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MODIFICATION OF A SIA-SYSTEM BY ADDITION OF A STIRRER DEVICE (MIXING FLOW CELL)

Vindevoghel Wouter

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Summary

Modification of a SIA-system by addition of a stirrer device.

The Sequential Injection Analysis (SIA) was developed by Ruzicka and Marshall at the University of Washington and is an automated continuous flow technique. The principle of this method is to measure a continuous flow of reagents and samples with a spectrometer. These measurements form a graph with a peak and the area under this peak is in proportion with the concentration of the solution you want to measure.

Phenols or phenolic compounds are chemical compounds that are comprehensive researched, more specifically in food products. In these products, they can be seen as a quality parameter. When phenols are present in food products, there is a relation with the flavor and the color of the food. The group phenols include a large number of compounds. To determine the entire group of phenolic compounds, the colorimetric Folin-Ciocalteu method is the most common method.

When using a flow system for the determination of phenols, they minimize the analyst intervention, increase the sampling rate and improve the precision of the measurements but they show also great potential to the development of cleaner analytical methods owing to the lower waste generation.

In previous theses, measurements are made with phenols and phenolic compounds. To determine these compounds, a sample and two reagents are needed, but due to bad mixing (no good reaction) between the reagents and the sample in the tubes of the SIA-system, this method is not optimum.

The goal of this work is to improve the measurements of one sample and two reagents. A stirrer device (mixing flow cell) is added to the SIA-system to improve the mixing between the sample and the two reagents. The mixing flow cell can also be used to make dilutions from a standard for calibration purposes.

First measurements with phenol red are done to introduce the mixing flow cell in the SIA-system and to make the first trials to optimize the stirring cell. For the determination of phenols, calibration lines are made with gallic acid standards. To determine phenols or gallic acid, the Folin-Ciocalteu reagent and sodium carbonate are needed. The reaction between phenolic compounds and the yellow F-C reagent takes place around a pH of 10, by adding sodium carbonate and forms a blue color that is measured at a wavelength of 760 nm.

The researched parameters are: accuracy, precision and linearity. When introducing the mixing flow cell into the SIA-system, the method for phenols with a waiting time of 2 minutes gave a good accuracy, linearity and precision from 10 until 100 ppm with an injection volume of 100 µl of the standard. If it is necessary to determine a lower concentration (lower than 10 ppm), the injection volume of the standard must be changed to 900 µl. This is accompanied with a bad precision, but the linear range and the accuracy are still good.

Acknowledgments

One year ago I was presented the choice to finish the final project of my studies abroad. I decide to do it, and four months ago I started with this amazing adventure. My stay in Girona was influenced by a lot of people, and here I would like to take the opportunity to thank a few people who helped me to make it a wonderful experience.

First of all I want to thank the KaHo Sint-Lieven (ODISEE), to give me the chance to go on Erasmus to Girona. I also want to thank Patrick Demeyere, my training mentor during this time, for his support, feedback and his help with my thesis.

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My internship would be completely different without Ben Van Elsuwé. I want to thank him for the amazing stay in Girona. I also would like to thank my lab partners at the Udg for helping when needed and for making the lab an amazing place to work.

Last, but not least, I want to thank my parents and my girlfriend for giving me the opportunity to go on Erasmus. They supported me from the beginning till the end and they were there when I need them. After this adventure I realize how much they care about me and how much they daily do.

I want to say that the hole Erasmus experience helped me to become a more mature and independent person. Thank you to everybody who made this an experience I will never forget.

Wouter

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1 INTRODUCTION (ADDITION OF A MIXING FLOW CELL)

Phenols or phenolic compounds are chemical compounds that are comprehensively researched, more specifically in food products. In these products, they can be seen as a quality parameter. When phenols are present in food products, there is a relation with the flavor and the color of the food. The group phenols include a large number of compounds. To determine the entire group of phenolic compounds, the colorimetric Folin-Ciocalteu method is the most common method.

The metals and Environment Research Group of the UDG is doing experiments with coffee and cork to adsorb organic compounds and metals in wastewaters. To determine these compounds, they also need to test the total phenolic content in a large number of samples. The classical Folin-method requires a long period (2 hours) for the colorimetric reaction which makes the analysis a tedious procedure. The use of a SIA continuous analysis system can be a reliable alternative for a fast, cheap and accurate analysis of total phenols in those samples.

1.1 PHENOLS

Phenols are aromatic compounds containing one or more hydroxy groups bonded on benzene ring. The aromatic compound with one hydroxy group is commonly known as the phenol and all other derivatives of phenol are called the phenolic compounds. In figure 1, the structure of the simplest phenolic compound (phenol, hydroxybenzene or carbolic acid) is shown. [University of Calgary, 2015]

1.1.1 Structure

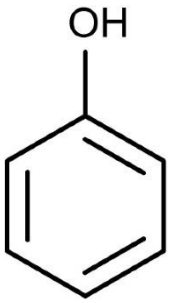

Figure 1: Structure of hydroxybenzene

The alcohol functional group consists of an O-atom (oxygen) bonded to a sp^2-hybridised aromatic C-atom (carbon) and a H-atom (hydrogen) via σ bonds. Both the C-O and O-H bonds are polar due to the high electronegativity of the O-atom.

In figure 2, the conjugation that happens between an unshared electron pair on the oxygen and the aromatic ring, is shown.

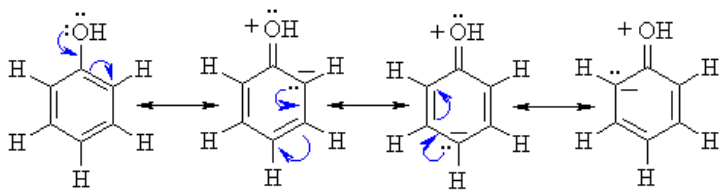

Figure 2: Conjugation of the phenol structure [University of Calgary, 2015]

This result in, compared to simple alcohols:

- a shorter C-O bond distance;
- a less basic hydroxyl oxygen;
- a more acidic hydroxyl proton.

1.1.2 Properties

1.1.2.1 Physical properties

Basic phenols with a low molecular weight are liquids. The reason of this can be seen in figure 3. These phenols have the capacity to form intermolecular H-bonds with other phenol molecules or other hydrogen bonding systems. [Chemguide, 2015]

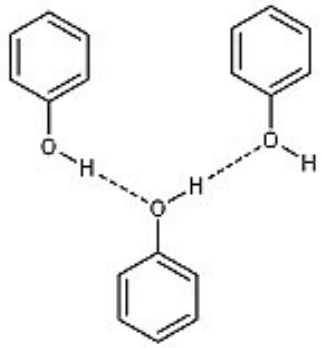

Figure 3: Intermolecular H-bonds [University of Hofstra, 2015]

In table 1, another characteristic of intermolecular H-bonds can be found. This characteristic is that phenols have a good solubility in aqueous media. This increases with more bonded hydroxyl groups. [Hugelier, 2013-2014]

Table 1: Chemical and physical properties of some phenols and alcohols

Name	Structural formula	Molecular weight (g/mol)	Solubility in water (g/100ml)	Boiling point (°C)	pKa
o-Catechol	$C_6H_5(OH)_2$	104	45	245	9,50
Phenol	C_6H_5OH	94,11	9,3	182	9,98
1-pentanol	$CH_3(CH_2)_4OH$	88,15	2,2	138	16,84
1-hexanol	$CH_3(CH_2)_5OH$	102,17	0,59	156	/

1.1.2.2 Acidity

In aqueous media, the phenolate ion or the phenoxide is formed after phenol gives its proton away. This can be seen in figure 4.

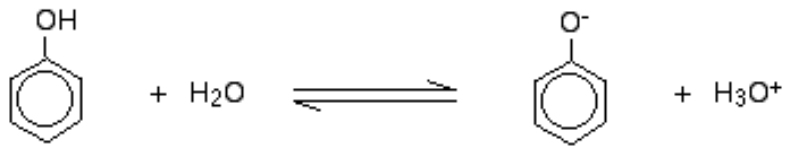

Figure 4: Acidic nature of phenol

Phenols are more acidic than alcohols, it can be seen from the pKa values in table 1: the lower the pKa value, the stronger the acidity of the compound.

In figure 5, the reason of increase in acidity is shown.

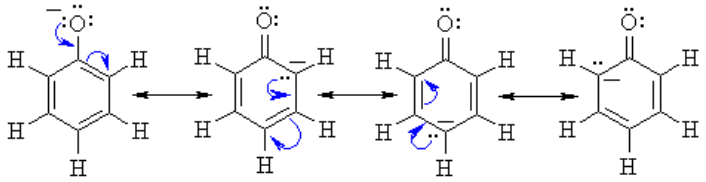

Figure 5: Stabilization of the phenolate ion by resonance [University of Calgary, 2015]

The increase of acidity is because of resonance due to electron delocalization onto the ring.

1.1.2.3 General antioxidant mechanisms of phenolics

Phenols are able to act as antioxidant in a number of ways. Phenolic hydroxyl groups are good H-donors: hydrogen-donating antioxidants can react with reactive oxygen and reactive nitrogen species in a termination reaction, which breaks the cycle of making new radicals.

A radical form of the antioxidant having a much greater chemical stability than the initial radical. The interaction of the hydroxyl groups, of phenolics with the π-electrons of the benzene ring gives the molecules special properties, most notably the ability to generate free radicals where the radical is stabilized by delocalization.

The antioxidant capacity of phenolic compounds is also attributed to their ability to chelate metal ions involved in the production of free radicals. However, phenolics can act as pro-oxidants by chelating metals in a manner that maintains or increases their catalytic activity or by reducing metals, thus increasing their ability to form free radicals. [PEREIRA, D.M., 2009]

1.1.3 Polyphenols

Polyphenols are a group of chemicals found in many fruits, vegetables and other plants such as berries, walnuts, …. They are classified as antioxidant, meaning that they remove free radicals from the body. These organic chemicals are characterized by the presence of large multiples of phenol structural units. The number and characteristics of these phenol structures determine the unique physical, chemical and biological properties of particular members of the class. [Phytochemicals, 2015]

Polyphenols are divided in three main subclasses:

- Flavonoids,
- Stilbenoids,
- Phenolic acids.

1.1.3.1 Flavonoids

Flavonoids are water soluble molecules containing 15 carbon atoms and they belong to the polyphenol family. Flavonoids can be visualized as two benzene rings which are joined together with a short three carbon chain. One of the carbons of the short chain is always connected to a carbon of one of the benzene rings, either directly or through an oxygen bridge, thereby forming a third middle ring, which can be five or six-membered. This can be seen in figure 6. [Phytochemicals, 2015]

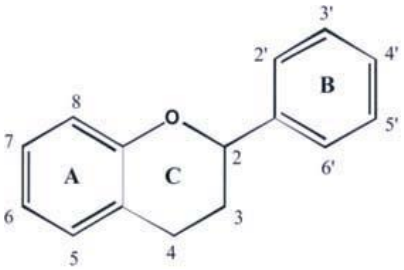

Figure 6: Flavonoids basic structure

The flavonoids consist of 6 major subclasses, as a function of the type of heterocycle that is involved and based on the position of the B ring relative to the C ring. Flavonoids are responsible for the coloring of fruits, vegetables and herbs. [Phytochemicals, 2015]

1.1.3.2 Stilbenoids

Stilbenoids are the smallest subclass of the three. Their basic structure consists out of 1,2-diphenylethylene or 1,2-diphenylethane. An example of a stilbenoid is shown in figure 7. [Wikipedia, 2015]

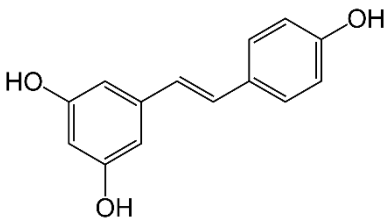

Figure 7: Structure of resveratrol

1.1.3.3 Phenolic acids

This group of polyphenols can be divided in two main groups: acids that are derived from benzoic acids, containing seven carbon atoms or from cinnamic acids, containing nine carbon atoms. One of the main members of this subclass is gallic acid (figure 8). [BORREMANS, R, 2014]

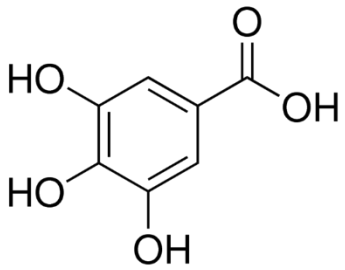

Figure 8: Structure of gallic acid

1.1.4 Analytical determination

The most common method for analyzing phenolics and polyphenols is the Folin-Ciocalteu method (F-C method). The F-C reagent consists of a mixture of phosphomolybdate and phosphotungstate. The reaction between the phenolic compounds and the yellow F-C reagent takes place around a pH of 10, by adding sodium carbonate. With these alkaline conditions, dissociation of a phenolic proton leads to the formation of a phenolate ion, which is capable of reducing the F-C reagent. When the F-C reagent is reduced, a blue color is formed. The change of colors can be seen in figure 9. [SANTOS, S.A.O, 2010] and [BLAINSKI, A, 2013]

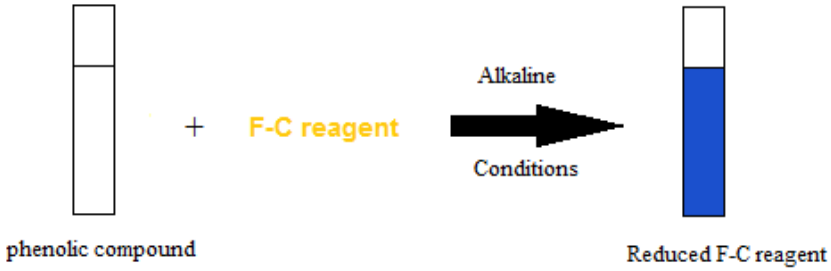

Figure 9: Phenolic determination with F-C reagent [BORREMANS, R, 2014]

In this thesis, the determination of phenolics is done by making a calibration line with gallic acid standards. Gallic acid is a common phenolic compound, it is soluble in water, it can be recrystallized easily from water and is stable in its dry form.

To make the calibration line, different amounts of gallic are prepared and mixed in the SIA-system with the reagents. At the end the same is done with the samples. After a waiting time of 2 hours, the reaction is fully completed and stable. The samples and standards are measured with a spectrophotometer at a wavelength of 760 nm. With the measurements of the standards, a calibration line can be made where each made standard of gallic acid represents an amount of phenolic molecules. The measurements of the samples are processed into the calibration line and the concentrations are calculated. The results of the phenolic samples are usually displayed as gallic acid equivalents (GAE). [SANTOS, S.A.O, 2010]

1.2 AUTOMATION IN ANALYTICAL CHEMISTRY

In the analytical chemistry is the automation of analytical analysis, due to the development of micro robotics, microelectronics, and computing for controlling multiple devices very important and there is also an widespread use of it. The automation of the analytical processes can be made in several ways. There are automated analyzers for discrete samples for example: flow analyzers and robotic analyzers.. The evolution in time is from discontinuous to continuous. These are more automatized, faster and spend less reagents.

1.2.1 Continuous methods

Continuous methods are automated chemical analyzers in which the samples and reagents are pumped continuously through a system of modules interconnected by tubing.

Continuous methods, that are explained in detail in chapter 1.3 (flow analysis techniques), are for example the AutoAnalyzer. This is an automated analyzer using a flow technique called flow analysis (CFA). The design is based on separating a continuously flowing stream with air bubbles. Other examples of continuous methods are FIA and SIA.

1.2.2 Discontinuous methods

These are methods where only one sample at the time can be measured, there is no continuous flow of reagents and samples. For example when using an automatic titrator, as can be seen in figure 10, the titrant is not continuous added to the solution of unknown concentration.

A titrator is an instrument which allows the automation of all operations involved in titration: titrant addition, monitoring of the reaction (signal acquisition), recognition of the endpoint, data storage, calculation and results storage.

Figure 10: Automatic titrator

1.2.3 Laboratory robotics

Laboratory robotics is the act of using robots in biology or chemistry labs. They are doing assay analysis and automating the movement of test tubes in research laboratories. Because of the high number of samples that need analysis and the amount of data collection required, the process and costs are easily validated with robotics. Advanced laboratory robotics can be used to completely automate the process of science. Robots in laboratory, life science and pharmaceutical applications perform tasks at rates beyond human capability.

With the use of robots in a laboratory, a discontinuous method can be "transformed" into a continuous method. For example the automatic titrator.

1.2.3.1 Advantages

One of the advantages to automation is faster processing, but it is not necessarily faster than a human operator. Other advantages are the repeatability and reproducibility that are improved in automated systems. Typically productivity is increased since human constraints, such as time constraints that are no longer a factor. Also the efficiency is generally improved as robots can work continuously and reduce the amount of reagents used to perform a reaction. There is also a reduction in material waste. [PATEL, S.N., 2014]

1.2.3.2 Disadvantages

Typically the costs of a single synthesis or sample assessment are expensive to set up and the startup cost for automation can be expensive. A second disadvantage is that many techniques for automation have not been developed yet. Additionally, there is difficulty automating instances where visual analysis, recognition or comparison is required such as color changes. Another potential disadvantage of robots is an increase in job shortage as automation may replace staff members who do tasks easily replicated by a robot. [PATEL, S.N., 2014]

1.3 FLOW ANALYSIS TECHNIQUES

1.3.1 Continuous Flow Analysis

CFA refers to any process in which the concentration of the analyte non-stop is measured in a stream of liquid or gas. The principle of continuous flow analysis is to eliminate chemical analysis by hand-mixing of reagent in individual items of glassware and to substitute a continuous flow stream of liquid reagents circulating through a closed system of tubing. In the CFA, the sample is converted into a flow stream by a pumping system and reagents additions are made by continuous pumping and merging of the sample and the reagent streams. The mixing and the chemical reactions take place while the sample solution is on its way toward a low-through cuvette, where the analytical signal is continuously monitored and recorded. [CHEREGI, M., C.]

The continuous flow methods can be classified into three groups.

1.3.1.1 Continuous mixing methods

This method (figure 11) involves the sample insertion into the system, mixing it with the carrier or reagent, measuring the reaction plug as it passes through a suitable detector and it sending to the waste.

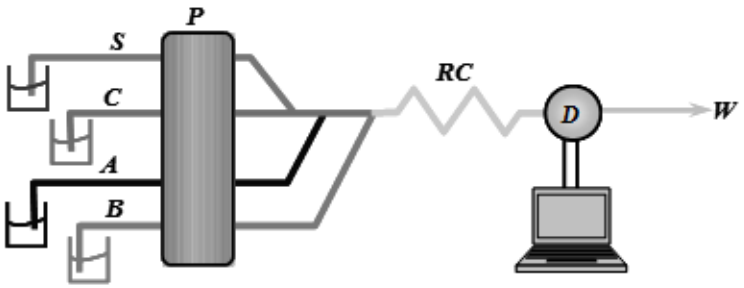

Figure 11: Continuous mixing method

S= sample; C= carrier; P= pump; A,B= reagents; RC= reaction coil; D= detector; W= waste

1.3.1.2 Stopped-flow continuous mixing methods

In this system the flow is stopped at various stages during the process in order to prevent air from entering the system between sample aspiration and reagent aspiration or washing.

1.3.1.3 Continuous-flow titrations

Here, the sample or titrant is continuously inserted into the system either by keeping its speed or that of the titrant constant and changing that of respectively other so measurements can be made as a function of the analytical signal obtained.

1.3.2 Segmented Flow Analysis

The segmented flow analysis (SFA) was the earliest contribution in the field of automated methods development. It originated from the scientific paper of Dr. L Skeggs (University of California, USA) published in 1957 and has found widespread use in almost every facet of analytical chemistry.

This system that is shown in figure 12, is characterized by the use of one or more several liquid streams where the samples are introduced by sequential aspiration and separated by means of air bubbles. Therefore, the final liquid stream is segmented into small discrete liquid slugs by bubbles of air or other gas that entirely fills the stream tubing bore.

The sample and the reagents are mixed by passing through glass coils and through a temperature controlled heating coil if heat is required to speed the development of the reaction before detection.

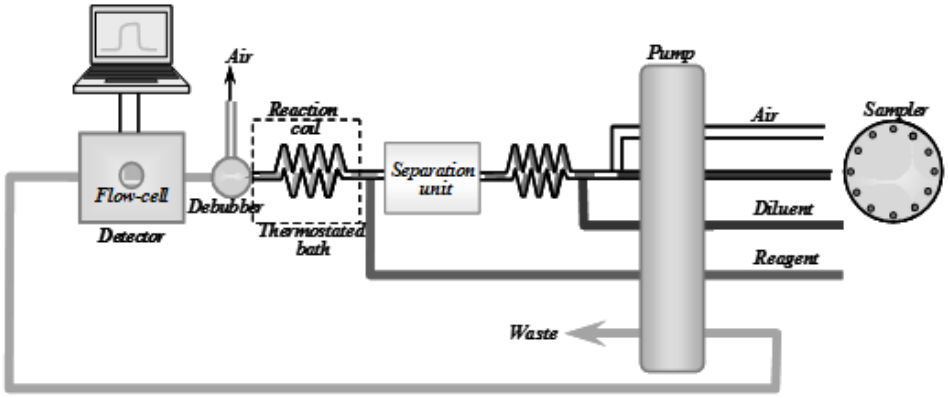

Figure 12: Schematic overview of the SFA [CHEREGI, M., C.]

1.3.3 Flow injection analysis (FIA)

FIA (Flow Injection Analysis) was defined by Ruzicka and Hansen in 1975. The first definition that they have given to FIA was: "FIA is a technique for information-gathering from a concentration gradient formed from an injected, well-defined zone of a fluid, dispersed into a continuous unsegmented stream of a carrier".

So FIA is an automated method in which a sample is injected into a continuous flow of a carrier solution. The stream has a purpose to deliver the sample to the detector and to mix the sample with the carrier and the reagent. A typical FIA manifold is comprised of a pump, injection valve and a flow cell with detector as can be seen in figure 13. [Globalfia, 2015]

All these element are connected with Teflon-tubes with a diameter of 0,5-0,8 mm.

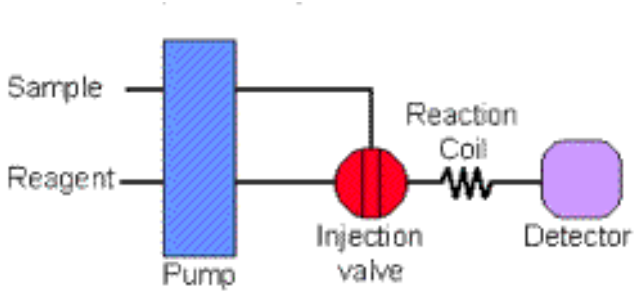

Figure 13: Schematic overview of FIA

The system has a constant flow of carrier and reagent in one direction due to the peristaltic pump. Because of the injection valve, it is possible to inject a sample volume in a reproducible way. The sample and the carrier flow together to the reaction coil. Here, both components are mixed with the reagent. Then, the detector can measure the resulting species. As a result, the detector will give a peak. The height of the peak or the area under the peak is in proportion with the analyte concentration. [GOMEZ, V.,2007]

FIA has many advantages such as automation, a high reproducibility and a lower sample and reagent use, so a lower waste of volume is the result.

In figure 14, the weakness of the FIA is shown. When the solution flows through the narrow tubes a physical phenomenon appears: dispersion.

Figure 14: Dispersion [Reunes, L., 2012]

When dispersion takes place, the solution takes a parabolic form during flow as can be seen in figure 14. This will have consequences for the determination of the analyte. Control of the degree of dispersion is needed for reproducible results.

Factors that will have an influence on the degree of dispersion are: flow rate, tubing ID, type of reactor (e.g. coil, knotted, static mixer, straight, serpentine), length of tubing, and internal architecture of components such as valves, detectors, and connectors, all of which are readily controlled.

When the signal is at a maximum, the following formula is applied:

$$D = {}^{C_0}\!/_{C_g}$$

C_0: analyst concentration before dispersion
C_g: concentration in the dispersed zone

There are actually two types of dispersion: axial and radial. Axial dispersion occurs in the direction of stream flow and causes greater dilution and peak broadening than radial dispersion. Axial dispersion dominates in a straight tube. Radial dispersion is caused by flow patterns in the stream that circulate normal to the direction of flow, and thus cause mixing with minimum dilution and peak broadening. Radial dispersion is promoted by serpentine and knotted tubing since they lead to greater sensitivity ad narrower peaks. [Globalfia, 2015]

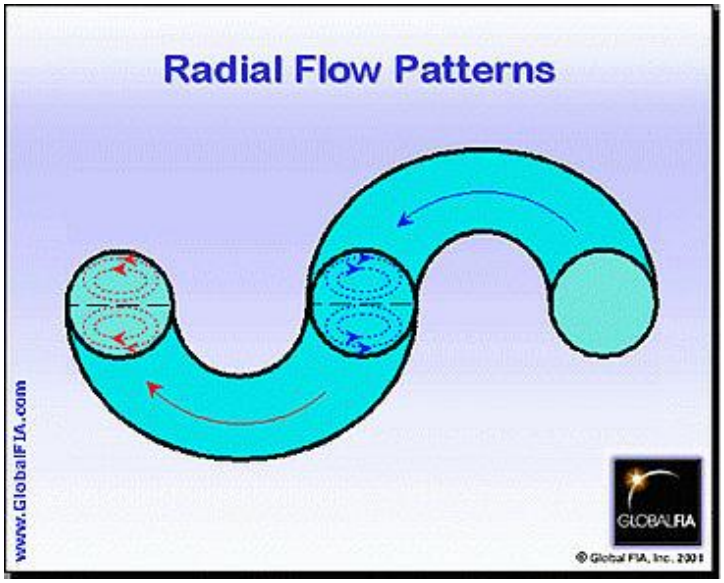

Figure 15: Radial flow patterns by turns in the flow path [Globalfia, 2015]

As shown in figure 15, the direction of the radial flow pattern reverses when the direction of the flow reverses.

Nowadays FIA is used for a lot more applications. By example pharmaceutical analysis and quality control, environmental monitoring and bio analytical applications.

1.3.4 Sequential injection analysis (SIA)

The Sequential Injection Analysis (SIA) was developed by Ruzicka and Marshall at the University of Washington in the laboratories of the Centre for Process Analytical Chemistry (CPAC°) and it is a generation of flow injections based techniques with a response to the problems of the first generation (FIA). The main problems were:

- In certain cases the flow manifold needs multichannel setups that must be reconfigured for every different analysis.
- A long term use of a peristaltic pump does not provide a stable flow rate, so changing the pump every time it breaks costs a lot.
- FIA has a lot of waste material because of its continuous flow.

The concept of SIA is based on the FIA-system but has a few adjustments (Figure 16). The biggest differences are:

- The use of a multiposition valve;
- Flow reversal.

