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# Calibration of a UV-Vis spectrophotometer for simultaneous estimation of nitrate and nitrite in nitrified urine

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Foto on title page: Urine nitrification columns at Eawag, Dübendorf (CH) (taken by Katja Briner)

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## Abstract

In the context of nutrient recovery research, urine is collected separately at Eawag Dübendorf (CH) and treated with biological nitrification. It is important to have an appropriate technique to measure the nitrite concentration in the reactor as nitrite, which is the intermediate product of nitrification, inhibits the process.

The objective of this project is to test UV-Vis spectrophotometry for the simultaneous estimation of nitrite and nitrate in the nitrified urine. For this purpose, the influence of changing nitrite and nitrate concentrations and of the urine background composition on the absorbance spectrum and the estimation accuracy is analysed. Furthermore, the stability of the estimation performance of the linear model under changes in the urine composition is evaluated.

In a first step, absorbance measurements focusing on the influence of changes in the nitrite and nitrate concentration are carried out in urine, and in pure water for comparison. For these measurements the urine background composition is kept constant. In a second step, measurements are conducted in nine urine samples taken over the course of three weeks to test the estimation accuracy when extrapolating in time. To estimate the nitrite and nitrate concentration, principal component analysis and principal component regression is used. As a concentration of around 50 mgN/L nitrite is critical for the operation, the required estimation accuracy for nitrite is ±20 mgN/L. For nitrate, no such limit is set as the influence of nitrate on the nitrite estimation is the main interest.

In urine with a constant background, an estimation accuracy of around ±10 mgN/L is reached, which clearly lies within the set limit. In comparison, the nitrite estimation accuracy is found to be around ±4 mgN/L in pure water. The difference in accuracy is partly caused by saturation effects. Saturation implies, that the relationship between absorbance and concentration is not strictly linear as in theory, but deviates from linearity at high concentrations. This effect is amplified by other light absorbing compounds in the urine such as organics. To estimate the concentrations, three principal components are used in the linear regression model. This number of principal components is chosen as it is the minimum number with which the estimation accuracy goal can be met in urine.

If the model is calibrated based on several measurements in three samples of nitrified urine collected over the course of one week, the estimation accuracy for nitrite lies within ± 20 mgN/L when extrapolating in time over two weeks.

Considering these results, UV-Vis spectrophotometry seems to be an appropriate technique to estimate the nitrite concentration in nitrified urine. Furthermore, the used model is sufficiently stable for the extrapolation over two weeks. However, in practice, the target extrapolation time is longer and more experiments are necessary to verify the stability of the model over time.

Future work should consequently focus on testing the stability of the model over a longer time span. Additionally, it would be interesting to use a smaller part of the absorbance spectrum for modelling and to analyse the impact on the estimation accuracy. Simpler sensors are less expensive and thus choosing the minimum absorbance spectrum needed to reach the required accuracy would be economic.

Transferring the conclusions of this project to other applications might be possible, however the influence of the background composition and of the variability thereof are liquid-specific and can thus not be deduced from the results presented here.

## Acknowledgement

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Finally, I am grateful to Flurina Zimmermann for proofreading this report.

# Contents

# 1. Introduction

This chapter provides information on the background of this project. In the first section, an explanation why nitrite estimation is important in the context of biological nitrification of urine is given. In the second and third section, the main method used in this project – UV-Vis spectrophotometry – and some results of previous work are presented. Lastly, the objectives of this project are introduced.

## 1.1 Source separation and treatment of urine

In the context of population growth and the associated increase in demand for resources, the significance of recycling is increasing. Recovering nutrients, water and energy from waste water is an important contribution to the recycling of resources. Considering nutrient recovery, a special focus lies on the urine as it contributes a large part of the nutrients found in waste water. Separating the urine at the source results in a more concentrated stream and thus allows to recover the nutrients more easily (Udert et al., 2012). Urine separation is being implemented in decentralized systems developed for regions in which the currently most applied technology to deal with waste water – centralized sewer-based waste water treatment – is not feasible. However, no-mix toilets or waterless urinals allow source separation and on-site treatment of urine in centralized systems as well.

At Eawag, the Swiss Federal Institute for Aquatic Science and Technology in Dübendorf (CH), urine is collected separately and treated biologically in order to avoid odour emissions and nitrogen loss by ammonia volatilization. After the treatment, the urine is distilled to a nitrate-ammonium-fertilizer (Etter at al., 2013). The usage of this fertilizer is currently investigated (Etter et al., 2015).

In Table 1 the composition of the fresh and the treated female urine are listed. In male urine, the expected concentrations are around twice as high as the concentrations found in female urine due to dilution in the women's toilets.

Table 1: Selected parameters of the fresh and treated female urine at Eawag (Etter et al., 2013).

| Parameter | Fresh urine | Treated urine | |
|---|---|---|---|
| Ammonium ($NH_4^+$) | 1790 ± 180 mgN/L | 899 ± 140 | mgN/L |
| Nitrite ($NO_2^-$) | - | 2 ± 1 | mgN/L |
| Nitrate ($NO_3^-$) | - | 914 ± 203 | mgN/L |
| Chemical oxygen demand (COD) | 2110 ± 390 mgCOD/L | 217 ± 35 | mgCOD/L |

The collected urine is treated in a pilot scale moving bed biofilm reactor with a volume of 120 L. The biomass degrades COD and partially nitrifies the influent ammonium, limited by alkalinity. Two types of bacteria are involved in the biological nitrification of ammonium. AOB (ammonium oxidizing bacteria) convert ammonium to nitrite and NOB (nitrite oxidizing bacteria) oxidize nitrite to nitrate. The intermediate product of the nitrification, nitrite, inhibits the NOB at elevated concentrations. As the inhibited NOB oxidize less nitrite, even more of it accumulates and further inhibits the NOB. Normally, nitrite is present in very small concentrations. However, if a sudden raise in the ammonium load occurs, nitrite can accumulate, as the activity of the AOB increases faster than the activity of the NOB (Etter et al., 2013).

As nitrite inhibits the growth of the NOB and thus the nitrification, it is vital to have the possibility to measure the nitrite concentration in the reactor and react to an accumulation as soon as possible, preferably in an automated way. Until a concentration of around 50 mgN/L nitrite is reached, the process can be recovered by cutting off the influent and waiting for the NOB to oxidize the accumulated nitrite. At higher concentrations, the process may fail. The accuracy of the measurement should suffice to detect an accumulation of nitrite before the critical concentration of 50 mgN/L is reached, so an accuracy of at most ± 20 mgN/L is requested (C. Thürlimann, personal communication, 11th August 2015). The measurement frequency should be above one hour as it takes a few hours for nitrite to accumulate to the critical concentration (Etter et al., 2013).

Different techniques are available to measure the nitrite concentration. Most of those techniques are either very accurate but too expensive, such as on-line analysers, or cannot reach the necessary time resolution, such as using strips or manual sampling. Other techniques are promising, but currently in research. Among these are soft-sensing, electrochemical methods and spectrophotometry. Soft-sensing makes use of readily measurable parameters such as pH or oxygen to indirectly estimate the nitrite concentration by use of process models (Mašić & Villez, 2014). Employing electrochemistry, the nitrite concentration can be estimated based on a well-defined relationship to the amperometric response if a constant potential is applied (Palanisamy et al., 2014). The focus of this project lies on the estimation of nitrite via UV-Vis spectrophotometry. The measurement principle, advantages and limitations of UV-Vis spectrophotometry are described in the next section.

## 1.2 UV-Vis spectrophotometry

UV-Vis spectrophotometry makes use of the principle that the absorbance of light increases, if the concentration of light-absorbing substances increases. Under ideal conditions, the relationship between absorbance and concentration is described by the Beer-Lambert law. According to the Beer-Lambert law (Equation 1), the absorbance of light is linearly dependent on the concentration of a substance.

$$A_\lambda = \varepsilon_\lambda * L * C \quad (1)$$

$A_\lambda$: Absorbance at wavelength λ [AU]
$\varepsilon_\lambda$: Absorptivity at wavelength λ [m²/mol]
L: Length of path [m]
C: Concentration [mol/m³]

Substances such as nitrate and nitrite do not absorb all wavelengths in the UV-Vis range (around 200 to 800 nm) equally, but show absorbance peaks (Burgess, 2007). In Table 2 the wavelengths of the primary and secondary absorbance peaks are listed for nitrate and nitrite.

Table 2: Absorbance peaks of nitrate and nitrite (Spinelli et al., 2007).

| | Nitrate | Nitrite |
|---|---|---|
| Primary absorbance peak | 206 nm | 213 nm |
| Secondary absorbance peak | 302 nm | 354 nm |

UV-Vis sensors have several advantages. They can measure in-situ and thus sampling errors can be excluded. Generally, once the sensor is properly calibrated, limited maintenance and no reagents are needed, apart from cleaning agents. Furthermore, if a sensor measuring the absorbance over the whole UV-Vis spectrum is chosen, multiple substances can be estimated and cross-sensitivities reduced. In addition, the measurement interval is in the range of minutes and thus short enough to detect rapid changes in composition (Van den Broeke et al., 2006).

However, UV-Vis spectrophotometry is limited by saturation effects. Saturation implies that the relationship between absorbance and concentration does not adhere to the Beer-Lambert law anymore. These effects occur if the absorbing compounds in the liquid absorb too much of the light emitted by the sensor, resulting in a loss of sensitivity to changes in the composition. To avoid saturation, the solution can be diluted, the path length can be reduced or, if existing, a weaker absorbance peak can be analysed.

## 1.3 Previous work

Previous work has shown that saturation is reached at the primary absorbance peaks of nitrate and nitrite in nitrified urine, because the concentration of light-absorbing compounds is very high. For nitrite however, it was found that at the weaker secondary peak the sensitivity is high enough to render an estimation possible. Particles present in the sample appear to have a minor influence on the estimation of nitrite with UV-Vis spectrophotometry (Santos, 2014).

Furthermore, the influence of biofilm formation on the sensor window was studied. From this, the conclusion was drawn that long-term estimation of nitrite in urine is feasible with an error smaller than 20 mgN/L (Hess, 2015).

Several questions are still to be answered. For instance it remains to be analysed how the presence of nitrate influences the estimation accuracy of nitrite and how accurately nitrate itself can be estimated in nitrified urine. Another open question is what nitrite estimation accuracy can be reached if only a few wavelengths of the spectrum are used.

## 1.4 Objectives

The overall objective of this project is to assess how well nitrate and nitrite can be estimated simultaneously in nitrified urine by UV-Vis spectrophotometry. The findings are meant to contribute to an automatic control system for biological urine nitrification. This overarching goal is composed of three intermediate objectives:

- How do changes in the nitrite and nitrate concentration affect the estimation of nitrite by UV-Vis spectrophotometry in the nitrified urine?
- How sensitive is the estimation of nitrite to changes in the composition of absorbing substances in the urine?
- Is extrapolation over time possible?

The approach to meet those objectives is described in the following paragraphs.

As a first step, the effects of changing nitrate and nitrite concentrations on the absorbance spectrum are analysed in pure water and in urine. First, the concentration range of 0 to 5000 mgN/L nitrate and 0 to 150 mgN/L nitrite is covered with a grid of absorbance measurements in pure water by adding nitrate and nitrite in a stepwise manner. Secondly, an analogous experiment series is conducted in urine. In nitrified urine, background concentrations of around 2000 mgN/L nitrate and around 2 mgN/L nitrite are expected. By adding nitrate and nitrite, the concentration range of around 2000 mgN/L to 5000 mgN/L nitrate and around 2 mgN/L to 152 mgN/L nitrite is covered with a grid of measurements. This is done using a single urine sample to keep the background composition constant. For both experiment series, the grid resolution is 1000 mgN/L nitrate and 30 mgN/L nitrite. The upper limits of the covered concentration ranges are process related. The maximum influent ammonium concentration is around 5000 mgN/L, so at most this concentration of nitrate can be reached in the reactor. At 150 mgN/L nitrite, the reactor is strongly inhibited.

In order to test the influence of changes in the urine composition on the absorbance spectrum, nine samples are taken in the course of three weeks and analysed. Each sample is split in half and spiked with a certain amount of nitrate and nitrite. This is done in a randomized manner to exclude any correlation of the samples. The same concentration range as in the first experiment row with urine is covered.

To estimate the nitrate and nitrite concentrations in pure water and in urine, a chemometric model is built based on the absorbance data of each of the three experiment series. In a first step, a principal component analysis (PCA) is carried out in order to reduce the dimensionality of the absorbance data and thus facilitate the interpretation. In a second step, principal component regression (PCR) is used to estimate the concentration of nitrate and nitrite based on the principal components. Only a part of the dataset is used for this calibration, and the obtained model is validated with the data not used in the calibration.

The stability of the model under changing urine composition is assessed by calibrating the model with the data from one of the three sampling weeks and validating it with the data from the remaining two weeks.

# 2. Material and Methods

In this chapter the experimental set-ups and the methods used to analyse the raw data obtained by UV-Vis spectrophotometry are explained. First, some details concerning the sensor used in the experiment series are presented. Next, the experiments series in pure water, in urine with a constant background composition, and in urine of varying composition are introduced. Finally, the chemometric model used to calibrate the sensor (PCR) and the validation procedure are explained.

## 2.1 UV-Vis spectrophotometer

For the absorbance measurements, a spectrophotometer of the type spectro::lyser™ from s::can Messtechnik GmbH (Austria) is used. This sensor is designed for a range of applications from ultra-pure water up to industrial waste water and is available in several configurations of path length, measuring range and measuring resolution. It can measure directly in the solution or in a by-pass using a flow-cell (s::can, 2011).

The used spectrophotometric sensor has a path length of 5 mm, is submerged in the liquid and measures the absorbance from 200 – 735 nm with a resolution of 2.5 nm. The path length was chosen based on a trade-off. High sensitivity at low concentrations is reached with a long path length, whereas less saturation at high concentrations occurs with a short path length. The absorbance spectrum is measured every minute. The automatic cleaning routing with pressurized air is disabled so as not to disturb the measurement. The experimental set-up and a zoom to the sensor window are shown in Figure 1 and Figure 2.

Figure 1: UV-Vis spectrophotometer recording absorbance spectra in a urine sample.

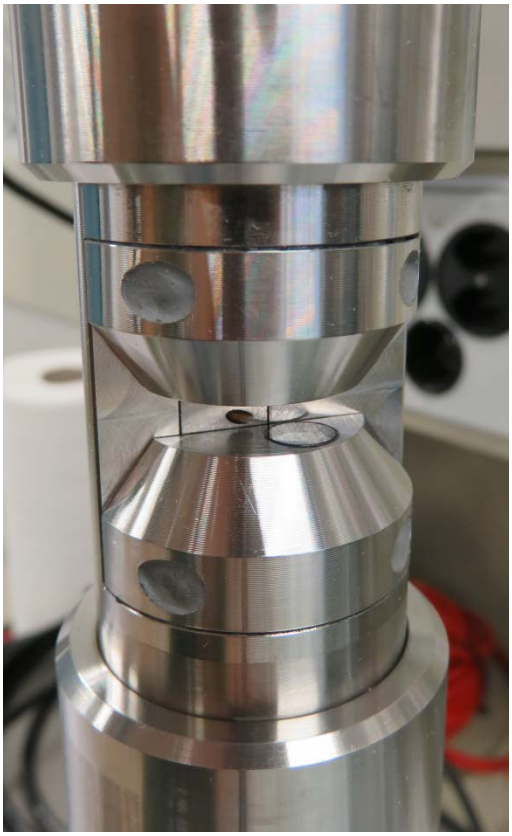

Figure 2: Sensor window in measuring gap of UV-Vis spectrophotometer.

## 2.2 Analysing the influence of nitrate and nitrite on the absorbance spectrum

### 2.2.1 Analysis in pure water

In order to assess the influence of changing nitrate and nitrite concentrations on the absorbance spectrum, absorbance measurements are conducted in pure water with different concentrations of nitrate and nitrite. Nitrate stock solutions with a concentration of 0, 1000, 2000, 3000, 4000 and 5000 mgN/L are prepared (details see protocol A1 on page II in the appendix). Based on each of these nitrate stock solutions, a nitrite stock solution is prepared (details see protocol A2 on page IV in the appendix). 500 mL of a nitrate stock solution are poured into a measuring cylinder. The UV-Vis sensor is then placed inside the liquid and six spectra are recorded. Next, a defined amount of nitrite stock solution is added to increase the nitrite concentration by 30 mgN/L and again six spectra are recorded. This addition of nitrite is repeated five times to reach a final concentration of 150 mgN/L of nitrite (details see protocol A3 on page VI in the appendix). Figure 3 visualizes the grid of combinations of concentrations that is obtained.

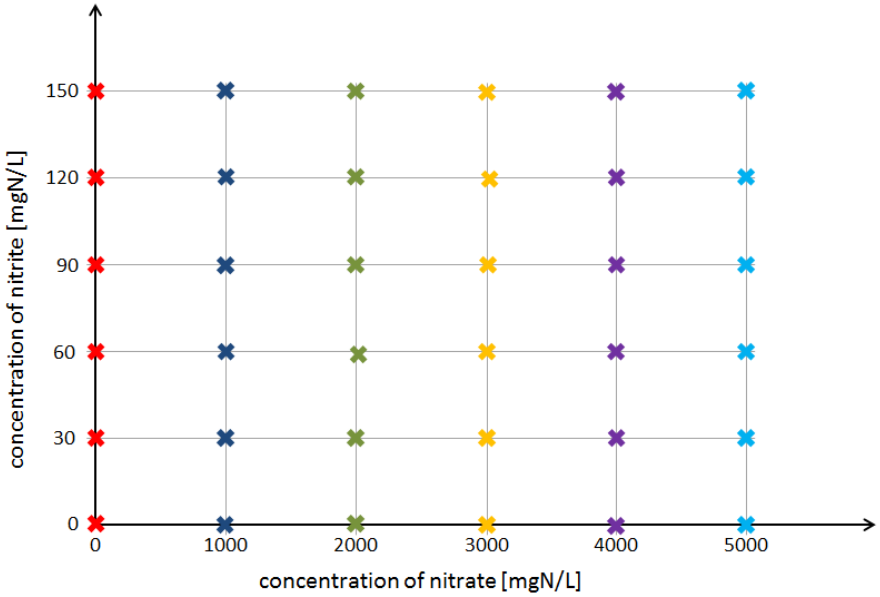


Figure 3: Visualization of the absorbance measurements in pure water: Each cross indicates a measurement at the corresponding concentration of nitrate on the x-axis and nitrite on the y-axis. Different colours indicate different nitrate stock solutions.

The range of concentrations is chosen based on process related boundary conditions. The maximal concentration of ammonium in the male urine is around 5000 mgN/L. Thus the maximal concentration of nitrate in the nitrified urine is around 5000 mgN/L, if all the ammonium is nitrified. At 150 mgN/L nitrite, the process is likely to fail and make a restart necessary (see section 1.1 on *Source separation and treatment of urine*).

### 2.2.2 Analysis in nitrified urine

Nitrified urine contains many substances which absorb light in the UV-Vis range, such as proteins or particles. Changes in the concentrations of those compounds influence the absorbance spectrum. In order to focus on the influence of nitrate and nitrite, one large sample of nitrified urine is taken to repeat the experiment procedure carried out in pure water (see section 2.2.1 on *Analysis in pure water*). This way the influence of the background composition is the same for the whole series of experiments and the influence of nitrate and nitrite on the spectrum can be analysed consistently.

Due to operational reasons, the sample was taken from the storage tank for nitrified urine downstream of the reactor. This ensures a homogeneous sample and reduces the impact on the reactor.

First, the urine sample is left settling for 60 minutes. The settled biomass is wasted and the clear liquid is used in the experiment series (details see protocol A4 on page VIII in the appendix).

