Electromembrane extraction followed by smartphone detection of methamphetamine - A feasibility study

Jelena Komnenic

Master Thesis in Pharmacy
45 credits

Section for Pharmaceutical Chemistry
Department of Pharmacy
Faculty of Mathematics and Natural Sciences

UNIVERSITY OF OSLO
April 2019
Electromembrane extraction followed by smartphone detection of methamphetamine - A feasibility study

Jelena Komnenic

The research and writing of this master thesis took place at the Section for Pharmaceutical Chemistry, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, University of Oslo in the period from August 2018 to April 2019.

Supervisors:
Professor Stig Pedersen-Bjergaard
Doctoral Research Fellow Magnus Saed Restan
Principal Engineer Marthe Lid
© Jelena Komnenic

2019

Electromembrane extraction followed by smartphone detection of methamphetamine -
A feasibility study

Jelena Komnenic

http://www.duo.uio.no/

Trykk: Reprocentralen, Universitetet i Oslo

IV
Abstract

In this work the possibility of developing a user-friendly and easy-to-handle device, which could provide information about medicine concentration in biological fluids, was investigated. The main idea was to implement electromembrane extraction (EME) as a sample preparation technique, Simon’s test as the indicator of medicine presence, and smartphone as a detector (colourimeter), for the purpose of quantifying methamphetamine based on RGB values obtained from digital images taken with the smartphone.

EME was investigated under stagnant conditions (without agitation), keeping the simplicity of practical application of such a device in mind. The effect of voltage, time and pH in the sample solution and in the acceptor solution on EME were tested. Extraction time of 15 minutes with 25 V, and 10 mM HCl in the acceptor solution and in the sample solution were found as optimal performance characteristics. However, recovery around 11% was not high enough to give enrichment as expected.

Different ratios of reagents in Simon’s test were investigated, as well as different volumes of the same ratio of reagents. Taking into consideration that colour of the blue complex changed with the concentration of methamphetamine, the quantification of this test was studied. The linear correlation between the concentration and the RGB values were investigated. The accuracy and precision obtained with highly concentrated samples were in accordance with EMA criteria.

With a smartphone as detector, limit of detection (LOD) and limit of quantification (LOQ) were calculated from the calibration curves to be around 0.05 mg/mL and 0.17 mg/mL, respectively. High LOD and LOQ with Simon’s test can be assigned to relatively high volumes of sample and reagents used. Thus, the coloured product of the reaction cannot be observed and measured at low concentrations.
Preface

Firstly, I would like to express my gratitude to Stig Pedersen-Bjergaard, Magnus Saed Restan and Marthe Lid. It was a true honour and privilege to work with you, and have you as my mentors. I am grateful for the continuous support, patience and motivation, as well as your guidance during the research and writing of this thesis.

My thanks also goes to fellow master students and other employees at the Section for Pharmaceutical Chemistry.

Last but not least, I would like to thank my husband, family and friends for all support, motivation and understanding.

Thank you!

Jelena Komnenic
# Table of contents

Abbreviation ............................................................................................................................... 1  
1 Introduction .................................................................................................................................. 3  
   1.1 Background .......................................................................................................................... 3  
   1.2 Aim of the work ................................................................................................................... 5  
2 Theory ........................................................................................................................................... 6  
   2.1 Electromembrane extraction (EME) .................................................................................. 6  
   2.2 Colourimetry – Quantification of colour .......................................................................... 10  
      2.2.1 Colour tests and their quantification .............................................................................. 11  
      2.2.2 Colour .......................................................................................................................... 11  
      2.2.3 Imaging devices ............................................................................................................ 12  
      2.2.4 RGB colour space ........................................................................................................ 12  
      2.2.5 Digital image processing and analysis ........................................................................... 13  
   2.3 Liquid chromatography ...................................................................................................... 14  
      2.3.1 Detector - mass spectrometry ...................................................................................... 15  
3 Experimental .................................................................................................................................. 16  
   3.1 Model analyte ..................................................................................................................... 16  
   3.2 Chemicals ............................................................................................................................ 16  
   3.3 Solutions ............................................................................................................................... 17  
   3.4 EME set-up .......................................................................................................................... 18  
   3.5 UHPLC-MS/MS .................................................................................................................. 19  
   3.6 Calculations .......................................................................................................................... 21  
   3.7 Imaging prerequisites and conditions ................................................................................... 21  
4 Results and discussion ................................................................................................................. 23  
   4.1 Colourimetry ....................................................................................................................... 23  
      4.1.1 Controlled environment for smartphone detection .......................................................... 23  
      4.1.2 Colourimetric testing with solutions of potassium dichromate .................................. 24  
      4.1.3 Colourimetric testing with solutions of 2, 6- dichloroindophenol .................................. 27  
   4.2 Quantification of colour tests with methamphetamine as analyte .................................... 29  
      4.2.1 Colour test with methamphetamine ............................................................................. 29  
   4.3 Electromembrane extraction .............................................................................................. 36
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3.1</td>
<td>Acceptor volume</td>
<td>38</td>
</tr>
<tr>
<td>4.3.2</td>
<td>Sample volume</td>
<td>38</td>
</tr>
<tr>
<td>4.3.3</td>
<td>Effect of voltage on EME</td>
<td>39</td>
</tr>
<tr>
<td>4.3.4</td>
<td>Time effect on EME</td>
<td>40</td>
</tr>
<tr>
<td>4.3.5</td>
<td>Effect of pH in the sample and in the acceptor solutions</td>
<td>40</td>
</tr>
<tr>
<td>4.4</td>
<td>EME followed by Simon’s test and colourimetry with smartphone</td>
<td>42</td>
</tr>
<tr>
<td>4.5</td>
<td>Recommendations</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>Conclusion</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Appendix</td>
<td>50</td>
</tr>
</tbody>
</table>
Abbreviation

A       Ampere
µA      Microampere
µg      Microgram
µl      Microliter
µm      Micrometre
cm      Centimetre
EME     Electromembrane extraction
eV      Electron Volt
HPLC    High Performance Liquid Chromatography
kV      Kilovolt
LC      Liquid Chromatography
LOD     Limit of detection
LOQ     Limit of quantification
M       Molar
mg      Milligram
min     Minute
mL      Millilitre
mM      Millimolar
mm      Millimetre
MS      Mass Spectrometer
m/z     Mass to charge ratio
ng      Nanogram
NPOE    2-nitrophenyl octyl ether
SRM     Selected reaction monitoring
RSD     Relative Standard Deviation
UHPLC   Ultra High Performance Liquid Chromatography
V       Volt
1 Introduction

1.1 Background

The affordability and portability of information technology and communications equipment, such as smartphones, digital cameras and computers, open a new possibilities in analytical chemistry [1]. Furthermore, the need for cost-effective point-of-care (POC) and on-site device applications and instruments for in situ and real-time monitoring is growing [2, 3]. Commercial self-tests, e.g. blood glucose testing or pregnancy tests, are good examples of such POC devices [4]. The advantage of this type of testing is the convenience of performing tests at home, and the simplicity and reduced response time.

Colour tests, spot tests or colourimetric tests are based on chemical reaction between an analyte and reagents which produces visible result – a colour [5, 6]. Colour tests are usually the simplest and quickest chemical tests applicable to the sample [7]. Since the introduction of colour tests in 18th century, known as naked eye tests [3], they have been considered solely as qualitative analysis or presumptive tests. However, the idea of quantification of these coloured reactions came with the appearance and development of imaging devices (smartphones, digital and video cameras) [3]. Colourimetry, in this respect, means quantification of a coloured solution (analyte) using imaging device and taking advantage of the colour intensities of red, green, blue (RGB) or a related magnitude as an analytical parameter [3]. As a proposal for further expansion of this technique, a POC or on-site device could be devised to measure medicine concentration in biological fluids by using smartphone.

The biggest challenges in the development of such device are complexity of biological samples, sample volume and low medicine concentration in the sample (ng/mL) [4]. Prior to any biological sample analysis, sample preparation is required. The purpose of sample preparation is to clean up the sample by removing all possible interference, and to make it compatible with analytical instruments. Another purpose of sample preparation is to enrich the concentration of the analyte which is to be detected. Ordinary methods used to that end are protein precipitation, liquid–liquid extraction, or solid-phase extraction [8].

In 2006 a new concept for sample preparation – Electromembrane extraction (EME) was published. EME is a liquid-phase microextraction intended for very fast and selective isolation...
and pre-concentration of target analytes from complex biological samples [9]. The EME system comprises two compartments separated by a supported liquid membrane – SLM, and the entire system is placed on an agitation platform [10]. Target analytes are charged and extracted from aqueous sample, through the SLM, to an aqueous acceptor solution under the influence of an electrical field. Taking into consideration pKa values of analytes, pH value of the sample and the acceptor solution are adjusted to ensure full ionization of analytes. Major operational parameters affecting the extraction efficiency are time, voltage and agitation. Extraction selectivity is controlled by the direction and magnitude of the electrical field, chemical composition of the SLM, and pH in the acceptor and sample solutions [10].

EME is compatible with all biological fluids such as human whole blood, plasma, urine, and saliva. Further, it can be interpreted as green chemistry, due to the use of only few microliter organic solvent per sample [10]. Pre-concentration obtained in EME depends on the acceptor volume and the sample volume. Recent work used EME in the lab-on-chip (LOC) device, where analytes were extracted from 70 μL biological fluid into 6 nL acceptor solution, with the enrichment factor of approximately 400 [11].

However, EME is not yet established as a routine technique for extraction. Since 2006 more than 270 articles relating to EME have been published [10]. This demonstrates the potential and attractiveness of EME. Some articles even show possible EME applicability with smartphone, for example extraction and quantification of lead (Pb) in water samples [12], and extraction of phenazopyridine in urine samples [13]. Namely, Pb was extracted by means of EME, as a LOC device, in acceptor solution with KI leading to formation of a yellow precipitate of PbI₂. Afterwards, a digital image of the precipitate was analysed by investigating its RGB intensities as an analytical parameter for concentration.