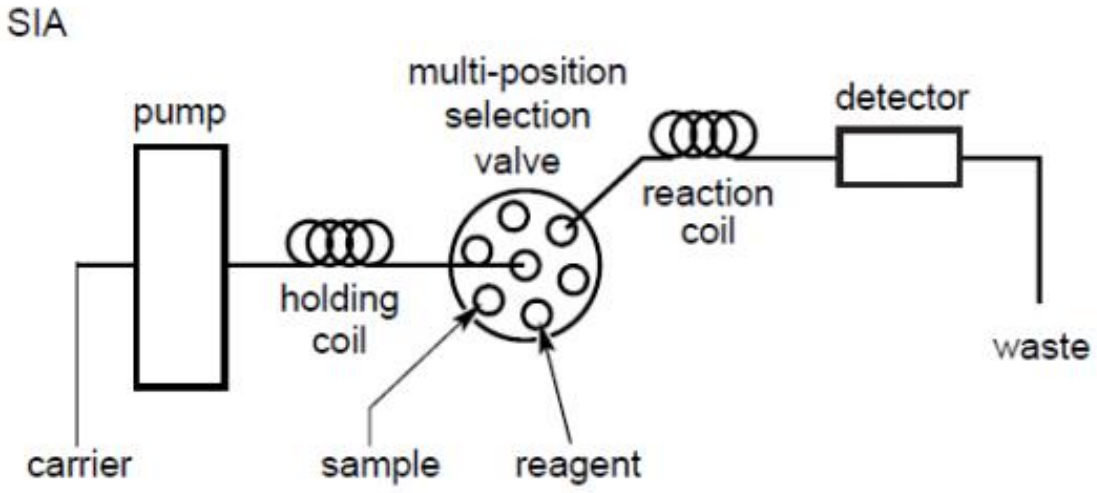

Figure 16: Schematic overview of SIA [REUNES, L, 2012]

In the SIA-system, the pump and injection valve work synchronously with the help of a computer. This system has, apart for the reaction coil, also a holding coil and a detector. Here, all the items are connected with Teflon tubes.

This system is working with a reversal flow, made possible by the pump, which can work in 2 directions. When the pump is working is reverse direction, precise volumes of samples and reagents can be taken. The samples and reagents will form different zones in the holding coil, as can be seen in figure 17a. Then the direction of the pump is changed and simultaneous, the valve from the reaction coil opens. This is flow reversal and the samples and reagents can flow to the reaction coil. Due to dispersion and diffusion, the sample and reagents diffuse in each other and form an overlapping zone. This phenomenon is shown in figure 17b. [BORREMANS, R, 2014]

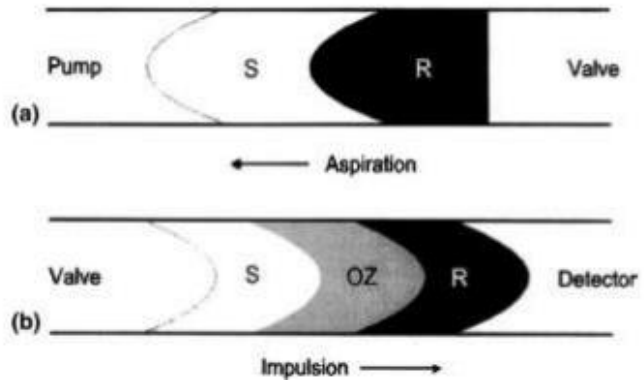

Figure 17: Zone forming a) Holding coil, b) Reaction coil; S= sample, R= reagent, OZ= overlap zone

Figure 18 is shown to understand the process of diffusion better.

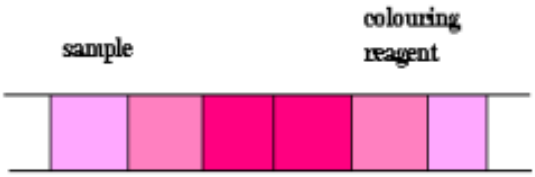

Figure 18: Development of the sample and reagent mixing

First the interfaces of both solutions will diffuse. If the diffusion is proposed in color intensity, the color of the interface will be the most intense. As the distance becomes larger, there will be less diffusion and the intensity will decrease.

As the solution flows through the SIA system, it reaches the detector, where the absorbance of the solution is measured. In the overlapping zone, the intensity is higher, so the signal will be higher as well. The detector in a SIA system can be a spectrophotometer, a voltametric detector, a mass spectrometer and others. [TEMMERMAN, T., 2009]

The advantages over FIA are the reduction of sample and reagent volumes, because it uses a syringe pump, the reagent flow can easily be stopped and reversed and due to the use of selection valve different sample and reagent streams can be selected. But there is also a downside on the SIA-system. The disadvantages of this system are the lower sample rate and the unavailability of software. But in the last case, software is developed by many institutes, so soon there will be universal software available. [WEIHONG, X., 2005]

Nowadays SIA is used for single-component analysis, but it's a very powerful technique. Multi component analysis can be done by this technique in just one single step by using different strategies such as solid-phase extraction, sequential injection chromatography, … [GOMEZ, V., 2007]

1.3.5 Lab-on-valve system (LOV)

LOV is the third generation flow injection technique. It's a miniature version of the sequential injection analysis, therefore it's fast and efficient. This system uses volumes of liquids but also gases. An advantage of this is the reduction of waste and of the amount of reagents needed.

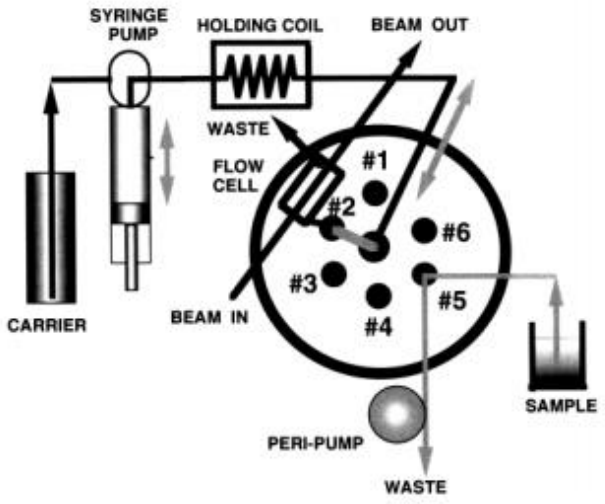

Figure 19: Schematic overview of LOV [RUZICKA, J., 2000]

In figure 19 the basic concept of the LOV-system is shown. It's very similar to the SIA-system. It has also a syringe pump to transport the carrier solution through the system and it can manipulate the flow.

The LOV has a couple of disadvantages like clogging of the system. This can be prevented by a good sample pretreatment of solid components. The second disadvantage is the absence of temperature control. This can be solved by placing the LOV manifold in an air thermostatic incubator. The use of organic solvents should be avoided if the LOV is fabricated from Plexiglas. [DE CASTRO, M.D., 2008]

1.4 ANALYSIS OF PHENOLS IN SIA-SYSTEMS

Phenols are extensively researched, for example in food products. Where they are seen as a quality parameter. Their presence is related to flavors, colors and sometimes to nutritive properties of foods. So in general the analyses of phenols are really needed. In this work the analysis of phenols are done with the SIA-system.

When using a flow system for the determination of phenols, they minimize the analyst intervention, increase the sampling rate and improve the precision of the measurements but they show also great potential to the development of cleaner analytical methods owing to the lower waste generation. [DOLATTO, R.G., 2012]

With the SIA-system there are some limitations when determine different compounds. A first limitation of the SIA-system is that only 1 sample and 2 reagents (3 compounds) can be used because otherwise there is no good mixing between the reagents and the sample in the tubes of the SIA-system. For example these problems occur when determine chromium (III) and phenols or phenolic compounds.

With the introduction of a stirrer device (mixing flow cell), a possible solution is tried for the bad mixing between the sample and the 2 reagents (improve the measurements). The mixing flow cell can also be used to make dilutions from a standard for calibration purposes.

There are different methods for the spectrometric determination of phenols or phenolic compounds. In this thesis the F-C method is used. When using this method, the reagents are the Folin-Ciocalteu reagent and sodium carbonate because the reaction between phenolic compounds and the yellow F-C reagent takes place around a pH of 10 and forms a blue color. This blue color will be spectrometric determined at a wavelength of 760 nm.

The other limitation of phenols analysis is the 2 hours waiting time that is needed before the absorbance can be measured. This can be overcome with the SIA-system, provided that the reaction is not completed but the time is very reproducible, so we can analyse a sample in 2-5 minutes even if the reaction did not reach the 50% of completion, but with the same time for all samples and standards.

Another example of the spectrometric determination of phenols or phenolic compounds is a method that is based on the reaction between phenol and 4-aminoantypirine in presence of potassium persulfate. The absorbance of the colored compound obtained in this reaction was measured at 520 nm. [DOLATTO, R.G., 2012]

2 OBJECTIVE

The objective of this thesis is to include a new device in the SIA system: magnetic stirrer and evaluate the functioning of this mixer for phenols determination.

In order to reach this goal, the following steps were taken:

1. Cr(III) determination to be accustomed with the SIA-system.

2. Introduce mixing flow cell into SIA-system. Change of manifold.

3. Optimizing tests with phenol red. Check for the rinsing procedures, check the dead volumes and reproducibility times. Determine accuracy of dilutions and range of dilutions allowed.

4. Optimization of phenols determination:

    A. Determination of optimum reagents concentrations and injection volumes
    B. Range of linearity
    C. Accuracy and precision
    D. Detection limit

3 EXPERIMENTAL

3.1 REAGENTS AND SOLUTIONS

3.1.1 Determination of chromium (III)

3.1.1.1 1,5-Diphenylcarbazide: DPC (0,5g/l) (Panreac, PA)

Dissolve 0,050 g DPC with 30 ml ethanol in a flask of 100,0 ml. When everything is dissolved with the aid of the ultrasonic bath, add 10 ml H_2SO_4 2,0 M and dilute with Milli-Q water until 100,0 ml.

3.1.1.2 Stock solution of chromium (III) (100 ppm) (Merck)

Take 50,00 ml of a 1000 ppm standard of Cr(III) ($CrCl_3$ in 4,2% HCl) and add 5,00 ml of concentrated HCl in a flask of 500,0 ml.

10 ppm solution:

Take 5,00 ml of a 100 ppm solution of Cr(III) and put it in a flask of 200,0 ml and add Milli-Q water until 200,0 ml.

20 ppm solution:

Take 5,00 ml of a 100 ppm solution of Cr(III) and put it in a flask of 100,0 ml and add Milli-Q water until 100,0 ml.

3.1.1.3 Cerium (IV) (Panreac, PA)

For 0,1 g/l stock solution, dissolve 0,02874g $Ce(SO_4)_2.4H_2O$ in a flask of 100,0 ml. Add 10 ml H_2SO_4 2,0 M. Dilute with Milli-Q water.

3.1.2 Determination of phenol red

3.1.2.1 Phenol red solution (0,0113 mol/l) in 0,1 M Sodium hydroxide (Scharlau)

Weigh 1,0010 g of phenol red on an analytical balance. Add it in a flask of 250,0 ml and add 100 ml of Milli-Q water.
Weigh 1,00 g of sodium hydroxide and put it in the flask of 250,0 ml. Add Milli-Q water until almost the mark. Put the flask in a ultrasonic bath until everything is dissolved. After everything is dissolved add Milli-Q water until the mark. This solution must be made once a week and is kept in the dark.

$5{,}65 * 10^{-5}$ M solution:

Take 1,00 ml of the stock solution of phenol red (0,0113 M), put it in a flask of 200,0 ml and add Milli-Q water until 200,0 ml.

$1{,}695 * 10^{-4}$ M solution:

Take 3,00 ml of a 0,0113 M stock solution of phenol red and put it in a flask of 200,0 ml and add Milli-Q water until 200,0 ml.

$2{,}825 * 10^{-4}$ M solution:

Take 5,00 ml of a 0,0113 M stock solution of phenol red and put it in a flask of 200,0 ml and add Milli-Q water until 200,0 ml.

3.1.2.2 Sodium hydroxide solution (10^{-5} mol/l) (Scharlau)

Weigh 0,0200 g of sodium hydroxide. Add it in a flask of 500,0 ml and add Milli-Q water until the mark. For a 10^{-5} mol/l sodium hydroxide solution, make a 100 times dilution.

3.1.3 Determination of phenols

3.1.3.1 Stock solution of 2000 ppm gallic acid (Acros organics)

Weigh 1,0000 g of gallic acid on an analytical balance. Add it in a flask of 500,0 ml and add Milli-Q water until almost the mark. Put a magnetic stirrer in the flask and let it mix until the gallic acid is totally dissolved because it forms a clinker. After everything is dissolved add Milli-Q water until the mark. This solution must be made every 2 or 3 days and is kept in the fridge.

20 ppm solution:

Take 1,00 ml of a 2000 ppm solution of gallic acid, put it in a flask of 100,0 ml and add Milli-Q water until 100,0 ml.

100 ppm:

Take 5,00 ml of a 2000 ppm solution of gallic acid, put it in a flask of 100,0 ml and add Milli-Q water until 100,0 ml.

3.1.3.2 Folin-Ciocalteu reagent (Scharlau)

This reagent is already made by Scharlau.

3.1.3.3 7,5% Na_2CO_3 solution (Scharlau; extra pure)

Weigh 15 g of Na_2CO_3. Put into a container and add 200 ml of Milli-Q water.

3.2 SIA-MANIFOLD: "SET UP COMMON TO ALL TRIALS"

3.2.1 The automatic dilutor

The automatic dilutor can be used to make samples with a great accuracy and repeatability. The biggest advantages are that it saves time and reduces the volume of the sample and reagents, so there is less waste.
Disadvantages of the dilutor are that the dilutions have a limited volume and supervision is necessary.
The automatic dilutor consists of 2 parts: a sample changer and a syringe pump.

3.2.1.1 Gilson sample changer 223

The Gilson sample changer 223, shown in figure 20, is a programmable sampler for automated sample preparation and transfer. It is the perfect solution for protocols such as serial dilutions, addition of samples, sampling into vials, timed reactions and tube-to-tube transfers. [GILSON, 2015]

Figure 20: The Gilson sample changer 223 [GILSON, 2015]

The most important part of this sampler is the robotic arm. It can move in x, y and z direction.
The sampler is connected to the Gilson syringe pump 402, this is handy because dilutions can be made by taking Milli-Q water with the syringe from a reservoir.

3.2.1.2 Gilson syringe pump 402

The second part of the automatic dilutor is the syringe pump 402 of Gilson. It can take a maximum of 5 ml at one time and has a reproducibility of 0,8 % and also a high accuracy of 98,2 % at 10 µl. The syringe has 3 inlet tubes. The first one is to take a sample from the carrier reservoir. The second goes to the mobile arm of the arm and the last one is a tube that goes to a waste container.

Figure 21: Gilson syringe pump 402 [GILSON, 2015]

Before using the automatic dilutor, it is important to have no bubbles in the tubes. A solution for this problem is to put the eluent first in a ultrasonic batch to remove the bubbles. After using the system, it is advised to do a cleaning step with the eluent. When the system is not used for 2 or more days, the cleaning step must be done with Milli-Q water and after this with ethanol and air to prevent algae grow.

3.2.1.3 Control software [REUNES, L, 2012]

These two parts of the automatic dilutor, the Gilson sample changer 223 and the Gilson syringe pump 402 are both controlled by the 735 Sample Software program of Gilson. In this program the user can write methods and applications to let the dilutor perform different tasks.

3.2.2 The SIA-system

In figure 22, the schematic overview of the SIA-system for the determination of phenols can be found.

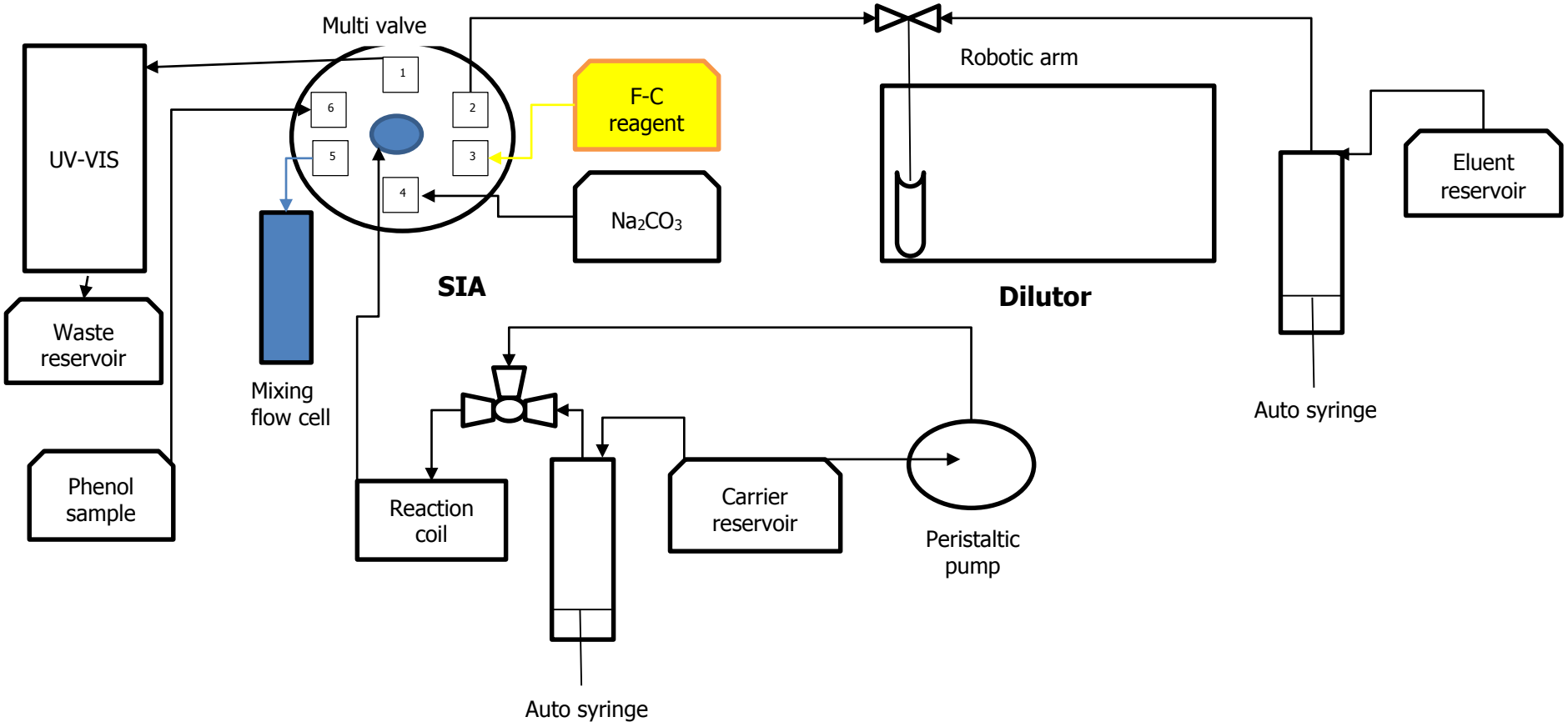

Figure 22: Schematic overview of the SIA system for the determination of phenols

3.2.2.1 The 6-ways multi-position injection valve of Bio-Chem (080T6)

This 6-ways multi-position injection valve connects the different devices of the SIA and the dilutor. With this it is possible to introduce the different reagents and sample into the system. The valve can make any combination of opening and closing the 6 different valves. Also the central connection is always open, so it is a common connection to the 6 valves that can be open or closed simultaneously any combination [BIO-CHEM FLUIDICS INC.].

Figure 23: The 6-ways multi-position injection valve of Bio-Chem (080T6)

The central common connection is always connected to the holding coil, which is the tube that comes the reservoir of carrier. The valve 1 is connected to the tube of the spectrometer. The connection of the other valves depends of the type of analysis done:

3.2.2.1.1 Determination of Cr(III)

When the multi-position injection valve of Bio-chem is used for the determination of Cr(III), the tubes are connected like this. In these tests the mixing flow cell is not used.
The tube of valve 1 is connected to the spectrometer.
The tube of valve 3 is placed in a vessel with DPC (1,5-diphenylcarbazide).
The tube of valve 4 is placed in a vessel with the Cr(III) solution.
The tube of valve 5 is placed in a vessel with Ce(IV).

3.2.2.1.2 Determination of phenol red

When the multi-position injection valve of Bio-chem is used for the determination of phenol, the tubes are connected like this.
The tube of valve 1 is connected to the spectrometer.
The tube of valve 4 is placed in a vessel with the phenol red solution.
The tube of valve 5 is connected with the mixing flow cell.

3.2.2.1.3 Determination of Phenols

When the multi-position injection valve of Bio-chem is used for the determination of phenols, the tubes are connected like this.
The tube of valve 1 is connected to the spectrometer.
The tube of valve 3 is placed in a vessel with Folin-Ciocalteu.
The tube of valve 4 is placed in a vessel with the Na_2CO_3 solution.
The tube of valve 5 is connected with the mixing flow cell.
The tube of valve 6 is placed in a vessel with the gallic acid solution.

3.2.2.2 Crison automatic burette (micro BU 2030)

In figure 24 the automatic burette that is used in the SIA system can be seen. It has a glass syringe with a capacity of 1000 µl and is very accurate. This burette is used to measure the exact amount of reagents, standard and samples that are injected into the SIA system for sending to the spectrophotometer.

Figure 24: Crison automatic burette (micro BU 2030)

3.2.2.3 Peristaltic pump minipuls 3 M312 of Gilson

The peristaltic pump minipuls 3 M312 of Gilson is shown in figure 25. It consists of 3 parts: the first one is the pump head, the second is the speed control module and the third is the flexible PE tube. This flexible PE tube of Tygon (R3607) has an internal diameter of 1,52 mm and a wall of 0,86 mm with a color code of yellow/blue.

The pump has 2 advantages. The direction of the flow can be reversed and the flow is pulse free. When the pump moves clockwise the carrier solution goes back to the reservoir, so the sample is absorbed into the holding coil. If it goes counterclockwise the carrier solution moves towards the spectrometer. In the determination of Cr(III), phenol red and phenols, a pump speed of 30 rpm is used.

Figure 25: Gilson M312 peristaltic pump minipuls 3

3.2.2.4 Spectrophotometer: USB2000 OceanOptics

In this thesis the compounds are spectrophotometrically determined. The spectrophotometer that is used is a UV/VIS molecular absorption spectrophotometer. With the use of a continuous flow cell the absorbance of the flowing through the solution can be measured at the desired wavelength.

In figure 26 the USB2000 spectrophotometer of OceanOptics is shown.

Figure 26: USB2000 spectrophotometer

This spectrometer makes a measurement of the whole spectra at the same time by using a led. By software, the desired range of wavelengths can be selected for the absorption reading.

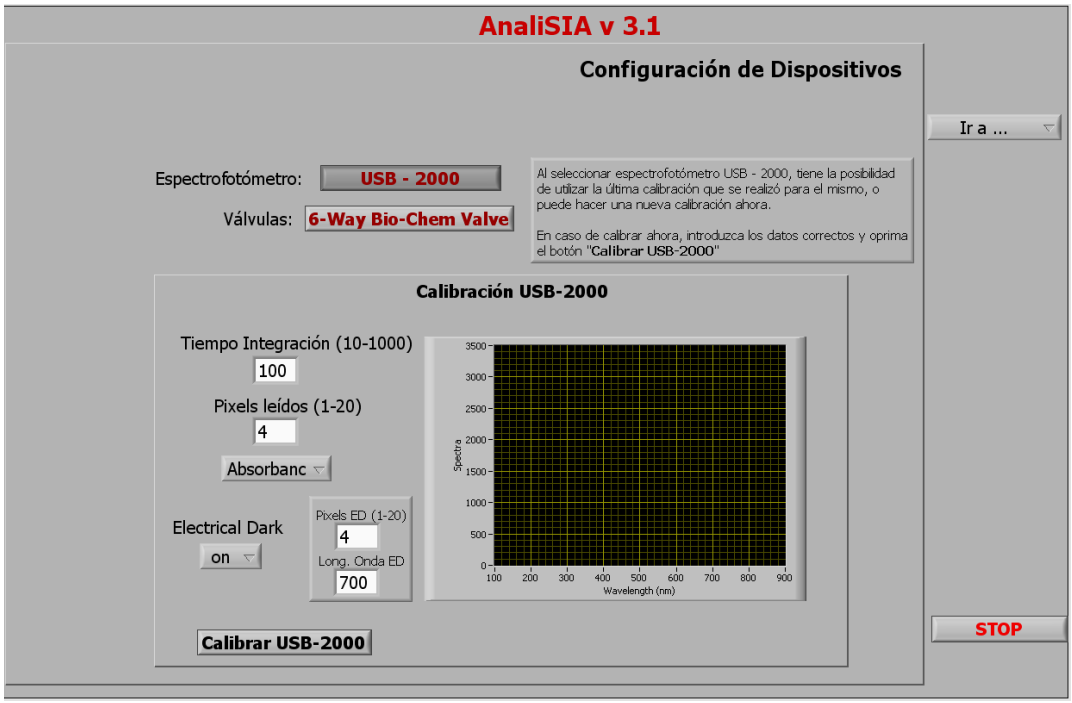

Figure 27: Settings USB2000 spectrophotometer

As can be seen in figure 27, different settings can be introduced for the USB2000.

The first parameter is the integration time, the normal setting is 100 ms. The integration time is the time taken to scan a single full range spectrum using photodiode arrays. The second parameter is the number of pixels. Normally it is 4. The USB200 has 2048 pixels to represent the whole spectra from 200 to 1100 nm. The last parameter is the settings for the electrical dark, this settings are usually 4 pixels and 700 nm. The spectrophotometer must be calibrated when the program is started for calculating the absorbance values of readings. The instructions for these calculations are in the WikiInstructions file.

3.2.2.4.1 Determination of Cr(III)

In table 2, the different settings of the spectrophotometer for measuring Cr(III) are shown.

Table 2: Settings spectrophotometer to determine Cr(III)

Integration time	20 ms
Pixels	4
Electrical dark	4 pixels and 700 nm
Wavelength	550 nm
Intensity	3000-3500
Frequence of measuring	200 ms

3.2.2.4.2 Determination of phenol red

In table 3, the different settings of the spectrophotometer for measuring phenol red are shown.

Table 3: Settings spectrophotometer to determine phenol red

Integration time	20 ms
Pixels	4
Electrical dark	4 pixels and 700 nm
Wavelength	550 nm
Intensity	3000-3500
Frequence of measuring	500 ms

3.2.2.4.3 Determination of Phenols

In table 4, the different settings of the spectrophotometer for measuring phenols are shown.