The expected background concentration of nitrate and nitrite in the urine is around 2000 mgN/L and around 2 mgN/L, respectively. Based on the conditioned urine (clear liquid from previous step), nitrate stock solutions with a concentration of around 2000, 3000, 4000 and 5000 mgN/L are prepared (details see protocol A5 on page IX in the appendix). Based on each of these stock solutions, a nitrite stock solution is made in order to increase the concentration of nitrite in steps of 30 mgN/L (details see protocol A6 on page XI in the appendix). Basing the stock solutions on the urine sample prevents dilution in the experiment. The absorbance measurements are carried out in analogy to the experiment series in pure water (see section 2.2.1 on *Analysis in pure water*).

Additionally, a sample of the conditioned urine is filtered and the concentration of nitrate, nitrite and COD is measured (details see protocol A7 on page XIII in the appendix). COD is measured to obtain a more complete assessment of the fractions contributing to the measured spectrophotometric absorbance.

## 2.3 Analysing the influence of changes in the urine composition on the absorbance spectrum

To look into changes in the background composition and their influence on the estimation accuracy of nitrite and nitrate, nine samples of nitrified urine are taken over a period of three weeks. In contrast to the sample for the experiments series with a constant background, these samples are taken directly from the nitrification reactor. The samples are left settling for 60 minutes, the settled biomass is wasted and the clear liquid is used in the experiment. A sample of this conditioned urine is filtered and the concentration of nitrate, nitrite and COD is measured.

Each conditioned urine sample is split in two. After the absorbance spectrum has been measured in both parts (background), each part is spiked with a certain amount of nitrate and nitrite and the absorbance spectrum is recorded (details see protocol A8 and A9 in on page XIV and XVII in the appendix). The goal of this procedure is to cover the same concentration range as in the experiment in urine with constant composition (see section 2.2.2 on *Analysis in nitrified urine*). To reduce the measuring effort but avoid correlation of the samples, the amount of nitrate and nitrite added to each sample is chosen by Latin Hypercube sampling (Zio, 2013). Table 3 shows which combinations of nitrate and nitrite concentration are covered with each sample.

Due to time limitation, only the samples number 1.1-3.3 in the measuring grid (Table 3) can be covered. The measurements should thus be complemented by another sampling period of three weeks.

Table 3: Experimental set-up for the measurement series in urine of varying composition, based on Latin Hypercube sampling. The number indicates the sample in which the concerned combination of nitrate and nitrite is adjusted by adding stock solutions and analysed (first number: measurement week, second number: sample within the concerned week). Only the samples shaded in green can be covered within the scope of this project.

| concentration of $NO_2$ [mgN/L] | concentration of $NO_3$ [mgN/L] | | | | | |
|---|---|---|---|---|---|---|
| | ≈2500 | ≈3000 | ≈3500 | ≈4000 | ≈4500 | ≈5000 |
| ≈17 | 6.1 | 3.3 | 4.2 | 5.3 | 2.2 | 1.3 |
| ≈32 | 3.1 | 5.1 | 6.3 | 4.1 | 1.2 | 2.3 |
| ≈62 | 2.2 | 1.1 | 5.2 | 3.1 | 6.2 | 4.3 |
| ≈92 | 5.2 | 4.3 | 2.1 | 1.1 | 3.2 | 6.3 |
| ≈122 | 1.2 | 6.2 | 3.2 | 2.1 | 4.2 | 5.1 |
| ≈152 | 4.1 | 2.3 | 1.3 | 6.1 | 5.3 | 3.3 |

## 2.4 Calibration and validation of the UV-Vis sensor

### 2.4.1 Data pre-processing

The six absorbance spectra from every measurement are checked visually for outliers and are averaged. The calibration of the model is based on the averaged spectra. This pre-processing reduces noise and random errors in the spectra. Only the part of the spectrum between 260 and 700 nm is considered for the following analyses. The motivation is to exclude the primary absorbance peaks for which the saturation is strong (Mašić et al., 2015).

### 2.4.2 Data preparation for the experiments with constant background

For the experiments in pure water and in urine with a constant background, half of the dataset is used for principal component analysis and regression (calibration). The other half of the dataset is used for validation. The estimation error is determined by comparing the estimated and the observed concentrations of nitrite and nitrate in the validation part of the dataset.

The dataset is divided based on a chequerboard approach, using every other measurement in the nitrate and nitrite concentration range (visualization of the chequerboard division see Figure A 4 in the appendix on page XXIII). The obtained halves of the dataset are both used once for calibration and once for validation. This procedure is carried out separately for the experiment series in pure water and urine with a constant background and the results are compared.

### 2.4.3 Data preparation for the experiment with varying background

The data collected from the experiment series with urine of varying composition is split into subsets as well. The dataset of each week is once used in the principal component analysis and regression (calibration). The data of the other two weeks are used for validation. The estimation accuracy is then analysed in order to assess the stability of the model under changing urine composition.

### 2.4.4 Principal Component Analysis

Principal components analysis (PCA) is a procedure used to reduce the dimensionality of the data and thus facilitate the interpretation while preserving most of the variance of the data. The original set of possibly correlated variables is converted into an uncorrelated set of variables. These variables are called principal components and are ordered by the amount of variance in the data they explain. The first few principal components account for most of the variance (Jolliffe, 2002).

The absorbance measurement at all wavelengths for each sample in the calibration set is the input data. First, centring is needed. For each wavelength, the average absorbance over all samples is calculated. Then this average is subtracted from the absorbance measured for every sample at the respective wavelength, resulting in the centred data matrix $\boldsymbol{X}$.

A singular value decomposition of the centred data matrix is then carried out:

$$\boldsymbol{X} = \boldsymbol{U} \cdot \boldsymbol{S} \cdot \boldsymbol{P}^T \tag{2}$$

$\boldsymbol{U}$ and $\boldsymbol{P}$ are orthogonal matrices and contain the left and right singular vectors, respectively. $\boldsymbol{S}$ is a diagonal matrix and its diagonal entries are called the singular values of $\boldsymbol{X}$. The right singular vectors are called principal components and the matrix $\boldsymbol{T}$

$$\boldsymbol{T} = \boldsymbol{U} \cdot \boldsymbol{S} \tag{3}$$

contains the scores (Hastie, 2001).

### 2.4.5 Principal Component Regression

In the principal component regression (PCR), the scores matrix $\boldsymbol{T}$ is used in a linear regression instead of the absorbance data matrix $\boldsymbol{X}$. A regression coefficient vector $\boldsymbol{\beta}$ is calculated for nitrite and nitrate separately as follows:

$$\boldsymbol{\beta} = (\overline{\boldsymbol{T}}^T \overline{\boldsymbol{T}})^{-1} \overline{\boldsymbol{T}}^T \boldsymbol{y} \tag{4}$$

With

$$\overline{\boldsymbol{T}} = [1\ \boldsymbol{T}] \tag{5}$$

Where $\boldsymbol{y}$ is the outcome vector, that is the known concentrations of nitrate or nitrite in the calibration subset.

As the first few principal components account for most of the variance of the data, only a small number of principal components is used in the principal component regression. This is accomplished by using only the first few columns of the scores matrix $\boldsymbol{T}$ (Hastie, 2001).

In order to determine the optimal number of principal components, the error of the estimation would have to be calculated in function of the number of principal components used. The number of principal components leading to the minimum error should be chosen. To simplify the modelling process, the number of principal components is chosen based on the variance explained by each principal component. Only the principal components explaining more than 1% of the variance of the original data are used in PCR. In order to assess the implications of this choice, the effect on the estimation accuracy is analysed by using one principal component more and less.

### 2.4.6 Estimation of nitrate and nitrite

Based on the principal component analysis and regression, the concentrations of nitrate and nitrite in the validation part of the dataset are estimated.

The scores for the estimation are calculated as follows:

$$\boldsymbol{T_{test}} = \boldsymbol{X_{test}} \cdot \boldsymbol{P} \tag{5}$$

Where $\boldsymbol{X_{test}}$ is the centred absorbance matrix of the validation part of the dataset. The concentration estimates $\check{\boldsymbol{y}}$ for nitrate or nitrite in those samples are calculated with the respective regression coefficient vector:

$$\check{\boldsymbol{y}} = \overline{\boldsymbol{T}}_{\boldsymbol{test}}\boldsymbol{\beta} \quad \text{with} \quad \overline{\boldsymbol{T}}_{\boldsymbol{test}} = [1\, \boldsymbol{T_{test}}] \tag{6}$$

As the concentrations of nitrate and nitrite in the validation part of the dataset are known, the estimated and observed concentrations are compared to assess the accuracy of the model. For nitrite, the required estimation accuracy is ±20 mgN/L. For nitrate, no such limit is set as mainly the influence of nitrate on the nitrite estimation is of interest.

# 3. Results

In the first section of this chapter, the results of the absorbance measurements in pure water and in urine with a constant background are presented. The second section delivers insight into the variation of the urine composition over time and its influence on the absorbance spectrum. In the third section, the results of the calibration and validation of the chemometric model (PCR) are presented.

## 3.1 Influence of nitrate and nitrite on the absorbance spectrum

In pure water, the concentration range from 0 to 5000 mgN/L nitrate and 0 to 150 mgN/L nitrite was covered with a grid of absorbance measurements with a resolution of 1000 mgN/L for nitrate and 30 mgN/L for nitrite.

In urine, background concentrations of around 2000 mgN/L nitrate and 2 mgN/L nitrite are expected. The concentration range from this background until around 5000 mgN/L nitrate (additional 3000 mg$NO_3$-N/L) and from the background to around 150 mgN/L nitrite (additional 150 mg$NO_2$-N/L) was to be covered in urine with a grid of absorbance measurements (details see section 2.2.2 on *Analysis in nitrified urine*). This experiment series was carried out on the 3rd to 5th August 2015 in one sample of nitrified urine taken on the 16th June 2015 which had been stored at 4°C. The sample contained 12.4 mgN/L nitrite, 1250 mgN/L nitrate and 465 mg/L COD. Taking this background into account, the actually covered concentration range was 1250 mgN/L to 4250 mgN/L for nitrate and 12.4 mgN/L to 162.4 mgN/L for nitrite.

Figure 4 shows the absorbance spectra recorded for the highest measured nitrate concentration and varying nitrite concentration in pure water (solid lines) and in urine (dashed lines). As the focus of this project lies on the secondary absorbance peaks, only the range between 260 and 420 nm is shown. The full spectra can be found in the appendix (Figure A 1 and Figure A 2) on page XX.

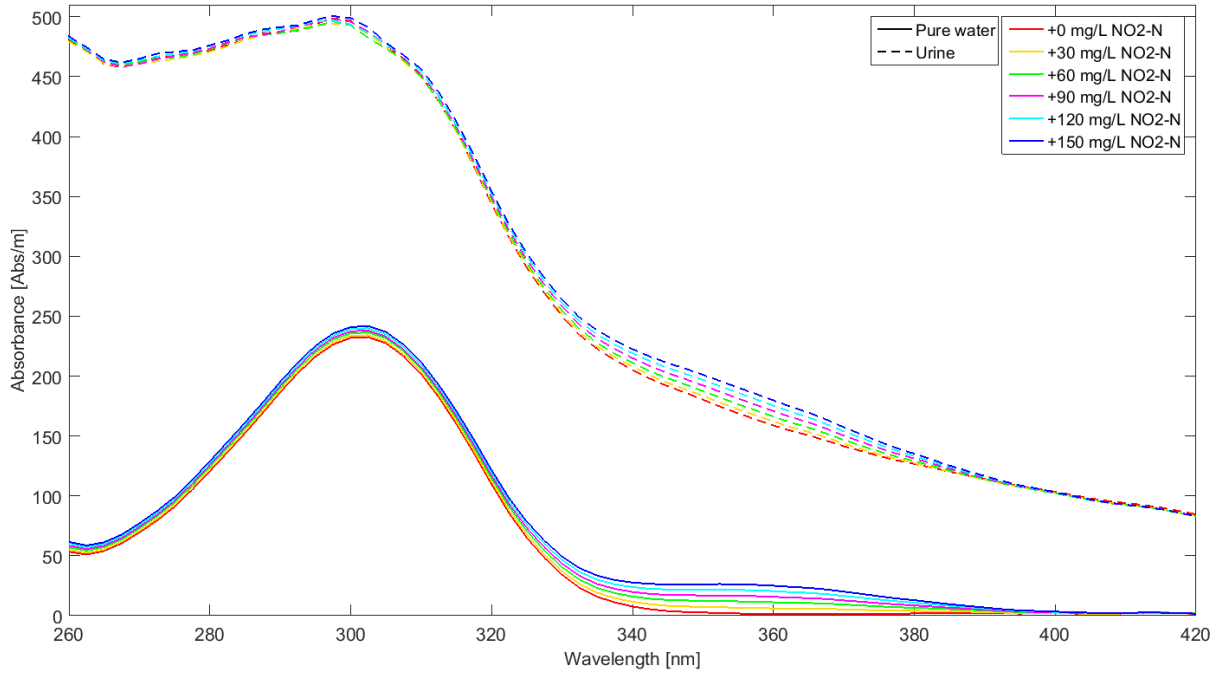


Figure 4: Absorbance spectra recorded in pure water and urine at a constant nitrate concentration and varying nitrite concentrations. Nitrate concentration: pure water: 5000 mg$NO_3$-N/L, urine: background plus 3000 mg$NO_3$-N/L (background urine: 1250 mg$NO_3$-N/L). Nitrite concentrations: pure water: 0 to 150 mg$NO_2$-N/L, urine: background plus 0 to 150 mg$NO_2$-N/L (background urine: 12.4 mg$NO_2$-N/L).

Figure 5 shows the spectra for the highest measured nitrite concentration and varying nitrate concentration.

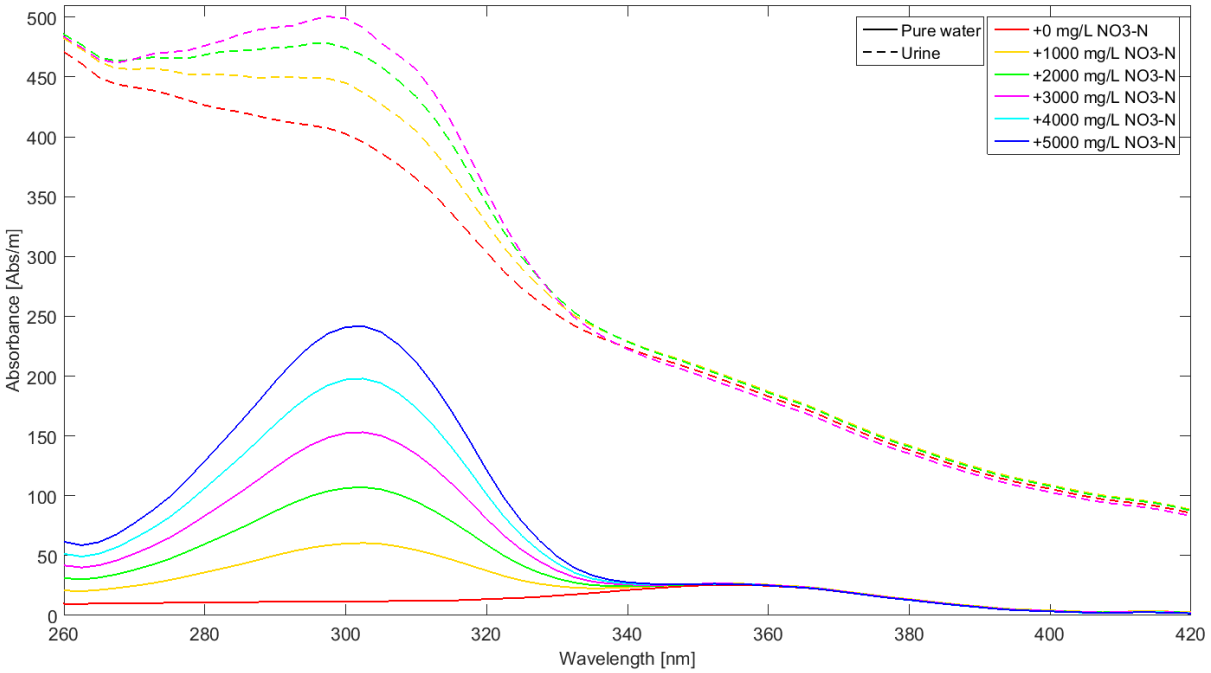


Figure 5: Absorbance spectra recorded in pure water and urine at a constant nitrite concentration and varying nitrate concentrations. Nitrite concentration: pure water: 150 mg$NO_2$-N/L, urine: background plus 150 mg$NO_2$-N/L (background urine: 12.4 mg$NO_2$-N/L). Nitrate concentrations: pure water: 0 to 5000 mg$NO_3$-N/L, urine: background plus 0 to 3000 mg$NO_3$-N/L (background urine: 1250 mg$NO_3$-N/L).

Figure 6 and Figure 7 show the adherence to the Beer-Lambert law over the covered concentration range for nitrite at the wavelength recorded that lies the closest to the secondary absorbance peak (355 nm) in pure water and in urine, respectively. Figure 8 and Figure 9 show the adherence to the Beer-Lambert law for nitrate at the wavelength recorded that lies the closest to the secondary absorbance peak (302.5 nm) in pure water and in urine, respectively. In all graphs, the straight line is fitted through the first and second data point and does not indicate the theoretical relationship between the concentration and the absorbance. It is meant as a guideline to observe potential non-linearity.

In water, the absorbance shows an almost linear relationship to the nitrite concentration up to 150 mgN/L. In the case of nitrate in water, the deviation from the Beer-Lambert law, caused by saturation at high concentrations, is visible. In urine, the non-linearity of the relationship between concentration and absorbance is more prominent than in water for both nitrite and nitrate.

The measurement accuracy, which is indicated in the four graphs below by the average ± two times the standard deviation, was calculated from the set of six absorbance spectra recorded for each combination of nitrite and nitrate concentration. In urine, the measurement is less accurate than in water at both secondary absorbance peaks.

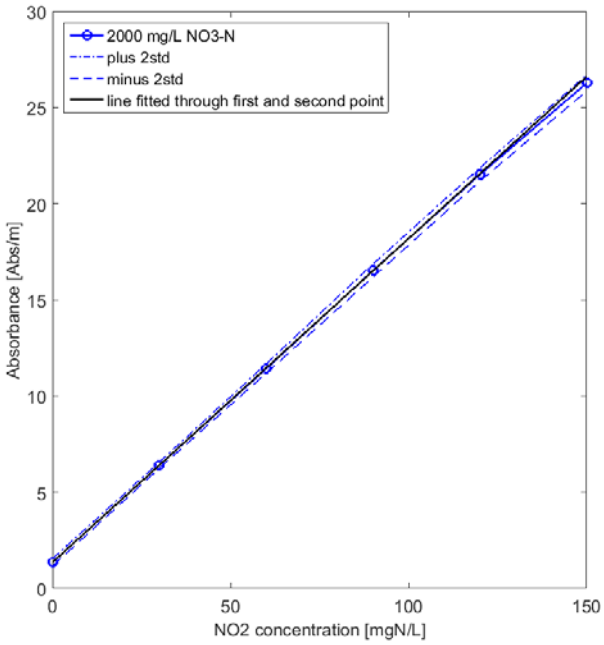


Figure 6: At 355 nm, adherence to Beer-Lambert law for nitrite in pure water at a nitrate concentration of 2000 mgN/L.

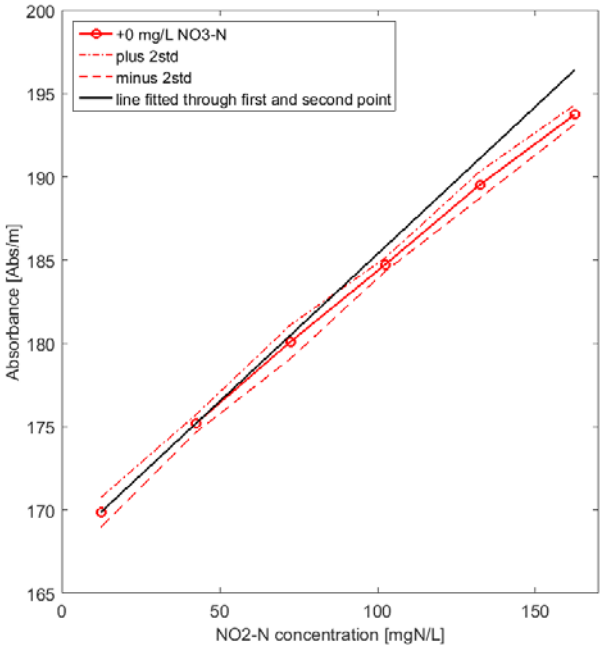


Figure 7: At 355 nm, adherence to Beer-Lambert law for nitrite in urine with a constant background at a nitrate concentration of 1250 mgN/L (background, no further nitrate added).

