On-Chip HPLC analysis of drugs of abuse

Analysis of cocaine, morphine and their major metabolites using an on-chip HPLC system

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Summary

Being able to analyse drugs of abuse in blood at a crime scene can save a lot of time and money. Lab-on-a-chip devices could possibly be used to achieve this goal. A microchip based HPLC system coupled to UV-Vis has been used to developed a method for simultaneous measurements of cocaine, benzoylecgonine, morphine and morphine-3ß-D-glucorinide in water. Methods to prepare blood for on-chip analyses are also discussed in this thesis. The research showed concentrations of 12.5 mg/L could be measured, with a high possibility of being able to measure much lower concentrations as well.

Content

List of abbreviations

AR	Aspect ratio
COC	Cyclic olefin copolymer
COP	Cyclic olefin polymer
DLLME	Dispersive liquid/liquid microextraction
EME	Ecgonine methyl ester
HCl	Hydrogen chloride
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
LOC	Lab-on-a-chip
LSD	Lysergic acid diethylamide
MeOH	Methanol
MS	Mass spectrometry
NaCl	Sodium Chloride
NFI	Netherlands Forensic Institute
NICC	Belgium National Institute of Criminalistics and Criminology
PAC	Pillar array column
PPM	Porous polymeric monolithic
SEM	Scanning electron microscopy
SPE	Solid phase extraction
UGT	Uridine diphosphate glucuronosyltransferase
UV	Ultraviolet
Vis	Visible

Introduction

When police forces deal with crime scenes, they will on occasion find the remains of a human being. In order to find out whether drugs, poison or something else was the cause of death, blood is one of the biological samples that needs to be analysed. In the current situation, this means blood needs to be taken by a professional, after which the blood has to be analysed in a laboratory. Being able to analyse blood at the crime scene, without the help of a professional would be a great way to reduce unnecessary testing in laboratories. With the help of a 'lab-on-a-chip' (LOC) system, this could possibly be achieved. Conventional analyses of blood samples is done by first preparing the blood, followed by high-performance liquid chromatography (HPLC) separation, followed usually by analysis with mass spectroscopy. When translating these steps to LOC several challenges have to be addressed. Tackling these challenges is the topic of this thesis. This will be done by first getting some insight into cocaine and morphine, and their most important metabolites, which will be discussed in chapter 1. This is followed by a short explanation on the workings of HPLC, Ultraviolet-Visible (UV-Vis) spectroscopy and LOC devices in chapter 2. Chapter 3 will talk about blood analyses; it will discuss some specific off-chip blood preparation methods and it will talk about on-chip blood analyses as well. This is followed by an overview of different microchip techniques and their advantages/disadvantages in chapter 4; the microchips used for the experiments are also discussed in this section. The first experimental step is discussed in chapter 5, which is creating a method to analyse cocaine and morphine in water on a regular HPLC system, to see if any issues arise. The results and discussion for the regular HPLC measurements can also be found in this chapter. Chapter 6 will then discuss material and methods for microchip based HPLC measurements of cocaine, benzoylecgonine, morphine and morphine-3ß-D-glucorinide in water, followed by results and discussion for these measurements. To finish everything up, chapter 7 will summarize conclusions and chapter 8 will talk about recommendations for possible further research.

1 Drugs of abuse

There are many sorts of drugs of abuse with different effects on a human being. They can be categorized into several categories: opiates (e.g. heroine, morphine), hallucinogens (e.g. LSD), stimulants (e.g. cocaine, amphetamine), barbiturates (e.g. secobarbital), alcohols/diols and metabolites (e.g. ethanol, methanol), and sedatives/hypnotics/anaesthetics (e.g. oxazepam) [1]. Screening of said drugs is an essential technique in forensic science, because the voluntary or forced intake of drugs of abuse has many detrimental effects on individuals such as reduced consciousness (with possible negative effects on behaviour), long term health issues such as liver disease , and in the ultimate case premature death to an overdose, but also to society as a whole [2] [3]. This literature study will focus on cocaine and morphine detection, because these drugs are commonly used/abused and are readily available for experimental purposes. For a more comprehensive list on drugs of abuse, see Appendix A [1].

1.1 Cocaine

Cocaine is a potent central nervous system stimulant and a widespread drug of abuse, making it of high importance to identify and quantify its level in forensic toxicology. Cocaine is metabolized in blood by hydrolysis of the ester linkages to benzoylecgonine and ecgonine methyl ester (EME), respectively (Figure 1). These are the two major metabolites, which are inactive but very useful in detection of cocaine abuse due to their longer half-lives in biological matrices (approx. five times longer than cocaine). To a lesser extent cocaine is hydrolysed to norcocaine, an active metabolite, or by presence of ethanol in the blood to the toxic ethylene cocaine. All metabolites are expected to be metabolized to the common product ecgonine, which is difficult to isolate from biological fluids and therefore only rarely done [4]. The way cocaine is inactivated makes it rather tricky to analyse the compound, because of the polar analytes at the beginning and the relatively nonpolar compounds at the end [4]. Cocaine is toxic at blood levels greater than 1 µg/mL, and its therapeutic range is in between 100 and 500 ng/mL [1]. Relevant concentrations for forensics is at blood levels greater than 0.10 mg/L.

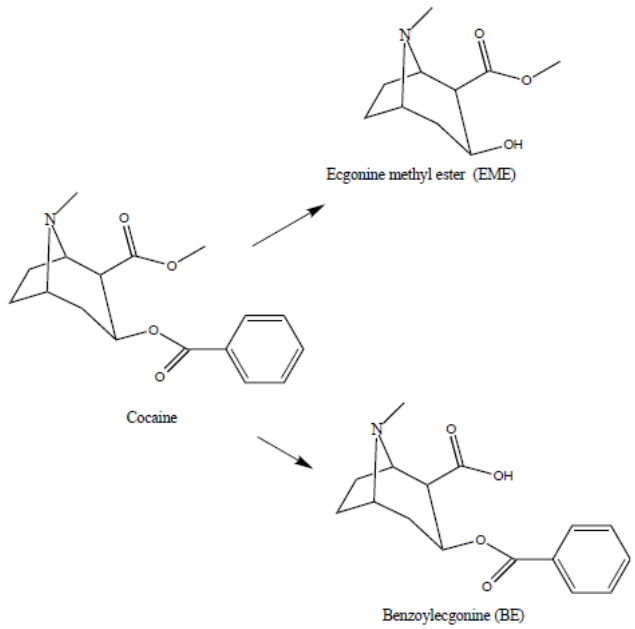

Figure 1: Molecular structures of cocaine and its two major metabolites, ecgonine methyl ester and benzoylecgonine.

1.2 Morphine

Morphine is a powerful opioid analgesic commonly used for management of severe pain. Morphine has a high potential for addiction as well as for the development of tolerance to the medication, which requires increases in dosage and/or frequency of administration [5] [6]. Morphine is metabolized to form morphine-3-β-glucuronide, morphine-6-β-glucuronide via liver uridine diphosphate glucuronosyltransferase (UGT) enzymes 2B7 and 1A3 (Figure 2), and in very small quantities normorphine, and hydromorphone. Morphine-6-β-glucuronide, which comprises approximately 10% of the metabolites formed, is bioactive and therefore toxicologically relevant [6] [7]. Bogusz et al. have discussed the possibility of correlating the blood levels of morphine and it is two glucuronides in, for example, fatal cases for the estimation of the survival time after drug intake [8].

Morphine is toxic at blood levels greater than 200 ng/mL, and its therapeutic range is in between 10 and 80 ng/mL [1]. Relevant concentrations for forensics is at blood levels greater than 0.010 mg/L.

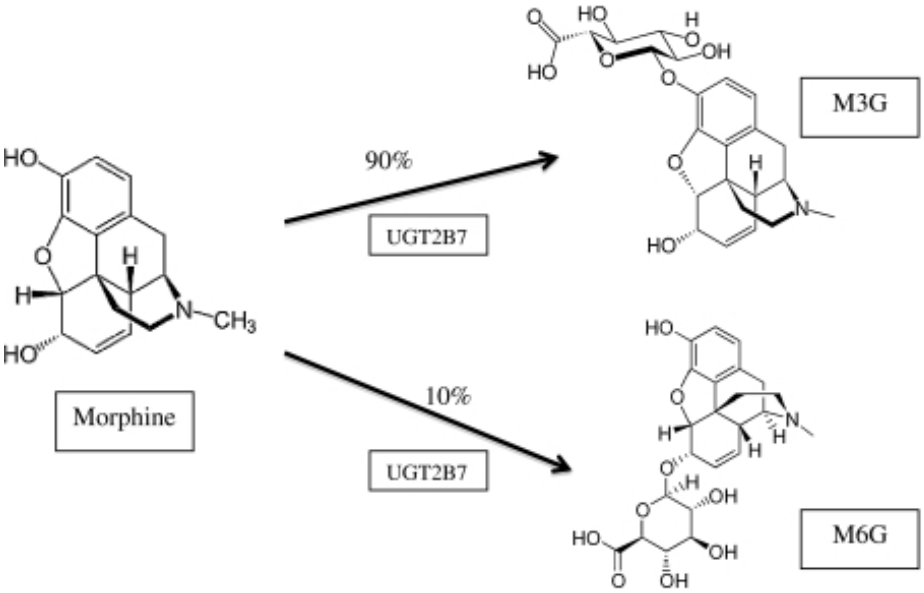

Figure 2: Molecular structures of morphine and its two major metabolites, morphine-3-β-glucuronide and morphine-6-β-glucuronide.

2 Drug Analyses

Analyses for this research has been done using high-performance liquid chromatography coupled to ultraviolet-visible spectroscopy. The final goal is to be able to perform this analyses on a LOC device. This chapter will shortly discuss the different techniques used.

2.1 HPLC

Chromatography is a separation process in which the sample mixture is distributed between two phases. In case of HPLC, a column is coated with a solid, porous, surface-active material in small particle form, and the second phase is a liquid that runs through the column. Components will move through the column at varying rates, based on their affinity towards the mobile and stationary phases. Phase preference can be expressed by the distribution coefficient, K:

$$K_x = \frac{C_{stat}}{C_{mob}}$$

Where x is the compound in the stationary phase, C_{stat} is the concentration of compound x in the stationary phase and C_{mob} is the concentration of compound x in the mobile phase. Figure 3 shows the representation of chromatographic separation, where the compound represented by a triangle has a stronger preference towards the stationary phase, and the compound represented by a circle has a stronger preference towards the mobile phase. In this sample, Figure 3c shows it takes about 3.5 particle diameters of the stationary phase to establish an equilibrium. This distance represents a theoretical plate. The higher the number of theoretical plates a column has, the better the degree of separation will be. Longer columns will allow for more theoretical plates, but this effect is partly compensated by band broadening [9] [10].

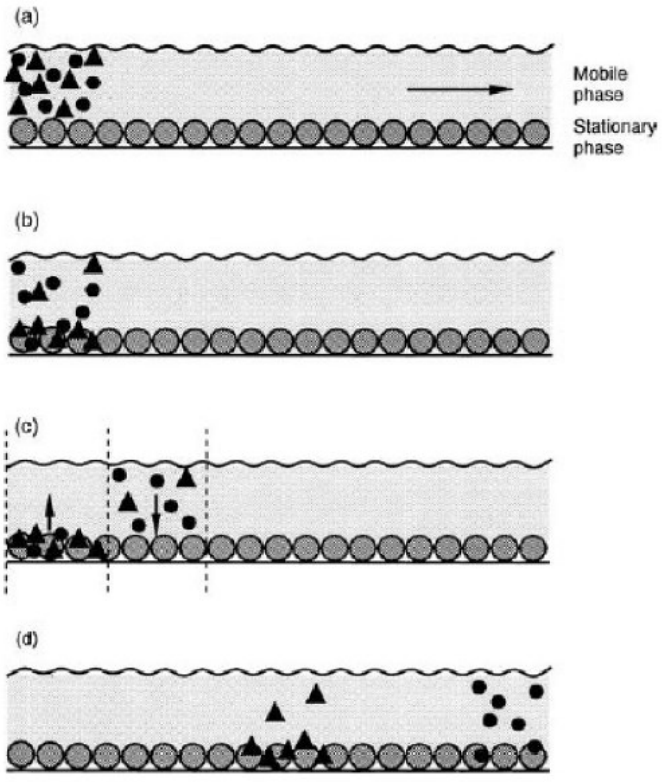

Figure 3: Representation of chromatographic separation [9].

HPLC analyses can be performed using 'normal phase HPLC' and 'reversed phase HPLC'. Normal phase HPLC uses a polar stationary phase and a non-polar mobile phase, where reversed phase HPLC uses a non-polar stationary phase and a polar mobile phase. Reversed phase HPLC is more common, as it offers more options for analyses [10].

Band broadening is influenced by several different factors. First, there is Eddy diffusion (A), which is diffusion caused by different travel paths through a packed column. Some molecules will get through in a mostly straight line, while others might have taken several diversions before leaving the column. Second, there is the diffusion coefficient (B) of the eluting particles in the longitudinal direction, which will result in dispersion. Third, there is resistance to mass transfer (C) of the analyte between mobile and stationary phase. These factors coupled with linear velocity (u), or the flow rate in the column, make for the van Deemter equation:

$$H = A + \frac{B}{u} + (C_s + C_m) * u$$

With H being plate height, which is optimal the lower it is. Figure 4 shows the dependence of band broadening on flow rate. Eddy diffusion is not influenced by the flow rate, and will therefore be absent in the case of a capillary column. Optimal plate height will not be used in most practical situations, because results can generally be obtained without reaching optimal plate height, which results in lower analyses time [9, 10].

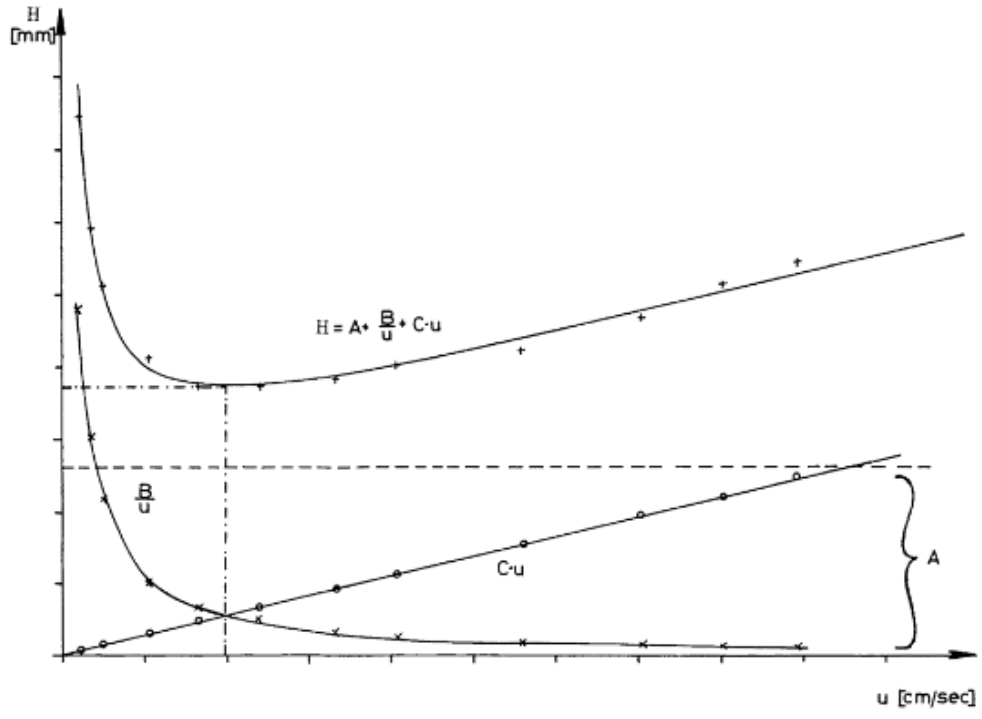

Figure 4: Dependence of band broadening on flow rate [10].

Once the compounds leave the column with the mobile phase and go through a detector, bell-shaped peaks can be observed, which can be used for both qualitative and quantitative analyses. The time it takes between sample injection and recording of the sample (retention time) is always the same under the same chromatographic conditions, and can therefore be used for qualitative analyses. The peak area and height are proportional to the amount of compound injected, so when compared to a peak from a known quantity, this can be used for quantitative analyses. Figure 5 shows an example peak which could be obtained through HPLC analyses [9].

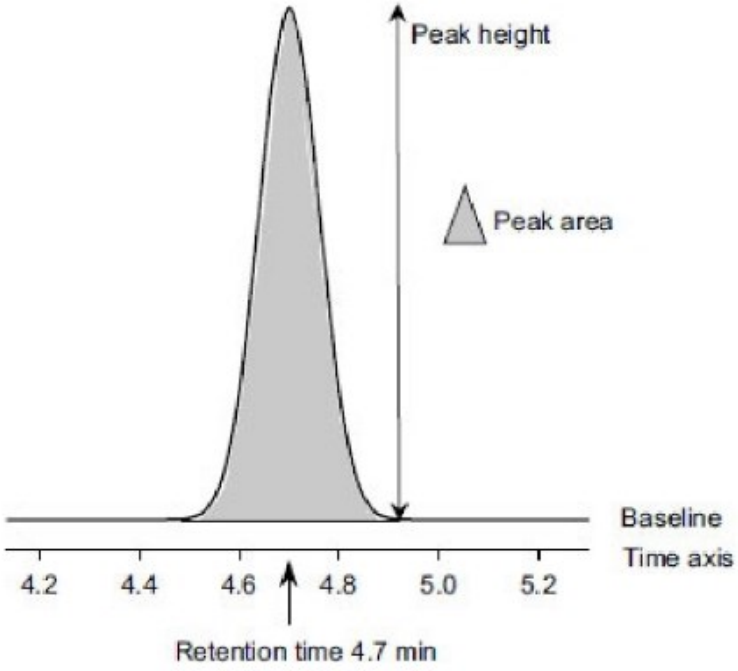

Figure 5: Example of a peak obtained through HPLC analyses [9].