Phenazopyridine was extracted with EME in a LOC device as well. However, this LOC device is somewhat more complex, and includes the use of syringe as a pump for the sample solution, and the use of a microsyringe for the acceptor solution withdrawal after extraction. After extraction, acceptor solution was absorbed on a strong cation exchanger. Colouring of the analyte was not necessary taking into consideration that phenazopyridine darkens the colour of urine to an orange or red colour. The concentration of the analyte was quantified based on RGB analysis of the digital image taken with smartphone. Despite the complexity of this system and the fact that only a few medicines colour biological fluids, this research signalizes the
possibility of development of a POC or on-site device which includes EME as sample preparation technique and smartphone as analytes concentration detector.

1.2 Aim of the work

Following the trends mentioned above, the aim of this work was to investigate the possibility of developing a user-friendly and easy-to-handle POC or on-site device which would provide information about medicine concentration in biological fluids. The main idea was to implement EME as a sample preparation technique, colour test as an indicator of medicine presence and smartphone as a detector (colourimeter).

Given a limited number of colour tests and their detection limits, methamphetamine was selected as a model analyte, and Simon’s test as a colour test. In the first set of experiments the focus was on:

- Understanding how the RGB values changed with the change of colour (calibration)
- Investigating if all three components of a colour followed the same trend
- Finding the optimal ratio of reagents for Simon’s test
- Studying correlation between methamphetamine concentration and the RGB values (quantification of the test)
- Investigating the accuracy and precision of quantitative measurements.

EME was investigated under stagnant conditions. In order to obtain the optimal performance of EME, the experiments were focused on:

- Effect of voltage on EME
- Effect of time on EME
- Effect of pH in the sample and in the acceptor solution
2 Theory

2.1 Electromembrane extraction (EME)

EME is a liquid-phase microextraction technique intended for selective extraction and pre-concentration of target analytes (drug substances) from aqueous samples (blood and urine) [14]. It is based on migration of charged substances in solution with the use of electrical potential. This type of transport is called electrokinetic migration. The use of electrical potential as driving force ensures considerably faster extraction of analytes compared to passive diffusion [9].

The EME is a three-phase system comprising of two compartments separated by a layer of organic solvent. One of the compartments is envisaged for the sample solution while the other one serves for the acceptor solution. In order to obtain pre-concentration, the volume of acceptor solution is typically smaller than the volume of sample solution. The layer of organic solvent is immobilized by capillary forces inside the pores of a porous polymeric membrane [14]. This has been termed supported liquid membrane – SLM [15]. The porous polymeric membrane can be a hollow fibre or a flat sheet [14].

An illustration of a single EME-cell in a 96-well format is illustrated in Figure 1. Aqueous sample solution is placed in a compartment below the SLM, and an aqueous acceptor solution is located above the SLM. An electrode is placed in each compartment. By connecting the electrodes to an external power supply the system becomes an electrical circuit.

Figure 1 Schematic illustration of the EME system, modification of [16]

In a typical EME system, strong agitation is necessary for efficient operation [9]. Agitation of EME system ensures convection of the sample solution and reduces the stagnant boundary layer in the area near the SLM [17]. This is considered essential for high extraction recovery and short extraction time [14, 18, 19].
In order to obtain migration of analytes in the EME system, analytes need to be charged (ionized). For basic substance (cations) pH in the sample solution and the acceptor solution should be acidic, whereas for acidic substance (anions) pH in the sample and acceptor solutions should be alkaline. By controlling pH conditions in the sample and acceptor it is possible to obtain selectivity of the extraction.

In the extraction of basic substances, the cathode is located in the acceptor solution and the anode in the sample solution, forcing positively charged target analytes to migrate towards the cathode. Likewise, in the extraction of acid substances the anode is in the acceptor solution and the cathode in the sample solution, causing migration of negatively charged target analytes to the anode.

The electrical field is the major driving force in the EME system. Passive diffusion is negligible. Without electrical field ionized analytes cannot migrate through the layer of organic solvent and into the acceptor solution. With external power supply the direction and magnitude of the electrical field can be manipulated, providing thus EME selectivity [14].

Pursuant to Ohm's law, the current through a conductor between two points is directly proportional to the voltage across the two points and introduces the constant of proportionality, the resistance:

\[ I = \frac{U}{R} \]  \hspace{1cm} (2.1)

where I is the current through the conductor, U is the voltage measured across the conductor, and R is the resistance of the conductor. Ohm's law further states that the R in this relation is constant, independent of the current.

In the EME system, where the major electrical resistance in the system is the SLM, selection of the organic solvent in membrane is crucial. Therefore, the solvent should have certain polarity or water content in order to provide electrical conductance and ensure penetration of the electrical field. Transport of analytes (through membrane) increases with decreased electrical resistance of the SLM. Regardless of the type of membrane and whether the analytes are inert to electrode reaction or not, electrolysis will occur in the sample and acceptor solutions, respectively [9]:

Sample solution (anode): \( \text{H}_2\text{O} (l) \rightarrow \frac{1}{2}\text{O}_2 (g) + 2\text{H}^+ (aq) + 2e^- \)  \hspace{1cm} (2.2)
Acceptor solution (cathode): \(2H^+ (aq) + 2e^- \rightarrow H_2 (g)\) \hspace{1cm} (2.3)

As a result, hydrogen and oxygen gas, generated at electrodes, can form bubbles and pH conditions may change in case of insufficient buffer capacity [14]. Change of pH can also be due to creation of a boundary layer of ions, at the interfaces at both sides of the artificial liquid membrane [9].

Excessive bubble formation can result in a reduced contact area between the SLM and both solutions, causing a reduction in electrokinetic migration and stability, and consequently a less effective EME system. Furthermore, excessive electrolysis will induce partial loss of the solutions which can be significant considering that the EME is a micro extraction technique applied to small volumes. Electrolysis and the resulting bubble formation increases as a function of current, which in turn are controlled by the electrical resistance of the organic solvent of the SLM and the applied voltage. Thus, the electrical conductance of the organic solvent and the applied voltage should not be too high, but rather a compromise between efficient analyte transport and low electrolysis rate in order to suppress substantial bubble formation and pH changes [9]. As a rule of thumb, current should not exceed 50 µA [14]. 2-nitrophenyl octyl ether (NPOE) as organic solvent was found to be a successful compromise [9, 20].

Selection of organic solvent is crucial for the cross-membrane transport of analytes through SLM [9]. The solvent should be hydrophobic to avoid leakage into the sample and acceptor, but at the same time the solvent should provide extraction current [14, 15]. Since the introduction of EME in 2006, the extraction has predominantly been conducted with NPOE [14], which satisfies these conditions. Choosing the ideal organic solvent for the SLM should be based on the hydrophilic and hydrophobic properties of analytes (log P), as well as key chemical properties, such as hydrogen donor and acceptor groups. By facilitating the extraction of some analytes while discriminating other compounds, the organic solvent has the highest impact on selectivity in the EME system [18].

For non-polar basic analytes with log P > 2, NPOE is an ideal solvent. In the case of more polar basic analytes with log P < 2, the use of pure solvent has been unsuccessful [14, 15]. However by adding the hydrophobic ion-pair reagents such as di(2-ethylhexyl) phosphate (DEHP) to the SLM facilitated transport [15]. For acidic analytes, aliphatic alcohols, such as 1-octanol, have been the most successful SLM, but less stable than NPOE in contact with biological fluids [14].
As a result of the electrical potential across SLM, the steady state flux $J_i$ of an the $i$th cationic substance can be calculated by a modified the Nernst-Planck flux equation [21]:

$$J_i = \frac{D_i}{h} \left( 1 + \frac{v}{ln \chi} \right) \left( \frac{x - 1}{\chi - \exp(-v)} \right) (c_i - c_{i0} \exp(-v))$$  \hspace{1cm} (2.4)

where $D_i$ is the diffusion coefficient for the ion, $h$ the thickness of the membrane, $v$ is a dimensionless driving force (defined in equation 2.5), $\chi$ is the ratio of the total ionic concentration on the sample side and the total ionic concentration on the acceptor side (ion balance) as defined in equation 2.6, $c_i$ and $c_{i0}$ are the concentrations of the $i$th cationic substance at the SLM/sample interface and at the acceptor/SLM interface, respectively [21].

$$v = \frac{z_i e \Delta \phi}{kT}$$  \hspace{1cm} (2.5)

where $z_i$ is the charge of the $i$th cationic substance, $e$ is the elementary charge, $\Delta \phi$ is the electrical potential across the SLM, $k$ is the Boltzmann's constant, $T$ is the absolute temperature,

$$\chi = \frac{\sum c_{ih} + \sum c_{k0}^*}{\sum c_{io} + \sum c_{k0}}$$  \hspace{1cm} (2.6)

where $c_{ih}$ and $c_{io}$ the concentration of the $i$th cationic substance in the sample solution and in the acceptor solution, $c_{k0}^*$ and $c_{k0}$ the concentration of the $k$th anionic substance in the sample solution and in the acceptor solution [21].

As demonstrated in the equations above, voltage, ion balance, temperature and thickness of SLM are crucial parameters for the flux of analytes over the membrane [21]. Manipulation with temperature and voltage are limited. Temperature over 40ºC can cause partial degradation of the SLM [21] and high voltage stimulates electrolysis [22]. With regards to ion balance, low ion balance (ion concentration in the acceptor solution should be higher compared to ion concentration in the sample solution) is beneficial for the EME [21]. Finally, recoveries increases with the increase of agitation speed, as discussed above [17].