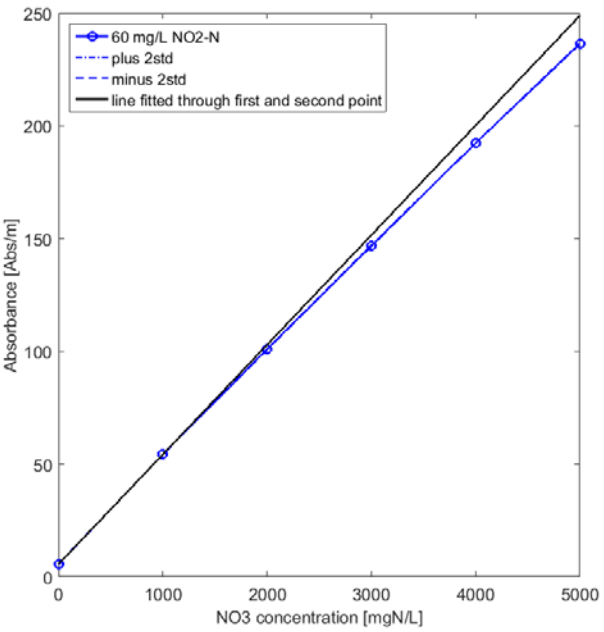


Figure 8: At 302.5 nm, adherence to Beer-Lambert law for nitrate in pure water at a nitrite concentration of 60 mgN/L.

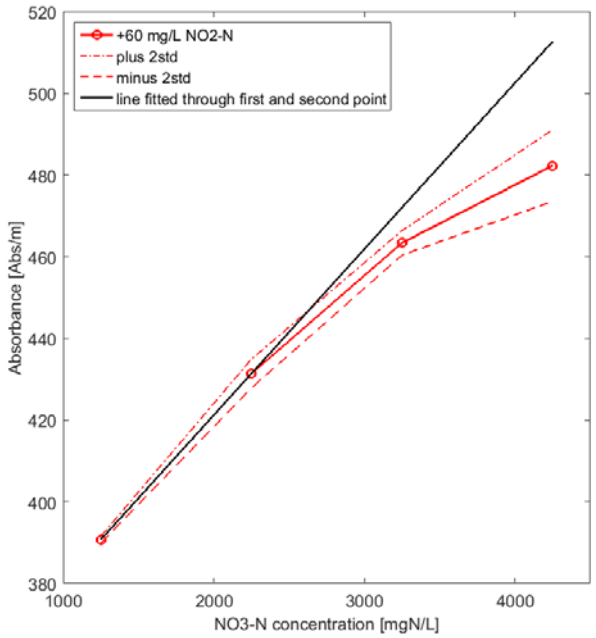


Figure 9: At 302.5 nm, adherence to Beer-Lambert law for nitrate in urine with a constant background at a nitrite concentration of 72.4 mgN/L (background of 12.4 mg$NO_2$-N/L plus 60 mg$NO_2$-N/L added).

## 3.2 Influence of changing urine composition on the absorbance spectrum

### 3.2.1 Change in urine composition

In Table 4 the measured concentrations of nitrite ($NO_2$), nitrate ($NO_3$) and chemical oxygen demand (COD) in the nine analysed samples are listed. Three samples were collected each week. An extended table containing the measured composition and the amount of nitrite and nitrate added to each sample can be found on page XXI in the appendix (Table A 1). The sample that was collected first was analysed as if it were sample N° 3.3. It is not possible to use this data as sample N° 1.1, as the set-up ensuring randomized samples during one week loses sense if shifted.

Table 4: Measured background concentrations in the nine urine samples ($NO_2$, $NO_3$ and COD).

| Sample N° | Sampling date | $NO_2$ concentration [mgN/L] | $NO_3$ concentration [mgN/L] | COD [mg/L] |
|---|---|---|---|---|
| 1.1 | 07.08.2015 | 1.31 | 2690 | 461 |
| 1.2 | 10.08.2015 | 1.30 | 2660 | 542 |
| 1.3 | 12.08.2015 | 1.22 | 2630 | 457 |
| 2.1 | 14.08.2015 | 1.21 | 2660 | 456 |
| 2.2 | 17.08.2015 | 1.07 | 2590 | 444 |
| 2.3 | 19.08.2015 | 0.99 | 2530 | 432 |
| 3.1 | 21.08.2015 | 0.85 | 2560 | 429 |
| 3.2 | 24.08.2015 | 0.77 | 2540 | 427 |
| 3.3 | 04.08.2015 | 1.24 | 2690 | 454 |

The concentration of all three measured compounds decreases over time.

### 3.2.2 Influence on the absorbance spectrum

For each measuring week one absorbance spectrum of the urine background is drawn in Figure 10. The samples N° 1.2 and 3.2 were selected because they exhibit the highest and lowest COD concentration. The decrease in the measured concentrations is mirrored in a decrease in absorbance (see Table 4).

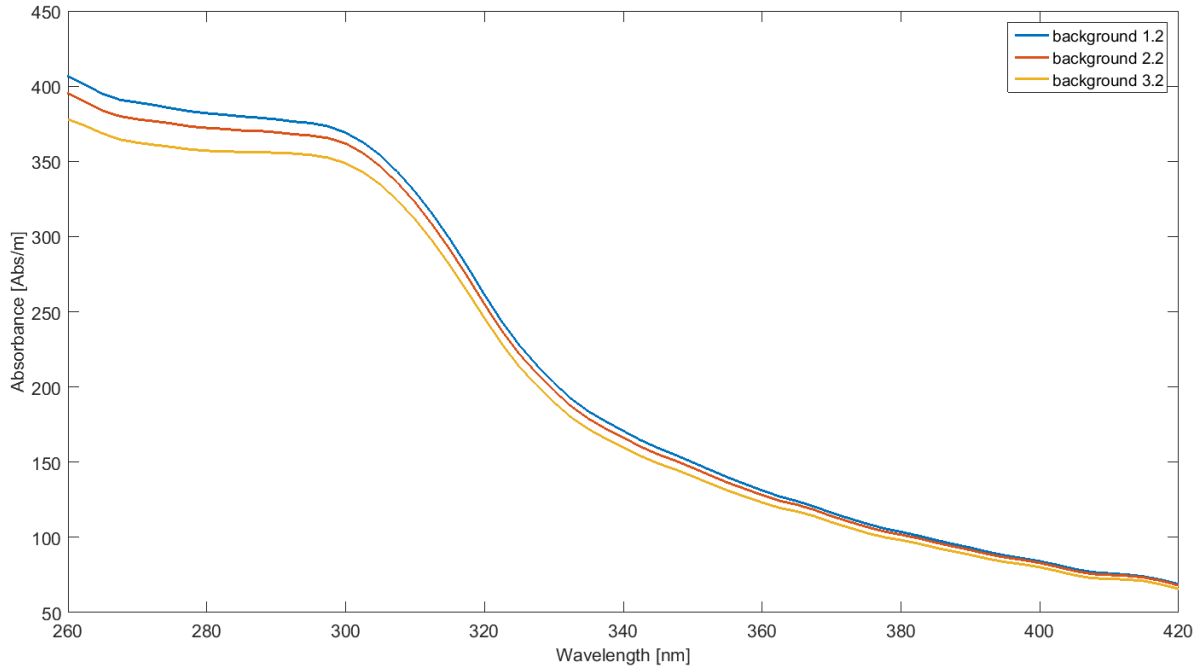


Figure 10: Absorbance spectra recorded in the urine samples N° 1.2, 2.2 and 3.2 (see Table 4) before adding further nitrite or nitrate (background).

## 3.3 Calibration and validation of the UV-Vis spectrophotometer

### 3.3.1 Calibration and validation for a constant background

Based on the data from the experiment series in pure water and in urine with a constant background, principal component analysis and regression (PCA and PCR) was carried out separately for the two experiments. In Table 5 the amount of variance explained by each of the first four principal components (PCs) in pure water and in urine is listed. In pure water, only the first and the second PC account for more than 1% of the variance in the absorbance data, whereas in urine the first three account each for more than 1%. To maintain comparability, three PCs are chosen for PCR for urine and pure water.

Table 5: Variance in the absorbance data explained by the first four principal components in pure water and urine with a constant background.

| | Variance explained by principal component [%] | | | |
|---|---|---|---|---|
| | N° 1 | N° 2 | N° 3 | N° 4 |
| Pure water | 98.57 | 1.43 | 0.004 | 0.001 |
| Constant urine | 92.53 | 6.05 | 1.34 | 0.04 |

To identify the meaning of the principal components, the loadings were inspected. Figure 11 and Figure 12 show the loadings of the first three principal components in pure water and in urine with a constant background. As the peak of the first principal component lies in water and in urine at around 300 nm and thus at the secondary absorbance peak of nitrate, it is identified as the PC accounting for the variability in the data caused by changes in the nitrate concentration. The second PC has its peak at the secondary absorbance peak of nitrite (around 350 nm) and thus accounts for the variability in the data caused by changes in the nitrite concentration. The loadings of the first and the second PC are alike in water and urine, apart from some roughness around 300 nm in the urine principal components. The third one exhibits a wavy course which is more pronounced in the left hand side of the spectrum.

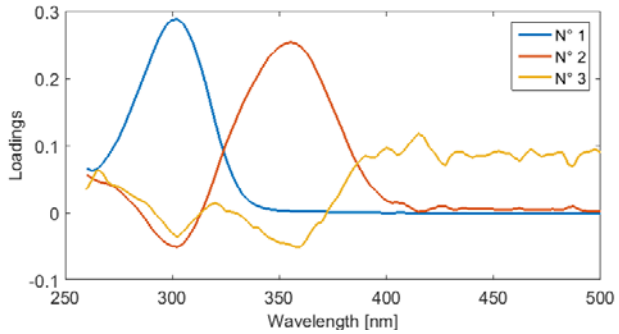


Figure 11: Loadings of the first three principal components in pure water.

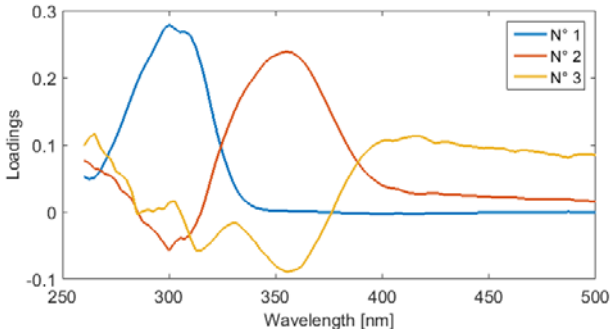


Figure 12: Loadings of the first three principal components in urine with a constant background.

As mentioned, the chemometric model was then calibrated using three principal components. The calibration and validation procedure is explained in section 2.4.2 (*Data preparation for the experiments with constant background*). Figure 13 and Figure 14 show the statistical distribution of the estimation error for nitrite and nitrate in pure water and in urine with a constant background. Reading example: for nitrite in urine, the median of the estimation error is around +1 mgN/. The upper and the lower quartiles of the distribution of the errors are around +4 and -4 mgN/L, respectively. The highest and lowest data points, which are less than 1.5 times the interquartile range away from the box, are around +10 and – 12 mgN/L, respectively. Points lying beyond this range are outliers and marked with a cross.

For both substances, the estimation is less accurate in urine than in water.

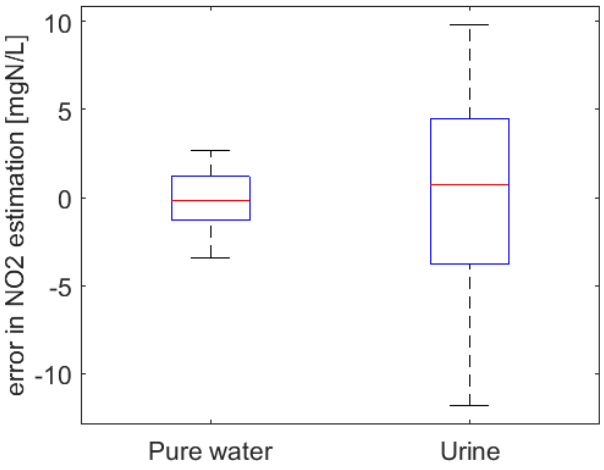


Figure 13: Estimation error for the nitrite concentration in pure water and urine with a constant background (pure water: N=36, urine: N=24).

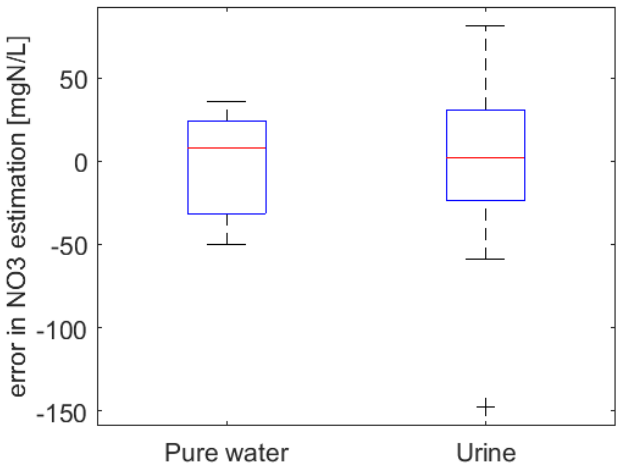


Figure 14: Estimation error for the nitrate concentration in pure water and urine with a constant background (pure water: N=36, urine: N=24).

For urine, one outlier in the nitrate estimation errors can be observed (+-sign in Figure 14). In the cold urine sample, bubble formation on the whole sensor was observed at the start of the measurement. Analysing the data, it was stated that the measured absorbance increased during bubble formation by around 20 Abs/m within 10 minutes. The spectra most influenced by bubble formation were not used for the analysis, however this outlier stems from one of those experiments. Further information is available in the appendix on page XXII.

The estimation accuracy reached with the two different calibration subsets is similar. Graphs showing the estimation accuracy of nitrite and nitrate separately for the different calibration subsets in pure water and urine can be found in the on page XXIII in the appendix (Figure A 5 to Figure A 8).

Comparing the median of the estimation errors at different concentrations, it is observed that the lower concentrations are generally underestimated, middle concentrations overestimated and high concentrations again underestimated (see Figure A 9 to Figure A 16 on page XXIVf in the appendix). For nitrite, the differences between the estimation accuracy at different concentrations are more prominent in urine than in water.

To review the choice of using three PCs in the regression, Table 6 shows the average absolute error for nitrite and nitrate in urine and pure water if two to four principal components are used. Box plots showing the statistical distribution of the estimation error using two or four instead of three principal components can be found on page XXVIII in the appendix (Figure A 17 to Figure A 20).

Table 6: Average absolute error depending on number of principal components used in principal component regression in pure water and in urine with a constant background.

| | Number of principal components used in regression [-] | Average absolute estimation error for nitrite [mgN/L] | Average absolute estimation error for nitrate [mgN/L] |
|---|---|---|---|
| Pure water | 2 | 2.0 | 24 |
| | 3 | 1.3 | 24 |
| | 4 | 0.8 | 9 |
| Urine with a constant background | 2 | 21 | 94 |
| | 3 | 4 | 35 |
| | 4 | 2 | 23 |

### 3.3.2 Stability of the model in urine with varying composition

To assess the stability of the model in urine with varying composition, the chemometric model was calibrated using the absorbance data from one week at a time. For each week, the background of the first sample (1.1, 2.1, 3.1, see Table 4) was added to the dataset to complement the range of nitrite and nitrate concentration covered. Resulting from the principal component analysis of each week's dataset, the variance in the data explained by the first four principal components (PCs) is listed in Table 7.

Table 7: Variance in the absorbance data explained by the first four principal components based on each of the three measuring weeks.

| | Variance explained by principal component [%] | | | |
|---|---|---|---|---|
| | N° 1 | N° 2 | N° 3 | N° 4 |
| Week 1 | 92.57 | 4.92 | 2.27 | 0.22 |
| Week 2 | 90.04 | 7.30 | 2.48 | 0.16 |
| Week 3 | 96.00 | 3.30 | 0.68 | 0.02 |

For the first and second week, the first three PCs account for more than 1 % of the variance in the data. In the third week, the third PC accounts for around 0.7 % of the variance, nevertheless it is included in the modelling procedure to ensure comparability. To identify and compare the meaning of the PCs, the loadings of the first three PCs were inspected. Figure 15 to Figure 17 show the loadings of the first three PCs based on data from one measuring week each. The loadings based on the first and second week are similar to the loadings in constant urine (see Figure 12). In the third week the order is different: not the second but the third PC seems to account for the variance caused by nitrite.

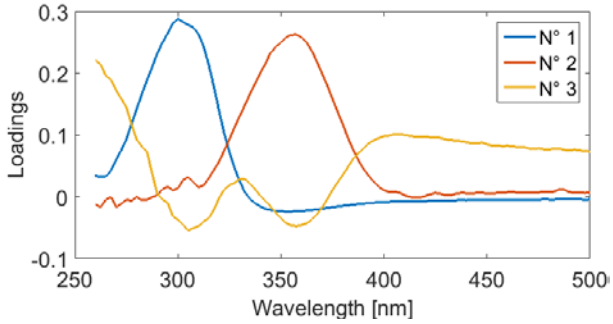


Figure 15: Loadings of the first three principal components based on the data from the first week.

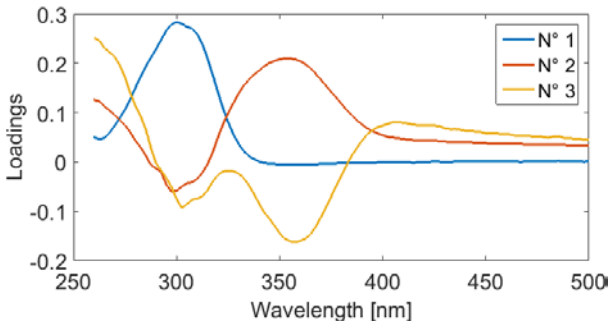


Figure 16: Loadings of the first three principal components based on the data from the second week.

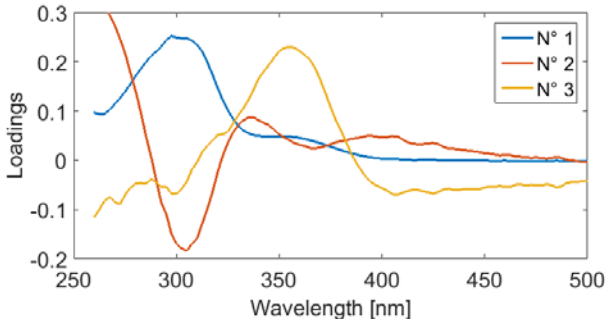


Figure 17: Loadings of the first three principal components based on the data from the third week.

As mentioned, to calibrate the model the first three PCs were subsequently used in the principal component regression. Based on the calibration with each week's dataset, the accuracy of the estimation of nitrite and nitrate in the samples of the other two weeks was analysed. The statistical distributions of

the estimation error are shown in Figure 18 and Figure 19. For nitrite, the model based on the third weeks is less accurate when extrapolating by one week than the model from the first or second week.

