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Continuous-flow production of aspirin using droplet microfluidics

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Abstract

Droplet based microfluidics is a promising method for the synthesis of drugs combining biochemistry, soft matter physics and microsystems engineering. Precise control of reaction conditions and droplet volume will allow safer, easier and cheaper synthesis of chemicals. This paper reviews a method for the benchtop fabrication of a SU-8 microfluidic system and its capability to synthesize aspirin inside microdroplets. Aspirin is soluble in fatty solvents, indicating that oil droplets have to be made on the microfluidic device. The wetting properties of the PDMS surface and the viscosity of the two liquid phases are key parameters for the generation of microdroplets. Altering these two parameters in the microfluidic device allowed successful formation of oil microdroplets in water. After 15 minutes reaction time, we were able to successfully produce aspirin inside microdroplets. Evaporation of ethyl acetate prevented achieving higher yields than observed in the in-batch synthesis, it is therefore recommended to use a disperse phase with a higher boiling point for future research.

Introduction

Aspirin

General theory on aspirin

For thousands of years, therapy for rheumatism was based on decoctions or extracts from plants or herbs containing salicylates (1). In 1874, Maclagan (2) reported that the usage of salicin on several rheumatic patients reduced fever, reduced swelling of the joints, reduced stiffness in the joints, and that especially the pain was abated. 1874 was also an important year for the development of aspirin as the organic synthesis of salicylic acid from phenol was described by Kolbe (3). In 1895, Felix Hoffman, who was a chemist at the chemical research laboratories at Bayer, discovered a method to acetylate the hydroxyl group of the benzene ring of salicylic acid forming acetylsalicylic acid. Bayer gave the newly formed drug the name Aspirin® in order to prevent confusion with salicylic acid (1). At that moment, little was known about the mode of action of the aspirin-like drugs.

Just like ibuprofen, naproxen and diclofenac, aspirin belongs to the nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs inhibit cyclo-oxygenase (COX), which is the enzyme responsible for the production of prostaglandins (4). COX-1 produces prostaglandins which are mainly responsible for physiological processes such as platelet aggregation, kidney function and protection of stomach mucosa (1)(4). COX-2 mainly produces prostaglandins responsible for contributing to swelling and pain in musculoskeletal injury and inflammation (4). Aspirin is able to selectively acetylate a hydroxyl group on a single serine residue (Ser 530) which is very close to the C-terminus of the protein. Aspirin thereby irreversibly inhibits COX enzymes; this immediately indicates that new COX enzymes have to be formed before prostaglandins can be produced (1).

According to Figure 1, aspirin is non-selective for COX, and even inhibits COX-1 more than it inhibits COX-2 (5).

Aspirin inhibiting COX-1 more than COX-2 combined with the theory about both COX isoenzymes, gives rise to the hypothesis that aspirin will inhibit important physiological processes, e.g. production of cytoprotective prostaglandins in the gastric mucosa. Treatment with non-selective COX inhibitors, like aspirin, shows higher incidence of gastrointestinal side-effects than treatment with COX-2 selective inhibitors, e.g. celecoxib, rofecoxib and meloxicam (5).

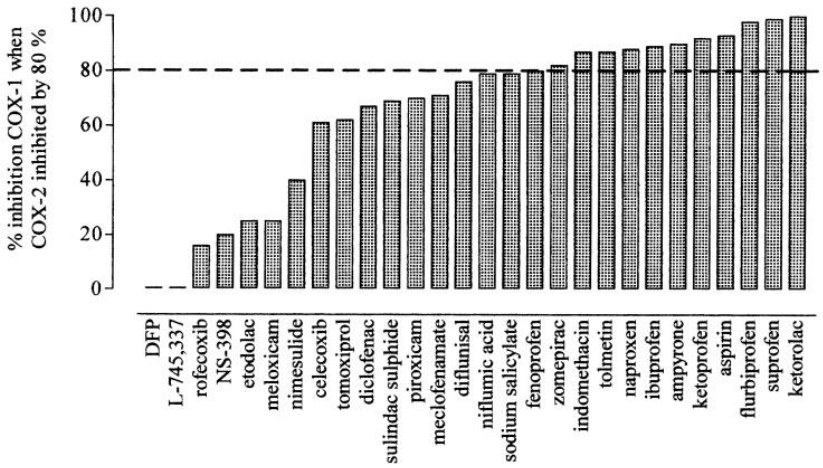


Figure 1. Percentage inhibition of COX-1 when 80% of COX-2 is inhibited per drug. The line in dots represents equal activity, i.e. an 80% inhibition for both COX-1 and COX-2. Aspirin inhibits COX-1 for more than 80% when COX-2 is inhibited by 80%. Adapted from Warner et al. (5)

The cardioprotective effect of aspirin is mediated through irreversible inhibition of platelet COX-1, thereby blocking the production of Thromboxane A2 (TXA_2) (6). In order to understand how aspirin has anti-thrombotic effects it is important to review the pathway leading to production of prostanoids in platelets. Phospholipase A_2 is able to release arachidonic acid from membrane phospholipids (6). This arachidonic acid can be converted by prostaglandin H synthase, also known as COX, into prostaglandin G_2 (PGG_2) (6). The PGG_2 can also be converted by peroxidases into prostaglandin H_2 (PGH_2). PGG_2 and PGH_2 are called the prostaglandin endoperoxides and are the substrates for secondary enzyme systems that produce functional prostanoids (6). The prostaglandin endoperoxides act on the TXA_2 receptors, thereby also having biological effects themselves. The PG endoperoxides are unstable substrates quickly converted into TxA_2, PGE_2 or PGI_2 by TxA_2 synthase, PGE_2 isomerase or PGI_2 synthase respectively. PGI_2 is produced in the systemic blood vessels and has anti-thrombotic effects by decreasing the blood vessel tone and platelet reactivity. PGE_2 is released from the vascular smooth muscle and endothelium cells, and has partially pro and anti-thrombotic effects. TxA_2 is produced in the platelets, and by a lesser extent by vascular smooth muscle cells, and increases the blood vessel tone and platelet reactivity (6).

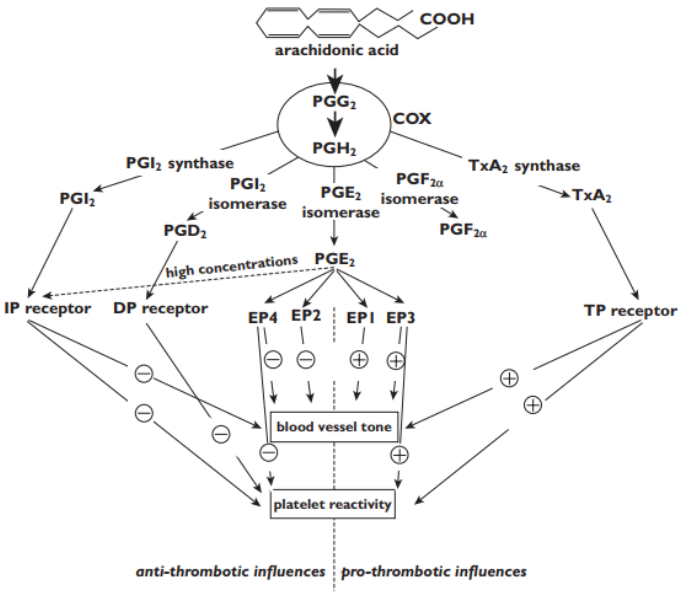


Figure 2. schematic representation of the formation of prostanoids from arachidonic acid with regard to the effects on blood vessels and platelets. Adapted from Warner et al. (6)

Aspirin is able to covalently bind to COX-1 present in the platelets, thereby irreversibly inhibiting the enzyme. Inhibited COX-1 enzymes in the platelets are not able to produce any TxA_2 anymore leading to anti-thrombotic effects. The acetylation of COX-1 by aspirin lasts for the lifetime of the platelet as platelets are unable to produce new COX-1 proteins. The anti-thrombotic effects of aspirin are therefore influenced by the platelet renewal speed and plasma exposure (6).

Synthesis of aspirin

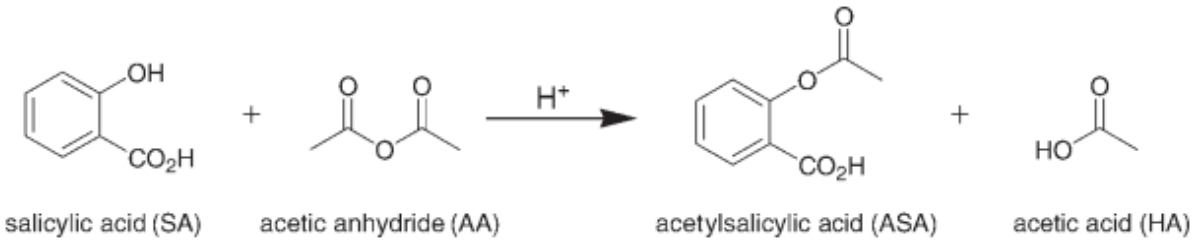


Figure 3. Reaction of salicylic acid with acetic anhydride forming aspirin and acetic acid. Adapted from Puxty et al. (7)

Figure 3 shows that acetic anhydride is able to acetylate salicylic acid forming acetylsalicylic acid and acetic acid under acidic circumstances.

Salicylic acid reacts with acetic anhydride with molar ratio 1:1. If acetic anhydride is added in excess, it is possible that acetylsalicylic acid reacts with the acetic anhydride forming doubly acetylated salicylic acid (7).

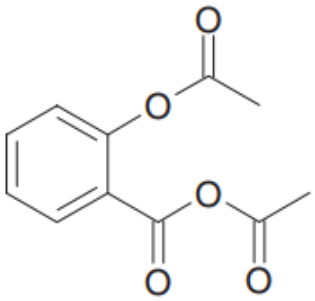


Figure 4. Doubly acetylated salicylic acid. Adapted from Puxty et al. (7)

Analysis of aspirin

Aspirin and salicylic acid can be analysed using a high-performance liquid chromatography (HPLC). An HPLC device separates substances based on polarity, which is specific for a molecule. The logP value of a molecule is an important parameter for HPLC. The log P value is the logarithm of the ratio of a compounds concentration in octanol phase to its concentration in the aqueous phase of a two-phase octanol/water system. Aspirin has a logP of 1.19 (8), meaning that aspirin molecules are about 10 times more likely to be present in the organic phase. Salicylic acid has a partition coefficient of 2.21 (9), meaning that salicylic acid molecules are about 100 times more likely to be present in the organic phase.

Fourier transform infrared spectroscopy (FT-IR) can be used to qualitatively analyse samples of aspirin or salicylic acid. The principle of FT-IR is based ability of molecules to absorb electromagnetic radiation at certain frequencies. Molecules absorb radiation on certain frequencies based on which functional groups are present in the structure of the molecule. Figure 5 shows functional groups corresponding to absorption in IR spectra.

wavelength of absorption (cm^{-1})	mode of vibration	functional group
2852–2852	C–H stretching	$-CH_2$ and CH_3 of the saturate
2922–2923	C–H stretching	$-CH_2$ and CH_3 of the saturate
1460	C–H deformation	$-CH_2$ and CH_3 of the saturate
1376	C–H symmetric deformation	$-CH_2$ of the saturate
722	C–H bending	–C–H of substituted benzene
1640–1800	C═O stretching	–C═O of carbonyl/carboxylic groups
3700–3100	O–H stretching	–OH of phenolic groups
1470–1590	C═C ring stretching	–C═C of aromatics

Figure 5. Functional groups and the places in the IR spectrum where absorption can be detected (17).

Aspirin differs from salicylic acid with respect to their functional groups. Aspirin contains an acetate group attached to the benzene ring, whereas salicylic acid contains a hydroxyl group attached to the benzene ring. According to figure 5, the hydroxyl group present in salicylic acid should absorb light between wavenumbers 3700 - 3100 cm^{-1}. This hydroxyl group is not present is aspirin, indicating that a distinction can be made between the two molecules.

Degradation of aspirin

Figure 6 shows that the amount of salicylic acid in an aqueous sample increases after time. This can be concluded from the figure as salicylic acid absorbs light at 265 and 296 nm, whereas aspirin only absorbs light at 265 nm. An increased absorption at 296 nm therefore indicates that the amount of salicylic acid in the sample has increased. This increase in salicylic acid in aqueous solution can be explained by the hydrolyzation of aspirin (10).

Degradation of aspirin also occurs in organic solvents. According to Skibinski and Komsta (11), the most stable solvents for aspirin are acetonitrile and 1,4-dioxane. Aspirin has a half-life of 83.35 hours in 1,4-dioxane and a half-life of 63.37 hours in acetonitrile. Aspirin is degraded the fastest in ethanol and methanol, with half-life of 8.15 and 8.83 hours respectively (11).

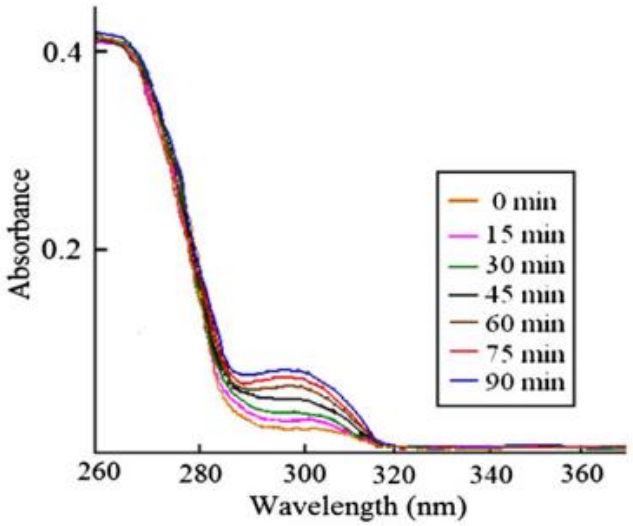


Figure 6. ultraviolet absorption of aspirin at given time points. Y-axis represents the absorbance which has no unit. X-axis represents the wavelength of the light in nanometre (nm). This figure is adapted from Wang et al. (10)

Microfluidics

General theory on microfluidic systems and flow chemistry

Microfluidics can be best described as the science and engineering of systems in which fluid behaviour deviates from large-scale flow theory, owing to the system's tiny length scale (12).

Surface tension

Figure 7 shows an illustration of contact lines between a solid, liquid and a gas. In this figure, the contact angle, θ, is indicated. The contact angle between the solid and the liquid determines whether a liquid wets the surface, thereby determining if the surface is hydrophilic or hydrophobic in the case of water. Contact angles smaller than 90 ˚C are corresponding to liquid that wets the surface. If the contact angle is larger than 90 ˚C, the liquid is not wetting the surface. A hydrophilic surface, is a surface that can be wetted with water. The opposite is true for a hydrophobic surface, which cannot be wetted by water (12).

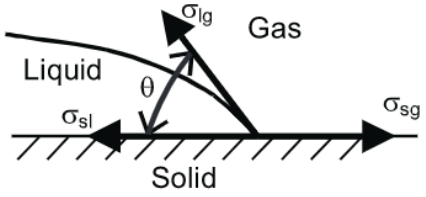


Figure 7. Contact line arrangement. Contact angle is labelled ϑ, tension between phases are labelled σ_{sl}, σ_{lg}, and σ_{sg} for solid-liquid, liquid-gas and solid-gas respectively. Figure adapted from Nguyen et al. (12)

Microreactors

Micromachining technology provides interesting room for biological, chemical and pharmaceutical analysis. Integration of building blocks and reactor miniaturization ensure new functionalities and capabilities, exceeding traditional counterparts. Low-costs production and high throughput can be achieved by using microreactors. For application in the industry, the advantages of microreactors can be seen in four aspects; cost, safety, scientific merit and functionality (12).

Cost

The reduced number of reagents used in microreactors minimized the costs caused by expensive reagents. Microreactors can achieve high throughput and fast screening with a relative low fabrication cost, this makes microreactors much more cost-effective than industrial reactors. Microreactors can cheaply be fabricated in batch and multiplexed synthesis can be achieved by replicating reaction units. This is especially beneficial for small-scale chemical and pharmaceutical industries as the results from multiplexed reaction units produced in the lab can directly be used for commercial production. Usage of microreactors is also flexible, as the reactor units are very small, they can be exposed to changing environments much more rapidly. Also, the production capacity can be easily increased by changing the number of reaction units (12).

Safety

Small-scale production and usage of chemicals leads to a small quantity of chemicals released from the system accidentally, these small quantities can be contained easier and will not lead to large-scale dangers compared to larger-scale reactor accidents. In microreactors, it is possible to apply sensors contributing to the overall safety, because failed reactors can be identified and replaced (12). In addition, the conditions (e.g., temperature) in a microfluidic device can be controlled more easily. Occurrence of an outlier for the conditions of a microfluidic device will happen less frequently than in larger-scale systems, which also contributes to the safety of a microfluidic device.