**EME under stagnant conditions**

As explained above, agitation in EME system is used to promote extraction efficiency [14, 17-19]. Since the sample solution is not in contact with the SLM the need for agitation is crucial in order to maintain contact between the sample solution and the SLM.
Taking into consideration the importance of agitation, it is expected that EME under stagnant conditions, i.e. without agitation, would not be as interesting to explore. However, some recent works have shown that this concept is very interesting, especially for small volumes promising high recoveries in a matter of a few minutes [19, 23].

In EME under stagnant conditions the contact between SLM and the sample is ensured by squeezing the sample with SLM, and providing liquid contact with the sample [23]. As noted earlier, the only major driving force in EME system is electrokinetic migration. However, the electrical field, which is responsible for electrokinetic migration, decreases with the increase in the distance between the electrodes. Thus, in the small compartment the field strength can be strong enough to draw the analytes through the membrane, even without agitation [17].

The theoretically expected recovery, as a function of time, can be represented with the following equation [23]:

\[ R_i(t) = 100\% \times \frac{2A}{V_s} \sqrt{\frac{D_i}{\pi}} \frac{t}{t} \]  
(2.7)

Where \( A \) is the cross-sectional area, \( V_s \) is the volume of the sample, \( D_i \) is the diffusion constant and \( t \) is the time of extraction. This implies that higher recoveries can be expected with longer extraction time and larger cross-sectional area. With regards to sample volumes, the smaller the sample volumes is, the higher the recoveries can be expected to be. This is in accordance with the fact that the impact of the electrical field decreases due to increase in the distance between the electrodes, as discussed above.

### 2.2 Colourimetry – Quantification of colour

Use of colour for the purpose of determining concentration of an analyte is a technique already recognized in analytical chemistry. Colourimetry is used to determine concentration of coloured solution (analyte) by measuring its absorbance in a specific wavelength of light with a colourimeter. This technique requires calibration of the device (colourimeter) and creation of a standard curve with standard samples of known concentration.
2.2.1 Colour tests and their quantification

Colour tests, spot tests or colourimetric tests are based on chemical reaction between an analyte and reagents which produces visible result – a colour [5, 6]. Since the introduction of colourimetric tests in 18th century, known as naked eye tests [3], they have been considered solely as qualitative analysis or presumptive tests. Colour tests are usually the simplest and the quickest chemical tests applicable to the sample [7]. The majority of those tests are, on the one hand, quite sensitive with respect to quantities of the sample necessary to complete a successful test [7], but, on the other hand, not very sensitive due to subjective aspect of colour evaluation, as it can differ from one analyst to analyst [3, 6, 7].

With appearance and development of imaging devices (mobile phones, digital and video cameras), the idea of colour quantification for colour tests by means of such devices was generated [3]. Colourimetry in this respect means quantification of coloured solution (analyte) using imaging device, and taking advantage of the colour coordinate or a related magnitude as the analytical parameter [3]. This method also requires a standard curve, while calibration of imaging device is not necessary.

Mobile phone characteristics like sensitivity and portability enable making of a low-cost analytical tool which can be used as out-of-lab applications (for on-site and real-time monitoring), whereas accessibility and availability can improve point-of-care (POC) testing, which can be performed e.g. at the bedside [1, 3].

2.2.2 Colour

Colour is a characteristic of the human visual perception and a colour of an object, as human eyes see it, is a result of light, in the visible electromagnetic spectrum, reflected from the object [24]. Around 6 to 7 million cones in the retina of the human eye are responsible for colour vision. The cones are colour-sensitive elements in the human eye and there are three sets of them. Approximately 65% of all cones are sensitive to red, 33% to green and about 2% to blue. The remaining colours are a combination of these three primary colours [24, 25]. The colour perceived in the eye depends not only on light reflected from an object and its intrinsic colour, but also on the power spectrum of the light source [25].
2.2.3 Imaging devices

Similar to the human eye, a camera gathers light reflected from objects by means of image sensors CCD (charge-coupled device) or CMOS (complementary metal-oxide semiconductor). The image sensor converts absorbed light energy (optical image) into electrical energy (electrical signal). The electrical signal is converted to a digital code [3, 24]. In the same manner as the human eye, image sensors have photosites with a colour filter over each photosite that permit only particular wave of light (red, green or blue). The most commonly used sensors in mobile phone cameras are CMOS sensors [3, 24].

The digital code is actually a matrix i.e. a numerical form of a two-dimensional digital image. Each element of the matrix, which has a particular location and value, is called an image element or a pixel [24]. Also, each pixel contains information about colour quantification, represented by three or four components, depending on the colour space used.

2.2.4 RGB colour space

RGB stands for red, green and blue, and the RGB colour space is the most common colour space in capture devices [3]. Generally speaking, the purpose of colour spaces, also known as colour models, is to present colours in a standard way. Each colour has a unique number or an address – a coordinate or a code in a coordinate system. In the RGB space, colour subspace is the cube (shown in Figure 2) where each point in the cube represents a colour [24].

![RGB Colour cube](image)

Figure 2 RGB Colour cube, points along the main diagonal have grey values [26]

The coordinates can vary from (0, 0, 0) which is black to (255, 255, 255) which is white. The cube encompasses 256 x 3 or 16,777,216 colours. Any point in the cube where the values for
red, green and blue are equal represents grey colour. An image represented in the RGB colour space is a 24-bit RGB image, where each colour is an 8-bit image, where the total number of colours is \( (2^8)^3 \) or 16,777,216 which corresponds to the RGB colour space.

The RGB colour space has been widely used in the purpose of the quantification of the analytes in the solutions and as immobilized substances. The quantification was done by using digital processing of images and a specific software which enables colourimetric information extraction [3].

### 2.2.5 Digital image processing and analysis

As mentioned previously, a digital image comprises of a limited number of pixels, also called resolution. Not all pixels are subjects of interest. Image segmentation or selecting the region of interest (ROI) in the picture is the first step in image analysis [3], and it is thus essential and critical in view of the fact that ROI contains information about analytes. For that reason, it is crucial to select a proper size of the region. If only one pixel is chosen for the analyses, it may not genuinely represent the colour of the analyte, since it can be a ‘noisy’ pixel. Selecting an average of e.g. 31 x 31 pixels, or more, usually gives more accurate information of the colour value of the analyte [27]. Averaging reduces random image noise [24]. There are different techniques for obtaining ROI such as thresholding, edge-based segmentation, region-based technique, neural networks, etc. [3, 26]. All of these techniques are mathematical operations or algorithms and constitute a part of a specific softer, but ROI can be selected (pointed) manually, for example with provided tool (eyedropper) in an application (e.g. Photoshop).

Once ROI is defined and obtained, the next step is digital image analysis or obtaining an analytical parameter - extracting colourimetric information of the analyte. The analytical parameter can be a value for single colour channel (red, green and blue), difference in values of red, green and blue values of the sample and red, green and blue values of the blank (if the blank is used), normalized RGB values, magnitude (also called intensity), image conversion to grayscale, etc [3]. The following step is finding a function (relation) between RGB values obtained from digital image of chemical reaction and concentration of the analyte, and creating calibration curves.
### 2.3 Liquid chromatography

Chromatography is a technique used to separate components in a mixture. It is a two-phase system, where one phase is mobile (mobile phase) and the other one is stationary (stationary phase). Target analytes should have different affinity for the two phases in order to be separated. Partition of analytes between two immiscible phases is the main principle in chromatography [8].

In liquid chromatography (LC) the mobile phase is liquid, forced through the stationary phase of a column. Depending on the pressure and the dimensions of the system used, there are mainly two types of liquid chromatography, HPLC (high performance liquid chromatography) where high pressure is used (30-300 bar (3-30 MPa)), and UHPLC (ultrahigh performance liquid chromatography) where very high pressures is used (up to 1200 bar (120 MPa)) [8]. The main components used in this technique are illustrated in Figure 3.

![Figure 3 Main components of LC system, modification of [8]](image-url)

The role of the pump in this system is to deliver mobile phase at a constant flow rate through the column. Target analytes are injected in the mobile phase, just before the column. The composition of the mobile phase may be constant (isocratic elution) or varied (gradient elution). Gradient elution is used to separate analytes with notable differences in retention [8].

The separation occurs when the mobile phase is pumped through the column carrying the analytes. The length of the column depends on the size of porous particles. The HPLC column is a 15–25 cm long steel tube packed with a 5µm particle, while the UHPLC column is a 3-5 cm long steel tube packed with smaller particles between 1.5 and 2.0 µm. The use of the shorter
column results in shorter separation time and reduced mobile phase consumption, which is one of the main differences between HPLC and UHPLC [8].

Depending on the nature of the stationary and mobile phase, there are two types of chromatography - normal phase chromatography and reversed-phase chromatography. In the normal phase chromatography, the stationary phase is more polar than the mobile phase. The stationary phase, typically silica, consists of porous particles with polar groups on the surface, and the mobile phase is an organic solvent. Retention is enabled by polar interactions, leaving the most polar analytes the most strongly retarded, which can be changed with the strength of the mobile phase. [8]

On the other hand, in reversed-phase chromatography, the mobile phase is more polar than the stationary phase. The stationary phase comprises porous particles with nonpolar groups on the surface. Retention is defined by nonpolar interactions. The mobile phase is typically a mixture of aqueous buffer solution and organic solvent which is miscible with water. Retention of neutral analytes is not affected by pH, while retention of ionic analytes is highly affected by pH, and for this reason it is important to select a pH based on pKa values of analytes.[8]

2.3.1 Detector - mass spectrometry

Mass spectrometry is a fundamental technique for identification and quantitative analysis of analytes. Unknown analytes are identified based on their corresponding mass spectra, and quantification is performed through measurement of peak areas on chromatograms. Mass spectrometry is carried out using a mass spectrometer (MS), an instrument serving primarily as a detector in gas chromatography (GC-MS) or liquid chromatography (LC-MS) [8].