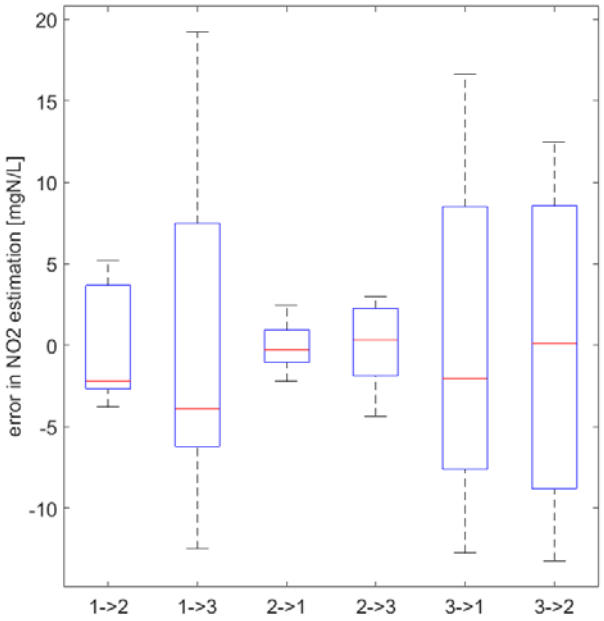


Figure 18: Estimation errors for the nitrite concentration in urine with a variable background, (1->2: calibration based on data from week 1, estimation for samples in week 2) (for each box N=7).

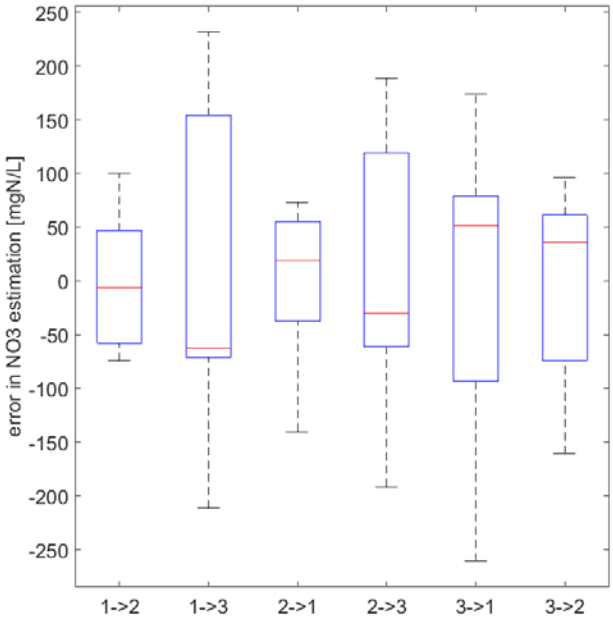


Figure 19: Estimation errors for the nitrate concentration in urine with a variable background, (1->2: calibration based on data from week 1, estimation for samples in week 2) (for each box N=7).

As the role of the principal components is less clear than in a constant background, the regression was also carried out using zero to five principal components to look at the effect on the estimation accuracy. At least three principal components are needed to reach a sufficient nitrite estimation accuracy, as shown exemplary for week 2 in Figure 20. Similar plots for week 1 and 3 and for nitrate can be found in the appendix on page XXIXf (Figure A 21 to Figure A 26).

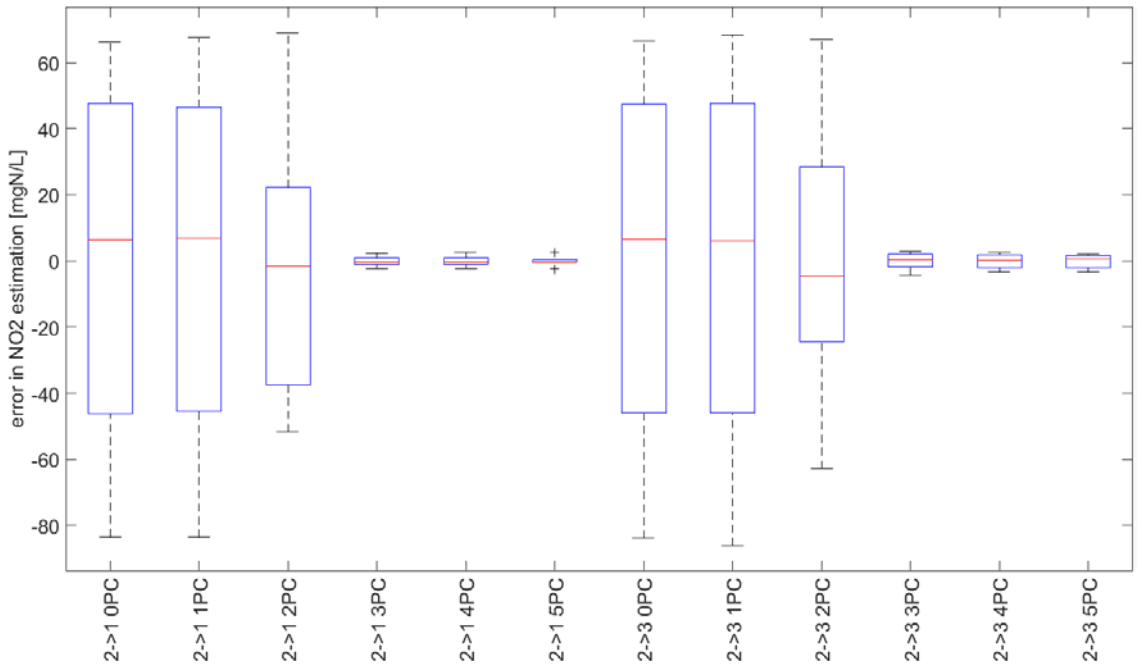


Figure 20: Estimation accuracy for the nitrite concentration using different numbers of principal components in regression, (2->1: calibration based on data from week 2, estimation for samples in week 1) (for each box N=7).

# 4. Discussion

In this chapter, the results presented previously are discussed and put into context. First, the implications of the applied data pre-processing are described. Next, the various influences on the absorbance spectrum are discussed and compared. After this, the estimation accuracy reached in a constant background is reviewed. Afterwards, the stability of the model under the influence of changes in the urine background composition is discussed. Finally, the transferability of the results to other applications is judged.

## 4.1 Implications of the applied data pre-processing

The raw absorbance data was pre-processed by averaging the six spectra recorded for each data point, after removing any outliers. This procedure reduces random errors of the measurement and thus the accuracy reached by such a calibration is more stable. To estimate nitrite automatically, an automatic method to detect outliers online is needed.

In practice, concentrations might change over a measuring time of six minutes and thus the averaging would smoothen the concentration change. The accumulation of nitrite to critical concentrations in the urine nitrification reactor takes a few hours however (Etter et al., 2013). In relation to this timespan a measuring time of six minutes appears acceptable.

After averaging, the absorbance spectrum was censored to exclude noise and saturation effects in the range of the primary absorbance peaks of nitrate and nitrite. The parts of the spectrum not considered in the analyses need not be recorded and therefore a simpler sensor measuring absorbance only in the range from 260 nm to 700 nm would suffice. Further reducing the spectrum or picking only a few single wavelengths might be possible, but was not tested within the scope of this project.

## 4.2 Influences on the absorbance spectrum

### 4.2.1 Influences of changes in the nitrite and nitrate concentration and sensor choice

In urine the absorbance is generally higher than in pure water. This makes sense as various light-absorbing substances are present in the urine apart from nitrite and nitrate, such as amines or aromatic compounds (Spinelli et al., 2007). Comparing the spectra for different nitrite and nitrate concentrations it is clearly visible that an increase in the concentration of nitrite or nitrate leads to an increase in the absorbance around the respective absorbance peak.

Linearity of the increase in absorbance to the increase in concentration, and thus adherence to the Beer-Lambert law, cannot be observed in urine. In pure water, practically no deviation from linearity is observed for nitrite up to 150 mgN/L, which means that saturation effects play a minor role. At high concentrations of nitrate in pure water, the deviation from the Beer-Lambert law is evident, but, as discussed below, the estimation accuracy when using a linear model is still good. In urine with a constant background, saturation effects are more prominent, especially for nitrate.

Choosing a sensor with a shorter measuring path would diminish the influence of saturation. However, using a shorter measuring path impairs the sensitivity at low concentrations. As saturation effects should have a small influence at the concentrations of nitrite and nitrate usually present in the reactor (at the low end of the covered range for nitrite and in the middle of the covered range for nitrate, see Table 1), the path length of the sensor seems to be well suited for the intended purpose. As discussed

below, this is backed by the sufficient estimation accuracy. In order to apply UV-Vis spectrophotometry in other concentration ranges, using a longer or shorter path should be considered.

### 4.2.2 Influences of a variable urine background

Comparing the nitrite, nitrate and COD concentrations, it seems that the urine was continuously diluted over the tree weeks during which the samples were taken. The background absorbance spectra of samples taken with a week's time difference are clearly different. As male urine was fed to the reactor during the whole sampling time, the observed dilution is probably an inherent fluctuation of the urine composition (B. Sterkele, personal communication, 1st September 2015). As the male urine is much more concentrated than the female urine, such a dilution could also be caused by an increasing percentage of female urine in the influent of the reactor.

## 4.3 Modelling and accuracy of the estimation for constant background

### 4.3.1 Estimation accuracy for constant background

The estimation accuracy that can be reached based on data recorded in a constant background is very high. With a maximal estimation error below 4 mgN/L for nitrite (and below 55 mgN/L for nitrate), the required accuracy of ±20 mgN/L nitrite is well met in pure water. The accuracy in pure water could be seen as the technical accuracy limit of the UV-Vis spectrophotometry. In urine, the maximal error of below 12 mgN/L for nitrite (and below 150 mgN/L for nitrate) lies clearly within the required accuracy limit as well. In a constant background, UV-Vis spectrophotometry is thus an appropriate technique to measure the nitrite concentration under changing nitrite and nitrate concentrations.

The decrease in accuracy from pure water to urine seems plausible. The absorbance measurement is generally less accurate, which might be due to particles in the sample. Probably more important, saturation effects are stronger in urine than in water, resulting in increasing uncertainty as a linear model is fitted to increasingly non-linear data.

The observation that lower concentrations are generally underestimated, middle concentrations overestimated and high concentrations again underestimated, is due to the linear regression of a dataset influenced by saturation. As the expected nitrite concentration in the urine is at the low end of the concentration range covered in the experiments, it would consequently be underestimated. In the case of an accumulation, the concentration of nitrite would shift into the range where it is overestimated and thus lead to a more pronounced increase in estimated concentration than in reality. This effect might have to be considered when implementing an automatized control system to avoid an overcompensating control action.

### 4.3.2 Details of the modelling procedure

The saturation effects leading to the under- and overestimation of different concentrations by using linear regression should be diminished by calibrating the model within a lower concentration range. In the lower concentration range, the influence of saturation is smaller. The dataset of this project is too small to test this hypothesis meaningfully.

Under normal operation conditions the expected concentration range in the reactor lies in the lower half of the concentration range covered in the experiments. Using a lower range for modelling should thus improve the estimation accuracy in this expected range. However, as extrapolation is not possible, the estimation accuracy of higher concentrations would be significantly worse. Consequently, the cali-

bration should be carried out within the whole concentration range expected for the concerned process, if a reasonable and consistent estimation accuracy for this range is needed. For the specific case of this project, the focus lies on maintaining a reasonable estimation accuracy up to 150 mgN/L nitrite and 5000 mgN/L nitrate. Thus calibrating the model beyond the usual concentration range is appropriate.

In urine, using three principal components in the regression increases the accuracy significantly compared to using only two. As this effect is less prominent in pure water, the third principal component has to be connected to a phenomenon more important in urine than in pure water. As the background was constant in both cases, this phenomenon is most probably the saturation effect, which is especially distinct at the secondary absorbance peak of nitrate in urine. Testing this hypothesis would again be possible by basing the calibration on a part of the dataset containing only the lower half of the concentration range. If then the increase in estimation accuracy from using two to using three principal components was significantly smaller, it would be confirmed that the third principal component compensates non-linearity caused by saturation. However, this confirmation would only apply to the case of a constant background, as the meaning of the principal components might be different in a variable background.

Using four principal components instead of three again increases the accuracy, but to a smaller extent only. An exception to this is the estimation of nitrate in pure water, which is much more accurate using four instead of three principal components. It seems that the fourth principal component is linked to the changes in nitrate concentration in the case of pure water. This observation was not investigated in detail, as the estimation accuracy of nitrate is of lesser importance than the estimation accuracy of nitrite.

The number of principal components used in the regression has a direct influence on the computational effort. Thus the decision how many principal components to use depends on the trade-off between estimation accuracy and computation time. In urine, three is the minimum number of principal components with which the required nitrite estimation accuracy of ±20 mgN/L can be reached. As the main focus lies on the estimation in urine, three principal components seem an appropriate choice.

The relationship between absorbance and concentration is not linear due to saturation effects. This limits the estimation accuracy possible with a linear model. Applying a more complicated model might improve the estimation accuracy. However, more complicated models increase the computational effort, thus leading to a trade-off between estimation accuracy and computational effort.

## 4.4 Stability of the model under changing urine composition

The models based on the dataset of one week meet the required nitrite estimation accuracy of ± 20 mgN/L when extrapolating by two weeks' time. For this extrapolation time, UV-Vis spectrophotometry is thus an appropriate technique to estimate the nitrite concentration under the influence of the background composition variability found in the scope of this project. In practice however, the extrapolation time is supposed to be much longer and thus further experiments are necessary. Nevertheless the following paragraphs analyse several aspects of the results obtained so far.

As the dataset of each week covers the possible concentration range of nitrate and nitrite only partly and exhibits a different background than the datasets of the other weeks, the estimation was expected to be less accurate than in urine with constant composition. This is only partly confirmed. The nitrite estimation for the samples in the first and third week, based on the calibration with the data from the second week (2->1 and 2->3), is as accurate as in urine with a constant background. Based on the calibration with the data from the first week, the extrapolation to the second week (1->2) reaches a similar accuracy, however the extrapolation to the third week (1->3) is significantly less accurate. If the model is calibrated with the data from the third week, the nitrite estimation for both the samples in the first and second week (3->1, 3->2) is only around as accurate as the 1->3 validation. The observation that the nitrite estimations 1->2, 2->1 and 2->3 are about equally accurate and more accurate than 1->3 and 3->1 might be connected to the time difference. 1->2, 2->1 and 2->3 all extrapolate to a next or previous week, whereas 1->3 and 3->1 extrapolate over two weeks' time. However, this thesis is not supported by the estimation 3->2.

To analyse the observed contradiction, it has to be considered that one sample belonging to week three was actually taken before all the other samples. Thus, the background variability in the dataset of week three is larger than in the other two weeks. In the third week, the nitrite variability is explained by the principal component N° 3 and not by N° 2. This order of the principal components indicates that the variability of nitrite is less important than the variability of another phenomenon. Here, the variability explained by the principal component N° 2 might be caused by saturation and / or changes in the background composition of the urine. Furthermore, the amount of variance explained by the principal component accounting for nitrite is much smaller in week 3 than in week 1 or 2. The cause of this is unknown. Taking this and the order of the principal components into account, the model built on week 3 might show a better performance in compensating changes in the background, but a weaker performance in nitrite estimation. This explanation would resolve the contradiction mentioned above, however the dataset is too small to assess its correctness.

In the model calibrated with the data from the third week only the third principal component seems to account for the nitrite variability. Using only two principal components in the regression, the estimation of nitrite is therefore very inaccurate. However, this decrease in accuracy is observed for all three weeks to an about equal extent. For calibration with principal component regression, three principal components are chosen, as it is the minimum number with which the target estimation accuracy for nitrite can be reached. Assessing the role of the second and the third principal component would require a larger dataset, with which the influence of saturation is minimized to see which principal component compensates for changes in the background composition.

Looking at the median of the error distributions of nitrate, the model based on week 1 underestimates the concentrations in week 3 and also slightly the concentrations in week 2. The model based on week 3 overestimates the concentrations in week 1 and 2. The reason is probably the dilution of the back-

ground over time. In the first week, the background is more concentrated than in week 2 and 3, resulting in a higher absorbance at the same nitrate concentration. The model calibrated with the concentrated background in week 1 thus underestimates the concentrations in the weeks with a less concentrated background, because there the actual nitrate concentration is higher at the same absorbance. This comparison is based on the nitrate estimation as the accuracy of the nitrate estimation is more constant over the calibration weeks than the nitrite estimation. These effects cannot be observed consistently in the estimation errors for nitrite.

## 4.5 Transfer to other applications

Transferring the findings of this project to other applications might be possible. Measuring light-absorbing substances of interest in similarly concentrated liquids with a similar variability could be tested. Examples might be digester supernatant or food processing waste water. However, as the background composition of such a liquid is specific, no direct transfer of the conclusions based on urine can be made.

# 5. Conclusions

In this project UV-Vis spectrophotometry was tested for the simultaneous estimation of nitrite and nitrate in nitrified urine. The main question was how changes in the concentration of nitrite and nitrate and changes in the background composition affect the absorbance spectrum and thus the estimation accuracy. To this purpose it was analysed if UV-Vis spectrophotometry, combined with principal component regression, enables an estimation of nitrite with an accuracy of ±20 mgN/L and if the model provides a stable estimation accuracy despite a dynamic urine background.

When assessing the influence of the nitrite and nitrate concentration on the absorbance spectrum in pure water and in urine, it was observed that the absorbance is saturated at high concentrations of both substances. In urine, the effect is stronger, as a variety of light-absorbing substances are present. As saturation implies a non-linear relation between the absorbance and the concentration, it impairs the estimation accuracy that can be reached with a linear regression model.

Despite the influence of saturation, the required estimation accuracy for nitrite was clearly met in urine with a constant background. Even if the nitrite estimation accuracy of around 10 mgN/L in constant urine is worse than in pure water (around 5 mgN/L), UV-Vis spectrophotometry seems an appropriate technique for the nitrite estimation in urine.

If the model is calibrated based on measurements in three samples of nitrified urine collected over the course of one week, the estimation accuracy for nitrite lies within ± 20 mgN/L when extrapolating in time over two weeks. Thus the model is sufficiently stable for the extrapolation over this time. However, in practice, the target extrapolation time is longer and more experiments are necessary to verify the stability of the model over time.

For calibration with principal component regression, three principal components were found to be necessary to reach the target estimation accuracy for nitrite. However, the meaning of the third principal component could not be confirmed. It is assumed that it compensates for saturation effects, changes in the background composition or a combination thereof.

# 6. Outlook

To reach the goals of this project, taking urine samples over another three weeks' time would be needed. Half of the measuring grid proposed for the experiment series with urine of varying composition could not be included in this project's plan. Furthermore, by repeating the experiment after some time the stability of the model over time could be analysed in detail. The background composition of the urine will likely be more dynamic over a longer period of time.

Testing the estimation accuracy for nitrite and nitrate using only a small part of the spectrum or even only a few wavelengths in modelling would be interesting. For a smaller spectrum simpler sensors are sufficient and the computational effort is reduced. Comparing the estimation accuracy reached with sensors with different path lengths might be a useful project to examine the trade-off between avoiding saturation at high concentrations and reaching high sensitivity at low concentrations.

By repeating the urine experiment with a constant background with a finer resolution in the low nitrite and nitrate concentration range, the role of the third principal component could be further analysed. With a fine enough resolution, the dataset would be large enough to use only the part containing the lower concentrations in a meaningful calibration and validation. The difference in accuracy reached with this refined dataset when using two, three or four (or any other number of) principal components should then be compared to the results of this project. Thus, the hypothesis that the third principal component compensates saturation effects could be confirmed or rejected.

As the relationship between absorbance and concentration is not linear due to saturation effects, the estimation accuracy possible with a linear model is limited. If would therefore be interesting to apply a more complicated model and assess the effect on the estimation accuracy. As the computational effort increases with the complexity of the model, the trade-off between accuracy and computational effort needs to be analysed to find the most economic model.