2.2 UV-Vis spectroscopy

UV-Vis spectroscopy is the observation of the absorption of electromagnetic radiation in the UV and visible regions of the spectrum (~200 nm-~800 nm)(Figure 6) [11].

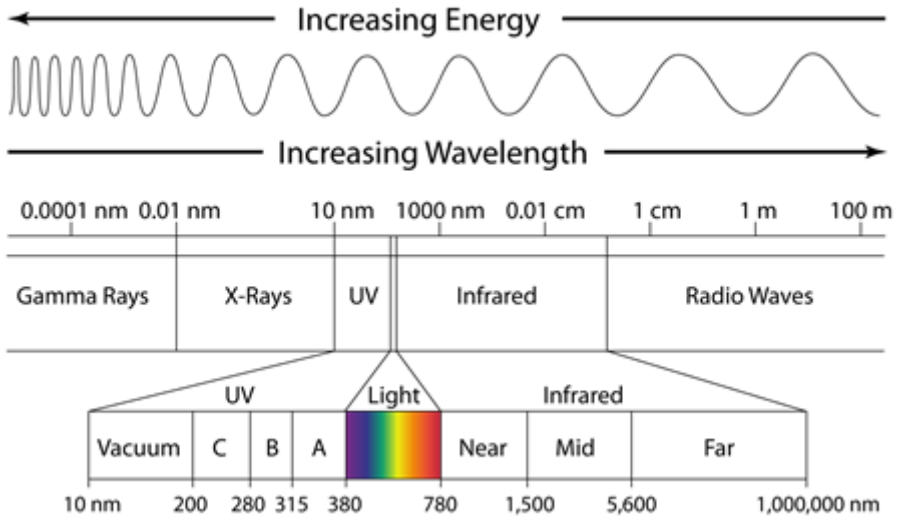

Figure 6: The electromagnetic spectrum with wavelengths and techniques that make use of the different regions [11].

The liquid exiting the HPLC column goes through a cell, constructed of an optically transparent material such as silica. Light will first go through a monochromator to separate the different wavelengths of light. After this the beam of incident radiation is split into two, one passing through the sample cell and one passing through an identical cell without the sample (reference). The beams are then compared at the detector (a photodiode) and the absorption is obtained as a function of wavelength. Figure 7 shows a simplified schematic of a UV-Vis spectrometer [11].

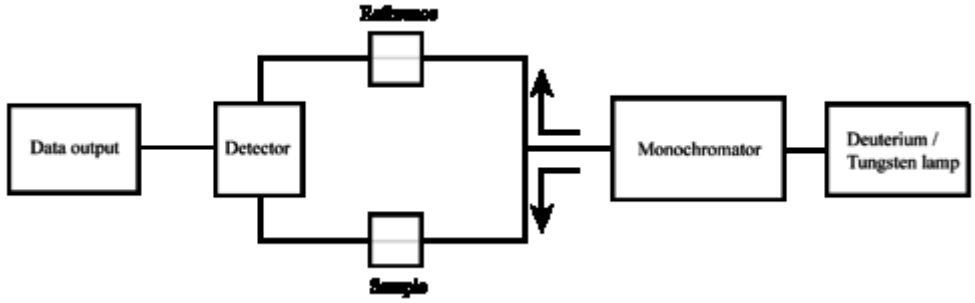

Figure 7: Simplified schematic of a UV-Vis spectrometer [11].

The intensity of absorption is measured as the absorbance, A, defined as:

$$A = \log_{10} \frac{I_0}{I} = \varepsilon[J]L$$

Where I_0 is the incident intensity and I is the measured intensity after passing through the sample. The Beer-Lambert law is used to relate the absorbance to the molecular concentration [J] of the absorbing species J and optical pathlength L, with ε being the molecular absorption coefficient [11].

2.3 Lab-on-a-chip

Being able to perform laboratory operations on a small scale using miniaturized (LOC) devices can be very appealing. LOC devices make use of microfluidic techniques, which use small amounts of fluids (10^{-9} to 10^{-18} litres), and channels with dimensions of tens to hundreds of micrometres. The small scale allows for several advantages over normal scale operations. Small volumes reduce the time needed to synthesize or analyse a product; liquids at the microscale behave in a unique way, which allows for greater control of molecular concentrations and interactions; quantities used are smaller, so reagents costs and the amount of chemical waste can be reduced. Another great advantage is being able to take a LOC device anywhere, which could, for example, allow for drug analyses at the point of need, rather than at a centralized laboratory [12].

3 Blood analyses

Quality of analysis can be greatly affected by the sample matrix. In a laboratory setting the composition of the matrix is often well known, but with real samples, the matrix can be very complicated. Some components might mask the presence of an analyte, while others might induce an additional detector response. Other possible effects can be clogging of the column, and fouling of the surfaces in the separation system [13].

3.1 Off-Chip blood analyses

Because of the complexity of the blood matrix, many methods involve pre-treatments of the sample. The methods vary widely, and most are fairly complex and time consuming. Below are three different methods used to prepare blood samples for further analyses.

Johansen et al. uses the following method for sample preparation:

0.200 g whole blood was mixed with 200 µL water and 100 µL 0.10 mg/L internal standard in acetonitrile, thereafter with 2.5 mL 0.2 M HCl and afterwards centrifuged for 10 min at 3600 rpm at 5 °C. The supernatant was transferred to a solid phase extraction (SPE) column for extraction. The column was first activated with 500 µL methanol and 500 µL 0.1 M HCl before applying the supernatant. The column was then washed with 1000 µL 0.1 M HCl and 1000 µL 20% methanol in H_2O, then dried for approximately 10 minutes and finally eluted with 500 µL freshly prepared solution of concentrated ammonia, dichloromethane and isopropanol (1:10:40, v/v). The eluate was evaporated to dryness at 50 °C under a steam of pure nitrogen. The remainder was reconstituted in 200 µL mobile phase and transferred to an autosampler vial of which 5 µL was injected into the chromatographic system [4].

Jagerdeo et al. uses the following method for sample preparation:

Pretreatment of the samples to precipitate plasma proteins was carried out by adding 1 mL of zinc sulphate heptahydrate/methanol solution to 0.5 mL of each blood sample. The samples were then vortexed and centrifuged for 15 minutes at 4000 rpm. 0.5 mL of the supernatant was removed and transferred to a labelled centrifuge tube with a filter, after which 0.5 mL of phosphate buffer was added and the tubes were centrifuged at approximately 3000 rpm for 5 minutes. Once completed, filtered samples were transferred to autosampler vials for analysis [14].

Fisichella et al. uses the following method for sample preparation:

500 µL of whole blood, previously spiked with a mix of drugs and internal standards, was deproteinized with 500 µL of methanol. After centrifugation at 10000 rpm for 10 minutes, 500 µL of the supernatant were transferred into a 15 mL conical tube containing 1 mL of water, 0.2 g of NaCl and 100 µL of saturated carbonate buffer (45g sodium bicarbonate, 30g sodium carbonate in distilled water) in order to reach pH9. DLLME was performed through the rapid injection of a binary solvent system constituted of 100 µL of chloroform as extractant and 250 µL of methanol as disperser solvent. The sample was gently shaken for 1 minute using an ultrasonic water bath and after centrifugation at 4000 rpm for 5 minutes the organic sediment phase (about 50 ±5 µL) was transferred into a vial and evaporated to dryness under a gentle nitrogen stream. The sample was then reconstituted in 100 µL of mobile phase A (0.1% formic acid in water) [15].

The Netherlands Forensic Institute (NFI) and the Belgium National Institute of Criminalistics and Criminology (NICC) both use a much simpler method; they only centrifuge the blood samples and inject the upper layer into the system. While the method used by the NFI and NICC is much less time consuming, it will mean the sample contains more possible interferences and is more likely to clog the column.

Mobile phase and columns used for blood analyses were mostly the same across various articles, using mostly C18 columns and a gradient mobile phase consisting of both acetonitrile and water with low amounts of formic acid (~0.1%) [4] [14] [15]. Results obtained were well defined (Figure 8), however, mass spectrometry was used for all these methods. UV detection has a higher limit of detection, which likely means results obtained using UV detection will not be as good.

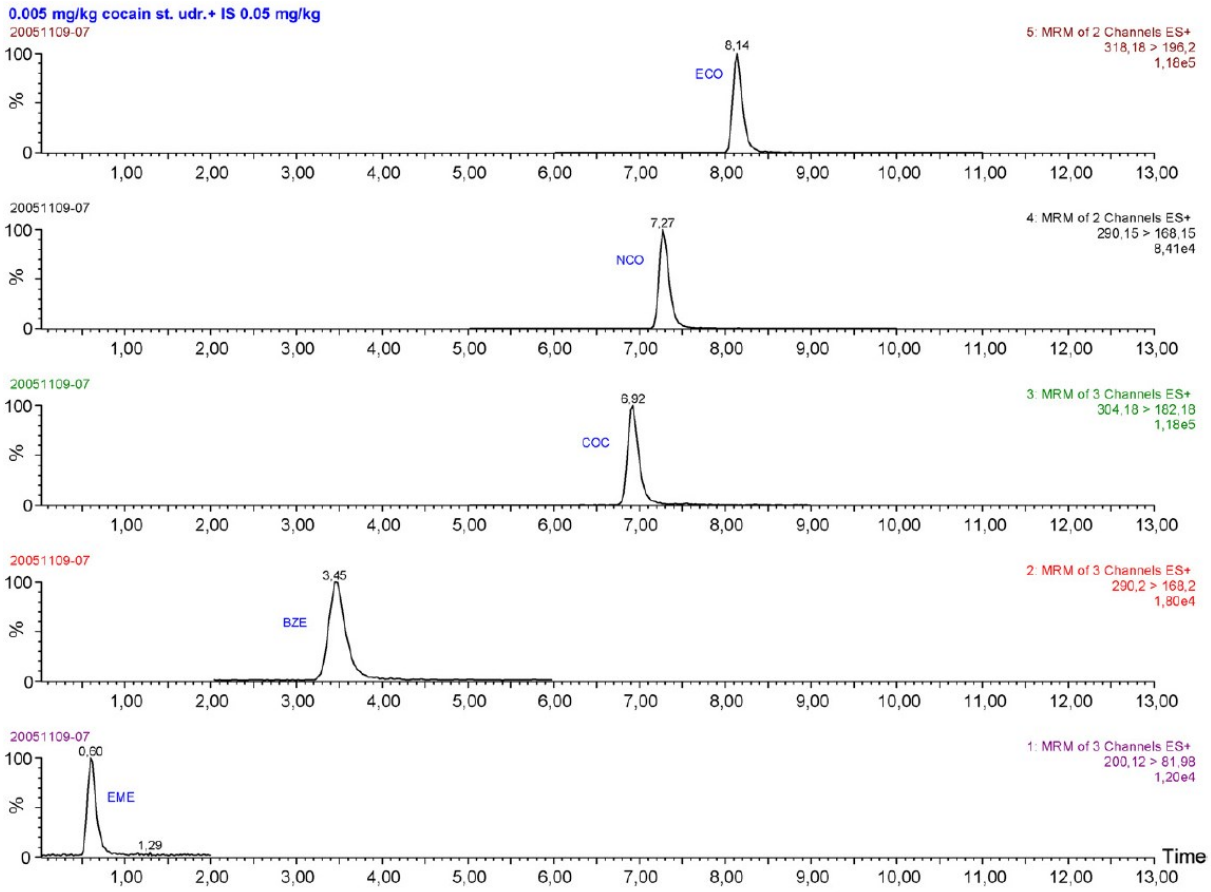

Figure 8: Ion chromatograms of cocaethylene, norcocaine, cocaine, benzoylecgonine and ecgonine methyl ester at 0.005 mg/kg in whole blood – analysed by LC/MS/MS after SPE [4].

3.2 On-Chip blood analyses

Sample clean-up is a more important step on chip-based systems, because of the much smaller channels, which consequently clog easier. Manual sample clean-up as discussed in chapter 3.1 is always an option, but there are several systems available for possible on-chip sample preparation.

Depending on the clean-up, different techniques such as physical particle filtering, diffusion-based filters, dialysis and cell lysis have been implemented on-chip. Sample clean-up techniques are typically used at the front end of an analysis system, but intermediate sample clean-up steps further down the line might be required in some situations. Centrifugally pumped microfluidic systems, which utilize microchannel networks laid out on compact disc-like platforms, can be spun at high rotation rates to drive solutions through the microchannels, allowing for on-chip implementation of analytical steps that would normally require a centrifuge. Filtering particles is especially important in microchip systems to avoid channel clogging. An overview of the size ranges relevant to filtering systems Is given in Figure 9 [13].

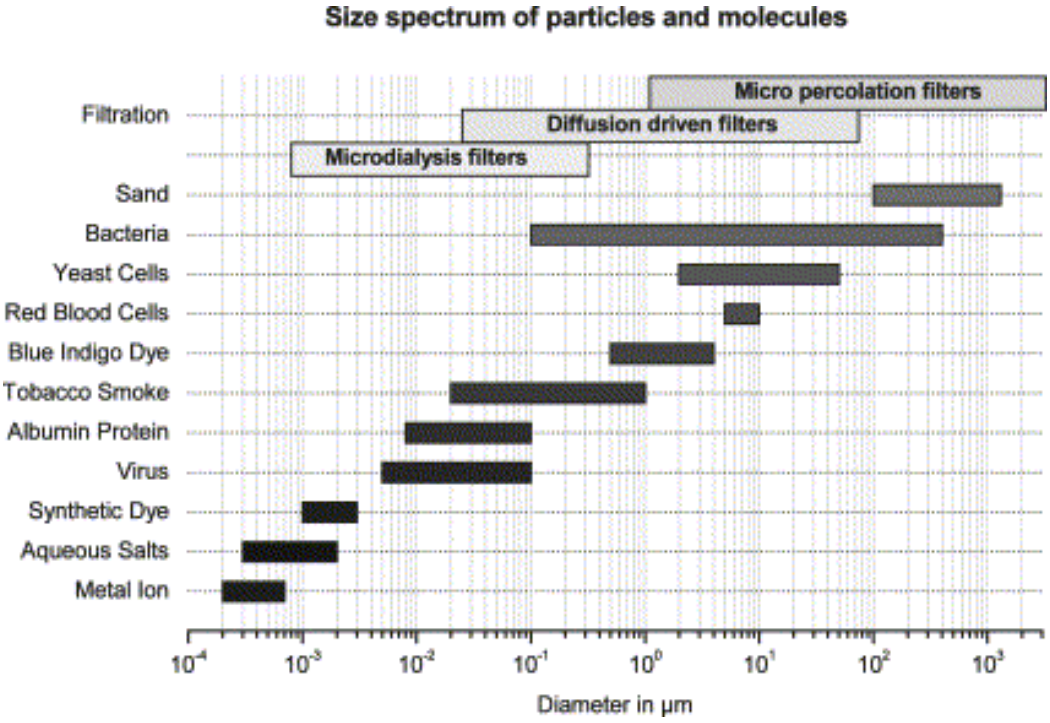

Figure 9: Typical dimensions of particles or analyte entities encountered in microfluidic systems [13]

4 Microchips

Microchip liquid chromatography chips have been constructed in various ways, each with their own advantages and disadvantages (Table 1). Three commonly used stationary-phase supports are particle-packed columns, monolithic columns and pillar array columns.

Table 1: Advantages and disadvantages of three different stationary-phase supports [16].

Support Type	Advantages	Disadvantages
Particles	• Many stationary-phase selectivities available • High batch-to-batch reproducibility • High loadability	• Packing quality dependent on packing skills • Retaining frit required • Higher back pressures generated
Monoliths	• Lower back pressure than particle-packed beds • No packing or frits (mostly) required • Different base chemistries available	• Inherent variations in synthetic approach (lower batch-to-batch reproducibility) • Synthesis procedure can depend on chip substrate
Pillar Arrays	• Ordered structure can result in higher efficiency than random-packed bed • Can be fabricated by nanoprint lithography (for mass production)	• Limited loadability • Hard to make porous (and doing so can affect performance) • Sophisticated fabrication techniques required

4.1 Particle-packed columns

One of the drawbacks of packed beds on microchip is the necessity for retaining frits to keep the particles in place, and advantage is obtained if the packed beds can be removed and refilled (allowing re-use of chip). Most chip-LC frits have relied on blocking particles through channel constrictions or weirs. These frits are usually only found at the outlet of the column, resulting in being limited to a single flow direction and possible particle loss at the inlet. Recently however, a two-weir column channel has been described that eliminates both particle loss and the need for a connecting channel between the sample injector and the head of the column. Filling of the channel is done via a side-packing channel, which is then completely closed to flow using and UV-polymerized monolith solution. This technique was later modified such that the actual monolith could be used as a frit. With these frits (Figure 10) beds can be packed in reverse from the column outlet and then fritted in a specific desired position, which allows for different column lengths and increases flexibility of the location of the column on the device [16].

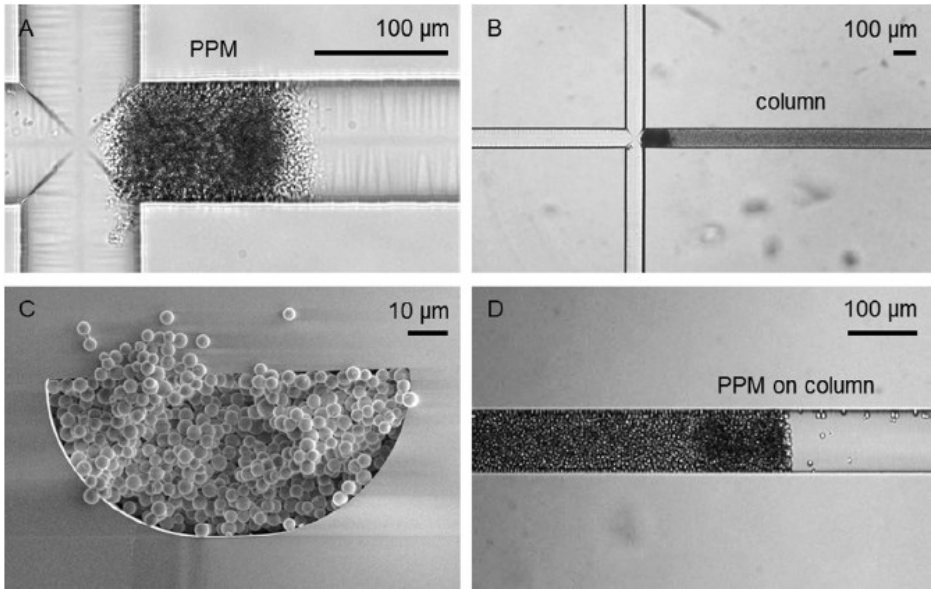

Figure 10: Fabrication of a packed bed using porous polymeric monolithic (PPM) frits. First, the inlet frit is formed adjacent to an injection cross (A), and a column bed is packed in reverse against this frit (B, with column cross section shown in C). Then, an outlet frit is polymerized at a distance equal to the desired length of the column, and the remaining particles are flushed out of the outlet (D) [16].