The technique is based on ionization of analytes. The ionization of analytes in LC-MS is conducted at atmospheric pressure by electrospray ionization or at atmospheric pressure by chemical ionization. The formed molecular ions are completely or partially fragmented into ions with lower masses, which are characteristic for each compound. The ions are separated according to their mass to charge ratio (m/z value) in a mass analyzer and are detected by a detector [8].
3 Experimental

3.1 Model analyte

As a model analyte methamphetamine was used, obtained from NMD (Oslo, Norway) with unknown purity. Methamphetamine is a secondary amine, with molar mass of 149.237 g/mol and pKa 9.87 [28].

3.2 Chemicals

All chemicals used during this work are listed in Table 1.

Table 1 List of chemicals

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Purity</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium nitroprusside</td>
<td>99.5%</td>
<td>Sigma-Aldrich (Steinheim, Germany)</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>99.999%</td>
<td>Merck KGaA (Darmstadt, Germany)</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>≥ 99.0%</td>
<td>Merck KGaA (Darmstadt, Germany)</td>
</tr>
<tr>
<td>Water</td>
<td>Deionized</td>
<td>Millipore (Oslo, Norge)</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>37%</td>
<td>Merck KGaA (Darmstadt, Germany)</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>99.3%</td>
<td>VWR International, Radnor, PA, USA</td>
</tr>
<tr>
<td>Potassium dichromate</td>
<td>100%</td>
<td>Merck KGaA (Darmstadt, Germany)</td>
</tr>
<tr>
<td>2,6-Dichloroindophenol sodium salt</td>
<td>&gt;98%</td>
<td>Sigma-Aldrich (Steinheim, Germany)</td>
</tr>
<tr>
<td>Selenious acid</td>
<td>98%</td>
<td>Sigma-Aldrich (Steinheim, Germany)</td>
</tr>
<tr>
<td>Ammonium vanadate</td>
<td>≥99%</td>
<td>Sigma-Aldrich (Steinheim, Germany)</td>
</tr>
<tr>
<td>Sulphuric acid</td>
<td>98-97%</td>
<td>Merck KGaA (Darmstadt, Germany)</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>35%</td>
<td>Merck KGaA (Darmstadt, Germany)</td>
</tr>
<tr>
<td>Methanol</td>
<td>100%</td>
<td>Merck KGaA (Darmstadt, Germany)</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>98%</td>
<td>Sigma-Aldrich (Steinheim, Germany)</td>
</tr>
<tr>
<td>NPOE</td>
<td>&gt;99%</td>
<td>Sigma-Aldrich (Steinheim, Germany)</td>
</tr>
</tbody>
</table>
3.3 Solutions

Stock solutions

A stock solution containing 1 mg/mL of potassium dichromate in deionized water and 0.04 mg/mL 2, 6- dichloroindophenol in 0.2 mM NaOH were prepared. Also, a stock solution containing 1 mg/mL methamphetamine was prepared in both 10 mM HCl and deionized water.

Standard solutions

For potassium dichromate six sets of standard solutions (1.0 mg/mL, 0.50 mg/mL, 0.25 mg/mL, 0.13 mg/mL, 0.063 mg/mL, and 0.031 mg/mL) were prepared from the stock solution.

For methamphetamine six sets of standard solutions (1.0 mg/mL, 0.50 mg/mL, 0.25 mg/mL, 0.13 mg/mL, 0.063 mg/mL, and 0.031 mg/mL) were prepared from the stock solution.

For 2,6- dichloroindophenol five sets of standard solutions (0.04 mg/mL, 0.02 mg/mL, 0.01 mg/mL, 0.005 mg/mL, and 0.0025 mg/mL) were prepared from the stock solution.

Quality control samples

The quality control sample solutions were prepared by dilution of stock solution.

Simon’s reagent 1 (Reagent 1)

0.1 g of sodium nitroprusside was dissolved in 10 mL of deionized water; 0.1 mL of acetaldehyde was dissolved in solution of sodium nitroprusside up to 10 mL.

Simons’ reagent 2 (Reagent 2)

0.2 g sodium carbonate was dissolved in 10 mL of deionized water.

10 mM HCl

83 µL of 37% hydrochloric acid was dissolved up to 100 mL of deionized water.

20 mM HCl

166 µL of 37% hydrochloric acid was dissolved up to 100 mL of deionized water.
50 mM HCl

41.5 µL of 37% hydrochloric acid was dissolved with deionized water up to 10 mL.

1 mM HCl

10 ml of 10 mM HCl was dissolved with deionized water up to 100 mL.

0.2 mM NaOH

1 ml of 0.01 M NaOH was dissolved with deionized water up to 50 mL.

3.4 EME set-up

The equipment used for EME is illustrated in Figure 4.

Figure 4 Image of devices used (8-well sample plate with electrodes, 96-well filter plate, aluminium 96 electrode plate, EME set-up)

The sample compartment was a custom-made 8-well sample plate with 0.5 mm diameter silver electrodes (K. A. Rasmusen, Hamar, Norway) [16]. For housing the acceptor solution and for immobilization of the organic solvent a 96-well filter plate with 0.45 µm pore size Hydrophobic PVDF membrane (MAIPN4550, MultiScreen-IP Filter Plate, Merck, Darmstadt, Germany) was used. An aluminium 96 electrode plate (made at the University of Oslo) was used as the top electrode. The electrodes were coupled to a DC power supply model ES 0300-0.45 (Delta
Elektronika V.B, Zierikzee, Nederland) with programmable voltage in the range from 0 to 300 V. The multimeter METRAHIT X-tra (Gossen Metrawatt Nuremberg Germany) was connected in parallel with power supply in order to monitor current.

**Procedure for EME**

Primarily, 230 µL sample of methamphetamine in HCl, was pipetted into the well of the 8-well sample plate. Secondly, 3 µL of NPOE (organic solvent) was pipetted from below into the pores of the hydrophobic PVDF membrane of 96-well filter plate, hereinafter referred to as SLM. After 10 seconds the organic solvent was evenly distributed, and 96-well filter plate was embedded in 8-well sample plate. Thirdly, 50 µL of HCl constituting the acceptor solution was pipetted into the 96-well filter plate, above the SLM. Set up for EME was positioned onto a used 96-well filter plate for balance purpose and to avoid leaning. Finally, the electrical field was coupled by connecting the 96-electrode plate and silver electrodes to the power supply via banana plugs and clips, so that top electrode would have negative potential and the bottom one positive potential. After extraction, the acceptor solution was collected with a pipette and transferred into a micro vials for LC-MS analysis or into an Eppendorf for colourimetry.

In some experiments different sample and acceptor solutions and volumes were used, as well as a different period of time and voltage, which is specified for each experiment. Current was held under 50 µA, per extraction unit.

**3.5 UHPLC-MS/MS**

After EME, the acceptor solution was collected with a pipette, and then transferred to a micro insert for the analysis. The analysis was performed on UHPLC-MS/MS instrument with ion trap. The list of equipment used is given in Table 2.
Table 2 Equipment used in UHPLC-MS/MS

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Description</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Software</td>
<td>Xcalibur 2.2 SPI.48</td>
<td>Thermo Scientific, San Jose, CA, USA</td>
</tr>
<tr>
<td>Column</td>
<td>HSS T31.8 μm 2.1x100 mm</td>
<td>Waters Milford, MA, USA</td>
</tr>
<tr>
<td>UHPLC</td>
<td>Dionex UltiMate 3000 RS</td>
<td>Thermo Scientific, San Jose, CA, USA</td>
</tr>
<tr>
<td>MS/MS</td>
<td>LTQ XL (linear ion trap)</td>
<td>Thermo Scientific, San Jose, CA, USA</td>
</tr>
</tbody>
</table>

UHPLC

Liquid chromatography was performed on Dionex UltiMate 3000 RS. The time of analysis was 10 minutes, with column temperature of 65 °C. The injection volume was 2 μl. Description of gradient elution used in analysis is presented in Table 3.

Table 3 Description of gradient elution for UHPLC

<table>
<thead>
<tr>
<th>Retention (min)</th>
<th>Mobile phase A (%)</th>
<th>Mobile phase B (%)</th>
<th>Flow (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0,3</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td>60</td>
<td>0,3</td>
</tr>
<tr>
<td>7.1</td>
<td>0</td>
<td>100</td>
<td>0,3</td>
</tr>
<tr>
<td>8.4</td>
<td>0</td>
<td>100</td>
<td>0,3</td>
</tr>
<tr>
<td>8.5</td>
<td>100</td>
<td>0</td>
<td>0,3</td>
</tr>
<tr>
<td>9.5</td>
<td>100</td>
<td>0</td>
<td>0,3</td>
</tr>
</tbody>
</table>

**Mobil phase A:** Formic acid: methanol 95:5 (v/v); 50 ml of methanol dissolved in 950 ml of 20 mM formic acid

**Mobile phase B:** Formic acid: methanol 5:95(v/v); 50 ml of 20 mM formic acid dissolved in 950 ml of methanol

MS/MS

Mass Spectrometry was performed on LTQ XL linear ion trap.

Ionization of methamphetamine was performed with electrospray in positive modus:

- Capillary voltage: 5 kV
- Capillary temperature: 350 °C
- Nebulization: Nitrogen (26AU)
Detection of methamphetamine was set up in the selected reaction monitoring mode (SRM) to detect the ion transition m/z 150.12 → m/z 118.92 at collision energy of 35 eV with dwell time of 1 ms.

### 3.6 Calculations

#### Calculations of concentration and extraction recovery

Calculation of the concentration of methamphetamine in the acceptor \(c_{a,\text{final}}\) after EME was calculated in accordance to Equation 3.1

\[
c_{a,\text{final}} = \frac{A_a}{A_{std}} \times c_{std}
\]  

(3.1)

where \(A_a\) and \(A_{std}\) are peak areas of the analyte and the standard solution, respectively, while \(c_{std}\) is concentration of the standard solution.