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# A Protocols

## Overview

PROTOCOL A1: Preparation of 1 liter nitrate solution
Christian Thürlimann, Eawag, June 2015

Required products:

- $NaNO_3$ powder
- Nanopure water

Required hardware:

- Funnel
- 1 litre glass container
- 500 ml standardized flask
- 2 x 100ml standardized flask
- Magnet stirrer with magnet
- Pipette (10, 5, 1, 0.2, 0.1 ml)

Plan:

Make 1000 ml solutions for X = 1 or 2 or 3 or 4 or 5 gNO3-N/l

FOR 1000ml nitrate stock solution

| | Unit | 1 g $NO_3$-N | 2 g $NO_3$-N | 3 g $NO_3$-N | 4 g $NO_3$-N | 5 g $NO_3$-N |
|---|---|---|---|---|---|---|
| **A** | **$NaNO_3$[g]** | 6.06801385 | 12.1360277 | 18.2040416 | 24.2720554 | 30.3400693 |
| **B** | **Rounded amount for scale [g]** | 6.068 | 12.136 | 18.204 | 24.272 | 30.34 |
| **C** | **Dilution for Lange Test** | 1:500 | 1:1000 | 1:1000 | 1:2000 | 1:2000 |
| **D** | **Expected concentra-tions [mg$NO_3$-N/l]** | 2.0 | 2.0 | 3.0 | 2.0 | 2.5 |

Steps:

1) Nitrate stock solution (goal: 1000 ml à X mg $NO_3$-N/L)
   a) Weigh X [g] of the $NaNO_3$ powder (see Table **row B**)
   b) Dissolve powder into water
      i) Take approx. 250 ml of nanopure water and add it to the standardized 500ml flask
      ii) Use a funnel to add the $NaNO_3$ powder to the container
      iii) Flush the funnel with some nanopure water to collect all the powder remaining on the surface of the funnel (make sure you don't reach the 500ml when flushing the funnel!!!)
      iv) Add stirring magnet to the flask
      v) Place the flask onto a magnetic stirrer
      vi) Switch the magnetic stirrer on and let it mix until all powder is dissolved
      vii) Switch the magnetic stirrer off
      viii) Remove the magnet and rinse it with nanopure in the flasks neck
      ix) Fill the container with nanopure water until exactly 500 ml is reached

x) Close the flask with a stopper, shake it slowly to mix the added water with the remaining solution.
xi) Fill the solution into the 1 litre glas container
xii) Fill the 500ml flask of nanopure again and fill the 1 liter glass container.
xiii) Close the glass container and shake it slowly for 1 minute.
xiv) Add the dry magnetic stirrer to the glass container, close it and let it stir for another 30 minutes.

c) Measure the $NO_3$-N sample with a Hach-Lange cuvette test (LCK 340)
   i) Make a dilution according to **row C** in the table above. Either choose (1),(2) or (3) depending on the requested dilution:
      (1) 1:500 (5 ml stock solution into a 100 ml flask (shake the flask), from this solution 2.5 ml into 100 ml flask (shake the flask))
      (2) 1:1000 (2 ml stock solution into a 100 ml flask (shake the flask), from this solution 5 ml into 100 ml flask (shake the flask))
      (3) 1:2000 (2 ml stock solution into a 100 ml flask (shake the flask), from this solution 2.5 ml into 100 ml flask (shake the flask))

d) Close the container

e) Proceed with Lange Test LCK 342

   i) Pipette 0.2 ml of the diluted solution from step c) slowly into the cuvette
   ii) Pipette 1.0 ml of the solution A slowly into the cuvette
   iii) Close cuvette and invert a few times until no more streaks can be seen.
   iv) Wait 15 minutes
   v) Clean the cuvette
   vi) Measure

f) Evaluate the quality of the solution (Expected concentration: See table **row D** ). Repeat steps a-h if the obtained quality is insufficient.

g) Label the flask with your name and content description
h) Store the flask in the fridge in B74

<u>Calculations:</u>

Nitrate:
Desired concentration: X g $NO_3$-N/L
Desired volume 1 L

$$M_{NaNO3} = X\frac{gN}{L} \cdot 1\ L \cdot \frac{84.994\frac{gNaNO3}{mol}}{14.007\frac{gN}{mol}} = X * 6.068\frac{gNaNO3}{L}$$

PROTOCOL A2: Preparation of nitrite stock solution with nitrate.
Christian Thürlimann, Eawag, June 2015

Required products:

- $NaNO_2$ powder
- $NO_3$ solution à 1000, 2000, 3000, 4000, 5000 mg/l $NO_3$-N (depending on the experiment) See protocol A1!
- Nanopure water

Required hardware:

- Funnel
- 2x 100 ml flask
- 500 ml flask
- Magnet stirrer with magnet
- Pipette (10, 5, 1, 0.2, 0.1 ml)
- LCK 342 Nitrite Lange Tests

Plan:

Make 100ml nitrite stock solution with constant nitrate solution.
Read the whole protocol before starting with step 1.

Steps:

1) Nitrite stock solution (goal: 505 ml nanopure with X mg $NO_3$-N/L and 150mg $NO_2$-N/l)
   a) Weigh 7.4625 g of the $NaNO_2$ powder
   b) Dissolve powder into water
      i) Take approx. 50 ml of nitrate solution (Protocol A1) and add it to the standardized 100 ml flask
      ii) Use a funnel to add the $NaNO_2$ powder to the container
      iii) Flush the funnel with a small amount of nitrate solution to collect all the powder remaining on the surface of the funnel (make sure you don't fill the flask to a 100ml!!!!)
      iv) Add stirring magnet to the flask
      v) Place the flask onto a magnetic stirrer
      vi) Switch the magnetic stirrer on and let it mix until all powder is dissolved
      vii) Switch the magnetic stirrer off
      viii) Remove the magnet and rinse it with nitrate solution in with nanopure in the flasks neck
      ix) Fill the container with nitrate solution until exactly 100 ml is reached
      x) Close the flask with a stopper, shake it slowly to mix the added water with the remaining solution.
   c) Take a sample to measure the $NO_2$-N concentration with a Hach-Lange cuvette test
   d) Close the flask with a stopper
   e) Label the flask with your name and content description
   f) Measure the $NO_2$-N sample with a Hach-Lange cuvette test (LCK 342)
      i) 1:5000 (1 ml stock solution into a 100 ml flask (shake the flask), from this solution 2 ml into 100 ml flask (shake the flask))
      ii) Carefully remove the foil from the zip
      iii) Remove the cap without turning it upside down
      iv) Pipette 0.2 ml of the diluted sample
      v) Immediately screw the cap back, fluting at the top.
      vi) Shake until all compounds are dissolved
      vii) Wait 10 minutes

viii) Invert some times more, clean the cuvette
ix) Measure

g) Store the flask in the fridge in B74

Evaluate the quality of the solution (Expected concentration: 3.03 mgNO2-N/L). Repeat steps a-g if the obtained quality is insufficient

Calculations:

Nitrite:
Desired concentration: 150 mg $NO_2$-N/L
Amount of $NaNO_2$ for 100 ml stock solution:

$$M_{NaNO2} = \frac{150\frac{mgN}{L} \cdot 505\,mL}{5mL} \cdot \frac{0.1L}{100mL} \cdot \frac{1gN}{1000mgN} \cdot \frac{68.995\frac{gNaNO2}{mol}}{14.007\frac{gN}{mol}} = 7.4625\frac{gNaNO2}{100ml}$$

Cross interference with nitrate in LCK 342 Lange-test is not existent up to a concentration of 2000mg/l NO3-N! Which is not the case for this protocol as we dilute the solution for the Lange Test by 1:5000.

PROTOCOL A3: Testing simultaneous nitrate and nitrite estimation with UV-Vis sensor
Christian Thürlimann, Eawag, June 2015

Required products:

- Nanopure water
- Stock solution Nitrite (goal: 150 mgN/L, 15.14997gN/L solution ) Protocol A2
- Solution Nitrate (X mgN /L) Protocol A1

Required hardware:

- 2 narrow measurement cylinder (1000 ml)
- 2 standardized 500 ml flask
- Magnet stirrer with magnet
- Pipette (2 ml)
- s::can Sensor "blue"
- Laptop

Plan:
Test UV-Vis sensor under the same idealized conditions for estimating nitrite and nitrate.
Experimental plan: $
0-5 nitrate concentration 0-5000mg$NO_3$-N/L
R-W nitrite concentration 0-150 mg$NO_2$-N/L
Example. Experimental row 3R-3W nanopure water with 3000mgNO3-N/L und 0 to 150mgNO2-N/L

| Nitrat mg/l / Nitrite mg/l | **R** | **S** | **T** | **U** | **V** | **W** |
|---|---|---|---|---|---|---|
| **5** | 5000/0 | 5000/30 | 5000/60 | 5000/90 | 5000/120 | 5000/150 |
| **4** | 4000/0 | 4000/30 | 4000/60 | 4000/90 | 4000/120 | 4000/150 |
| **3** | 3000/0 | 3000/30 | 3000/60 | 3000/90 | 3000/120 | 3000/150 |
| **2** | 2000/0 | 2000/30 | 2000/60 | 2000/90 | 2000/120 | 2000/150 |
| **1** | 1000/0 | 1000/30 | 1000/60 | 1000/90 | 1000/120 | 1000/150 |
| **0** | 0/0 | 0/30 | 0/60 | 0/90 | 0/120 | 0/150 |

Steps:
1) Prepare the sensors for measurement. Clean it with nanopure water and dry it.
2) Fill 500 ml Nanopure water in the first measurement cylinder
   ***Skip this step for all "0R-0W" experiments and go to step 4)***
   Measure the nanopure absorbance with the sensors.
   a) Insert the sensor until a volume on the measurement container reads about 900 ml.
   i) Start the stirrer again (1000 rpm)
   ii) Note the time and facts in the corresponding Logbook
   iii) Ensure that at least 5 measurements are recorded (7-10 minutes)
   iv) Switch stirrer off
   v) Remove the sensor
3) Put the narrow measurement cylinder with nanopure water aside and take the second cylinder from now on.
4) Measure the absorbance spectra with the sensor (see Figure 1 below)
   a) Prepare the UV sensor for measurement. Dry it.
   b) *For all experiments **except** 0R-0W:* Take 500 ml of the nitrate solution by means of a standardized flask and add it to the narrow measurement cylinder.
   *For the 0R-0W experiment:*
   Take 500ml of nanopure water by means of a standardized flask and add it to the narrow measurement cylinder.

c) Add a stirring magnet and place the container on a magnetic stirrer. Let it stir.
d) Switch stirrer off to avoid bubbles, insert the UV probe until the volume on the measurement container reads around 900 ml, start the stirrer again.
e) Check that no bubbles are caught under the sensor.
f) Note the time, start the measurements. Ensure at least 5 measurements (approximately 6-7 minutes in total) are recorded.
g) Lift the sensor from the measurement container.
h) Add 1ml of nitrite stock solution.
i) Insert the probe again (check for bubbles) and record at least 5 measurements.
j) Remove the sensor from the measurement container.
k) Add four more times 1ml of the nitrite stock solution and measure every time at least 5 spectra.
l) Remove the container from the stirrer and empty the solution into the sink, without losing the magnet.

5) Dry the probe. Tidy up the lab place or start new experimental series.
6) Make a PDF scan of the lab journal and update the digitized log file.

## Protocol A4: Preparation of urine samples

Katja Briner, Eawag, June 2015

Required products:

- Approximately 10L nitrified urine

Required hardware:

- 4x Imhoff cone
- 1000mL beaker
- 3x 2000mL container

Objective:

Let the biomass of around 10L of nitrified urine settle to prepare around 5L of urine for subsequent use in protocol A5.

Plan:

1. Collect and prepare urine sample (adapted from step 1 of protocol A.2 Angelika Hess).

a) Take 10L of nitrified urine from the tap on the nitrification reactor.
b) Separate at least 1 liter of clear liquid from 2L of sample as follows:
    i. Shake the 10L container to mix it.
    ii. Fill two 1L Imhoff cones with nitrified urine.
    iii. Let the biomass settle for 60 minutes.
    iv. Collect the settled biomass by removing at least 150mL from each cone by opening the bottom tap of the Imhoff cone. This liquid is wasted.
    v. Collect the remaining supernatant of both cones into a 2000mL container.
    vi. Repeat as necessary until at least 1 liter of bulk liquid is obtained.
c) Repeat step b) five times to treat the whole sample.

## Protocol A5: UV/Vis-measurements in urine spiked with nitrate and nitrite

Katja Briner, Eawag, July 2015

Required products:

- around 1.3L prepared urine (see protocol A4)
- Nanopure water
- $NaNO_3$ salt
- 2x Hach Lange Cuvette test LCK 340 (nitrate)
- Hach Lange Cuvette test LCK 342 (nitrite)

Required hardware:

- funnel
- 1000mL standardized flask
- 4x 100mL standardized flask
- pipette (0.2mL, 1mL, 10mL)
- stirrer und stirring magnet

Objective:

Conduct UV-Vis measurements in the same urine sample with different nitrate and nitrite concentrations to capture the background effects within the range of 2000 – 5000 mg/L NO3-N and 0 – 150 mg/L NO2-N.

Spike a part of the prepared urine sample (see protocol A4) with nitrate. Prepare a nitrite stock solution based on the spiked urine. Conduct UV-Vis measurements in the spiked urine while increasing the nitrite concentration stepwise by adding some nitrite stock solution.

Plan:

1. Take a sample of the prepared urine and measure nitrite and nitrate concentration with Hach Lange Cuvette tests (adapted from step 2 of protocol A.2 Angelika Hess).

    a) Filter the sample with a 0.4 µm glass microfiber filter to remove suspended particles.
    b) Nitrate: expected concentration around 2000 mg$NO_3$-N/L, dilution 1:200
        i. Pipette 5mL of the sample into a 100mL standardized flask, fill up with Nanopure water to 100mL, shake.
        ii. Pipette 10mL of the first dilution into a 100mL standardized flask, fill up with Nanopure water to 100mL, shake.
        iii. Pipette 0.2mL of the second dilution into the Hack Lange LCK 340 test tube, add 1mL "A", invert a few times until no more streaks can be seen and wait 15 minutes. Wipe the test tube and measure.
    c) Nitrite: expected concentration around 2 mg$NO_2$-N/L, no dilution
        i. Pipette 0.2 mL of the sample into the Hach Lange LCK 342 test tube, remove the foil from the dosicap, screw on the dosicap, shake and wait 10 minutes. Wipe the test tube and measure.

2. Spike the filtered urine with nitrate, according to the experiment row (adapted from protocol A.1 Christian Thürlimann).

   a) Weight Xg of $NaNO_3$ salt (see table below).
   b) Add around 500mL of prepared urine to the 1000mL standardized flask.
   c) Add the salt via funnel and rinse the funnel with urine.
   d) Add a stirring magnet and stir until all salt is dissolved.
   e) Remove the stirring magnet and rinse it with urine over the bottleneck of the flask.
   f) Fill up to 1000mL with prepared urine, shake well.
   g) Prepare dilution for Hach Lange Cuvette test:
      i. 1:200: Pipette 5mL of spiked urine into 100mL standardized flask, fill up with Nanopure water, shake. Pipette 10mL of the first dilution into 100mL standardized flask, fill up with Nanopure water, shake.
      ii. 1:500: Pipette 5mL of spiked urine into 100mL standardized flask, fill up with Nanopure water, shake. Pipette 4mL of the first dilution into 100mL standardized flask, fill up with Nanopure water, shake.
   h) Pipette 0.2mL of the diluted sample into the Hach Lange LCK 340 test tube, add 1mL "A", invert a few times until no more streaks can be seen and wait 15 minutes. Wipe the test tube and measure. If the result is insufficient, repeat from step 1.

| Target concentration $NO_3$-N | ≈2000 mg/L | ≈3000 mg/L | ≈4000 mg/L | ≈5000 mg/L |
|---|---|---|---|---|
| Concentration increase $NO_3$-N | 0 mg/L | 1000 mg/L | 2000 mg/L | 3000 mg/L |
| Required amount $NaNO_3$ (rounded) X | 0 g | 6.068 g | 12.136 g | 18.204 g |
| Dilution for LCK 340 | 1:200 | 1:200 | 1:200 | 1:500 |
| Expected concentration | result from step 2.b)iii. | 5 mg/L + result from step 2.b)iii. | 10 mg/L + result from step 2.b)iii. | 6 mg/L + ⅗ result from step 2.b)iii |

3. Use protocol A6 to prepare nitrite stock solution based on nitrate-spiked urine.

4. Use protocol A7 to measure the absorbance spectra in the urine spiked with nitrate after adding 0 to 5 mL of the urine based nitrite stock solution.

PROTOCOL A6: Preparation of nitrite stock solution based on urine spiked with nitrate (based on protocol A2 Christian Thürlimann)

Katja Briner, Eawag, July 2015

Required products:

- $NaNO_2$ powder
- urine spiked with $NO_3$, $NO_3$-N concentrations of around 2000, 3000, 4000, 5000 mg/l $NO_3$-N (depending on the experiment) See protocol A5!
- Nanopure water

Required hardware:

- Funnel
- 3x 100 ml flask
- Magnet stirrer with magnet
- Pipette (0.2, 0.1 ml)
- LCK 342 Nitrite Lange Tests

Plan:

Make 100ml nitrite stock solution based on urine with constant nitrate concentration.
Read the whole protocol before starting with step 1.

Steps:

1) Nitrite stock solution (goal: 505 ml urine sample with X mg $NO_3$-N/L and 150mg $NO_2$-N/l)
   a) Weigh 7.4625 g of the $NaNO_2$ powder
   b) Dissolve powder into urine spiked with nitrate
      i) Take approx. 50 ml of spiked urine (Protocol A5) and add it to the standardized 100 ml flask
      ii) Use a funnel to add the $NaNO_2$ powder to the container
      iii) Flush the funnel with a small amount of spiked urine to collect all the powder remaining on the surface of the funnel (make sure you don't fill the flask to a 100ml!!!!)
      iv) Add stirring magnet to the flask
      v) Place the flask onto a magnetic stirrer
      vi) Switch the magnetic stirrer on and let it mix until all powder is dissolved
      vii) Switch the magnetic stirrer off
      viii) Remove the magnet and rinse it with spiked urine in the flasks neck
      ix) Fill the container with spiked urine until exactly 100 ml is reached
      x) Close the flask with a stopper, shake it slowly to mix the added urine with the remaining solution.
   c) Take a sample to measure the $NO_2$-N concentration with a Hach-Lange cuvette test
   d) Measure the $NO_2$-N sample with a Hach-Lange cuvette test (LCK 342)
      i) 1:5000 (1 ml stock solution into a 100 ml flask (shake the flask), from this solution 2 ml into 100 ml flask (shake the flask))
      ii) Carefully remove the foil from the zip
      iii) Remove the cap without turning it upside down
      iv) Pipette 0.2 ml of the diluted sample
      v) Immediately screw the cap back, fluting at the top.
      vi) Shake until all compounds are dissolved
      vii) Wait 10 minutes
      viii) Invert some times more, clean the cuvette
      ix) Measure

Evaluate the quality of the solution (Expected concentration: 3.03 mgNO2-N/L, effect of nitrite in urine negligible). Repeat steps a-g if the obtained quality is insufficient

Calculations:

Nitrite:
Desired concentration: 150 mg $NO_2$-N/L
Amount of $NaNO_2$ for 100 ml stock solution:

$$M_{NaNO2} = \frac{150\frac{mgN}{L} \cdot 505\,mL}{5mL} \cdot \frac{0.1L}{100mL} \cdot \frac{1gN}{1000mgN} \cdot \frac{68.995\frac{gNaNO2}{mol}}{14.007\frac{gN}{mol}} = 7.4625\frac{gNaNO2}{100ml}$$

Cross interference with nitrate in LCK 342 Lange-test is not existent up to a concentration of 2000mg/l NO3-N! Which is not the case for this protocol as we dilute the solution for the Lange Test by 1:5000.

PROTOCOL A7: Testing simultaneous nitrate and nitrite estimation with UV-Vis sensor in urine (adapted from protocol A3 Christian Thürlimann)

Katja Briner, Eawag, July 2015

Required products:

- Stock solution Nitrite (goal: 150 mgN/L, 15.14997gN/L solution ) Protocol A6
- Urine spiked with nitrate (X mgN /L) Protocol A5

Required hardware:

- narrow measurement cylinder (1000 ml)
- standardized 500 ml flask
- Magnet stirrer with magnet
- Pipette (1 ml)
- s::can Sensor with 5mm gap
- Memory Stick

Plan:

Test UV-Vis sensor with a constant urine background conditions for estimating nitrite and nitrate.

