university of groningen

The extraction of hippuric acid from cow urine using Liquid/Liquid extraction

Research Project
MSc Industrial Engineering and Management

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Abstract

The emissions of ammonia (NH_3) are rising, which is causing a variety of problems within the environment. This could cause a decrease in human and animal health. Ammonia is produced when urine and faeces are in contact. Research has shown that the separation of animal urine and faeces is possible in barns. The rising ammonia emissions are a great motivation to separate the urine from the faeces in barns. Another aspect that can help aid the motivation for such systems is that hippuric acid can be extracted out of cow urine. This hippuric acid can then be used in the chemical industry.

This project aims to optimize the extraction of hippuric acid from cow urine using the technique of Liquid/Liquid Extraction. Experiments are performed using hippuric acid dissolved in water which resulted in 91.9% of hippuric acid being extracted from the initial sample while 53.4% is extracted to the final water phase using back extraction. Using the parameters determined during the initial stage of research, experiments were performed using cow urine. This resulted in 52.4% of hippuric acid being extracted out of the cow urine while 28.1% of the hippuric acid was extracted to the final water phase.

Keywords: Hippuric acid, Cow Urine, Ammonia, Liquid/Liquid extraction.

Contents

List of Figures

List of Tables

Abbreviations

LLE Liquid-Liquid Extraction

TLC Thin Layer Chromatography

HA Hippuric Acid

Chemical formulas

NH_3 Ammonia

1 Introduction

Ammonia (NH_3) emissions are rising, causing all kinds of problems such as acidification, eutrophication of ecosystems and decreased animal and human health (Wyer et al., 2022; Vaddella et al., 2010). Ammonia is a precursor to $PM_{2.5}$, which is a collective name quantification of the number of particles or droplets in the air with a diameter of less than 2,5 microns. Therefore a rise in NH_3 emissions causes a rise in $PM_{2.5}$ as well. This is an important aspect as a high level of $PM_{2.5}$ can cause a variety of health issues such as lung cancer, chronic obstructive pulmonary disorder (COPD) and even increases the risk of premature mortality (Wyer et al., 2022). Agriculture contributes to over 80% of the global emissions of ammonia and in Europe and it is the cause of 50% of the $PM_{2.5}$. The main sources of these emissions from agriculture are livestock and animal production, manure handling, livestock housing and artificial fertilizers that are used on the land (Wyer et al., 2022; Vaddella et al., 2010; Sigurdarson et al., 2018).

Research has shown that ammonia is produced by multiple reactions when the urine and faeces of cattle are in contact (Wyer et al., 2022; Vaddella et al., 2010). It has been shown that the separation of the urine and faeces in barns is a possibility (Lachance et al., 2005; Stewart et al., 2004; Von Bernuth et al., 2005; Swierstra et al., 2001). The formation of ammonia forms a great motivation to separate the urine and faeces as well as that the hippuric acid that can be extracted from the urine can be used in other aspects.

The hippuric acid that is extracted can be hydrolysed to benzoic acid, which in turn is a widely used chemical that has an inhibitory effect on the growth of bacteria (Kalpana and Rajeswari, 2019). Besides that benzoic acid can decrease the pH value in food, increasing the nutrient digestibility (Mao et al., 2019).

In order to be able to use the hippuric acid in cow urine it needs to be separated from the other components in the urine. Within this research, the possibilities for extracting hippuric acid out of cow urine by liquid/liquid extraction will be explored.

2 Conceptual Research Design

2.1 System description

2.1.1 Content of cow urine

When considering the extraction of hippuric acid from cow urine it must be considered that there are multiple chemicals in cow urine besides the main component of water. Table 1 shows a list of these components. In order to obtain the end product of pure hippuric acid, it needs to be extracted from the mixture without extracting the other components.

Table 1: *Content of cow urine (Sharma et al., 2020).*

Number	Chemical
1	Ammonia
2	Aurum Hydroxide
3	Calcium
4	Carbolic acid
5	Cupper
6	Creatinin
7	Enzymes
8	Hippuric acid
9	Iron
10	Lactose
11	Manganese
12	Nitrogen
13	Other minerals
14	Phosphates
15	Potassium
16	Salt
17	Sodium
18	Sulfur
19	Urea
20	Uric acid
21	Vitamins (A,B,C,D,E)

2.1.2 Liquid/liquid extraction

The aim of this project is to separate the hippuric acid from the other components in cow urine using the technique of Liquid-Liquid Extraction (LLE). LLE is a technique used to extract components from liquid solutions. This is done by the addition of another solution, which is immiscible with the starting solution but in which the to-be-extracted component does dissolve (Hanson, 2013; Pedersen-Bjergaard and Rasmussen, 2008; Hu et al., 2017). This means that LLE has two bulk phases: the aqueous phase in which the wanted product is dissolved and an organic phase, in which the product is to be dissolved. The product will move from the aqueous phase to the organic phase as a result of a thermodynamic driving force of chemical or electrochemical potential. This is performed through the mechanism of convective-diffusive mass transfer in which the concentration in the aqueous phase and inorganic phase will reach an equilibrium (Cantwell and Losier, 2002; Hu et al., 2017). This technique is described in the two-film theory, in which it is said that the different phases are separated by means of an interface. The mass transfer between these two phases occurs in two liquid films by molecular diffusion. This theory assumes that the interface is a sharp boundary at which an equilibrium occurs. It is assumed that the mass transfer resistance between the two phases occurs because of the films on either side of the interface and not because of the interface itself (Hu et al., 2017).

LLE is often used in cases where enhanced selectivity is required, extracting and isolating a specific compound from the matrix of interfering compounds (Silvestre et al., 2009; Pedersen-Bjergaard and Rasmussen, 2008). The use of LLE in the industry happens because of multiple reasons some of which being: The separation of systems with similar boiling points in which distillation becomes more difficult, the separation of systems with high boiling points, the separation of temperature-sensitive systems and the extraction of organic compounds from salts, salts from polymer solutions or metal salts from wastewater (Müller et al., 2000). Another advantage of this technique is that the organic extract can be directly used in quantitative analytical measurements. It also has the advantage of extracting the compound from the organic phase into the water phase, also known as back extraction, which can increase the selectivity towards a specific compound (Cantwell and Losier, 2002).

Figure 1: *Schematic overview of a separatory funnel used for LLE (Kelly, 1993).*

Besides having multiple advantages LLE has its disadvantages, such as high consumption of organic solvents, difficulties of automation and limited flexibility in extraction chemistry (Pedersen-Bjergaard and Rasmussen, 2008). Because of the high volume of organic solvents used, research has focused on limiting this by using techniques such as single-drop micro extractions or finding ways to recycle the used solvents (Pedersen-Bjergaard and Rasmussen, 2008).

The simplest form of LLE is performed in a separatory funnel in which the two phases should be mixed or shaken in some way to enhance the mass transfer of the compound to the other phase by enhancing the area of the interface between the two liquid phases (Cantwell and Losier, 2002). A schematic overview of this is shown in Figure 1. As using this method is batch focused and requires high amounts of labour research has been performed to use the technique of LLE in continuous flow systems such as systems with liquid membrane or closed-loop systems (Silvestre et al., 2009). Since this research is focused on lab-scale experiments using only a separatory funnel, the continuous flow systems will not be further discussed.

2.1.3 Liquid/liquid extraction of hippuric acid

This research is aimed at the extraction of hippuric acid from cow urine. As mentioned in subsection 2.1.1 there are many components within the cow urine which need to be separated from the hippuric acid. Hippuric acid is a colourless crystal and is used as a biological indicator of exposure to toluene, polyhydrophenols, glycine and benzoic acid. Hippuric acid can be measured in urine. Hippuric acid is a weak acid with a pK_a of 3.62 (Toulabi et al., 2010). In a solution with a high pH hippuric acid is a charged compound as a hydrogen atom is separated from the molecule and causes a negative charge on the hippuric acid. When the pH of this solution is lowered it causes the hippuric acid to become a neutral compound in the solution which does not have a charge (Kendall and Booge, 1917). Therefore, hippuric acid becomes more soluble in organic solutions when the pH of the aqueous solution is lowered significantly. For hippuric acid, this occurs around the pH of 3 (Toulabi et al., 2010).

In order to successfully transfer hippuric acid from the aqueous phase of the urine to the organic phase used during LLE the pH of the urine should be lowered. Once this is done the first LLE out of the two needed for the purifying of hippuric acid can be performed. For this step, a suitable organic compound should be selected. Once these two phases have been mixed and the first LLE has been performed the hippuric acid resides in the organic phase. In order to purify this solution even further a back extraction, as mentioned in subsection 2.1.2, can be performed. In order to make the aqueous phase more preferred for the hippuric acid to dissolve the pH of this phase should be increased in order to cause the hippuric acid molecule to become charged again and therefore become more hydrophilic (Kendall and Booge, 1917). This creates the right conditions to back-extract the hippuric acid from the organic phase into an aqueous phase. Once both, the extraction and the back-extraction have been performed a solution of hippuric acid in water should

be obtained.

2.1.4 Analysis methods

For this research, multiple methods have been used to analyse the samples created. These methods will be discussed in this section.

2.1.4.1 UV-VIS spectrometry

UV-VIS Spectrometry is an analytical technique in which the absorption of light is measured through the sample as a function of the wavelength. When light is cast through a sample, excitation occurs when an electron residing in a low-energy molecular orbital is promoted to a higher-level orbital. This absorbs part of the light as energy. The remainder of the light that is not absorbed is measured by the equipment, therefore knowing the change that occurred within the sample. This is then converted to absorption values. These can be shown in a spectrum. (Douglas A. Skoog, 2013)

2.1.4.2 Thin Layer chromatography

Thin-layer chromatography (TLC) is used in this research to determine whether or not hippuric acid has transferred to the final stage of the LLE. With TLC a stationary phase is used as a thin layer on the surface of a plate. In the case of this research, it is a silica plate that is used. A liquid phase is used to drag the sample across the plate, using capillary action.

To use TLC, a line is drawn 1-2 cm from the edge of the plate. On this line drops of the to-be-measured samples are placed. In order to achieve a clear measurement multiple layers of drops can be placed to increase the concentration.