Table 4: Settings spectrophotometer to determine phenols

Integration time	20 ms
Pixels	4
Electrical dark	4 pixels and 700 nm
Wavelength	760 nm
Intensity	3000-3500
Frequence of measuring	500 ms

3.2.2.5 AnaliSIA 3.1

The software used to control all the devices and to obtain the results measured by the spectrophotometer is analiSIA 3.1. This software has been made at the MiMA research group of the University of Girona. In this program different methods (SIA-methods) can be programmed to control all the devices of the SIA, by example the valves, the burette, ... The spectrophotometer readings are saved in a .txt file, which is imported to an Excel template to process the data. For the determination of phenol red one example of a SIA-method is explained here (method 8), all the other methods can be found in the annex.

This method is used for a volume of 600 µl, when changing volumes the method can also be used for volumes of 1000 and 1500 µl.

0 va 1
1 bu 1 1 500
10 va 8
11 bu 1 0 300
19 va 16
20 bu 1 1 300
31 bu 1 0 300
43 va 1
44 bo 63 1
45 es 550 500
75 bo 0 0
76 va 0

Time (seconds)	Action
1	Empty burette
11	Loading phenol red into reaction coil
20	Phenol red from reaction into stirrer cell
31	Phenol red from stirrer cell into reaction coil
44	Peristaltic pump, 63%, anticlockwise, phenol red from reaction coil to spectrophotometer
45	Turn on spectrophotometer, 550 nm, 500 ms
75	Peristaltic pump stops
76	Close valves

Method 1: LoadCell600BUR.txt

3.2.2.5.1 General SIA-methods

These methods are used for each determination in all trials done in this work. Between brackets, the name of the method on the computer of the SIA-system can be found.

- Cleaning mail line (clean1.txt) [Annex 1.1]
- Cleaning burette (cleanbureta.txt) [Annex 1.2]
- Cleaning tubes 3, 4 and 6 (clean3-4-5.txt) [Annex 1.3]
- Emptying of the cell (EmptyCell.txt) [Annex 1.4]
- Flushing of the cell (FlushCell.txt) [Annex 1.5]

3.2.2.5.2 Methods for the determination of Cr(III)

These methods are used for each determination of chromium(III). When explaining each particular trial the SIA- method that is used will be indicated. Between brackets, the name of the method on the computer of the SIA-system can be found.

- Cr(III) calibration (CrIII100CeSDpc.txt) [Annex 2.1]

3.2.2.5.3 Methods for the determination of phenol red

These methods are used for each determination of phenol red. Between brackets, the name of the method on the computer of the SIA-system can be found.

- Loading stirrer cell with peristaltic pump (LoadCell600.txt) [Annex 3.1]
- Loading stirrer cell with automatic burette (LoadCell600BUR.txt) [Annex 3.2]
- Direct injections (TestPhenolRed.txt) [Annex 3.3]
- Indirect injections (IndirectInjections.txt) [Annex 3.4]
- Different volumes taken out of the cell (PhenolRedDifVol.txt) [Annex 3.5]
- Linearity of dilutions (DilutionPR.txt) [Annex 3.6]

3.2.2.5.4 Methods for the determination of phenols

These methods are used for each determination of phenols. Between brackets, the name of the method on the computer of the SIA-system can be found.

- Initial tests (TestPhenols.txt) [Annex 4.1]
- Calibration gallic acid (Phenol200uLStd.txt) [Annex 4.2]

3.3 CALCULATIONS

3.3.1 Limit of detection

The LOD or limit of detection is the lowest concentration of a sample that still can be measured with a good certainty. It can be calculated with the equation below.

$$L_D = \frac{2 * t_{1-\alpha,v} * S_B}{b}$$

b: calibration slope
S_B : standard deviation of the blank, in the units of the measured values obtained
$t_{1-\alpha,v}$: statistical t-value on signification level α and liberty grades v, the level of significance adopted usually is 95%

3.3.2 Range of linearity

This parameter indicates the maximum concentration in which the relationship between signal and concentration is still linear. The lowest value of the linear range is the limit of detection and the upper value can be calculated with the equation below.

$$|y - y_1| = 0{,}03\ y$$

y: measured value
y1: calculated value from calibration equation

If the difference between the measured and the calculated value is lower than 3% there is a good linearity.

3.3.3 Accuracy

This parameter determines how close the measured value lies related to the real value.

$$\varepsilon = \Delta\ (x\ measured - x\ real)$$

It can be estimated if x measured is the value obtained from the calibration equation and x real is the known value of a standard.

3.3.4 Precision

The precision can be calculated by calculating the RSD. The relative standard deviation is defined as the degree that measurements, done under the same conditions, show the same results. It is a measurement of the variability of several replications. Other terms used are reproducibility or repetitivity.

$$\%\ RSD = \frac{Standard\ deviation}{Average} * 100\%$$

The lower the RSD the better the results because than the differences between the measurements are lower.

4 RESULTS AND DISCUSSION

4.1 PRELIMINARY ANALYSIS WITH CHROMIUM(III)

These first trials are done to get accustomed to the SIA-system and the software of analiSIA 3.1. In table 5, the experimental conditions of the samples and reagents are shown. If there is a change in setting, it is mentioned.

Table 5: Conditions preliminary analysis with Cr(III)

Carrier solution	H_2SO_4 0,2M
Cr(III) concentration	10 ppm
DPC concentration	0,5 g/l
Ce(IV) concentration	0,5 g/l
Volume of sample taken	100 µl
Volume of DPC taken	100 µl
Volume of Ce(IV)	100 µl
Method	CrIII100CeSDpc.txt [Annex 2.1]
Replications	3

Sequence of injection:

1. Ce(IV)
2. Cr(III)
3. DPC

4.1.1 Different injection volumes of a 10 ppm Cr(III) solution

In this test 2 different volumes are tested: 100 and 200 µl.

In figure 28, the graph using volumes of 100 µl of Cr(III), Ce(IV) and DPC is shown.

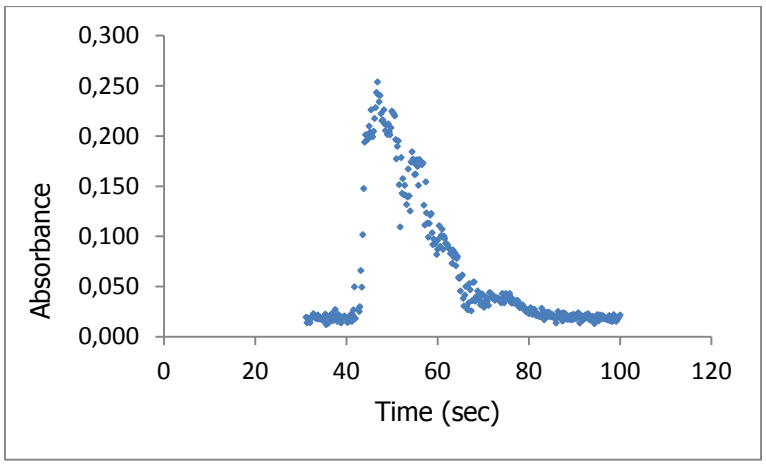

Figure 28: 100 µl of a 10 ppm solution Cr(III)

In this second trail, volumes of 200 µl of Ce(IV), Cr(III) and DPC are tried to see if there is a difference in sharpness between the peak of different volumes.

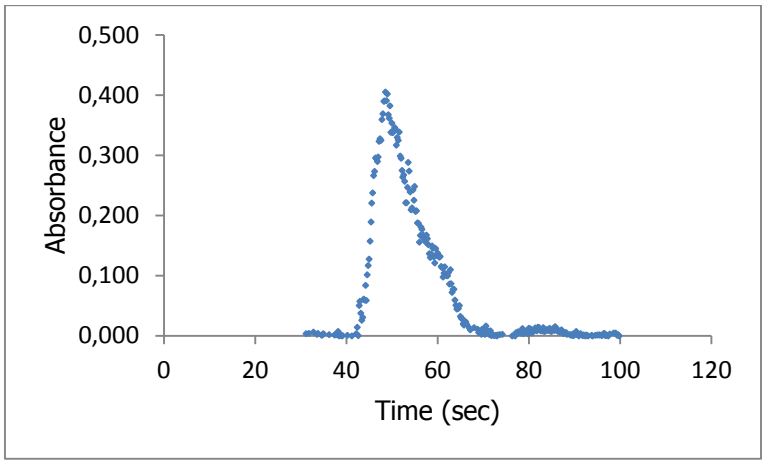

Figure 29: 200 µl of a 10 ppm solution Cr(III)

As can be seen in the graphs, when changing the volumes of Ce(IV), Cr(III) and DPC from 100 to 200 µl, there is a big difference is the sharpness of the peak. When using a volume of 200 µl, the peak is much better. In peak area there is no difference between the different volumes but when injecting a volume of 200 µl, there absorbance is higher. This can be seen in table 6.

Table 6: Summary table different injection volumes a 10 ppm Cr(III) solution

Injection volume (µl)	100	200
Peak area	4,143	4,141
Absorbance	0,260	0,332

4.1.2 Different injection volumes of a 20 ppm Cr(III) solution

In this experiment are also two different volumes (100 and 200 µl) of Ce(IV), Cr(III) and DPC tested. But now, a standard solution of 20 ppm Cr(III) is used.

Same instrumental conditions than before.

The graph of testing a volume of 100 µl of Ce(IV), Cr(III) and DPC is shown in figure 30.

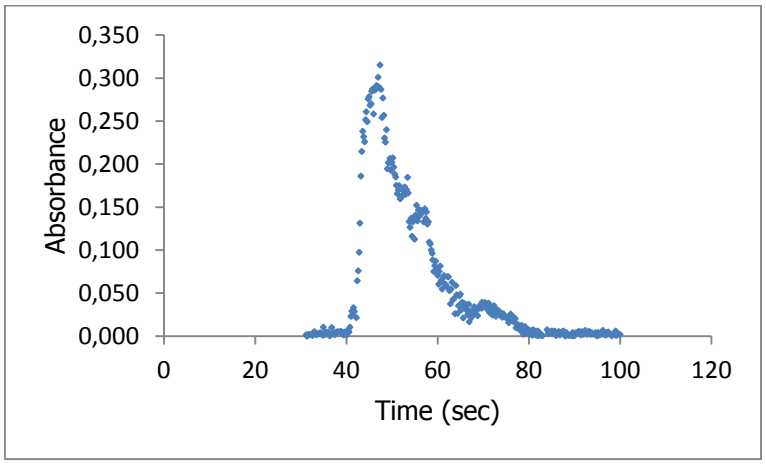

Figure 30: 100 µl of a 20 ppm solution of Cr(III)

In figure 31, the graph of using volumes of 200 µl can be seen.

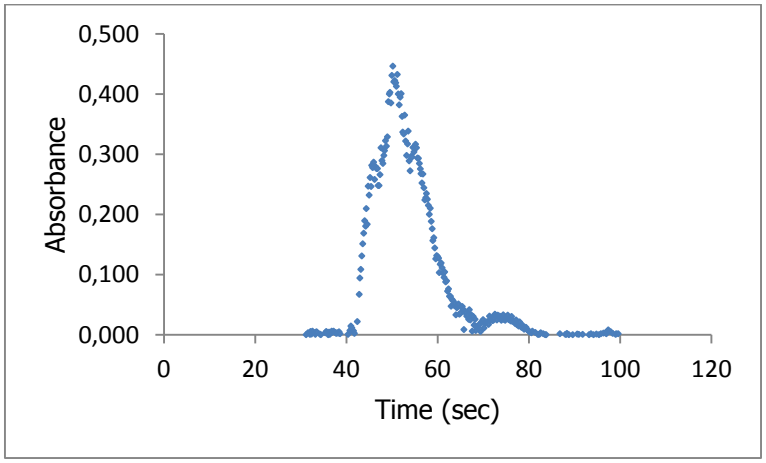

Figure 31: 200 µl of a 20 ppm solution Cr(III)

From this test, the same conclusion of testing a 10 ppm solution can be taken. With a volume of 200 µl Ce(IV), Cr(III) and DPC the peak is better. But here, there is also a difference in peak area between the different volumes. This can be seen in table 7.

Table 7: Summary table different injection volumes a 20 ppm Cr(III) solution

Injection volume (µl)	100	200
Peak area	3,826	5,413
Absorbance	0,311	0,458

When comparing the peaks of 10 and 20 ppm with the volumes of 200 µl, there is no big difference between the peaks but in general can concluded that the curves are not very smooth.

4.1.3 Cr(III) calibration

6 dilutions were made from 0 until 20 ppm from a stock of 100 ppm of Cr(III). The dilutions-table can be found in table 60 [Annex 6]. The dilutions ware handmade solutions.

In table 8, the changed conditions for the determination of Cr(III) are shown.

Table 8: Changed conditions determination of Cr(III)

Volume of sample taken	200 µl
Volume of DPC taken	200 µl
Volume of Ce(IV)	200 µl
Method	CrIII100CeSDpc.txt [Annex 2.1]
Replications of the blank	9
Replications of the standards	6

In figure 32, the graph of the calibration of Cr(III) is shown with the equation and the coefficient of determination. A poor linearity can be observed with a low regression coefficient. This was also observed in previous works. [REUNES, L., 2012]

It is likely due to the difficulty of mixing the 3 reagents in the tubes of the SIA-system.

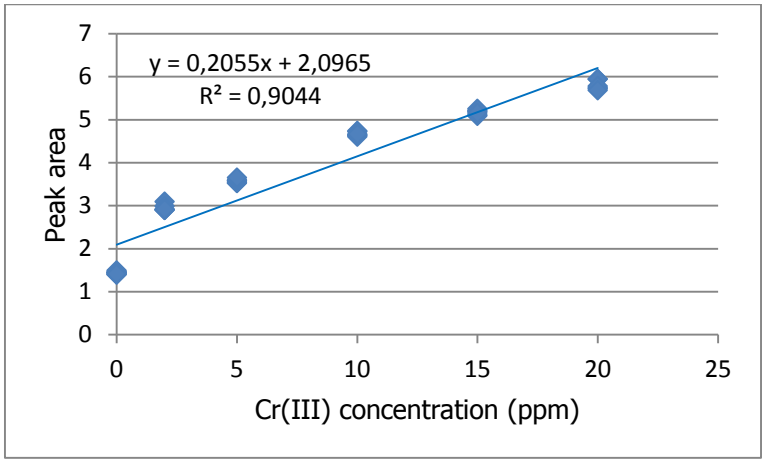

Figure 32: Cr(III) calibration (linear relation)

The result to calculate the LOD is shown in table 9.

Table 9: LOD Cr(III) calibration

Average peak area blank	Standard deviation blank	LOD
1,436	0,032	0,710

The linearity of the Cr(III) calibration is presented in table 10.

Table 10: Linearity Cr(III) calibration

Cr(III) concentration	y (measured)	y1 (calculated)	|y-y1|	% difference
0	1,436	2,097	0,661	46,031
2	2,950	2,508	0,442	14,983
5	3,575	3,124	0,451	12,615
10	4,668	4,152	0,516	11,054
15	5,166	5,179	0,013	0,252
20	5,801	6,207	0,406	6,999

The Accuracy of the linear relation of the Cr(III) calibration can be seen in figure 33 and the one for the polynomic relation is shown in figure 34.

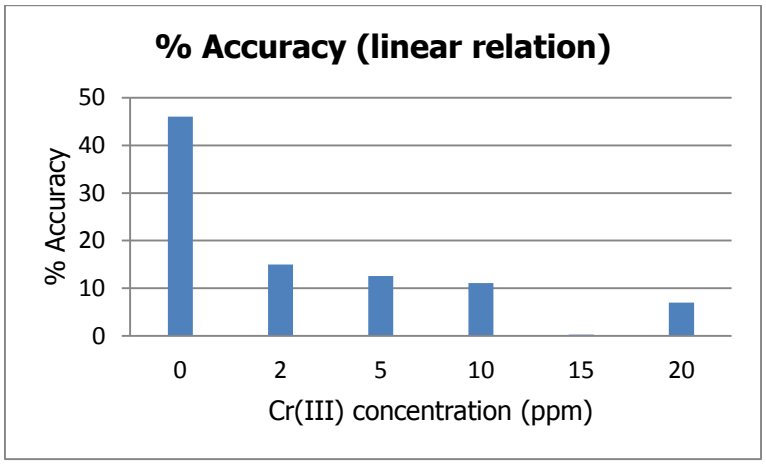

Figure 33: Accuracy linear relation Cr(III) calibration4

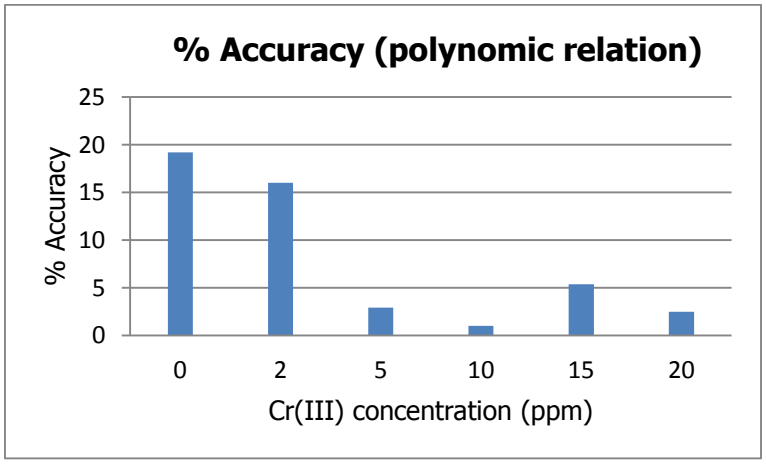

Figure 34: Accuracy polynomic relation Cr(III) calibration

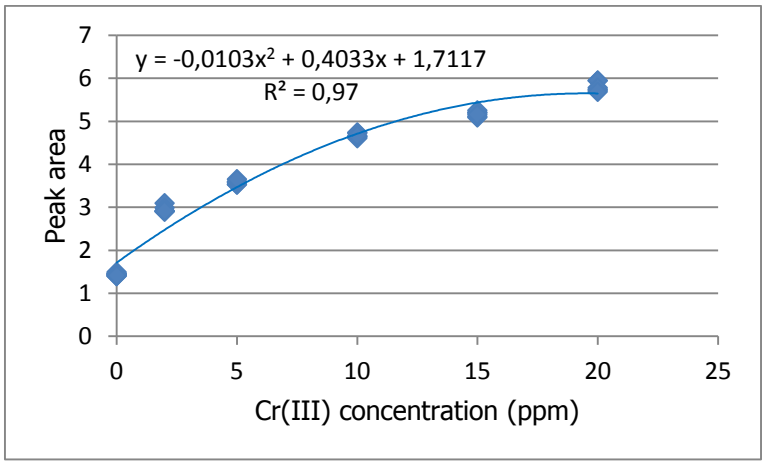

Figure 35: Cr(III) calibration (polynomic relation)

Conclusions:

LOD= 0,710

The limit of detection is 0,710 ppm. This means that this is the lowest concentration that can still be measured with a good certainty.

This value is different of the LOD in previous works for example in Lindy's thesis. There a LOD of 1,468 was found. But in this test is the limit of detection not very reliable due to the poor regression coefficient obtained. When using a polynomic relation, the regression coefficient is much better.

For a good linearity the % difference must be below 3%, so as can be seen in table 10, there is no good linearity. The Accuracy is better if polynomic relation is used than with the linear relation. Also the coefficient of determination is better.

4.2 OPTIMIZATION OF THE MIXING FLOW CELL

4.2.1 Relation between RPM, time and volume of liquid

The purpose of this test is to calculate the flow of the peristaltic pump at 3 different velocities, namely 20, 25 and 30 revolutions per minute.

In figure 36 the graph is shown with the equation and the coefficient of determination for the three velocities. The values of these measurements can be found in table 59 [Annex 5].

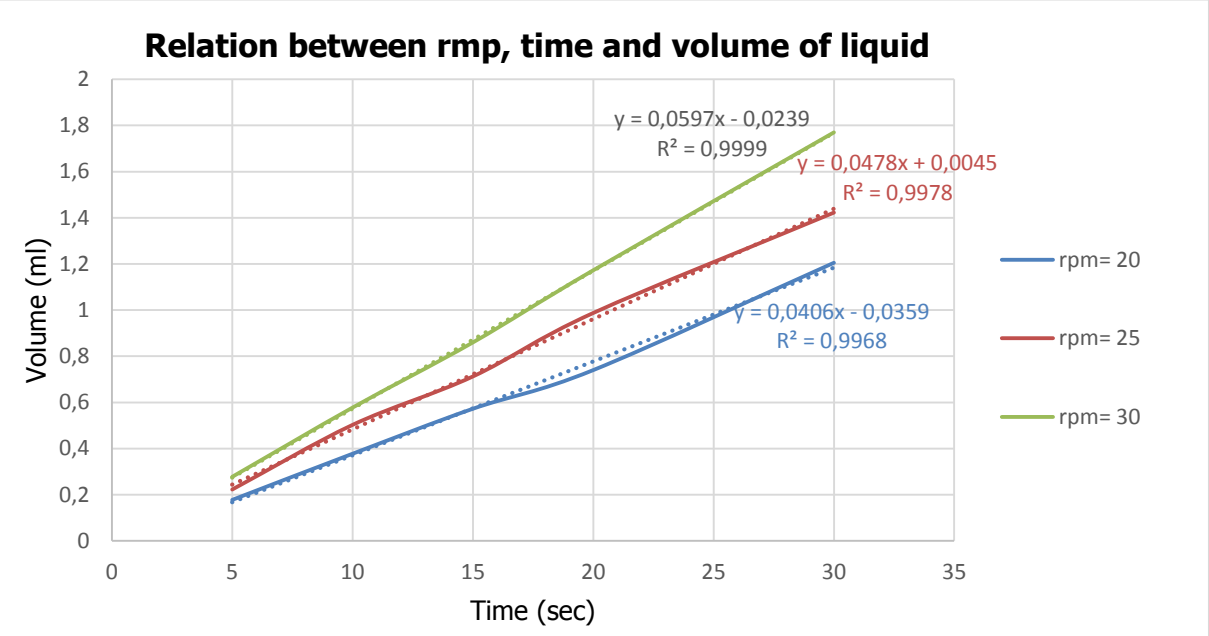

Figure 36: Relation between rpm, time and volume of liquid

In the summary (table 11), the results of the calculations of the flow rate can be found.

Table 11: Summary table of the relation between rpm, time and volume of liquid

Vel. (rpm)	Regression lines	Correlation coefficient	Q (ml/min)
20	y= 0,0406x - 0,0359	0,9968	2,43891892
25	y= 0,0478x + 0,0045	0,9978	2,86864865
30	y= 0,0597x -0,0239	0,9999	3,58256757

From this experiment can be concluded that the flow rate varies between 2,44 and 3,58 ml/min for respectively 20-30 rpm of the pump. In next trials, a flow rate of 3,58 ml/min (30 rpm) is used in order to reduce as much as possible the length of one measurement.

4.2.2 Types of stirring magnets and minimum quantity of liquid

When using the stirrer, a good mixing of the standards and the reagents is needed.

In this experiment different volumes of Milli-Q water are added in the mixing flow cell to see what quantity of liquid gives the best mixing in relation with the magnet. The tested volumes are 0,5; 1, 2, 3, 4 and 5 mL and in figure 37, the three different types of magnets that were be used are shown.

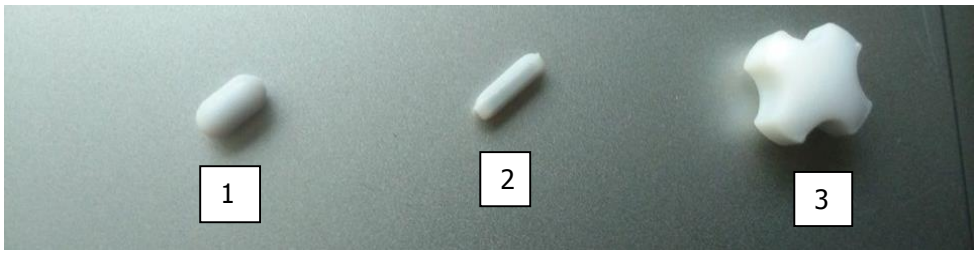

Figure 37: Different types of magnets (1: 4 mm large; 2: 6 mm large; 3: 10 mm large)

Table 12: Results minimum quantity of liquid for good stirring

V (ml)/ Type of magnet	1	2	3
0,5	Good mixing	Good mixing	Too small amount of liquid
1,0	Good mixing	Good mixing	Good mixing
2,0	Good mixing	Good mixing	Good mixing
3,0	Good mixing	Good mixing	Good mixing
4,0	Too big amount of liquid	Too big amount of liquid	Good mixing
5,0	Too big amount of liquid	Too big amount of liquid	Good mixing

There are no big differences between the different volumes and magnets.

When using the little magnets (4 and 6 mm), volumes of 4 and 5 ml are too big for a good mixing. If it is necessary to have big volumes, it's better to use the greatest magnet (10 mm).

The best volumes to use in general are 1 or 2 ml.

4.2.3 Washing of the cell

This study is done to see how the cell must be cleaned. Phenol red was used as a colorant to measure the accuracy of rising the cell by passing the solutions through the spectrometer. To flush the cell, we try 2 different devices of the SIA-system. In the first trial, the volumes to flush the cell are taken by the peristaltic pump. In the second trial, the volumes are taken by the automatic burette.

In table 13, the experimental conditions of the samples and reagents are shown.

Table 13: Conditions washing of the cell

Concentration of phenol red	5,65 * 10-5 M
Carrier solution	NaOH 10-5 M
Injection volumes of phenol red	600, 1000 or 1500 µl
Flushing volumes of carrier solution	600, 1000 or 1500 µl
Method (trial with peristaltic pump)	LoadCell600.txt [Annex 3.1]
Method (trial with automatic burette)	LoadCell600BUR.txt [Annex 3.2]

4.2.3.1 Trial with the peristaltic pump

Table 14: Trial with the peristaltic pump

	600 µl	**1000 µl**	**1500 µl**
Injection	Absorbance= 0,521	Absorbance= 0,663	Absorbance= 0,718
Flushing 1	Absorbance= 0	Absorbance= 0	Absorbance= 0
Flushing 2	Absorbance= 0	Absorbance= 0	Absorbance= 0

Here absorbance stands for the maximum absorbance of the peak obtained by the SIA.

4.2.3.2 Trial with the automatic burette

Table 15: Trial with automatic burette

	600 µl	**1000 µl**	**1500 µl**
Injection	Absorbance= 0,390	Absorbance= 0,654	Absorbance= 0,417
Flushing 1	Absorbance= 0	Absorbance= 0	Absorbance= 0
Flushing 2	Absorbance= 0	Absorbance= 0	Absorbance= 0

Here absorbance stands for the maximum absorbance of the peak obtained by the SIA.