Scientific merit

Flows inside microreactors are laminar, which is well studied and tools for computational fluid dynamic models can be used. If the flow enters the molecular-size regime, new computational models are required for design and analysis (12).

Functionality

The small system of a microreactor leads to small thermal inertia, as the temperature inside a microsystem can be well controlled and temperature is one the most important reaction parameters. The tiny size of the microreactor system also leads to higher heat and mass transfer rates which allow reactions to occur under more uniform temperature conditions (12).

A small system also accounts for shorter residence times in the reactors. This enables less stable intermediates to be quickly converted into the next compound. New reaction pathways are thereby accessible in microreactors that are not accessible in conventional reaction method. (12)

Laminar flow

The Reynolds number is a dimensionless parameter in fluid mechanics describing the ratio between inertial forces and viscous forces (13).

Turbulent or laminar flow can be predicted for flows in long straight channels in microfluidic systems using Reynolds number. The Reynolds number, Figure 8, can be calculated using the hydraulic diameter (D_h), characteristic velocity in the channel (u), the density (ρ), the kinetic viscosity (ν) and the dynamic viscosity (η). The transitional Reynolds number is the number above which flow is turbulent and below which is laminar, and this number is between 1500 and 2500 (12).

$$\mathrm{Re} = \frac{\rho D_\mathrm{h} u}{\eta} = \frac{D_\mathrm{h} u}{\nu}$$

Figure 8. Equation for determining Reynolds number. The different parameters are explained in the text. Adapted from Nguyen et al. (12)

The Reynolds number is calculated for the PDMS chip used in the experiment in appendix 6. The Reynolds number for the channels after droplet formation is between 0.628 – 0.947. The Reynolds number cannot be calculated for the channels containing droplets as two phases are present here, it is thus expected that Reynolds number for these channels are values in between the Reynolds number corresponding to the channel containing disperse or continuous phase. Reynolds number for the channels containing only disperse phase is 0.947. Reynolds number for the channels containing only continuous phase is 0.628. Considering this number is not higher than 1500, only laminar flow is present in the chip.

Flow Chemistry

Instead of chemistry in batch, flow chemistry describes a chemical reaction which is run in a continuous flow system. Flow chemistry gives access to unusual reaction conditions (e.g., high pressures and temperature), safely handling hazardous intermediates, and easy mass and heat transfer (14). This has led to inclusion of flow chemistry steps in the production process of pharmaceuticals. Besides the industry, the capability to synthesize pharmaceuticals on a small to medium scale with flow chemistry influences a lot of daily laboratory research as well. On a larger scale droplet-based reactors operate well, and the main advantage of this kind of system is that reaction is isolated from the channel walls preventing fouling by the channel walls (15).

Droplet microfluidics

The formation of droplets is dependent on the capillary number and the sample fraction (12). The formula for capillary number can be written as; $Ca = {}^{u * v}/_{\sigma}$, with Ca as capillary number, u as the mean velocity, v as the viscosity, and σ being the surface tension (12). At medium-high capillary number, the disperse phase will form a droplet. At lower capillary number, the continuous phase will be able to form two separate, alternating droplets and these droplets will be unstable at even higher capillary numbers, as long as the volumetric flow rates of the two phases (r) stay the same. The usage of polyvinyl alcohol (PVA) in aqueous solution will increase the viscosity of the aqueous phase with regard to concentration (16). In aqueous solutions there are two intermolecular interactions between water and PVA, which increase the viscosity; H-bonding between the hydroxyl group of PVA and water molecules and H-bonding between the hydroxyl groups on PVA chains. The sample fraction, r, can be calculated as following; $r = {}^{Q_{con}}\big/_{(Q_{con} + Q_{dis})}$ with Q_{con} being the flow rate of the continuous phase and Q_{dis} being the flow rate of the disperse phase (12). The sample fraction used in this experiment is equal to 0.5 as both flow rates were set to be the same.

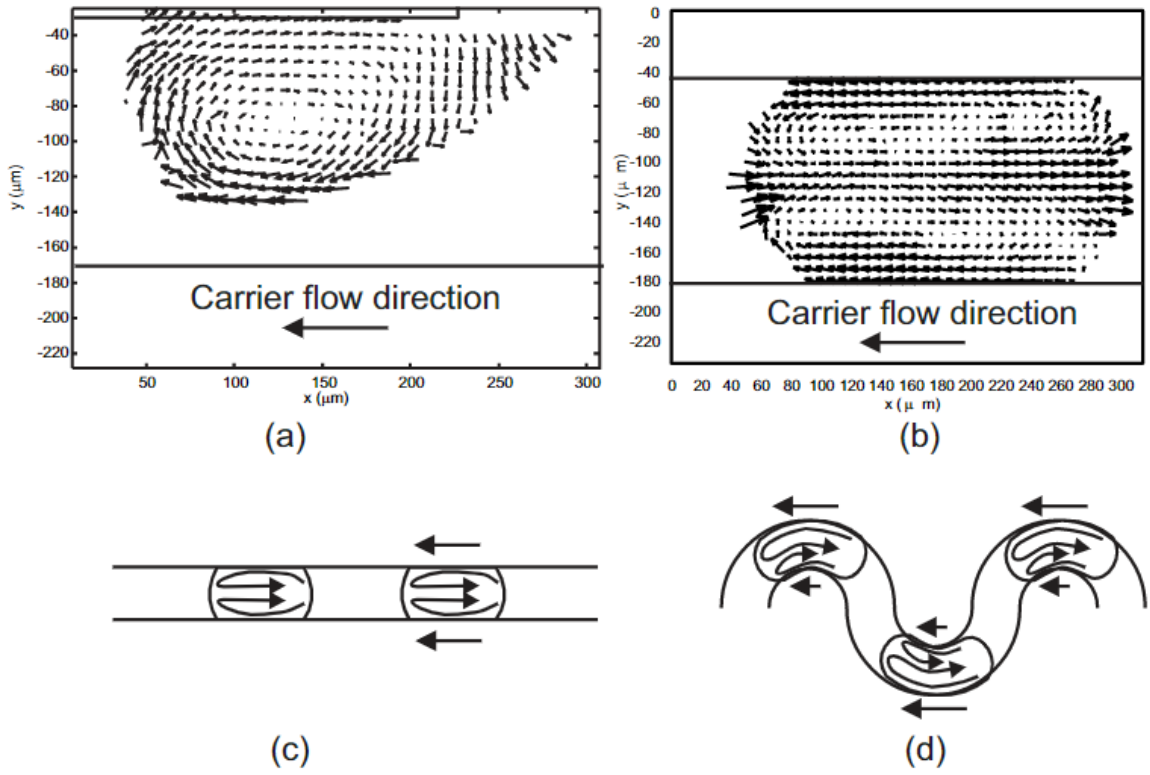


Figure 9. Patterns of flow inside a microdroplet. (a) flow pattern during the formation of the droplet. (b) flow pattern of the microdroplet passing a straight rectangular channel. (c) illustrated flow inside a microdroplet that is passing a straight channel and (d) inside a channel with turns. Adapted from Nguyen et al. (12)

Figure 9 shows the patterns of flow created in a microdroplet when forming and moving through channels. Figure 9c shows that inside a straight microchannel, the two vortices exist in both halves of the droplet, thereby indicating that mixing is not improved. Figure 9d shows that inside a microchannel containing turns, the flow patterns differ from that of straight channels. The vortices differ in each side of the droplet, with one side of the droplet having a shorter vortex than the other side while the droplet is going through a turn. The difference in flows in microchannels containing turns creates the possibility of faster mixing inside a microdroplet (12).

Aim

Synthesis of aspirin on a microfluidic device would be of great interest for the pharmaceutical industry as this opens up possible faster and safer methods of production. However, the possibility to produce aspirin on a microfluidic device has not been investigated extensively. This experiment will provide a method to produce aspirin on a microfluidic device using continuous flow.

Subgoal 1: synthesis of ASA in microdroplets

The first goal in this experiment is to investigate whether it is possible to produce aspirin inside of a microdroplet. In order to do so, a PDMS device was designed that is able to produce oil in water droplets. Oil in water droplets are needed for the synthesis of aspirin as salicylic acid and aspirin are both soluble in fatty solvents, and it is desired for the reaction to occur inside of a microdroplet.

Subgoal 2: comparing the microdroplets to the in-batch synthesis

The second goal in this experiment is to determine which reaction model is the most effective in producing aspirin from salicylic acid and acetic anhydride. This is done by performing the synthesis of aspirin both in batch and in microdroplets and comparing the yield and purity between the two different methods.

Materials and methods

Batch synthesis of aspirin

Chemicals and reagents

Aspirin, salicylic acid, ethanol 96%, acetic anhydride, sulfuric acid and acetonitrile (ACN) HPLC quality were obtained from Sigma Aldrich Chemicals Company (Zwijndrecht, the Netherlands). Ultra-pure (UP) water was obtained from Milli-Q apparatus.

In-batch synthesis of aspirin

An Erlenmeyer containing 6 mL acetic anhydride, 2.98 gram salicylic acid and 5 drops of sulfuric acid was prepared. The Erlenmeyer was placed on top of the water bath thereby heating the solution at 95 ˚C for 15 minutes. The solution was placed in an ice bath and crystallisation was induced by scratching the bottom of the Erlenmeyer with a glass rod. This process of crystallisation was stopped after 1.5 hours and the crystals were filtered using a Büchner funnel and a glass filter. The crystals on the filter paper were redissolved in ethanol and placed on the water bath at 70 ˚C to speed up the dissolution. After all crystals were dissolved, crystallisation was induced again in an ice bath containing household spiritus. The final product with filter paper and a watch glass was placed on the analytical balance to determine the yield.

Analysis of aspirin

Iron chloride test

A 1% iron chloride solution was prepared by adding 0.108 g iron chloride to 10 mL UP-water. A drop of the iron chloride solution was then added into test tubes containing the sample, pure aspirin, pure salicylic acid and to an empty test tube.

HPLC analysis

Analysis by HPLC was performed using a VWR HITACHI HPLC with l-2455 detector, l-2300 column oven, l-2200 autosampler, l-2130 pump and organizer. A C18 column with 5 µm pores was used with an inner diameter of 4 mm and a total length of 125 mm. Eluent containing acetonitrile and UP-water with volume ratios of 30/70 respectively was prepared. The UP-water used to prepared the eluent contains 0.2% phosphoric acid, this was prepared by adding 1.4 mL H_3PO_4 to 700 mL water. The sample was prepared by adding 20.3 mg to 10 mL ACN and diluting this 1,000 times prior to injection into the HPLC, resulting in a theoretical expected concentration of 0.0203 mg/mL of aspirin. Separate stock solutions for comparison containing 0.01 mg/mL salicylic acid and aspirin were prepared. In order to make the calibration curve, different samples containing 20 mg salicylic acid or aspirin were prepared in 10 mL ACN. These samples were diluted 100 times in ACN creating the stock solutions used for the calibration curve. The stock solutions were used to prepare the calibration samples according to Table 1.

Table 1. Contents and concentration of the calibration samples used in HPLC analysis.

HPLC Vial	SA or ASA Stock solution (µL)	ACN (µL)	Concentration (mg/mL)
1	1000	0	0.2
2	800	200	0.16
3	600	400	0.12
4	400	600	0.08
5	200	800	0.04

In order to select an optimal eluent for the analysis of aspirin and salicylic acid, the two compounds were analysed using different composed eluents. Eluents containing different contents of ACN and water were used. The volumetric ratios of H_2O / ACN in the eluents used are 80/20, 70/30, 60/40 and 40/60. In order to calculate the plate number and resolution for the two peaks in each eluent the following formulas were used:

$$N = 5.54 * \left({}^{t_r}\!/\!_{w_{\frac{1}{2}}} \right)^2 = 5.54 * \left({}^{1.4}\!/\!_{0.09} \right)^2 = 1341$$

$$Rs = \frac{0.589 * (|t_{rSA} - t_{rASA}|)}{\frac{1}{2} * (W_{\frac{1}{2}SA} + W_{\frac{1}{2}ASA})} = \frac{0.589 * (|1.4 - 1.7|)}{\frac{1}{2} * (0.09 + 0.1)} = 1.86$$

Each formula contains an example calculation from eluent water/ACN 40/60.

UV-spectrophotometry

Analysis by UV was performed using a VWR UV-6300PC spectrophotometer. Phosphate buffer with pH of 6.83 was prepared by adding 3.473 g HPO_4^{2-} and 3.085 g $H_2PO_4^-$ to 1L UP-water. 20 mg of sample was added to 10 mL of phosphoric buffer and diluted an extra 1,000 times before analysis, resulting in a final concentration in the sample of 0.0020 mg/mL. This was also done for pure aspirin and salicylic acid.

IR spectroscopy

Analysis by IR was performed using a SCHIMADZU FT-IR spectrophotometer. A sample of 100 mg was analysed as solid without adding or removing any substituents. Different solid mixtures of salicylic acid and aspirin were prepared using a pestle and mortar and adding the components using 50/50 mixing. Table 2 shows the weights of contents of each solid sample.

Table 2. Contents of samples used in infrared spectroscopy

Sample	Weight aspirin (mg)	Weight salicylic acid (mg)
Aspirin	200	0
ASA/SA 80/20	168	43
ASA/SA 60/40	119	77
ASA/SA 40/60	82	120
ASA/SA 20/80	41	160
Salicylic acid	200	0

Continuous-flow synthesis of aspirin

Chemicals and reagents

Perfluorooctyl trichlorosilane (PFOTCS), polyvinyl alcohol (PVA), aspirin, salicylic acid, ethanol 96%, mineral oil, isopropanol, acetic anhydride, sulfuric acid and acetonitrile (ACN) HPLC quality were purchased from Sigma-Aldrich Sigma (Zwijndrecht, the Netherlands). Polyvinyl alcohol was also purchased from Sigma Aldrich Chemicals Company. Sylgard™ 184 silicone elastomer base as well as the curing agents were purchased from Dow Chemical (Midland, MI, USA). Ultra-pure (UP) water was obtained from Milli-Q apparatus.

Preparing the photomask for the glass mould

Software program CleWin3.0 (Wieweb, Hengelo, the Netherlands) was used to create a photomask. The photomask consists of four chip designs, visible in Figure 20, each differing in junction area. Each design contains two inlets, followed by 200-µm-wide channels. These channels are organized in such a way that the continuous phase moves through two 200-µm-wide channels entering the junction from the top and the bottom, and the disperse phase enters the junction from the left channel. The junction was designed as the spot where the three channels meet and continue in one channel directing the flow towards the broader channels containing turns. These channels are 1.8 mm wide and contain turns at each end, a space of 500 µm was placed between the larger channels. The total length of the entire PDMS structure containing the channel in its surface was 5 cm, the total width was 1.2 cm.

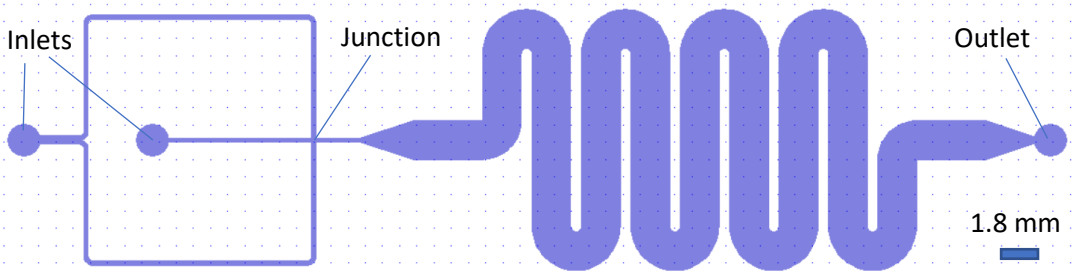


Figure 10. Photomask design of one of the chips. Image was created in CleWin3.0.

Preparing the glass mould

The fabrication of the glass mould in the clean room is described in appendix 7.

Glass mould coating

Perfluorooctyl trichlorosilane was applied to the glass mould using a vacuum chamber. Perfluorooctyl trichlorosilane is able to create an anti-adhesive layer on the glass, making it easier to detach the PDMS from the glass mould.

Preparing the PDMS chips

PDMS base and curing agent were mixed for two minutes with in a 10:1 weight ratio, respectively. A total weight of PDMS of 45 grams was used per glass mould. The mixed liquid PDMS was degassed under vacuum for 30 minutes. The PDMS was poured over the glass mould and gas present in the PDMS was removed by degassing with air. The mould was heated at 70 ˚C for 2 hours. The PDMS was peeled off the mould and cut into the desired shapes. The inlets and outlets were punched with a 1.5 mm biopsy puncher. The cut pieces of PDMS were attached to a microscope slide using a HARRICK oxygen plasma cleaner (310 - 320 mTorr, 30 seconds; Harrick Plasma, Ithaca, NY, USA).