High chemical resistivity and optical transparency are important for analytical separation and detection, which is why glass is a common substrate for microfluidics. However, the pressure limits of such devices are often far lower than those of capillary columns. In order to allow for higher pressure limits, metal substrates have been used to improve device strength. A downside to 3D printed metal chips is low chromatographic efficiency due to surface roughness. Although on-column optical detection is limited when using a metal substrate, coupling one with MS would work well. The lower flow rates are more compatible with electrospray ionization than standard LC columns and lower injection volumes can be used [16].

4.2 Monolithic columns

Chip-LC monolith columns are often used in low-cost polymer substrate devices. These low cost substrates cannot hold pressures as high as glass or silicon substrates, but they often have lower flow resistance than particle-packed beds, making it possible to work with low cost substrates. Cyclic olefin polymers (COPs) and cyclic olefin copolymers (COCs) are two low cost substrates becoming more popular because of their high chemical resistance, good optical transparency, and easy of fabrication. These materials can be useful with monolithic columns, because of their compatibility with the solvents needed to form monoliths and high transmission of UV light for photopolymerization. A technique for the ex situ formation of a variety of PPMs that can then be integrated into COC chips was developed. Trapezoid-shaped monoliths were fabricated to easily align into similarly shaped channels, after which the monoliths were bonded and sealed into place with the substrate (Figure 11) [16].

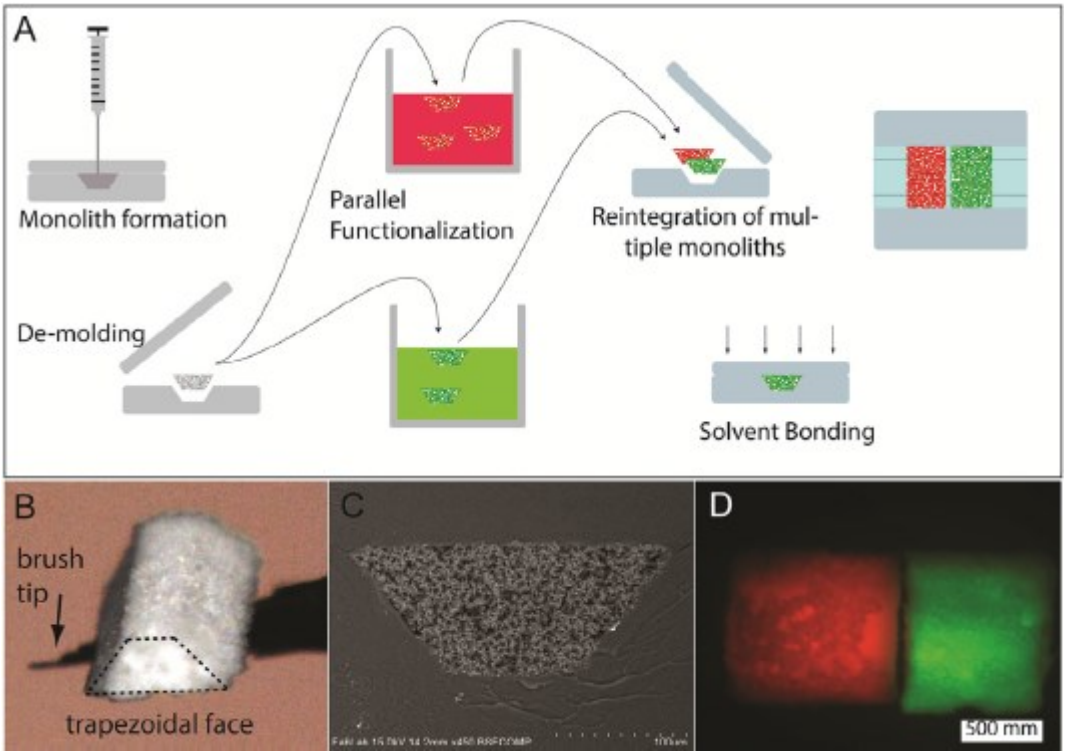

Figure 11: Formation of an ex situ monolith, monolith functionalization, and integration into a microfluidic chip (A). Optical micrograph of an ex situ monolith is show in (B) with a scanning electron microscopy (SEM) image of the chip cross section after the monolith has been integrated into a chip and bonded (C). In (D), two monoliths (with a different dye covalently attached to each) are embedded in the same channel [16].

4.3 Pillar array columns in microchips

Pillar arrays are generated using photolithography and deep reactive-ion etching. These techniques allow for highly reproducible structures and high production throughput, giving pillar array columns (PACs) high potential as a stationary-phase support.

Pillar array columns are nearly completely devoid of eddy diffusion (A-term) due to their near perfect order. Another advantage compared to packed beds is the much more open and uniform structure of their flow-through pores, lowering flow resistance significantly. Flow resistance can be further reduced by increasing external porosity of the pillar array [17]. The other main source of dispersion is axial diffusion (B-term). Suppressing this type of diffusion requires a strong degree of anisotropy , which is not possible with the strong isotropic nature of monoliths and packed columns, but can be achieved with pillar array columns. Pillar array columns were mostly created using either cylindrical, pillar shaped or axially elongated pillars. These arrangements were mostly chosen based on flow resistance and C-term dispersion considerations, as this would provide the most uniform flow profile [17]. Radially elongated pillars have been created to suppress the effective diffusion in the axial direction. Only a fraction of the sample band can diffuse in axial direction, due to the low number of exit points in axial direction [16] [17]. Two drawbacks to these types of columns are that the velocity at which the lower plate height is reached is decreased and there is a larger pressure drop due to larger flow obstruction [18]. Figure 12 shows pillar arrangement and velocity field of (a) a conventional cylindrical pillar array column and (b) a radially elongated pillar column.

One of the drawbacks of pillar array columns is their low sample loadability. Two approaches can be used to achieve the same level of loadability with PACs as with porous-shell particle columns. Sol-gel deposition has a number of drawbacks, such as the fact that the procedure is additive and specific surface area dependent, resulting in a uneven form and thickness of the generated silica along the column. A second drawback lies in regions with insufficient confinement, where no conformal coating will occur and predominantly loose particles will be formed, which can result in a decrease in permeability or even clogging. The second approach to generate porous layers is electrochemical anodization, which will generate meso-pores growing inwards in the pillars and thus leaving the original flow profiles at the sidewall region unchanged (Figure 14) [19].

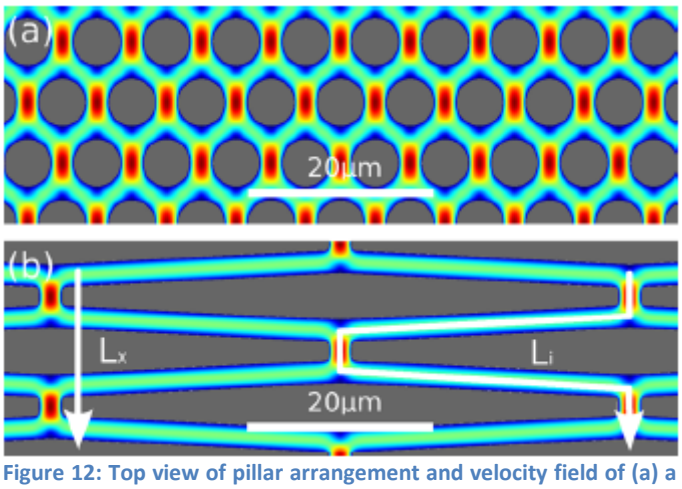

Figure 12: Top view of pillar arrangement and velocity field of (a) a conventional cylindrical pillar array column and (b) a radially elongated pillar column with aspect ratio = 9. White arrow L_x indicates the net direction of flow; white arrow L_i indicates the direction of flow along the tortuous path followed by the liquid [18].

4.4 Porous Pillar shaped PAC ('long chip')

Two different chips have been used for measurements, the first being a porous pillar shaped PAC as mentioned in chapter 4.3, which has the following dimensions: the channels were composed of 5 cm long channel tracks filled with 5 µm diameter pillars (spacing 2.5 µm) connected by turn channels. Turn channels can cause the so-called racetrack effect, as to minimize the losses caused by this effect, the width of the actual turn segments was minimized (20 µm wide) while the transition between the 300 µm wide separation channels and the turn channels was made via two flow distributors (Figure 13). Total length of the separation channel on the chip was 1 m [19]. Material used to fabricate the chip is silicon; for a full overview of the fabrication process of the chip, see Callewaert, M., et al. (2014).

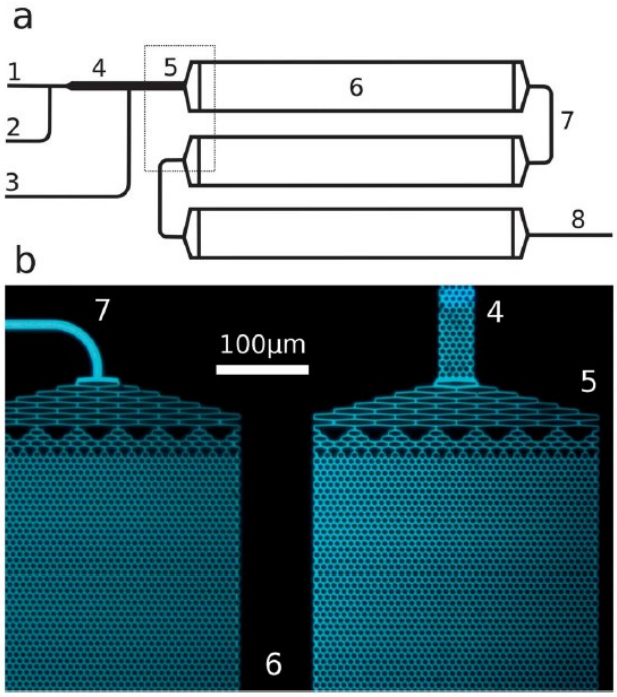

Figure 13: (a) Schematic overview of the different zones of the chip. 1: inlet mobile phase, 2: inlet sample phase, 3: outlet sample phase, 4: injection box, 5: inlet distributor, 6: channel track, 7: connecting turn, 8: mobile phase outlet. (b) Optical fluorescence microscope image of the box at the channel section (filled with 1 mM C480 dye) in (a) [19].

As mentioned before, one of the advantages of PACs is the ordered nature of the etched non-porous pillar arrays, which is why it is crucial to make sure that the added surface is uniformly distributed along the channel. Under the applied anodization conditions used for this microchip, the thickness of the porous layer was 300 nm for 10 min anodization (Figure 14a and b).

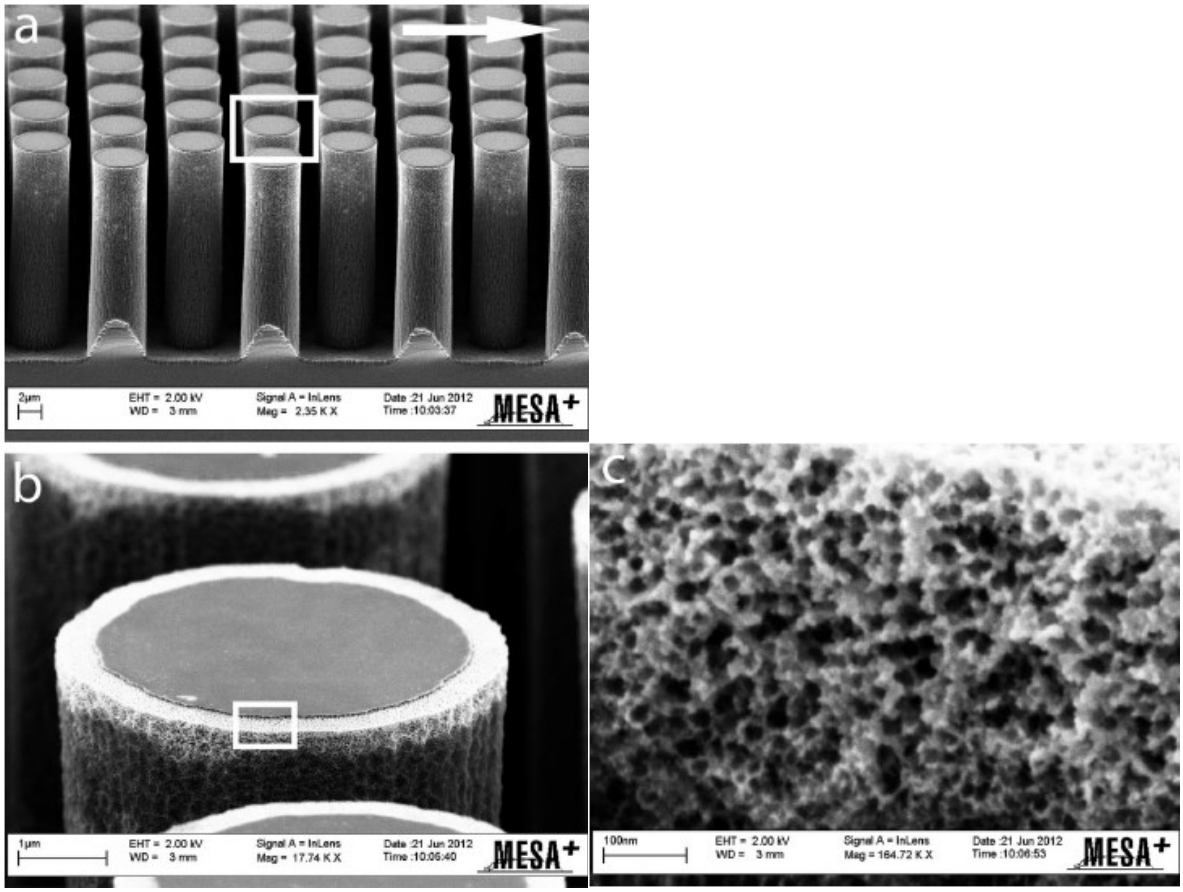

Figure 14: SEM images of the porous shell micro pillar array column. (a) The pillars are positioned on an equilateral triangle pattern with a distance of 2.5 µm between the pillars. The total diameter of the porous shell pillar is 5 µm, the height is 18 µm and the porous shell thickness is approximately 300 nm. The arrow indicates the flow direction, the white box shows the region used for higher magnification in the image below. (b) One single pillar in detail, the white box shows the magnification for the image below. (c) Zoom-in on the porous shell around the pillar. The pores are in the range of 30 nm in diameter [19].

As shown in Figure 14a and b, great uniformity can be obtained by using the before mentioned method. Another major advantage of this method, is that the pore size can be easily altered by varying the electrical potential. In this example, a sufficiently large pore size was aimed for, so that separation of relatively large analytes would be possible as well. As seen in Figure 14c, the current anodization conditions lead to an average pore size around 30 nm. A large discrepancy between the performance measured inside a pillar array with on-chip fluorescence microscopy and that measured with a commercial HPLC system is often seen. Dispersive effects related to sidewall-, turn- and interfacing can be excluded by using on-chip measurement. In previous off-chip detection systems, around 80% of the performance was lost. With the current set-up, where a porous shell layer of 300 nm thickness on a folded channel containing 5 µm diameter pillars was connected to a commercial capillary LC instrument using UV-Vis detection, excessive coupling losses that generally occur in classic capillary column operations were reduced to 10% for a 1 m column [19].

4.5 Porous Radially elongated structure PAC ('short chip')

The second chip used for measurements was a porous radially elongated structure PAC as mentioned in chapter 4.3, which has the following dimensions: Pillar array columns with radially elongated pillars (1.25 cm long, 1 mm wide) have been fabricated. The wafer contained channels filled with pillars with an aspect ratio (AR, equalling the ratio of their largest radial to the axial dimension) of AR = 15 (Figure 15). The axial length of the pillars was set at 5 µm and their radial length was 75 µm. They were positioned on an equilateral triangular grid at an interpillar distance of 2.5 µm throughout the whole array, except at the sidewall region where a distance of 2 µm between the sidewall and the adjacent pillar was maintained. Total length of the separation channel on the chip was 10 cm [17]. Material used to fabricate the chip is silicon; for a full overview of the fabrication process of the chip, see Op De Beeck, J., et al. (2013). The radially elongated pillars for this chip were also made porous, to gain the same advantages as discussed in chapter 4.4.

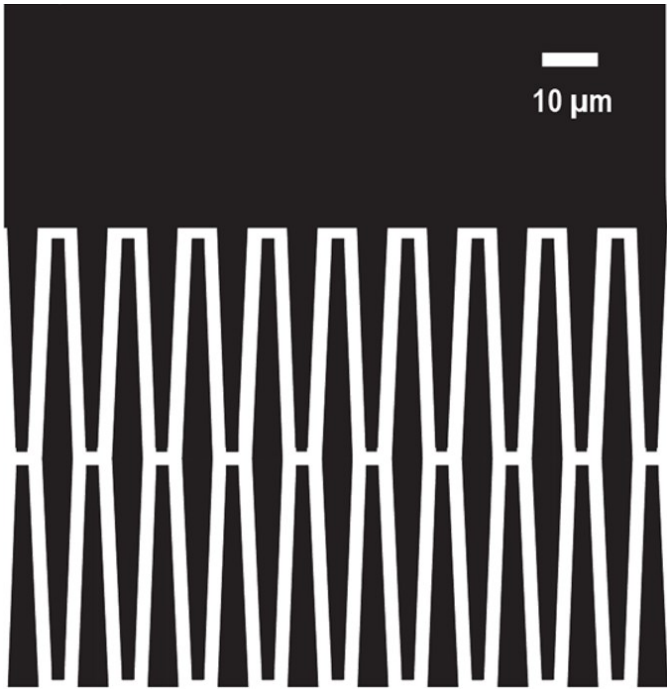

Figure 15: Wafer design of radially elongated pillars with AR = 15 of a pillar array column [17].