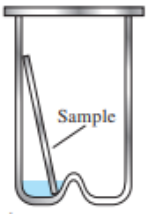

Figure 2: *Schematic overview of a development chamber used for TLC (Douglas A. Skoog, 2013).*

Once the sample is placed on the line the plate should be put in a specific liquid agent inside a developing chamber. A schematic overview of such a chamber is shown in Figure 2. The fluid is then rising onto the plate due to capillary action. This should be stopped before the fluid reaches the top of the plate. After this the plate should be dried in an oven and dye can be applied when needed to show the markings more clearly.

A sample of the pure component is used in order to determine the placement of this component on the plate. If a marking shows up on the same height for the other samples it shows that this component is present in those samples as well. (Douglas A. Skoog, 2013)

2.1.4.3 HPLC chromatography

HPLC chromatography, or High-Performance Liquid Chromatography, is a technique used for separating and determining components in a sample. A schematic overview of a standard HPLC system is shown in Figure 3.

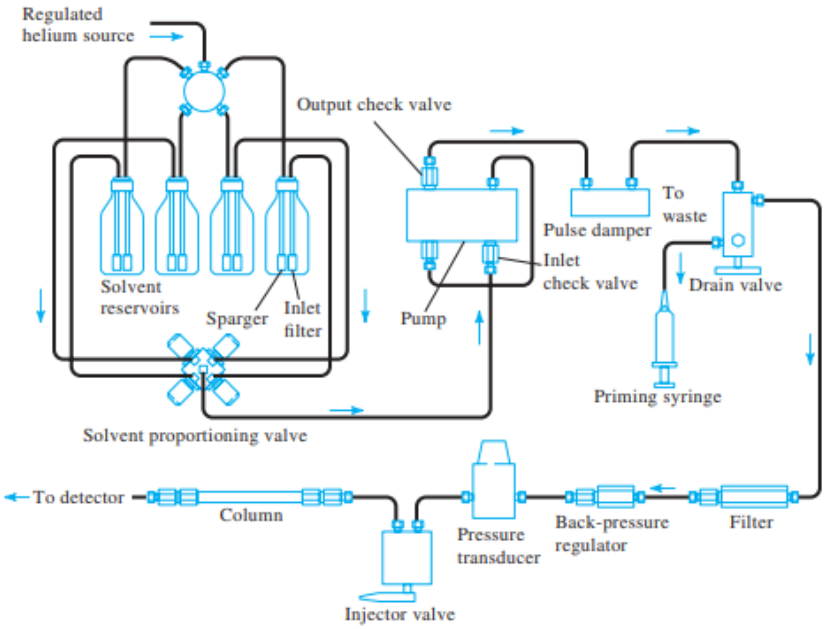

Figure 3: *A schematic overview of a basic HPLC apparatus (Douglas A. Skoog, 2013).*

An HPLC is used to detect and separate each compound in a sample. Within the HPLC two phases exist: the mobile phase and the stationary phase. The mobile phase is a solvent that dissolves the target compound. The stationary phase of an HPLC is part of the column which interacts with the compounds in the sample. The stronger the affinity of the compounds in the sample are with the stationary phase, the longer it remains in the column as the mobile phase is passing through.

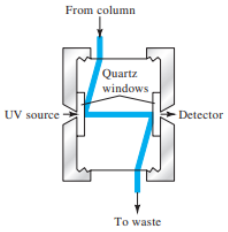

Figure 4: *Schematic overview of UV absorption detector within a HLPC (Douglas A. Skoog, 2013)*

Once the sample has traveled through the column it goes past a detector. A schematic overview of a detector is shown in Figure 4. Absorption or ultraviolet detectors are most commonly used. The absorptions measured within the detector are set as absorption values and will be plotted against time in a graph. In this graph, peaks will show the different components in a sample. The area underneath this peak correlates to the concentration of the compound within the sample.

$$Concentrationcomponent(mg/L) = \frac{Areapeaksample \times 100(mg/L)}{Areapeakpurecomponent} \tag{2.1}$$

Equation 2.1 shows the formula that should be used to calculate the concentration of the components within the sample.

2.2 Project context

2.2.1 Ammonia emissions

As previously mentioned in chapter 1 agriculture causes over 80% of the global ammonia emissions. It was shown that the emission of ammonia from agriculture doubled in Europe between 1950 and 1986, while the ammonia emissions in the Netherlands has grown by a factor of 2.5 (Monteny and Erisman, 1998; Sommer and Hutchings, 2001; Webb et al., 2005). This has caused critical loads of nitrogen to depose onto land, causing eutrophication and soil acidification. Which in turn causes environmental stress (Monteny and Erisman, 1998). In the Netherlands 46% of the potential acid deposition is caused by ammonia emissions in the late 1900's (Monteny and Erisman, 1998).

When considering the emissions from manure handling in agriculture there are multiple microbiological processes that are involved in the production of ammonia. The following two reactions are to be considered:

$$C_5H_4O_3N_4 + 1.5O_2 + 4H_2O \rightarrow 5CO_2 + 4NH_3 \tag{2.2}$$

$$CO(NH_2)_2 + H_2O \rightarrow CO_2 + 2NH_3 \tag{2.3}$$

As shown by these formulas ammonia is produced. The first reaction is the conversion of uric acid in the presence of oxygen and water, which is catalyzed by the enzyme uricase. In the second equation, urea is decomposed in the presence of water. This is catalyzed by the enzyme urease (Wyer et al., 2022; Vaddella et al., 2010). Urea can be found in urine, and the urease that catalyses the reaction is present in faecal matter, therefore the second reaction will only take place once the urine and faeces are in contact (Vaddella et al., 2010; Rotz, 2011). Research has shown that separating the urine from the faeces could cause a reduction of ammonia emissions ranging from 5 - 99% when it is done in barns (Vaddella et al., 2010). There are multiple articles that show that it is possible to separate the urine from the faeces such as Lachance et al. (2005); Stewart et al. (2004); Von Bernuth et al. (2005); Swierstra et al. (2001). These articles show possibilities such as separating the urine from the faeces by means of a grid on which the faeces will remain while the urine seeps through, as well as a method to teach cows how to use a so-called cow toilet on which the faeces will be transported up a slope using a conveyor belt while the urine is captured in a tank at the bottom.

2.2.2 Hippuric acid

The aim of this project is to extract and purify hippuric acid from cow urine. As mentioned in subsection 2.1.3 Hippuric acid, otherwise known as 4-hydroxybenzoic acid is a weak organic acid (Toulabi et al., 2010). This acid is a valuable intermediate for multiple other bio products with varying applications, such as food, cosmetics, pharmacy and fungicides (Wang et al., 2018).

Hippuric acid is currently produced based on petroleum-based chemicals under harsh reaction conditions such as high temperature and pressure. It also produces by-products, which makes the chemical synthesis of this product expensive (Wang et al., 2018). Therefore the research of Wang et al. (2018) looked into multiple pathways of producing hippuric acid using biosynthesis. The ability to extract hippuric acid from a waste stream such as cow urine is, therefore, an interesting approach as well as it not only produces pure hippuric acid without the use of fossil fuels and high energy consumption because of reaction conditions, it minimizes the emissions of ammonia at the same time.

Hippuric acid can be hydrolysed to benzoic acid (Kalpana and Rajeswari, 2019). Benzoic acid has been proven to have antimicrobial activity (Park et al., 2001). Benzoic acid is a carboxylic acid which can be added to food in order to acidify it and therefore extend the shelf life of these products. This benzoic acid is then metabolized to hippuric acid in the body and excreted through urine (Mao et al., 2019; Langenbeck and Seegmiller, 1973).

The research of Amdare et al. (2017) shows that pyrazole molecules can be synthesised from hippuric acid as well. These molecules can in turn be used in the treatment of inflammation as the current method of treatment has drawbacks such as ulceration and bleeding. Weak organic acids have been shown to have antibacterial effects. The research of Burns et al. (2021) shows hippuric acid activity against bacteria such as E. Coli.

2.3 Stakeholder analysis

To achieve a better overview of the involved parties for this project a stakeholder analysis is performed. Within this research multiple stakeholders are interested these are shown in Figure 5 and will be further discussed.

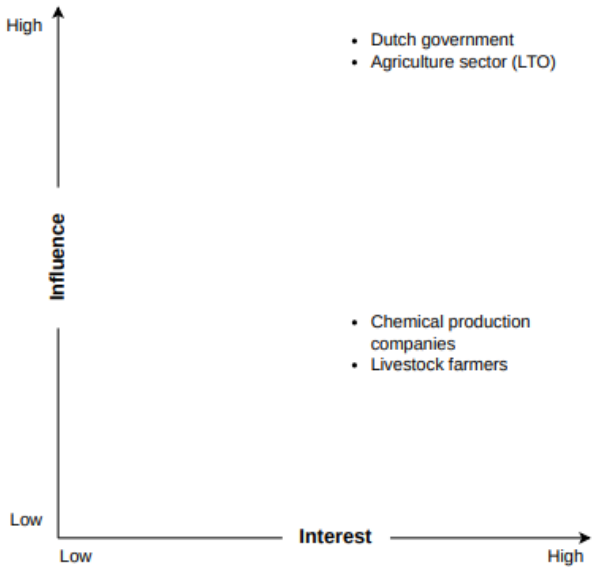

Figure 5: *A Mendelow's diagram on the stakeholders of extraction of hippuric acid from cow urine.*

The first mentioned stakeholder is the government of the Netherlands. The government has shown great interest in minimizing ammonia emissions to increase the quality of nature. Their goal is to ensure that 74% of the nitrogen-sensitive nature in Natura-2000-Areas will no longer exceed the "Critical deposition values" by 2030. In order to achieve this goal they intend to minimise the emissions in various aspects including agriculture (Rijksoverheid, 2023).

The second stakeholder is the Agriculture sector represented by the agriculture association in the Netherlands, the LTO. The LTO has stated that since 1990 the emissions of ammonia from agriculture have been reduced by 65%. Yet, they still want to reduce these emissions even further while taking the farmers and their business into consideration (Nederland, 2023).

Another stakeholder in this research is the chemical production companies as they will benefit from the production of hippuric acid in an environmentally friendly manner.

The last mentioned stakeholder are the farmers themselves. If regulations force them to minimize the emissions of ammonia it is of importance to them that they can oblige to these regulations while not losing profit to do so. Finding a way for the farmers to profit off of their waste streams while helping the farm to comply to possible regulations will be of great benefit to them.

2.4 Problem statement

Ammonia emissions from agriculture should be reduced as well as that a sustainable production method for hippuric acid should be designed to reduce emissions overall. Therefore, a method to sustainably extract hippuric acid from cow urine which in turn motivates the separation of cow urine from faeces is required.