Experimental plan:

2-5 nitrate concentration 2000-5000mg$NO_3$-N/L

R-W nitrite concentration 0-150 mg$NO_2$-N/L

Example. Experimental row 3R-3W urine with 3000mgNO3-N/L und 0 to 150mgNO2-N/L

| Nitrat mg/l / Nitrite mg/l | **R** | **S** | **T** | **U** | **V** | **W** |
|---|---|---|---|---|---|---|
| **5** | 5000/0 | 5000/30 | 5000/60 | 5000/90 | 5000/120 | 5000/150 |
| **4** | 4000/0 | 4000/30 | 4000/60 | 4000/90 | 4000/120 | 4000/150 |
| **3** | 3000/0 | 3000/30 | 3000/60 | 3000/90 | 3000/120 | 3000/150 |
| **2** | 2000/0 | 2000/30 | 2000/60 | 2000/90 | 2000/120 | 2000/150 |

Steps:

1) Prepare the sensor for measurement. Clean it with nanopure water and dry it.
2) Take 500 ml of the urine spiked with nitrate solution by means of a standardized flask and add it to the narrow measurement cylinder.
3) Add a stirring magnet and place the container on a magnetic stirrer. Let it stir.
4) Switch stirrer off to avoid bubbles, insert the UV probe until the volume on the measurement container reads around 830 ml, start the stirrer again.
5) Check that no bubbles are caught under the sensor.
6) Note the time, start the measurements. Ensure at least 5 measurements (approximately 6-7 minutes in total) are recorded.
7) Lift the sensor out of the measurement container but keep it above the container to collect any dripping solution.
8) Add 1ml of nitrite stock solution, let it stir for 2min.
9) Insert the probe again (check for bubbles) and record at least 5 measurements.
10) Add four more times 1ml of the nitrite stock solution and measure every time at least 5 spectra.
11) Remove the sensor from the measurement container, rinse it with nanopure water and dry it.
12) Remove the container from the stirrer and empty the solution into the sink, without losing the magnet.

## Protocol A8: Preparation of nitrate and nitrite stock solutions for UV-Vis-measurements in urine samples with changing background

Katja Briner, Eawag, August 2015

Required products:

- Nanopure water
- $NaNO_3$ salt
- $NaNO_2$ salt
- Hach Lange Cuvette test LCK 340 (nitrate)
- Hach Lange Cuvette test LCK 342 (nitrite)

Required hardware:

- 36x 100mL standardized flask
- 12x 100mL bottle
- pipette (0.2mL, 1mL)
- stirrer und stirring magnet

Objectives:

Prepare nitrate and nitrite stock solutions to increase the nitrate concentration in the prepared urine stepwise by 500 - 3000 mg/L $NO_3$-N and the nitrite concentration by 15 - 150 mg/L $NO_2$-N. The total volume of sample after addition of the stock solutions is 506 mL (500 mL prepared urine, 1 mL nitrite stock solution, 5 mL nitrate stock solution).

Plan:

1. Prepare nitrate stock solution according to table below, goal: initial concentration + X mg/L $NO_3$-N in 522 mL sample.
    a) Weigh Xs $NaNO_3$ salt.
    b) Fill approximately 50mL Nanopure water into a 100mL standardized flask.
    c) Add the salt via funnel and rinse the funnel.
    d) Add a stirring magnet and stir until all salt is dissolved.
    e) Remove the stirring magnet and rinse it with Nanopure water over the bottleneck of the flask.
    f) Fill up to 100mL with Nanopure water, shake well.
    g) Prepare dilution for Hach Lange Cuvette test 340 (according to plan below):
        i. 1:1000: Pipette 5mL of the stock solution into a 100mL standardized flask, fill up with Nanopure water, shake. Pipette 2mL of the first dilution into a 100mL standardized flask, fill up with Nanopure water, shake.
        ii. 1:5000: Pipette 1mL of the stock solution into a 100mL standardized flask, fill up with Nanopure water, shake. Pipette 2mL of the first dilution into a 100mL standardized flask, fill up with Nanopure water, shake.

h) Pipette 0.2mL of the diluted sample into the Hach Lange LCK 340 test tube, add 1mL "A", invert a few times until no more streaks can be seen and wait 15 minutes. Wipe the test tube and measure. The expected result is x mg/L $NO_3$-N. If the result is insufficient, repeat from step 1.a).

2. Prepare nitrite stock solutions according to the table below, goal: initial concentration + Y mg/L $NO_2$-N in 522mL sample.

   a) Weigh Ys $NaNO_2$ salt.
   b) Fill approximately 50mL Nanopure water into a 100mL standardized flask.
   c) Add the salt via funnel and rinse the funnel.
   d) Add a stirring magnet and stir until all salt is dissolved.
   e) Remove the stirring magnet and rinse it with Nanopure water over the bottleneck of the flask.
   f) Fill up to 100mL with Nanopure water, shake well.
   g) Prepare dilution for Hach Lange Cuvette test 342 (according to plan below):
      i. 1:5000: Pipette 1mL of the stock solution into a 100mL standardized flask, fill up with Nanopure water, shake. Pipette 2mL of the first dilution into a 100mL standardized flask, fill up with Nanopure water, shake.
      ii. 1:10'000: Pipette 1mL of the stock solution into a 100mL standardized flask, fill up with Nanopure water, shake. Pipette 1mL of the first dilution into a 100mL standardized flask, fill up with Nanopure water, shake.
   h) Pipette 0.2mL of the diluted sample into the Hach Lange LCK 342 test tube, remove the foil from the dosicap, screw on the dosicap, shake and wait 10 minutes. Wipe the test tube and measure. The expected result is y mg/L $NO_2$-N. If the result is insufficient, repeat from step 2.a).

3. Fill the stock solutions into a 100mL bottle each, label them and store them in the fridge.

Plan for NO3-N stock solutions:

| Target concentration mgNO3-N/L | X: Added concentration mgNO3-N/L | Xs: Salt needed for 100mL g $NaNO_3$ | Dilution for LCK 340 1: | x: Expected concentration mgNO3-N/L |
|---|---|---|---|---|
| around 2000 | 0 | 0 | 0 | 0 |
| around 2500 | 500 | 7.919 | 1000 | 13.05 |
| around 3000 | 1000 | 15.837 | 1000 | 26.10 |
| around 3500 | 1500 | 23.756 | 5000 | 7.83 |
| around 4000 | 2000 | 31.675 | 5000 | 10.44 |
| around 4500 | 2500 | 39.593 | 5000 | 13.05 |
| around 5000 | 3000 | 47.512 | 5000 | 15.66 |

Plan for NO2-N stock solutions:

| Target concentration mgNO2-N/L | Y: Added concentration mgNO2-N/L | Ys: Salt needed for 100mL g $NaNO_2$ | Dilution for LCK 342 1: | y: Expected concentration mgNO2-N/L |
|---|---|---|---|---|
| around 2 | 0 | 0 | 0 | 0 |
| around 15 | 15 | 1.928 | 5000 | 0.783 |
| around 30 | 30 | 3.857 | 5000 | 1.566 |
| around 60 | 60 | 7.714 | 10'000 | 1.566 |
| around 90 | 90 | 11.571 | 10'000 | 2.349 |
| around 120 | 120 | 15.427 | 10'000 | 3.132 |
| around 150 | 150 | 19.284 | 10'000 | 3.915 |

Calculations:

$$M_{NaNO3} = \frac{X\frac{mgN}{L} \cdot 522\,mL}{20mL} \cdot \frac{0.1L}{100mL} \cdot \frac{1gN}{1000mgN} \cdot \frac{84.994\frac{gNaNO3}{mol}}{14.007\frac{gN}{mol}} = Xs\ \frac{gNaNO3}{100ml}$$

$$M_{NaNO2} = \frac{Y\frac{mgN}{L} \cdot 522\,mL}{2mL} \cdot \frac{0.1L}{100mL} \cdot \frac{1gN}{1000mgN} \cdot \frac{68.995\frac{gNaNO2}{mol}}{14.007\frac{gN}{mol}} = Ys\ \frac{gNaNO2}{100ml}$$

## Protocol A9: UV-Vis-measurements in urine samples to assess the influence of changing background

Katja Briner, Eawag, August 2015

Required products:

- around 2L nitrified urine
- Nanopure water
- Hach Lange Cuvette test LCK 514 (COD)
- Hach Lange Cuvette test LCK 340 (nitrate)
- Hach Lange Cuvette test LCK 342 (nitrite)

Required hardware:

- 1x 2L bottle
- 2x Imhoff cone
- 2x 1000ml beaker
- 1x 100mL standardized flask
- pipette (0.2mL, 1mL, 10mL)
- stirrer und stirring magnet
- 2x 1000mL measuring cylinder
- 1x 500mL standardizes flask

Objectives:

Take a sample of nitrified urine three times a week and carry out the following UV-Vis measurements. Compare the results of different samples to assess the influence of changing urine background composition on the UV-Vis measurement of nitrite and nitrate.

Plan:

1. Collect and prepare urine sample (adapted from step 1 of protocol A.2 Angelika Hess).

    i) Take 2L of treated urine from the tap on the nitrification reactor according to the measurement schedule below.
    j) Separate at least 1.2 liter of clear liquid as follows:
        i. Fill two 1L Imhoff cone with nitrified urine.
        ii. Let the biomass settle for 60 minutes.
        iii. Collect the settled biomass by removing at least 150mL from each cone by opening the bottom tap of the Imhoff cone. This liquid is wasted.
        iv. Collect the remaining supernatant of the cone into the 1000mL containers.
        v. Repeat as necessary until at least 1.2 liter of bulk liquid is obtained.

2. Take a sample of the prepared urine and measure COD, nitrite and nitrate concentration with Hach Lange Cuvette tests (adapted from step 2 of protocol A.2 Angelika Hess).

    d) Filter the sample with a 0.4 µm glass microfiber filter to remove suspended particles.

e) COD: expected concentration around 250 mg COD/L, no dilution
   i. Pipette 2mL of the filtered sample into the Hach Lange LCK 514 test tube, invert a couple of times and put into the oven. Bake 2h at 148° C (according to the instructions in the LCK 514-package) . Invert twice and wait until it has cooled down. Wipe the test tube and measure.
f) Nitrate: expected concentration around 2000 mg$NO_3$-N/L, dilution 1:100
   i. Pipette 1mL of the sample into a 100mL standardized flask, fill up with Nanopure water to 100mL, shake.
   ii. Pipette 0.2mL of the dilution into the Hack Lange LCK 340 test tube, add 1mL "A", invert a few times until no more streaks can be seen and wait 15 minutes. Wipe the test tube and measure.
g) Nitrite: expected concentration around 2 mg$NO_2$-N/L, no dilution
   i. Pipette 0.2 mL of the sample into the Hach Lange LCK 342 test tube, remove the foil from the dosicap, screw on the dosicap, shake and wait 10 minutes. Wipe the test tube and measure.

3. Use protocol A8 to prepare nitrite and nitrate stock solutions.

4. Measure absorbance spectra according to plan (see below)

a) Prepare the sensor for measurement. Clean it with ethanol, acid, base and nanopure water and dry it.
b) Take 500mL of the urine sample by means of a standardized flask and add it to the narrow measurement cylinder.
c) Add a stirring magnet and place the container on a magnetic stirrer. Let it stir.
d) Switch off the stirrer to avoid bubbles, insert the UV probe until the volume on the measurement container reads around 830 mL, start the stirrer again.
e) Check that no bubbles are caught under the sensor.
f) Note the time, start the measurement. Ensure that at least 5 measurement (around 6-7 minutes in total) are recorded.
g) Lift the sensor out of the measurement container but keep it above the container to collect any dripping solution.
h) Add 2mL of the required nitrite stock solution and 20mL of the required nitrate stock solution (according to plan below, measurement 1), let it stir for 2min.
i) Insert the probe again (check for bubbles) and record at least 5 measurements.
j) Remove the sensor from the measurement container, rinse it with nanopure water and dry it.
k) Remove the container from the stirrer and empty the solution into the sink, without losing the magnet.
l) Repeat steps a) to k) with new 500mL of prepared urine sample, adding stock solutions according to measurement 2 (see plan below)
m) Clean the sensor with ethanol, acid, base and nanopure water and dry it.

5. Measurement plan:

| measuring week | sample date | concentrations for measurement 1 | | concentrations for measurement 2 | |
|---|---|---|---|---|---|
| | | NO2 mgN/L | NO3 mgN/L | NO2 mgN/L | NO3 mgN/L |
| 1 | Friday, 07.08.2015 | +60 | +1000 | +90 | +2000 |
| | Monday, 10.08.2015 | +120 | +500 | +30 | +2500 |
| | Wednesday, 12.08.2015 | +15 | +3000 | +150 | +1500 |
| 2 | Friday, 21.08.2015 | +90 | +1500 | +120 | +2000 |
| | Monday, 24.08.2015 | +60 | +500 | +15 | +2500 |
| | Wednesday, 26.08.2015 | +150 | +1000 | +30 | +3000 |
| 3 | Friday, 14.08.2015 | +60 | +2000 | +30 | +500 |
| | Monday, 17.08.2015 | +120 | +1500 | +90 | +2500 |
| | Wednesday, 19.08.2015 | +150 | +3000 | +15 | +1000 |

Latin Hypercube for measurements:

| concentration of NO2 [mgN/L] | concentration of NO3 [mgN/L] | | | | | |
|---|---|---|---|---|---|---|
| | +500 | +1000 | +1500 | +2000 | +2500 | +3000 |
| +15 | 6 | 3 | 4 | 5 | 2 | 1 |
| +30 | 3 | 5 | 6 | 4 | 1 | 2 |
| +60 | 2 | 1 | 5 | 3 | 6 | 4 |
| +90 | 5 | 4 | 2 | 1 | 3 | 6 |
| +120 | 1 | 6 | 3 | 2 | 4 | 5 |
| +150 | 4 | 2 | 1 | 6 | 5 | 3 |

The numbers indicate the measuring week in which the measurement point is covered. Each week three samples are taken and used cover two points each. The whole grid could be covered by measuring for 6 weeks. As this measuring campaign only lasts for three weeks, additional three weeks of measurements are needed to complete the grid.

# B Additional results

In this section, additional results are presented as referenced in chapter 3 on *Results*.

## B.1 Additional spectra

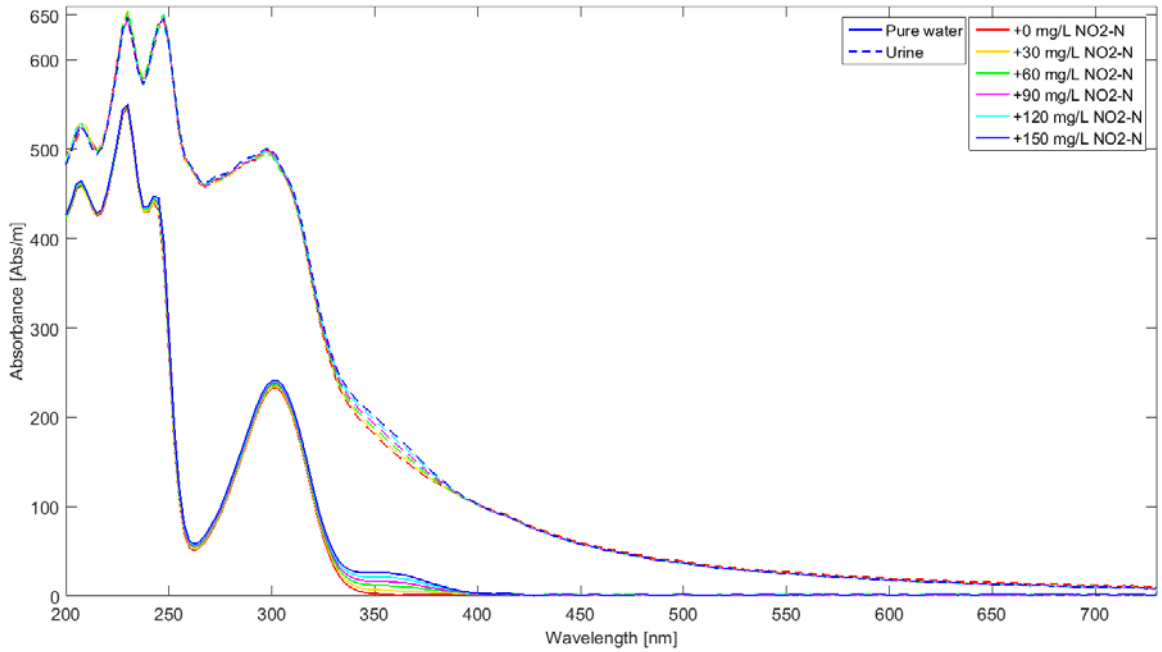


Figure A 1: Full spectra recorded in pure water and urine at a constant nitrate concentration and varying nitrite concentrations. Nitrate concentration: pure water: 5000 mg$NO_3$-N/L, urine: background plus 3000 mg$NO_3$-N/L (background urine: 1250 mg$NO_3$-N/L). Nitrite concentrations: pure water: 0 to 150 mg$NO_2$-N/L, urine: background plus 0 to 150 mg$NO_2$-N/L (background urine: 12.4 mg$NO_2$-N/L).

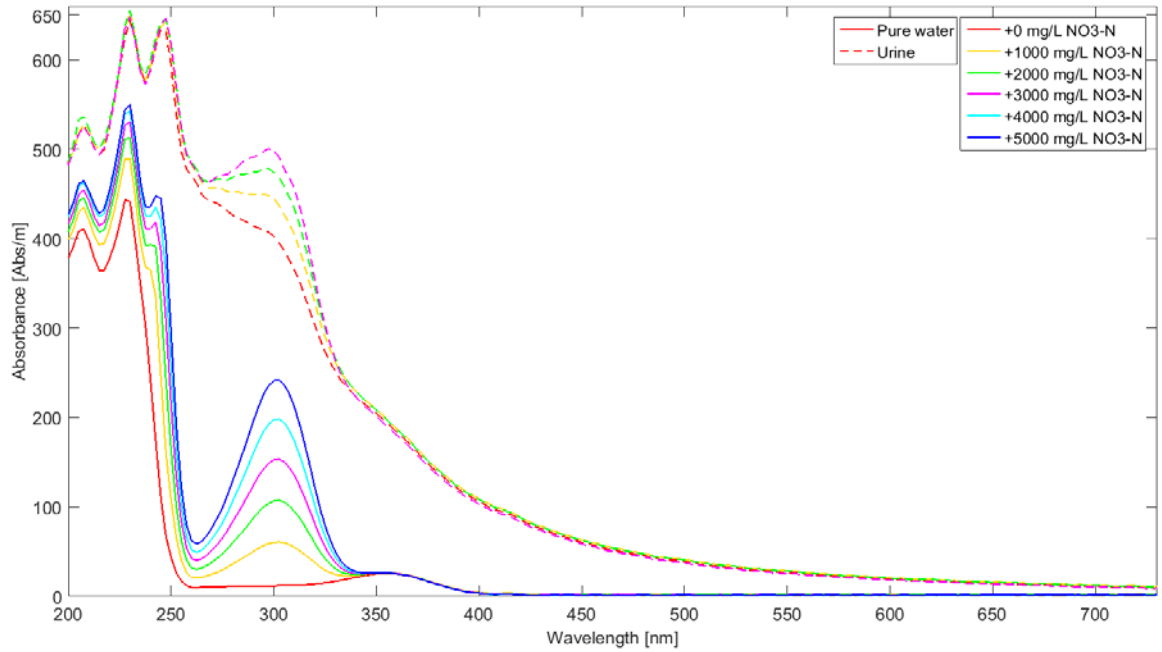


Figure A 2: Full spectra recorded in pure water and urine at a constant nitrite concentration and varying nitrate concentrations. Nitrite concentration: pure water: 150 mg$NO_2$-N/L, urine: background plus 150 mg$NO_2$-N/L (background urine: 12.4 mg$NO_2$-N/L). Nitrate concentrations: pure water: 0 to 5000 mg$NO_3$-N/L, urine: background plus 0 to 3000 mg$NO_3$-N/L (background urine: 1250 mg$NO_3$-N/L).