Testing the solubility of salicylic acid in mineral oil

16.7 mg of salicylic acid was added to an empty 250 mL Erlenmeyer flask. Mineral oil was added first in quantity steps of 10 mL but when more of the salicylic acid started to dissolve steps were reduced to 2 mL. The Erlenmeyer was placed in the ultrasonic bath to induce faster dissolvement.

Changing the hydrophobicity of the PDMS surface inside the chips

The chips were placed into the oxygen plasma (390-410 mTorr, 10 minutes), and afterwards 50 µL of UP-water was added in the outlet of the chip.

Preparation of the continuous and disperse phase

The continuous phase was prepared by adding 100 mg of polyvinyl alcohol to 10 g of UP-water. This does not dissolve properly without heating and stirring. Therefore, the solution was first stirred at 250 rpm for 40 minutes, then heated at 100 ˚C while stirring at 250 rpm for another 40 minutes. The solution was then left to cool down to 65 ˚C while stirring at 250 rpm. The PVA was weighed and UP-water was added to compensate for any water that was evaporated during the dissolving process.

The disperse phase was prepared by adding 1.0 gram of salicylic acid, 0.211 mL acetic anhydride and 0.1 mL sulfuric acid to 10 mL ethyl acetate.

Synthesis of aspirin on the chip

The prepared chip was placed on the water bath which was set at 57 ˚C, in order to prevent any ethyl acetate to evaporate. Ethyl acetate has a boiling point of 77 ˚C, so the temperature on the water bath was initially chosen 20 ˚C lower. A 3D printed mould was used to fit the chip into the water bath.

The continuous phase was dispensed into the chip using a plastic 1 mL syringe with blunt needle attached to about 15 cm of plastic tubing. The syringe was placed in to a syringe pump from ProSense B.V (Oosterhout, The Netherlands) using a flow rate of 250 µL/h.

The disperse phase was dispensed into the chip using a 1 mL glass syringe with Kevlar inner tip attached to about 15 cm of plastic tubing. The syringe was placed in to a laboratory syringe from ProSense B.V. (Oosterhout, The Netherlands) using a flow rate of 250 µL/h.

A HPLC vial was attached to the outlet of the chip using plastic tubing of about 5 cm. This vial served as the collection vial for the reaction on the chip. The collected sample in this vial was diluted 1,000 times with ACN before injection into the HPLC.

Results & Discussion

The results section of the experiment can be divided into a section discussing the batch synthesis and a section discussing the synthesis on the PDMS device. The results of the batch synthesis include determination of yield and purity by different methods. IR spectroscopy, UV-Vis spectroscopy, Iron-chloride testing, and HPLC analysis results are described. The results of the synthesis on a microfluidic device include results from the generation of oil-in-water droplets and results from the analysis of the product using HPLC.

Batch synthesis of aspirin

Yield determination

2.98 grams of salicylic acid was used in the reaction. Using the molecular weight of salicylic acid (138.121 g/mol), a chemical quantity can be calculated using the following equation:

$$n = {}^{W}/_{MW} = {}^{2.98}/_{138.121} = 0.02158\ mol = 21.58\ mmol$$

So, 2.98 grams of salicylic acid is equivalent to 21.58 mmol. The reaction mechanism described in the theory states that salicylic acid is converted into acetylsalicylic acid with mol ratio 1:1. In theory, a maximum of 21.58 mmol of acetylsalicylic acid can be produced.

The total weight of the product was 3.72 grams. Using the molecular weight of acetylsalicylic acid (180.158 g/mol), a chemical quantity can be calculated using the following equation:

$$n = {}^{W}/_{MW} = {}^{3.72}/_{180.158} = 0.02065\ mol = 20.65\ mmol$$

The yield can be calculated by comparing the chemical quantity of acetylsalicylic acid to the theoretical maximum of acetylsalicylic acid that could be produced:

$$yield\ (\%) = \left({}^{n_{measured}}/_{n_{theoretical}}\right) * 100\ \% = \left({}^{20.65}/_{21.58}\right) * 100\ \% = 95.7\ \%$$

Iron chloride test

In Figure 11 it is visible that iron chloride turns dark purple when it comes into contact with salicylic acid, but does not change colour and remains transparent when it comes into contact with acetylsalicylic acid.

Sample R contains the product of the batch synthesis. Sample R remained transparent when in contact with iron chloride solution but a slight tint of purple was visible by eye.

This indicates that the product formed from the batch synthesis contains a small portion of salicylic acid but the majority was acetylsalicylic acid.

Figure 11. (From left to right) Iron chloride test of sample Roel (R), pure acetylsalicylic acid (ASA), blanco iron chloride solution (B), sample Mirjam (M) and pure salicylic acid (SA).

Infrared spectroscopy

Mixture analysis

In order to determine whether IR can be used to analyse samples with aspirin, it was first determined if IR is a suitable method to quantify and identify contents in the sample.

Table 3. Results from analysing mixtures of SA and ASA with IR spectroscopy. For the exact weights of contents in the mixture view methods section 'IR spectroscopy'. Measurement data can be viewed in appendix 1.

Content of the mixture (SA/ASA w%)	Content according to literature	Highest literature matching score (max. 1000)
Salicylic acid (100/0)	Salicylic acid	992
Salicylic acid/ acetylsalicylic acid (80/20)	Salicylic acid	970
Salicylic acid/ acetylsalicylic acid (60/40)	Salicylic acid	925
Salicylic acid/ acetylsalicylic acid (40/60)	Acetylsalicylic acid Salicylic acid	894 818
Salicylic acid/ acetylsalicylic acid (20/80)	Acetylsalicylic acid	903
acetylsalicylic acid (0/100)	Acetylsalicylic acid	957

In all mixture samples, the literature spectrum with the highest matching score was indeed the spectrum from the component that made up the majority of the mixture. In one of the four mixture samples, the content of the sample was correctly identified as containing both acetylsalicylic acid and salicylic acid. In three of the four mixture samples, the spectrum of the major component was detected in the literature.

These results indicate that the major component in the mixture can be identified by using IR. Infrared spectroscopy can however not be used to give an accurate quantity of the contents in the sample. However, it may be possible to identify both compounds in the mixture when using IR. This can be explained by reviewing the spectra of acetylsalicylic acid and salicylic acid. The spectrum of SA is shown in appendix 1.1 and the spectrum of ASA is shown in appendix 1.6. Both spectra contain mostly similarities but differ at 3250 cm^{-1} as here a small peak was shown for salicylic acid whilst this peak was not present in the spectrum of acetylsalicylic acid. This makes the detection of salicylic acid in mixtures easier than the detection of acetylsalicylic acid in mixtures with salicylic acid. As presence of salicylic acid will give a peak at 3250 cm^{-1} and it will become harder to detect acetylsalicylic acid due to presence of this peak, as ASA can be identified by lack of the peak at 3250 cm^{-1}. According to the theory (17) the peak at wavenumber 3250 cm^{-1} can be linked to a phenolic OH group, which is present in salicylic acid but not in acetylsalicylic acid. This is in accordance with the structure of the two molecules as the phenolic hydroxyl group in salicylic acid can be replaced by an acetate group to form acetylsalicylic acid. Acetylsalicylic acid may be hard to identify when looking at lack of a peak for phenolic hydroxyl groups, it is much better visible when looking at the IR spectrum of an ester bond, which should be visible at a wavenumber of 1745 cm^{-1} according to the theory (18). At 1745 cm^{-1}, it is clearly visible that a sharp peak becomes visible already with low contents of ASA in the mixtures.

Sample analysis

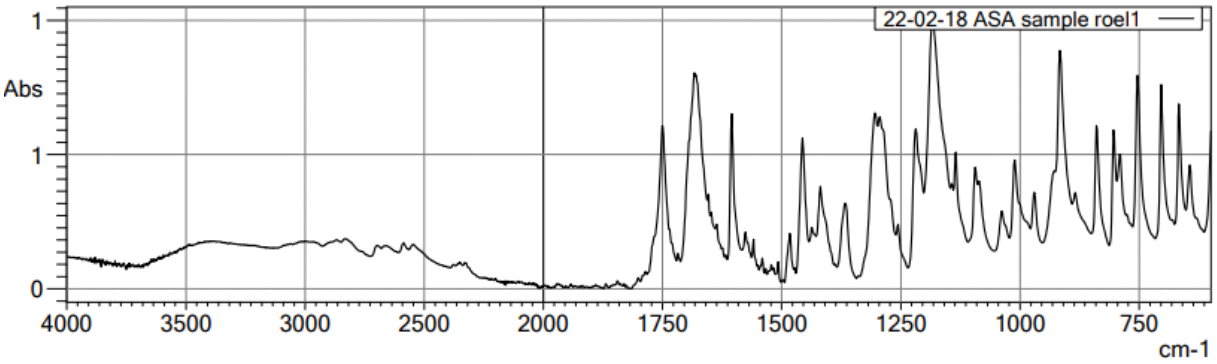


Figure 12. Infrared spectrum of in batch produced acetylsalicylic acid. The Y-axis represents the absorption which has no unit. The X-axis represents the wavenumber in cm^{-1}.

Figure 5 shows the IR spectrum of the in batch produced aspirin. Appendix 1.1, the IR spectrum of salicylic acid, and appendix 1.6, the IR spectrum of acetylsalicylic acid, are used as reference samples to compare with the sample IR spectrum.

It is visible that a sharp peak is present at 1750 cm^{-1}, there is no peak present at 3250 cm^{-1} and a broad small peak is present between 3700 and 3300 cm^{-1}.

The sharp peak at 1750 cm^{-1} is an indicator for presence of aspirin due to presence of an ester bond, this is also described in the section 'mixture analysis'.

At 3250 cm^{-1}, there was no peak visible. Presence of a peak here would indicate the presence of salicylic acid. However, as seen in appendix 1.2 - 1.5, it can be hard to decide whether a peak was present here in a mixture, especially if the content of salicylic acid in the sample becomes lower. This therefore does not conclude that the sample contains purely acetylsalicylic acid and that salicylic acid was not present.

A difference between this sample spectrum and the spectra of ASA and SA is visible at 3700 - 3300 cm^{-1}. Where a broad small peak appears in the sample, no peak was present in the literature nor mixture spectra created with pure ASA and SA. The theory describes that OH groups can cause broad peaks at wavenumbers of 3100 - 3700 cm^{-1}. It is therefore possible that another product was formed containing more OH groups than salicylic acid and acetylsalicylic acid. From all possible impurities as stated by the European Pharmacopoeia (Ph. Eur.) (19), only 4-hydroxybenzoic acid contains more OH groups than salicylic acid. 4-hydroxybenzioc acid contains three hydroxyl groups whereas salicylic acid contains two hydroxyl groups. NH groups also absorb radiation around the same wavenumber as OH groups, but in the reagents, there was no compound present containing nitrogen atoms, therefore the option of a molecule present with N-H groups can be excluded. Thus, it was possible that 4-hydroxybenzoic acid was also formed in the in-batch production of aspirin.

UV/Vis spectroscopy

Sample analysis

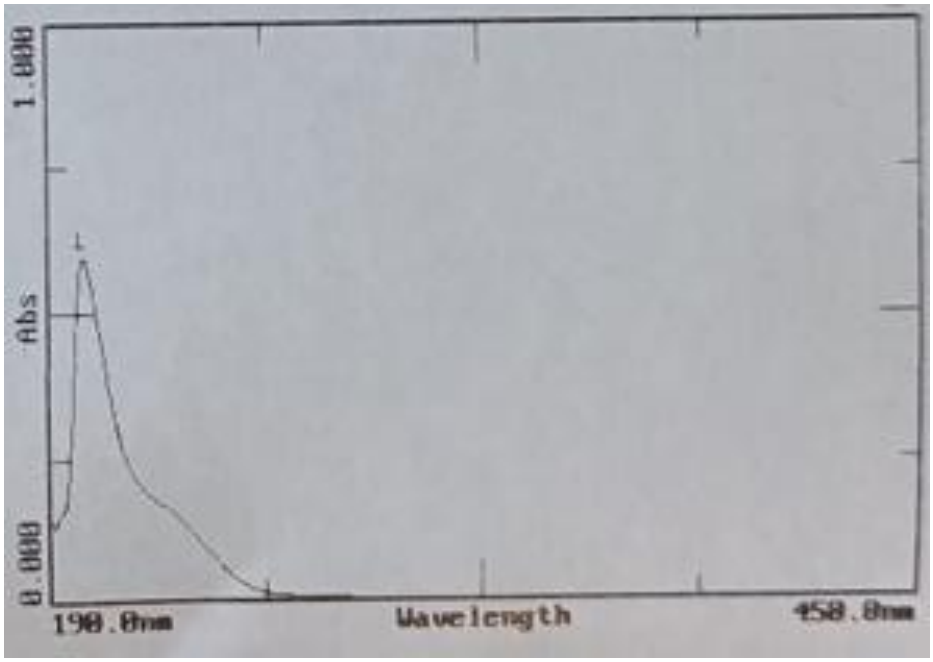


Figure 13. UV spectrum of in batch produced sample on a print. The Y-axis represent the absorption which has no unit. The X-axis represents the wavelength of the radiation used in nanometre.

Figure 13 shows the UV spectrum of the sample that was produced in batch. Appendix 2 contains the spectra of salicylic acid and acetylsalicylic acid, which are used to compare with the sample spectrum.

Figure 13 shows that the sample absorption is the highest at a wavelength of 201 nm, where the absorption reaches 0.591. Furthermore, the absorption increases between wavelength 190 and 201 nm and decreases between 201 and 250 nm.

The peak in the sample spectrum at 201 nm does not provide sufficient information to make a distinction in whether the sample contains ASA or SA. The spectra in appendix 2 show that the absorption is the highest around 200 - 203 nm for both aspirin and salicylic acid. A distinction can be made when reviewing the second and third peak in the spectrum of salicylic acid, as these two peaks are absent in the spectrum of acetylsalicylic acid. The second peak in the spectrum of salicylic acid is present at 230 nm and the third peak is present at 290 - 300 nm. The sample spectrum does not have peak at 230 nm or 290 - 300 nm. With regard to this information, it can be concluded that the sample contains acetylsalicylic acid.

HPLC analysis

Eluent selection

Table 4. Important parameters for determining the best eluent. Data used to determine the parameters is stated in appendix 3.

Eluent (v%)	Highest retention time (min)	Lowest plate number	Resolution
H_2O/ACN 40/60	1.7	1341	1.86
H_2O/ACN 60/40	2.807	2227	3.47
H_2O/ACN 70/30	4.653	2999	5.71
H_2O/ACN 80/20	10.427	1679	6.45

Table 4 shows all important parameters to determine the optimum eluent in order to separate salicylic acid from acetylsalicylic acid using an HPLC. In all four eluents, a mixture of salicylic acid and acetylsalicylic acid was analysed. The highest retention time refers to the peak in the chromatogram with the highest retention time, which was salicylic acid in all four eluents. The lowest plate number is an index to determine the column efficiency, and was determined for all peaks in the chromatograms. The resolution determines whether the two components were separated in the column, complete separation is considered at resolutions of 2 or higher.

The four parameters were used to select the best eluent for the separation of acetylsalicylic acid and salicylic acid.

The eluent H_2O/ACN 40/60 v% does provide short retention times (max. 1.7 minutes), but the resolution is not higher than 2, therefore the ASA and SA were not separated. The lowest plate number (1341) is the lowest of all four eluents as well, therefore this eluent was not selected as optimal.

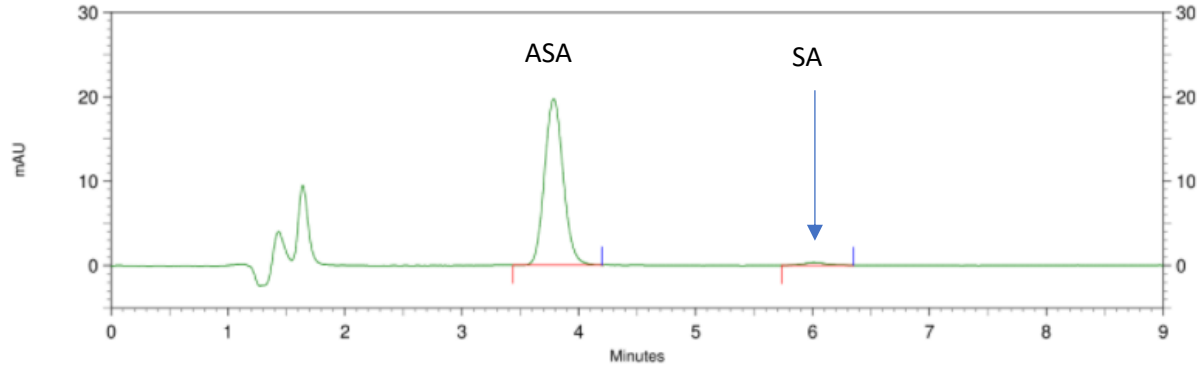
The eluent H_2O/ACN 60/40 v% does have a resolution higher than 2, the plate number is 2227 which is decent, and the highest retention time is 2.807 minutes. Which makes this eluent optional for the analysis of ASA and SA.