2.5 Research objective

To find an optimal procedure to extract and purify hippuric acid from cow urine by using the technique of liquid/liquid extractions.

2.6 Cycle choice

The desirable outcome of this research is to find a procedure in which the extraction of hippuric acid using Liquid/Liquid extractions is optimized. Since this is a project based on gathering more knowledge on this specific procedure the empirical cycle was chosen. A schematic overview of this specific cycle is shown in Figure 6.

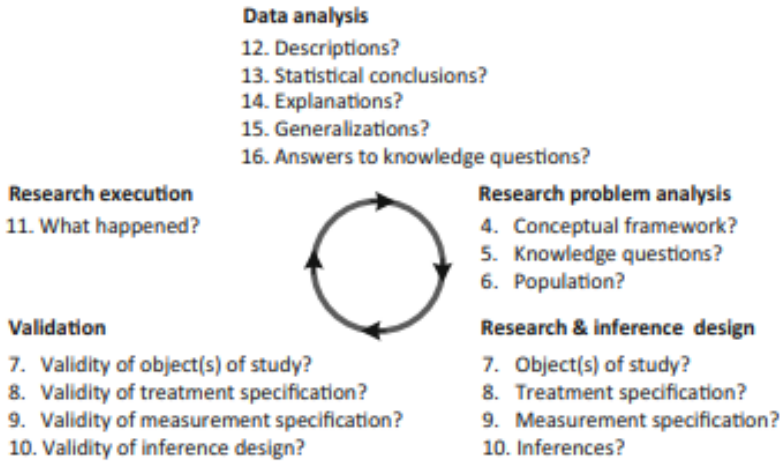

Figure 6: *A schematic overview of the empirical cycle (Wieringa, 2014)*

2.7 Research questions

In order to reach the goal of the project, which is answering the main research question, multiple subquestions will be formulated. The goal of these subquestions is to guide the research.

The main research question of this project is:

What is the optimal procedure to extract hippuric acid from cow urine while achieving a pure product using Liquid/Liquid Extraction methods?

In order to answer this research question, the following sub-questions should be answered:

Process parameters

SQ 1 *Which solvent is most compatible to perform the extraction of hippuric acid from cow urine?*

SQ 2 *What volume of this solvent should be used in order to achieve a high extraction efficiency?*

SQ 3 *What volume of water should be used during the back-extraction in order to achieve a high extraction efficiency?*

SQ 4 *To what pH should the urine sample be lowered in order to achieve a high extraction efficiency?*

SQ 5 *To what pH should the water be increased in order to achieve a high extraction efficiency?*

SQ 6 *What contact time should be maintained in order to achieve a high extraction efficiency?*

SQ 7 *How many extraction cycles should be performed on a urine sample in order to extract a sufficient amount of hippuric acid?*

Sustainability considerations

SQ 8 *Can the solvent be reused multiple cycles?*

Purity of the end product

SQ 9 *If the obtained hippuric acid needs to be further purified, how could this be achieved?*

Once these sub-questions are answered during the project it should also answer the main question formulated at the start of this chapter.

3 Technical Research Design

3.1 Operationalisation

This section will contain the tasks that need to be performed in order to be able to answer the research questions formulated in section 2.7.

3.1.1 Overall strategy

First, the overall strategy of the research will be discussed. In order to research the before mentioned research questions a solution of hippuric acid in water will be used to perform the initial tests. This is done in order to speed up the lab research as the concentration of hippuric acid in water can be measured using UV-VIS spectrometry. This allows for quicker measurements as opposed to HPLC chromatography measurements for every step. These samples will from now on be called "water samples". In order to achieve a good overview of what is happening within these phases a sample of the initial hippuric acid in water solution should be measured as well as the solution which forms the end product. The hippuric acid that is not found in either of these samples has remained in the organic solvent.

Once a research question is answered the result of this is taken into the used procedure in order to achieve a better outcome with every step of this research. The starting procedure will be discussed in a later section.

After the initial measurements with hippuric acid and water are performed the optimal procedure will be performed on cow urine samples as well as multiple experiments that could not sufficiently be measured using the hippuric acid in water samples.

3.1.2 Starting procedure

In order to have a starting point for these experiments a volume of 50 mL of water sample was extracted using 50 mL of organic solvent. The back extraction was then performed using 50 mL of demi water.

The extractions are performed by shaking the separation funnel for two minutes and are rested until a clear separation of the two phases is visible. Both of the water phases are then measured with UV-VIS spectrometry to determine their hippuric acid concentration.

3.1.3 Operationalisation of the research questions

SQ 1 In order to determine the optimal organic solvent to use for the extraction of hippuric acid literature research needs to be performed on the solubility of hippuric acid in different organic solvents. From this literature research, a selection of solvents should be made which then can be tested using the water samples. With the results of these initial extractions, a definite choice of solvent can be made.

SQ 2 & 3 Since both of these sub-questions regard the volume of solvent used the same approach can be used to answer them. With the solvent selected after answering SQ 1 experiments can be run using different volumes of organic solvent and water for both extractions. These experiments will be performed using water samples.

SQ 4 & 5 As described in subsection 2.1.3 the urine sample should be lowered in pH while the pH should be increased again for the back extraction. The pH range tested is based on the research of Toulabi et al. (2010), which performed similar research on the extraction of hippuric acid from cow urine using Liquid/Liquid micro-extraction. The pH range of the urine ranged from 2-6 while that of the back extraction ranged from 8-13.

SQ 6 The contact time of the two phases can influence the mass transfer of hippuric acid between the two. Therefore a range of contact times should be tested in order to select the optimal contact time.

SQ 7 As mentioned in subsection 2.1.2 the technique of LLE reaches an equilibrium. By performing multiple extractions on the same sample the amount of hippuric acid extracted from the cow urine can be increased. An experiment should be performed in which the same water sample is used multiple times. Once this has been done an optimal amount of washes with solvent or water can be determined.

SQ 8 In order to minimize the volume of solvent used to extract the hippuric acid from the cow urine it should be determined whether or not the same organic solvent can be used for multiple extractions. This can be researched by performing the experiment multiple times using the same solvent. The efficiency of each run can then be determined.

SQ 9 Once all other research questions are answered and the final product is measured using HPLC it can be determined whether or not the hippuric acid should be further purified. This can be done, if needed using the technique of crystallisation.

Once these tasks are performed an optimal extraction procedure can be formulated using the results.

3.2 Research Materials

In this section the materials used to perform the tasks described in subsection 3.1.3 will be described.

3.2.1 Chemicals

For this research hippuric acid from Sigma Aldrich was used (98%, CAS 495-69-2). Furthermore, the following organic solvents were used: Diethyl ether (Sigma Aldrich, 99.7% CAS 60-29-7), Dimethyl carbonate (Sigma Aldrich, 99% CAS 616-38-6) Ethyl acetate (Macron, CAS 141-78-6), butyl acetate (BDH chemicals, 96% CAS123-86-4). Lastly cow urine was used, collected from a local farm.

3.2.2 Analytical equipment

The analytical equipment used in this research was:

- Spectrophotometer

  QS-Cuvette

- HPLC Chromatogram

  Rezex ROA-organic acid H+ (8%)

  Dimensions: 300 x 7.8 mm ID

  Eluens of 2.5 mM H_2SO_4

  Detection at 254 nm

- Thin layer Chromatography

  Silica gel plate

  Mobile phase of Acetone:Water:Chloroform:Ethanol:Acetic acid of 60:2:6:10:22

- Rotary Evaporator

  Vacuum pump

  Water bath

  Round bottom flask

4 Results

Within this section, the results of the previously discussed experiments will be analysed.

4.1 Hippuric acid in water samples

As mentioned previously the initial tests to extract hippuric acid were performed using a hippuric acid in water solution. For this initial sample 1.00 gram of hippuric acid was dissolved in 1 Liter of water. In order to be able to measure the concentration of hippuric acid in the water samples a calibration curve was made using the linear part of the results given out of a range of solution. This calibration curve can be seen in Figure 16 in Appendix A.

4.1.1 Solvent choice

The first step in this research was to determine the optimal solvent to use for the extraction. The selection of solvents was determined by considering the research of Toulabi et al. (2010). After which Ethyl acetate, butyl acetate, diethyl ether and dimethylcarbonaat were selected. The optimal solvent that this research found was a mixture of ethyl acetate and butyl acetate. This mixture is therefore included in the experiments. The experiments were performed as described in subsection 3.1.2.

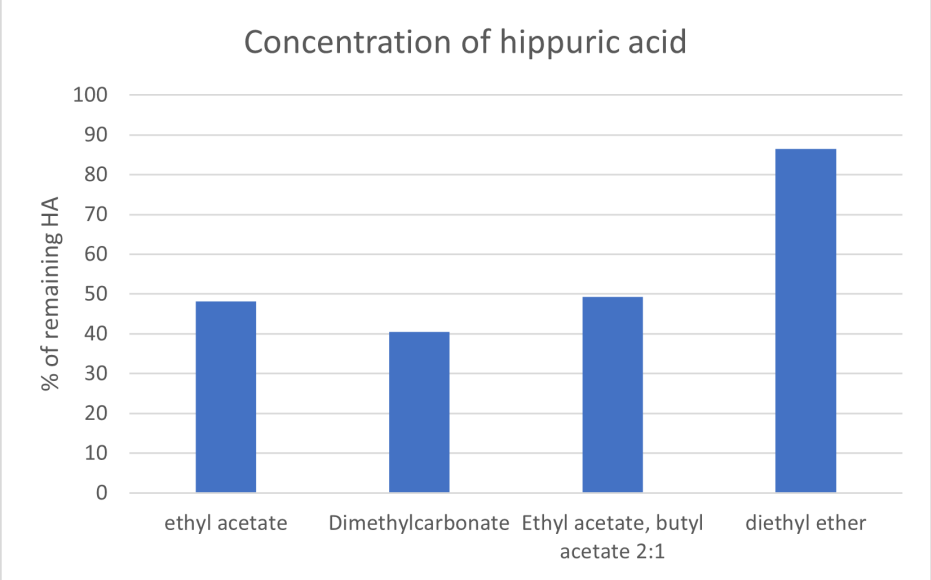

Figure 7: *The results of the experiments to determine the optimal solvent.*

Figure 7 shows the results of the tests using different solvents. This shows the percentage of hippuric acid that remained in the water sample after extraction. As can be observed in this graph dimethyl carbonate shows the lowest amount of hippuric acid remaining in the initial sample. However, this solvent caused the formation of a third layer in the separatory funnel and will therefore not be chosen to move forward with.