## B.2 Samples in urine with varying background

Table A 1: Samples with varying urine composition: sample number, sampling date, background concentrations of nitrite, nitrate and COD and added amount of $NO_2$ and $NO_3$ in the two halves of each sample.

| Sample N° | Sampling date | background $NO_2$ concentration [mgN/L] | background $NO_3$ concentration [mgN/L] | background COD [mg/L] | added amount of $NO_2$ [mgN/L] | added amount of $NO_3$ [mgN/L] |
|---|---|---|---|---|---|---|
| 1.1 | 07.08.2015 | 1.31 | 2690 | 461 | 60<br>90 | 1000<br>2000 |
| 1.2 | 10.08.2015 | 1.30 | 2660 | 542 | 120<br>30 | 500<br>2500 |
| 1.3 | 12.08.2015 | 1.22 | 2630 | 457 | 15<br>150 | 3000<br>1500 |
| 2.1 | 14.08.2015 | 1.21 | 2660 | 456 | 90<br>120 | 1500<br>2000 |
| 2.2 | 17.08.2015 | 1.07 | 2590 | 444 | 60<br>15 | 500<br>2500 |
| 2.3 | 19.08.2015 | 0.99 | 2530 | 432 | 150<br>30 | 1000<br>3000 |
| 3.1 | 21.08.2015 | 0.85 | 2560 | 429 | 60<br>30 | 2000<br>500 |
| 3.2 | 24.08.2015 | 0.77 | 2540 | 427 | 120<br>90 | 1500<br>2500 |
| 3.3 | 04.08.2015 | 1.24 | 2690 | 454 | 150<br>15 | 3000<br>1000 |

## B.3 Bubble formation on the sensor

In the cold urine sample used in the urine experiment with constant background, bubble formation on the whole sensor was observed at the start of the measurement. Analysing the data, it was stated that the measured absorbance increased during bubble formation by around 20 Abs/m within 10 minutes. To investigate the contribution of the temperature to the extent of bubble formation, an absorbance measurement was carried out in cold pure water. A similar effect could be observed, however the increase of the absorbance was much lower. Gasing out of $CO_2$ as the temperature rises might be the cause of the strong bubble formation in the urine sample. However, this has not been pursued. The spectra in Figure A 3 show the increase in absorbance in cold urine due to bubble formation.

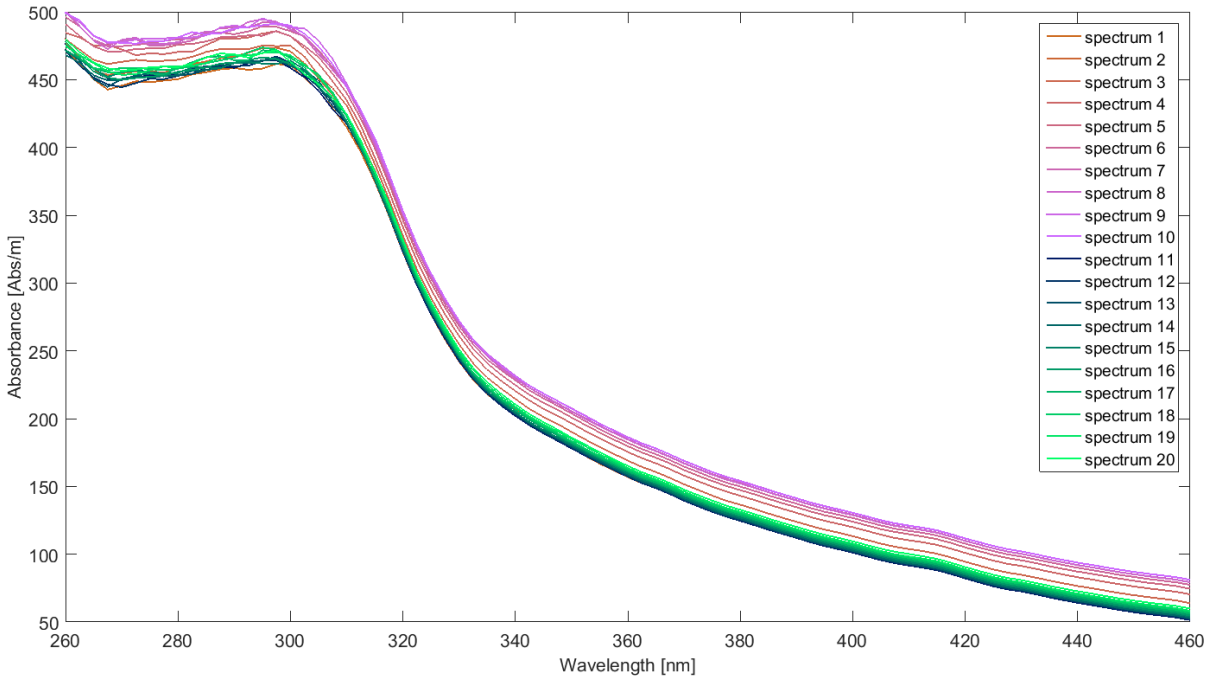


Figure A 3: Increasing absorbance during 20 minutes due to bubble formation on the sensor in cold urine with a concentration of 3250 mg/L nitrate and 12.4 mgN/L background nitrite. After recording spectrum 10, the sensor was shaken to remove the bubbles.

This effect was observed while measuring at background nitrite and 3250 mgN/L nitrate (background plus 2000 mg$NO_3$-N/L) as well as at background nitrite and 4250 mgN/L nitrate (background plus 3000 mg$NO_3$-N/L). For the calibration and validation of the chemometric model, only the first two spectra recorded in each of those two measurements were used, as they were least affected by bubble formation. Two instead of one spectra were chosen to reduce random errors.

## B.4 Estimation accuracy for the experiment series with constant background

The following plots (Figure A 5 Figure A 8) show the estimation accuracy reached for nitrite and nitrate in pure water and in urine with a constant background using different calibration subsets. The dataset was divided in a chequerboard manner. In analogy to the following sketch (Figure A 4), all the measurements in a black cell belong to calibration subset “a” and all the measurement in a white cell to calibration subset “b”. The estimation accuracy is then determined in the remaining part of the dataset (validation subset).

Figure A 4: Visualization of the division of a dataset into subsets (chequerboard approach, only extract).

| | | | |
|---|---|---|---|
| $NO_2$ concentration 3 | | | |
| $NO_2$ concentration 2 | | | |
| $NO_2$ concentration 1 | | | |
| | $NO_3$ concentration 1 | $NO_3$ concentration 2 | $NO_3$ concentration 3 |

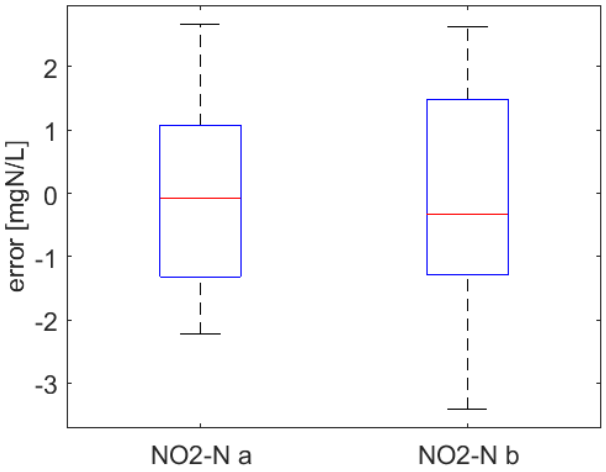


Figure A 5: Estimation accuracy of nitrite in pure water based on the two different calibration subsets a and b (for both boxes N=18).

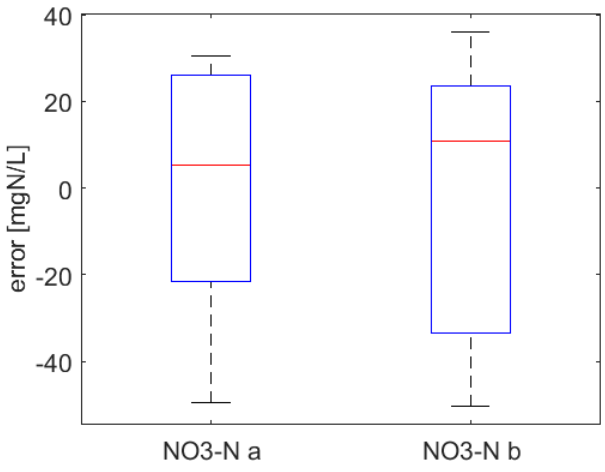


Figure A 6: Estimation accuracy of nitrate in pure water based on the two different calibration subsets a and b (for both boxes N=18).

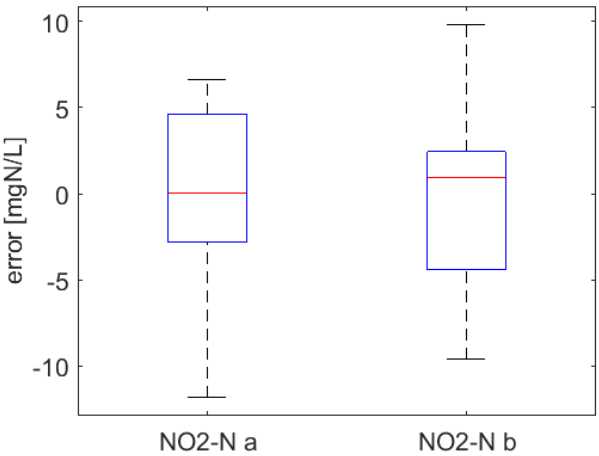


Figure A 7: Estimation accuracy of nitrite in urine with a constant background based on the two different calibration subsets a and b (for both boxes N=12).

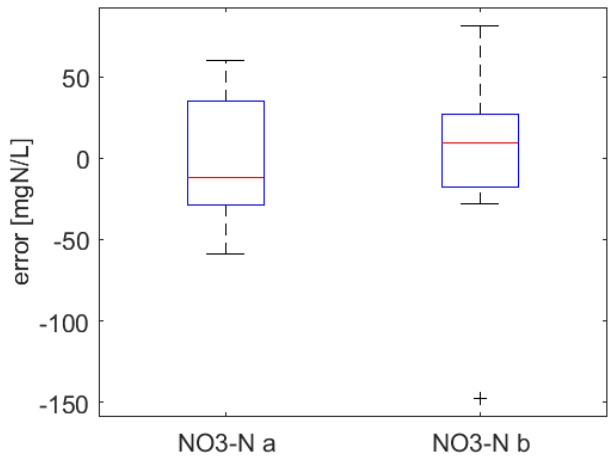


Figure A 8: Estimation accuracy of nitrate in urine with a constant background based on two different calibration subsets a and b (for both boxes N=12).

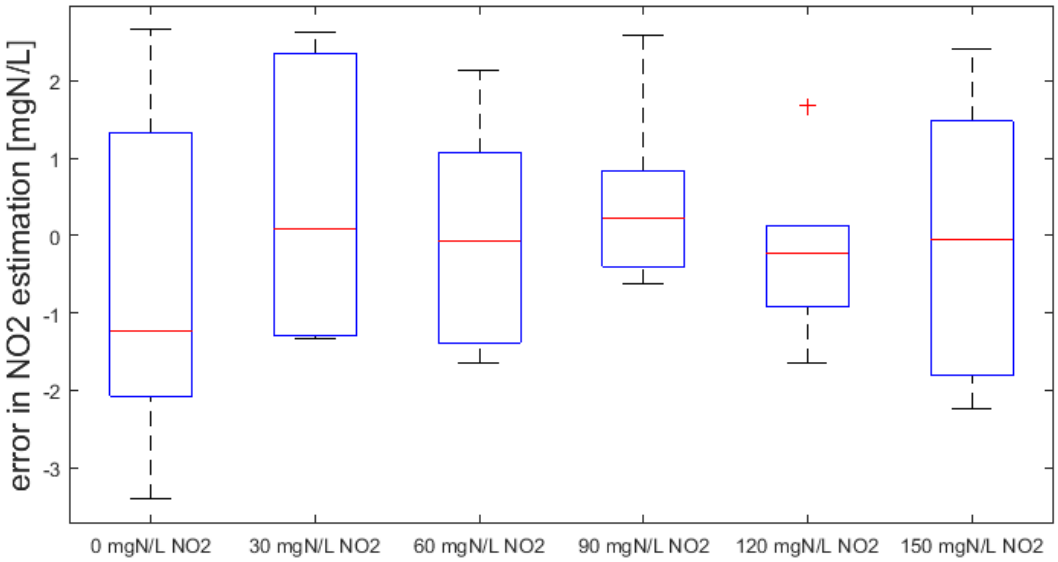


Figure A 9: Estimation accuracy of nitrite in pure water at different nitrite concentrations (for every box N=6).

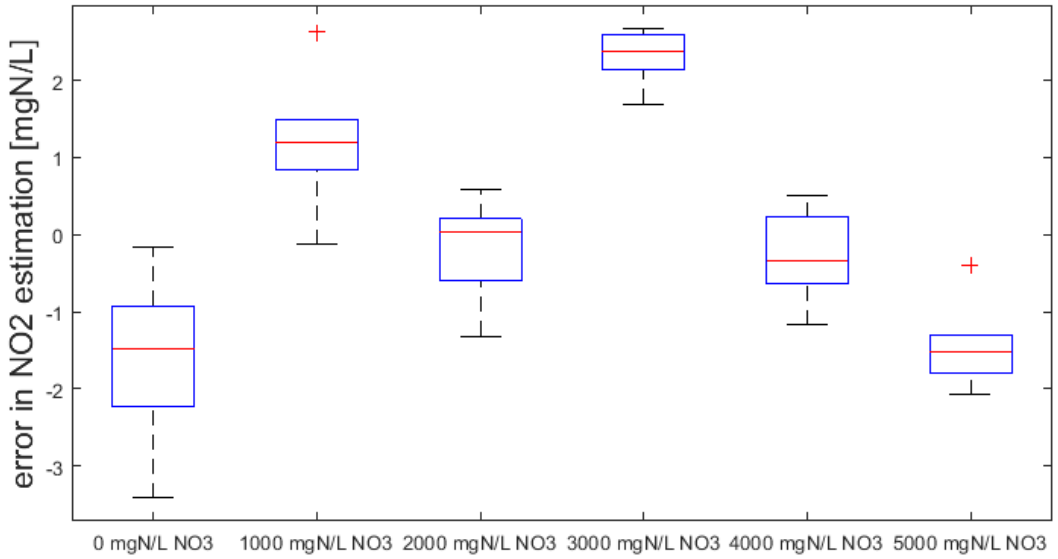


Figure A 10: Estimation accuracy of nitrite in pure water at different nitrate concentrations (for every box N=6).

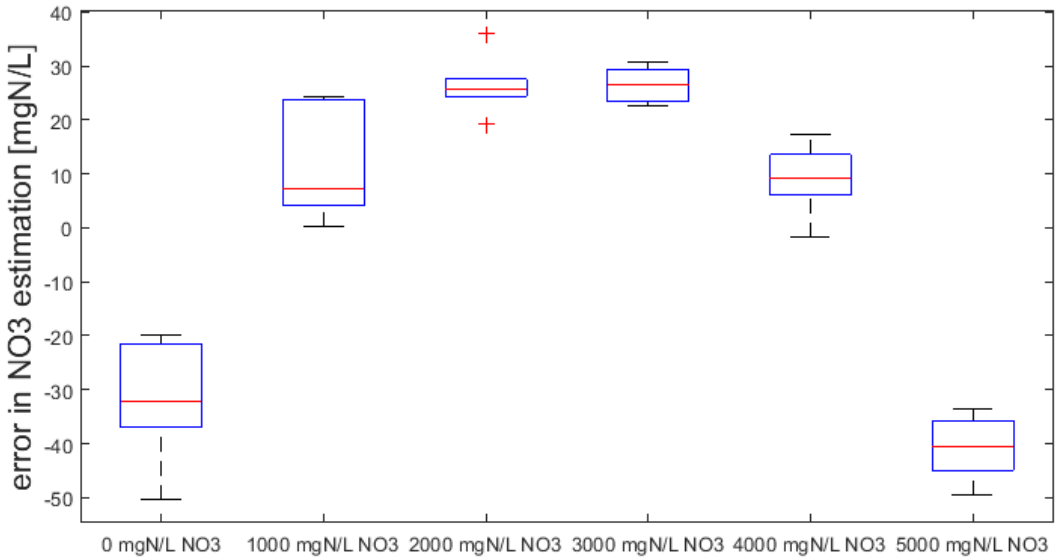


Figure A 11: Estimation accuracy of nitrate in pure water at different nitrate concentrations (for every box N=6).

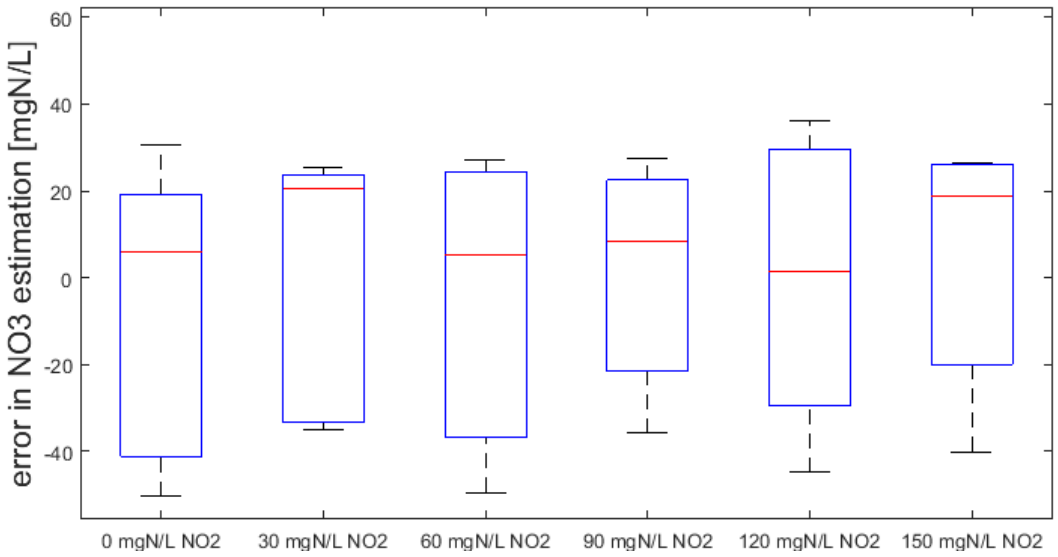


Figure A 12: Estimation accuracy of nitrate in pure water at different nitrite concentrations (for every box N=6).

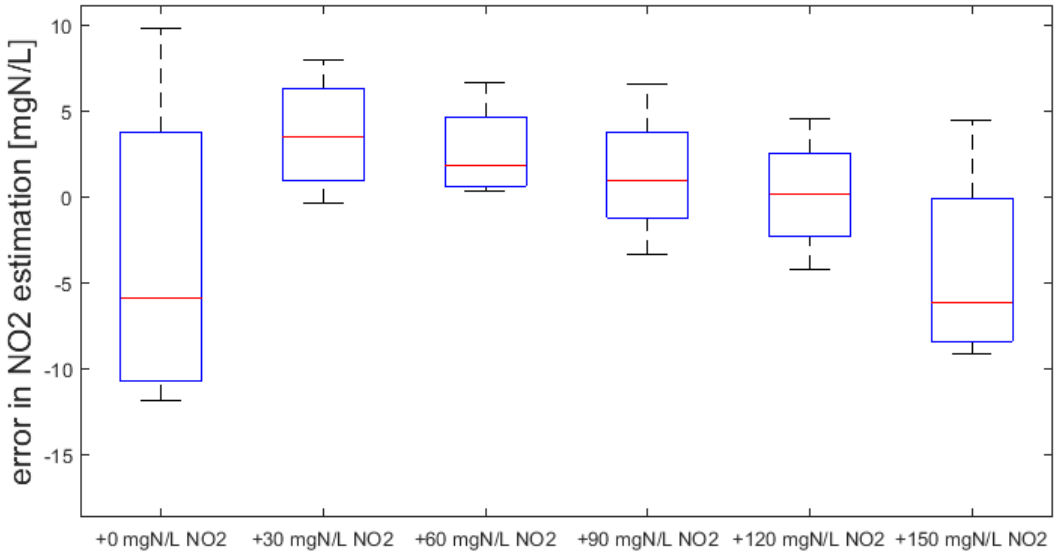


Figure A 13: Estimation accuracy of nitrite at different nitrite concentrations in urine with a constant background (background concentration of nitrite: 12.4 mgN/L) (for every box N=4).