Recovery (R) for the methamphetamine after EME was calculated according to the following Equation 3.2:

\[
R = \frac{n_{a,\text{final}}}{n_{s,\text{initial}}} \times 100\% = \frac{V_a \times c_{a,\text{final}}}{V_s \times c_{s,\text{initial}}} \times 100\%
\]  

(3.2)

where \(n_{a,\text{final}}\) is the number of moles of the analyte transferred to the acceptor, and \(n_{s,\text{initial}}\) is the number of moles of the analyte in the sample. \(V_a\) is the volume of acceptor, \(V_s\) is the volume of sample, whereas \(c_{a,\text{final}}\) is the final concentration of analyte in the acceptor phase, and \(c_{s,\text{initial}}\) is the initial analyte concentration in the sample.

### 3.7 Imaging prerequisites and conditions

#### The photo box

A custom-made aluminium photo box (20.5 cm x 13 cm x 16 cm) was developed at the University of Oslo. The photo box has an aperture of 2.5 cm diameter, intended for the
smartphone’s back camera. Image of the box and schematic illustration of the photo box used for taking images of sample solutions with the smartphone camera are presented in Figure 5.

![Image of the box and schematic illustration of the photo box](image)

Figure 5 Image (left) and schematic illustration (right) of the photo box

The inside of the photo box was coated with white paper (Bench surface protector, VWR International, Radnor, PA, USA). In the upper-left corner of the photo box a led bulb (1.2 W (11W), Ledsavers, Kjell & Company, Oslo, Norway) was installed. As a container for the sample solution a 100 µL dark quartz cuvette (VWR International, Randor, PA, USA) was used.

That way, the effect of the environment and light was held constant during all experiments. For the same reasons, the cuvette was used in order to obtain fixed path length of the light, bearing in mind that the colour of the solution can vary with different path length. The cuvette was placed 13 cm from the aperture for the smartphone.

**Smartphone**

The smartphone used for imaging was Samsung Galaxy J5, model SM-153O-F. All images were taken without flash and with auto white balance.

**RGB reader**

The application used for reading RGB values was The RGB Colour Detector, and it was downloaded for free, via Google Play Store. In some experiments Photoshop was used as well, and no difference was noted.
4 Results and discussion

4.1 Colourimetry

As mentioned earlier, one of the aims of this work was to test the concept where a smartphone is used as a detector (colourimeter) to measure a coloured analyte in solution. This basically means that a digital image of the solution, captured with a smartphone, is analysed in order to obtain an analytical signal, which establishes correlation with the analyte concentration.

The most common colour space used in smartphones is the RGB colour space, where RGB stands for red, green and blue. The analytical signal can be a value for single colour channel (red, green or blue), combinations of the RGB coordinates, normalized RGB values, magnitude (also called intensity), and image conversion to grayscale, to mention a few different possibilities. Because we had no previous experience with the RGB space and digital image analysis, the focus of the first set of experiments was on understanding the RGB space and changing of colour coordinates inside the space.

Solutions with low analyte concentrations have a light colour, while solutions with higher concentration have a darker colour. Thus, two colours (yellow and blue) with a different concentration were tested, using potassium dichromate and 2,6-dichloroindophenol as analytes. The aim of the testing was (i) to understand how the RGB values changed with the change of colour (calibration), (ii) to investigate if all three components of a colour followed the same trend, (iii) to study if there was any correlation between analyte concentration and the RGB values, and finally (iv) to investigate accuracy and precision of quantitative measurements.

4.1.1 Controlled environment for smartphone detection

The colour perceived in the eye depends not only on the light reflected from an object and its intrinsic colour, but also on the power spectrum of the light source [24, 25]. That also applies to images obtained with a smartphone. In practice, this means that taking picture of the same colour with different light power will result in the perception of different colours. Further, the same colour may appear progressively darker as the background becomes lighter [24]. Nonetheless, white background eliminates any potential interference with colour perception [29].
Taking into consideration that the lighting conditions during imaging can be difficult to control, especially outside the controlled environment, and that poor visibility can affect the actual colour, a custom-made photo box (20.5 cm x 13 cm x 16 cm) was developed with an aperture for the smartphone camera, as illustrated in Figure 6. The inside of the photo box was coated with white paper, and led light was installed in the upper-left corner. As a container for the sample solution a dark quartz cuvette with the volume of 100 µL was used (commercially available).

![Figure 6 Image (left), and schematic illustration (right) of the photo box used for photo of sample solutions with smartphone camera](image)

### 4.1.2 Colourimetric testing with solutions of potassium dichromate

Standard solutions of potassium dichromate (in deionized water) were prepared with the following concentrations: 1.0 mg/mL, 0.50 mg/mL, 0.25 mg/mL, 0.13 mg/mL, 0.063 mg/mL and 0.031 mg/mL. Deionized water was also tested as analyte-free (blank) sample. Two quality control samples (QC samples) of potassium dichromate in deionized water were prepared with the concentration of 0.33 mg/mL and 0.67 mg/mL, respectively. 100 µL of each standard solution was transferred with a pipette into the quartz cuvette, which was then placed inside the photo box. The underlying conditions (light, distance, position) were held constant during the testing, as described in Chapter 3.7.

Standard solutions and QC samples, were photographed three times. The RGB Colour Detector, used as the RBG reader, was downloaded for free, via Google Play Store (as already discussed in Chapter 3.7). RGB values were obtained from four individual points on each digital image and inserted in an Excel table, as illustrated in Figure 7.
The average values of 12 points (four points and three images) for each RGB value were calculated, and their calibration curves were plotted. This experiment was repeated on four days (Day 1-4). The calibration curves (Day 1-4) are summarized in Figure 8, illustrating the trend in RGB values as function of concentration of potassium dichromate.

Using linear regression, data were fitted to the equation $y = ax + b$, giving the equation for the calibration curve. In the equation, $x$ is the analyte concentration, $y$ is the red, green or blue
value we measure, $a$ is the slope and represents the sensitivity of the technique, and $b$ is the $y$ intercept.

In the evaluation of the calibration curves, we focused on two parameters (criteria), namely the square of the correlation ($R^2$), and the slope of the curve. The $R^2$ provides information about linear correlation. For bioanalytical methods $R^2$ value should exceed 0.995 [8]. On the other hand, the slope is an indicator of sensitivity, which provides information about the extent to which the measured signal changes with the small changes in concentration.

If we apply the criteria mentioned above to the calibration curves presented in Figure 8, the curves for red and green were not useful, due to poor sensitivity and poor linear correlation. On the other hand, the calibration curves obtained with blue values provided higher sensitivity with the slope values between -146 and -127. However, the linear correlation was relatively poor with $R^2$ in the range of 0.85-0.92.

RGB values for the QC samples were measured under identical conditions as used for standard solutions. The concentrations of potassium dichromate in the QC samples were obtained from the calibration curves. In order to investigate accuracy and precision, each of the three calibration curves were used in the calculation of the concentration. The obtained concentrations of QC samples were compared with the nominal (true) values. The accuracy, as percent of the nominal (true) value, and inter-day precision, expressed as the relative standard deviation, are presented in Table 4.

<table>
<thead>
<tr>
<th>Accuracy (%)</th>
<th>0.67 mg/mL</th>
<th>0.33 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Red</td>
<td>Green</td>
</tr>
<tr>
<td>Day 1</td>
<td>96%</td>
<td>112%</td>
</tr>
<tr>
<td>Day 2</td>
<td>38%</td>
<td>92%</td>
</tr>
<tr>
<td>Day 3</td>
<td>152%</td>
<td>47%</td>
</tr>
<tr>
<td>Day 4</td>
<td>36%</td>
<td>109%</td>
</tr>
<tr>
<td>Precision (%)</td>
<td>69</td>
<td>33</td>
</tr>
</tbody>
</table>

According to the EMA Guideline on bioanalytical method validation, the accuracy values of the QC samples should be within 85 and 115% of the nominal (true) values, whereas precision should not exceed 15% [30].
The accuracy values with QC samples obtained from red and green calibration curves were as expected very poor (in the range between 0% and 223%). This was due to poor sensitivity as discussed above. Using the blue values, accuracies obtained with the most concentrated sample ranged between 111% and 122%. Those values were very close to the EMA criteria. The inter-day precision between measurements performed on four days was 4% RSD. This was in compliance with the EMA criteria. However, with the less concentrated sample, accuracy values were not acceptable, and ranged between 135% and 157%. The inter-day precision was within 7 % RSD in this case.

Despite acceptable precision, accuracy was not in compliance with the EMA guideline requirements, and linearity was poor. Therefore, current measurements of potassium dichromate cannot be considered as a quantitative method. However, given that the blue value changed with the concentration, it may be considered as a semi-quantitative method.

**4.1.3 Colourimetric testing with solutions of 2, 6-dichloroindophenol**

Based on the experiences discussed above and in order to fully understand RGB colour space, experiment with another analyte was made. The main purpose of the next experiment was to analyse the trend in RGB colour space with blue sample solutions. For this purpose, the blue dye 2,6- dichloroindophenol was chosen as analyte.

Standard solutions of 2,6- dichloroindophenol (in 0.2 mM sodium hydroxide) were prepared with the following concentrations: 0.04 mg/mL, 0.02 mg/mL, 0.01 mg/mL, 0.005 mg/mL and 0.0025 mg/mL. Two quality control samples (QC samples) of 2,6- dichloroindophenol were prepared with the concentration of 0.027 mg/mL and 0.013 mg/mL, respectively, in 0.2 mM sodium hydroxide. 100 µL of each standard solution was transferred with a pipette into the quartz cuvette, which was later on placed inside the photo box. The underlying conditions (light, distance, position) were held constant during the experiment, as described in Chapter 3.7. The procedure for imaging and image analysis was the same as used for the potassium dichromate solutions.

