elevating the column temperature or using superficially porous columns in the second dimension could speed up this separation. Also, higher temperature could reduce the backpressure allowing the use of longer columns to obtain higher resolving power. The compatibility of mobile phases in orthogonal 2D-LC is also a difficult problem. Finding new strategies to overcome these limitations will increase the application of 2D-LC in environmental analysis.

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