Both chips discussed in chapter 4.4 and 4.5 are PACs and both of them use a C18 coating. The two main differences between the chips are the pillar shape and the length of the chips. Even though the B term in the van Deemter equation is smaller for the chip mentioned in chapter 4.5, the total number of plates is still greater for the chip discussed in chapter 4.4, due to it being much longer. To obtain results that are relevant for forensic applications, a high number of plates will greatly help, which means the chip discussed in chapter 4.4 will likely obtain better results.

5 Regular HPLC

Measurements were initially done on a regular HPLC setup, to get an idea of what retention times to expect and to see whether or not components could be separated using simple HPLC conditions. Only cocaine and morphine were measured with a regular HPLC, because ecgonine methyl ester, benzoylecgonine and morphine-3ß-D-glucorinide, that were later measured on a chip based HPLC could not be obtained at this point in time.

5.1 Material and methods regular HPLC

5.1.1 Chemicals

Cocaine-HCl (1 mg/mL) in methanol, morphine (1mg/mL) in methanol, methanol, HPLC-grade acetonitrile, sodium dihydrogen phosphate, and phosphoric acid, obtained from Acros Organics. Milli-Q water was obtained from an in-house Millipore purification system.

5.1.2 Stock Solutions

Solutions of 0.1, 1 and 10 mg/L were made for both cocaine and morphine, using a 1 mg/mL stock diluted with Milli-Q water to acquire the desired concentrations.

5.1.3 Instrumentation

For separation two setups have been used. First, an Instrument Solutions Lab Alliance series II/III HPLC system with a 150 mm x 4.6 mm reverse phase C18 column, packed with 5 µm particles. Elution was done at 1 mL/min with an isocratic mobile phase consisting of 30% acetonitrile and 70% phosphate-buffer (pH 3.0). An Instrument Solutions S3210 UV/Vis spectrometer was coupled to the HPLC system, and the wavelength was set at 239 nm. Data was obtained using Clarity Chromatography Software. The second setup was an Instrument Solutions Beta gradient pump, coupled to an Instrument Solutions Sapphire variable wavelength detector. All other variables were the same as used in the first setup.

5.1.4 Methods

The goal of these experiments was to see whether or not results could be obtained using simple HPLC conditions with a standard C18 column, for different concentrations of both cocaine and morphine. Samples were injected manually using a syringe, after which data acquisition was started manually through the Clarity Chromatography Software. The syringe was cleaned after every injection using methanol.

5.2 Results and discussion

Blank Milli-Q water was measured first in order to get a starting reference. As shown in Figure 16, at 2.23 minutes a peak can be observed. The same peak can be found in the results for cocaine (Figure 19), but it does not interfere with the cocaine peak. For morphine it does pose an issue, which will be discussed later. From the results for blank Milli-Q water (Figure 16) and cocaine (Figure 17,9,10), it becomes evident that the peak at 2.23 minutes is constant. Employees at Saxion Hogeschool, where the HPLC device used for these experiments was located, confirmed that the peak at 2.23 is a common occurrence with this device; the most likely cause being injection of the sample.

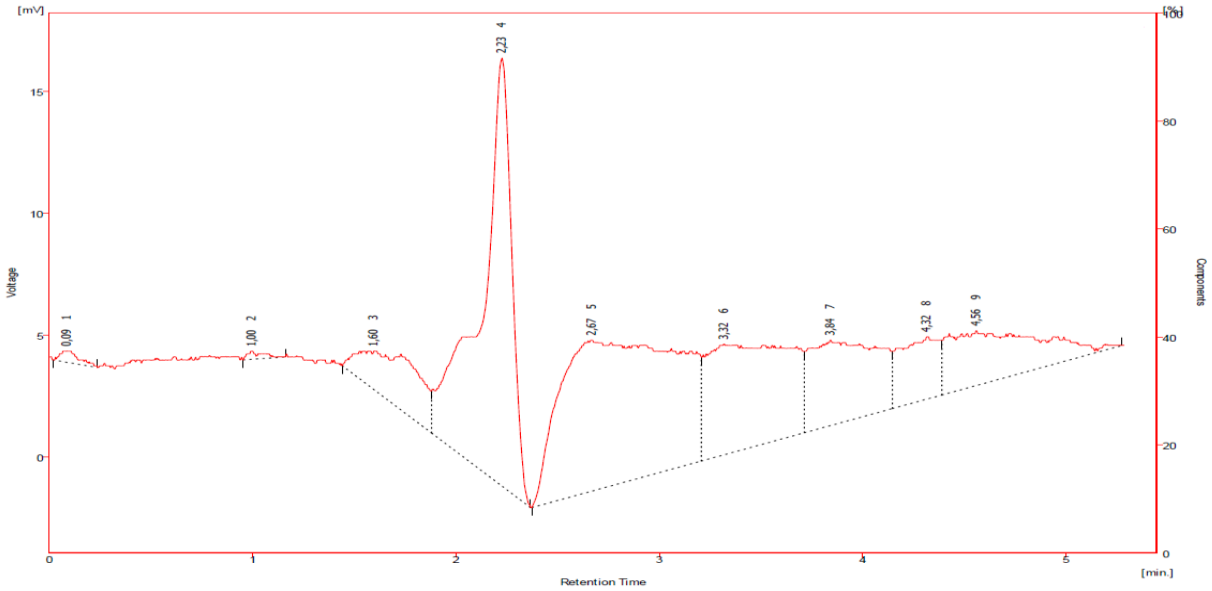

Figure 16: Chromatogram of a blank Milli-Q sample obtained using HPLC-UV/Vis.

5.2.1 Cocaine results

The chromatograms for cocaine (Figure 17,19,20) show the same peak at 2.23 minutes as earlier discussed for the blank Milli-Q chromatogram. The cocaine peak itself can be observed at around 4.40 minutes. Slight variations in retention times are most likely caused by manually starting the data acquisition. For all three chromatograms, the cocaine peak can be observed well (concentration value giving a ratio signal to noise S/N > 3). Figure 18 shows the chromatogram for cocaine (10 mg/mL) for setup 2. The peak at 2.23 minutes cannot be observed in this chromatogram, however, the retention time for the cocaine peak is also different from results from the first setup. The gradient pump was experiencing pressure drops, likely caused by a leak in the system (liquid could be seen dripping from the system near the column), which could account for the difference in retention time between the two setups.

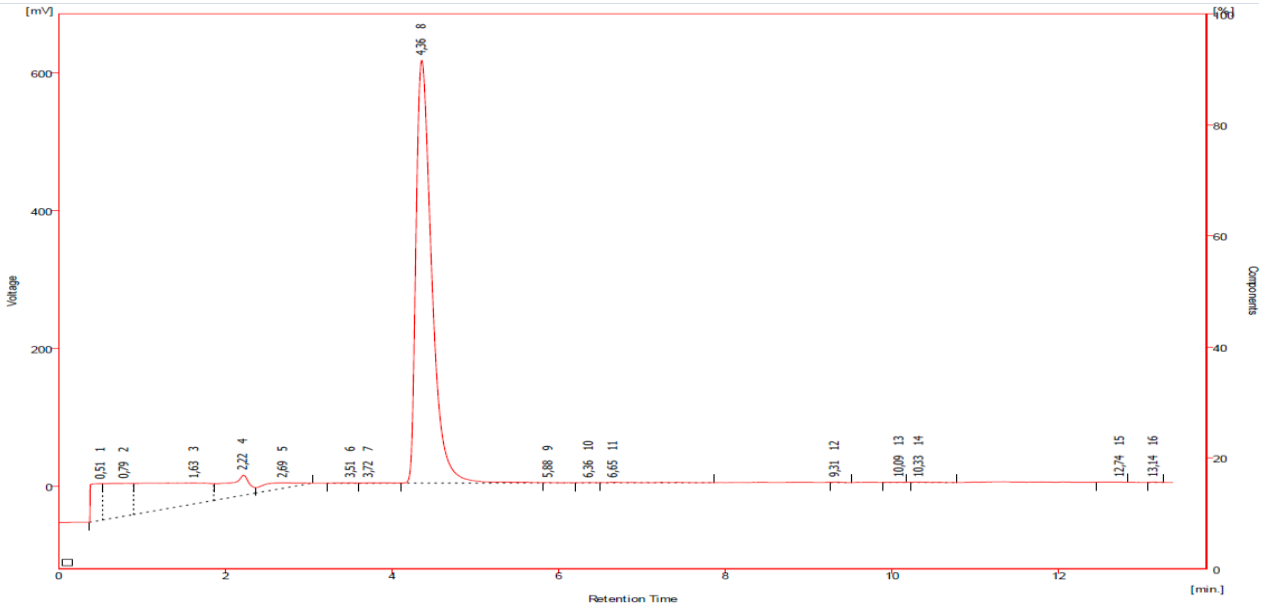

Figure 17: Chromatogram of cocaine (10 mg/mL) obtained using HPLC-UV/Vis setup 1.

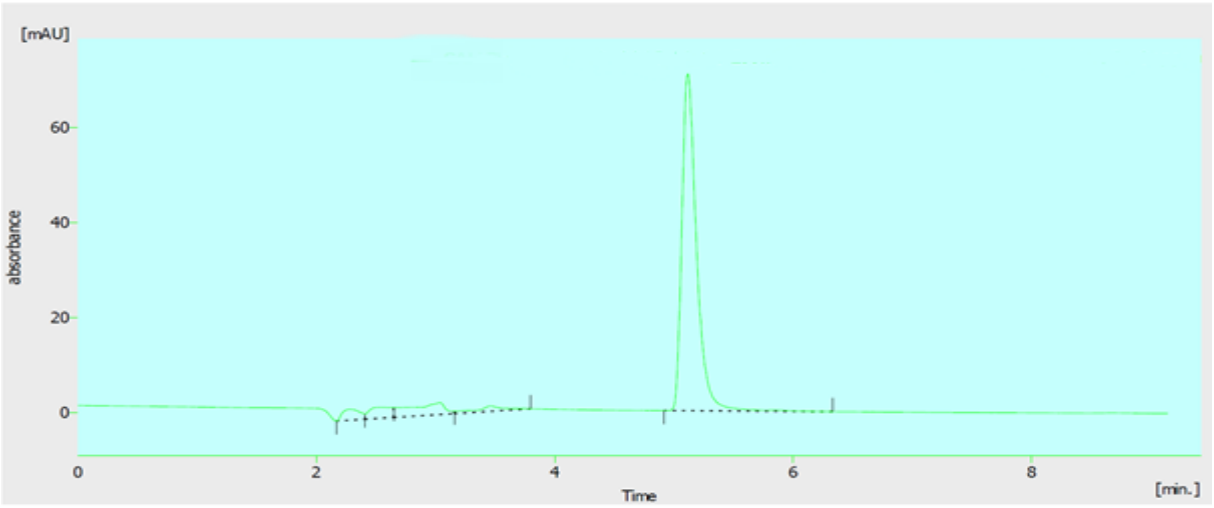

Figure 18: Chromatogram of cocaine (10 mg/mL) obtained using HPLC-UV/Vis setup 2.

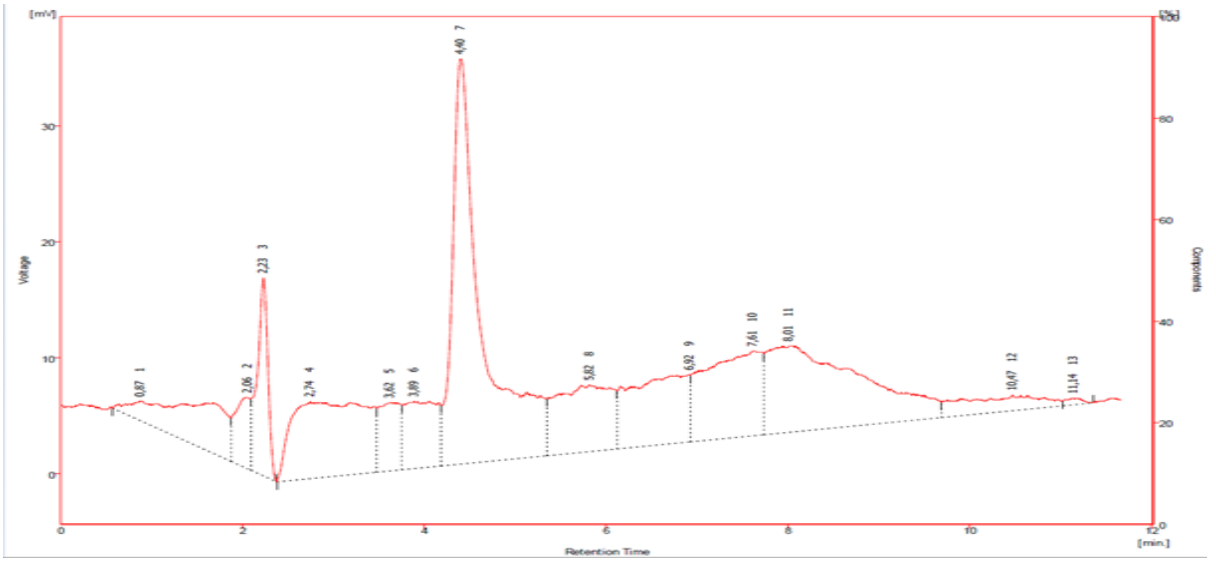

Figure 19: Chromatogram of cocaine (1 mg/mL) obtained using HPLC-UV/Vis setup 1.

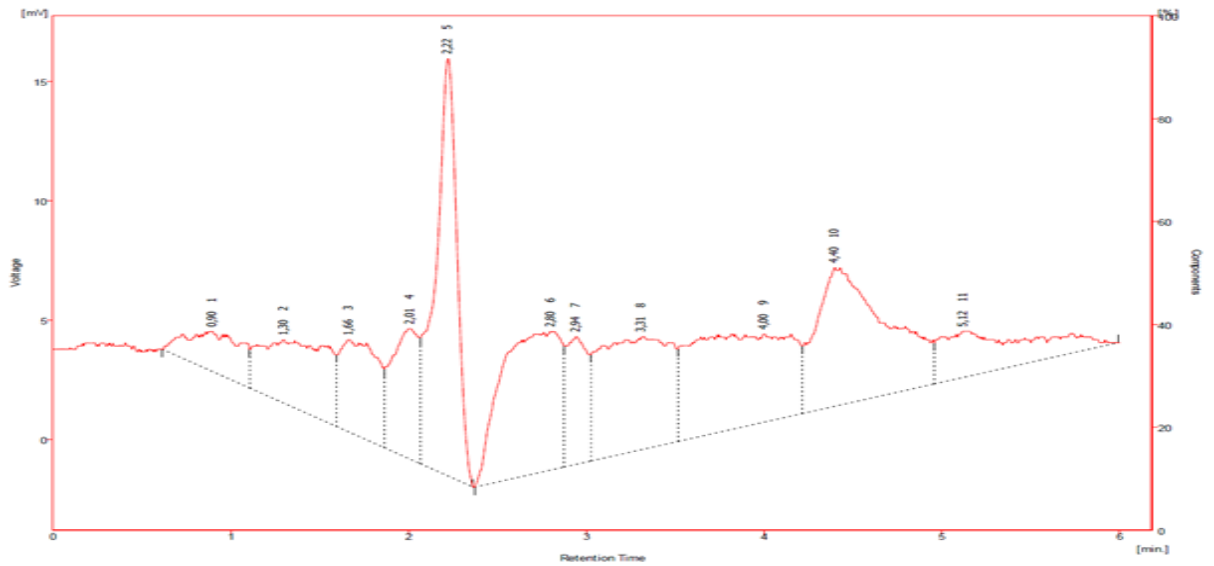

Figure 20: Chromatogram of cocaine (0.1 mg/mL) obtained using HPLC-UV/Vis setup 1.

5.2.2 Morphine results

The chromatograms for morphine (Figure 21,13,14) clearly show an issue with the unknown peak at around 2.23 minutes. Morphine has the same retention time as the unknown peak under the conditions used for these experiments. Figure 21 shows a peak much larger than shown before in Figure 16 which proves the morphine peak is actually there. For 10 mg/mL the unknown peak does not interfere with the intended results; it is clear morphine at that concentration can be measured well under the current experimental conditions. However, results for 1 mg/mL (Figure 23) become harder to interpret, although the peak at 2.23 minutes is still clearly bigger than in the Milli-Q blank. For the results for 0.1 mg/mL (Figure 24), it is no longer possible to see whether or not morphine can be detected, because the unknown peak is bigger than the morphine peak would be. In order to properly separate morphine from the injection peak, morphine would have to gain retention in the column, as it currently flows through the column without any. When just taking morphine into account this can be done by increasing the amount of water in the mobile phase using isocratic settings, however, this would significantly slow down analysis when other components like cocaine have to be measured as well. The best option to separate multiple components while still maintaining retention for morphine would be the use of gradient settings; with a high amount of water in the first few minutes, after which the composition would contain an increasing amount of acetonitrile. Figure 22 shows the chromatogram for morphine (10 mg/mL) for setup 2. The peak at 2.23 minutes cannot be observed in this chromatogram, however, the retention time for the morphine peak is once again different from the results from the first setup. The gradient pump was experiencing pressure drops, which could account for the difference in retention time between the two setups.