From table 14 and 15 can be concluded that at each injection volume, a one-time flushing with the same volume is enough to clean the stirrer cell. Between using the pump or using the automatic burette to take the flushing volumes, there are no big differences but when using the automatic burette, the volumes are more accurate. In table 15 can be seen that the absorbance for the injection of 1500 µl is very low. There is no explanation for this low value.

4.2.4 Direct and indirect injections of phenol red

The direct and the indirect injections are done to see how much the absorbance decreases when an indirect injection is executed because if the solution stays a longer time in the SIA-system, there is a little decrease of absorbance.

In table 16, the general conditions of the direct and indirect injections are shown. If there is something changed in further tests, it will be mentioned.

Table 16: Conditions direct and indirect injections of phenol red

Injection volumes	200, 400, 600, 800 and 1000 µl
Carrier solution	NaOH 10^{-5} M
Replications	2
Method (direct injection)	TestPhenolRed.txt [Annex 3.3]
Method (indirect injection)	IndirectInjection.txt [Annex 3.4]

The table how the dilutions are made can be found in table 61 [Annex 7]. And during the time always the same stock solution is used, when a new stock solution is used, it's mentioned. General conditions of the manifolds and reagents are explained in chapter 3 (experimental).

4.2.4.1 Direct injections

4.2.4.1.1 No adjusting of sodium hydroxide (0,1M)

In this test there was an error with the solutions. The sodium hydroxide (0,1M) was not added in the dilutions.

For further measurements, the absorbance need to be under 1,5. When the absorbance is above 1,5 a dilution of the phenol red solution is made.

Table 17: Direct injections (no adjusting of NaOH)

Injection volume/ Dilution	**0**	**10**	**100**	**200**	**250**
200 µl	Too high signal	Too high signal	2,269	1,401	1,090
400 µl	Too high signal	Too high signal	2,453	1,701	1,219
600 µl	Too high signal	Too high signal	2,471	1,621	1,246
800 µl	Too high signal	Too high signal	Too high signal	1,661	1,272
1000 µl	Too high signal	Too high signal	Too high signal	1,638	1,328

Dilutions of 200-250X of the stock solution (0,0113 M) give approximately an absorbance of 1,5 for injections of 1000 µl. In further measurements dilutions of 200 or 250 times will be used.

4.2.4.1.2 Adjusting of sodium hydroxide (0,1M)

In this experiment, the NaOH (0,1M) is added to the dilution that is used.

In table 18, the changed conditions for this trial are shown. How this dilution is made can be found in table 61 [Annex 7].

Table 18: Changed conditions direct injections (adjusting of NaOH)

Injection volumes of phenol red	200, 400, 600, 800, 1000 and 1500 µl
Phenol red solution	5,65 * 10^{-5} M (200 times dilution of the stock)
Replications	5

In figure 38 the graph with the equation and the coefficient of determination of the peak areas of direct injections (adjusting of sodium hydroxide) is shown. The absorbances can be seen in figure 39.

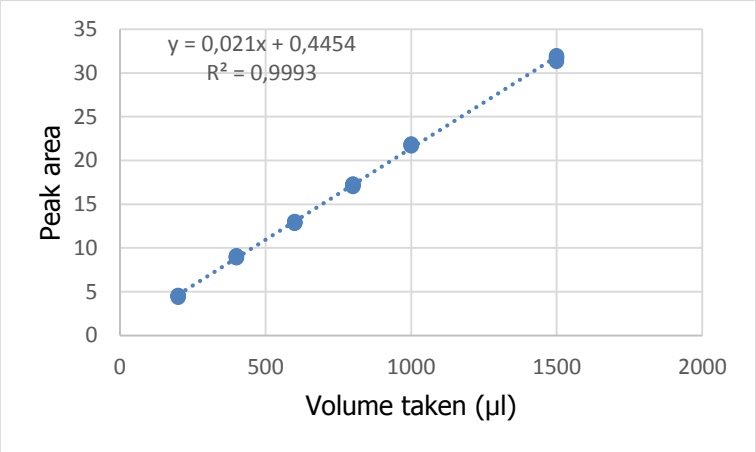

Figure 38: Peak areas of direct injections (adjusting of NaOH)

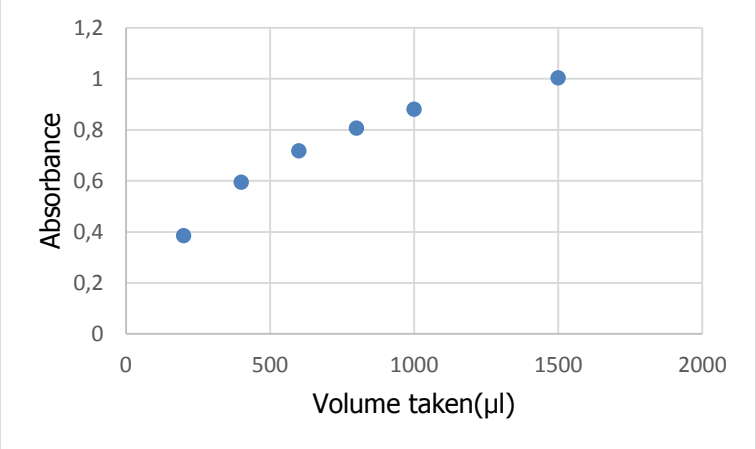

Figure 39: Absorbances of direct injections (adjusting of NaOH)

The accuracy of the direct injections (adjusting of NaOH) is presented in figure 40.

Figure 40: Accuracy direct injections (adjusting of NaOH)

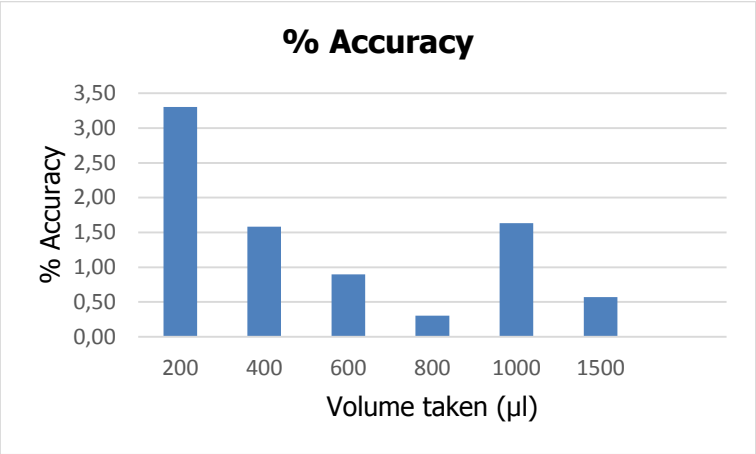

Table 19 is a summary table of the results of the direct injections (adjusting of NaOH).

Table 19: Summary table direct injections (adjusting of NaOH)

Volume taken (µl)	Absorbance	Peak area	% RSD	% Accuracy
200	0,386	4,49	1,8	3,30
400	0,595	8,973	1,2	1,58
600	0,718	12,908	0,7	0,90
800	0,807	17,165	0,7	0,30
1000	0,881	21,765	0,4	1,63
1500	1,004	31,711	0,8	0,57

When adjusting sodium hydroxide (0,1M) to the used dilution, there is a good linearity from 200 till 1500 µl because the % difference is below 3%. Also the % accuracy and the precision are very good.

4.2.4.2 Indirect injections

4.2.4.2.1 No adjusting of sodium hydroxide (0,1M)

In this test there was an error with the solutions. The sodium hydroxide (0,1M) was not added in the dilutions.

In table 20 the changed conditions for the trial of the indirect injections (no adjusting of NaOH) are presented. How this dilution is made can be found in table 61 [Annex 7].

Table 20: Changed conditions indirect injections (no adjusting of NaOH)

Injection volumes of phenol red	200, 400, 600, 800, 1000, 1500 and 2000 µl
Phenol red solution	$5{,}65 * 10^{-5}$ M (200 times dilution of the stock)
Replications	5

In figure 41 the graph with the equation and the coefficient of determination of the peak areas of indirect injections (no adjusting of sodium hydroxide) is shown. The absorbances can be seen in figure 42.

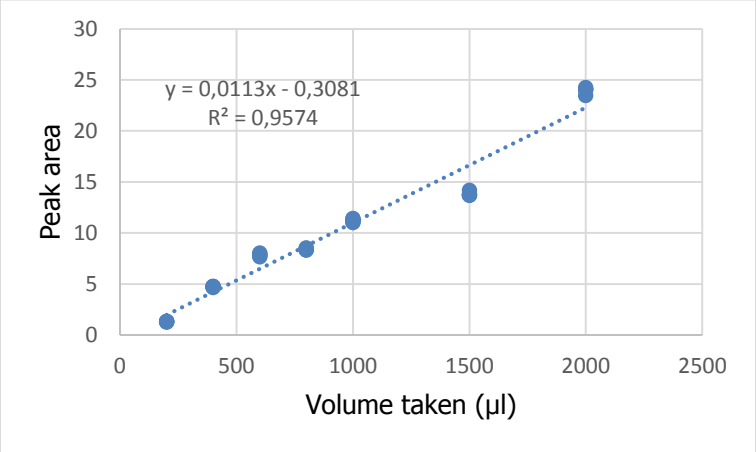

Figure 41: Peak areas of indirect injections (no adjusting of NaOH)

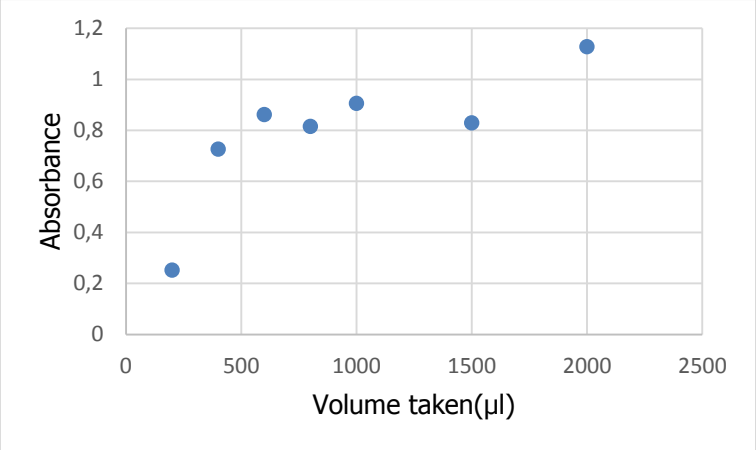

Figure 42: Absorbances of indirect injections (no adjusting of NaOH)

The accuracy of the indirect injections (no adjusting of NaOH) is presented in figure 43.

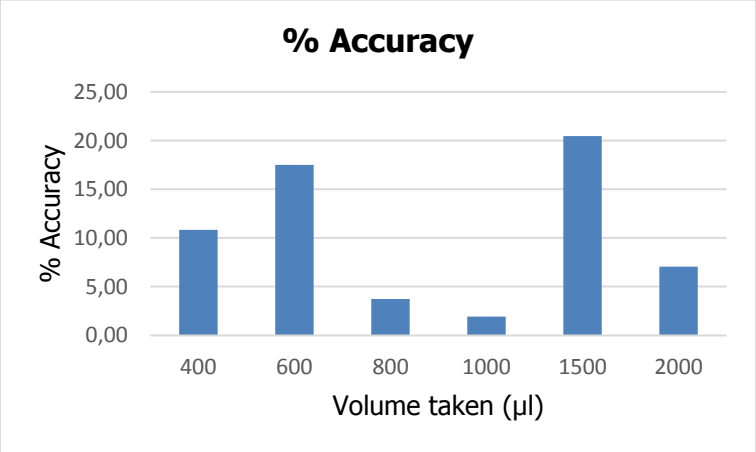

Figure 43: Accuracy indirect injections (no adjusting of NaOH)

Table 21 is a summary table of the results of the indirect injections (no adjusting of NaOH).

Table 21: Summary table indirect injections (no adjusting of NaOH)

Volume taken (µl)	Absorbance	Peak area	% RSD	% Accuracy
200	0,252	1,305	1,9	49,50
400	0,726	4,721	1,1	10,82
600	0,861	7,84	1,9	17,49
800	0,815	8,413	1,1	3,75
1000	0,906	11,203	1,6	1,93
1500	0,829	13,808	1,6	20,47
2000	1,127	23,972	1,3	7,05

If the sodium hydroxide is not adjusted to the phenol red solution, the precision is still good but there is no good linearity and accuracy.

4.2.4.2.2 Adjusting of sodium hydroxide (0,1M)

In this experiment, the NaOH (0,1M) is added to the dilution that is used.

In table 22, the changed conditions for this trial are shown. How this dilution is made can be found in table 61 [Annex 7].

Table 22: Changed conditions indirect injections (adjusting of NaOH)

Injection volumes of phenol red	200, 400, 600, 800, 1000, 1500 and 2000 µl
Phenol red solution	5,65 * 10^{-5} M (200 times dilution of the stock)
Replications	5

In figure 44 the graph with the equation and the coefficient of determination of the peak areas of indirect injections (adjusting of sodium hydroxide) can be seen. The absorbances can be seen in figure 45.

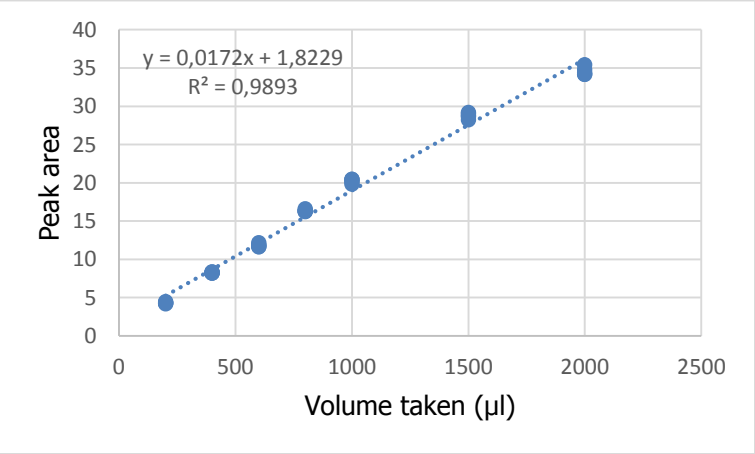

Figure 44: Peak areas of indirect injections (adjusting of NaOH)

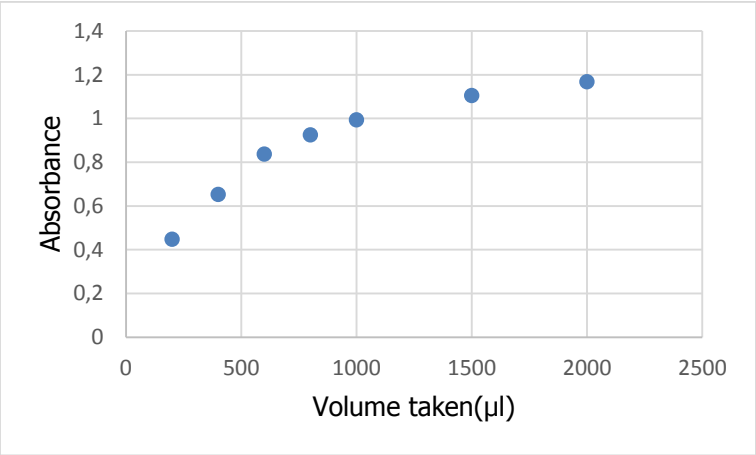

Figure 45: Absorbances of indirect injections (adjusting of NaOH)

The accuracy of the indirect injections (adjusting of NaOH) is presented in figure 46.

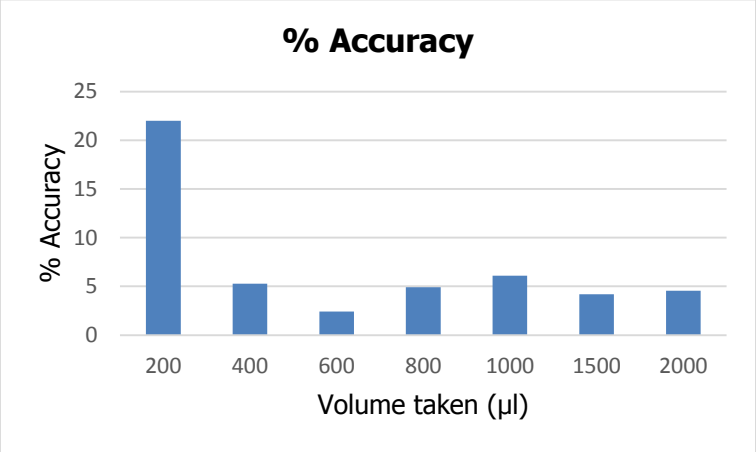

Figure 46: Accuracy of indirect injections (adjusting of NaOH)

Table 23 is a summary table of the results of the indirect injections (no adjusting of NaOH).

Table 23: Summary table indirect injections (adjusting NaOH)

Volume taken (µl)	Absorbance	Peak area	% RSD	% Accuracy
200	0,448	4,312	2,3	22
400	0,653	8,257	0,9	5,27
600	0,837	11,837	1,9	2,42
800	0,924	16,362	0,9	4,93
1000	0,994	20,223	1,4	6,11
1500	1,105	28,77	1,3	4,18
2000	1,168	34,57	1,6	4,56

Conclusion:

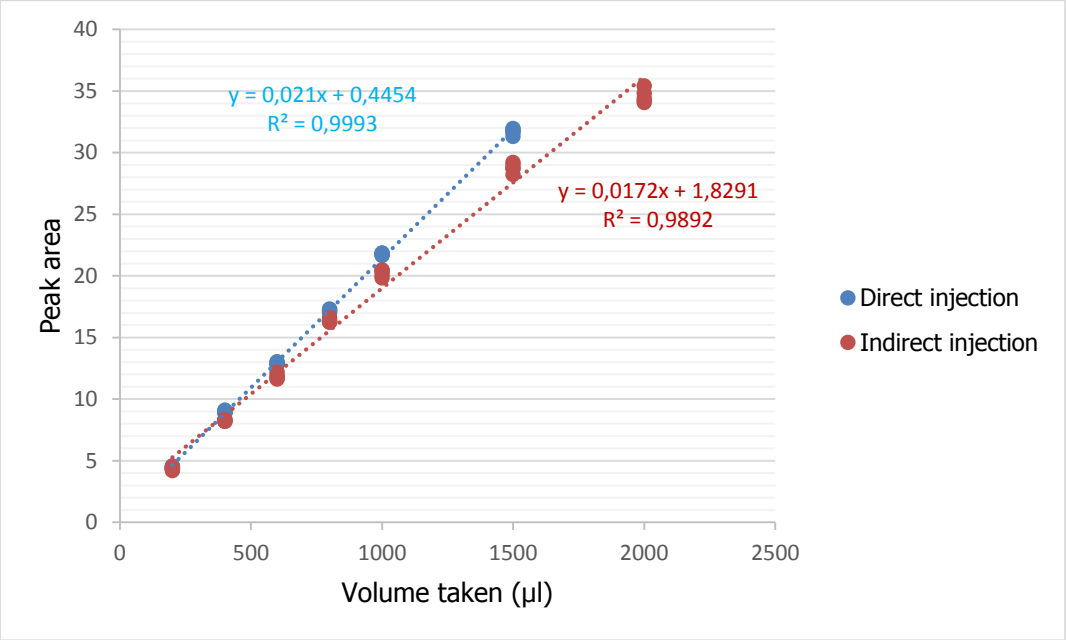

Figure 47: Comparing peak areas of direct and indirect injections (adjusting sodium hydroxide)

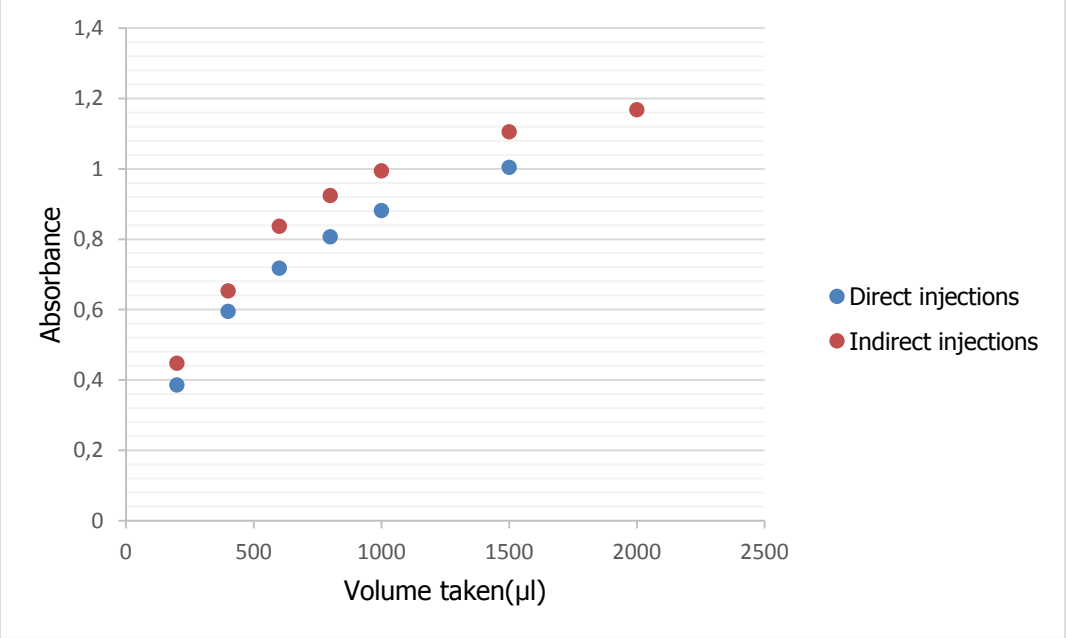

Figure 48: Comparing absorbances of direct and indirect injections (adjusting sodium hydroxide)

In general we can conclude that the linearity and accuracy are better with the addition of sodium hydroxide. The precision is still good. When comparing the results of this test with the trial of the direct injections (adjusting sodium hydroxide 0,1M), there is something very strange happened because the absorbances of the direct injections are lower than the absorbances of the indirect injections. A possible explanation for these values can be that always the same stock solution is used and there is a decrease of color of the stock solution during the time. It will be checked later.

4.2.4.2.3 Different carrier solution

Another carrier solution is tried to see if there is a difference in absorbance and peak area with a more concentrated carrier solution. The carrier solution is changed from 10^{-5} M to 10^{-3} M NaOH.

In table 24, the conditions for this test are presented. How this dilution is made can be found in table 61 [Annex 7]. In this trial indirect injections (using the stirrer cell) are done.

Table 24: Conditions using a different carrier solution

Injection volumes of phenol red	200, 400, 600, 800, 1000 and 1500 µl
Phenol red stock solution	0,0113 M
Phenol red standard solution	5,65 * 10^{-5} M
Carrier solution	NaOH 10^{-3} M
Replications	5
Method	IndirectInjection.txt [Annex 3.4]

The graph with the equation and the coefficient of determination is shown in figure 49. In figure 50, the absorbances when using another carrier solution can be seen.

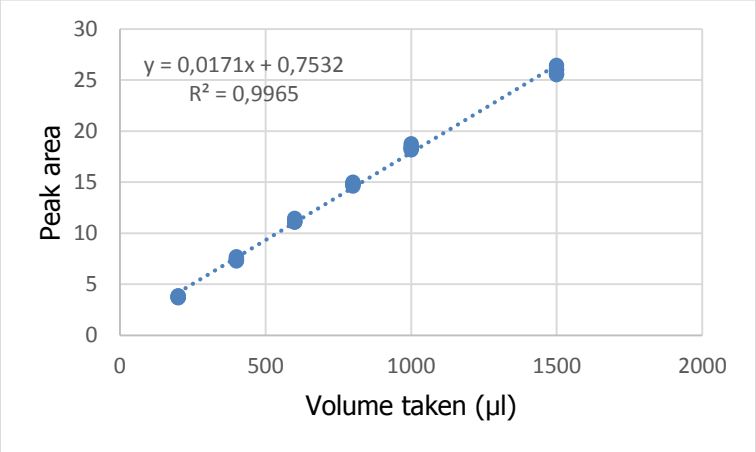

Figure 49: Calibration different carrier solution (peak areas)

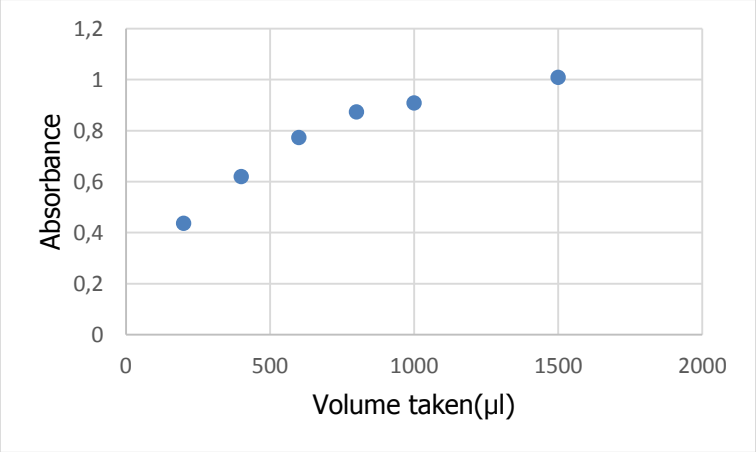

Figure 50: Absorbances when using a different carrier solution

The accuracy of the calibration with another carrier solution is shown in figure 51.

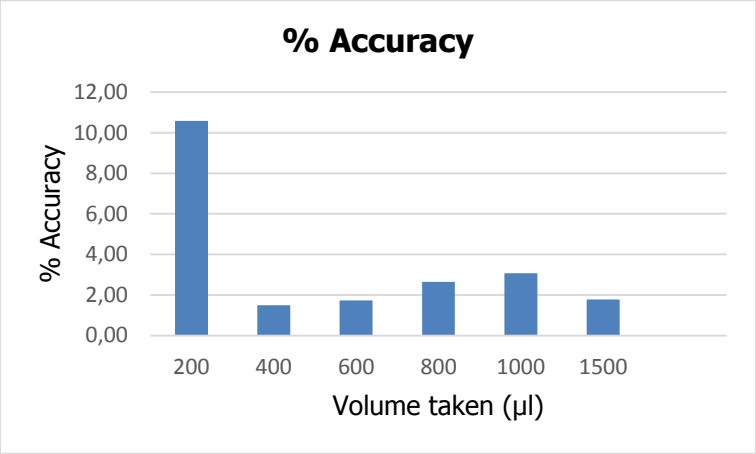

Figure 51: Accuracy when using a different carrier solution

Table 25 is a summary table of the results when using a different carrier solution.