The eluent H_2O/ACN 70/30 v% has high resolution (5.71), plate number (2999) is the highest of all four eluents, and the retention time (max. 4.653 minutes) will not be a problem for the analysis. Which makes this eluent an excellent choice for the analysis of ASA and SA.

The eluent H_2O/ACN 80/20 v% has the highest resolution of all four eluents (6.45), the plate number is the second lowest of all four (1679), the highest retention time is the highest of all four (10.427 minutes). The retention time is unnecessarily high with this eluent, plate numbers are higher in two other eluents with good retention time, therefore this eluent was not selected for analysis of ASA and SA.

The eluent H_2O/ACN 70/30 v% was selected as the best eluent for the analysis of acetylsalicylic acid and salicylic acid.

Sample analysis



DAD-CH1 237 nm Results

Retention Time	Area	Height
3.787	877453	78871
6.013	20168	1314

Figure 14. HPLC chromatogram of the in batch produced sample with table containing exact retention time, area under the curve and height. Y-axis represents the intensity in milli absorbance units (mAU). The X-axis represents the retention time in minutes.

Figure 14 shows that the sample chromatogram contains two peaks. The first peak has a retention time of 3.787 minutes, an area under the curve of 877,453 mAU*min and a height of 78,871. The second peak has a retention time of 6.013 minutes, an area under de curve of 20,168 mAU*min and a height of 1,314.

Figure 14 indicates that the majority of the in batch produced product consist of acetylsalicylic acid, and that there was also a small amount of salicylic acid still present in the product. The first peak corresponds to presence of acetylsalicylic acid as this compound elutes faster on a reversed phase HPLC than salicylic acid, because the logP value of acetylsalicylic acid (1.24) is lower than that of salicylic acid (2.26). This means that acetylsalicylic acid will adhere to the hydrophobic column shorter than salicylic acid, and thus elute faster.

The retention times in Figure 14 had shifted compared to the eluent selection chromatogram in appendix 3.3. This can be explained by the impurities that were present on the column during the analysis of the batch sample. The impurities stuck on the column might interact with the contents of the sample thereby changing the retention times of the contents in the sample. Interaction of the impurities with the salicylic acid or aspirin could occur by formation of physical barrier by the impurities, resulting in longer times for the aspirin or salicylic acid to pass the column. Impurities on the column could also cause hydrophobic interactions between the aspirin and salicylic acid and the hydrophobic impurities, this also results in an extended time on the column for aspirin and salicylic acid.

The impurities were removed from the column by reversing the column in the HPLC device and applying a mobile phase in gradient concentrations of ACN and water to the reversed column. In the gradient analysis, water concentration changed from 100% to 0%, while ACN concentration changed from 0% to 100%. This created a strong elution of impurities from the column.

Determining the purity

In order to analyse the contents of the sample both qualitatively and quantitatively, a calibration curve was made that is presented here. The data used to create the calibration curve are stated in appendix 4.

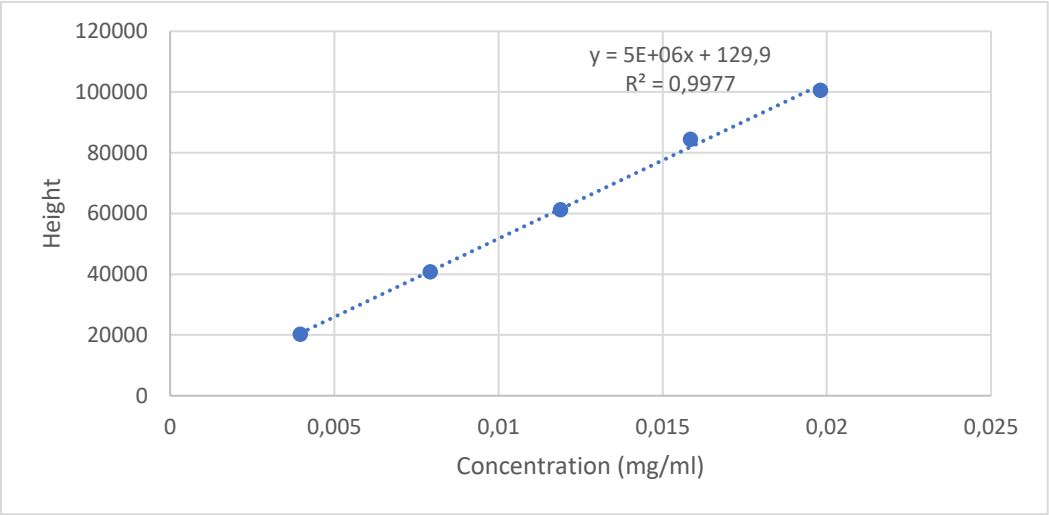


Figure 15. Calibration curve used to quantify ASA in the batch sample. The dotted line represents a trendline best corresponding to all individual measurements. The R^2 is stated in the graph and displays the proportion of variability between the measurements. The equation of the trendline is shown in the graph and is used to calculated the concentration in the sample. The Y-axis represents height which has no unit. The X-axis represents concentration of ASA in mg/mL. This calibration curve is created with data stated in appendix 4.

Figure 14 shows the calibration curve used to determine the concentration of acetylsalicylic acid in the batch sample. The trendline of the equation is described as $y = 5159874 * x + 129.9$.

The height of the ASA peak in the sample was 78871. In order to calculate a concentration from this, the trendline equation first has to be rebuild: $x = {(y - 129.9)}/{5159874}$

Filling in the height as Y into the equation gives: $x = {(78871 - 129.9)}/{5159874} = 0.0153\ mg/ml$

If the only content of the weighed powder was ASA than the expected concentration of ASA in the sample would be 0.0203 mg/mL. The measured sample contained 0.0153 mg/mL. This indicates that the product has a purity of 75.2%.

$$Purity\ (\%) = \left({measured\ [ASA]}/{expected\ [ASA]}\right) * 100\% = \left({0.0153}/{0.0203}\right) * 100\% = 75.2\%$$

The European Pharmacopoeia states that the content of aspirin should be 99.5% to 101.0% (19). The sample's purity does not meet the requirements of aspirin as stated by the Eur. Ph.

Droplet generation

Water with 0.2% tween 20 as continuous phase

Figure 16 shows a microscopic view of the PDMS chip. The water phase contained ultrapure (UP) water with 0.2% tween 20 and a trace of blue food dye. The oil phase was mineral oil without any further contents. The water phase was coloured blue and arrived at the junction from the top and bottom. The oil phase arrived at the left side of the junction which is not visible in this figure. Figure 16 shows that the droplets were formed from the water phase. The upper and lower water channels flowed alternatingly, meaning that a droplet formed out of the upper channel was followed by a droplet formed out of the lower channel. This process repeated itself forming a cluster of water droplets moving through the middle of the PDMS channels. Changing the flow rates of the different phases resulted at all times in water droplets being formed in the oil phase.

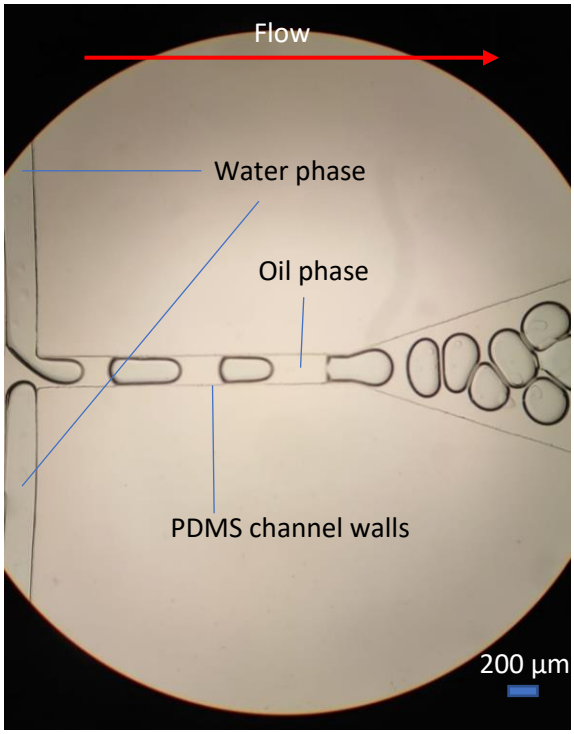


Figure 16. Microscopic view of the PDMS chip. The visible section contains the junction where the dispersed phase meets the continuous phase, but also the channel following the junction up. The droplets are made from the water phase which was coloured slightly blue. The right lower corner contains a bar indicating the scale. The different phases and flow direction are assigned in the figure.

Salicylic acid but also aspirin both dissolve better in the oil phase, thus it was important to create oil droplets in water as water droplets in oil would not contain the starting materials nor products of the reaction.

The water droplets are nicely formed and barely fuse together due to addition of the 0.2% tween 20. The critical micelle concentration (CMC) of tween 20 is 0.06 - 0.07%, indicating that a concentration higher than the CMC was achieved during the experiment. This means that micelles of tween 20 are formed in the continuous phase. The micelles have a hydrophobic inside and hydrophilic outside. Once the water with the micelles comes into contact with the ethyl acetate, it is theoretically possible that the ethyl acetate is stored in the inside of a micelle, creating a microdroplet of ethyl acetate inside the water.

Figure 16 indicates that no oil droplets are formed, which was desired for performing the reaction on the chip. Part of the explanation for this was that the PDMS used to produce the chip was hydrophobic and therefore surface of the chanaaanels in the chip are hydrophobic. The surface of the channels thereby interact better with the oil phase, resulting in the oil phase moving along the sides of the chip and the water phase moving through the middle of the chip. This does not allow the oil phase to be dispersed in the water phase, and therefore the oil phase cannot form droplets.

A change in flow rate changed the rate at which the droplets were formed. By changing the flow rates of the different phases, water droplets were created at all times, indicating that flow rate does not influence which phase was dispersed and forms droplets.

PDMS chip coated with polyvinyl alcohol

In order to alter the hydrophobicity of the surface so that the surface of the PDMS channels becomes more hydrophilic, the chips were coated with polyvinyl alcohol (PVA). The water phase consists of UP water with 0.2% tween 20 and a trace of blue food dye. The oil phase consists of mineral oil without any contents.

Figure 17 shows that the water phase still forms droplets and that these droplets adhere to the surface of the channels. The droplets are not spherical, and stick along the sides of the channels and slowly move forward. The oil phase moved through the middle of the channels without forming any droplets.

Figure 17 indicates that no oil in water droplets are made when using PVA to coat the surface of the channels and tween 20 as a surfactant in the continuous phase. Using PVA to coat the surface of the channels was sufficient for the water phase to flow along the side and the oil phase to flow through the middle. Inititially it was thought that the PVA coating was not enough to fully change the polarity of the channel surfaces.

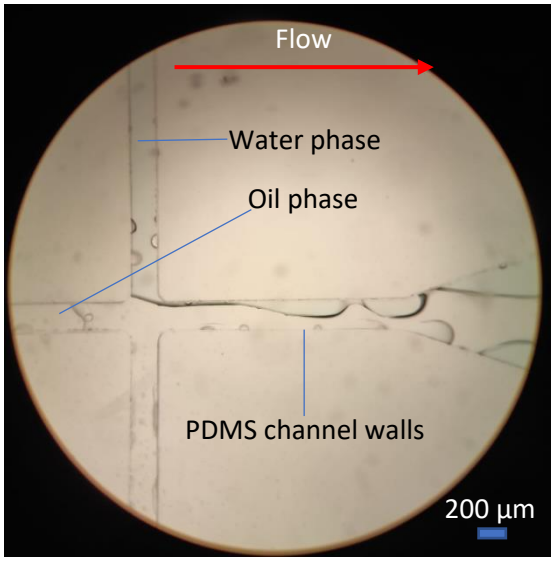


Figure 17. Microscopic view of the junction area of the PDMS chip. The continuous phase meets the dispersed phase at the junction. Prior to the experiment, the chip was coated with PVA to increase the hydrophilicity of the surface of the channels. Different phases and flow direction are assigned in the figure.

PDMS chip prepared with oxygen plasma and UP-water

Initially, it was thought that the PVA coating did not work as intended, because the water phase formed droplets and these droplets moved along the sides of the PDMS channels. Therefore another method to change the polarity of the channel surface was used prior to the experiment. The chip was placed into the oxygen plasma cleaner for an extra 10 minutes after manufacturing the chip. After these 10 minutes, the chip was filled with UP-water. The continuous phase consists of UP-water. The disperse phase consists of mineral oil.

Figure 18 shows that neither the water phase nor the oil phase formed droplets and two streams of the different phases were created. The water phase flowed along the sides of the PDMS channels. The oil phase flowed through the middle of the PDMS channels.

Figure 18 illustrates that changing the polarity of the channels was not sufficient to produce oil droplets in water. Even though the channel looks very hydrophilic, because the water phase completely covers the channel surface. Instead of one phase forming droplets, no droplets are formed, this was most likely due to absence of tween 20.

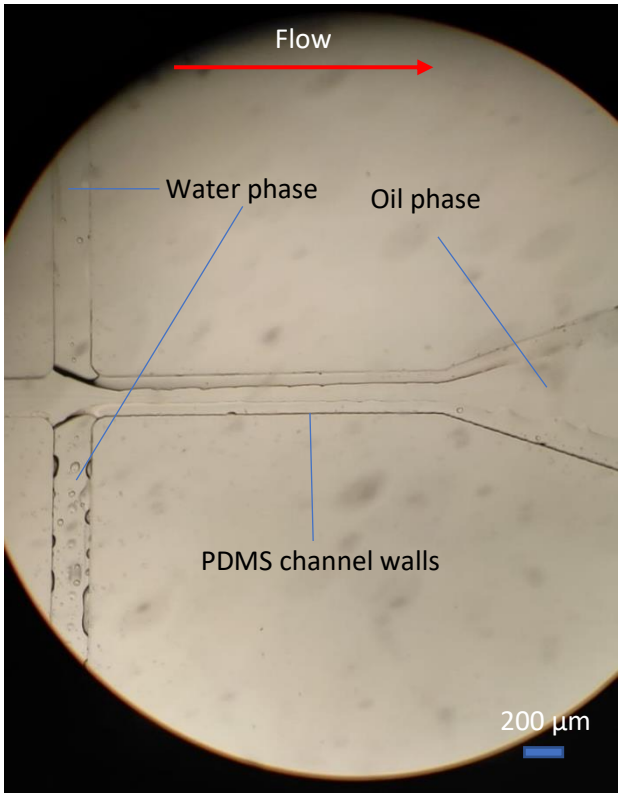


Figure 18. Microscopic view of the junction area of the PDMS chip. At this junction, the continuous phase meets the disperse phase. Prior to the experiment, the chip was placed in the oxygen plasma cleaner for 10 minutes and filled with UP-water after the 10 minutes. Different phases and flow direction are assigned in the figure.

Using mineral oil as a solvent for the reaction of salicylic acid with acetic anhydride

It was investigated whether mineral oil could be used as solvent for the reaction on the chip. The boiling range of mineral oil is 260 - 427 ˚C (20), which provides a possibility to let the starting compounds react on the chip at 95˚C. This was the same temperature used in the in-batch production. Ethyl acetate has a boiling point of 77˚C, which restricts the reaction on the chip, as the gaseous ethyl acetate on the chip was not desired.

Another aspect which is important for the reaction on the chip is whether salicylic acid is soluble in mineral oil. This was therefore tested in the lab.

122.5 mL of mineral oil was needed to dissolve 10 mg of salicylic acid. This means that a maximum concentration of 81.6 µg/mL can be achieved when salicylic acid was dissolved in mineral oil. This concentration was much lower than in ethyl acetate where concentrations of 100 mg/mL can be used. The reaction rate is also dependent on the concentration of the reagents in the solvent, therefore it was decided that ethyl acetate will be used for the reaction on the chip, and that the temperature needs to be lower than 77˚C to avoid evaporation of the ethyl acetate.

1% Polyvinyl alcohol in the continuous phase

Li et al. (21) described how 1% polyvinyl alcohol (PVA) can increase the viscosity of hydrophilic solvents, thereby contributing to the formation of oil droplets in water. In this experiment, the channel surfaces were made hydrophilic with the oxygen cleaner, and 1% PVA solution in UP-water was used as the continuous phase. The disperse phase consists of ethyl acetate containing acetic anhydride, salicylic acid and 1% sulfuric acid.

From this moment on, the continuous phase was 1% PVA in UP-water and the disperse phase was ethyl acetate containing the reagents. Previously, the terms water phase and oil phase were used because in those cases the disperse phase (oil) was not dispersed in the continuous phase (water).

Figure 19 and 13 shows that the ethyl acetate forms nice and spherical droplets in the continuous phase. The droplets remain the same size directly after they are produced, but shrink after about 15 minutes on the chip. Another important observation was made, namely that the droplets change colour as they flow through the channel.