After obtaining the average RGB values for each standard solution, the calibration curves were plotted. The calibration curves are presented in Figure 9, illustrating the trend in RGB values as function of concentration of 2,6- dichloroindophenol.
All three calibration curves presented in Figure 9, were steep with slope values between -2256 and -3611. Thus, both red, green, and blue values provided good sensitivity. However, the linear correlation ($R^2$) was in the range of 0.60 and 0.97, and was not in accordance with the criterion.

RGB values for the QC samples were measured under identical conditions as used for standard solutions. The concentrations of 2,6- dichloroindophenol in the QC samples were estimated from the calibration curve. In order to evaluate accuracy, each of the three standard curves (red, green, blue) were used in the calculation of the concentration. The obtained concentrations of QC samples were compared with the nominal (true) value. The accuracy, as percent of the nominal (true) value is presented in Table 5.

### Table 5 Accuracy with QC samples of 2, 6- dichloroindophenol

<table>
<thead>
<tr>
<th></th>
<th>0.027 mg/mL</th>
<th>0.013 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Red</strong></td>
<td>96 %</td>
<td>158 %</td>
</tr>
<tr>
<td><strong>Green</strong></td>
<td>105 %</td>
<td>155 %</td>
</tr>
<tr>
<td><strong>Blue</strong></td>
<td>99 %</td>
<td>150 %</td>
</tr>
</tbody>
</table>

The accuracy values with the 0.027 mg/mL QC sample obtained from red, green and blue calibration were in the range between 96% and 105%, which is in compliance with EMA requirements. However, the accuracy values with the 0.013 mg/mL QC sample obtained from red, green and blue calibration ranged between 150% and 158%, and was not in compliance with EMA requirements. Thus, also for 2,6- dichloroindophenol, smartphone detection based on red, green, or blue values may be considered a semi-quantitative method only.
4.2 Quantification of colour tests with methamphetamine as analyte

As mentioned earlier in the text, colour tests or spot tests are based on chemical reaction between an analyte and reagents, with coloured product as a result. These tests are used as quick presumptive tests, indicating presence or absence of a specific analyte in a given sample [7]. Many of these tests are standardized, and in practice used for on-site identifications [6, 31]. The idea of developing such tests for quantitative analysis came along with the development of image sensors and imaging devices [3].

4.2.1 Colour test with methamphetamine

Based on initial experiments with different colour reagents (see Appendix), Simon’s test was selected for the experiments reported in this section. Methamphetamine was selected as the model analyte, because this substance is detected with relatively high sensitivity down to approximately 10 µg [5], using Simon’s test. Furthermore, methamphetamine is a relevant model substance for drug of abuse applications.

Ratio investigation

In the first set of experiments, the ratio between methamphetamine and reagents was investigated. In Simon’s test two reagents are needed. The first reagent is acetaldehyde in aqueous sodium nitroprusside solution (Reagent 1), and the second one is sodium carbonate in water (Reagent 2), as described in Chapter 3.7. Figure 10 shows the reaction mechanism of Simon’s test with methamphetamine as the model analyte. Typically, one drop of Reagent 1 is added to the sample, followed by two drops of Reagent 2, resulting in the observation of blue colour in the presence of methamphetamine [31, 32].
Figure 10 Reaction mechanism of Simon’s test with methamphetamine, modified from [33, 34]

The amine group on methamphetamine and acetaldehyde form the enamine, which subsequently reacts with sodium nitroprusside to an immonium salt. This substance is finally hydrolysed to the blue Simon’s-Awe complex [33, 34]. The blue Simon’s-Awe complex is described as deep blue [35], blue [32, 33], dark blue [5] or brilliant violet blue [36].

First, methamphetamine, Reagent 1 and Regent 2 in the volume ratio of 2:1:8, respectively, were tested. This ratio was selected based on published work [29, 34, 37]. To this purpose, standard solutions of methamphetamine (in deionized water) were prepared with the following concentrations: 1.0 mg/mL, 0.50 mg/mL, and 0.25 mg/mL. Next, 200 µL of each standard solution was transferred with a pipette into an Eppendorf tube, followed by 100 µL of Reagent 1, and 800 µL of Reagent 2. The solution was mixed gently. 100 µL of this solution was transferred with a pipette into the quartz cuvette, and then placed inside the photo box. Each solution was photographed 3.0 minutes after adding Reagent 2. The underlying conditions (light, distance, position) were held constant during the testing, as described in Chapter 3.7. The procedure for imaging and image analysis was identical to the procedure described in Chapter 4.1. The results of the experiment, as well as the colours observed, are presented in Table 6.
The absence of blue colour was assigned to relatively low concentration of the standard solution, and large volume of reagents added. Reagent 1 is coloured due to red colour of sodium nitroprusside, which can interfere with the observing of blue colour of the complex. Further, colour of the mixture after the reaction changed with time. The initial colour was light rose, changing to light purple, and getting darker with time. Finally, after three minutes, the colour became dark brown-purple. It is certain that the reaction took place, but the coloured complex was not observed due to above mentioned conditions.

In the next experiment, the same ratio between the standard solutions and reagents was used, but with different volumes tested. The above procedure was repeated but with 20 µL of the standard solution, followed by 10 µL of Reagent 1 and 80 µL of Reagent 2. Each solution was photographed 2.0 minutes after adding Reagent 2, as suggested in published work [37]. The results of this experiment with the colours observed are presented in Table 7. The colours observed were very similar to previous experiment.

Taking into account the conventional use of this test, one drop of Reagent 1 followed by two drops of Reagent 2, a ratio of 2:1:2 was tested in the next experiment. Namely, 20 µL of Reagent 1 was added to the standard solution of 40 µL, followed by 40 µL of Reagent 2. With this ratio,
blue colour was observed almost immediately. Therefore, the solutions were photographed 60 seconds after adding Reagent 2. The colours observed are presented in Table 8.

Table 8 Colours observed in Simon’s test with methamphetamine

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Volume*</th>
<th>Reagent 1</th>
<th>Reagent 2</th>
<th>RGB code (red, green, blue)</th>
<th>RGB** Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/ml</td>
<td>µL</td>
<td>µL</td>
<td>µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>40 µL</td>
<td>20 µL</td>
<td>40 µL</td>
<td>(33, 28, 82)</td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>40 µL</td>
<td>20 µL</td>
<td>40 µL</td>
<td>(47, 39, 76)</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>40 µL</td>
<td>20 µL</td>
<td>40 µL</td>
<td>(62, 44, 70)</td>
<td></td>
</tr>
</tbody>
</table>

*Volume of standard solutions
** RGB Colours are presented with the use of “Custom colours” in Excel, and may differ from the actual colour

All experiments described above were repeated with standard solutions of methamphetamine in 10 mM HCl, and data were comparable with those with methamphetamine in deionized water.

For better understanding, the trend in RGB values as a function of the concentration of methamphetamine, and as a function of the volume ratio, is illustrated in Figure 11.

Figure 11 Measured RGB values as function of methamphetamine concentration, at different volume ratios (calibration curves)
As can be seen in Figure 11, calibration curves (sensitivities) were dependent on volumes and volume ratios. For the rest of this work, methamphetamine, Reagent 1 and Reagent 2 were mixed in the ratio 2:1:2, respectively.

**Quantification of Simon’s test**

In the next set of experiments, quantification of methamphetamine with Simon’s test was investigated. Bearing in mind that colour of the blue complex changed with the concentration of methamphetamine in the sample, the aim of the experiments was to study if there was any correlation between the analyte concentration and the RGB values, as well as to investigate the accuracy and precision of quantitative measurements.

Standard solutions of methamphetamine (in 10 mM HCl) were prepared with the following concentrations: 0.50 mg/mL, 0.25 mg/mL, 0.13 mg/mL, 0.063 mg/mL and 0.031 mg/mL. Two quality control samples (QC samples) of methamphetamine were prepared at 0.33 mg/mL and 0.17 mg/mL, respectively, in 10 mM HCl. As an analyte-free (blank) sample, 10 mM HCl was used. 40 µL of each standard solution was transferred with a pipette into an Eppendorf tube, followed by 20 µL of Reagent 1 and 40 µL of Reagent 2. The solution was mixed gently. Next, 100 µL of the solution was transferred with a pipette into the quartz cuvette, and then placed inside the photo box. Each solution was photographed 60 seconds after adding Reagent 2. The underlying conditions (light, distance, position) were held constant during the testing, as described in Chapter 3.7. The procedure for imaging and image analysis was the same as described in Chapter 4.1.

After obtaining average RGB values for each standard solution, the calibration curves were plotted. The experiment was repeated on three days (Day 1-3). The calibration curves (Day 1-3) are summarized in Figure 12, illustrating the trend in RGB values as a function of the concentration of methamphetamine.
After applying the criteria mentioned in Chapter 4.1.3 to the calibration curves presented in Figure 12, the curves for red and green provided better sensitivity comparing to the curve for blue, with slopes between -126 and -277. However, the linear correlation was relatively poor with $R^2$ in the range of 0.73-0.88, and not in accordance with the criterion.

RGB values for the QC samples were measured under identical conditions as used for the standard solutions. The concentrations of methamphetamine in the QC samples were obtained from the calibration curves. In order to investigate accuracy and precision, each of the three standard curves was used in the calculation of the concentration. The obtained concentrations of QC samples were compared with the nominal (true) values. The accuracy, as percent of the nominal (true) value, and inter-day precision, expressed as the relative standard deviation, are presented in Table 9.
Table 9 Accuracy and inter-day precision with QC samples of methamphetamine (n=3)

<table>
<thead>
<tr>
<th>Accuracy (%)</th>
<th>0.17 mg/mL</th>
<th>0.33 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Red</td>
<td>Green</td>
</tr>
<tr>
<td><strong>Day 1</strong></td>
<td>150</td>
<td>153</td>
</tr>
<tr>
<td><strong>Day 2</strong></td>
<td>158</td>
<td>133</td>
</tr>
<tr>
<td><strong>Day 3</strong></td>
<td>127</td>
<td>120</td>
</tr>
<tr>
<td><strong>Precision (%)</strong></td>
<td>11</td>
<td>12</td>
</tr>
</tbody>
</table>

The accuracy values with the most concentrated QC sample obtained from the calibration curves were in the range between 83% and 123%. However, the accuracy with the green curve was between 100% and 111%, and the inter-day precision within 5% RSD. These results were in compliance with the EMA criteria. With the less concentrated QC sample, the accuracy values, obtained from the calibration curves, were not acceptable, and ranged between 97% and 158%. The inter-day precision was between 11% and 20% RSD in this case. This result was not in compliance with the EMA criteria.