Table 25: Summary table using different carrier solution

Volume taken (µl)	Absorbance	Peak area	% RSD	% Accuracy
200	0,437	3,775	2,2	10,58
400	0,62	7,482	2,2	1,50
600	0,773	11,209	1,3	1,74
800	0,874	14,827	1	2,65
1000	0,909	18,422	1,2	3,08
1500	1,009	25,943	1,5	1,78

Conclusion:

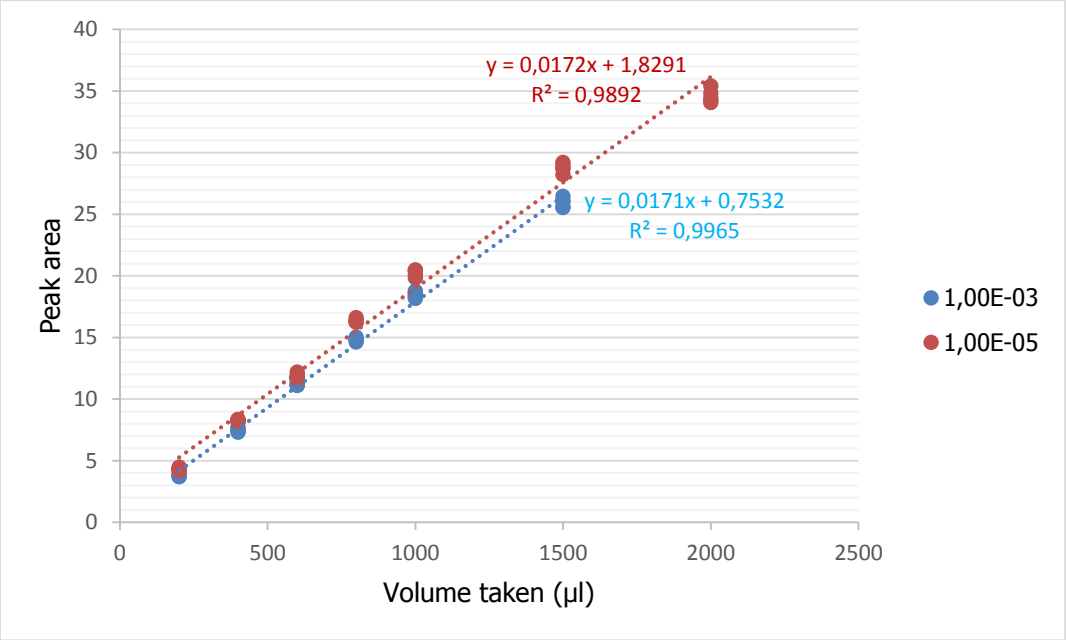

Figure 52: Comparing peak areas of different carrier solutions

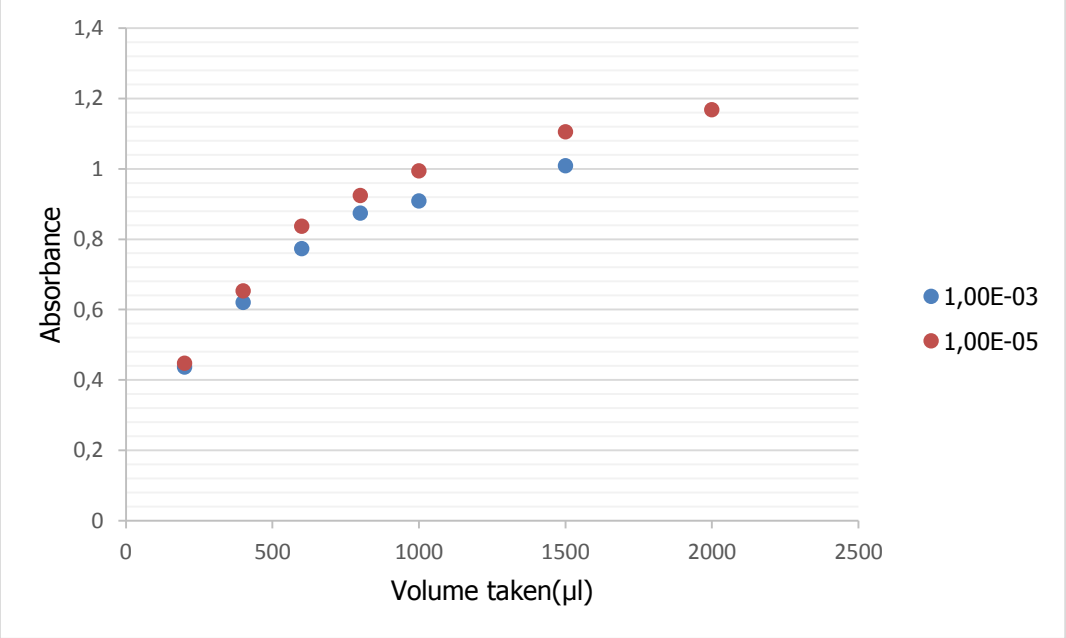

Figure 53: Comparing absorbances of different carrier solutions

Using a carrier solution of 10^{-3} M NaOH gives a fairly good linearity from 400 till 1500 µl. Also the precision of the measurements is good.

When comparing the results of using a 10^{-3} M NaOH carrier solution instead of a 10^{-5} M NaOH carrier solution can be seen that when a more concentrated carrier solution is used, the absorbances of the measurements are lower. So in further measurements, a carrier solution of 10^{-5} NaOH will be used.

4.2.4.2.4 Different volumes taken out of the cell

In this test a volume of 1400 µl of phenol red is injected into the stirrer cell and different volumes are taken out of the cell to measure the absorbance, in order to see if there is a linear behavior between the volume taken from mixing cell and the absorbance measured.

In table 26, the conditions for this test are presented. How this dilution is made can be found in table 61 [Annex 7].

Table 26: Conditions different volumes taken out of the cell

Phenol red stock solution	0,0113 M
Phenol red standard solution	5,65 * 10^{-5} M
Injection volume of phenol red into the cell	1400 µl
Volumes taken out of the cell to measure	200, 400, 800, 1200 and µl
Carrier solution	NaOH 10^{-5} M
Replications	5
Method	PhenolRedDifVol.txt [Annex 3.5]

The graph with the equation and the coefficient of determination is shown in figure 54.

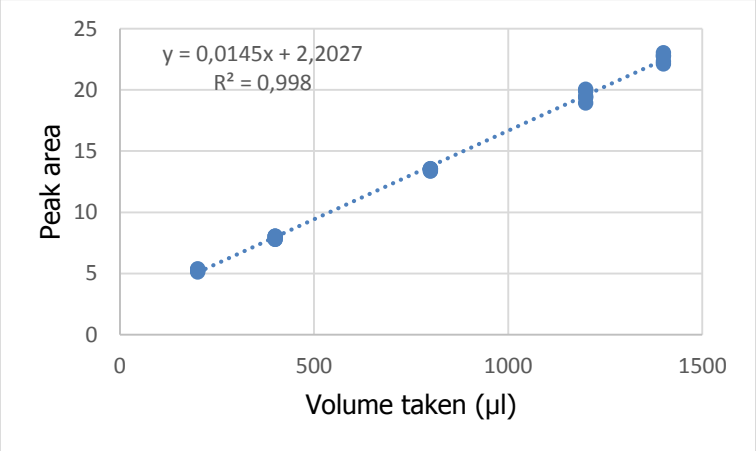

Figure 54: Different volumes taken out of the cell

The accuracy of this calibration is presented in figure 55.

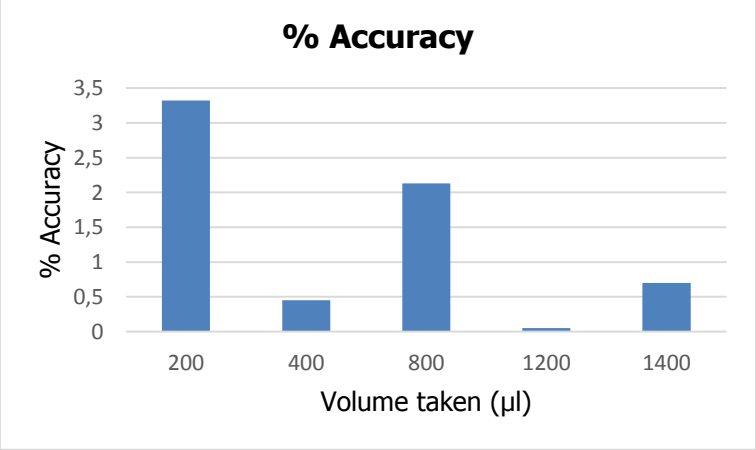

Figure 55: Accuracy different volumes taken out of the cell

In table 27 a summary of the results when taking different volumes out of the cell can be seen.

Table 27: Summary table (different volumes taken out of the cell)

Volume taken (µl)	Absorbance	Peak area	% RSD	% Accuracy
200	0,4	5,27	1,7	3,32
400	0,585	7,952	1,7	0,45
800	0,801	13,485	0,6	2,13
1200	0,857	19,548	2,3	0,05
1400	0,891	22,607	1,7	0,7

Conclusion:

If a volume of 1400 µl of phenol red is introduced into the mixing flow cell and less volume is taken out of the cell there is good linearity and accuracy from 200 till 1400 µl as well as a good precision. This is very interesting because it can be used to make higher dilutions.

A disadvantage of this test is that it takes a lot of time to make 5 replications of each volume because when introducing 1400 µl into the cell and take less volume out of the cell, the quantity of liquid that is inside the cell must be removed. So the stirrer cell and the main line must be cleaned behind each replication. It takes around a half an hour to make 5 replications.

4.2.5 Linearity of dilutions

In these 2 tests different dilutions of phenol red are tested to see until what concentration (or dilution) the measurements are still linear. In the first trial a total volume of 600 µl is sent to the spectrometer and in the second trial a total volume of 1200 µl is sent to the spectrometer to measure the absorbance. The reason of trying the high volume is to reach a higher dilution.

4.2.5.1 Total volume of 600 µl

In table 28, the conditions for this trial are shown.

In this experiment different volumes of concentrated phenol red solution and the corresponding quantity of carrier solution are introduced in the mixing flow cell to a final volume of 600 µl. Dilutions from 2 till 15 times are tested. How the dilutions are made can be found in table 62 [Annex 8].

Table 28: Conditions linearity phenol red 1

Phenol red stock solution	0,0113 M
Phenol red standard solution	1,695 * 10^{-4} M
Carrier solution	NaOH 10^{-5}
Volume taken to measure	600 µl
Times diluted tested	2, 3, 4, 5, 10 and 15
Replications	6
Method	DilutionPR.txt [Annex 3.6]

The graph with the equation and the coefficient of determination is shown in figure 56.

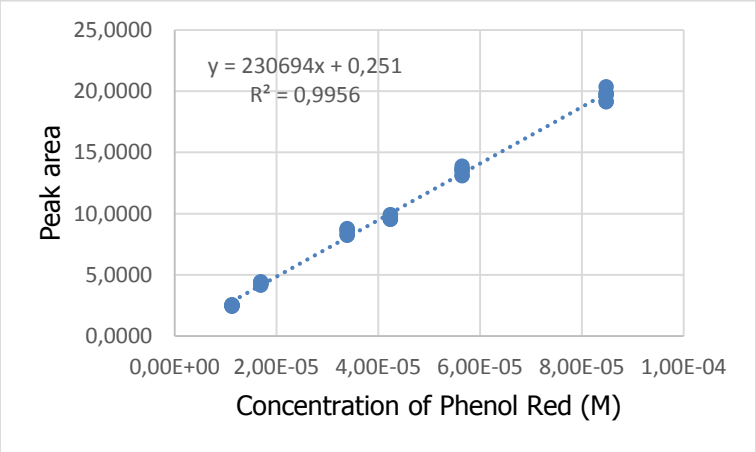

Figure 56: Dilutions phenol red (600 µl)

The % accuracy of this first test with a total volume of 600 µl can be seen in figure 57.

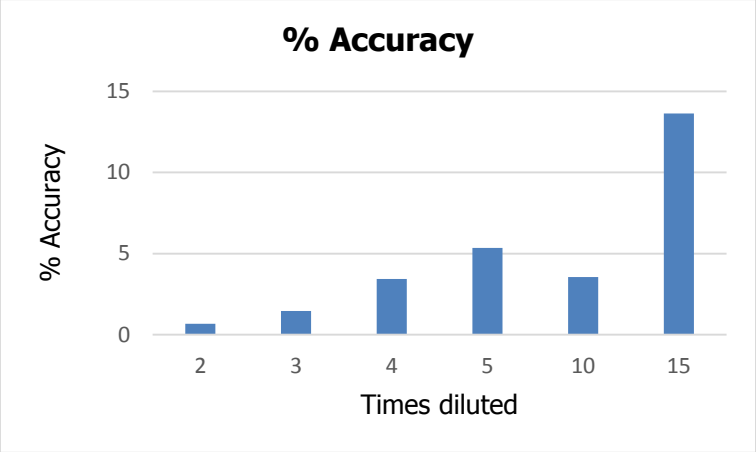

Figure 57: Accuracy dilutions phenol red (600 µl)

Table 29 is a summary table of the measurements of dilutions of phenol red with a total volume of 600 µl.

Table 29: Summary table dilutions phenol red (600 µl)

Times diluted	Concentration of Phenol red (M)	Absorbance	Peak area	% RSD	% Accuracy
2	8,48E-05	1,844	19,67	2,3	0,67
3	5,65E-05	1,388	13,482	2,3	1,46
4	4,24E-05	1,065	9,695	1,8	3,43
5	3,39E-05	0,859	8,529	2,8	5,36
10	1,70E-05	0,423	4,315	2,4	3,56
15	1,13E-05	0,237	2,515	1,9	13,63

Conclusion:

With a total volume of 600 µl, a dilution of 10 times or a concentration of 1,70 * 10^{-5} M of phenol red can be reached with a good linearity and accuracy. For higher dilutions a larger total volume of solutions in the cell must be used, provided that we can't make smaller dilutions with the automatic syringe. 30 µl in this case for a 15 times dilution with a syringe of 1000 µl.

4.2.5.2 Total volume of 1200 µl

In this test the same instrumental conditions than before (with a total volume of 600 µl) are used. Only the volumes injected into the mixing cell are different.

Dilutions of 5, 10, 15 and 20 times are made. So volumes of 240,0 ; 120,0 ; 80,0 ; and 60,0 µl of a concentrated phenol red solution are introduced in the cell and carrier solution is added till a total volume of 1200,0 µl. How the dilutions are made can also be found in table 63 [Annex 9].

The graph with the equation and the coefficient of determination is shown in figure 58.

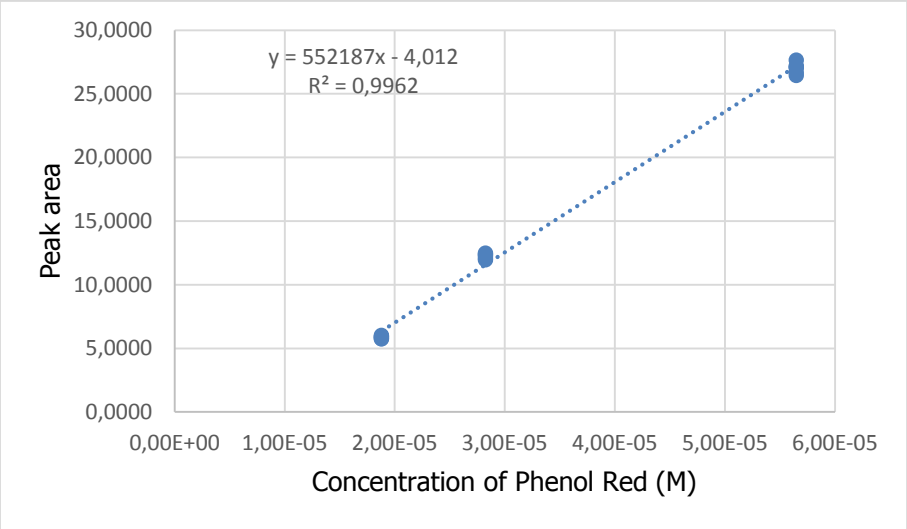

Figure 58: Dilutions phenol red (1200 µl)

The % accuracy of this second test with a total volume of 1200 µl can be seen in figure 59.

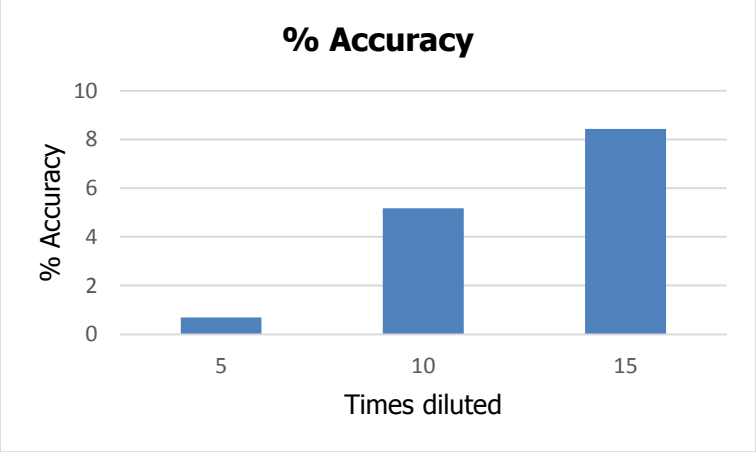

Figure 59: Accuracy dilutions phenol red (1200 µl)

Table 30 is a summary table of the measurements of dilutions of phenol red with a total volume of 1200 µl.

Table 30: Summary table dilutions phenol red (1200 µl)

Times diluted	Concentration phenol red (M)	Absorbance	Peak area	% RSD	% Accuracy
5	5,65E-05	1,623	27,021	1,6	0,61
10	2,83E-05	0,762	12,248	1,7	5,39
15	1,88E-05	0,399	5,874	1,9	8,43
20	1,41E-05	0,070	/	/	/

Conclusion:

The precision of these dilutions is good but the linearity and accuracy is not very good only with a dilution of 5 times (5,65 * 10^{-5} M phenol red). A dilution of 20 times was also measured, but the results of this dilution are not included because the absorbance of this was too low to have a reliable peak area.

With a total volume of 1200 µl, a dilution of 15 times can be reached but it is accompanied with a bad linearity and accuracy.

Maybe an effect of decreasing accuracy and precision with high volumes must be further check in future works.

With a dilutions of 20 times, there is no peak area because of the low absorbance. The .txt file cannot calculate the peak area. So there is no possibility to calculate the % RSD and the % accuracy.

4.2.6 New phenol red solution

During all previous tests the same stock solution was used. At the end of the tests a new phenol red solution was made because in the first tests there were some strange things happened for example: the absorbance of the direct injections was lower than the absorbance of the direct injections. With this test, it can be seen if there is a problem with the stock solution (for example a loss of color) or something is wrong with the SIA-system.

4.2.6.1 Direct injections 2 (adjusting sodium hydroxide)

In this experiment, the NaOH (0,1M) is added to the dilution that is used.

In table 31, the conditions for this trial are shown. How this dilution is made can be found in table 61 [Annex 7].

Table 31: Conditions direct injections 2

Injection volumes	200, 400, 600, 800 and 1000 µl
Carrier solution	NaOH 10^{-5} M
Phenol red solution	5,65 * 10^{-5} M
Replications	2
Method (direct injection)	TestPhenolRed.txt [Annex 3.3]

In tables 32 and 33 the absorbances and peak areas of the old and the new phenol red solution are compared.

Table 32: Comparing absorbances of old and new phenol red solution

Volume taken (µl)	New phenol red solution (Absorbance)	Old phenol red solution (absorbance)
200	1,1382	0,3860
400	1,8852	0,5950
600	2,2350	0,7180
800	Saturated	0,8070
1000	Saturated	1,0040

Table 33: Comparing peak areas of old and new phenol red solution

Volume taken (µl)	New phenol red solution (Peak area)	Old phenol red solution (Peak area)
200	15,4060	4,4900
400	29,6512	8,9730
600	40,8263	12,9080
800	/	17,165
1000	/	21,765

Conclusion:

When comparing the results of direct injections from the old and the new phenol red solution, we can conclude that there is a loss of color of the phenol red solution during the time. In the beginning of the measurements with tis colorant this problem was not expected because it also used as an acid-base indicator.

A solution for this problem is to make the stock solution once a week and keep it in the dark.

4.2.6.2 Indirect injections 2 (adjusting sodium hydroxide)

In this experiment, the NaOH (0,1M) is added to the dilution that is used.

In table 34, the conditions for the direct injections 2 are shown. How this dilution is made can be found in table 61 [Annex 7].

Table 34: Conditions indirect injections 2 (adjusting of NaOH)

Injection volumes of phenol red	200, 400, 600, 800 and 1000 µl
Phenol red solution	5,65 * 10^{-5} M (200 times dilution of the stock)
Replications	3
Method	IndirectInjection.txt [Annex 3.4]

In figure 60 the graph with the equation and the coefficient of determination of the indirect injections 2 (adjusting of sodium hydroxide) can be seen.

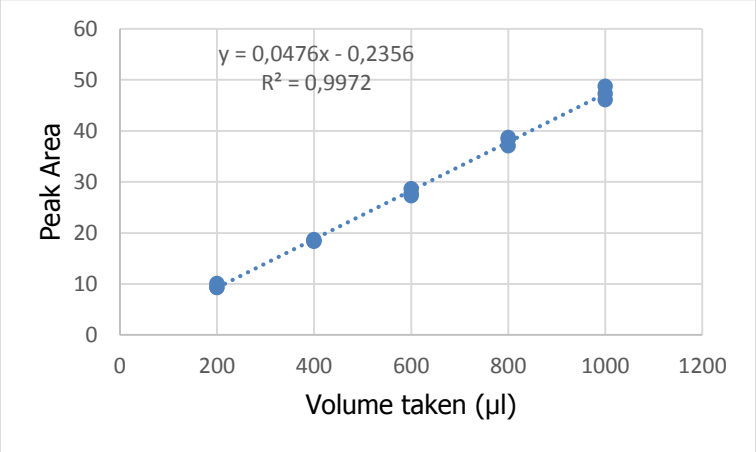

Figure 60: New phenol red solution indirect injections

The % accuracy of the second trial with the new phenol red solution can be seen in figure 61.

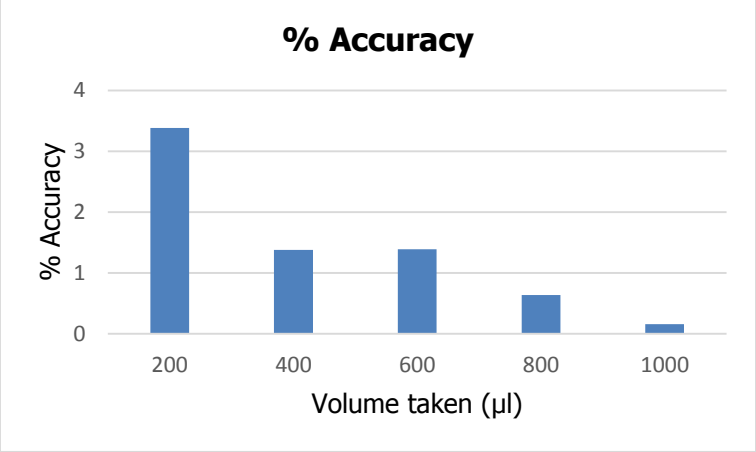

Figure 61: Accuracy new phenol red solution

Table 35 is a summary table of the results of indirect injections with a new phenol red solution.

Table 35: Summary table new phenol red solution

Volume taken (µl)	Absorbance	Peak area	% RSD	% Accuracy
200	0,934	9,6	4,7	3,38
400	1,412	18,532	1	1,38
600	1,882	27,911	2,5	1,39
800	2,167	38,054	2,3	0,64
1000	2,47	47,396	2,7	0,16

With the new phenol red solution there is a fairly good linearity from 200 until 1000 µl.

Conclusions:

In figures 62 and 63, the results of absorbances and peak areas of indirect injections of a new and an old phenol red solution are compared. In tables 64 [Annex 10] and 65 [Annex 11] the values of these 2 graphs can be seen.

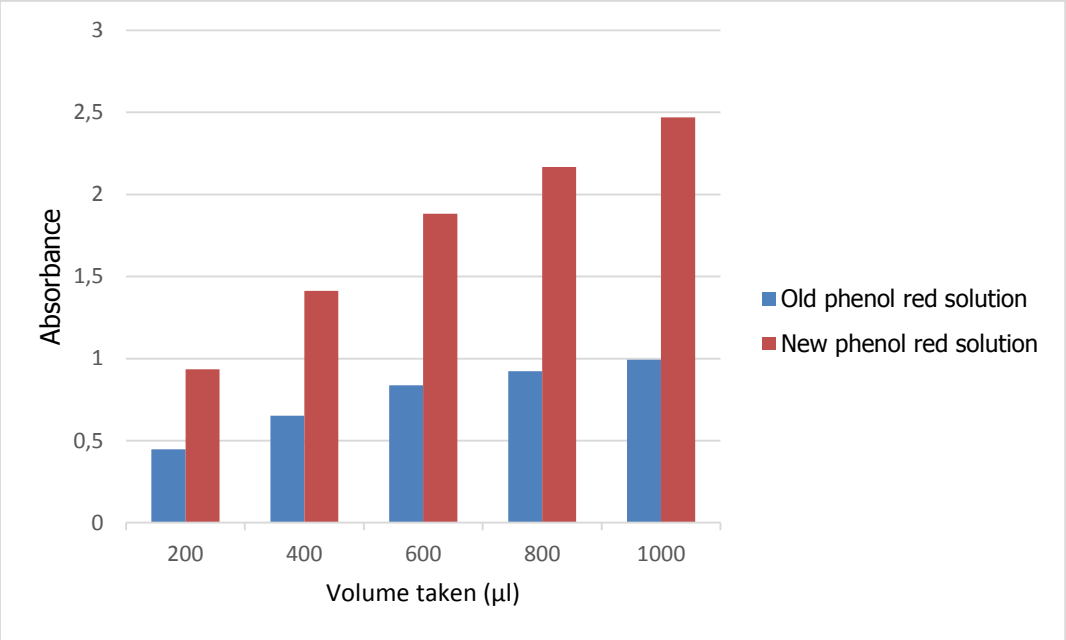

Figure 62: Comparing absorbances of indirect injections (old and new phenol red solution)

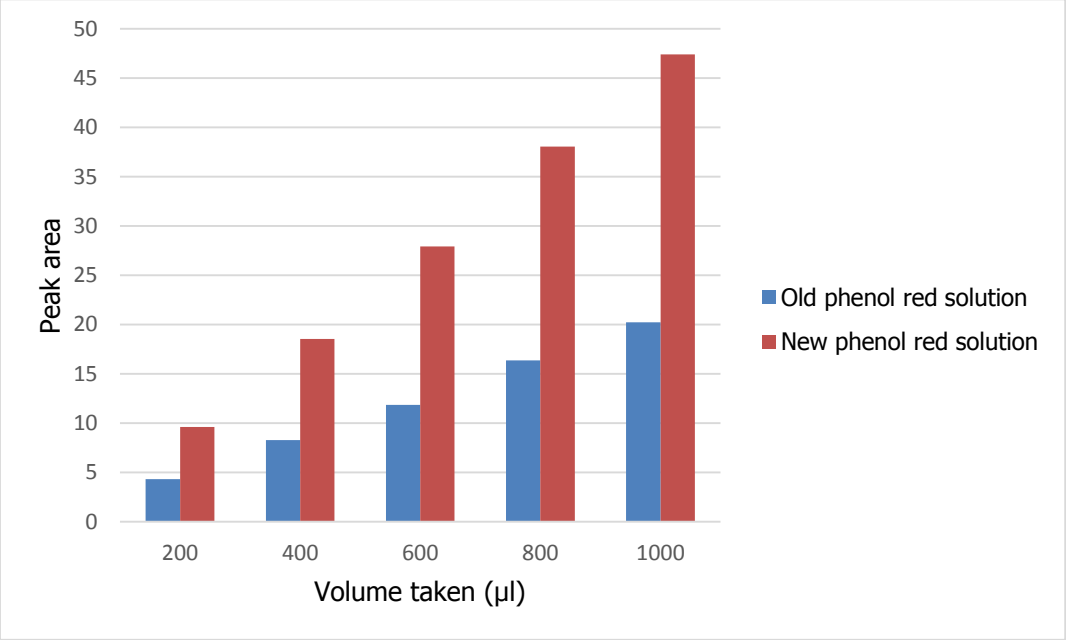

Figure 63: Comparing peak areas of indirect injections (old and new phenol red solution)

When comparing the results from the old and the new phenol red solution of indirect injections, we can conclude that there is a loss of color of the phenol red solution during the time because there are big differences in absorbance.