The difference in the results of ethyl acetate and the mixture of ethyl acetate and butyl acetate were small, however, the experiments performed with ethyl acetate resulted in slightly higher efficiencies. Ethyl acetate was chosen to use as the solvent for the extraction of hippuric acid.

4.1.2 Solvent volume

During the initial experiments, the ratio of the Hippuric acid sample to the solvent used was 1:1. In order to minimize the use of solvent it was tested whether it was feasible to use less solvent in order to extract the hippuric acid.

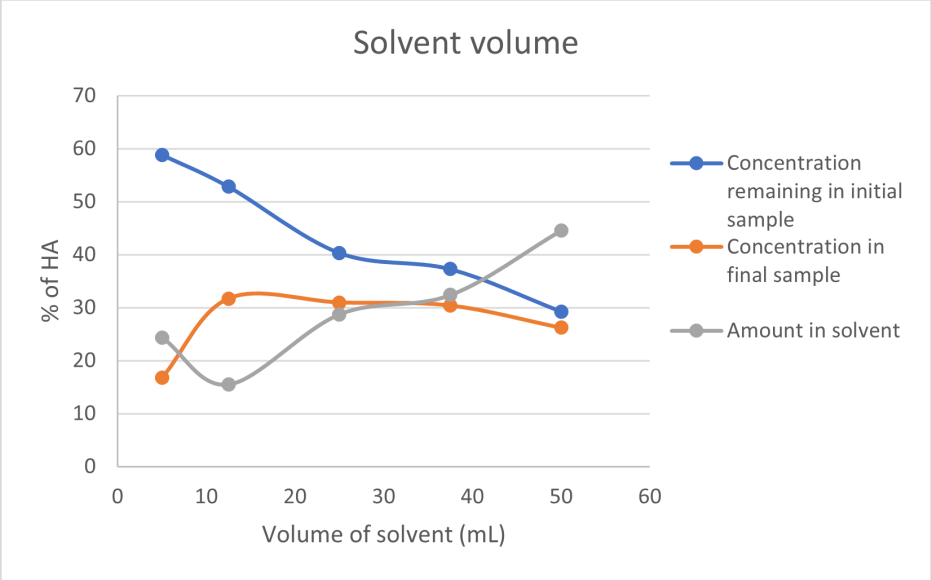

Figure 8: *The results of the experiments to determine the optimal solvent volume to be used.*

The volume of the solvent ranged from 5 to 50 mL. The results are shown in Figure 8. By increasing the solvent volume the amount of hippuric acid remaining in the initial sample decreased. The concentration of hippuric acid extracted from the final water phase remained the same. As shown in Figure 8 the concentration in the initial hippuric acid sample stagnates when comparing 25 and 35 mL of solvent used. Using a volume of 50 mL of the solvent caused a high amount of hippuric acid to remain in the solvent after back extraction. Therefore, it was determined that 25 mL of solvent was the optimal result.

4.1.3 Back extraction water volume

In order to be able to concentrate the hippuric acid already while performing the extractions an experiment was performed using a range of volumes for the water phase during the back extractions. The results of these experiments are shown in Figure 9.

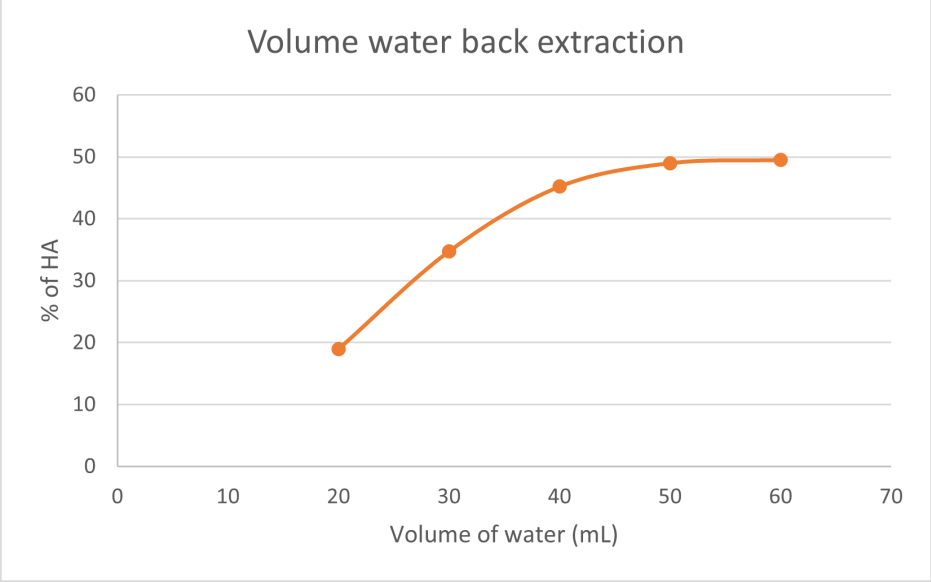

Figure 9: *The results of the experiments to determine the optimal water volume to be used during back extractions.*

The percentage of hippuric acid extracted from the solvent rises as the volume of the water phase increases. Comparing the results of 25 mL or 30 mL of water used shows only a slight increase

whereas the difference between 20 mL and 25 mL is larger. Therefore it was determined that 25 mL of water was the optimal volume to use for the back extractions. This does unfortunately mean that the hippuric acid mixture is not concentrated more during the extractions.

4.1.4 pH of the initial sample

To make sure that the hippuric acid molecules are neutral during extraction the pH of the initial sample is lowered. In the research of Toulabi et al. (2010) a range of a pH of 2-6 was used. The hippuric acid in the water sample that was used during this stage of experiments has an pH of 4 therefore a range of 2-4 will be used during these experiments. The results are shown in Figure 10.

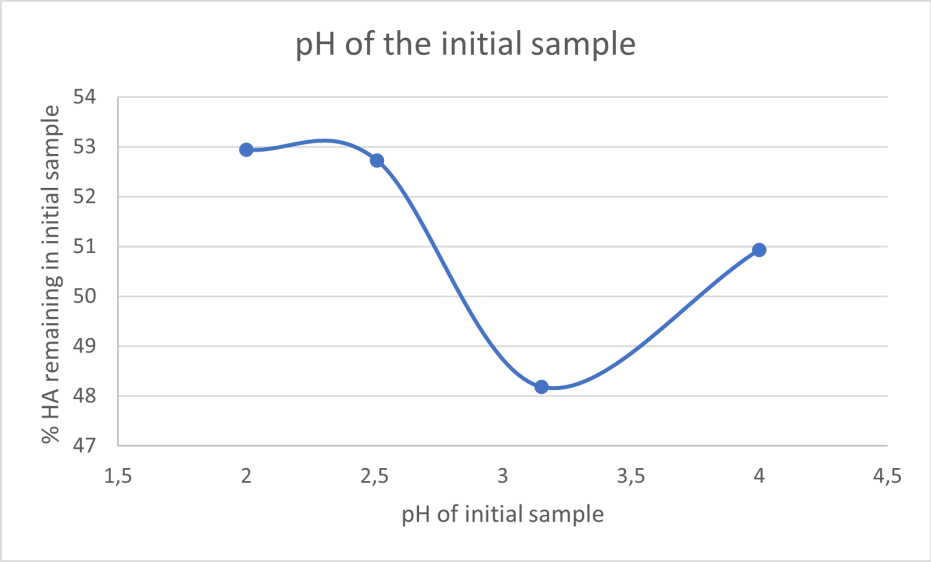

Figure 10: *The results of the experiments to determine the optimal pH of the initial sample during extraction.*

The figure clearly shows that at a pH of 3, the amount of hippuric acid remaining in the initial sample is the lowest. This has been chosen as the starting pH for the remainder of the experiments.

4.1.5 pH of the water phase for back extraction

To charge the hippuric acid molecule again in order to back extract them using a water phase the pH needs to be increased. A range of 10 to 13 was used during these experiments. Figure 11 shows the results.

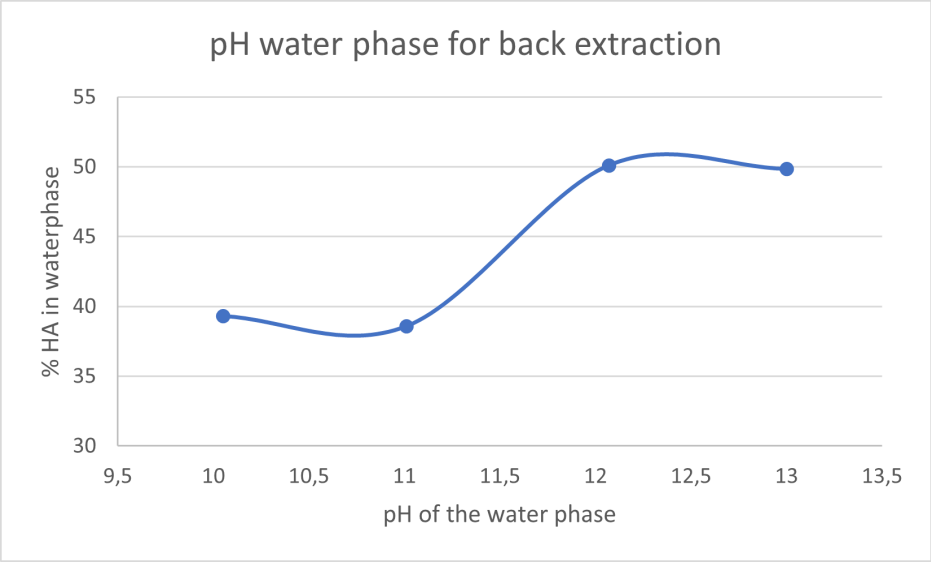

Figure 11: *The results of the experiments to determine the optimal pH of the water phase during back extraction.*

A pH of 12 was chosen as an optimal pH considering the results.