Increasing the viscosity of the continuous phase together with the hydrophilicity of the channel surfaces turned out be of great importance when producing oil droplets in water.

The formula for capillary number in the theory describes that an increased viscosity will lead to an increase in the capillary number, which determines which phase will form droplets. Addition of PVA into the continuous phase increases the viscosity of the continuous phase thereby increasing the capillary number, making it more likely for the disperse phase to form droplets.

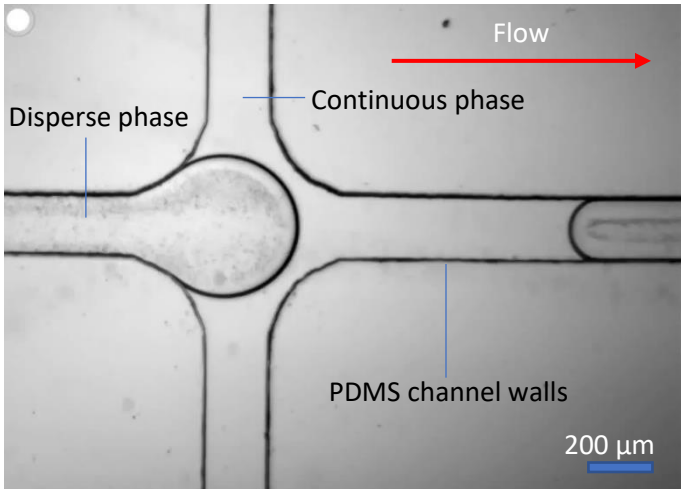


Figure 19. Microscopic view of the junction area of the PDMS chip. At the junction the continuous phase meets the disperse phase. The continuous phase consists of 1% PVA in UP-water. The disperse phase consists of ethyl acetate containing the reagents for the reaction of aspirin. Different phases and flow direction are assigned in the figure.

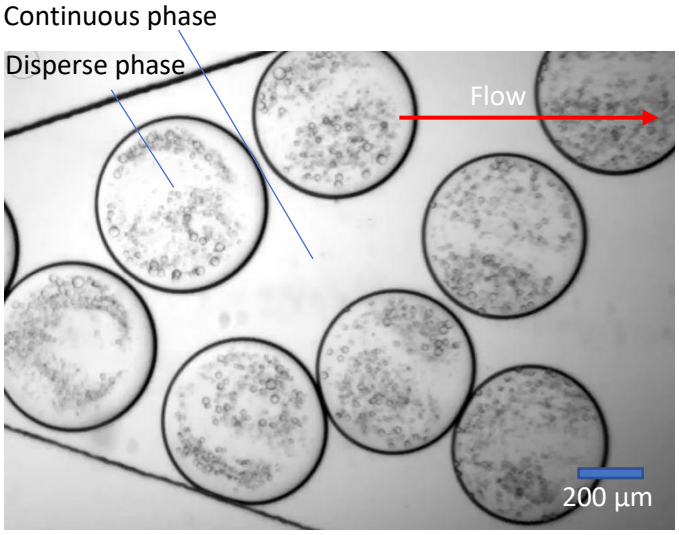


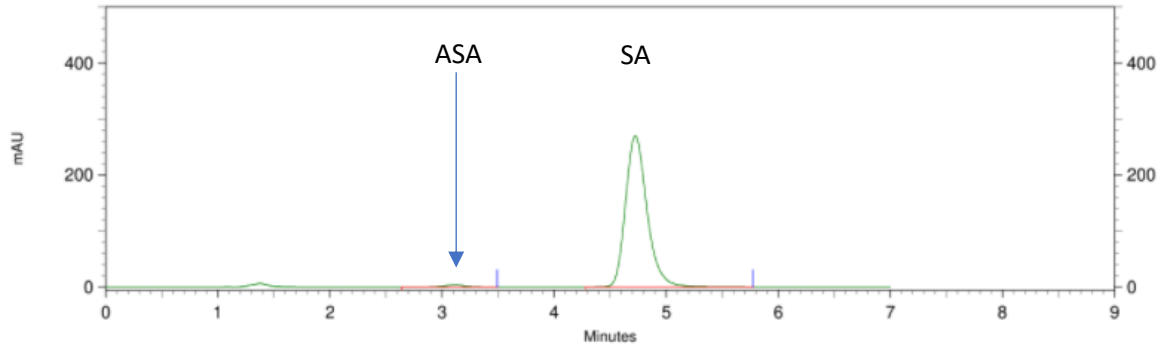
Figure 20. Microscopic view of the channel directly after the junction on the PDMS chip. The droplets visible are ethyl acetate surrounded by the continuous phase, which was 1% PVA in UP-water. Different phases and flow direction are assigned in the figure.

A possible explanation for the shrinkage of the droplets after 15 minutes is that the ethyl acetate partially evaporates on the chip. This can also be witnessed on the chip as gaseous bubbles are formed that do not originate from the inlets in the chip. PDMS is permeable to gases, so it could be possible that the gaseous ethyl acetate moves through the PDMS and forms a gas bubble elsewhere in the channels. In order for ethyl acetate to evaporate as minimum as possible a water bath temperature lower than 60 ˚C was set for the next experiments.

A possible explanation for the change of colour in the droplets is that the salicylic acid or aspirin starts to crystalize as the droplets flow through the chip.

Aspirin production in droplets using microfluidics

15 minutes reaction time



DAD-CH1 237 nm Results

Retention Time	Area	Height
3.120	182226	14833
4.727	14376937	1078344

Figure 21. Chromatogram of sample which was 15 minutes on the PDMS chip. The sample was diluted 1000 times prior to injection into the HPLC. The Y-axis represents the intensity in milli absorbance units (mAU). The X-axis represents the retention time in minutes. The figure also contains a table where the retention times, areas under the curve and heights of the peaks are stated.

Figure 21 shows two peaks. The first peak is visible at a retention time of 3.120 minutes, has an area under curve of 182,226 mAU*min and a height of 14,833. The second peak is visible at a retention time of 4.727 minutes, has an area under the curve of 14,376,937 mAU*min and a height of 1,078,344.

The first peak corresponds to aspirin, as aspirin has a lower logP value (1.24) than salicylic acid (2.26). In addition, the chromatogram of sample containing pure aspirin which is visible in appendix 5.1, has a retention time of around 3.1 minutes.

The second peak corresponds to salicylic acid, as this has a higher logP value than aspirin. In addition, the chromatogram of sample containing pure salicylic acid which is visible in appendix 5.2, has a retention time of around 4.7 minutes.

In order to calculate the concentration of aspirin in the sample from the chip, a calibration curve was made. This calibration curve is visible in appendix 5.3. The height of the peak corresponding to aspirin can be used together with the equation of the trendline in order to calculate the concentration in the sample. The equation of the trendline is described as:

$$y = 6053573 * x - 234.29$$

In order to calculate a concentration (x) with the height (y) the equation needs to be rewritten:

$$x = {(y + 234.29)}/{6053573}$$

Filling in the height of the aspirin peak from Figure 21 as Y, which is 14833 gives:

$$x = {(14833 + 234.29)}/{6053573} = 0.002489\ mg/mL$$

This concentration is compared to a theoretical maximum concentration of 0.1304 mg/mL. Appendix 5.4 contains the calculation and explanation of how this maximum concentration was determined. Overall, a recovery percentage can be calculated:

$$Recovery\ ASA\ (\%) = {[ASA]_{measured}\ \left(\frac{mg}{mL}\right)}\Big/{[ASA]_{max}\ \left(\frac{mg}{mL}\right)} * 100\% = {0.002489}/{0.1304} * 100$$

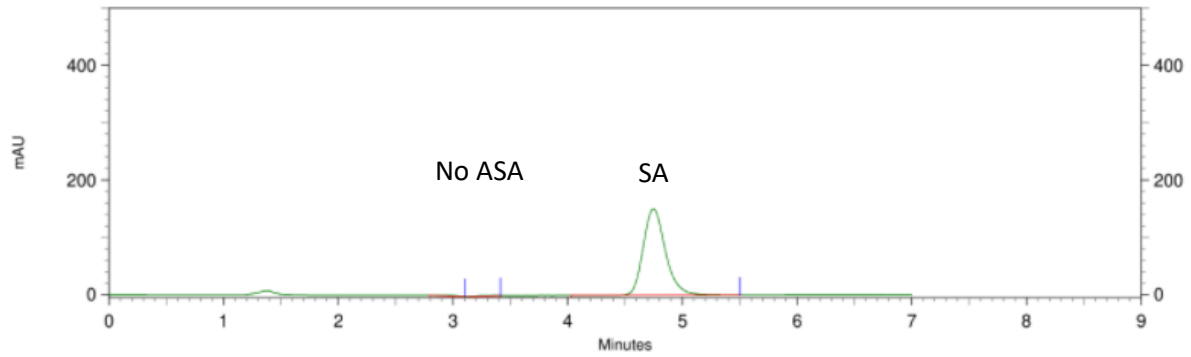
$$= 1.9\%$$

This means that 1.9% of the salicylic acid that was added in the disperse phase has reacted and formed aspirin in 15 minutes.

The low conversion is most likely explainable by the fact that some of the contents in the dispersed phase started to crystalize on the chip before they were collected in the collection vial. This also blocked the channels inside the chip and the droplets remaining on the chip could not be collected. Blockage of the channels is not an explanation for the low conversion percentage but does give extra bias, as the extra droplets could have served for increased amounts of droplets in the collection vial thereby decreasing the variability between the individual concentration in each droplet and the average concentration of all droplets on the chip.

Another explanation for the low conversion is that the temperature which was 57 ˚C was too low for the reaction to complete after 15 minutes. The in-batch production of aspirin takes 15 minutes but was heated at 95 ˚C. Heating at 57 ˚C on the chip will result in a slower reaction and thus a lower conversion of salicylic acid.

2 hours reaction time



DAD-CH1 237 nm Results

Retention Time	Area	Height
2.940	48242	4234
3.373	28975	1110
4.747	8140651	603475

Figure 22. Chromatogram of sample which was 2 hours on the PDMS chip. The sample was diluted 1000 times prior to injection into the HPLC. The Y-axis represents the intensity in milli absorbance units (mAU). The X-axis represents the retention time in minutes. The figure also contains a table where the retention times, areas under the curve and heights of the peaks are stated.

Due to a low conversion rate in the reaction on the chip for 15 minutes, it was tested whether 2 hours reaction time on the chip would lead to a higher conversion rate.

Figure 22 shows only one peak. The retention time of this peak was 4.747 minutes and corresponds to salicylic acid.

Figure 22 illustrates that aspirin was not formed after 2 hours reaction time on the chip. In the chromatogram, there was no peak at the retention time where aspirin peaks should be if aspirin was present. An explanation for this is that crystals were formed on the chip, these crystals did not reach the collection vial and therefore were not present in this HPLC sample. The crystals that were formed on the chip are visible in Figure 23.

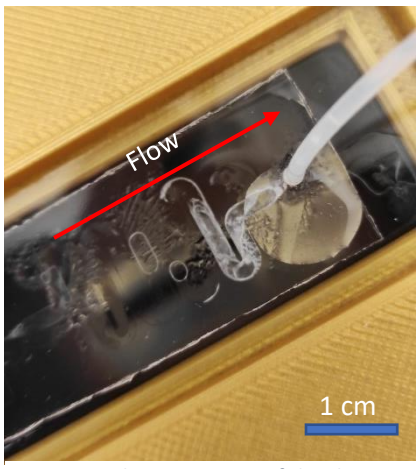
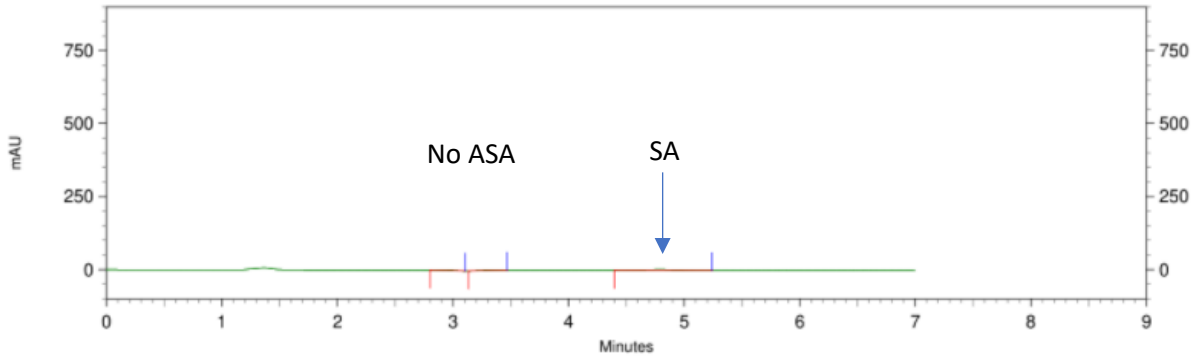


Figure 23. Close-up image of the chip during the two-hour reaction. White crystals are visible close to the outlet of the chip.

Analysis of the crystals remaining on the chip

The contents of the crystals that remained on the chip were analysis by dissolving them in acetonitrile (ACN) and diluting 1,000 times prior to injection in the HPLC.



DAD-CH1 237 nm Results

Retention Time	Area	Height
2.833	59488	1386
3.367	60652	3218
4.793	174437	11499

Figure 24. Chromatogram of sample made from crystals that remained on the chip. The sample was diluted 1,000 times prior to injection into the HPLC. The Y-axis represents the intensity in milli absorbance units (mAU). The X-axis represents the retention time in minutes. The figure also contains a table where the retention times, areas under the curve and heights of the peaks are stated.

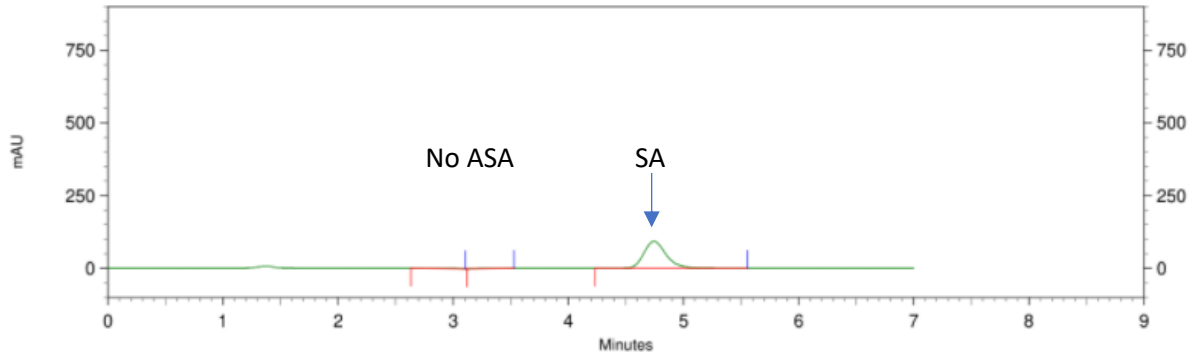
Figure 24 shows a peak at 4.793 minutes and no peak around 3.1 minutes.

The peaks seem to be very low, therefore it was most likely unnecessary to dilute this sample an extra 1,000 times.

Figure 24 does indicate that the contents of the crystals on the chip were no different than the contents of the sample collected after 2 hours. As both results show a peak for salicylic acid and no peak for aspirin. This indicates that salicylic acid was the compound responsible for crystallisation on the chip.

3 minutes reaction time

In order to exclude the possibility that aspirin was produced on the chip faster than 15 minutes and broken down already at 2 hours, it was tested whether aspirin was produced on the chip after 3 minutes.



DAD-CH1 237 nm Results

Retention Time	Area	Height
2.953	80752	5505
3.460	53124	1334
4.747	4986985	368343

Figure 25. Chromatogram of sample which was on the chip for 3 minutes. The sample was diluted 1000 times prior to injection into the HPLC. The Y-axis represents the intensity in milli absorbance units (mAU). The X-axis represents the retention time in minutes. The figure also contains a table where the retention times, areas under the curve and heights of the peaks are stated.

Figure 25 shows a peak at 4.747 minutes and no peak at around 3.1 minutes.

Figure 25 indicates that there was no aspirin formed when the droplets were on the chip for 3 minutes.

Most likely the temperature of the water bath at 2 hours and 3 minutes reaction time was too low for the reaction to occur. The temperature was set to 53 ˚C after crystals and gaseous ethyl acetate were visualized on the chip with reaction time 15 minutes. In this experiment, the highest amount of aspirin was produced on the chip at 15 minutes reaction time.