**Limit of detection (LOD) and limit of quantification (LOQ)**

In order to fully validate this method, limit of detection and limit of quantification were investigated. According to EMA Validation of Analytical Procedures [38], several approaches for determining limit of detection and limit of quantification are possible. One of them is determination based on standard deviation of the response of the blank ($\sigma$) and the slope (S). With this approach, by analysing an appropriate number of blank samples and calculating the standard deviation of these responses, background response is measured. Calculations for LOD and LOQ are represented with the following equations [38]:

$$LOD = \frac{3.3 \sigma}{S}$$

$$LOQ = \frac{10 \sigma}{S}$$

As a blank sample, 20 µL of Reagent 1 was added in 40 µL of 10 mM HCl, followed by 40 µL of Reagent 2. The solution was mixed gently, and transferred with a pipette into the quartz cuvette, and then placed inside the photo box, and photographed 60 seconds after adding Reagent 2. The underlying conditions (light, distance, position) were held constant during the
testing, as described in Chapter 3.7. The procedure for imaging and image analysis was the same as described in Chapter 4.1.

RGB values for the blank samples were measured under identical conditions as used for the standard solutions. In order to investigate sensitivity and compare results, each of the three standard curves, presented in Figure 12, was used in the calculation of LOD and LOQ. The experiment was repeated on three days (Day 1-3). The results are presented in Table 10.

<table>
<thead>
<tr>
<th></th>
<th>LOD (mg/mL)</th>
<th>LOQ (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Red</td>
<td>Green</td>
</tr>
<tr>
<td><strong>Day 1</strong></td>
<td>0.055</td>
<td>0.293</td>
</tr>
<tr>
<td><strong>Day 2</strong></td>
<td>0.061</td>
<td>0.356</td>
</tr>
<tr>
<td><strong>Day 3</strong></td>
<td>0.054</td>
<td>0.281</td>
</tr>
</tbody>
</table>

As seen in Table 10, red, green and blue gave different values for LOD and LOQ. That was due to different slope of the calibrations curve. The curve for red, with slope between -277 and -256, provided the best sensitivity with LOD around 0.05 mg/mL and LOQ around 0.17 mg/mL. Values for LOD and LOQ, differed with 6% from day to day.

High LOD and LOQ with Simon’s test can be assigned to the relatively high volumes of sample and reagents used. It is certain that the reaction takes place, but the coloured product of the reaction cannot be observed and measured at low concentrations.

### 4.3 Electromembrane extraction

One of the aims of this work was to find a simple EME system ensuring extraction of target analyte from a microliter volume of sample (biological fluid) and into an acceptor solution. The latter is then coloured with reagents that are specific for the analyte, and the colour intensity is measured with a smartphone.

In a typical EME system, agitation is used to promote convection in the sample. Convection reduces the stagnant boundary layer in the area near the SLM, and increases the extraction efficiency [19]. In this work agitation was disregarded in order to ensure simplicity, stability and easy application as point-of-care or on-site device. For the same reasons high voltages were
dismissed. EME in this work was performed in a traditional 96-well system [18, 39], and this is illustrated in Figure 13. Without agitation, this system was considered convenient for EME testing under stagnant conditions. It was not considered as a final device for on-site measurements.

The experiments were focused on (i) electrical contact across the system from cathode to anode, (ii) and optimal conditions for efficient extraction under stagnant conditions.

The EME set-up comprised the following (Figure 13):

- 96-well filter plate
- 8-well sample plate with electrodes
- Power supply
- Aluminium 96 electrode plate

Figure 13 Image (left) and schematic illustration (right) of the EME system, modification of [16]

Firstly, the sample solution was pipetted into the 8-well sample plate. Secondly, organic solvent (3 µL of NPOE) was pipetted into the filter of the 96-well filter plate to form the SLM. The cross-sectional area of each filter (SLM included) was 0.3 cm². Thirdly, acceptor solution was pipetted into the 96-well filter plate, above the SLM. The electrical field was coupled by one electrode inserted in the acceptor solution (cathode), and one inserted in the sample (anode).

Finally, after extraction, the acceptor solution was collected with pipette and transferred into micro vials for UHPLC-MS analysis. From this point on, UHPLC-MS will be referred to as LC-MS. Recovery was calculated as described in Chapter 3.6.
4.3.1 Acceptor volume

In the first experiment, acceptor volumes of 30, 50, and 100 µL were tested. The sample volume was 230 µL in all the experiments. The experimental data are shown in Table 11.

<table>
<thead>
<tr>
<th>Acceptor volumes</th>
<th>30 µL</th>
<th>50 µL</th>
<th>100 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery (%)</td>
<td>10</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>8</td>
<td>6</td>
<td>10</td>
</tr>
</tbody>
</table>

\( (n=4, 25 \text{ V}, 15 \text{ minutes extraction time, } 10 \text{ mM HCl in the acceptor solution, and } 1 \mu\text{g/mL methamphetamine in } 10 \text{ mM HCl in the sample solution}) \)

With 100 µL acceptor volume, recovery was 15%. As illustrated in Table 10, both 30 and 50 µL acceptor volume gave similar recoveries, and relative standard deviations under 10%. Despite the higher enrichment with 30 µL acceptor volume, 50 µL was selected due to lower relative standard deviation.

4.3.2 Sample volume

In the second experiment different sample volumes were investigated. As illustrated in Figure 13 the sample is located below the SLM. The sample volume is crucial to ensure contact between the SLM and the sample. Volumes tested were 200 and 230 µL, and the results are given in Table 12.

<table>
<thead>
<tr>
<th>Sample volume</th>
<th>230 µL</th>
<th>200 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery (%)</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>6</td>
<td>12</td>
</tr>
</tbody>
</table>

\( (n=4, 25 \text{ V}, 15 \text{ minutes extraction time, } 10 \text{ mM HCl in the acceptor solution and } 1 \mu\text{g/mL methamphetamine in } 10 \text{ mM HCl in the sample solution}) \)

As seen from the data, the precision was improved when the sample volume was increased from 200 to 230 µL. Furthermore, the recovery for methamphetamine was significantly higher with 230 µL (t-test, 95 % confidence level). Higher recovery and lower relative standard deviation were assigned to better contact between sample and SLM with 230 µL sample, as demonstrated.
in the Figure 14. The importance of good contact is described through Equation 2.7, stating that the recovery is directly proportional to the cross-sectional area of the SLM.

Hence, for the rest of this work 230 µL volume was selected for the sample.

4.3.3 Effect of voltage on EME

The applied voltage is highly important in EME as described by Equations 2.4 and 2.5. Therefore, the recovery of methamphetamine in the EME system was characterized as a function of applied voltage in the range 0-50 V. The results are summarized in Figure 15.

Without application of the electrical potential difference, methamphetamine was not detected in the acceptor. This result supported the absence of passive diffusion. With the use of voltage as the driving force, recovery increased. With only 5 V applied to the system, recoveries were around 6%. Recoveries continued to increase with increasing voltage. In order to ensure extra-
low voltage and low risk of electric shock for user, 25 V was selected as the optimal voltage, and was used in all further experiments.

4.3.4 Time effect on EME

In a fourth experiment, extraction recovery of methamphetamine was tested as a function of time. The results are presented in Figure 16. As can be seen from the figure, recovery increased gradually with time, reaching its maximum at 27% after 60 minutes. Experiments longer than 60 minutes were not performed.

![Figure 16 Recovery of methamphetamine as a function of the time](image)

Because the current work was performed as part of development towards on-site technology, short extraction times are important and therefore 15 minutes was used for the rest of this work.

4.3.5 Effect of pH in the sample and in the acceptor solutions

In order to optimize the performance of the EME system, different HCl concentrations in the sample and acceptor were considered.

Effect of pH in the sample solution

In a first set of experiments, methamphetamine was spiked into solution of 1 and 10 mM HCl. Recoveries from those samples are shown in Table 13.
Table 13 Recovery and RSD of methamphetamine with different sample solutions

<table>
<thead>
<tr>
<th>Sample</th>
<th>10 mM HCl</th>
<th>1 mM HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery (%)</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>6</td>
<td>11</td>
</tr>
</tbody>
</table>

(*n=4, 25V, 15 minutes extraction, 10 mM HCl in the acceptor solution, 1 µg/mL methamphetamine in the sample solution*)

Recoveries were not significantly different (t-test, 95 % confidence level), but difference in relative standard deviation was notable. Thus, 10 mM HCl was used for the rest of this work.

**Effect of pH in the acceptor solution**

In a next set of experiments, different HCl concentrations in the acceptor solution were tested. Table 14 shows the results obtained from these experiments. The same trend can be noticed as with different HCl concentration in the sample. Recoveries were similar, but precision was favoured with 10 mM HCl, and this was used for the rest of this work.