4.1.6 Contact time

Since the mass transfer of hippuric acid to the other phase needs to occur during extraction the contact time of those phases is of importance. During previous experiments, it was found that the transfer from the initial water sample to the organic solvent remained around 50% while the transfer from the solvent to the water phase during back extraction was around 90%. Therefore the contact time between the initial sample and the organic solvent was used for these experiments. The contact time was varied from 2 to 20 minutes with the addition of a sample that was stirred for 16 hours. The results of these tests are shown in Figure 12

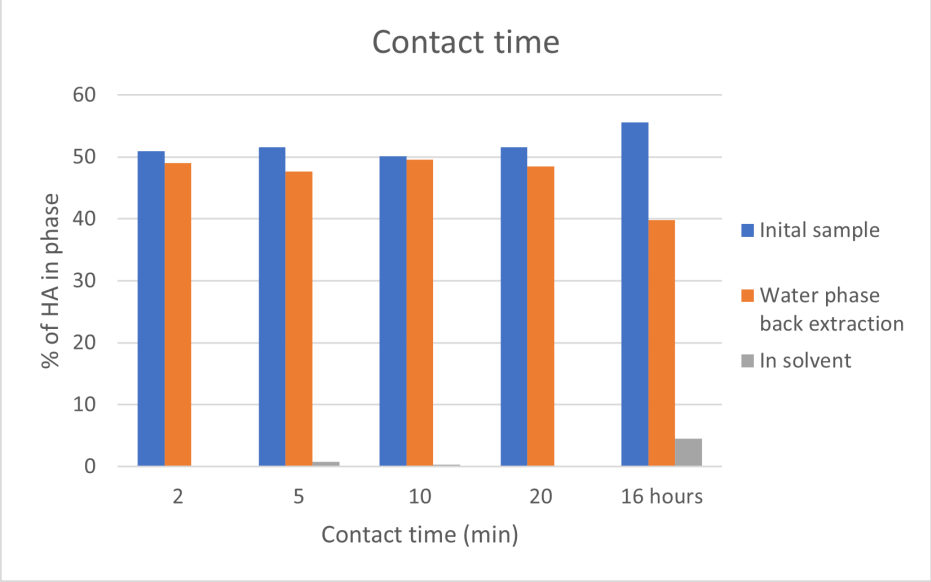

Figure 12: *The results of the experiments to determine the optimal contact time of the extraction of hippuric acid from the initial sample.*

As can be seen, the percentage of hippuric acid remaining in the initial sample does not vary along the different contact times. The experiment with a contact time of 16 hours is the only sample with a significant difference which is the opposite of what was expected when increasing the contact time between the phases. Therefore the contact time of the phases remained 2 minutes.

4.1.7 Multiple step extraction

Since the extraction using LLE is based on an equilibrium of the concentration of hippuric acid in both phases, using multiple extraction steps could increase the overall percentage of hippuric acid extracted out of the initial sample. First it was attempted to use the same volume of solvent, but separated in three different steps (3x 8,3 mL). This did not show any improvement compared to the previous experiments in which 25 mL was added at once.

Using 3 rounds of 25 mL of solvent gave the results shown in Figure 13

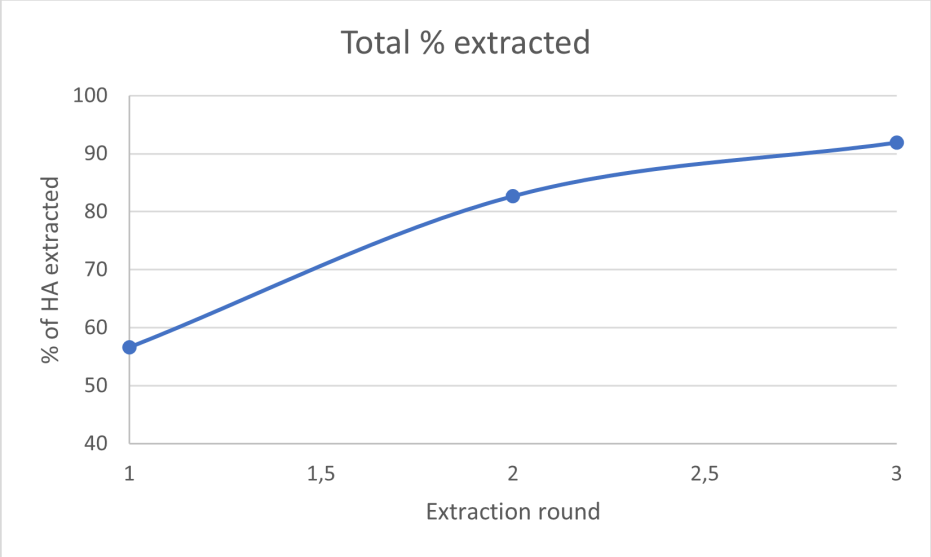

Figure 13: *The results of the experiments to determine the optimal amount of rounds using 25 mL of solvent.*

The amount of extracted hippuric acid increased with every round performed ending at a total extracted percentage of 91.9%. This was determined to be the last extraction step as using more steps would only increase the amount of hippuric acid extracted from the initial sample slightly.

4.1.8 Reuse of solvent

In consideration of the sustainability of the process, it was researched whether the used solvent could be reused for new samples. Three rounds of extractions were performed using the hippuric acid in water samples as the initial samples. This showed no decrease in efficiency. However, this was to be expected as there are no impurities within the initial sample. Therefore this test must be repeated using cow urine as a starting sample.

4.1.9 Optimal procedure

To summarize and give an overview of all experiments that have previously been discussed a list of the parameters chosen is given as an overview.

- Solvent choice: Ethyl Acetate
- Initial sample volume: 50 mL
- Solvent volume: 25 mL
- Water volume for back extraction: 50 mL
- pH initial sample: 3
- pH Water for back extraction: 12
- Contact time: 2 minutes
- Number of extraction steps: 3
- Reuse of solvent: undetermined

4.2 Cow urine samples

In this section, the results using cow urine as the sample will be discussed.

4.2.1 Optimal procedure

First, the optimal procedure previously tested on hippuric acid in water will be tested. In order to do so the Equation 2.1 should be adapted to fit the current experiments.

In the initial measurements of hippuric acid in water, a second peak was shown. This can be seen in Figure 17. After research, it was determined this must be a peak caused by benzoic acid. Figure 18 shows the peak of benzoic in roughly the same place as the second peak occurring in the pure hippuric acid sample. Therefore the initial amount of 0.1 mg/mL of hippuric acid should be adapted fittingly. After taking the presence of benzoic acid in the sample into account the pure hippuric acid sample contains 0.73 mg/mL. With the results of the hippuric acid in the HPLC Equation 2.1 can be adjusted as follows:

$$Concentrationcomponent(mg/L) = \frac{Areapeaksample \times 0.73(mg/L)}{840.149} \tag{4.1}$$

Firstly the results of the optimal procedure discussed in subsection 4.1.9 will be compared to those with the use of urine samples. The HPLC graphs are shown in section B.4. The results are summarized in Table 2

Table 2: *Results of using the optimal procedure on cow urine samples.*

	area (mAU.s)	concentration (mg/L)
urine	4438,628	3,846129
Urine after extraction	2111,004	1,829212
Water phase	1247,693	1,081142

This means that 52.4 % of the hippuric acid is taken out of the urine and 28.1 % of the hippuric acid ends up in the final water stage. This is significantly lower than the 91.9% that is removed from the hippuric acid in water samples. 24% of the hippuric acid remains in the solvent.

4.2.2 Reuse of solvents

When further analysing the results shown in Table 3 the following graph is made:

Table 3: *Results of reusing the same solvent for 5 cycles.*

	area (mAU.s)	concentration mg/L
urine	4438,628	3,846129
Urine after extraction round 1	2111,004	1,829212
Water phase round 1	1247,693	1,081142
Urine after extraction round 2	3071,076	2,661127
Water phase round 2	1086,453	0,941426
Urine after extraction round 3	1542,152	1,336295
Water phase round 3	761,189	0,65958
Urine after extraction round 4	2359	2,044104
Water phase round 4	826,743	0,716384
Urine after extraction round 5	3155,446	2,734235
Water phase round 5	608,128	0,526951

Table 4: *The results of high volume extraction of hippuric acid out of cow urine.*

	area (mAU.s)	concentration (mg/L)
Urine after extraction round 1	2824,886	2,4478
Water phase round 1	963,384	0,834785

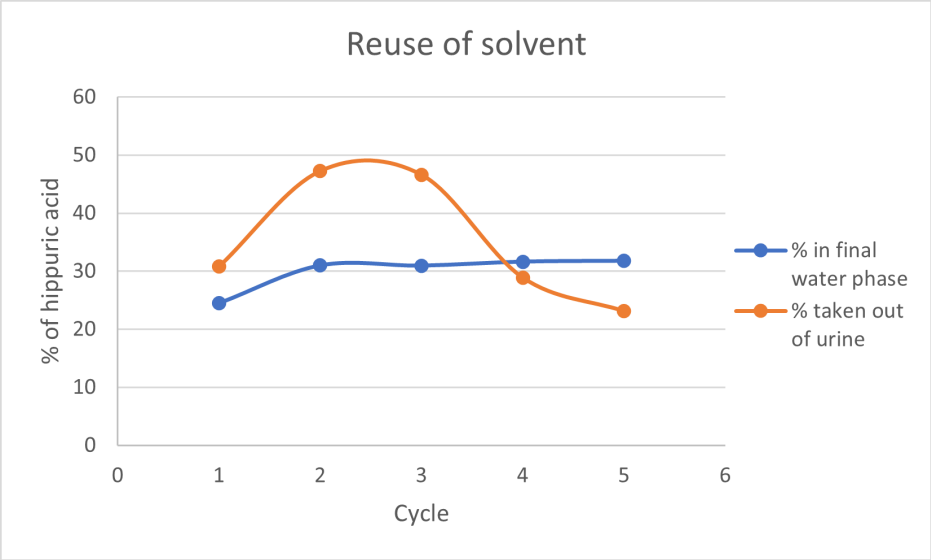

Figure 14: *An overview of the percentage of hippuric acid in the remaining urine and in the final water phase.*

The first measurement taken deviates from what is observed in the other measurements. When not considering the results of the first round it can be seen that the amount of hippuric acid taken out of the solvent remains the same over the 4 rounds. However, the amount of hippuric acid taken out of the urine sample decreases after round three. This might occur due to the fact that part of the hippuric acid remains in the solvent after back extraction. This could explain the decrease in hippuric acid taken out of the solvent while the amount of hippuric acid in the final water phase remains around the same levels. Overall the results show that it is possible to reuse the solvent for multiple cycles although the back extraction should be optimized in order to prevent the solvent from becoming saturated with hippuric acid.

4.2.3 Increased volume

To be able to predict the behaviour of the system when the volume is increased it was tested by increasing the initial urine sample from 50 mL to 500 mL, adapting the other volumes in ratio as well.