During the in-batch synthesis of aspirin, the highest yield can be achieved after reacting for 15 minutes at 95 ˚C. The reaction in the microdroplet was performed at 53 ˚C for 15 minutes. This difference in temperature can explain why a lower yield of aspirin was found in the microdroplets, as the reaction rate is directly dependent on the temperature of the reaction. Changing the time, the microdroplets undergo the reaction would normally increase the yield, as the lower reaction rate is then compensated by the longer reaction time. In the reaction on the chip that took 2 hours, it was clearly visible that ethyl acetate had evaporated and crystals were formed on the chip. As the crystals blocked the flow inside the microchannels, this could be a potential reason for the low yield during this experiment. If the ethyl acetate had not evaporated and no crystals were formed on the chip, it is expected that a higher yield can be achieved than seen in the in-batch synthesis. This can be achieved by using a disperse phase with a higher boiling point, preferably higher than 120 ˚C, as the reaction in the microdroplets can then be performed at 95˚C without any evaporation of the disperse phase.

Conclusion

A simple, yet successful method to synthesize aspirin both in-batch and in microdroplets is described in this article. The in-batch production of aspirin had a yield of 95,7% and the synthesized aspirin had a purity of 75,2%. A microfluidic device able to produce oil-in-water droplets was fabricated using photolithography, whereby the polarity of the PDMS was changed into hydrophilic using the oxygen plasma. A 1% polyvinyl alcohol solution was used as continuous phase and this ensured formation of spherical oil droplets from the disperse phase. This article demonstrates that this microfluidic device can be used to create microreactors, in which aspirin can be synthesized from salicylic acid. The production in the microreactors had a yield of 1,9% and no purification steps were performed. In this research, the in-batch synthesis was more effective in producing aspirin. For future research it is recommended to use another disperse phase, preferably a disperse phase with a boiling point higher than 120 ˚C as the reaction can then be performed at 95 ˚C without any evaporation. Another criterion for the disperse phase is that salicylic acid and aspirin can be well dissolved in the disperse phase and it has to be compatible with the material used in the microfluidic device. This will most likely increase the yield of the production of aspirin in the microdroplets. Another recommendation is to investigate whether a gas could be used as continuous phase. This will allow easier analysis of the contents of the microdroplet as the microdroplet is better extractable from the collection vial.

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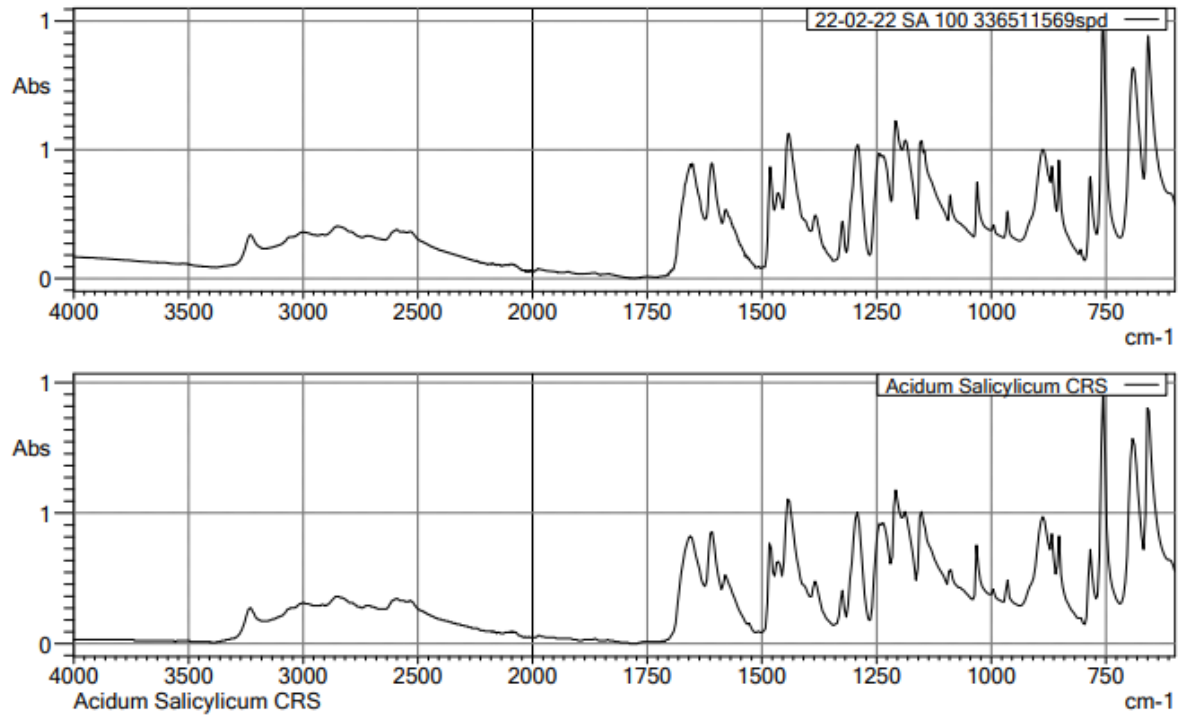
Appendix

Appendix 1: IR spectra of mixtures of salicylic acid and acetylsalicylic acid compared to literature

Appendix 1.1: IR spectrum of salicylic acid compared to spectra in literature

SHIMADZU

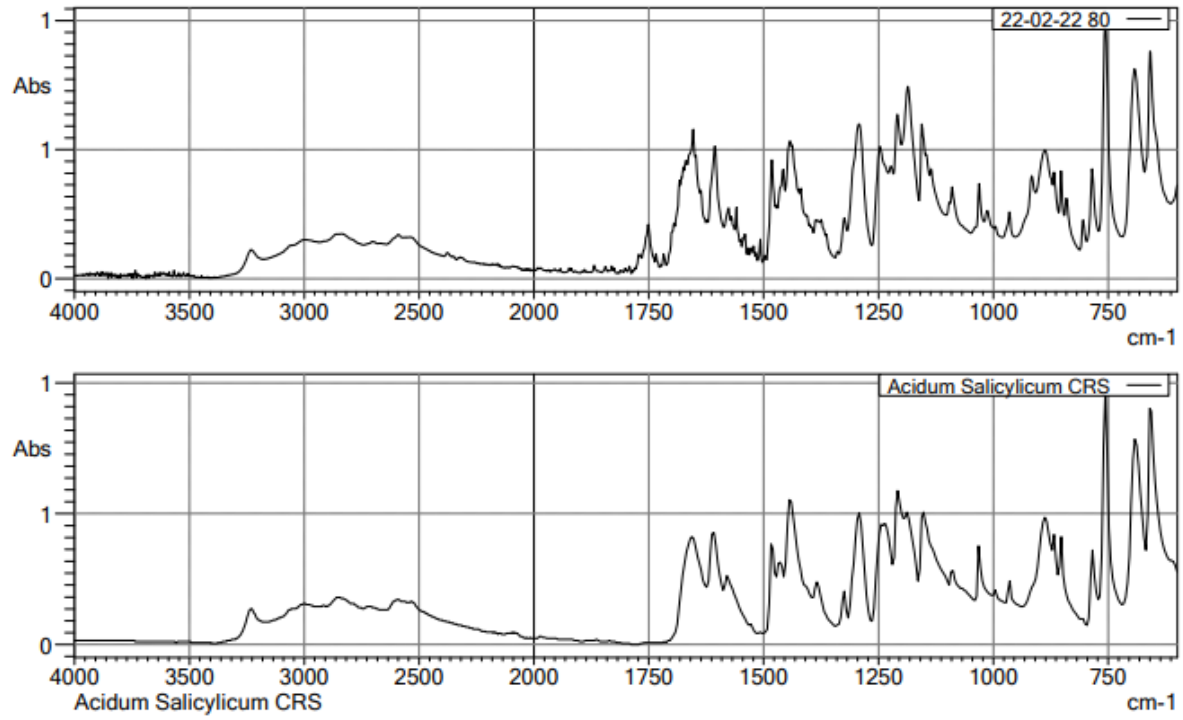


C:¥LabSolutions¥LabSolutionsIR¥Data¥22-02-22 SA 100 336511569spd.ispd

	Score	Library	Name	Comment
1	992	15 - RUG	Acidum Salicylicum CRS	Acidum Salicylicum CRS
2	962	76 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR
3	962	400 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR
4	962	79 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR
5	961	69 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM SALICYLICUM (180) - PH.EUR. - BUFA - 97D11FR	ACIDUM SALICYLICUM (180) - PH.EUR. - BUFA - 97D11FR

Appendix 1.2: IR spectrum of a mixture of 80% salicylic acid and 20% acetylsalicylic acid compared to spectra in literature

SHIMADZU

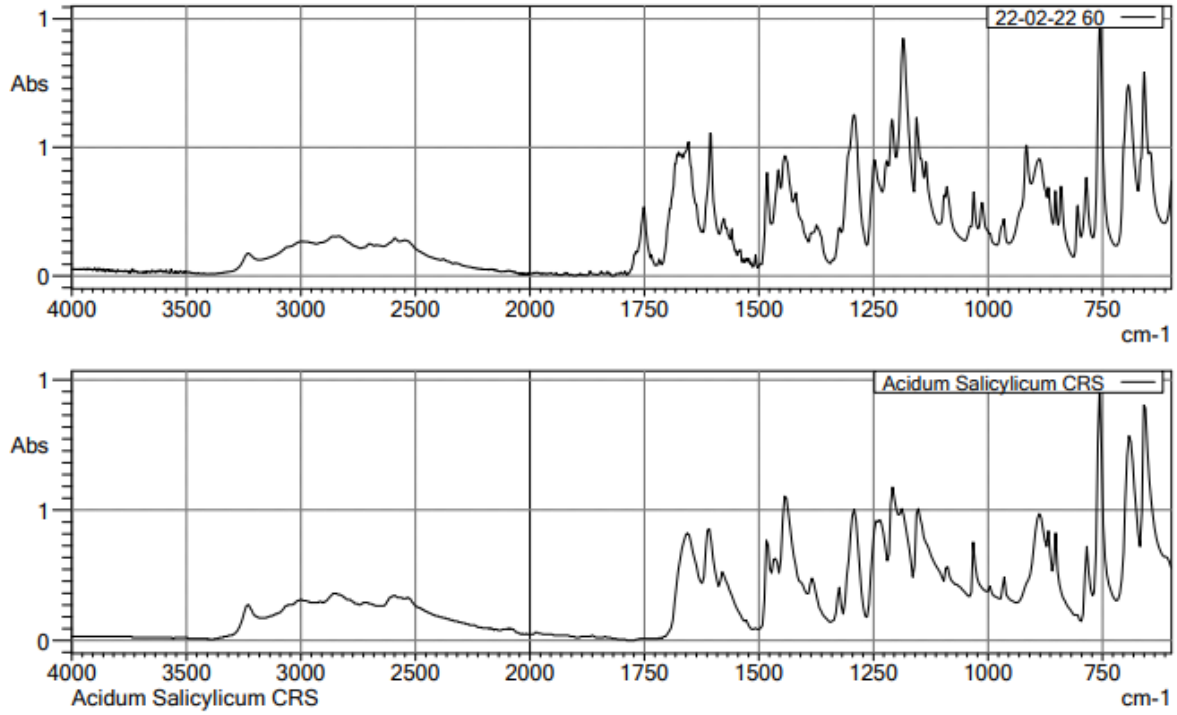


C:¥LabSolutions¥LabSolutionsIR¥Data¥22-02-22 80.ispd

	Score	Library	Name	Comment
1	970	15 - RUG	Acidum Salicylicum CRS	Acidum Salicylicum CRS
2	937	76 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR
3	937	400 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR
4	937	79 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR
5	934	69 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM SALICYLICUM (180) - PH.EUR. - BUFA - 97D11FR	ACIDUM SALICYLICUM (180) - PH.EUR. - BUFA - 97D11FR

Appendix 1.3: IR spectrum of a mixture of 60% salicylic acid and 40% acetylsalicylic acid compared to spectra in literature

SHIMADZU

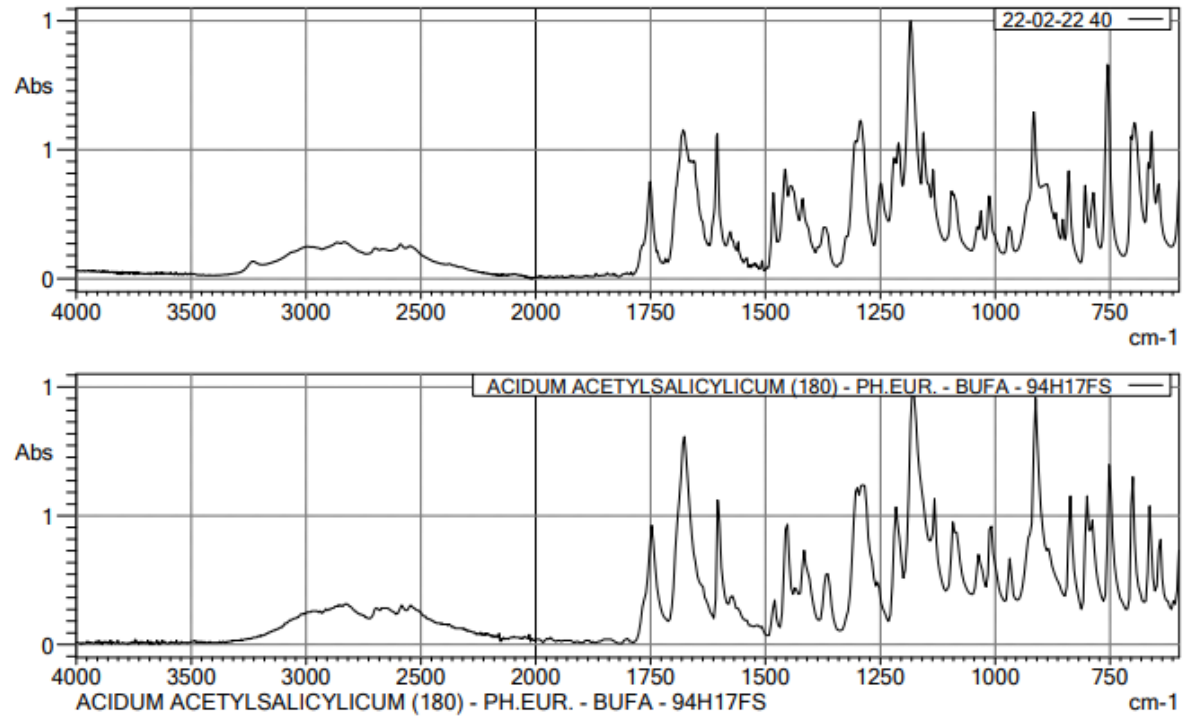


C:¥LabSolutions¥LabSolutionsIR¥Data¥22-02-22 60.ispd

	Score	Library	Name	Comment
1	925	15 - RUG	Acidum Salicylicum CRS	Acidum Salicylicum CRS
2	887	76 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR
3	887	400 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR
4	887	79 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR	ACIDUM SALICYLICUM (90) - PH.EUR. - BUFA - 97D16FR
5	885	69 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM SALICYLICUM (180) - PH.EUR. - BUFA - 97D11FR	ACIDUM SALICYLICUM (180) - PH.EUR. - BUFA - 97D11FR

Appendix 1.4: IR spectrum of a mixture of 40% salicylic acid and 60% acetylsalicylic acid compared to spectra in literature

SHIMADZU

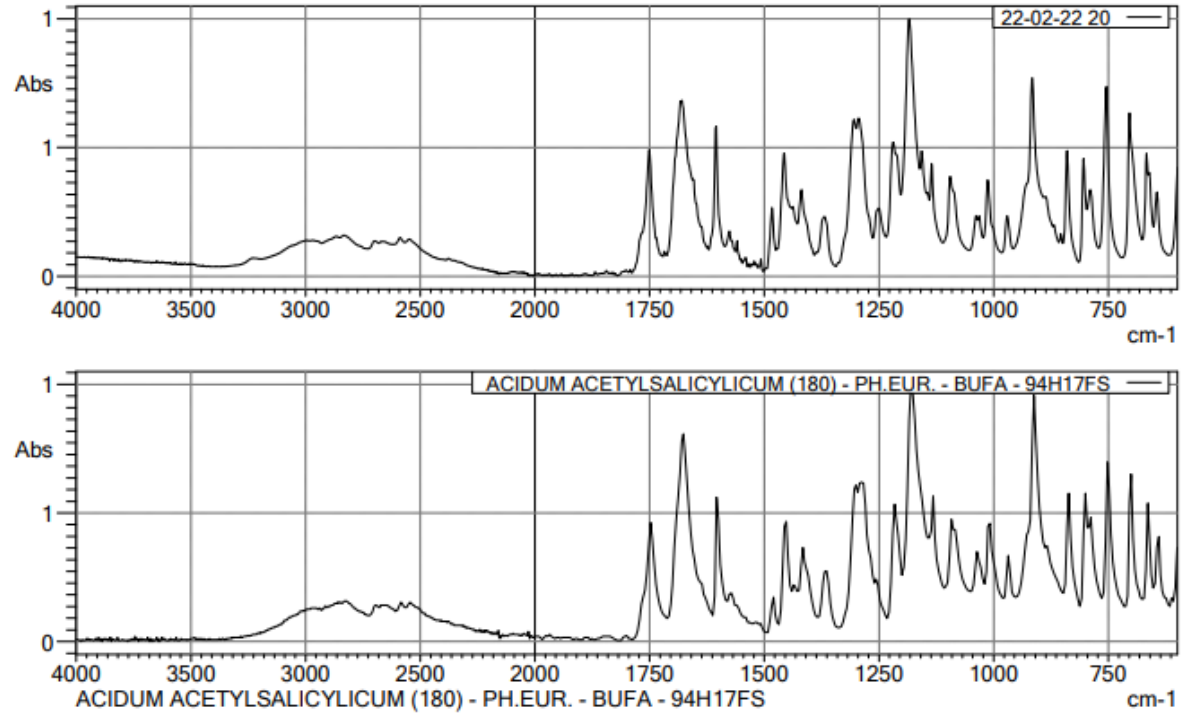


C:¥LabSolutions¥LabSolutionsIR¥Data¥22-02-22 40.ispd

	Score	Library	Name	Comment
1	894	140 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS
2	894	465 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS
3	894	144 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS
4	818	15 - RUG	Acidum Salicylicum CRS	Acidum Salicylicum CRS