Table 14 Recovery and RSD of methamphetamine with different acceptor solutions

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>10 mM HCl</th>
<th>20 mM HCl</th>
<th>50 mM HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery (%)</td>
<td>11</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>6</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

(*n=4, 25V, 15 minutes extraction, 1 µg/mL methamphetamine in 10 mM HCl in the sample solution*)

**Performance characteristics**

According to the results of the experiments discussed above, the optimal performance of EME for methamphetamine, as the model analyte, was obtained with:

- Acceptor solution of 50 µL 10 mM HCl
- Sample solution of 230 µL containing 10 mM HCl
- Extraction time of 15 minutes
- Voltage of 25 V
4.4 EME followed by Simon’s test and colourimetry with smartphone

The aim of this work was to investigate the possibility of developing a user-friendly and easy-to-handle POC or on-site device which could provide information about medicine concentration in biological fluids. The main idea was to implement EME under stagnant conditions as a sample preparation technique, colour test as an indicator of medicine presence and smartphone as a detector (colourimeter). However, whole blood was not tested. Taking into consideration LOD for methamphetamine with Simon’s test and recovery with EME under stagnant conditions, it was more than obvious that it was not possible to apply this concept to concentrations usually found in blood.

In the last set of experiments, we attempted to summarize all experiments mentioned above. That basically meant that the experiment encompassed three parts. First, methamphetamine was extracted with EME under stagnant conditions. Secondly, methamphetamine was coloured with Simon’s test, and, thirdly, colour was photographed and analysed.

**EME set-up**

The EME set-up was the same as explained in Chapter 3.4. Due to the high LOD for methamphetamine with Simon’s test, the concentration of methamphetamine in the sample solution was set to 0.50 mg/mL (in 10 mM HCl), and 10 mM HCl was used as the acceptor solution.

After 15 minutes of extraction with 15 V, the acceptor was collected and transferred into an Eppendorf tube, and was used as QC sample. One part of the acceptor (40 µL) was used for the colourimetric analysis, while the second part (5 µL) was diluted with deionized water and analysed with LC-MS for comparison. Subsequently the concentration was obtained with colourimetric analysis and LC-MS, and the recovery was calculated.

**Calibration curve - colourimetric analysis**

Standard solutions of methamphetamine (in 10 mM HCl) were prepared with the following concentrations: 0.50 mg/mL, 0.25 mg/mL, 0.13 mg/mL and 0.063 mg/mL. As an analyte-free (blank) sample, 10 mM HCl was used. 40 µL of each standard solution was transferred with a
pipette into an Eppendorf tube, followed by 20 µL of Reagent 1 and 40 µL of Reagent 2. The solution was mixed gently. 100 µL of this solution was transferred with a pipette into the quartz cuvette, and then placed inside the photo box. Each solution was photographed 60 seconds after adding Reagent 2. The underlying conditions (light, distance, position) were held constant during the testing, as described in Chapter 3.7. The procedure for imaging and image analysis was the same as described in Chapter 4.1.

After obtaining average RGB values for each standard solution, the calibration curves were plotted. The test was repeated on two days (Day 1-2). The calibration curves (Day 1-2) are summarized in Figure 17, illustrating the trend in RGB change with the change of the concentration of methamphetamine.

![Image](image.png)

Figure 17 Measured RGB values as a function of methamphetamine concentration (calibration curves)

The curves for red and green showed higher sensitivity compared to the curve for blue, with the slopes between -144 and -251. However, the linear correlation was relatively poor, with $R^2$ in the range of 0.85-0.93, and not in accordance with the criterion ($R^2 \geq 0.995$).

**Accuracy and precision**

As mentioned above, the acceptor solution, after extraction, was used as QC Sample. 40 µL of the acceptor was used for the colourimetric analysis, while 5 µL was diluted with deionized water and analysed with LC-MS for comparison. RGB values for the QC samples were measured under identical conditions as used for the standard solutions.

Recoveries were calculated from LC-MS data, as LC-MS is considered a reliable analytical technique. The accuracy with smartphone detection, as percent of the concentration obtained
with LC-MS, and intra-day precision, expressed as the relative standard deviation, are presented in Table 15.

Table 15 Accuracy and intra-day precision* with QC samples of methamphetamine (n=4)

<table>
<thead>
<tr>
<th>Day</th>
<th>Red</th>
<th>Green</th>
<th>Blue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>154</td>
<td>161</td>
<td>127</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precision (%)</td>
<td>5</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Day 2</td>
<td>128</td>
<td>153</td>
<td>168</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precision (%)</td>
<td>11</td>
<td>7</td>
<td>15</td>
</tr>
</tbody>
</table>

*Intra-day precision is calculated (and not inter-day) due to different dilution on Day 1 and Day 2. On Day 1, 5 µL of the acceptor solution was diluted 20 times, while on Day 2, it was diluted 200 times.

The accuracy values were in the range between 127% and 168%, which is not in compliance with EMA requirements. However, the intra-day precision was in the range between 3% and 15%, which is in compliance with the EMA criterion.

4.5 Recommendations

During the writing of this thesis, a key observation deserving additional attention was made and commented on, as it could be beneficial knowledge for prospective experiments. Simon’s test with highly concentrated solutions of methamphetamine gives blue colour. However, with lower concentration observed colour is purple to light brown, due to the influence of red colour of Reagent 1. Taking into account that RGB values are actually coordinates in the RGB cube that means that each colour has a unique location inside of the cube. That also means, if we are analysing a wider range of concentrations of methamphetamine with Simon’s test, as function of only one coordinate of the colour, for example red, linearity cannot be expected. However, if we narrow the analysis to the specific range of concentrations of methamphetamine which gives brown purple to light brown colour linearity can be expected.

For better understanding, correlations between concentrations in the range of 0 mg/mL to 1 mg/mL and RGB values, and concentrations in the range of 0 mg/mL and 0.25 mg/mL and RGB values, are presented in Figure 18.
As seen in Figure 18, with the analysis narrowing to the range of concentration from 0.0 to 0.25 mg/mL, linear correlation ($R^2$) and the slope changed dramatically for all three curves. The linear correlation green curve was $\geq 0.995$, in accordance with the criterion for the bioanalytical methods [8]. The slope values between -434 and -322 provided better sensitivity, which in practice mean lower LOD and LOQ.

In Figure 19 measured RGB values of coloured complex (concentrations in the range of 0 mg/mL to 1 mg/mL) are presented as the function of all three coordinates (red, green and blue).

Linearity in 3D coordinate system is much more complex than in 2D and difficult to discuss, considering that requires knowledge of advanced programs. However, the implementation of these programs is beyond of the scope of this study, since such analysis would require special knowledge.
5 Conclusion

The present work is a proof of concept study where electromembrane extraction (EME) under stagnant conditions was used as a sample preparation technique, Simon’s test as an indicator of medicine presence, and smartphone as a detector (colourimeter), for the purpose of quantifying methamphetamine based on RGB values obtained from digital images taken with the smartphone. Although precision obtained with highly concentrated samples were in accordance with EMA criteria, the concept suffered from the following issues: (i) low recovery with EME under stagnant conditions, (ii) high LOD and LOQ with Simon’s test, and (iii) limitations of smartphone as a measuring instrument.

The recovery obtained with EME under stagnant conditions was not high enough to give enrichment as expected. Our findings suggest that further experiments should focus on development of a smaller compartment for acceptor solution, with high cross-sectional area between sample and SLM, and between SLM and acceptor. Also, electrical field in the system and its geometry should be investigated, and this is currently under development in our research group.

High LOD and LOQ with Simon’s test can be assigned to the relatively high volumes of sample and reagents used. It is certain that the reaction takes place, but the coloured product of the reaction cannot be observed and measured at low concentrations. Additional work is therefore needed to down-scale the volumes of the reagent solutions.

Another important issue is the fact that smartphones are not intended to be used as analytical colourimeters, and the current work demonstrated challenges especially related to sensitivity.

In spite of the challenges identified in this work, EME coupled with smartphone detection is a very interesting and futuristic concept. Taking into consideration the expansion and development of smartphones and their optical parts, this technique may become a robust and reliable analytical technique in the future. By using this concept, measurements can be performed on-site, and not in analytical laboratories as is common practice today.
References


15. Gjelstad, A., K.E. Rasmussen, S. Pedersen-Bjergaard, and A. Gjelstad, Electrokinetic migration across artificial liquid membranes Tuning the membrane chemistry to different types of drug substances. 2006. p. 29-34.


36. Screening Colour Test and Specific Colour Tests for the Detection of Methylendioxyamphetamine and Amphetamine Type Stimulants. Laboratory and Scientific Section, Division of Policy Analysis; United Nations Office on Drugs and Crime, 2009.


Appendix

Experiments with Marquis, Mecke, and Mandelin reagents

Mecke and Mandelin reagents were selected due to fast chemical reaction, where colour develops instantly, and stays stable within few minutes [35]. On the other hand, Marquis test is considered to be one of the most important colour tests for amphetamine, methamphetamine and their analogues, by allowing the distinction between (meth)amphetamine and its ring-substituted analogues [7]. The composition of reagents is presented in Table 16.

Table 16 Composition of reagents used, with the expected colour of reaction

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Composition of reagent</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marquis reagent</td>
<td>10 ml of concentrated sulphuric acid added to 0.5 ml of 35% formaldehyde (formaldehyde: water, v:v)</td>
<td>Orange, slowly turning brown</td>
</tr>
<tr>
<td>Mecke reagent</td>
<td>0.1 g selenious acid dissolved in 10 mL of concentrated sulphuric acid</td>
<td>Yellow brown</td>
</tr>
<tr>
<td>Mandelin reagent</td>
<td>0.1 g ammonium vanadate in 10 mL of concentrated sulphuric acid</td>
<td>Yellow green</td>
</tr>
</tbody>
</table>

After a few experiments, these tests were discarded due to the corrosive effect of concentrated sulphuric acid, and high LOD. LOD for methamphetamine with Mandelin reagent is 100 µg [5, 31], which is not of great interest for analytical chemistry. LOD for methamphetamine with Mecke reagent is 5 µg [5, 31].

Further, reaction with a small amount of sample (20, 40, 60 and 100 µL) with a relatively low concentration (5 mg/mL, 2.5 mg/ml, 1 mg/ml and 0.5 mg/mL) took a long time for colour to develop. In one set of experiments the heating was tested, and it resulted in faster colour development. However, only light colours were observed.