The results in Table 4 show that 21.7% of the hippuric acid ends up in the final water phase while a total of 36.4% is removed from the cow urine. This shows a decrease in comparison to the smaller-scale experiments. This could be due to the larger volume not being mixed to the same degree as the smaller samples were.

4.2.4 Crystallisation

As can be seen in the HPLC graphs in section B.4 the final water phase of these experiments still consist of two large peaks. In order to be able to further purify the hippuric acid the technique of crystallisation is used. For this experiment, the final water phase of the large volume experiment was used. After crystalizing the hippuric acid using a rotary evaporator the product was dissolved in water in order to be able to analyze it. Figure 15 shows the result of this experiment.

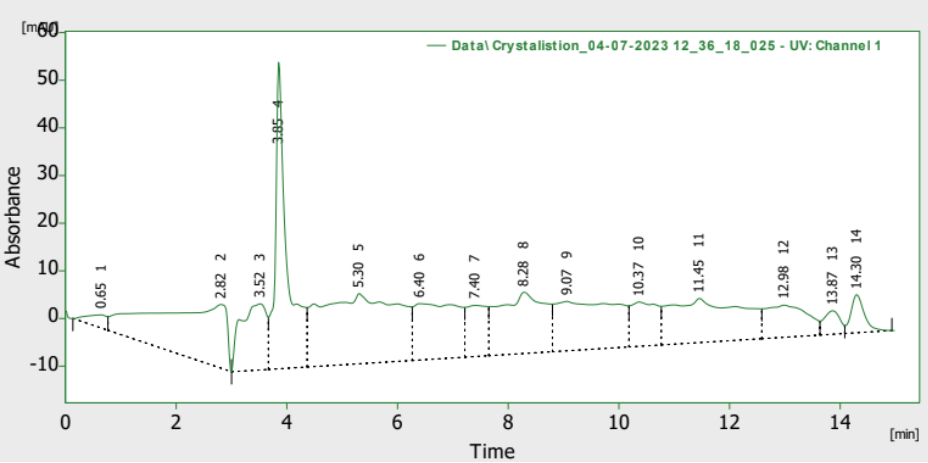

Figure 15: *The HPLC results of a sample taken after crystallisation dissolved in water.*

As can be seen only one peak remains which coincides with the peak of hippuric acid. No other impurities are shown in this graph. This shows that the aim of crystallisation to further purify the sample has achieved its goal.

5 Conclusion

The aim of this research is to determine the optimal procedure for extracting hippuric acid from cow urine. This was determined to be with the following parameters: Ethyl Acetate as solvent, a sample volume of 50 mL using 25 mL of solvent and 50 mL of water to perform the (back) extraction, a pH of 3 for the initial sample and a pH of 12 for the water phase, a contact time of 2 minutes and 3 extraction steps. This realised a total extraction of hippuric acid out of the water sample of 91.9% of which 53.4% ended up in the final water phase.

When using cow urine samples the results showed 52.4% of hippuric acid extracted out of the urine while 28.1% of the hippuric acid ended up in the final water phase. The difference between the water samples and urine samples most likely comes from the other impurities present in cow urine. The solvent can be reused multiple rounds while maintaining the same percentage of hippuric acid in the final water stage.

6 Discussion

The outcomes of this research have shown insight into the optimal parameters of the procedure to extract hippuric acid from cow urine. This section provides a reflection on the research process. When considering the solvent choice dimethyl carbonate was tested as it was shown to be the most effective for the extraction of hippuric acid into the solvent. However, during further experiments, a third layer appeared when using this solvent. No explanation has been found for this occurrence. The used materials has been cleaned to prevent the formation of this layer as well as letting it rest for extended amounts of time.

Furthermore, the measurements of pure hippuric acid with the HPLC showed two peaks instead of one. It was assumed that the second peak was due to benzoic acid being present in the sample as the peak of pure benzoic acid was close to that of the second peak in the hippuric acid sample. Other than that the peaks measured with the HPLC in the following cow urine samples deviated from that of pure hippuric acid slightly. Assumptions were made on which peak correlated to hippuric acid during the analysis of these results.

6.1 Future work

Within this section, a proposal for future work relating to this research will be discussed.

As mentioned in subsection 4.1.1 the results of dimethyl carbonate were initially better than those achieved with ethyl acetate. Therefore it would be of interest to further investigate dimethyl carbonate as well as the mixture of ethyl acetate and butyl acetate as those results were similar to that of ethyl acetate.

During initial experiments, the hippuric acid was almost fully extracted out of the solvent during the back extraction. During measurements with cow urine, this proved not to be the case. Therefore it will be of interest to research whether or not using multiple extraction cycles, like done for the first extraction step, could benefit the final outcome of the extraction. This could potentially also have a positive impact on the re-usability of the solvent as briefly discussed in subsection 4.2.2.

When considering the experiment performed on a larger volume the effect of contact time could be further explored as that might be the reason the results of this test are lower than those of smaller quantities.