In figures 64 and 65, the results of absorbances and peak areas of direct and indirect injections of a new phenol red solution are compared. In tables 66 [Annex 12] and 67 [Annex 13] the values of these 2 graphs can be seen.

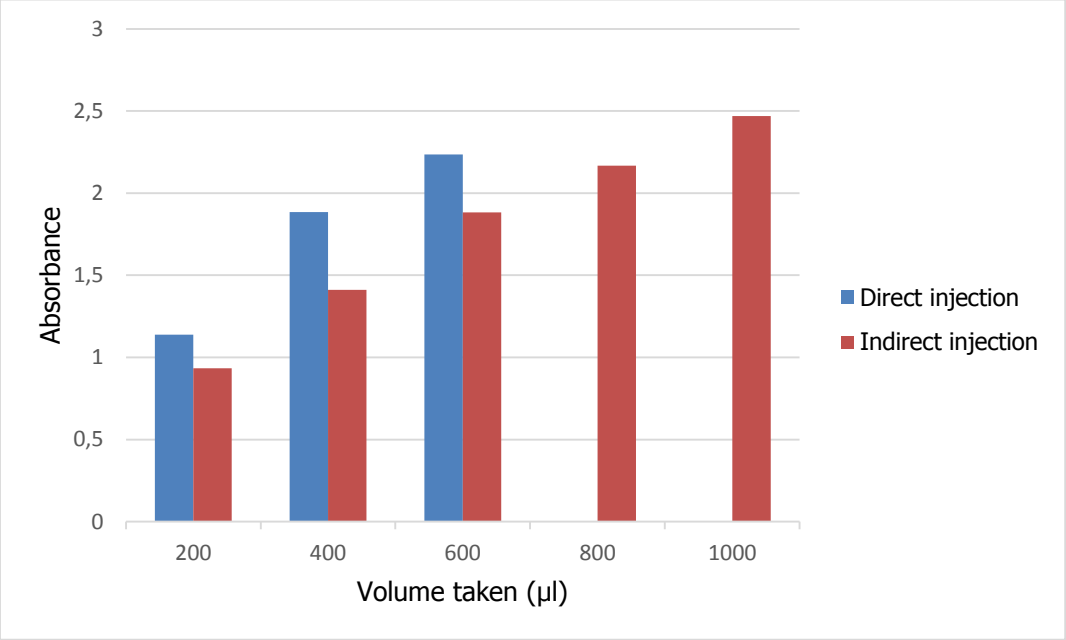

Figure 64: Comparing absorbances of direct and indirect injections (new phenol red solution)

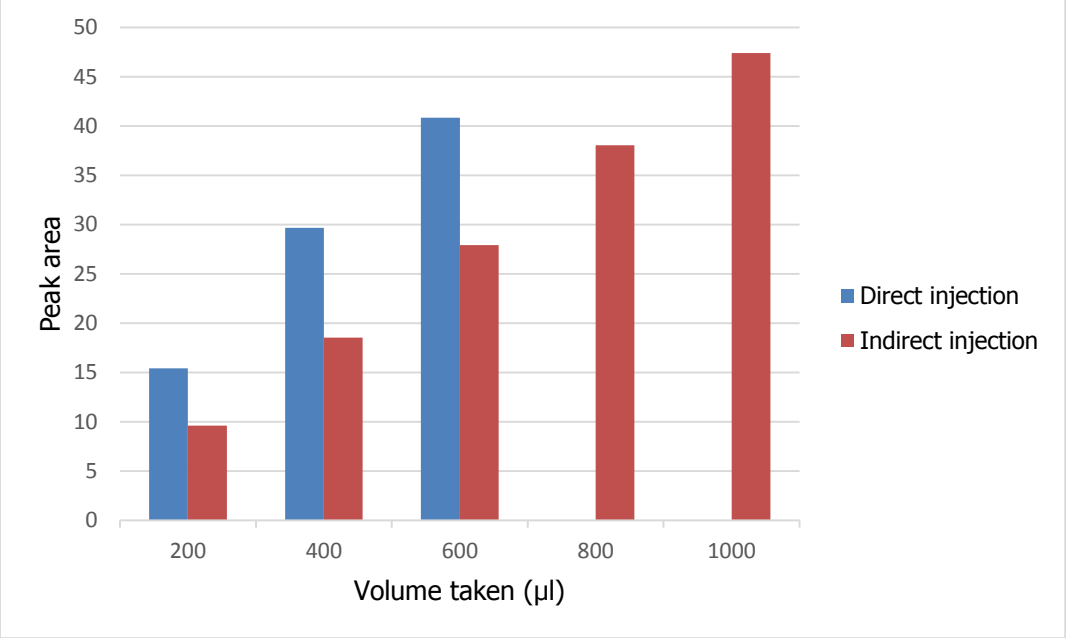

Figure 65: Comparing peak areas of direct and indirect injections (new phenol red solution)

When comparing the results from the direct and indirect injections with a new phenol red solution we can see that there is a difference in absorbance and in peak area between the two. With volumes of 800 and 1000 µl of direct injections, there are no values because there the absorbances and peak areas are too high (saturated).

From this results we can conclude that when it takes a longer time to send the solution to the spectrometer (send it first to the mixing flow cell), the absorbance of the phenol red solution decreases.

4.2.6.3 Linearity of dilutions 2

In these tests dilutions of phenol red are tested again with a new phenol red solution to see until what concentration (or dilution) the measurements are still linear. Dilutions from 5 to 20 times are tested and 3 different total volumes prepared. Therefore, in each experiment, a different quantity of liquid is sent to the spectrometer. In table 36 the injection volumes are shown.

In table 36, the conditions for the 3 different volumes can be seen. The dilutions that were used in these tests can be found in table 68 [Annex 14] till table 70 [Annex 16].

Table 36: Conditions linearity of dilutions phenol red 2

Phenol red stock solution	0,0113 M
Phenol red standard solution	5,65 * 10^{-5} M
Carrier solution	NaOH 10^{-5} M
Volumes taken to measure	1000, 2000 or 3000 µl
Times diluted tested	5, 10, 12,5; 16, 20 and 25 (only with 3000 µl)
Replications	5
Method	DilutionPR.txt [Annex 3.6]

4.2.6.3.1 Total volume of 1000 µl

The % accuracy of the trial with a total volume of 1000 µl can be seen in figure 66.

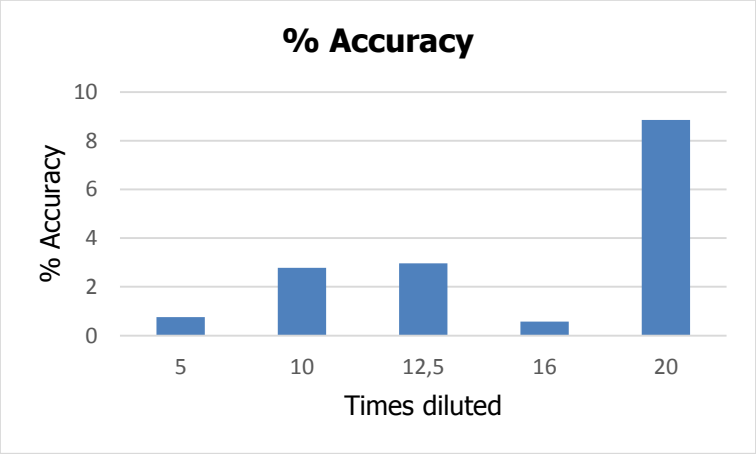

Figure 66: Accuracy dilutions phenol red 2 (1000 µl)

Table 37 is a summary table of the results of dilutions with a new phenol red solution (total volume of 1000 µl).

Table 37: Summary table dilutions phenol red (1000 µl)

Times diluted	Concentration Phenol Red (M)	Absorbance	Peak area	% RSD	% Accuracy
5	1,13E-05	0,414	8,721	0,6	0,76
10	5,65E-06	0,214	4,599	0,7	2,78
12,5	4,52E-06	0,178	3,718	1,3	2,96
16	3,53E-06	0,139	2,868	1,1	0,57
20	2,83E-06	0,103	2,125	1,5	8,86

When sending 1000 µl to the spectrometer to measure, there is a good precision until 20X diluted or concentration of 2,83 * 10^{-6} M phenol red. But there is only a good linearity until a dilution of 16 times (concentration of 3,53 * 10^{-6} M of phenol red).

4.2.6.3.2 Total volume of 2000 µl

The % accuracy of the trial with a total volume of 2000 µl can be seen in figure 67.

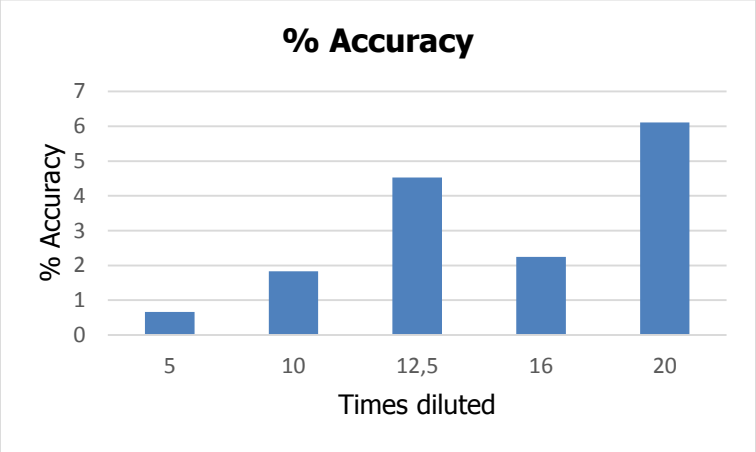

Figure 67: Accuracy dilutions phenol red 2 (2000 µl)

Table 38 is a summary table of the results of dilutions with a new phenol red solution (total volume of 2000 µl).

Table 38: Summary table dilutions phenol red (2000 µl)

Times diluted	Concentration Phenol Red (M)	Absorbance	Peak area	% RSD	% Accuracy
5	1,13E-05	0,444	14,470	1,5	0,66
10	5,65E-06	0,221	7,602	1,6	1,83
12,5	4,52E-06	0,179	6,329	0,9	4,53
16	3,53E-06	0,143	4,692	1,4	2,25
20	2,83E-06	0,116	3,686	0,7	6,11

When sending 2000 µl to the spectrometer, there is a fairly good linearity from 5 till 16 times diluted or from a concentration of 1,13 * 10^{-5} till 3,53 * 10^{-6}.

4.2.6.3.3 Total volume of 3000 µl

The % accuracy of the trial with a total volume of 3000 µl can be seen in figure 68.

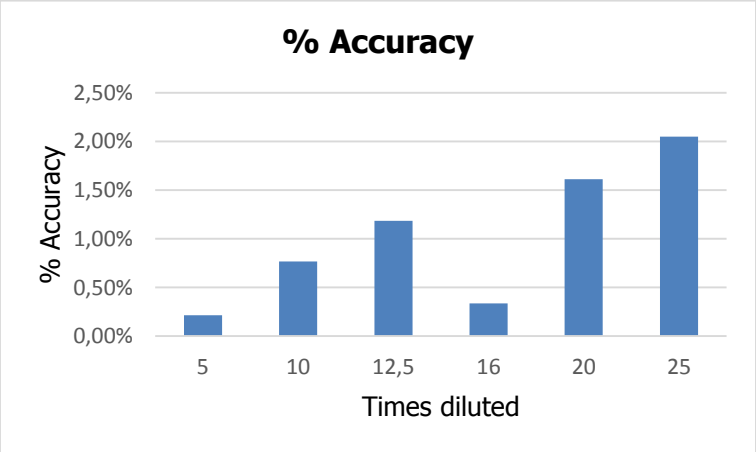

Figure 68: Accuracy dilutions phenol red 2 (3000 µl)

Table 39 is a summary table of the results of dilutions with a new phenol red solution (total volume of 3000 µl).

Table 39: Summary table dilutions phenol red (3000 µl)

Times diluted	Concentration Phenol Red (M)	Absorbance	Peak area	% RSD	% Accuracy
5	1,13E-05	0,348	16,677	2,3	0,22%
10	5,65E-06	0,175	8,557	2,1	0,77%
12,5	4,52E-06	0,145	7,103	1,1	1,18%
16	3,53E-06	0,118	5,595	1,3	0,34%
20	2,83E-06	0,099	4,689	2,6	1,61%
25	2,26E-06	0,081	3,735	1,9	2,05%

The regression lines and the coefficients of determination of the 3 different volumes are shown in figure 69.

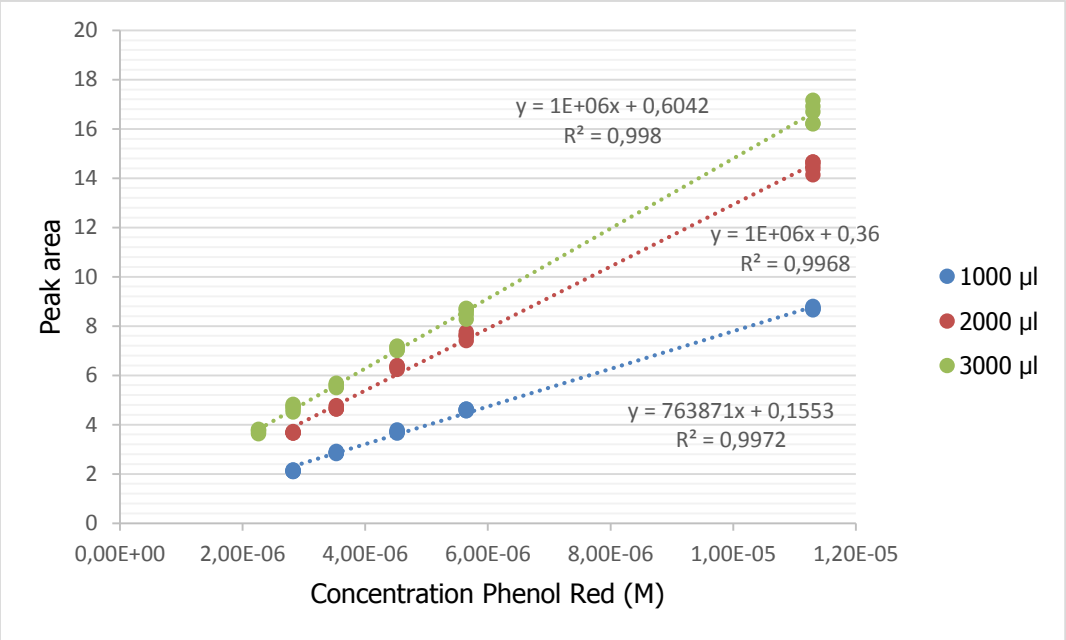

Figure 69: Regresision lines linearity phenol red 2

Conclusion:

All the calibration yield a god goodness of fit but, the best results have been obtained with a total volume of 3000 µl. In this test, there is a good linearity from 5 till 25 times diluted. When sending a volume of 3000 µl to the spectrometer a concentration of 2,26 * 10^{-6} M of phenol red can be reached. This higher volume of solutions allow a higher aliquot of concentrated solution to be injected by the syringe which decreases the error.

4.3 PHENOLS

4.3.1 Initial tests

The initial tests are done to see the effect of a waiting time before the spectrometric reading. In the classical test, a period of 2 hours is recommended before measuring. In the first trials a 100 ppm and a 20 ppm solution of gallic acid are measured directly and measured after waiting times of 2, 5, 10, 30 and 60 minutes.

In the two trials the total volume in the stirring cell is 2000 µl. The difference between these two tests are the volumes of the F-C reagent, the sodium carbonate and the carrier solution that are different. This can be seen in table 40.

Table 40: General conditions initial tests phenols

Carrier solution	Milli-Q water
Gallic acid stock solution	2000 ppm
Gallic acid standard solution	100 or 20 ppm
Volume of standard injected into the cell	100 µl
Volume of F-C reagent injected into the cell	100 or 200 µl
Volume of Na_2CO_3 injected into the cell	1000 or 1500 µl
Volume of carrier injected into the cell	800 or 200 µl
Volume taken to measure	2000 µl
Waiting times before measuring	0, 2, 5, 10, 30 or 60 minutes
Replications	2
Method	TestPhenols.txt [Annex 4.1]

4.3.1.1 Trial 1

- Injected volumes into stirring cell:

  100 µl of 20 or 100 ppm standard solution
  100 µl of Folin-Ciocalteu reagent
  1000 µl of Na_2CO_3
  800 µl H_2O

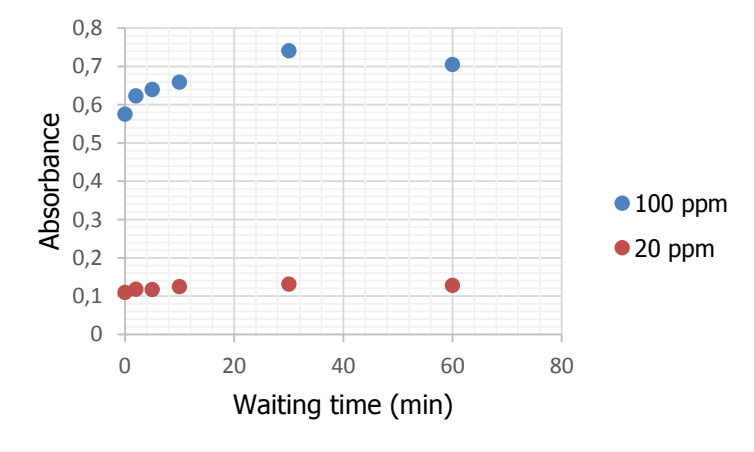

Figure 70: Absorbances of initial test 1 phenols

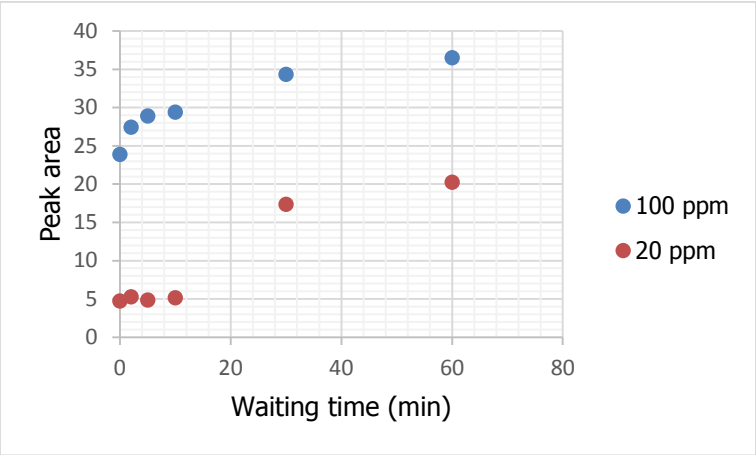

Figure 71: Peak areas of initial test 1 phenols

Conclusion:

The quantities introduced of standard and reagents are good enough. The gallic acid reacts and it is detected. Increase of time before sending the solution to the spectrometer, results in a higher absorbance and peak area. At 30 minutes a maximum is reached. When measuring directly (no waiting time), there are some bubbles going from the cell into the tubes. So in further measurements, it's better to introduce a waiting time before sending the solution to the spectrometer.

4.3.1.2 Trial 2

- Injected volumes into stirring cell:

  100 µl of 20 or 100 ppm gallic acid standard solution
  200 µl of Folin-Ciocalteu reagent
  1500 µl of Na_2CO_3
  200 µl H_2O

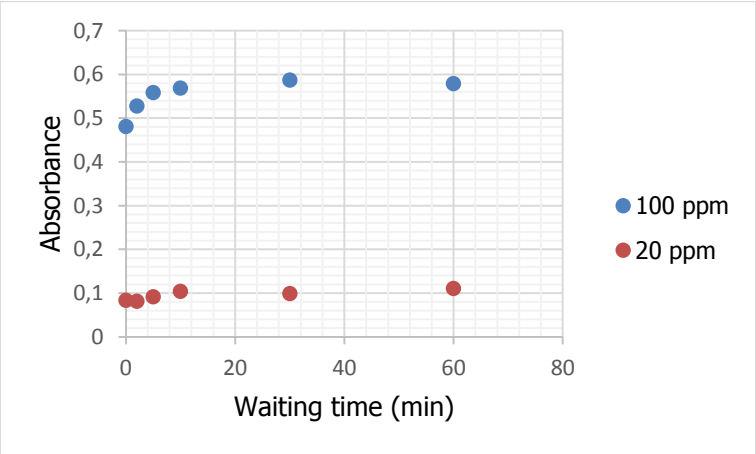

Figure 72: Absorbances of initial test 2 phenols

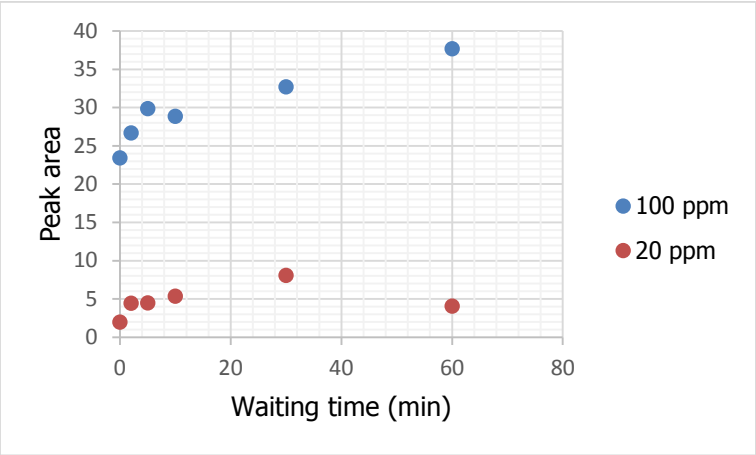

Figure 73: Peak areas of initial test 2 phenols

Conclusion:

The quantities introduced of standard and reagents are with these volumes also good enough. The gallic acid reacts and it is detected. Increase of time before sending the solution to the spectrometer, results in a higher absorbance and peak area. Here is also at 30 minutes a maximum reached. But after 5 minutes the increase of signal is small.

In general can we conclude that there is no big difference when changing the volumes of F-C reagent and the sodium carbonate. So the smaller dose of F-C reagent can be used (100µl). For the waiting time needed, 5 minutes seems to be a value from where the increase of signal is acceptable and it should give a great variability, but on the other hand 2 minutes seems to be a good lap of time when the reaction has proceeded significantly. Therefore, in further measurements a using time of 2 minutes is used to lose not too much time with the measurements.

4.3.2 Gallic acid

After the initial tests, different calibration lines are made with different conditions. First a calibration line was made from 10 till 100 ppm. Afterwards different volumes of lower concentrations than 10 ppm are tested to see until what volume of low concentration there is still linearity. In each trial, the used conditions are mentioned. The dilutions of these experiments can be found in table 71 [Annex 17].

Method: Phenol200µlStd.txt [Annex 4.2]

4.3.2.1 Calibration from 10 until 100 ppm

The conditions for the first calibration can be found in table 41.

Table 41: Conditions calibration 1 gallic acid

Carrier solution	Milli-Q water
Volume of standard injected into the cell	100 µl
Volume of F-C reagent injected into the cell	100 µl
Volume of Na_2CO_3 injected into the cell	800 µl
Volume of carrier injected into the cell	1000 µl
Volume taken to measure	2000 µl
Waiting time before measuring	2 minutes
Replications	5

In figure 74 the graph with the equation and the coefficient of determination of the calibration from 10 till 100 ppm can be seen.

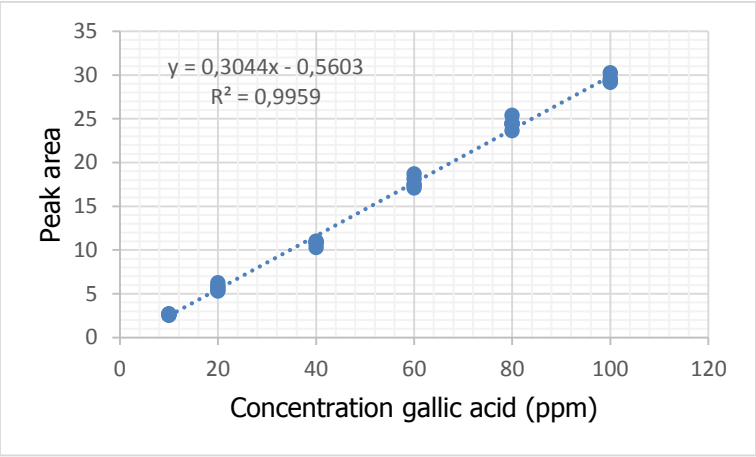

Figure 74: Calibration 1 gallic acid

The % accuracy of the first calibration line can be seen in figure 75.