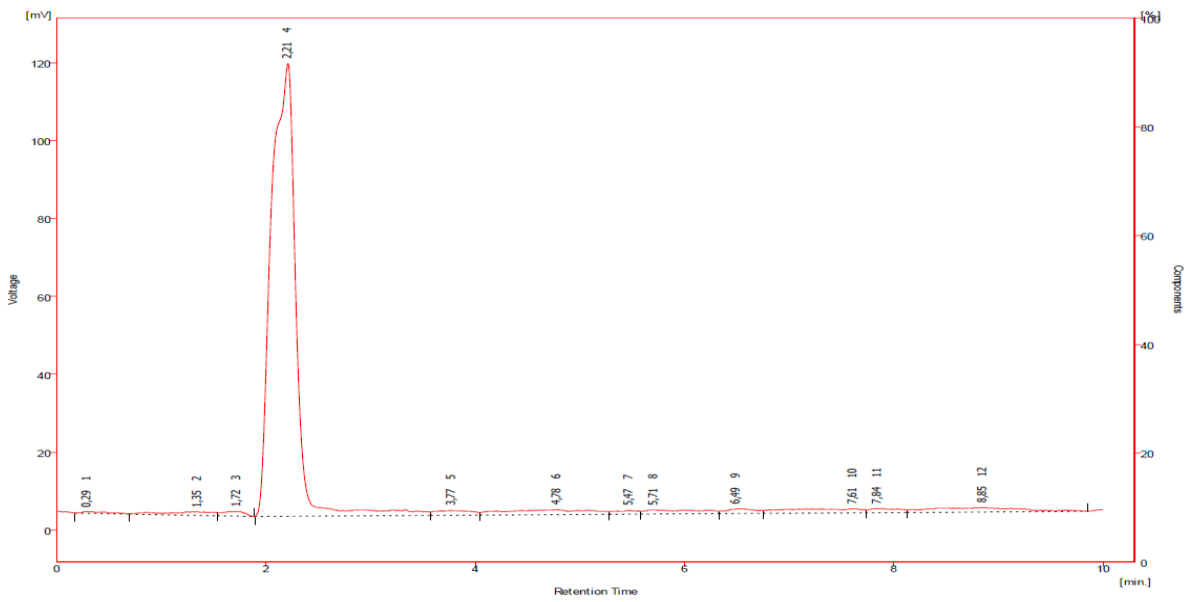

Figure 21: Chromatogram of morphine (10 mg/mL) obtained using HPLC-UV/Vis setup 1.

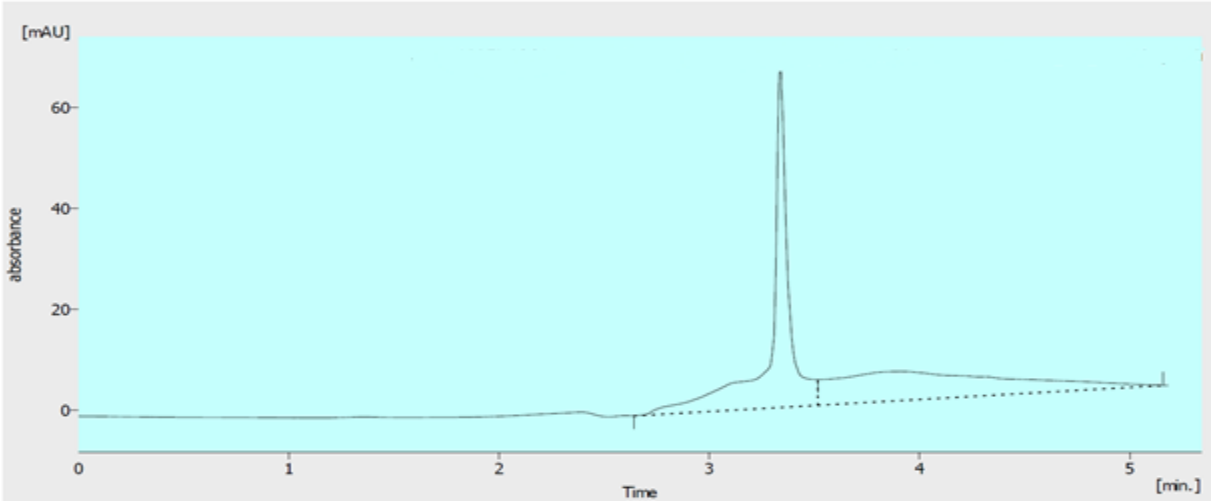

Figure 22: Chromatogram of morphine (10 mg/mL) obtained using HPLC-UV/Vis setup 2.

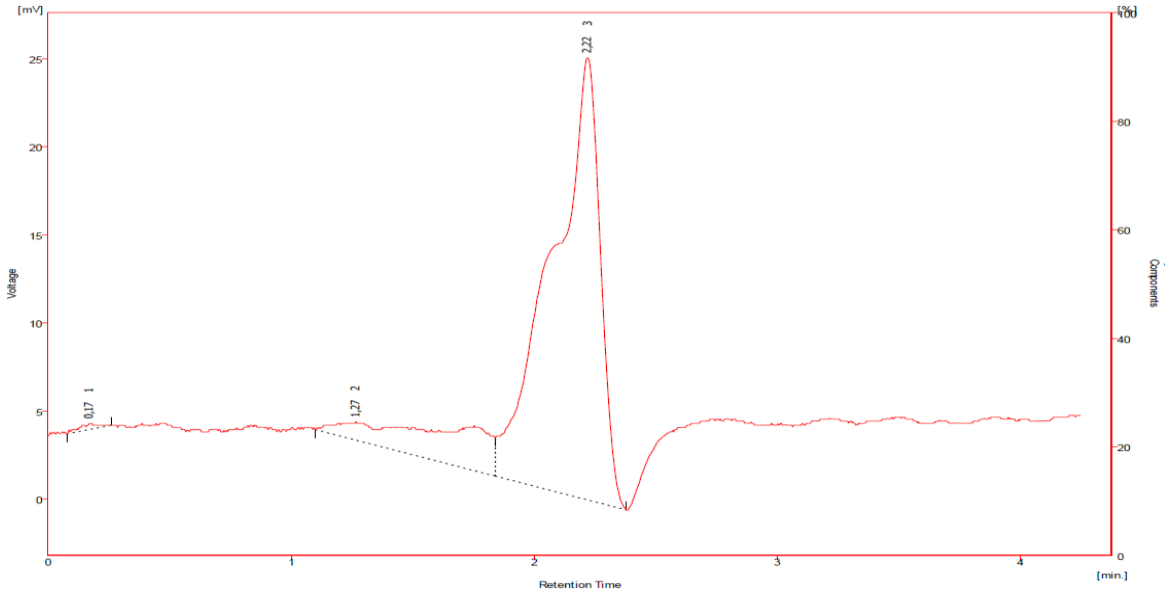

Figure 23: Chromatogram of morphine (1 mg/mL) obtained using HPLC-UV/Vis setup 1.

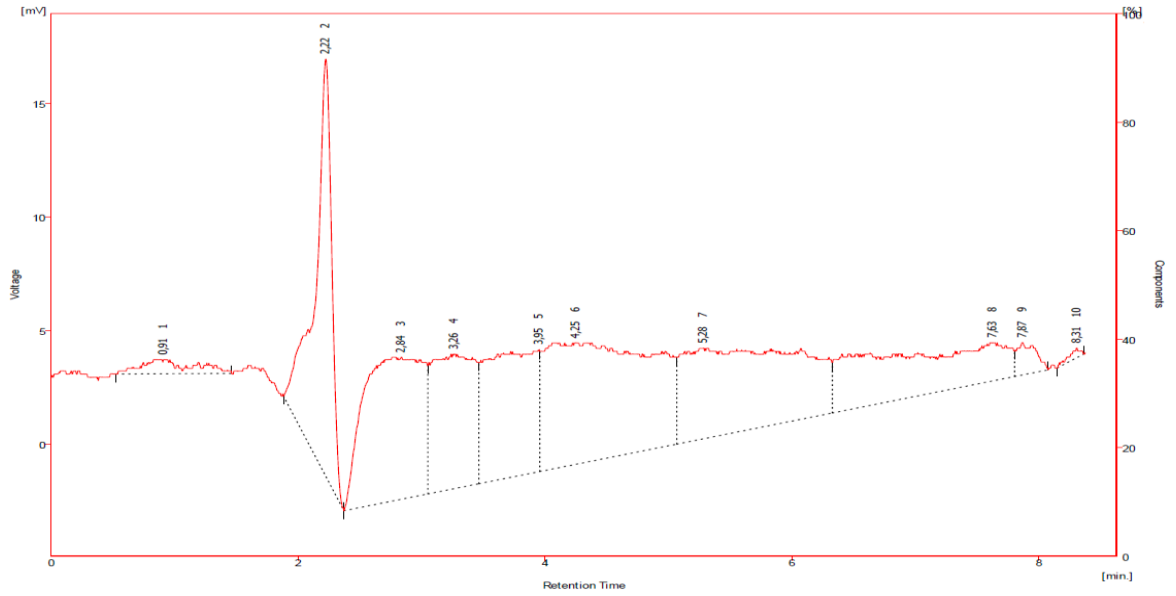

Figure 24: Chromatogram of morphine (0.1 mg/mL) obtained using HPLC-UV/Vis setup 1.

5.2.3 Gradient measurements

Initially, measurements using gradient settings on a regular HPLC system were planned as well, however, due to a large amount of issues encountered with several different HPLC systems these measurements were never done. It has become clear, however, that cocaine is quite easy to measure using simple parameters on a regular HPLC system. Morphine will likely need adjustments to create retention on the column, since with the settings used in the above discussed experiments, it seemed morphine had no retention time.

6 Microchip based HPLC

Measurements using a HPLC system with a microchip replacing the regular column are discussed in this section. Two different chips have been used, because the first chip broke during the experiments.

6.1 Material and methods microchip HPLC

6.1.1 Chemicals

Cocaine (1 mg/mL) in MeOH, benzoylecgonine (1 mg/mL) in MeOH, ecgonine methyl ester (1 mg/mL) in acetonitrile, morphine (1 mg/mL) in MeOH and morphine-3ß-D-glucorinide (1 mg/mL) in MeOH obtained from the NICC in Brussels, Belgium. Cocaine, benzoylecgonine and morphine were obtained in sealed vials with 0.5 mL of said substances. Ecgonine methyl ester and morphine-3ß-D-glucorinide was obtained in ampoules with 1 mL of said substances. HPLC-grade acetonitrile and formic acid, obtained from Vrije Universiteit Brussel. Milli-Q water was obtained from an in-house Millipore purification system.

6.1.2 Stock solutions

Solutions with a concentration of 5 mg/L and 50 mg/L were made for cocaine. Benzoylecgonine, ecgonine methyl ester, morphine and morphine-3ß-D-glucorinide were made into solutions with a concentration of 100 mg/L. Lastly, cocaine, benzoylecgonine, morphine and morphine-3ß-D-glucorinide were mixed into one solution with concentrations of 12.5 mg/L, 25 mg/L, 25 mg/L and 25 mg/L, respectively. All dilutions were made using Milli-Q water.

6.1.3 Instrumentation

For separation, a Dionex micro HPLC system was used with the following components: a Dionex LC packings LPG-3000 micropump, a Dionex Ultimate 3000 Flow Manager, a Dionex Ultimate 3000 RS Variable Wavelength Detector, and a Dionex LC Packings WPS-3000 Autosampler. The column was replaced with a microchip (longchip/shortchip reference). Elution was done at varying flowrates and pressure settings, using both isocratic and gradient mobile phase settings with varying ratios of acetonitrile (0.1% formic acid) and water (0.1% formic acid). Measurements for cocaine, benzoylecgonine and ecgonine methyl ester were done at a 230 nm wavelength; measurements for morphine and morphine-3ß-D-glucorinide were done at a 210 nm wavelength. The mixture of cocaine, benzoylecgonine, morphine and morphine-3ß-D-glucorinide were measured at both 210 and 230 nm. Data was obtained using Chromeleon software.

6.1.4 Optimization

Measurements were first done on 'long chip' using an isocratic mobile phase consisting of 70% acetonitrile (0.1% formic acid) and 30% water (0.1% formic acid). Pressure was set to be constant at 250 bar, with a flowrate of 2 µL/min. Actual column flowrate is lower because of the constant pressure setting. Constant pressure combined with a higher flow rate was used to prevent fluctuations in the base line, as normal flow rates used with microchips on this system were known to cause issues. Mobile phase was later changed to 100% acetonitrile (0.1% formic acid) to reduce measurement time.
After this, measurements continued on 'short chip' due to the 'long chip' breaking (liquid appeared on top of the chip, indicating a tear in one of the channels), also starting with an isocratic mobile phase consisting of 70% acetonitrile (0.1% formic acid) and 30% water (0.1% formic acid). Pressure was set to be constant at 200 bar, with a flowrate of 2 µL/min. Lower pressure was used with this chip to make sure it would not break as well. Mobile phase consistency was later changed to 80%-20% and 90%-10% acetonitrile (0.1% formic acid) and 30% water (0.1% formic acid), respectively, in order to improve peak shape.

To further optimize peak shape, measurements using gradient settings were used. Gradient measurements were started using a constant flow rate of 0.2 µL/min using the following program for acetonitrile (0.1% formic acid) (%A) and water (0.1% formic acid) (%B):

Table 2: Initial gradient settings used for cocaine and bezoylecgonine measurements.

Time (min)	Flow (µL/min)	%A	%B
-5	0,2	100	0
0	0,2	100	0
24	0,2	50	50
28	0,2	0	100
40	0,2	0	100
52	0,2	100	0
60	0,2	100	0

These settings were used for initial cocaine and benzoylecgonine measurements. For initial morphine and morphine-3ß-D-glucorinide measurements, the following gradient was used:

Table 3: Initial gradient settings used for morphine and morphine-3ß-D-glucorinide measurements.

Time (min)	Flow (µL/min)	%A	%B
-5	0,2	100	0
0	0,2	100	0
24	0,2	50	50
28	0,2	10	90
40	0,2	10	90
52	0,2	100	0
60	0,2	100	0

Measurements for 4 components were done with constant flow (Table 4), and constants pressure (200 bar)(Table 5), the gradient program was not changed.

Table 4: Constant flow gradient setting for the 4 component mixture.

Time (min)	Flow (µL/min)	%A	%B
-5	0.2	97	3
0	0.2	97	3
5	0.2	97	3
10	0.2	75	25
12	0.2	10	90
55	0.2	10	90
60	0.2	97	3

Table 5: Constant pressure gradient setting for the 4 component mixture.

Time (min)	Flow (µL/min)	%A	%B
-5	2.0	97	3
0	2.0	97	3
5	2.0	97	3
10	2.0	75	25
12	2.0	10	90
55	2.0	10	90
60	2.0	97	3

6.1.5 Methods

Samples were injected using a Dionex LC Packings WPS-3000 Autosampler, after which data acquisition started automatically with the Chromeleon software. The syringe was automatically cleaned with methanol by the autosampler. Results were interpreted, after which parameters were changed in order to optimize analysis.

6.2 Results and discussion

Results have been split into several categories; ‘long chip’, ‘short chip isocratic’, ‘short chip gradient’ and ‘short chip 4 components’. Results for each category are discussed, and reasoning behind HPLC parameter changes are explained.

6.2.1 Long Chip

Measurements were started using an isocratic mobile phase consisting of 30% A and 70% B, as used before for the measurements done on a normal HPLC. Figure 25 shows the first measurement for cocaine (5 mg/L), which showed a lot of irregular peaks in the first 5 minutes, after which a larger peak can be seen at around 5.2 minutes. Figure 26 shows the second measurement for cocaine (50 mg/L) under the same circumstances, but the results are completely different from the first measurement. The only visible peak showed around 9.7 minutes this time. A likely cause for this discrepancy, is pressure issues within the system caused by clogging, which will become more apparent later on (Figure 43).

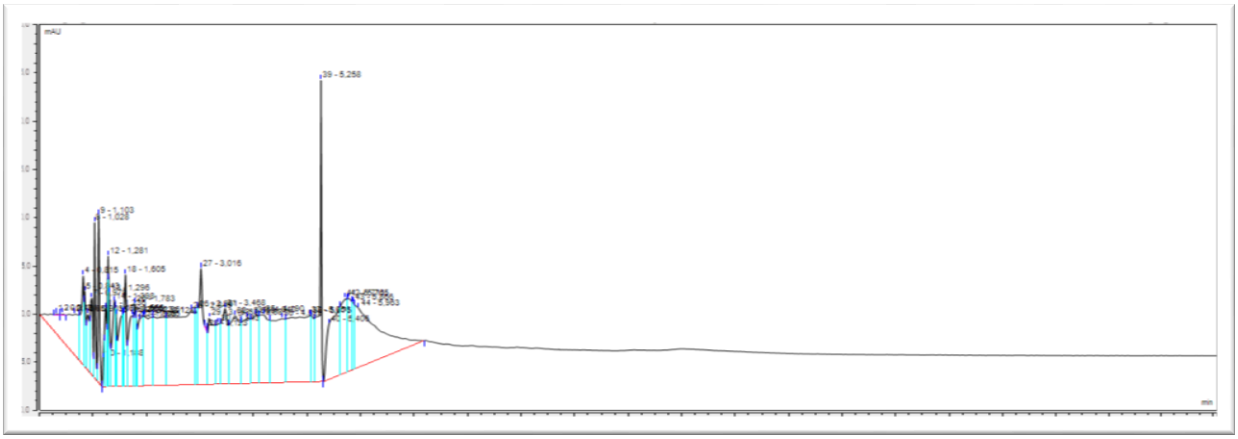

Figure 25: Cocaine 5 mg/L, isocratic 30% A 70% B

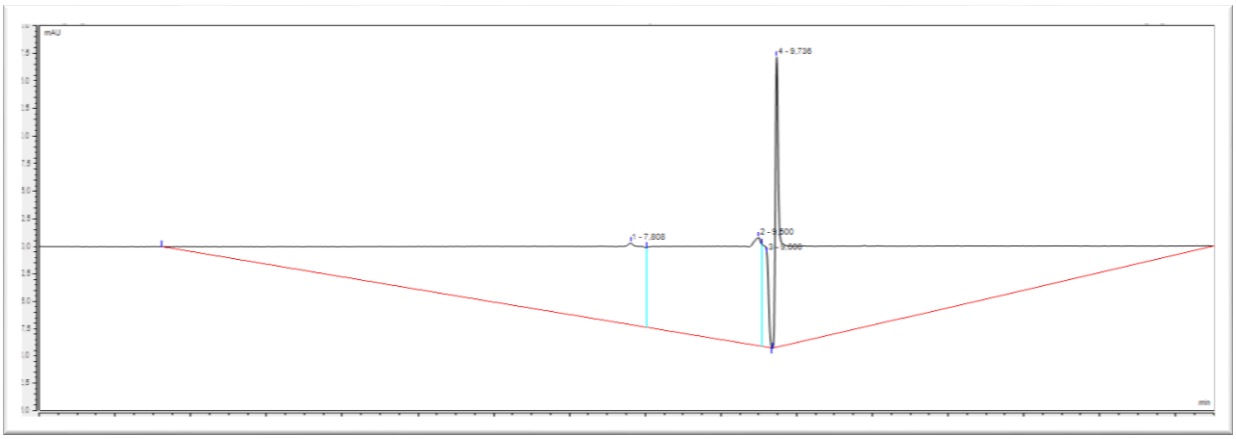

Figure 26: Cocaine 50 mg/L, isocratic 30% A 70% B

A third measurement was done with cocaine 5 mg/L using a mobile phase consisting of 100% B, results are shown in Figure 27. The chromatogram showed several different peaks compared to earlier measurements; a large peak around 4.7 minutes and two smaller peaks at around 5.7 and 8.4 minutes.