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A Calibration curve

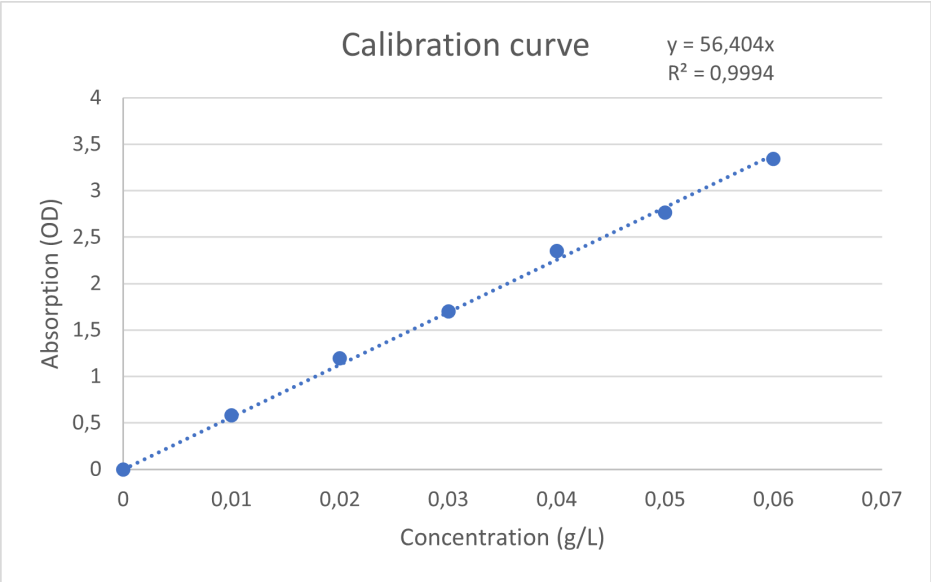

Figure 16: *The data used for the calibration curve for the spectrophotometer.*

B HPLC graphs

B.1 Pure Hippuric acid

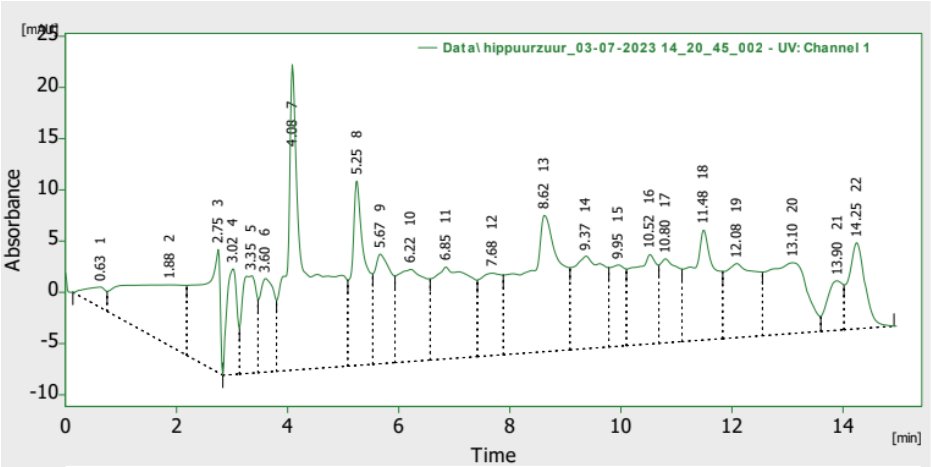

Figure 17: *The HPLC results of hippuric acid dissolved in water.*

B.2 Pure benzoic acid

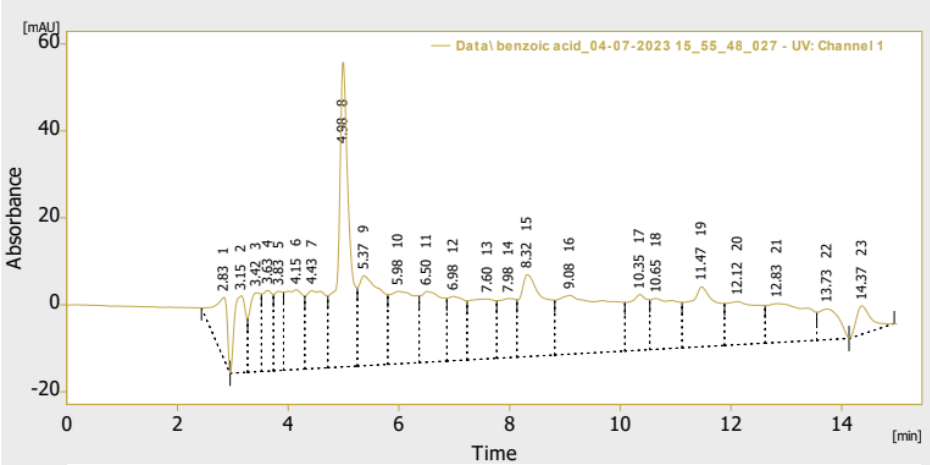

Figure 18: *The HPLC results of benzoic acid dissolved in water.*

B.3 Untreated cow urine

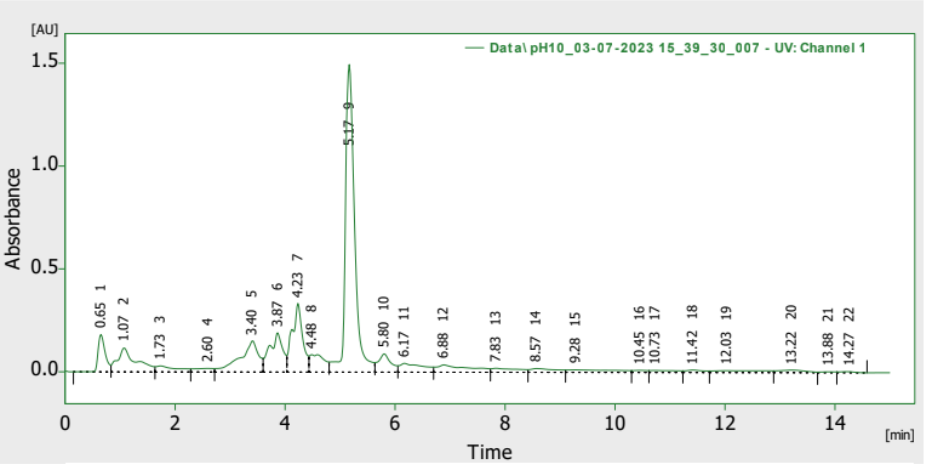

Figure 19: *The HPLC results of untreated cow urine*

B.4 Optimal procedure

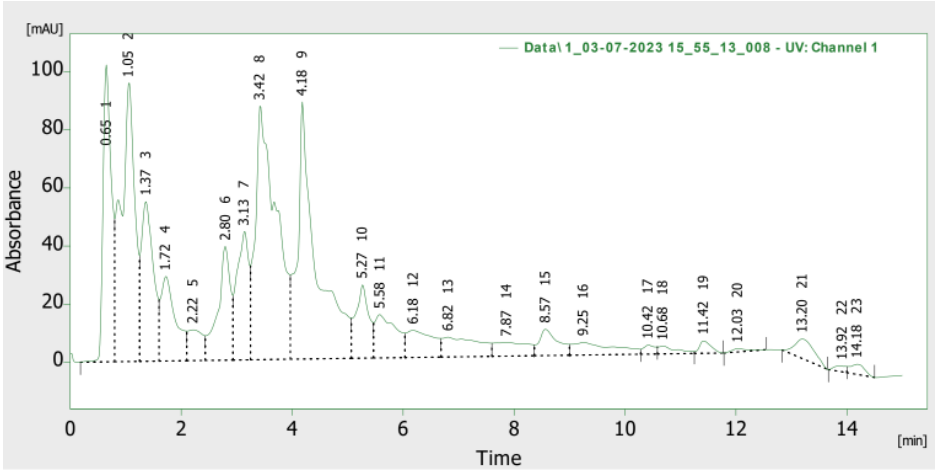

Figure 20: *The HPLC results of the urine after extraction following the optimal procedure.*

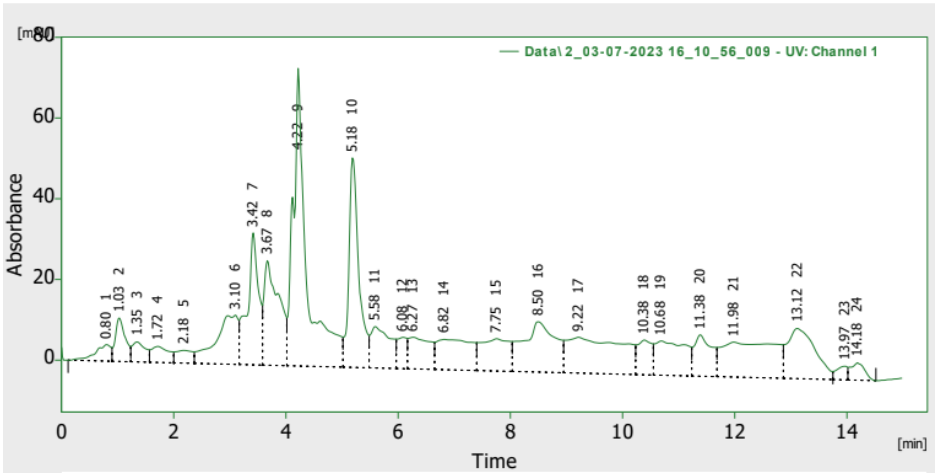

Figure 21: *The HPLC results of the water phase after extraction using the optimal procedure.*

B.5 Solvent reuse

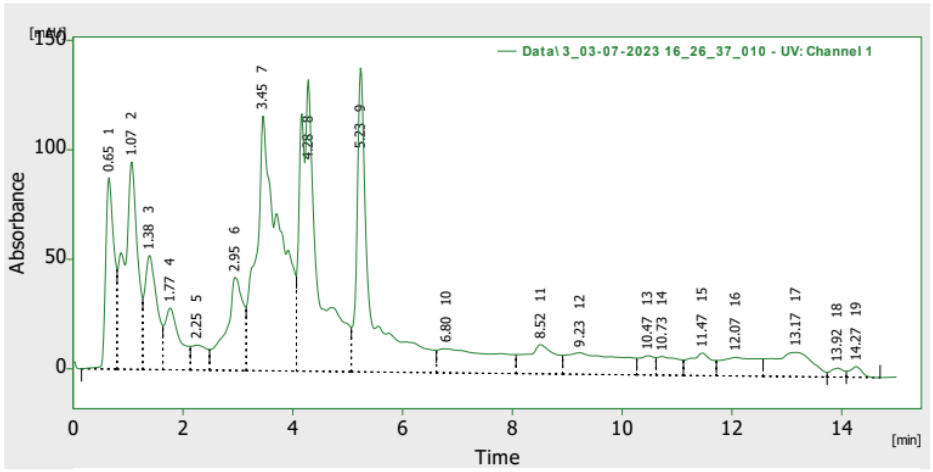

Figure 22: *The HPLC results of the urine after extraction following the optimal procedure.*

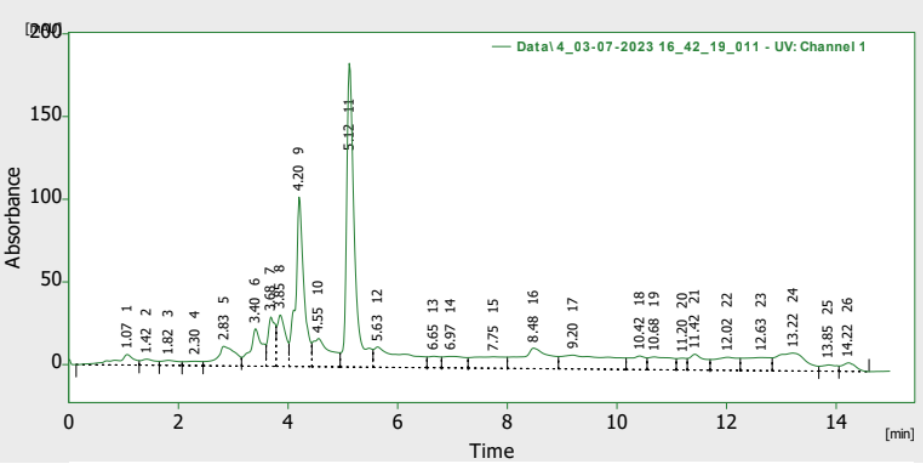

Figure 23: *The HPLC results of the water phase after extraction using the optimal procedure.*

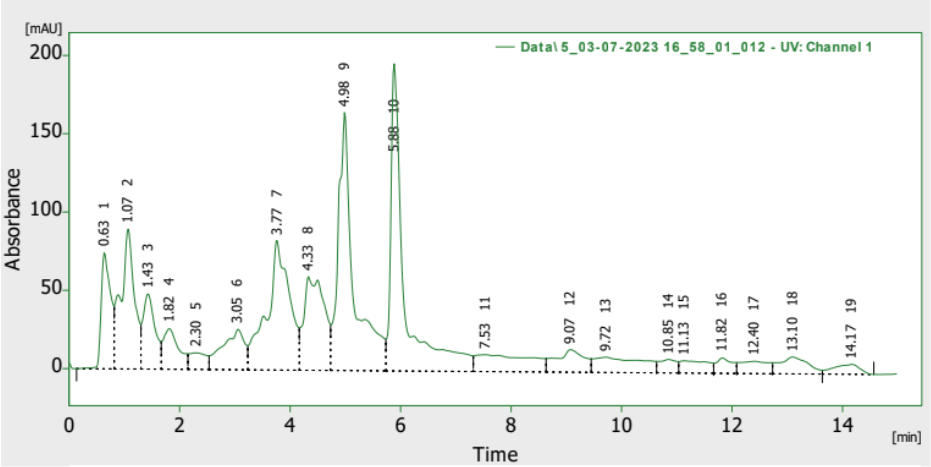

Figure 24: *The HPLC results of the urine after extraction following the optimal procedure in the second round.*

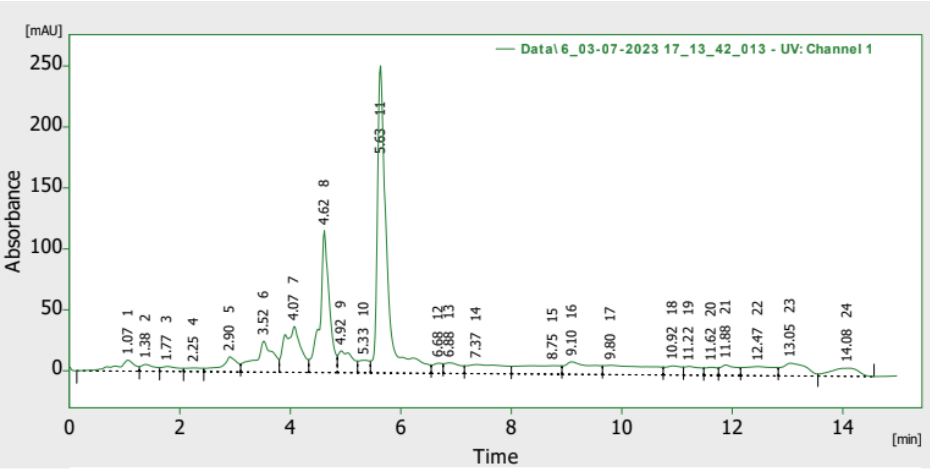

Figure 25: *The HPLC results of the water phase after extraction using the optimal procedure in the second round.*