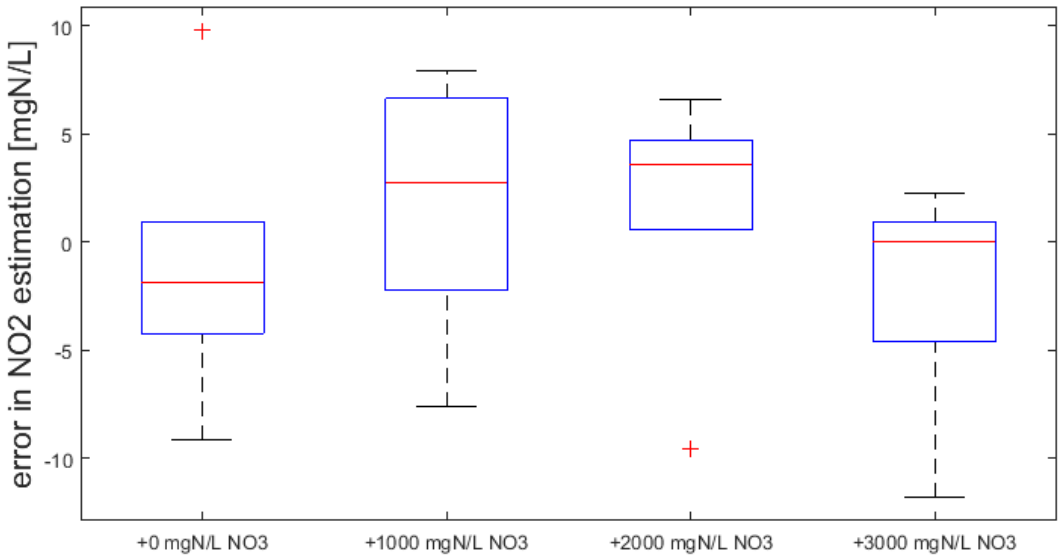


Figure A 14: Estimation accuracy of nitrite at different nitrate concentrations in urine with a constant background (background concentration of nitrite: 12.4 mgN/L, background concentration of nitrate: 1250 mgN/L) (for every box N=6).

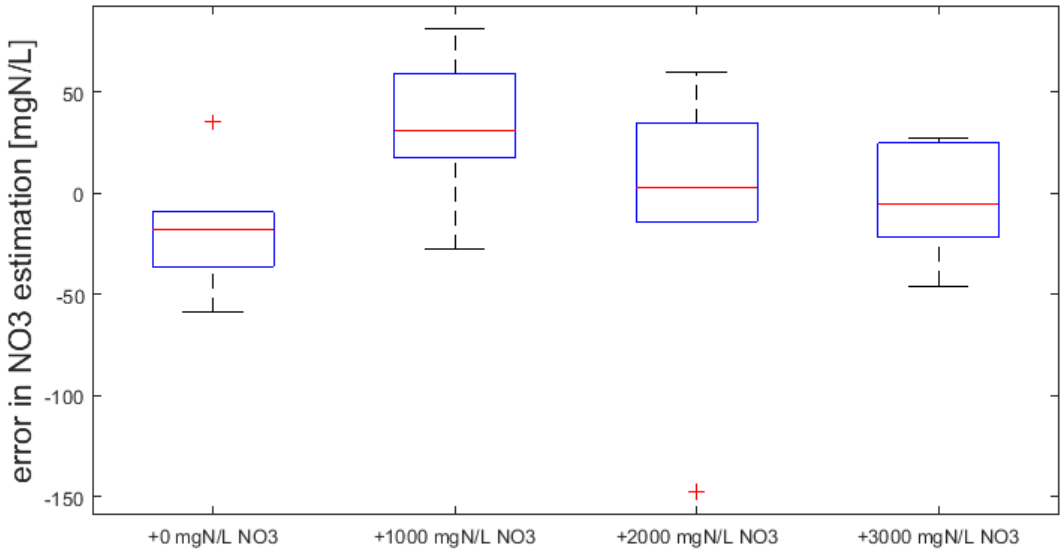


Figure A 15: Estimation accuracy of nitrate at different nitrate concentrations in urine with a constant background (background concentration of nitrate: 1250 mgN/L) (for every box N=6).

The concentration of nitrate for the measurement at +2000 mgN/L nitrate and +0 mgN/L nitrite in urine with a constant background is underestimated by 148 mgN/L, which can be seen as an outlier in Figure A 15. The spectra of this measurement were influenced by the bubble formation (see section B.3 on *Bubble formation on the sensor*), which is most probably the cause for the deviation of the estimation.

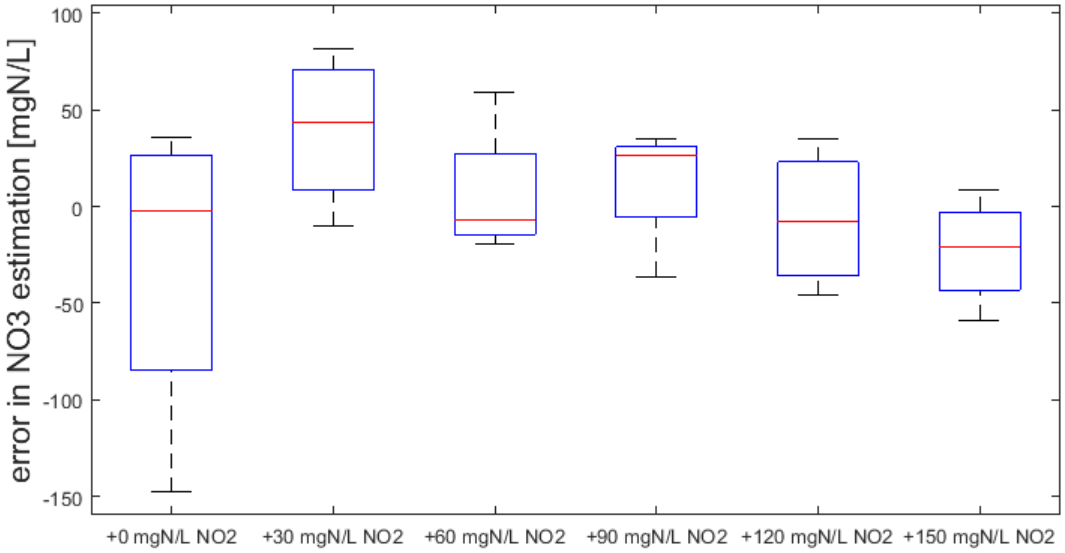


Figure A 16: Estimation accuracy of nitrate at different nitrite concentrations in urine with a constant background (background concentration of nitrite: 12.4 mgN/L, background concentration of nitrate: 1250 mgN/L) (for every box N=4).

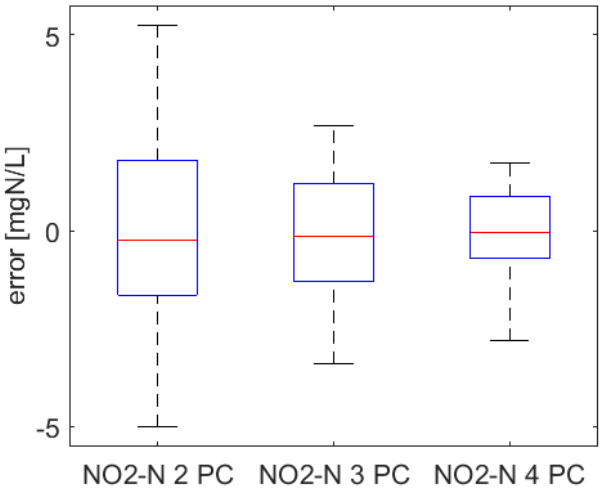


Figure A 17: Estimation accuracy for nitrite in pure water using 2, 3 or 4 principal components in the regression (N=36 for each box).

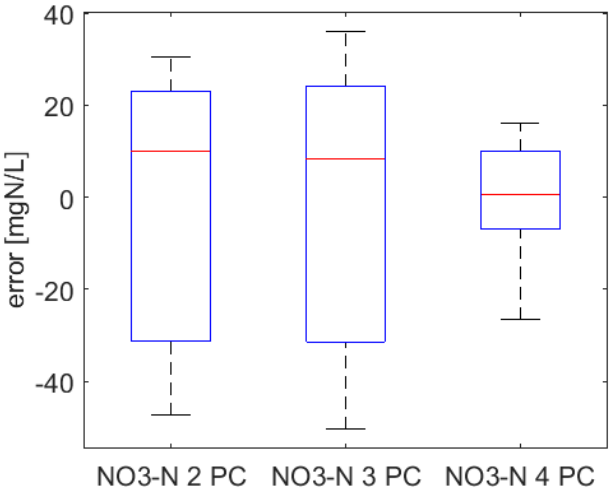


Figure A 18: Estimation accuracy for nitrate in pure water using 2, 3 or 4 principal components in the regression (N=36 for each box).

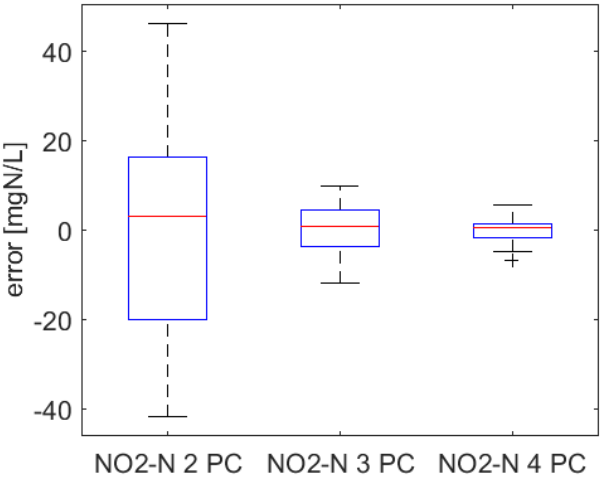


Figure A 19: Estimation accuracy for nitrite in urine with a constant background using 2, 3 or 4 principal components in the regression (N=24 for each box).

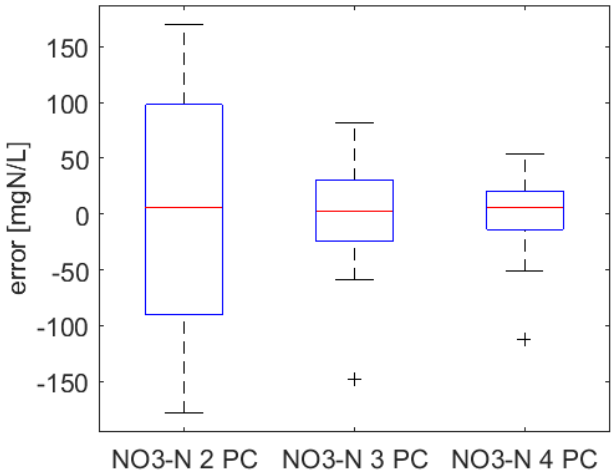


Figure A 20: Estimation accuracy for nitrate in urine with a constant background using 2, 3 or 4 principal components in the regression (N=24 for each box).

## B.5 Estimation accuracy for the experiment series in urine with variable background

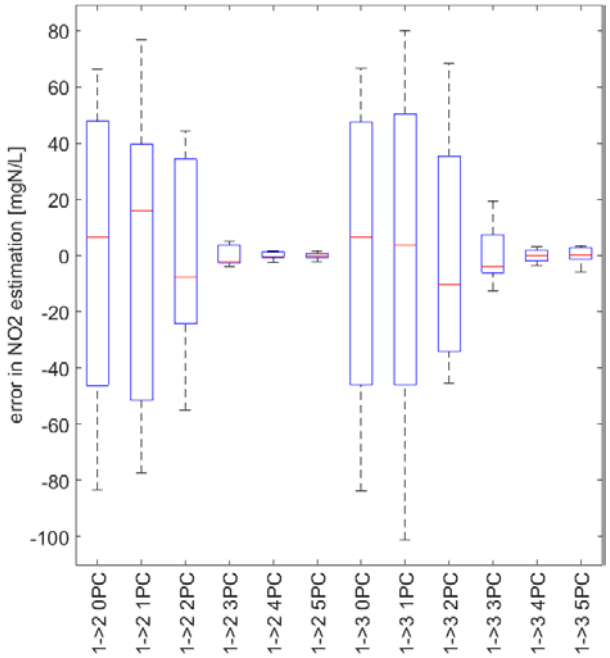


Figure A 21: Based on week 1: Estimation accuracy for the nitrite concentration using different numbers of principal components in regression, (1->2: calibration based on data from week 1, estimation for samples in week 2) (for each box N=7).

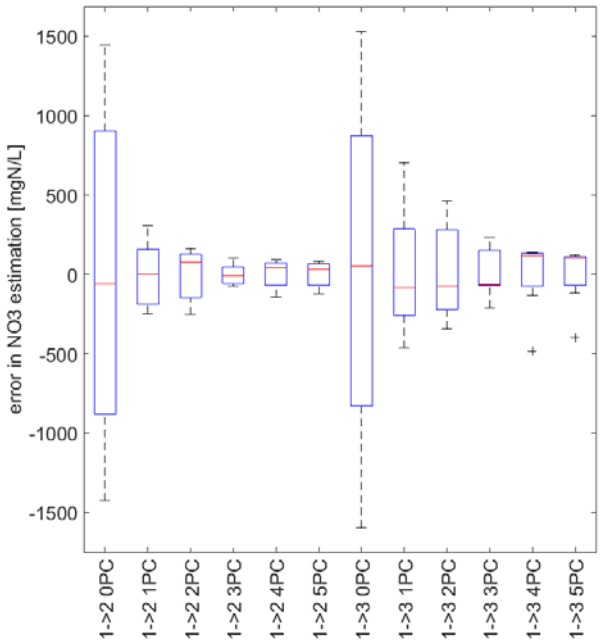


Figure A 22: Based on week 1: Estimation accuracy for the nitrate concentration using different numbers of principal components in regression, (1->2: calibration based on data from week 1, estimation for samples in week 2) (for each box N=7).

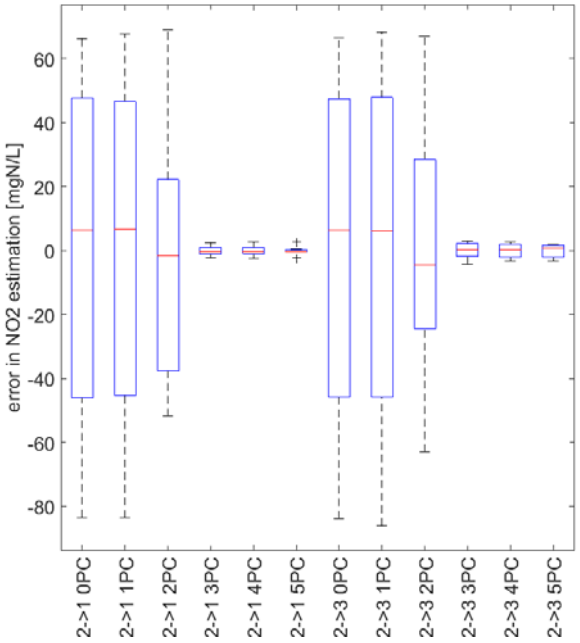


Figure A 23: Based on week 2: Estimation accuracy for the nitrite concentration using different numbers of principal components in regression, (2->1: calibration based on data from week 2, estimation for samples in week 1) (for each box N=7).

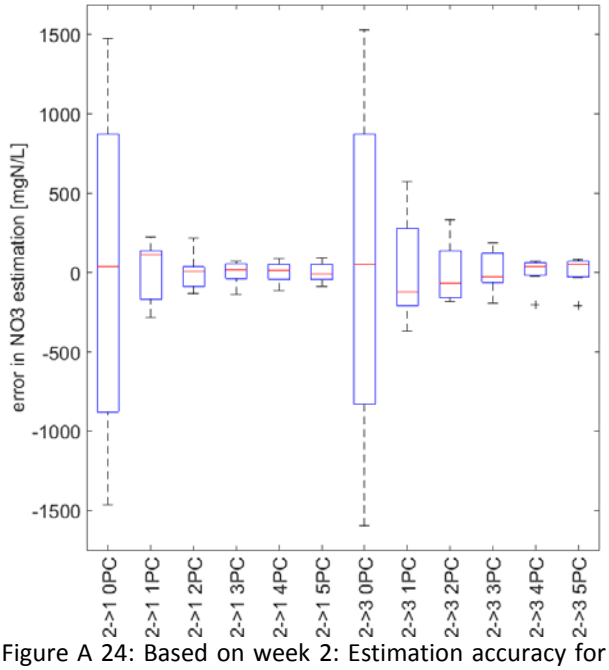


Figure A 24: Based on week 2: Estimation accuracy for the nitrate concentration using different numbers of principal components in regression, (2->1: calibration based on data from week 2, estimation for samples in week 1) (for each box N=7).

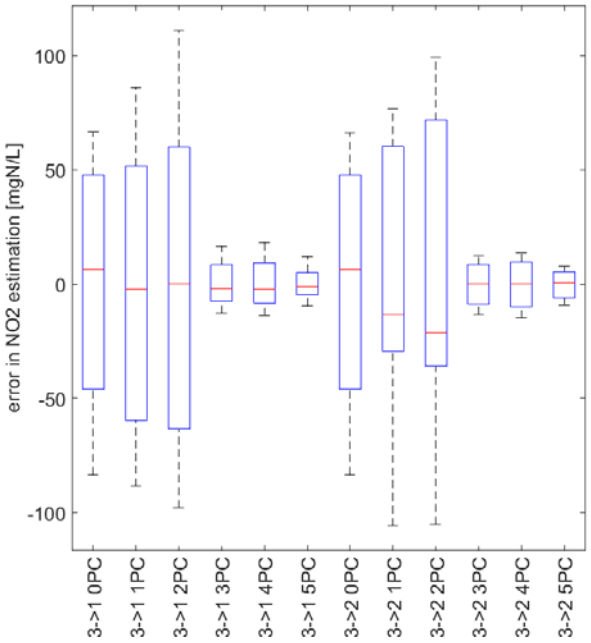


Figure A 25: Based on week 3: Estimation accuracy for the nitrite concentration using different numbers of principal components in regression, (3->1: calibration based on data from week 3, estimation for samples in week 1) (for each box N=7).

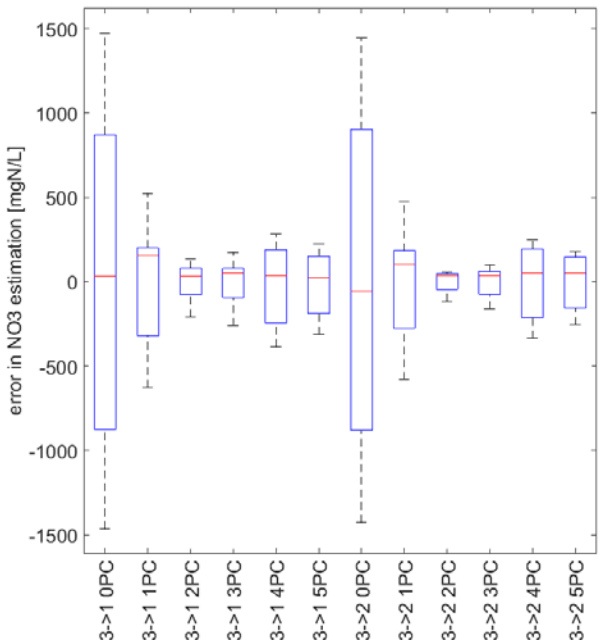


Figure A 26: Based on week 3: Estimation accuracy for the nitrate concentration using different numbers of principal components in regression, (3->1: calibration based on data from week 3, estimation for samples in week 1) (for each box N=7).