Appendix 1.5: IR spectrum of a mixture of 20% salicylic acid and 80% acetylsalicylic acid compared to spectra in literature

SHIMADZU

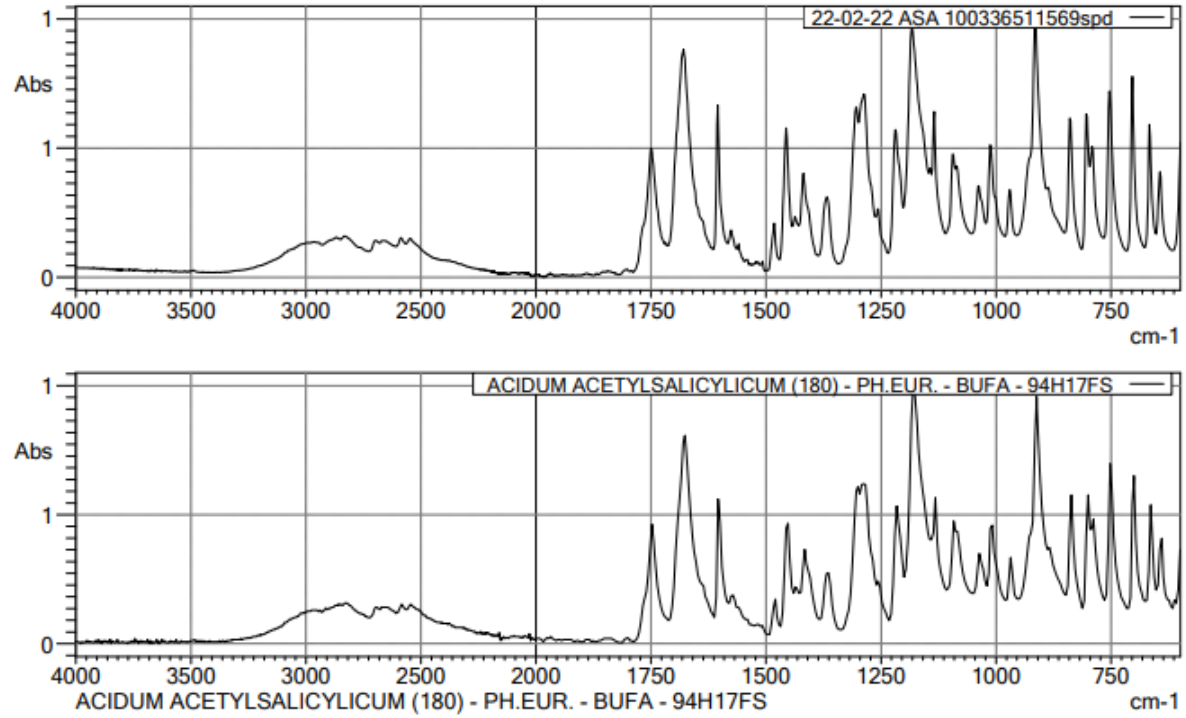


C:¥LabSolutions¥LabSolutionsIR¥Data¥22-02-22 20.ispd

	Score	Library	Name	Comment
1	903	140 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS
2	903	465 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS
3	903	144 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS

Appendix 1.6: IR spectrum of acetylsalicylic acid compared to spectra in literature

SHIMADZU

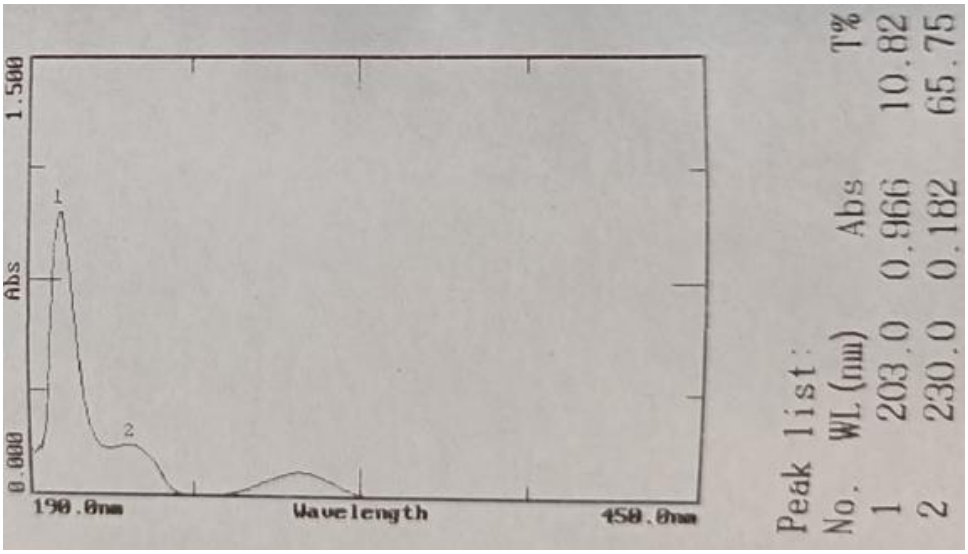


C:¥LabSolutions¥LabSolutionsIR¥Data¥22-02-22 ASA 100336511569spd.ispd

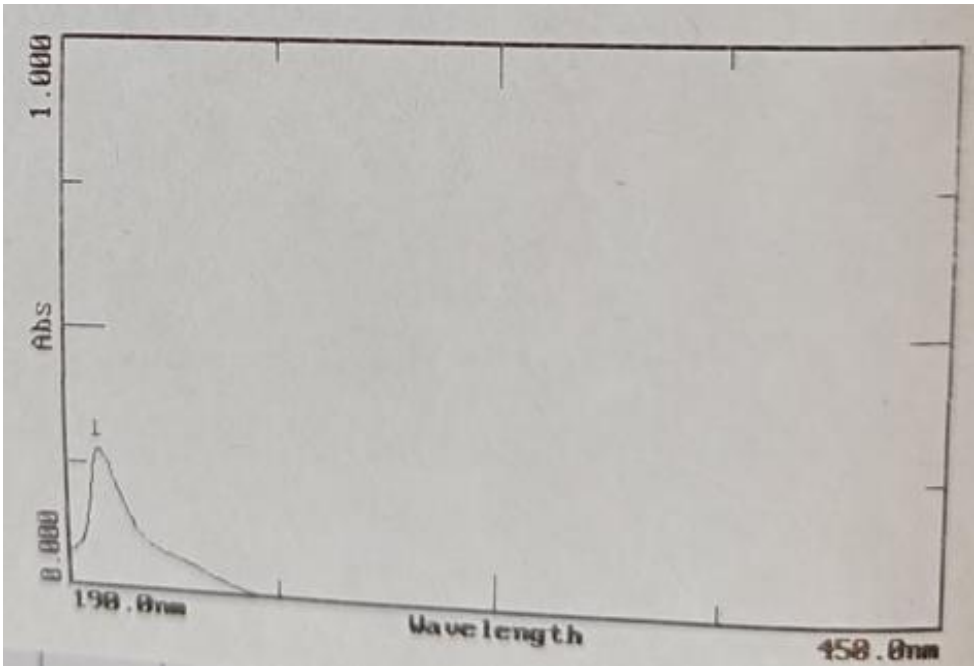
	Score	Library	Name	Comment
1	957	140 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS
2	957	465 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS
3	957	144 - Pharmaceutische grondstoffen Leverancier BUFA	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS	ACIDUM ACETYLSALICYLICUM (180) - PH.EUR. - BUFA - 94H17FS

Appendix 2: UV spectra of salicylic acid and acetylsalicylic acid

Appendix 2.1: UV spectrum of salicylic acid

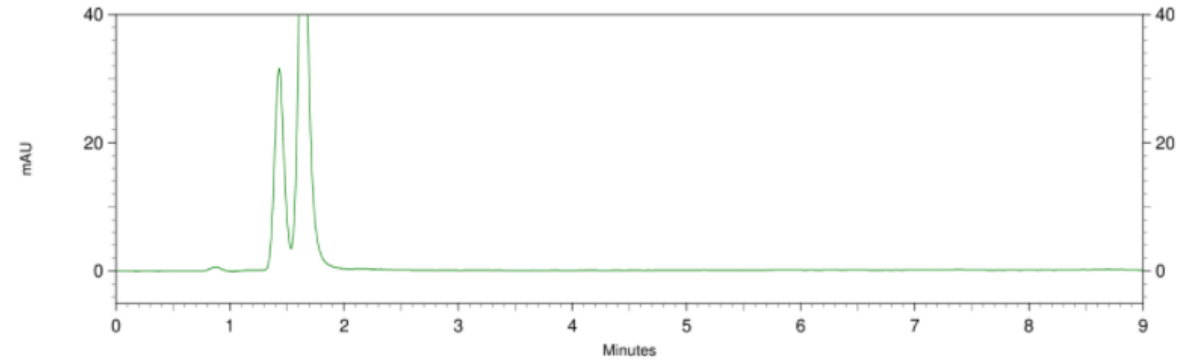


Appendix 2.2: UV spectrum of acetylsalicylic acid

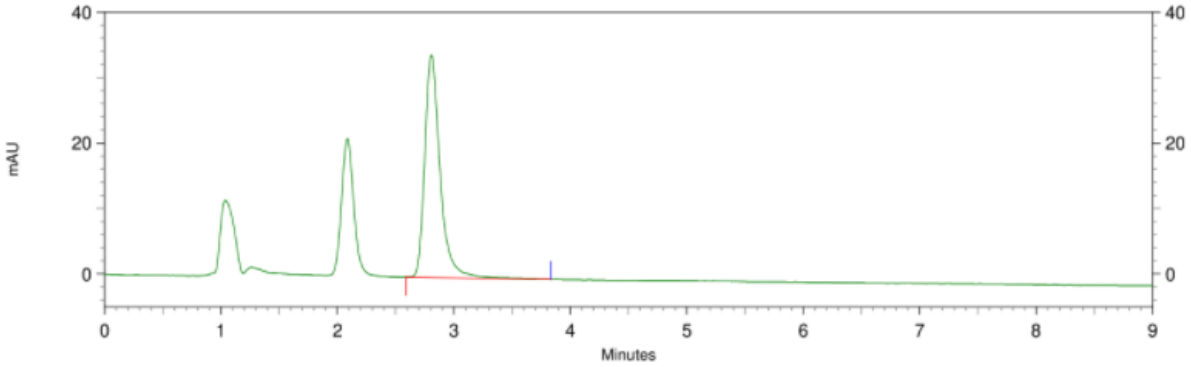


Appendix 3: Chromatograms of ASA and SA for eluent selection

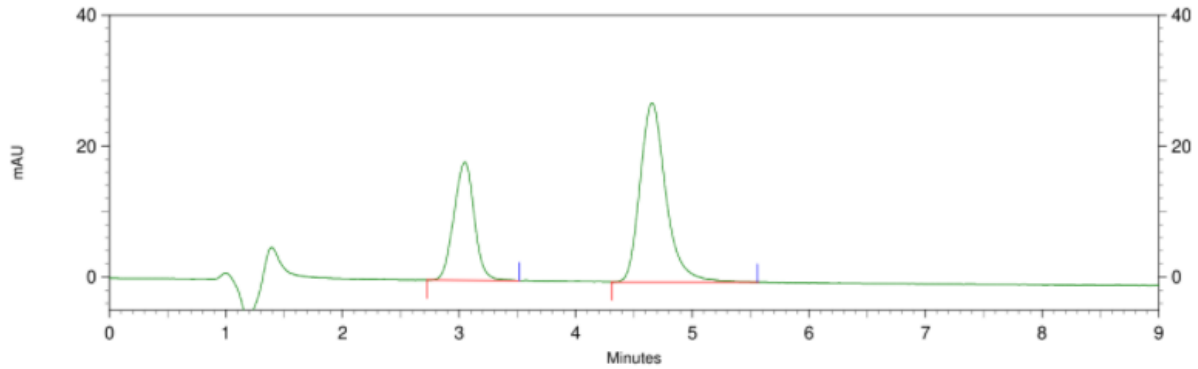
Appendix 3.1: Chromatograms of ASA and SA in H_2O/ACN 40/60 v%



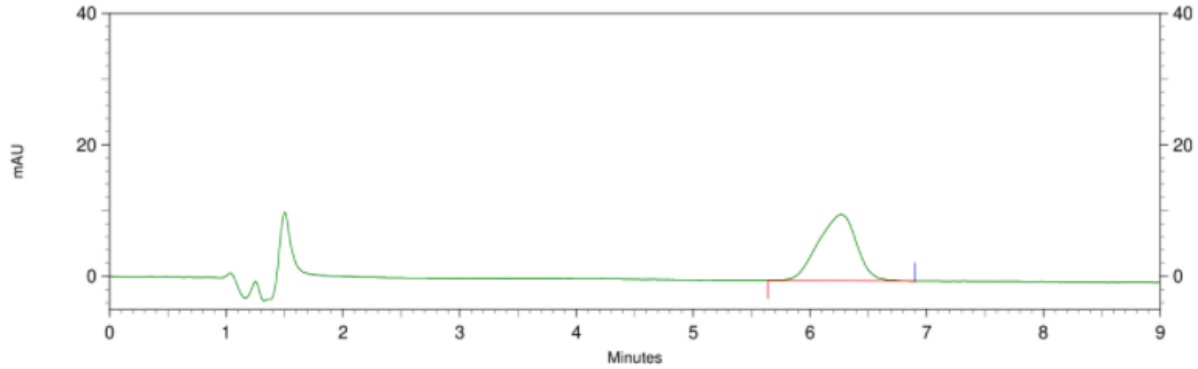
Appendix 3.2: Chromatograms of ASA and SA in H_2O/ACN 60/40 v%



Appendix 3.3: Chromatograms of ASA and SA in H_2O/ACN 70/30 v%

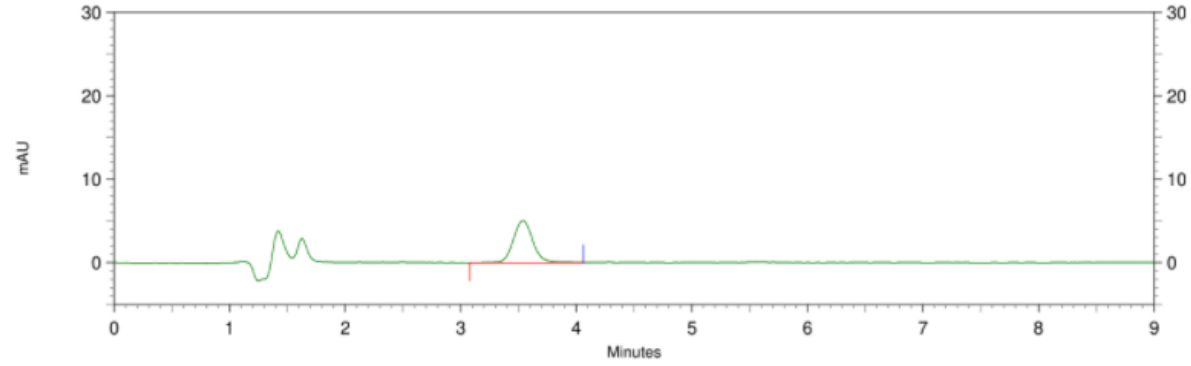


Appendix 3.4: Chromatograms of ASA and SA in H_2O/ACN 80/20 v%



Appendix 4: Chromatograms of ASA for the calibration curve

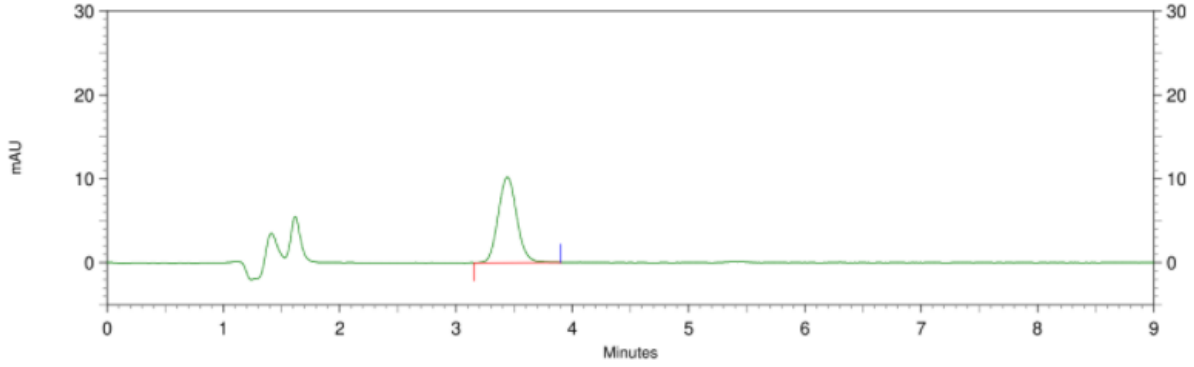
Appendix 4.1: Chromatogram of 0.00396 mg/ml ASA