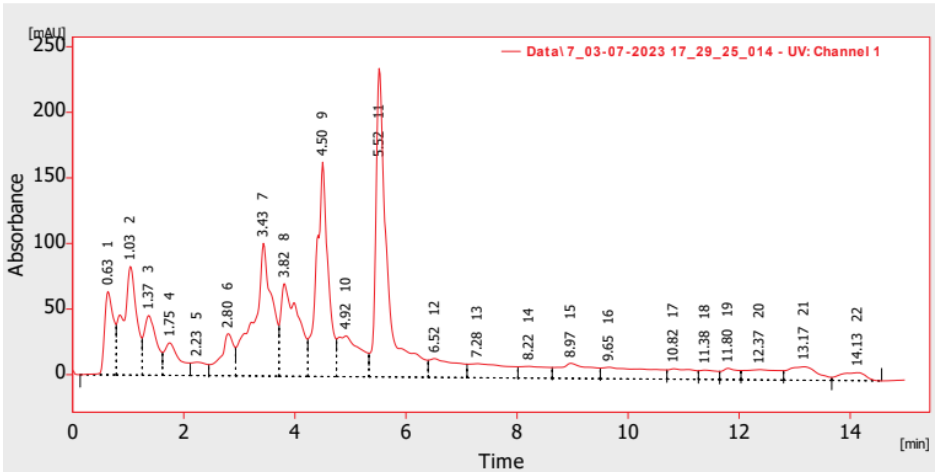

Figure 26: *The HPLC results of the urine after extraction following the optimal procedure in the third round.*

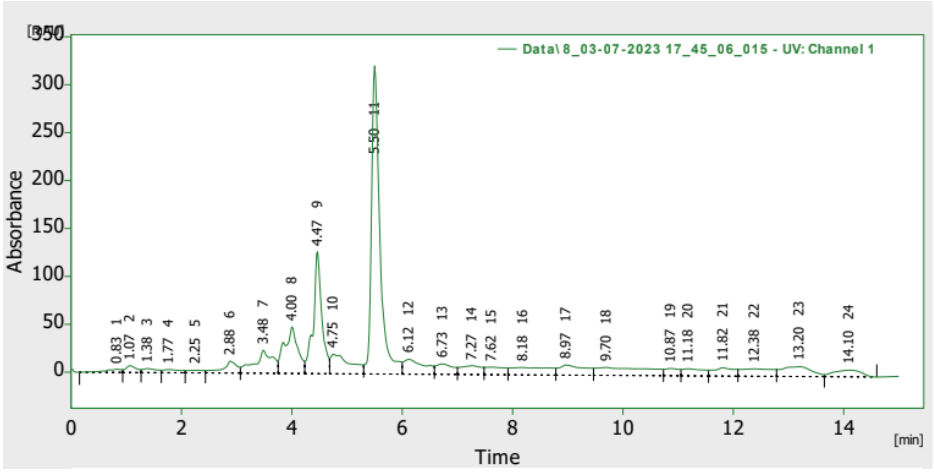

Figure 27: *The HPLC results of the water phase after extraction using the optimal procedure in the third round.*

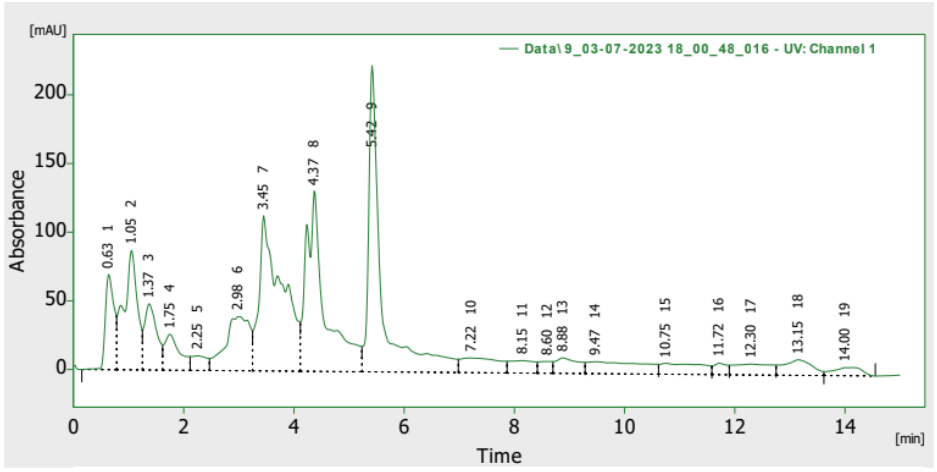

Figure 28: *The HPLC results of the urine after extraction following the optimal procedure in the fourth round.*

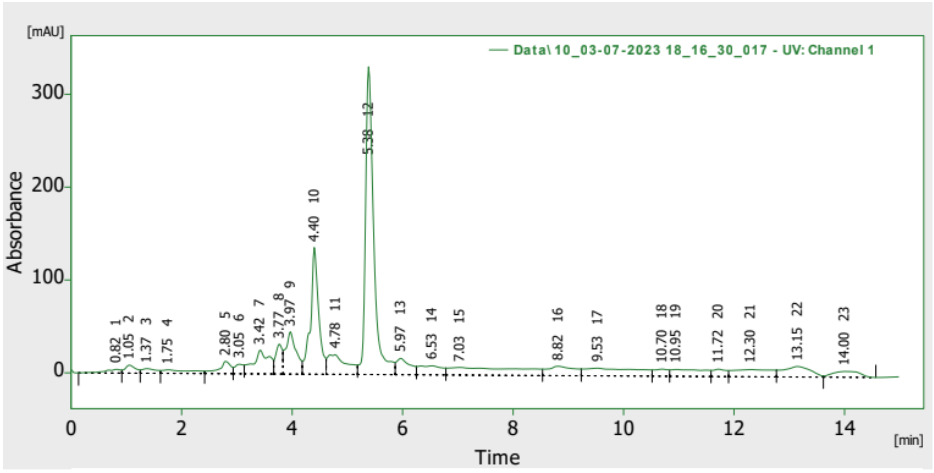

Figure 29: *The HPLC results of the water phase after extraction using the optimal procedure in the fourth round.*

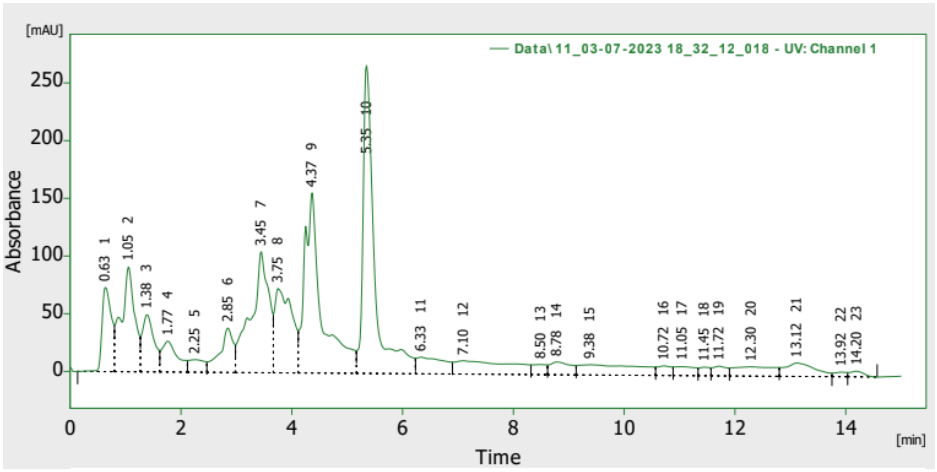

Figure 30: *The HPLC results of the urine after extraction following the optimal procedure in the fifth round.*

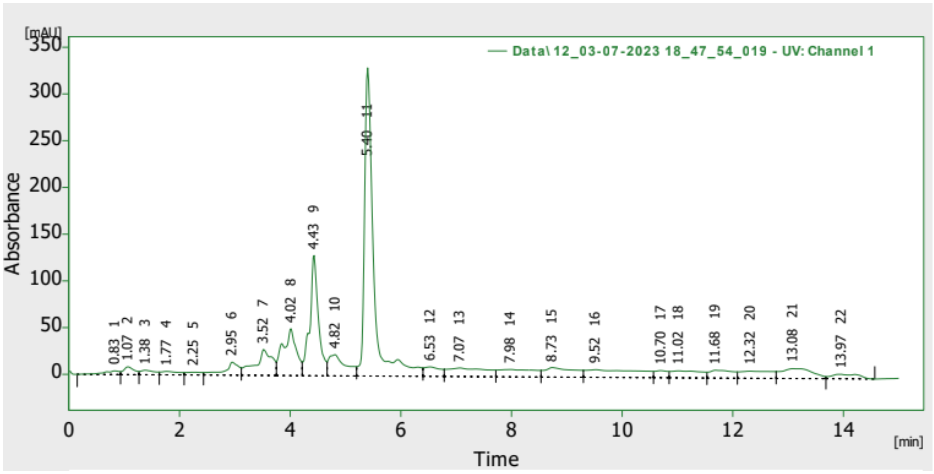

Figure 31: *The HPLC results of the water phase after extraction using the optimal procedure in the fifth round.*

B.6 Crystallisation

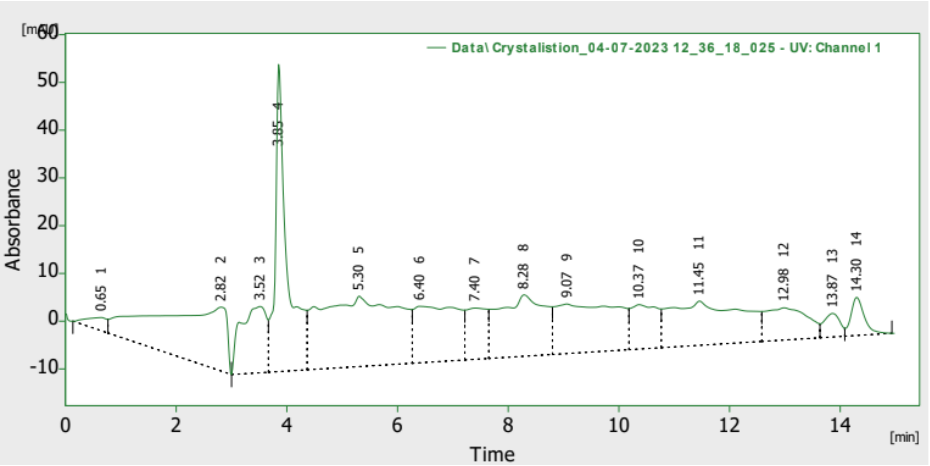

Figure 32: *The HPLC results of a sample taken after crystallisation dissolved in water.*