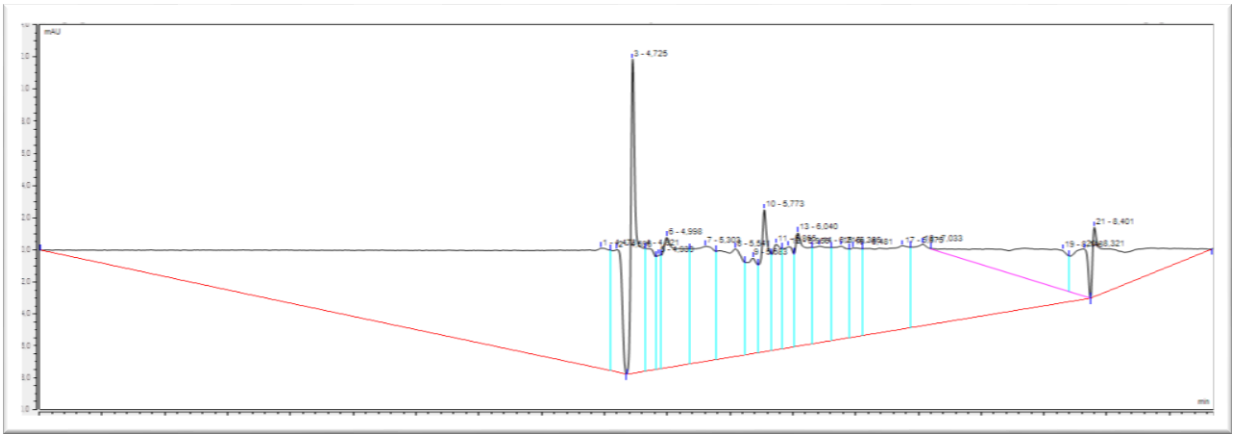

Figure 27: Cocaine 5 mg/L, isocratic 100% B

At this point another measurement was started using just MeOH, to see whether or not this would produce consistent results. As shown in Figure 28, however, the baseline started to show constant fluctuations, pointing towards a pressure issue. When the chip was inspected, it was clear there was a crack in one of the channels, rendering the chip unusable. A different chip (short chip), was then connected to the system, to continue measurements.

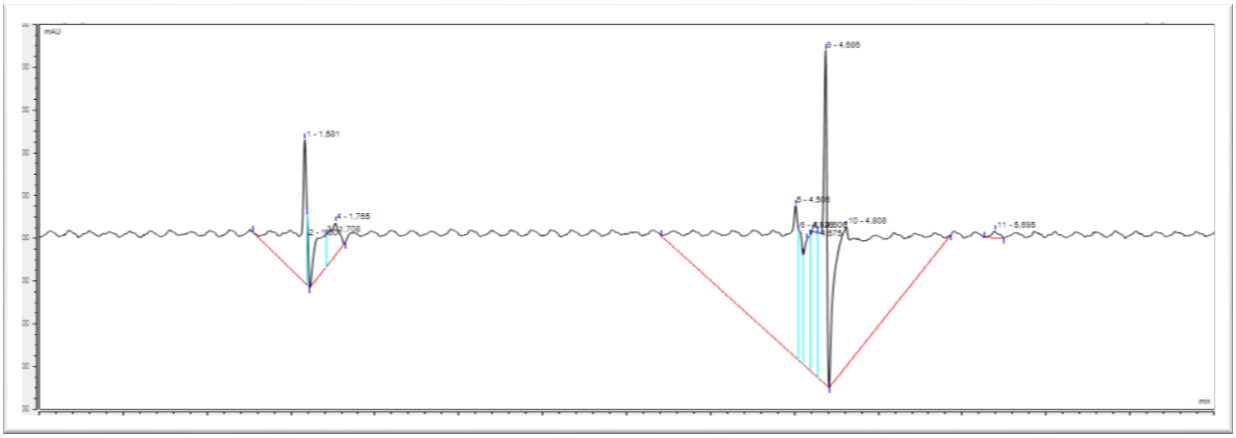

Figure 28: MeOH, isocratic 70% B

6.2.2 Short Chip Isocratic

Measurements with the new chip were started using MeOH, to check for a consistent baseline. Pressure was lowered to 200 bar to lower the chance of the chip breaking to high pressure. As shown in Figure 29, the baseline was consistent and the only visible peak is at 1.4 minutes, followed by a large drop. This was consistent for all runs, and is most likely caused by injection into the system.

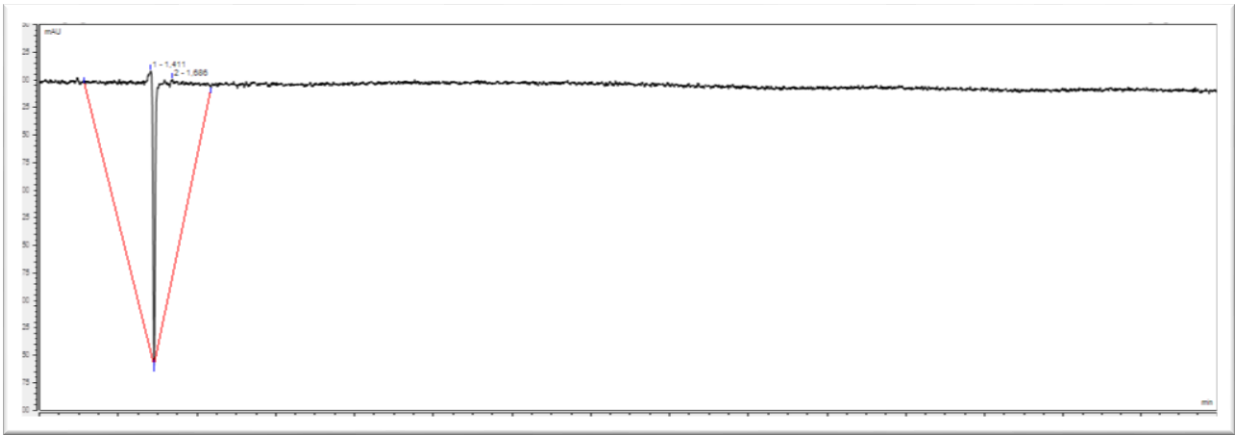

Figure 29: MeOH, isocratic, 90% B

With this chip showing consistent results for MeOH, new measurements for cocaine were started, starting at 50 mg/L and a mobile phase consisting of 30% A and 70% B. Results for this measurement are shown in Figure 30. Besides the injection peak at around 2.5 minutes, another peak at around 8 minutes can be observed. The second peak shows a significant amount of tailing, however. The tailing problem is likely caused by the same pressure issues that were mentioned earlier in chapter 6.2.1, as cocaine should be easily measurable on a C18 column based on results from earlier measurements on a regular HPLC system (Figure 17). Another observation was the low intensity of the peak, considering the concentration of the sample was relatively high. Even considering the bad peak shape, when comparing these results with earlier results obtained with the long chip (Figure 26), peak intensity is much lower.

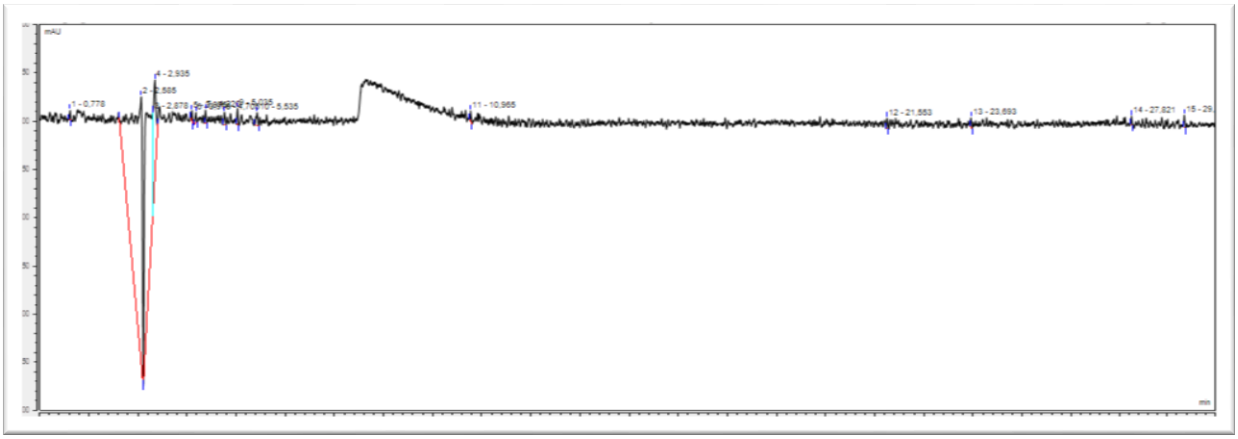

Figure 30: Cocaine 50 mg/L, isocratic, 70% B

In an attempt to improve the peak shape, two more runs where done with the mobile phase consistency changed to 20% A 80% B, and 10% A 90% B. Results for these runs are show in Figure 31 and Figure 32, respectively. Peak shape for both runs did not improve, and retention times for the peaks were not as expected. With greater amounts of acetonitrile, the cocaine peak should show earlier. While this was true for the measurement with 80% B, the measurement with 90% B had the cocaine peak showing at 8 minutes, which is similar to the first measurement with 70% B. The injection peak did show earlier for increasing amounts of acetonitrile.

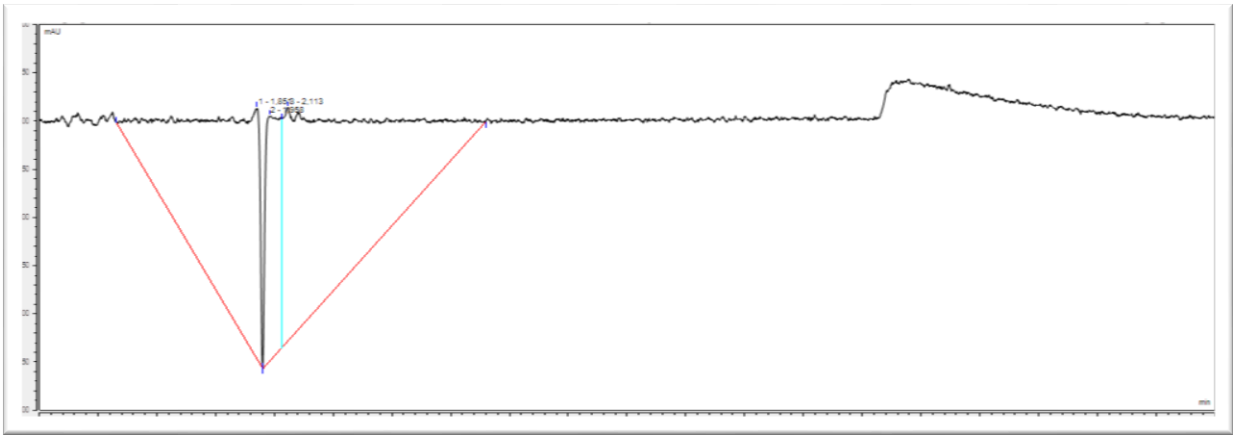

Figure 31: Cocaine 50 mg/L, isocratic, 80% B

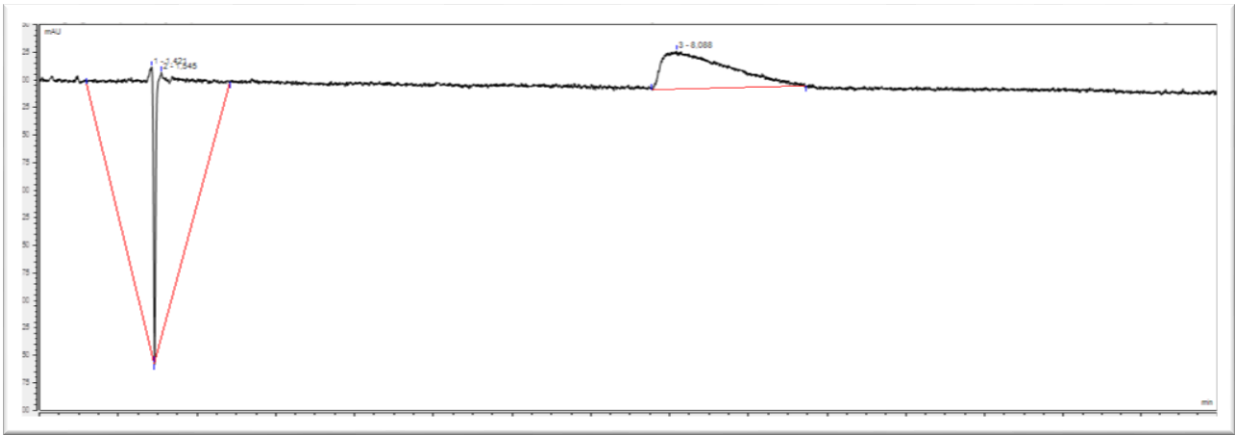

Figure 32: Cocaine 50 mg/L, isocratic, 90% B

To check for peak intensity at lower concentrations, a measurement with cocaine (5 mg/L) was done. No other parameters were changed. Results for this measurement are shown in Figure 33. Retention times are similar to the measurement done with 50 mg/L with the same mobile phase. The cocaine peak shown at around 8 minutes is very small now, however, as expected with a concentration ten times lower than in previous measurements. Without the significant tailing, peak intensity would be much better.

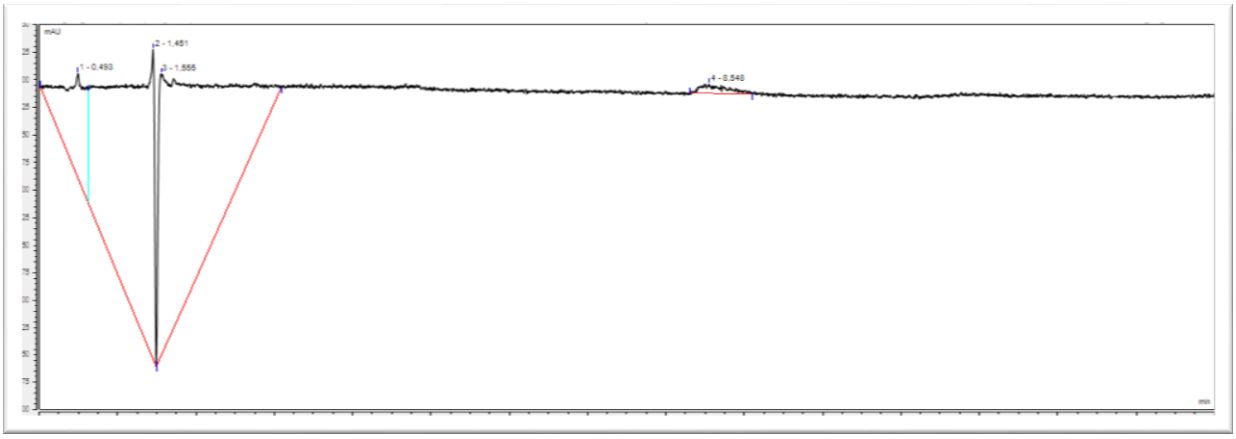

Figure 33: Cocaine 5 mg/L, isocratic, 90% B

With the tailing issues observed with cocaine measurements, a different component was measured to see whether or not tailing issues would be observed again. Ecgonine methyl ester was chosen because this was the only component dissolved in acetonitrile. Because EME is known to have low UV absorption, a high concentration of 100 mg/L was used for the initial measurement. As shown in Figure 34 no significant peaks can be observed. This is likely because of the low intensity issue mentioned earlier for cocaine measurements, combined with the fact that EME has low UV absorption.

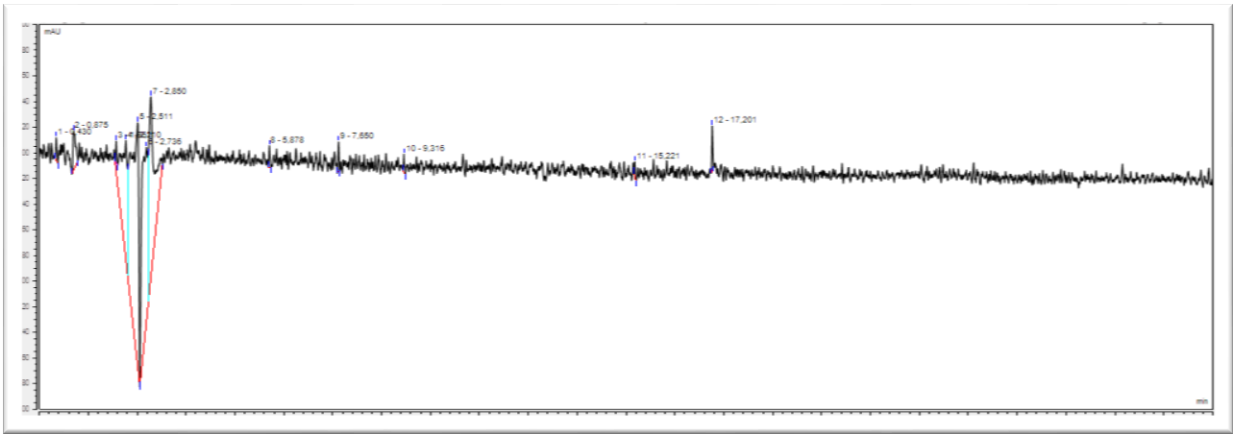

Figure 34: EME 100 mg/L, isocratic, 70% B

At this point the choice was made to start measuring using a gradient mobile phase, as this would be necessary to separate the different components, based off of literature and earlier measurements done on a normal HPLC.