DAD-CH1 237 nm Results

Retention Time	Area	Height
3.533	237906	20210

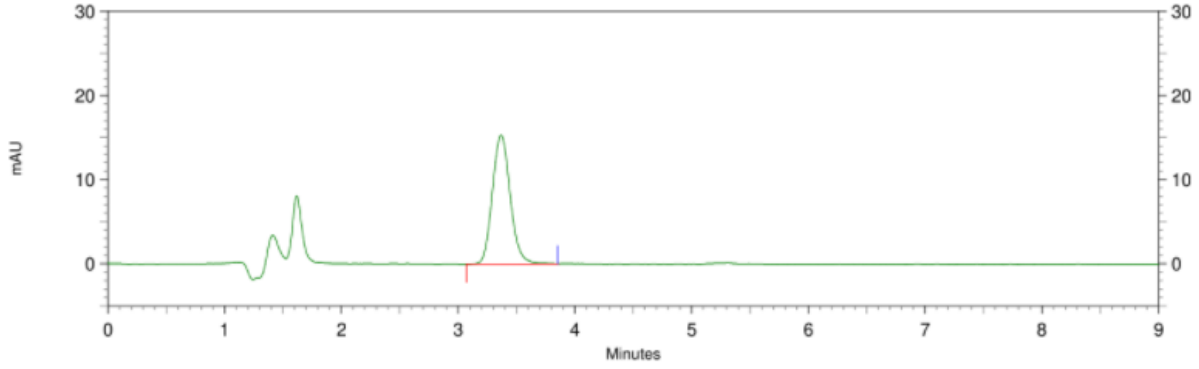
Appendix 4.2: Chromatogram of 0.00792 mg/ml ASA



DAD-CH1 237 nm Results

Retention Time	Area	Height
3.440	452779	40757

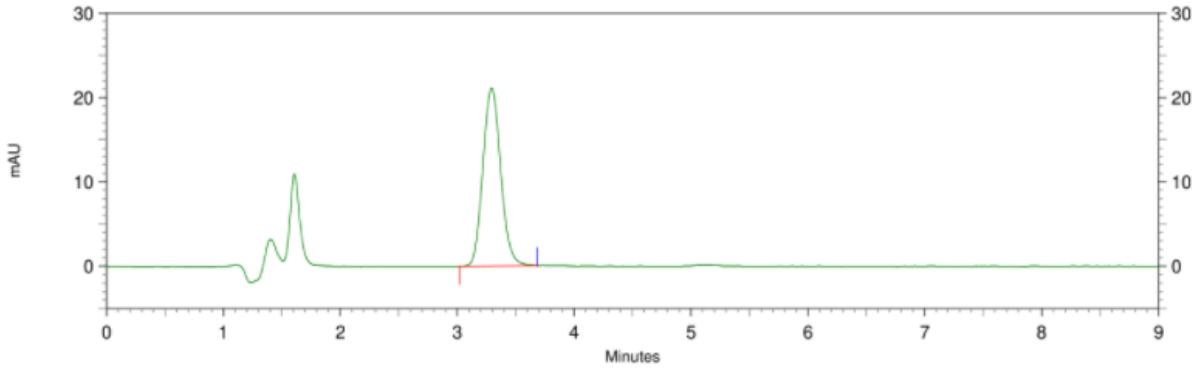
Appendix 4.3: Chromatogram of 0.01188 mg/ml ASA



DAD-CH1 237 nm Results

Retention Time	Area	Height
3.367	650711	61221

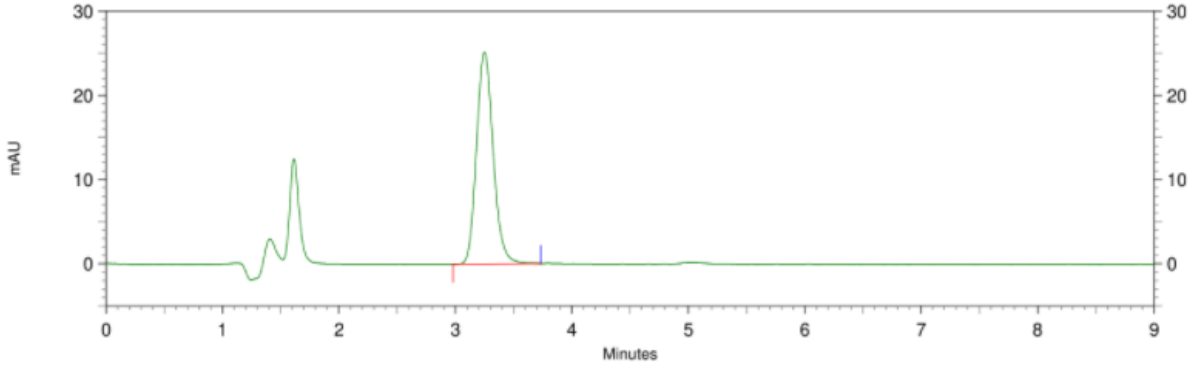
Appendix 4.4: Chromatogram of 0.01584 mg/ml ASA



DAD-CH1 237 nm Results

Retention Time	Area	Height
3.293	895791	84408

Appendix 4.5: chromatogram of 0.0198 mg/ml ASA

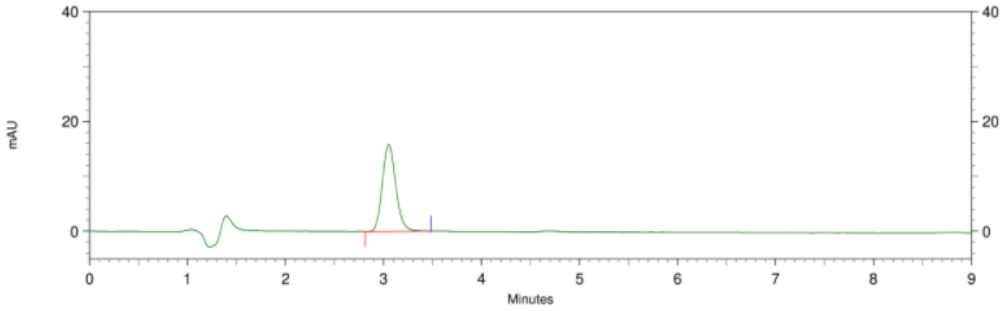


DAD-CH1 237 nm Results

Retention Time	Area	Height
3.253	1001959	100550

Appendix 5: Aspirin production on the chip

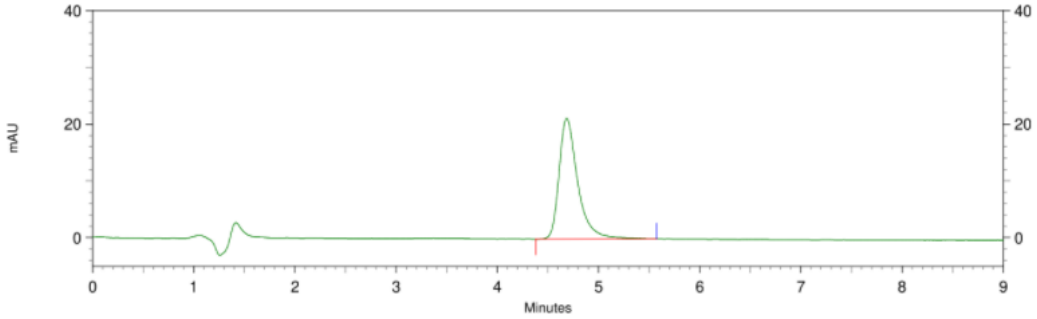
Appendix 5.1: Chromatogram of sample containing pure aspirin



DAD-CH1 237 nm Results

Retention Time	Area	Height
3.053	606612	63252

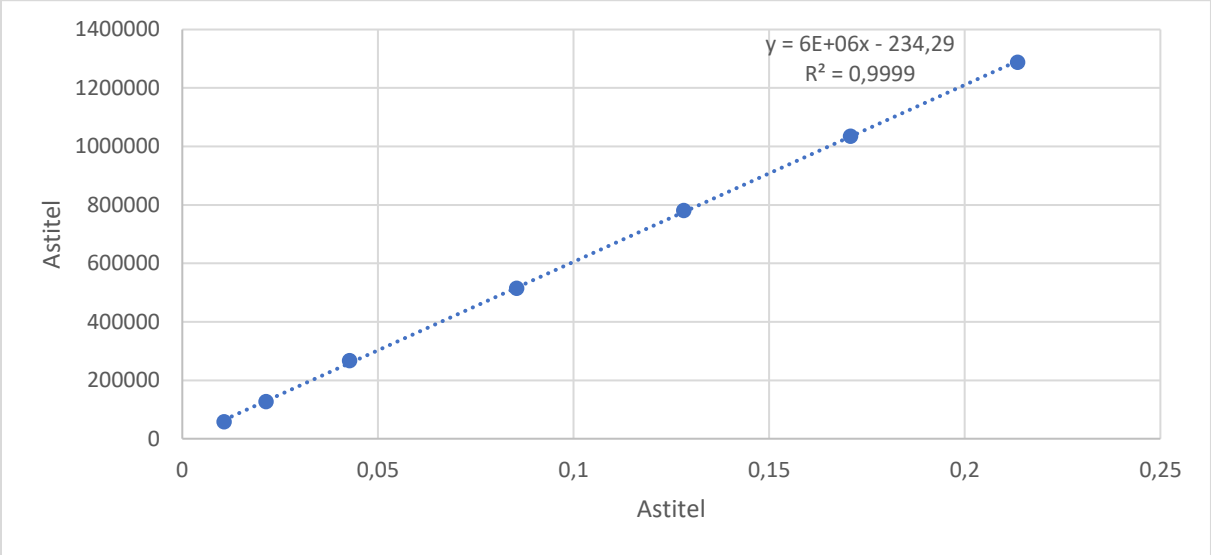
Appendix 5.2: Chromatogram of sample containing pure salicylic acid



DAD-CH1 237 nm Results

Retention Time	Area	Height
4.687	1093375	85219

Appendix 5.3: Calibration curve for aspirin used for on chip production



The files from which this calibration curve is created can be found on the drive at the department of pharmaceutical analysis (RUG, Groningen, The Netherlands), and have the following file names:

C:\Users\MWP-WKS034511\Desktop\BA 2021 Data\Catchup\22-03-17 ASA c0.01.dat

C:\Users\MWP-WKS034511\Desktop\BA 2021 Data\Catchup\22-03-17 ASA c0.02.dat

C:\Users\MWP-WKS034511\Desktop\BA 2021 Data\Catchup\22-03-17 ASA 0.04.dat

C:\Users\MWP-WKS034511\Desktop\BA 2021 Data\Catchup\22-03-17 ASA 0.08.dat

C:\Users\MWP-WKS034511\Desktop\BA 2021 Data\Catchup\22-03-17 ASA 0.12.dat

C:\Users\MWP-WKS034511\Desktop\BA 2021 Data\Catchup\22-03-17 ASA 0.16.dat

C:\Users\MWP-WKS034511\Desktop\BA 2021 Data\Catchup\22-03-17 ASA C0.2.dat

The files were placed into the Y-drive and can be found with regard to their date, content which is ASA and concentration quantity, which are also in the file names stated above.

Appendix 5.4: Calculation for the theoretical maximum concentration of aspirin in the sample from the chip.

1 gram of salicylic acid was dissolved in 10 ml ethyl acetate, resulting in a concentration of 100 mg/ml salicylic acid.

This concentration needs to be converted into moles/ml in order to calculate a maximal theoretical conversion. This is done with the molecular weight of salicylic acid, 138.121 g/mol or mg/mmol.

$$[SA]\left(\frac{mmol}{ml}\right) = {[SA]\left(\frac{mg}{ml}\right)}\Big/{Mw_{SA}\left(\frac{mg}{mmol}\right)} = {100}/{138.121} = 0.724\left(\frac{mmol}{ml}\right)$$

This means that a concentration of aspirin in the sample is 0.724 mmol/ml if salicylic acid is 100% converted. The molecular weight of aspirin, 180.158 mg/mmol, can then be used to calculate a concentration in mg/ml. This is done as following:

$$[ASA]_{max}\left(\frac{mg}{ml}\right) = [ASA]_{max}\left(\frac{mmol}{ml}\right) * Mw_{ASA}\left(\frac{mg}{mmol}\right) = 0.724 * 180.158 = 130.4\ mg/ml$$

The sample is diluted 1000 times prior to injection into the HPLC, so the theoretical maximum concentration should be divided by 1000. This results in maximum concentration of aspirin of 0.1304 mg/ml.

Appendix 6: Calculation of Reynolds numbers

In order to calculate Reynolds number, the following equation is used:

$$\mathrm{Re} = \frac{\rho D_\mathrm{h} u}{\eta} = \frac{D_\mathrm{h} u}{\nu}$$

This equation is explained in the theory section.

In order to determine the Reynolds number using this equation for ethyl acetate in the channels of the PDMS chip the following parameters were used.

D_h = 2 * 10^{-4} m

v = 3.663 * 10^{-7} m^2/s

$$u = {velocity\ disperse\ phase\ (mm^3/h)}\Big/{diameter\ channel\ (mm^2)} = {250}/{0.04} = 6250\frac{mm}{h} = 0.001736\ m/sec$$

$$Re_{Ethyl\ acetate} = {0.0002 * 0.001736}/{3.663 * 10^{-7}} = 0.947$$

The continuous phase differs in kinetic viscosity from the ethyl acetate:

v = 0.553 * 10^{-6} m^2/s

$$Re_{water} = {0.0002 * 0.001736}/{0.553 * 10^{-6}} = 0.628$$

Because we cannot calculate the Reynolds number after formation of droplets, the Reynolds number is expected to be between 0.947 and 0.628 after the formation of droplets.

Appendix 7: Fabrication protocol of glass moulds in the clean room

Fabrication of the wafer for channels, 150 µm depth

1. Design mask with CleWin 3 and print at ProArt (resolution 2000 dpi / 12.7 µm)
2. Clean a 4-inch borofloat wafer with acetone, isopropanol and water
3. Dry by spinning the water off and baking at 150 °C for 30 min
4. Cool down on a paper in the wet bench for 2 min
5. Center wafer on 4-inch chuck
6. Pour SU-8 2100 directly from the bottle onto the wafer, forming a ~4 cm diameter puddle. Prevent the formation of air bubbles by pouring firmly and cutting the flow by rotating the bottle of SU-8. Wipe off the bottle with acetone on a cleanroom tissue.
7. Spin-coat:
   500 rpm, 13 s, +100 rpm/s
   1800 rpm, 30 s, +300 rpm/s
8. Soft bake on two leveled hotplates in the wet bench, covered by an inverted glass Petri dish:
   65 °C, 5 min (no ramping!)
   95 °C, 30 min (no ramping!)
   Cool down on a tissue in the wet bench for 2 min
9. Expose with UV light, 400 mJ/cm^2; covering the mask firstly with the 10.2 mm thick glass disk and secondly with the square glass plate
10. Post-exposure bake:
    65 °C, 5 min (no ramping!)
    95 °C, 12 min (no ramping!)
    Cool down on a tissue in the wet bench for 2 min
11. Develop by shaking in SU-8 developer:
    15 min
    Refresh solvent, another 5 min
    Development of narrow troughs can be enhanced by flushing developer on the surface with a disposable 3 mL Pasteur's pipette.
12. Rinse the wafer with isopropanol and dry with nitrogen gun
13. Hard bake: 20 min at 150 °C
    Cool down passively on the hotplate to <65 °C
14. Coat the wafer with trichloro(1H,1H,2H,2H-perfluorooctyl)silane (PFOCTS) by pipetting 10 µL of PFOCTS in an aluminum cup and placing both in a desiccator with vacuum pump for at least 1 h.