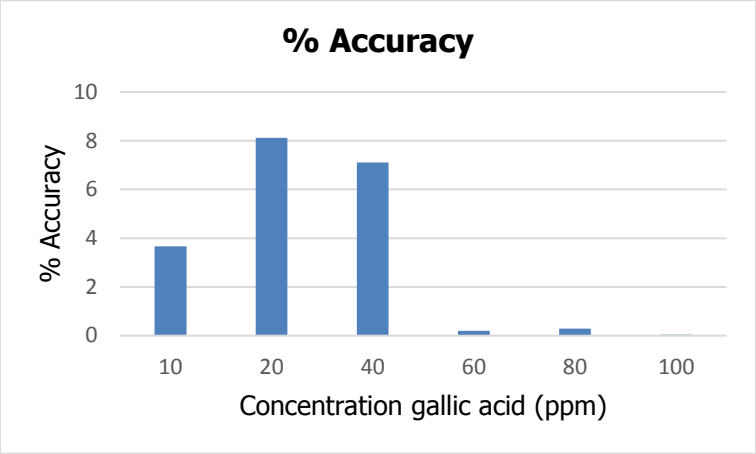

Figure 75: Accuracy calibration 1 gallic acid

In table 42, the results of the first calibration of gallic acid are shown in a summary table.

Table 42: Sumarry table calibration 1 gallic acid

Concentration gallic acid (ppm)	Absorbance	Peak area	%RSD	% Accuracy
10	0,07	2,648	2,7	3,67
20	0,109	5,739	6,4	8,12
40	0,215	10,798	2,7	7,11
60	0,35	17,763	3,7	0,2
80	0,472	24,486	2,5	0,29
100	0,575	29,538	1,5	0,043

Conclusion:

In this test there is a only a fairly good linearity from 10 until 100 ppm. Normally it would be a calibration line from 0 till 100 ppm but when using a blank solution there was a problem of diffraction between the blank and the different reagents accompanied with a negative peak area. So a blank solution cannot be used in this tests. After this calibration, different tests are done to see until what concentration the problem of diffraction takes place or how to avoid this problem.

4.3.2.2 Different volumes of a 10 ppm solution

In this experiment a calibration line is made with different volumes a of 10 ppm solution of gallic acid. The used conditions can be seen in table 43.

Table 43: Conditions calibration 2 gallic acid

Carrier solution	Milli-Q water
Volume of standard injected into the cell	100, 200, 400 or 600 µl
Volume of F-C reagent injected into the cell	100 µl
Volume of Na_2CO_3 injected into the cell	1000 µl
Volume of carrier injected into the cell	800, 700, 500 or 300 µl
Volume taken to measure	2000 µl
Waiting time before measuring	2 minutes
Replications	5

The % accuracy of the second calibration line can be seen in figure 77.

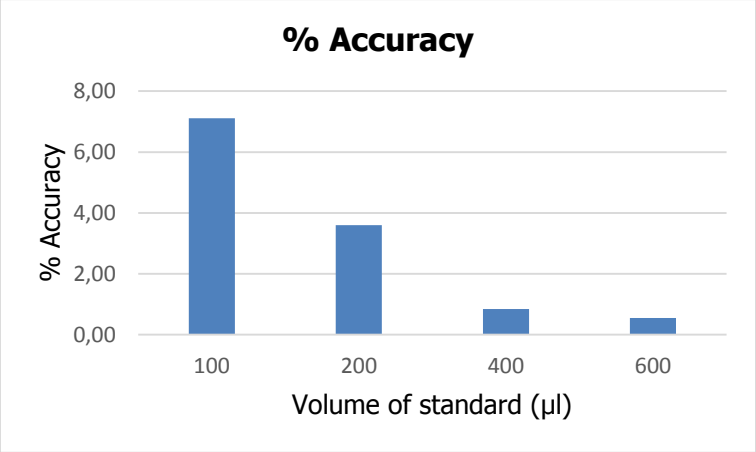

Figure 76: Accuracy calibration 2 gallic acid

4.3.2.3 Different volumes of a 5 ppm solution

In this experiment different volumes of a 5 ppm solution are tried to see if using bigger volumes avoid the problem of the diffraction.

In table 44 the conditions used in this test are shown.

Table 44: Conditions calibration 3 gallic acid

Carrier solution	Milli-Q water
Volume of standard injected into the cell	100, 200, 400 or 600 µl
Volume of F-C reagent injected into the cell	100 µl
Volume of Na_2CO_3 injected into the cell	1000 µl
Volume of carrier injected into the cell	800, 700, 500 or 300 µl
Volume taken to measure	2000 µl
Waiting time before measuring	2 minutes
Replications	5

The accuracy of taking different volumes of a 5 ppm solution gallic acid is shown in figure 77.

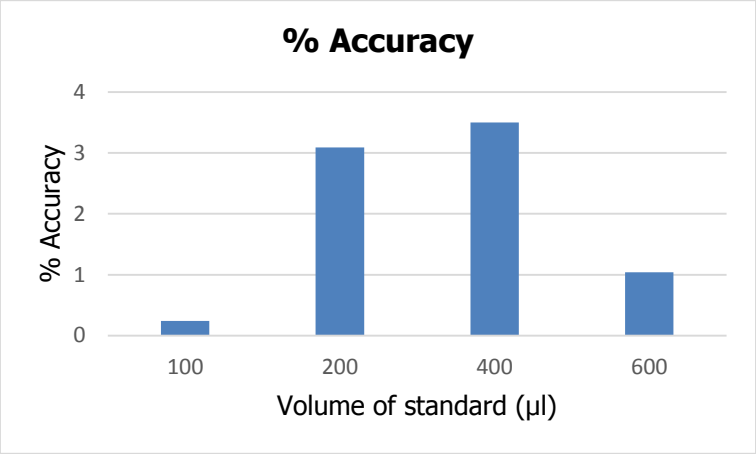

Figure 77: Accuracy calibration 3 gallic acid

4.3.2.4 Different volumes of a 2 ppm solution

Because of the low concentration of gallic acid, other (bigger) volumes are used for the calibration. The conditions of using a 2 ppm solution gallic acid are shown in table 45.

Table 45: Conditions calibration 4 gallic acid

Carrier solution	Milli-Q water
Volume of standard injected into the cell	100, 500, 700 or 900 µl
Volume of F-C reagent injected into the cell	100 µl
Volume of Na_2CO_3 injected into the cell	1000 µl
Volume of carrier injected into the cell	800, 400, 200 or 0 µl
Volume taken to measure	2000 µl
Waiting time before measuring	2 minutes
Replications	5

The accuracy of taking different volumes of a 2 ppm solution gallic acid is shown in figure 78.

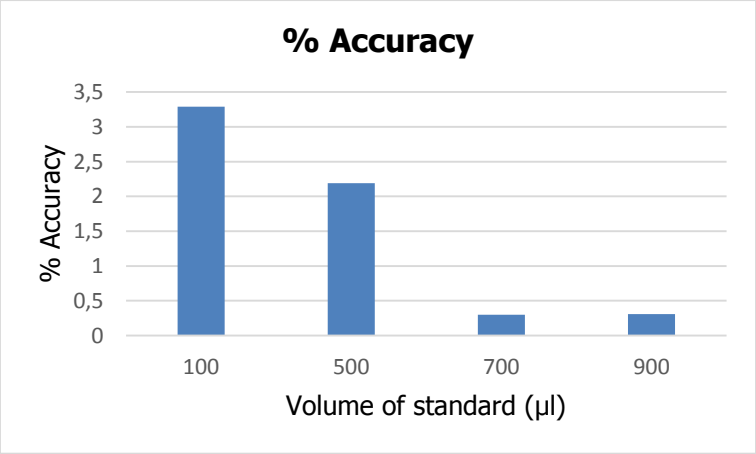

Figure 78: Accuracy calibration 4 gallic acid

4.3.2.5 Different volumes of a 1 ppm solution

In this experiment are the same volumes used as in the trial with a 2 ppm solution.

In table 46 are the conditions used in this test shown.

Table 46: Conditions calibration 5 gallic acid

Carrier solution	Milli-Q water
Volume of standard injected into the cell	100, 500, 700 or 900 µl
Volume of F-C reagent injected into the cell	100 µl
Volume of Na_2CO_3 injected into the cell	1000 µl
Volume of carrier injected into the cell	800, 400, 200 or 0 µl
Volume taken to measure	2000 µl
Waiting time before measuring	2 minutes
Replications	5

The accuracy of this calibration can be seen in figure 79.

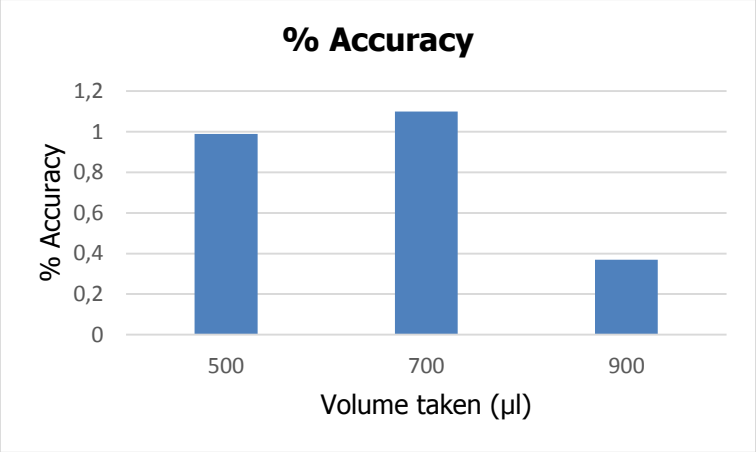

Figure 79: Accuracy calibration 5 gallic acid

On the next page, a summary table with all the results can be seen. Also 2 graphs, the first one (figure 80) with the regression lines and the coefficients of determination of the peak areas of the different concentrations using different volumes. And the right one (figure 81) is a graph with the absorbances of the different concentrations using different volumes.

Concentration gallic acid (ppm)	10				5				2				1			
Volume of std. (µl)	100	200	400	600	100	200	400	600	100	500	700	900	100	500	700	900
Absorbance	0,055	0,110	0,218	0,321	0,042	0,061	0,102	0,149	0,031	0,056	0,061	0,075	/	0,039	0,042	0,041
Peak area	2,430	5,485	10,747	15,939	1,701	2,797	5,447	7,551	0,713	2,389	3,088	3,896	/	0,393	1,002	1,643
% RSD	6,7	2,2	1,2	1,6	3,8	4,4	3,1	1,0	16,1	3,5	2,7	2,3	/	14,1	5,0	5,6
% Accuracy	7,11	3,6	0,84	0,55	0,24	3,09	3,5	1,04	3,29	2,19	0,30	0,31	/	0,99	1,1	0,37

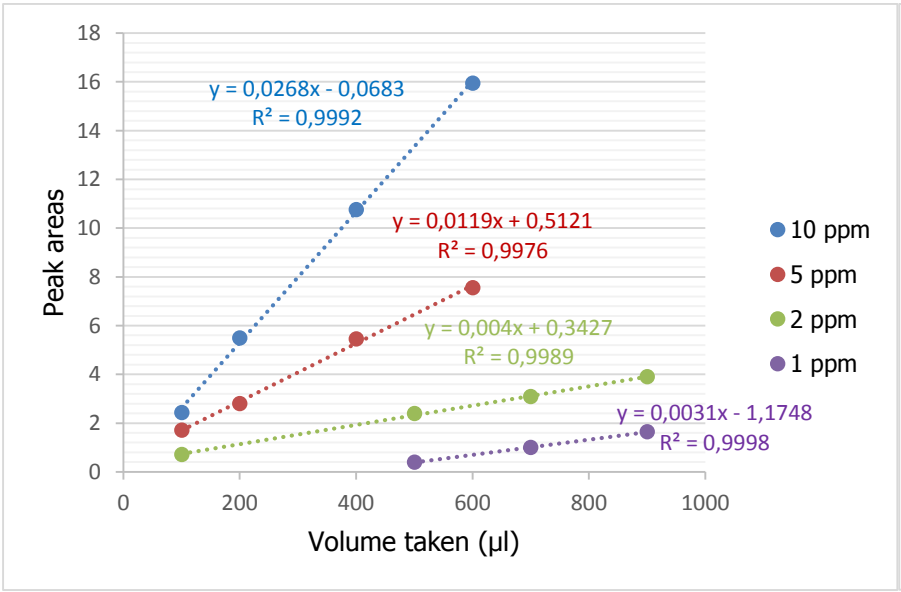

Figure 80: Regression lines of different concentrations of gallic acid

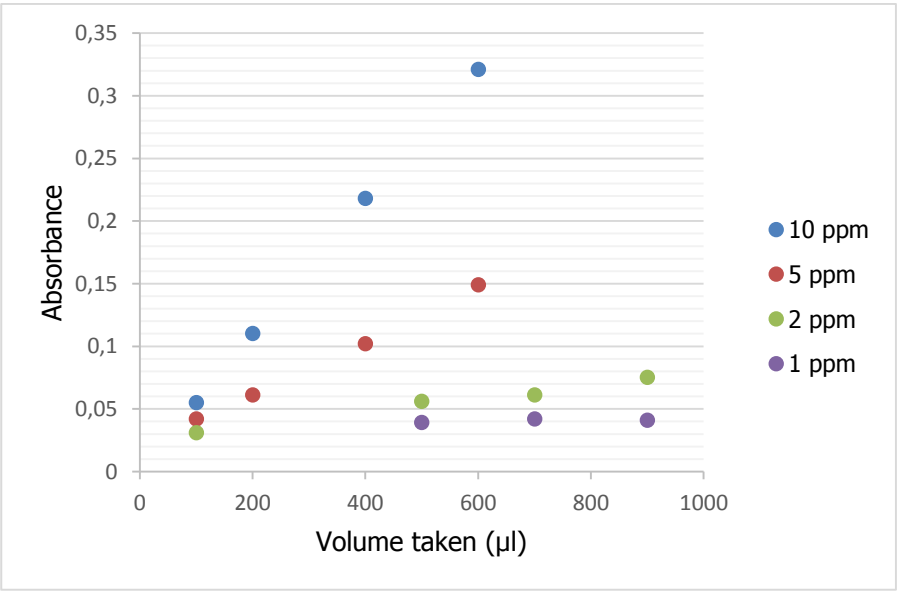

Figure 81: Absorbances of different concentrations of gallic acid

Conclusion:

When using a 5 ppm solution of gallic acid and taking different volumes of this solution, there is a good linearity from 100 until 600 µl. There is no problem of diffraction between the sample and the reagents, if volumes of 100 till 600 µl of a 5 ppm solution are used. So the solutions of 1 and 2 ppm were tested to see at which concentration diffraction occurs. With a solution of 2 ppm there is no diffraction with volumes from 100 till 900 µl. But with a volume of 100 µl, the precision is not good.

When taking 100 µl of a 1 ppm solution, the phenomenon of diffraction takes place. So there are too many negative points to have a reliable peak area.

Taking volumes from 500 till 900 µl gives good linearity but it's accompanied with a very bad precision.

In general we can conclude that if low concentrations of phenols must be determined, it's better to take big volumes of the standard or sample. For example 700 or 900 µl.

4.3.3 Real samples

Two different types of real samples are measured with the SIA-system. The first measurements were with samples of cork extraction and the second were samples of coffee extraction. In these tests, the concentrations are determined with 2 different methods to compare them. First the absorbances of the samples are measured with a classical spectrophotometer method and in the second method the absorbances samples are determined with the SIA-system.

4.3.3.1 Cork samples 1

The samples of cork have a low concentration of phenols, so 2 different calibration lines are made. The first one is to try if it is possible to make a calibration line from 20 till 100 ppm and interpolate the measurements of the samples. And the second one is a calibration line from 2 till 10, normally the concentration of these samples are between this 2 values.

How the dilutions of these calibration lines are made can be found in table 72 [Annex 18].

The dilutions of the classic method were made with the automatic dilutor and also the standards and samples that were measured with the classic spectrophotometer are mixed with the F-C reagent, the sodium carbonate in the dilutor and after a waiting time of 2 hours the absorbance was measured.

The dilutions that were used in the SIA-system are handmade and the standards and samples that were measured with this system are mixed with the reagents (Folin-Ciocalteu and sodium carbonate) in the stirrer cell and the absorbance was measured after a waiting time of 2 minutes.

Table 47: Conditions cork samples

Gallic acid stock solution	2000 ppm
Gallic acid standard solutions	20, 40, 60, 100 ppm and 2, 4, 6, 10 ppm
Carrier solution	Milli-Q water
Volume of standard or sample injected into the cell	100 µl (line 1) and 900 µl (line 2 + samples)
Volume of F-C reagent injected into the cell	100 µl (line 1, 2 and samples)
Volume of Na_2CO_3 injected into the cell	1000 µl (line 1, 2 and samples)
Volume of carrier injected into the cell	800 µl (line 1) and 0 µl (line 2 + samples)
Volume taken to measure	2000 µl
Waiting time before measuring	2 minutes
Replications	3
Method	Phenol200uLStd.txt [Annex 4.2]

4.3.3.1.1 Calibration line 1

The first calibration line is from 20 till 100 ppm.

In figure 82, the first calibration line that is used to calculate the concentration of phenols is shown.

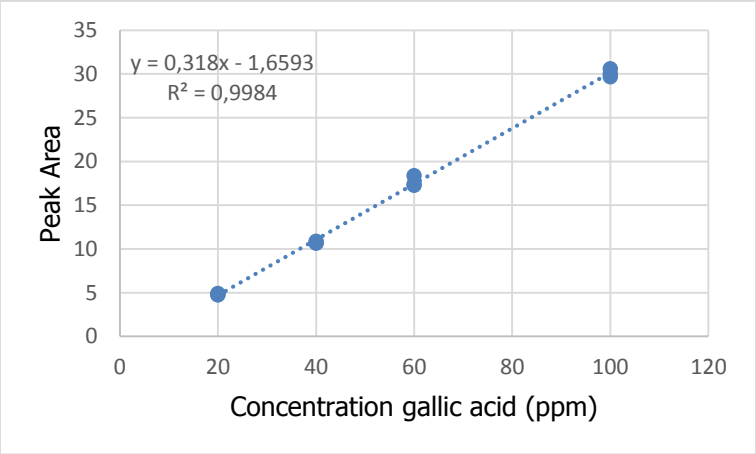

Figure 82: Calibration 1 cork samples

Table 48 is a summary table of the results of the first calibration line.

Table 48: Summary table calibration 1 cork samples

Concentration Gallic acid (ppm)	Peak area	Absorbance	%RSD	% Accuracy
20	4,818	0,102	1,9	2,413
40	10,746	0,217	1	2,947
60	17,669	0,334	3,4	1,388
100	30,103	0,569	1,6	0,142

In table 49 and 50, the phenol concentrations of the cork samples of the 2 different methods (SIA and classic spectrometric method) can be seen.

Table 49: Sample concentrations from SIA-analysis

Samples	Peak area	Absorbance	Concentration of phenols (ppm)
1	0,33	0,021	0,69
10	0,666	0,042	0,81
15	0,508	0,048	0,76
19	4,513	0,102	2,16
35	5,086	0,137	2,36
38	5,199	0,138	2,40

Table 50: Sample concentrations from the classic spectrometric method

Samples	Concentration of phenols (ppm)
1	0,62
10	1,53
15	4,08
19	2,17
35	5,18
38	6,36

4.3.3.1.2 Calibration line 2

The second calibration line is from 2 till 10 ppm and can be seen in figure 83.

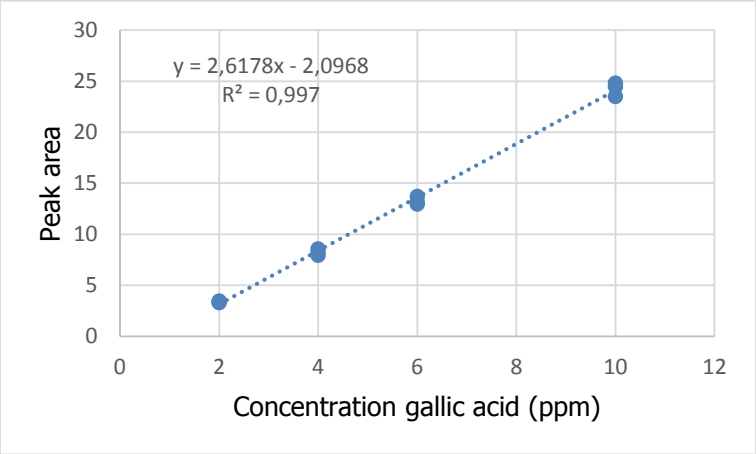

Figure 83: Calibration 2 cork samples

Table 51 is a summary table of the results of the second calibration line.

Table 51: Summary table calibration 2 cork samples

Concentration Gallic acid (ppm)	Peak area	Absorbance	%RSD	% Accuracy
2	3,391	0,062	2,4	7,438
4	8,256	0,143	3,7	1,434
6	13,283	0,232	3,1	2,462
10	24,274	0,412	2,9	0,794

In table 52 and 53, the phenol concentrations of the cork samples of the 2 different methods (SIA and classic spectrometric method) can be seen.

Table 52: Sample concentrations from SIA-analysis

Samples	peak area	Absorbance	Concentration of phenols (ppm)
1	0,33	0,021	0,93
10	0,666	0,042	1,06
15	0,508	0,048	1,00
19	4,513	0,102	2,52
35	5,086	0,137	2,74
38	5,199	0,138	2,79

Table 53: Sample concentrations from the classic spectrometric method

Samples	Concentration of phenols (ppm)
1	0,62
10	1,53
15	4,08
19	2,17
35	5,18
38	6,36

4.3.3.2 Cork samples 2

The second samples of cork have also a low concentration of phenols, so 2 different calibration lines are made. The first one is to try if it is possible to make a calibration line from 20 till 100 ppm and interpolate the measurements of the samples. And the second one is a calibration line from 2 till 10, normally the concentration of these samples are between this 2 values.

How the dilutions of these calibration lines are made can be found in table 72 [Annex 18].

The same conditions of the first cork samples are used.

4.3.3.2.1 Calibration line 1

The first calibration line is from 20 till 100 ppm.

In figure 84, the first calibration line that is used to calculate the concentration of phenols is shown.

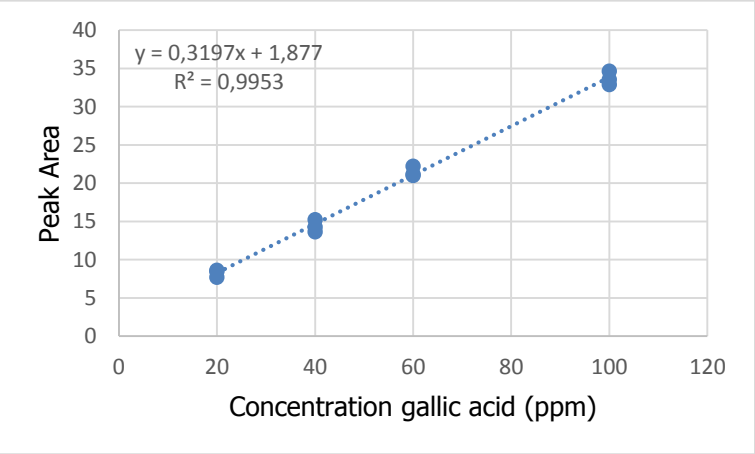

Figure 84: Calibration 1 cork samples 2

Table 54 is a summary table of the results of the first calibration line.

Table 54: Summary table calibration 1 cork samples 2

Concentration Gallic acid (ppm)	Peak area	Absorbance	%RSD	% Accuracy
20	8,257	0,115	5,9	0,178
40	14,400	0,228	5,5	1,85
60	21,490	0,337	3,1	1,996
100	33,702	0,558	2,6	0,44

In table 55 and 56, the phenol concentrations of the second cork samples of the 2 different methods (SIA and classic spectrometric method) can be seen.

Table 55: Sample concentrations from SIA-analysis

Samples	Peak area	Absorbance	Concentration of phenols (ppm)
1	0,871	0,026	-0,35 (0)
10	1,83	0,043	-0,02 (0)
15	3,414	0,058	0,53
19	4,661	0,094	0,97
35	9,35	0,14	2,60
38	9,244	0,143	2,56

Table 56: Sample concentrations from the classic spectrometric method

Samples	Concentration of phenols (ppm)
1	0,85
10	1,37
15	1,68
19	3,08
35	4,46
38	3,77

4.3.3.2.2 Calibration line 2

The second calibration line is from 2 till 10 ppm and can be seen in figure 85.

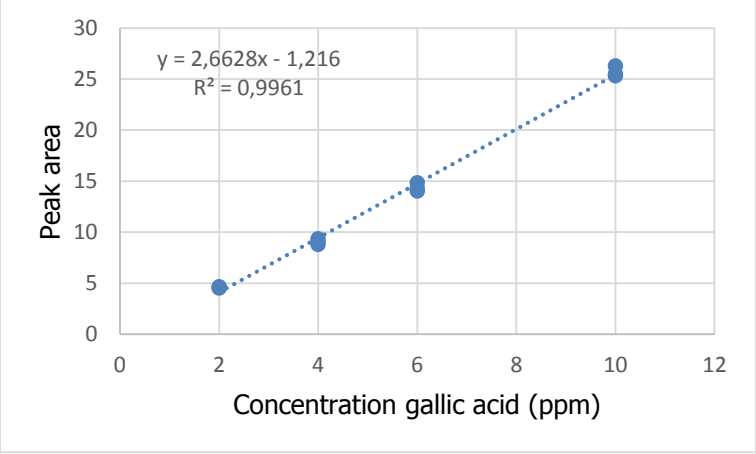

Figure 85: Calibration 2 cork samples 2

Table 57 is a summary table of the results of the second calibration line.

Table 57: Summary table calibration 2 cork samples 2

Concentration Gallic acid (ppm)	Peak area	Absorbance	%RSD	% Accuracy
2	4,591	0,075	1,3	10,484
4	9,048	0,166	3,1	4,281
6	14,378	0,228	2,9	2,664
10	25,701	0,408	2	1,123

In table 58 and 59, the phenol concentrations of the second cork samples of the 2 different methods (SIA and classic spectrometric method) can be seen.

Table 58: Sample concentrations from SIA-analysis

Samples	Peak area	Absorbance	Concentration phenols (ppm)
1	0,871	0,026	0,78
10	1,83	0,043	1,14
15	3,414	0,058	1,74
19	4,661	0,094	2,21
35	9,35	0,14	3,97
38	9,244	0,143	3,93

Table 59: Sample concentrations from the classic spectrometric method

Samples	Concentration of phenols (ppm)
1	0,85
10	1,37
15	1,68
19	3,08
35	4,46
38	3,77

Conclusions:

From al the cork samples the same conclusions can be taken.

When comparing the concentrations of the phenols of cork samples measured with the SIA-system or measured with the classic spectrophotometer, there are big differences in the two calibration lines. It seems that the low concentration calibration yields results of phenols concentrations in samples that are more coherent with the classical method. This first trial gives results with very different values between both analytical methods, but the second trial give a better reproducibility.

Also it is observed that the interpolations of higher volumes of sample into the high concentration curve does not give reproducible results. This effect should be further studied.

The differences can be explained to interferences due to solid particles that were present in the samples. Mainly in the first trial, as it could be observed with a simple view of the solutions.