6.2.3 Short Chip Gradient

Triple measurements were done for cocaine (50 mg/L) using the initial gradient settings mentioned in the optimization chapter. The result were consistent, with one of them shown in Figure 35. The baseline was highly unstable for all measurements done, similar to the one shown in Figure 35. The pressure profile was checked and showed unexpected results. Pressure went up during the run, even though the amount of water in the mobile phase was reduced later on in the run, which should result in lower pressure. Besides the unstable baseline, results were fairly consistent, with an injection peak showing at around 6 minutes and a cocaine peak showing at around 32 minutes. The cocaine peak shape using a gradient mobile phase was better than with isocratic settings, but still was not as sharp as it should be.

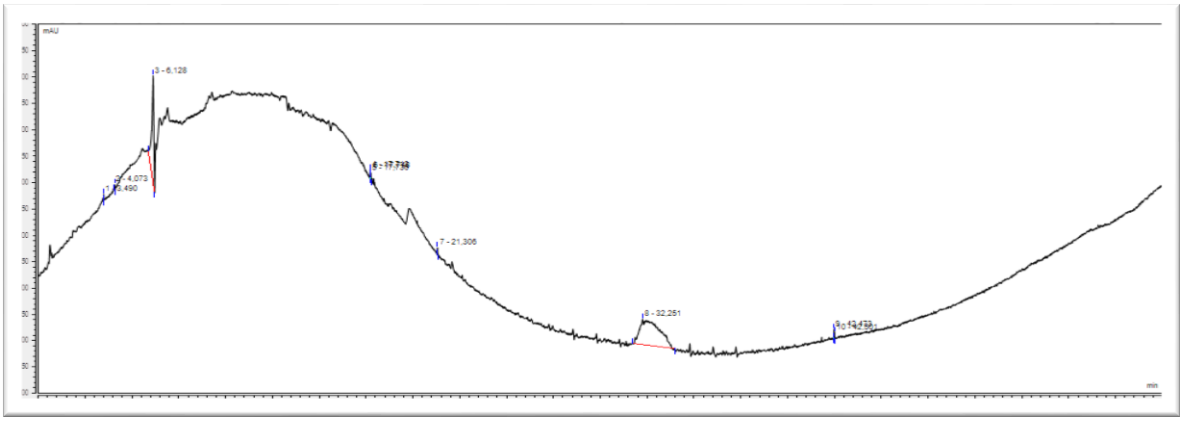

Figure 35: Cocaine 50 mg/L, gradient

With fairly consistent cocaine results obtained, next measurements for benzoylecgonine were started using the same gradient setting as used for the previous cocaine measurements. Sample concentration was chosen at 100 mg/L, to make sure the peak would be well visible. Again, triple measurements were done, showing consistent results, with one of them shown in Figure 36. The results show a similar unstable baseline, with an injection peak at around 6 minutes and a benzoylecgonine peak at around 16 minutes. Results were fairly consistent just as with cocaine. The peak shape for benzoylecgonine was much better than for cocaine, but a small amount of tailing could still be observed.

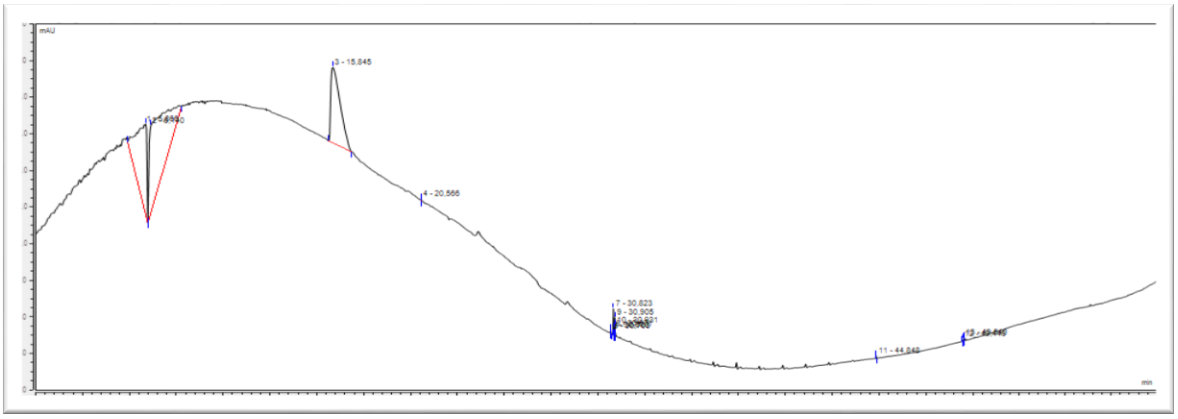

Figure 36: Benzoylecgonine 100 mg/L, gradient

The next component that was measured was morphine, using the gradient settings mentioned in the optimization chapter. Sample concentration was chosen at 100 mg/L again. Triple measurements were done, showing consistent results, with one of them shown in Figure 37. The baseline is much more stable this time around with an injection peak showing at around 5.5 minutes and the morphine peak can be observed at around 6.5 minutes. Isocratic measurements for cocaine performed earlier on a normal HPLC system showed no retention at all, but with these gradient settings morphine does have a retention time. The peak for morphine is sharp, and looks much better than for the previous measurements done for cocaine and benzoylecgonine.

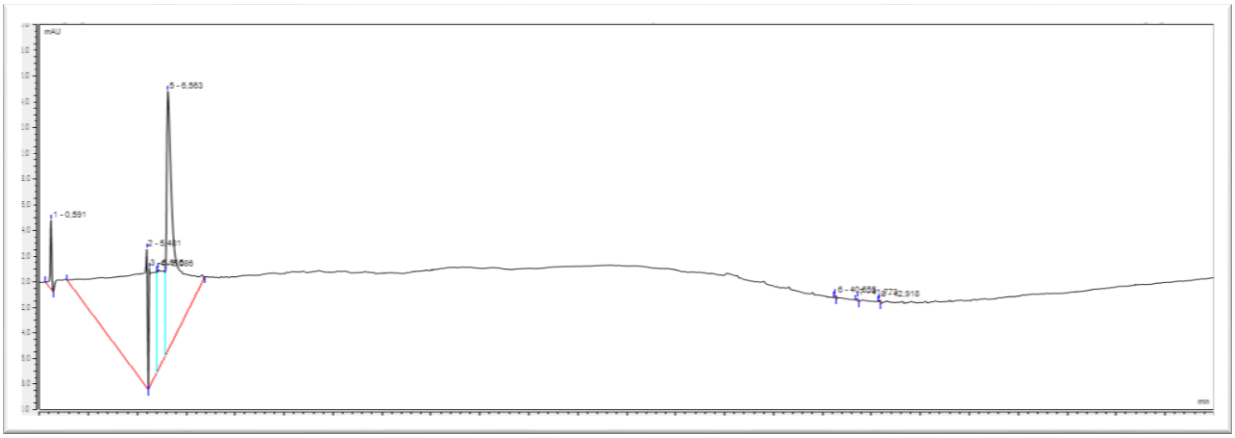

Figure 37: Morphine 100 mg/L, gradient

With consistent results for morphine, the next component measured was morphine-3ß-D-glucorinide, using the same settings and same sample concentration as for the morphine measurements. Triple measurements were done again, showing consistent results, with one of them shown in Figure 38. Once again a stable baseline can be observed, with an injection peak showing at around 6 minutes and the morphine-3ß-D-glucorinide can be observed at around 6.5 minutes. Peak shape looks sharp and the component has some retention in the chip.

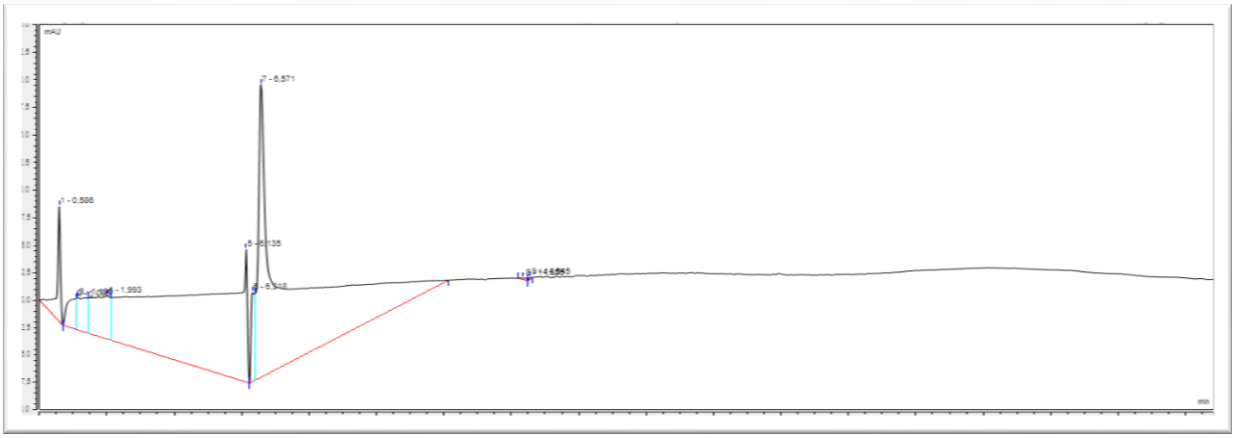

Figure 38: Morphine-3ß-D-glucorinide 100 mg/L, gradient

6.2.4 Short chip 4 components

With 4 components now individually measured and having obtained fairly consistent results for them, measurements for 4 components in one sample were started. First, triple measurements were done using constant flow mode, showing consistent results, with one of them showing in Figure 39 (210 nm) and Figure 40 (230 nm). Both figures show an unstable baseline once again, with peaks showing around 20 and 30 minutes. Pressure during the run showed similar unexpected results as discussed earlier for cocaine measurements. Peak shapes are not well defined, and it is hard to tell what peak belongs to which component. Peaks around 20 minutes are likely from the morphine components and are better defined at 210 nm (Figure 39), while peaks around 30 minutes are likely from the cocaine components and are better defined at 230 nm (Figure 40).

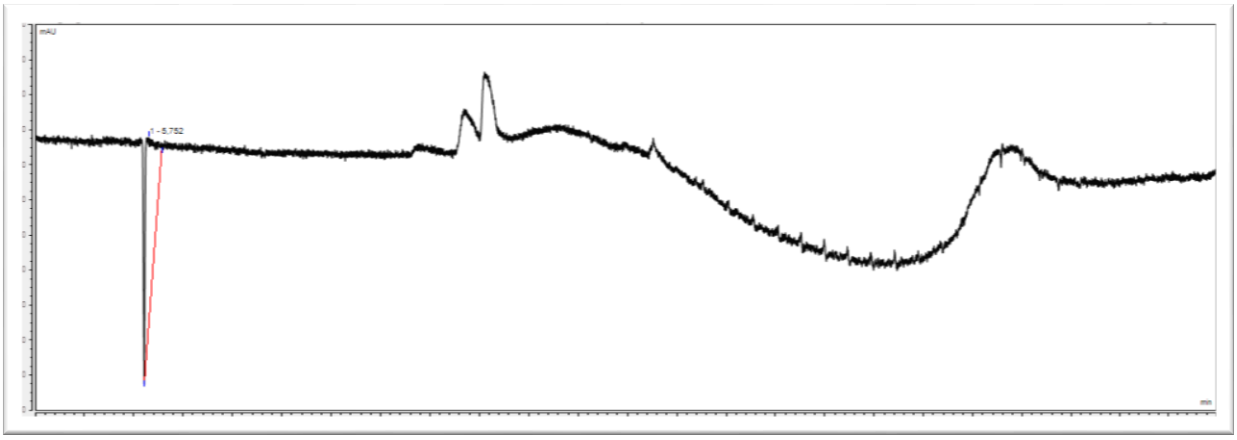

Figure 39: Cocaine, benzoylecgonine, morphine, morphine-3ß-D-glucorinide (12.5 mg/L, 25 mg/L, 25 mg/L, 25 mg/L, respectively), gradient, constant flow, 210 nm.

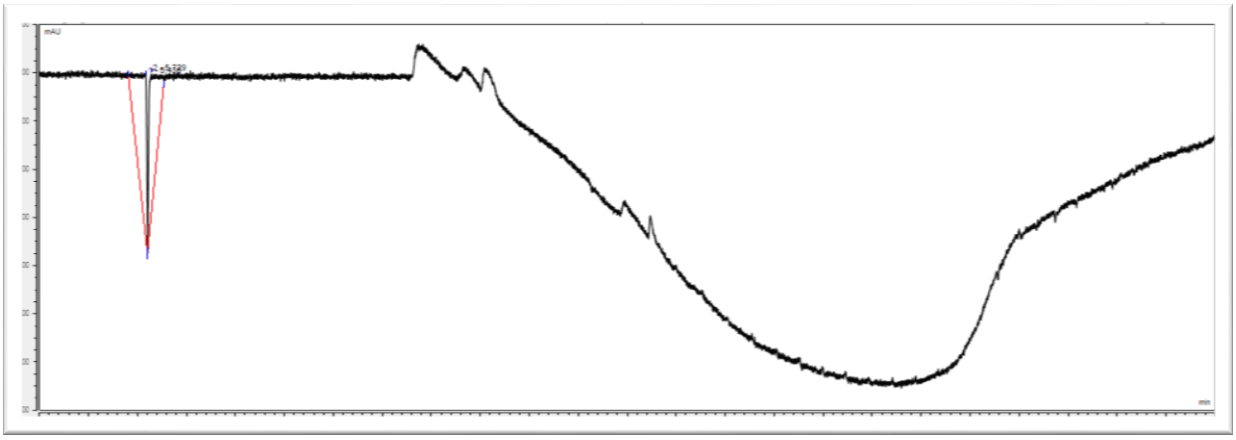

Figure 40: Cocaine, benzoylecgonine, morphine, morphine-3ß-D-glucorinide (12.5 mg/L, 25 mg/L, 25 mg/L, 25 mg/L, respectively), gradient, constant flow, 230 nm.

In an attempt to stabilize the baseline, measurements using constant pressure of 200 bar and initial flow rate of 2 µL/min were started. No other parameters were changed. Results are shown in Figure 41 (210 nm) and Figure 42 (230 nm). The baseline was much more stable this time around, indicating that using constant pressure with this setup is likely the better way to measure. Both wavelengths show clearly defined peaks for the injection peak (6 minutes), morphine-3ß-D-glucorinide (6.5 minutes), morphine (7.8 minutes), benzoylecgonine (32 minutes) and cocaine (37.5 minutes). Sample concentration is still fairly high (12.5 and 25 mg/L), but judging from these results, a ten times lower sample concentration should be measurable with the current settings.

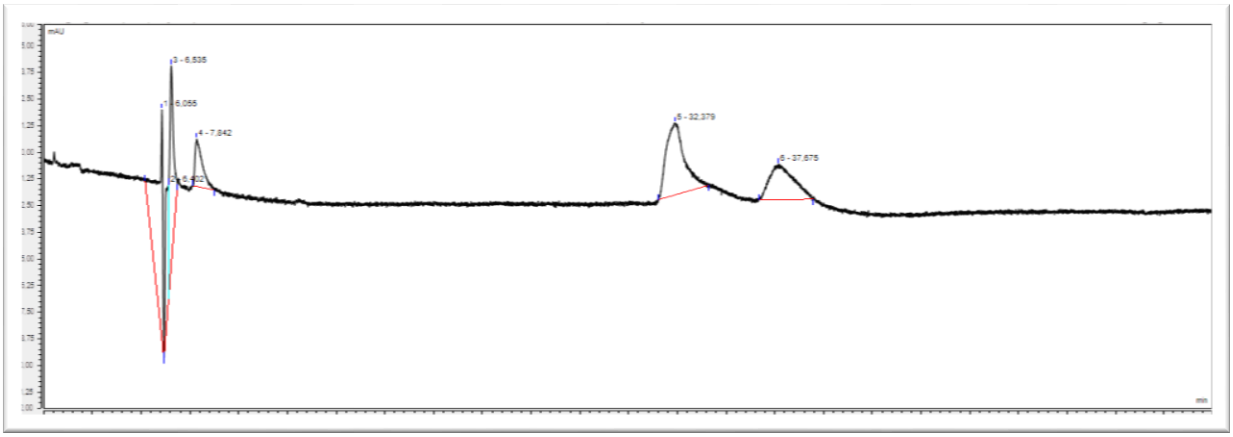

Figure 41: Cocaine, benzoylecgonine, morphine, morphine-3ß-D-glucorinide (12.5 mg/L, 25 mg/L, 25 mg/L, 25 mg/L, respectively), gradient, constant pressure, 210 nm.