Also some differences can be due to unknown interferences of other substances in the media, that can have a big effect at such a low concentration. A spiked sample trial should be done to evaluate this interferences.

4.3.3.3 Coffee samples

The conditions that are used for the determination of phenols in coffee samples are shown in table 60. The samples of coffee have a high concentration so a calibration line is made from 200 till 1000 ppm. How these dilutions are made can be found in table 73 [Annex 19].

The dilutions of the classic method were made with the automatic dilutor and also the standards and samples that were measured with the classic spectrophotometer are mixed with the F-c reagent, the sodium carbonate in the dilutor and after a waiting time of 2 hours the absorbance was measured.

The dilutions that were used in the SIA-system are made with the dilutor and the standards and samples that were measured with this system are mixed with the reagents (Folin-Ciocalteu and sodium carbonate) in the stirrer cell and the absorbance was measured after a waiting time of 2 minutes.

Table 60: Conditions coffee samples

Gallic acid stock solution	2000 ppm
Gallic acid standard solutions	200, 400, 600, 800 and 1000 ppm
Carrier solution	Milli-Q water
Volume of standard or sample injected into the cell	100 µl (calibration line and samples)
Volume of F-C reagent injected into the cell	100 µl (calibration line and samples)
Volume of Na_2CO_3 injected into the cell	1000 µl (calibration line and samples)
Volume of carrier injected into the cell	800 µl (calibration line and samples)
Volume taken to measure	2000 µl
Waiting time before measuring	2 minutes
Replications	3
Method	Phenol200uLStd.txt [Annex 4.2]

In figure 86, the calibration line that is used to calculate the concentration of phenols in coffee samples is shown.

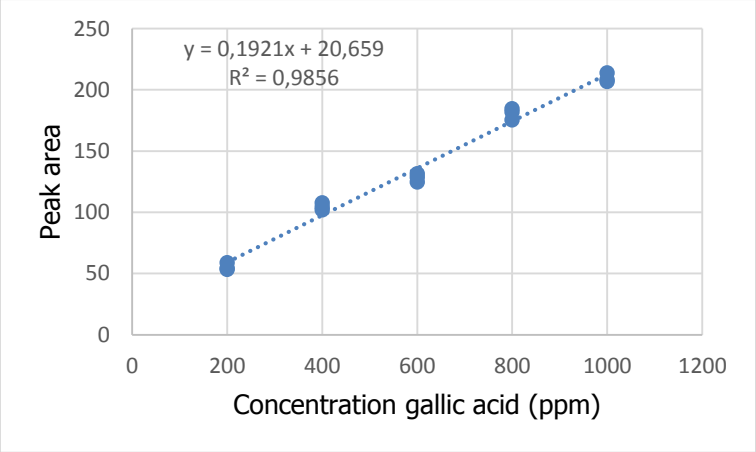

Figure 86: Calibration coffee samples

Table 61 is a summary table of the results of the calibration line.

Table 61: Summary table calibration coffee samples

Concentration Gallic acid (ppm)	Peak area	Absorbance	%RSD	% Accuracy
200	55,646	1,081	5,3	6,157
400	104,836	2,223	2,6	7,012
600	128,284	2,595	2,9	5,936
800	180,785	2,965	2,6	3,615
1000	209,738	3,386	1,8	1,424

In table 62 and 63, the phenol concentrations of the cork samples of the 2 different methods (SIA and classis spectrometric method) can be seen.

Table 62: Sample concentrations from SIA-analysis

Sample	Peak area	Absorbance	Concentration of phenols (ppm)
1	128,470	2,326	648,2
2	129,927	2,426	655,8
3	140,207	2,441	709,3
4	137,754	2,472	696,6
5	130,173	2,301	657,1

Table 63: Sample concentrations from the classic spectrometric method

Sample	Absorbance	Concentration of phenols (ppm)
1	1,2578	642,7
2	1,2778	653,2
3	1,2578	642,7
4	1,2428	634,7
5	1,1878	605,7

Table 64: % Difference between sample concentrations of the 2 different methods

In table 64, the % difference between the sample concentrations of the 2 different methods can be seen. It's the difference between the 2 measurements divided by the average and multiplied by 100.

Sample	% Difference
1	0,85
2	0,40
3	9,85
4	9,30
5	8,14

Conclusion:

When comparing the concentrations of the phenols of coffee samples measured with the SIA-system or measured with the classic spectrophotometer, there are not that big differences. With the classic method the concentrations are a little bit lower than with the SIA-system.

When comparing the 2 methods we can see that there is a general % of difference lower than 10% which is good for these real samples. The reliability of the SIA-method at such high concentrations is very good, and results are very reproducible to the classical method.

5 CONCLUSION

- The mixing flow cell works very well with a good reliability for determinations of phenol concentrations of 1-10 ppm and from 10-100 ppm. This is based on a good accuracy (under 5%), a good precision (under 3%) and good coefficients of determination (over 0,998…) obtained. The limit of detection is between 0,5 and 1 ppm.

- Good dilutions can be made with the mixing cell from 5 till 20 times with a good accuracy (under 2%) and a good precision (under 3%).

- One time flushing is enough for cleaning the mixing flow cell.

- A very good reproducibility of different injection volumes in the mixing flow cell have been observed.

- Good reproducibility of the determination of phenols in coffee extracts (high concentrations of phenols).

- Not so good reproducibility of the determination of phenols in cork extracts because of the low concentrations of phenols in the samples. No good extrapolation can be done in the high concentration calibration from injections of high volumes of samples.

- For the optimal conditions of phenols determination differences between the conditions of concentrations above or under 10 ppm must be made. In table 58 the best conditions are shown.

Table 58: Best conditions to measure phenol concentrations above 10 ppm

Condition	**Optimal values (1-10 ppm)**	**Optimal values (10-100 ppm)**
Volume of standard or sample injected into the cell	900 µl	100 µl
Volume of F-C reagent injected into the cell	100 µl	100 µl
Volume of Na_2CO_3 injected into the cell	1000 µl	1000 µl
Volume of carrier injected into the cell	0 µl	800 µl
Volume taken to measure	2000 µl	2000 µl
Waiting time before measuring	2 minutes	2 minutes
Carrier solution	Milli-Q water	Milli-Q water
Concentration of sodium carbonate	7,5%	7,5%

6 ANNEX

Annex 1: General SIA-methods

Annex 1.1: Cleaning main line

When measuring phenols, the wavelength is changed from 550 to 760 nm.

0 va 1
1 es 550 500
2 bo 60 1
60 bo 0 0
60 va 0 0

Time	**Action**
0	Open valve 1
1	Turn on spectrophotometer, 550 nm, 500 ms
2	Peristaltic pump, 60%, Anticlockwise
60	Peristaltic pump stops and close valves

Method 2: Clean1.txt

Annex 1.2: Cleaning burette

0 va 1
1 es 550 500
2 bu 1 1 500
12 bu 0 0 500
22 bu 1 1 500
32 bu 0 0 500
42 bu 1 1 500
52 bu 0 0 500
62 bu 1 1 500
72 bu 0 0 500
82 bu 1 1 500
92 va 0

Time	**Action**
0	Open valve 1
1	Turn on spectrophotometer, 550 nm, 500 ms
2	Empty burette
12	Loading burette
92	Close valves

Method 3: Cleanbureta.txt

Annex 1.3: Cleaning tubes 3, 4 and 6

0 va 32
1 bo 50 1
50 va 4
51 bo 50 1
95 va 8
96 bo 50 1
140 bo 0 1
141 va 0 0

Time	**Action**
0	Open valve 6
1	Peristaltic pump, 50%, anticlockwise
50	Open valve 3
51	Peristaltic pump, 50%, anticlockwise
95	Open valve 4
96	Peristaltic pump, 50%, anticlockwise
140	Peristaltic pump stops
141	Close valves

Method 4: Clean3-4-6.txt

Annex 1.4: Emptying of the cell

When measuring phenols, the wavelength is changed from 550 to 760 nm.

0 va 16
1 bo 63 0
19 bo 0 0
20 va 1
21 bo 63 1
45 es 550 500
76 bo 0 0
76 va 0

Time	**Action**
0	Open valve 5
1	Peristaltic pump, 63%, clockwise
20	Open valve 1
21	Peristaltic pump, 63%, anticlockwise
45	Turn on spectrophotometer, 550 nm, 500 ms
76	Peristaltic pump stops and close valves

Method 5: Emptycell.txt

Annex 1.5: Flushing of the cell

When measuring phenols, the wavelength is changed from 550 to 760 nm.

0 va 16
1 bo 63 1
12 bo 63 0
23 bo 0 0
24 va 1
25 bo 63 1
26 es 550 500
70 bo 0 0
71 va 0

Time	**Action**
0	Open valve 5
1	Peristaltic pump, 63%, anticlockwise
12	Peristaltic pump, 63%, clockwise
24	Open valve 1
25	Peristaltic pump, 63%, anticlockwise
26	Turn on spectrophotometer, 550 nm, 500 ms
70	Peristaltic pump stops
71	Close valves

Method 6: FlushCell.txt

Annex 2: Methods for the determination of Cr(III)

Annex 2.1: Cr(III) calibration

This test is for volumes of 100 µl of Ce(IV), standard and DPC. These volumes are changed to 200 µl for another test.

0 va 1
1 bu 1 1 500
11 va 16
12 bu 1 0 100
15 va 8
16 bu 1 0 100
19 va 4
20 bu 1 0 100
30 va 1
31 es 550 200
32 bo 50 1
100 bo 0 1
100 va 0

Time	**Action**
1	Empty burette
12	Loading Ce(IV) into reaction coil
16	Loading Cr(III) into reaction coil
20	Loading DPC into reaction coil
31	Turn on spectrophotometer, 550 nm, 200 ms
32	Peristaltic pump, 50%, anticlockwise (solution to spectrophotometer)
100	Peristaltic pump stop and close valves

Method 7: CrIII100CeSDpc.txt

Annex 3: Methods for the determination of phenol red

Annex 3.1: Loading stirrer cell with peristaltic pump

This test is used for a volume of 600 µl, when changing time the method can also be used for volumes of 1000 and 1500 µl.

0 va 8
1 bo 63 0
12 bo 0 1
13 va 16
14 bo 63 1
25 bo 63 0
36 bo 0 0
37 va 1
38 bo 63 1
39 es 550 500
80 bo 0 0
81 va 0

Time	**Action**
1	Peristaltic pump, 63%, clockwise, loading phenol red into reaction coil
14	Peristaltic pump, 63%, anticlockwise, phenol red from reaction coil into stirrer cell
25	Peristaltic pump, 63%, clockwise, phenol red from stirrer cell into reaction coil
38	Peristaltic pump, 63%, anticlockwise, phenol red from reaction coil to spectrophotometer
39	Turn on spectrophotometer, 550 nm, 500 ms
80	Peristaltic pump stops
81	Close valves

Method 8: LoadCell600.txt

Annex 3.2: Loading stirrer cell with automatic burette

This method is used for a volume of 600 µl, when changing volumes the method can also be used for volumes of 1000 and 1500 µl.

0 va 1
1 bu 1 1 500
10 va 8
11 bu 1 0 300
19 va 16
20 bu 1 1 300
31 bu 1 0 300
43 va 1
44 bo 63 1
45 es 550 500
75 bo 0 0
76 va 0

Time	Action
1	Empty burette
11	Loading phenol red into reaction coil
20	Phenol red from reaction into stirrer cell
31	Phenol red from stirrer cell into reaction coil
44	Peristaltic pump, 63%, anticlockwise, phenol red from reaction coil to spectrophotometer
45	Turn on spectrophotometer, 550 nm, 500 ms
75	Peristaltic pump stops
76	Close valves

Method 9: LoadCell600BUR.txt

Annex 3.3: Direct injections

Here a volume of 1000 µl of phenol red is used. For changing volume, the time and volumes were be changed.

0 va 1
1 bu 1 1 500
11 va 8
12 bu 1 0 500
22 va 1
23 es 550 500
24 bo 50 1
105 bo 0 1
106 va 0

Time	Action
1	Empty burette
12	Loading phenol red into reaction coil
23	Turn on spectrophotometer, 550 nm, 500 ms
24	Peristaltic pump, 50%, anticlockwise, phenol red from reaction coil to spectrophotometer
105	Peristaltic pump stops
106	Close valves

Method 10: TestPhenolRed.txt

Annex 3.4: Indirect injections

These test is described for a volume of 400 µl of phenol red. When it is needed to use other volumes, the times and volumes must be changed.

0 va 1
1 bu 1 1 500
11 va 8
12 bu 1 0 200
22 va 16
23 bu 1 1 200
33 bu 1 0 200
43 va 1
44 bo 63 1
45 es 550 500
100 bo 0 0
101 va 0

Time	Action
1	Empty burette
12	Loading phenol red into reaction coil
23	Phenol red from reaction coil into stirrer cell
33	Phenol red from stirrer cell into reaction coil
44	Peristaltic pump, 63%, anticlockwise, phenol red from reaction coil to spectrophotometer
45	Turn on spectrophotometer, 550 nm, 500 ms
100	Peristaltic pump stops
101	Close valves

Method 11: IndirectInjection.txt

Annex 3.5: Different volumes taken out of the cell

In this test, a quantity a liquid is injected in the stirrer cell and less volume is taken out and send to the spectrophotometer to measure the absorbance. This is an example of injecting 1400 µl of phenol red into the stirrer cell and take 800 µl out of the cell to send to the spectrophotometer.

0 va 1
1 bu 1 1 500
11 va 8
12 bu 1 0 350
22 bu 0 1 350
32 bu 1 0 350
42 va 16
43 bu 1 1 350
53 bu 0 0 350
63 bu 1 1 350
73 bu 1 0 400
83 va 1
84 bo 63 1
85 es 550 500
140 bo 0 0
141 va 0

Time	Action
1	Empty burette
12	Loading phenol red into reaction coil
32	Loading phenol red into reaction coil
43	Phenol red from reaction coil into stirrer cell
63	Phenol red from reaction coil into stirrer cell
73	Phenol red from stirrer cell into reaction coil
84	Peristaltic pump, 63%, anticlockwise, phenol red from reaction coil to spectrophotometer
85	Turn on spectrophotometer, 550 nm, 500 ms
140	Peristaltic pump stops
414	Close valves

Method 12: PhenolRedDifVol.txt

Annex 3.6: Linearity of dilutions

This method is used for a total volume of 1000 µl. When the different times and volumes are changed, it can be used for the other tests.

0 va 1
1 bu 1 1 500
11 bu 0 0 400
21 va 8
22 bu 1 0 100
24 va 16
25 bu 1 1 500
35 bu 1 0 500
45 va 1
46 bo 63 1
47 es 550 500
115 bo 0 0
116 va 0

Time	**Action**
1	Empty burette
11	Loading carrier into reaction coil
22	Loading phenol red into reaction coil
25	Phenol red from reaction coil into stirrer cell
35	Phenol red from stirrer cell into reaction coil
46	Peristaltic pump, 63%, anticlockwise, phenol red from reaction coil to spectrophotometer
47	Turn on spectrophotometer, 550 nm, 500 ms
115	Peristaltic pump stops
116	Close valves

Method 13: DilutionPR.txt

Annex 4: Methods for the determination of phenols

Annex 4.1: Initial tests

This is the general method for the determination of phenols. A lot of things can be changed on this method, for example a waiting time can be introduced or the volumes of the sample or reagents can be changed. An example of a changed method can be seen in Annex 14, where a waiting time of 2 minutes is introduced and a volume of 200 µl of standard.

```
0 va 1
1 bu 1 1 500
11 va 32
12 bu 1 0 50
17 va 4
18 bu 1 0 50
23 va 16
24 bu 1 1 100
30 bu 0 1 100
36 va 8
37 bu 1 0 500
47 va 16
48 bu 1 1 500
58 bu 0 0 400
68 bu 1 1 400
78 bu 1 0 500
88 bu 0 1 500
98 bu 1 0 500
108 va 1
108 es 760 500
109 bo 63 1
200 bo 0 1
201 va 0
```

Time	**Action**
1	Empty burette
12	Loading standard into reaction coil
18	Loading F-C reagent into reaction coil
24	Solution from reaction coil into stirrer cell
37	Loading sodium carbonate into reaction coil
48	Sodium carbonate into stirrer cell
68	Loading carrier into stirrer cell
78	Solution from stirrer cell into reaction coil
98	Solution from stirrer cell into reaction coil
108	Turn on spectrophotometer, 760 nm, 500 ms
109	Peristaltic pump, 63%, anticlockwise, solution from reaction coil to spectrophotometer
200	Peristaltic pump stops
201	Close valves

Method 14: TestPhenols.txt

Annex 4.2: Calibration gallic acid

0 va 1
1 bu 1 1 500
11 va 32
12 bu 1 0 50
17 va 4
18 bu 1 0 50
23 va 16
24 bu 1 1 100
30 bu 0 1 100
36 va 8
37 bu 1 0 500
47 va 16
48 bu 1 1 500
58 bu 0 0 400
68 bu 1 1 400
188 bu 1 0 500
198 bu 0 1 500
208 bu 1 0 500
219 es 760 500
220 bo 63 1
371 bo 0 1
372 va 0

Time	**Action**
1	Empty burette
12	Loading standard into reaction coil
18	Loading F-C reagent into reaction coil
24	Solution from reaction coil into stirrer cell
37	Loading sodium carbonate into reaction coil
48	Sodium carbonate into stirrer cell
68	Loading carrier into stirrer cell
68-188	*Waiting time of 2 minutes*
188	Solution from stirrer cell into reaction coil
208	Solution from stirrer cell into reaction coil
219	Turn on spectrophotometer, 760 nm, 500 ms
220	Peristaltic pump, 63%, anticlockwise, solution from reaction coil to spectrophotometer
391	Peristaltic pump stops
392	Close valves

Method 15: Phenol200uLStd.txt

Annex 5: Values measurements relation between rpm, time and volume of liquid

Table 59: Values of the relation between rpm, time and volume of liquid

Vel. (rpm)	t (s)	V (ml)	Vel. (rpm)	t (s)	V (ml)	Vel. (rpm)	t (s)	V (ml)
20	5	0,17	25	5	0,22	30	5	0,28
20	5	0,18	25	5	0,23	30	5	0,28
20	5	0,17	25	5	0,22	30	5	0,27
20	5	0,19	25	5	0,22	30	5	0,28
20	10	0,38	25	10	0,5	30	10	0,59
20	10	0,36	25	10	0,5	30	10	0,58
20	10	0,39	25	10	0,52	30	10	0,56
20	10	0,38	25	10	0,49	30	10	0,58
20	15	0,57	25	15	0,72	30	15	0,87
20	15	0,57	25	15	0,7	30	15	0,85
20	15	0,58	25	15	0,73	30	15	0,85
20	15	0,57	25	15	0,7	30	15	0,87
20	20	0,75	25	20	0,98	30	20	1,18
20	20	0,73	25	20	0,99	30	20	1,16
20	20	0,75	25	20	0,99	30	20	1,17
20	20	0,73	25	20	0,99	30	20	1,18
20	30	1,2	25	30	1,44	30	30	1,77
20	30	1,22	25	30	1,42	30	30	1,79
20	30	1,18	25	30	1,41	30	30	1,77
20	30	1,22	25	30	1,42	30	30	1,75

Annex 6: Dilutions Cr(III) calibration

Table 60: Dilutions Cr(III) calibration

Stock solution: 100 ppm

Cr(III) concentration (ppm)	Volume of stock solution (ml)	Volume of Milli-Q water (ml)
2	2,00	98,00
5	5,00	95,00
10	10,00	90,00
15	15,00	85,00
20	20,00	80,00

Annex 7: Dilutions direct and indirect injections of phenol red.

The dilutions that were used in the direct and indirect injections of phenol red can be seen in table 15.

Stock solution: 0,0113 M phenol red in 0,1 M NaOH

Table 61: Dilutions direct and indirect injections of phenol red

Dilution	Volume of stock solution (ml)	Volume of Milli-Q water (ml)
10 ($1{,}13 * 10^{-3}$ M)	10,00	90,00
100 ($1{,}13 * 10^{-4}$ M)	1,00	99,00
200 ($5{,}65 * 10^{-5}$ M)	0,50	99,50
250 ($4{,}52 * 10^{-5}$ M)	0,40	99,60

Annex 8: Dilutions linearity of phenol red 1

Initial volume= volume of phenol red into the cell
Final volume= volume send into the detector to measure the absorbance
Final volume – Initial volume= volume of carrier into the cell

Table 62: Dilutions phenol red (total volume of 600 µl)

Stock solution (M)	Final concentration (M)	Final volume (µl)	Initial volume (µl)	Dilution
1,695E-04	8,478E-05	600,0	300,0	2,0
1,695E-04	5,650E-05	600,0	200,0	3,0
1,695E-04	4,238E-05	600,0	150,0	4,0
1,695E-04	3,390E-05	600,0	120,0	5,0
1,695E-04	1,695E-05	600,0	60,0	10,0
1,695E-04	1,130E-05	600,0	40,0	15,0

Annex 9: Dilutions linearity of phenol red 2

Initial volume= volume of phenol red into the cell
Final volume= volume send into the detector to measure the absorbance
Final volume – Initial volume= volume of carrier into the cell

Table 63: Dilutions phenol red (total volume of 1200 µl)

Stock solution (M)	Final concentration (M)	Final volume (µl)	Initial volume (µl)	Dilution
2,825E-04	5,650E-05	1200,0	240,0	5,0
2,825E-04	2,825E-05	1200,0	120,0	10,0
2,825E-04	1,883E-05	1200,0	80,0	15,0
2,825E-04	1,143E-05	1200,0	60,0	20,0

Annex 10: Comparing absorbances of indirect injections of new and old phenol red solution

Table 64: Comparing absorbances of indirect injections (old and new phenol red)

Volume taken (µl)	New phenol red solution (Absorbance)	Old phenol red solution (absorbance)
200	0,934	0,448
400	1,412	0,653
600	1,882	0,837
800	2,167	0,924
1000	2,470	0,994

Annex 11: Comparing peak areas of indirect injections of new and old phenol red solution

Table 65: Comparing peak areas of indirect injections (old and new phenol red)

Volume taken (µl)	New phenol red solution (Peak area)	Old phenol red solution (Peak area)
200	9,600	4,312
400	18,532	8,257
600	27,911	11,837
800	38,054	16,362
1000	47,396	20,223

Annex 12: Comparing absorbances of direct and indirect injections of a new phenol red solution

Table 66: Comparing absorbances of direct and indirect injections (new phenol red)

Volume taken (µl)	Direct injections of new phenol red solution (absorbance)	Indirect injections of new phenol red solution (absorbance)
200	1,138	0,934
400	1,885	1,412
600	2,235	1,882
800	Saturated	2,167
1000	Saturated	2,470

Annex 13: Comparing peak areas of direct and indirect injections of a new phenol red solution

Table 67: Comparing peak areas of direct and indirect injections (new phenol red)

Volume taken (µl)	Direct injections of new phenol red solution (peak areas)	Indirect injections of new phenol red solution (peak areas)
200	15,406	9,600
400	29,651	18,532
600	40,826	27,911
800	Saturated	38,054
1000	Saturated	47,396

Annex 14: Dilutions phenol red (total volume of 1000 µl)

Initial volume= volume of phenol red into the cell
Final volume= volume send into the detector to measure the absorbance
Final volume – Initial volume= volume of carrier into the cell

Table 68: Dilutions phenol red (total volume of 1000 µl)

Stock solution (M)	Final concentration (M)	Final volume (µl)	Initial volume (µl)	Dilution
5,650E-05	1,130E-05	1000,0	200,0	5,0
5,650E-05	5,650E-06	1000,0	100,0	10,0
5,650E-05	4,520E-06	1000,0	80,0	12,5
5,650E-05	3,531E-06	1000,0	62,5	16,0
5,650E-05	2,825E-06	1000,0	50,0	20,0

Annex 15: Dilutions phenol red (total volume of 2000 µl)

Initial volume= volume of phenol red into the cell
Final volume= volume send into the detector to measure the absorbance
Final volume – Initial volume= volume of carrier into the cell

Table 69: Dilutions phenol red (total volume of 2000 µl)

Stock solution (M)	Final concentration (M)	Final volume (µl)	Initial volume (µl)	Dilution
5,650E-05	1,130E-05	2000,0	400,0	5,0
5,650E-05	5,650E-06	2000,0	200,0	10,0
5,650E-05	4,520E-06	2000,0	160,0	12,5
5,650E-05	3,531E-06	2000,0	125,0	16,0
5,650E-05	2,825E-06	2000,0	100,0	20,0

Annex 16: Dilutions phenol red (total volume of 3000 µl)

Initial volume= volume of phenol red into the cell
Final volume= volume send into the detector to measure the absorbance
Final volume – Initial volume= volume of carrier into the cell

Table 70: Dilutions phenol red (total volume of 3000 µl)

Stock solution (M)	Final concentration (M)	Final volume (µl)	Initial volume (µl)	Dilution
5,650E-05	1,130E-05	3000,0	600,0	5,0
5,650E-05	5,650E-06	3000,0	300,0	10,0
5,650E-05	4,520E-06	3000,0	240,0	12,5
5,650E-05	3,531E-06	3000,0	187,5	16,0
5,650E-05	2,825E-06	3000,0	150,0	20,0
5,650E-05	2,260E-06	3000,0	120,0	25,0

Annex 17: Dilutions used in calibrations of gallic acid

Table 71: Dilutions used in calibrations of gallic acid

Stock solution: 2000 ppm of gallic acid

Concentration gallic acid (ppm)	Volume of stock solution (ml)	Volume of Milli-Q water (ml)
10	0,50	99,50
20	1,00	99,00
40	2,00	98,00
60	3,00	97,00
80	4,00	96,00
100	5,00	95,00

Annex 18: Dilutions used in calibration 1 of real samples (cork)

Table 72: Dilutions used in calibrations of real samples (cork)

Stock solution: 2000 ppm of gallic acid

Concentration gallic acid (ppm)	Volume of stock solution (ml)	Volume of Milli-Q water (ml)
20	1,00	99,00
40	2,00	98,00
60	3,00	97,00
100	5,50	95,00

For the calibration line from 2 till 10 ppm, dilutions of 10 times of this solutions were made.

For example:

Take 10,00 ml of this 100 ppm solution, put it in a flask of 100,0 ml and add Milli-Q water until 100,0 ml.

Annex 19: Dilutions used in calibration of real samples (coffee)

Table 73: Dilutions used in calibrations of real samples (coffee)

Stock solution: 2000 ppm of gallic acid (these dilutions are made with the dilutor)

Concentration gallic acid (ppm)	Volume of stock solution (µl)	Volume of Milli-Q water (µl)
200	500	4500
400	1000	4000
600	1500	3500
800	2000	3000
1000	2500	2500

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