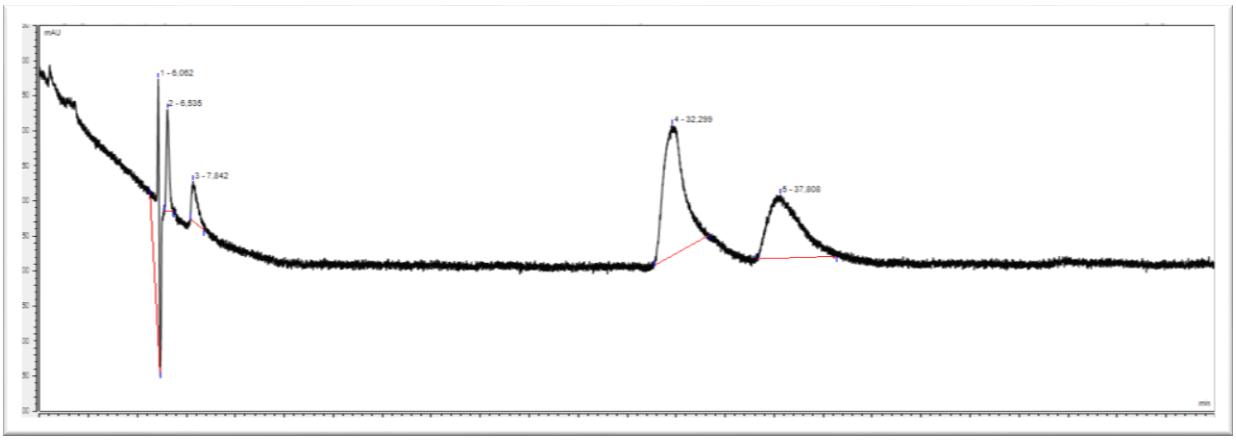

Figure 42: Cocaine, benzoylecgonine, morphine, morphine-3ß-D-glucorinide (12.5 mg/L, 25 mg/L, 25 mg/L, 25 mg/L, respectively), gradient, constant pressure, 230 nm.

More measurements with lower concentrations were planned, but the earlier mentioned pressure issue caused more severe issues, resulting in just noise in the chromatogram (Figure 43).

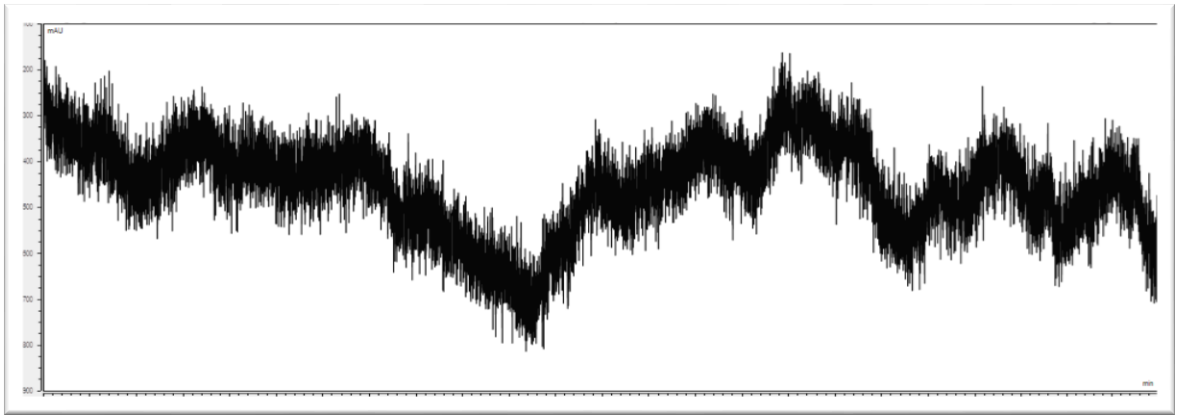

Figure 43: Pressure issue, system clogged.

7 Conclusions

The aim of the research conducted for this thesis was to separate cocaine, benzoylecgonine, ecgonine methyl ester, morphine, morphine-3ß-D-glucorinide and morphine-6ß-D-glucorinide in water, using a chip based HPLC system. Based off of the results obtained for these measurements, blood samples with the same components would possibly be analysed as well.
The first step in this research was the collection of results using a normal HPLC system, to obtain a reference point for future on-chip measurements. The second step was finding a usable method for the on-chip setup, starting with single components. The third step was to use the suitable method in order to separate all components in one water sample. A fourth step doing these same measurements in blood would have been added, but due to issues with the HPLC system and a lack of time these measurements were never performed.

Initial measurements on a normal HPLC system were only done for cocaine and morphine, which showed no problems with measuring cocaine up to a concentration of 0.1 mg/L. Morphine measurements showed morphine not having any retention in the column, so in order to properly analyse this component, gradient settings would have to be used.

Finding a suitable method for on-chip measurements showed gradient measurements were necessary, as expected based on measurements done on the normal HPLC system. Ecgonine methyl ester and morphine-6ß-D-glucorinide could not be measured due to low UV absorption.
The remaining four components were successfully separated using an on-chip HPLC system, for concentrations of 12.5 and 25 mg/L. Due to issues with the system, lower concentrations could not be measured at this point in time, but it is likely this would be possible under normal circumstances. Whether or not relevant concentrations for forensic applications can be measured is hard to say based on the data that has been obtained in this research.

8 Recommendations

While working on creating a method to analyse cocaine, benzoylecgonine, morphine and morphine-3ß-D-glucorinide, several issues were encountered. A large part of the issues were related to issues with equipment. Some common issues were leaks in the system, causing the pressure to drop, or a clogged system, causing pressure to rise. Whenever the pressure profile is not as expected, it is recommended to find the issue to the deviation first, before continuing measurements. Even when decent results can be obtained with pressure deviations, it will be very hard to create consistent results, which makes it hard to draw any conclusions. Pressure issues are especially noticeable when working with gradient settings, due to baseline inconsistencies when the composition of the mobile phase gradually changes. Another issue that was encountered had to do with the obtained results having a lower than expected intensity, which was caused by an old UV lamp in the detector. This might cause problems when trying to measure lower concentrations, so it is recommended to replace the lamp if this issue occurs. Besides these issues it is also recommended to work with one system, because having to work with multiple systems makes it harder to compare results between them.

In order to develop a method to analyse cocaine, benzoylecgonine, morphine and morphine-3ß-D-glucorinide in blood, it is recommended to start with analyses on a normal HPLC system. Blood is a complex matrix, which will increase the difficulty of analyses; especially for lower concentrations (<1.0 mg/L). As discussed in chapter 3.1, because of the complexity of the blood matrix, many methods involve pre-treatments of the sample [4] [14] [15]. Starting with the simple sample pre-treatment used by the NFI and NICC is recommended, as this method is not very time consuming and inexpensive. Use of a pre-column to protect the main column is recommended when using this method, as there will likely be a lot of contaminants. For on-chip analyses, channel clogging is a greater issue [13], so use of a pre-column is once again recommended. If the simple sample pre-treatments is used for on-chip measurements as well, make sure to regularly check the pressure profile to see whether or not unexpected pressure rises occur.

References

1. Baer, D.M. and R.A. Paulson. *Cutoff and toxicity levels for drugs-of-abuse testing*. 2015; Available from: www.clr-online.com.
2. Li, X., et al., *Rapid screening of drugs of abuse in human urine by high-performance liquid chromatography coupled with high resolution and high mass accuracy hybrid linear ion trap-Orbitrap mass spectrometry.* J Chromatogr A, 2013. **1302**: p. 95-104.
3. Roy, D.N. and R. Goswami, *Drugs of abuse and addiction: A slippery slope toward liver injury.* Chem Biol Interact, 2015.
4. Johansen, S.S. and H.M. Bhatia, *Quantitative analysis of cocaine and its metabolites in whole blood and urine by high-performance liquid chromatography coupled with tandem mass spectrometry.* J Chromatogr B Analyt Technol Biomed Life Sci, 2007. **852**(1-2): p. 338-44.
5. Ghazi-Khansari, M., et al., *Determination of morphine in the plasma of addicts in using Zeolite Y extraction following high-performance liquid chromatography.* Clin Chim Acta, 2006. **364**(1-2): p. 235-8.
6. Sartori, D., et al., *The development of a high-performance liquid chromatography-tandem mass spectrometric method for simultaneous quantification of morphine, morphine-3-beta-glucuronide, morphine-6-beta-glucuronide, hydromorphone, and normorphine in serum.* Clin Biochem, 2015.
7. Skarke, C. and J. Lötsch, *Morphine metabolites: Clinical implications.* Seminars in Anesthesia, Perioperative Medicine and Pain, 2002. **21**(4): p. 258-264.
8. Maurer, H.H., *Liquid chromatography–mass spectrometry in forensic and clinical toxicology.* Journal of Chromatography B: Biomedical Sciences and Applications, 1998. **713**(1): p. 3-25.
9. Meyer, V.R., *Practical High-Performance Liquid Chromatography*. 2013: Wiley.
10. Gutnikov, G. and H. Engelhardt, *High Performance Liquid Chromatography*. 2012: Springer Berlin Heidelberg.
11. Atkins, P., *Shriver and Atkins' Inorganic Chemistry*. 2010: OUP Oxford.
12. Whitesides, G.M., *The origins and the future of microfluidics.* Nature, 2006. **442**(7101): p. 368-73.
13. Lichtenberg, J., et al., *Sample Preparation on Microchips*, in *Separation methods in microanalytical systems*, J.P. Kutter and Y. Fintschenko, Editors. 2006, Taylor & Francis: Boca Raton. p. 359-431.
14. Jagerdeo, E., et al., *An automated SPE/LC/MS/MS method for the analysis of cocaine and metabolites in whole blood.* J Chromatogr B Analyt Technol Biomed Life Sci, 2008. **874**(1-2): p. 15-20.
15. Fisichella, M., S. Odoardi, and S. Strano-Rossi, *High-throughput dispersive liquid/liquid microextraction (DLLME) method for the rapid determination of drugs of abuse, benzodiazepines and other psychotropic medications in blood samples by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and application to forensic cases.* Microchemical Journal, 2015. **123**: p. 33-41.
16. Grinias, J.P. and R.T. Kennedy, *Advances in and prospects of microchip liquid chromatography.* TrAC Trends in Analytical Chemistry, 2015.
17. Op De Beeck, J., et al., *On the advantages of radially elongated structures in microchip-based liquid chromatography.* Anal Chem, 2013. **85**(10): p. 5207-12.
18. Desmet, G., et al., *Merging Open-Tubular and Packed Bed Liquid Chromatography.* Anal Chem, 2015. **87**(14): p. 7382-8.
19. Callewaert, M., et al., *Integration of uniform porous shell layers in very long pillar array columns using electrochemical anodization for liquid chromatography.* Analyst, 2014. **139**(3): p. 618-25.

Appendix A

	Drug (and trade name)	Some common street names	Detectable duration in urine after	Urine cutoff points for reporting positive last dose	Toxic blood level by screening assay*	Blood reference (therapeutic) range
OPIATES	Heroin (Diacetylmorphine)	Horse, Stuff, Smack, Junk	1-2 days (total opiate)	2000 ng/mL (as morphine) 150 ng/mL (as 6-monacetyl morphine)	>200 ng/mL	None detected
	Morphine (Duramorph)	M, Junk, Morpho, White stuff	2 days	2000 ng/mL 300 ng/mL	>200 ng/mL	10-80 ng/mL
	Methadone (Dolophine, Amidone)	Methadose	3 days	300 ng/mL 200 ng/mL 150 ng/mL	>2000 ng/mL	For narcotic stabilization: 300-1000 ng/mL For pain: 100-400 ng mL
	Meperidine (Demerol, Pethidine)	Fortis, Demies	2-3 days	200 ng/mL	>1000 ng/mL	70-500 ng/mL
	Codeine (Analgesics with codeine)	Rabo, School boy	2 days	2000 ng/mL 300 ng/mL	>1000 ng/mL	10-100 ng/mL
	Tramadol (Ultram, Tramal Ultracet)	Ultra T	6 hours to 2 days	not established	not established	Variable by patient
	Oxycodone (Oxycontin, OxyIR, Percocet, Percodan)	Oxy, OC, Oxycotton, Killer	1-3 days	100 ng/mL 300 ng/mL	>200 ng/mL	10-100 ng/mL
	Hydrocodone (Lorcet, Vicodin, Lortab, Hycodan)	Vikes, Hydro, Norco	1-2 days	300 ng/mL 100 ng/mL 50 ng/mL	>100 ng/mL	10-40 ng/mL
	Hydromorphone (Dilaudid)	Dust, Juice, Smack, D, Footballs	1-2 days	2000 ng/mL 300 ng/mL	>100 ng/mL	10-30 ng/mL
	Fentanyl (Sublimaze, Duragesic, Actiq, Fentora)	Percopop, Apache, China girl, China white, Dance fever, Friend, Goodfella, Jackpot, Murder 8, TNT, Tango and Cash	1-2 days	5 ng/mL	>34 ng/mL >3 ng/mL (naive patients)	1-3 ng/mL (highly variable, depends on dose and route of administration)
HALLUCINOGENS	Lysergic acid, diethylamide (LSD)	Acid, Microdot, White lightning	1-5 days	0.5 ng/mL 100 pg/mL	>2 ng/mL	None detected
	Marijuana and cannabinoids	Mary Jane, Hashish, Bhang, Ganja, Sensemilla	Single use: 2-7 days (as Δ9-THC-COOH) Prolonged use: 1-2 months (as Δ9-THC-COOH)	100, 50, 25, or 20 ng/mL 15 ng/mL	50-200 ng/mL	None detected
	Phencyclidine	PCP, Angel dust, Killer weed, Hog	Single use: 1 week Prolonged use: 2-4 weeks	25 ng/mL	100 ng/mL	None detected
STIMULANTS	Cocaine	Coke, Crack, Flake, Snow	Single use: 3 days Prolonged use: 4 days	300 ng/mL 150 ng/mL (as metabolite benzoylecgonine)	>1000 ng/mL	100-500 ng/mL
	Amphetamine (Benzedrine, Dexedrine)	Speed, Bennies, Uppers, Dexies	Single use: 48 hours Prolonged use: 7-10 days	100 ng/mL 500 ng/mL	>200 ng/mL	20-30 ng/mL
	Methylene-3,4 dioxy-Methamphetamine (MDMA)	Ecstasy, Adam, XTC, Love drug, Hug drug	Single use: 24 hours	3000-5000 ng/mL 5 ng/mL	100-1000 ng/mL	20-30 ng/mL
	Methamphetamine (Desoxyn, Methedrine)	Speed, Meth, Crystal ice, Crank	Single use: 48 hours Prolonged use: 7-10 days	100 ng/mL 500 ng/mL	>500 ng/mL	10-50 ng/mL

	Drug (and trade name)	Some common street names	Detectable duration in urine after last dose	Urine cutoff points for reporting positive by screening assay*	Toxic blood level	Blood reference (therapeutic) range
BARBITURATES	Pentobarbital (Nembutal)	Goof balls, Downers, Nembies, Yellow jackets, Yellow submarine	2 days	300 ng/mL 200 ng/mL	>10 µg/mL	1-5 µg/mL
	Secobarbital (Seconal)	Bullets, Pink ladies, Reds	2 days	300 ng/mL 200 ng/mL	>5 µg/mL	1-2 µg/mL
	Butabarbital (Butisol)	Goof balls, Candy, Peanuts, Stoppers	2 days	300 ng/mL 200 ng/mL	20 µg/mL	3-25 µg/mL
	Butalbital (Fiorinal)	Goof balls, Sleepers, Stoppers, Peanuts	2 days	300 ng/mL 200 ng/mL	20 µg/mL	5-15 µg/mL
	Phenobarbital	Barbs, Downers	1-3 weeks	300 ng/mL 200 ng/mL	>40 µg/mL	10-40 µg/ml
ALCOHOLS, DIOLS, & METABOLITES	Ethanol	Booze, Hooch	<1 day	10 mg/dL	80-400 mg/dL	100-150 mg/dL (for treatment of toxic alcohols)
	Methanol	Wood alcohol	<1 day	5 mg/dL (GC)	>20 mg/dL	<0.15 mg/dL
	Isopropanol	Rubbing alcohol	<1 day	5 mg/dL (GC)	>50 mg/dL	None detected
	Acetone		<1 day	5 mg/dL (GC)	>33 mg/dL	<1.0 mg/dL
	Ethylene Glycol	Antifreeze	<1 day	5 mg/dL (GC)	>50 mg/dL	None detected
SEDATIVES/HYPNOTICS/ANESTHETICS	Diazepam (Valium)	Tranks, Downers, Blues, Yellows, Blue Magoo, V	Single use: Not detected Prolonged use: 5-7 days (up to 30 days)	300 ng/mL 200 ng/mL 150 ng/mL	Drug plus Metabolite: >5.0 µg/mL	Drug plus Metabolite: 0.1-1.0 µg/mL
	Oxazepam (Serax)	Tranks, Downers, Blues, Yellows	Single use: Not detected Prolonged use: 5-7 days	300 ng/mL 200 ng/mL 150 ng/mL	>2.0 µg/mL	0.2-1.4 µg/mL
	Alprazolam (Xanax)	Tranks, Downers, Blues, Yellows	Single use: Not detected Prolonged use: 5-7 days	300 ng/mL 200 ng/mL 150 ng/mL	>350 ng/mL	20-30 ng/mL
	Clonazepam (Klonopin)	Tranks, Downers, Blues, Yellows	Single use: Not detected Prolonged use: 5-14 days	300 ng/mL 200 ng/mL 150 ng/mL	>80 ng/mL	20-70 ng/mL
	Chlordiazepoxide (Librium)	Tranks, Downers, Blues, Yellows	Single use: Not detected Prolonged use: 5-7 days	300 ng/mL 200 ng/mL 150 ng/mL	>5 µg/mL	0.7-1.0 µg/mL
	Lorazepam (Ativan, Loraz)	Tranks, Downers, Blues, Yellows	Single use: Not detected Prolonged use: 5-7 days	300 ng/mL 200 ng/mL 150 ng/mL	0.3-0.6 µg/mL	50-240 ng/mL
	Flunitrazepam (Rohypnol)	Roofies, Rib, Rope, Roach-2, R-2	72 hours	0.1-60 ng/mL (Immunoassay)	50 ng/mL	5-15 ng/mL
	Gamma-Hydroxybutyrate (Somatomax)	GHB, G-Caps, Geebers, Fantasy, Liquid Ecstasy	12 hours	1-10 mg/L (GC; GC-MS)	>250 mg/L	48-125 mg/L (for narcolepsy)
	Ketamine Hydrochloride (Ketajet)	Special K, Lady Kay, Vitamin K, Jet, Cat Valium	<72 hours	5-10 ng/mL (GC-MS)	>7-27 µg/mL (highly variable)	0.5-5.0 µg/mL

[1]