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Development and validation of a GCMS method for the quantitation of potential genotoxic impurities in pharmaceutical substances

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Abstract

The company LOBA Feinchemie GmbH is specialized in the production of naphazoline hydrochloride and naphazoline nitrate. Those salts are used as active pharmaceutical ingredients in nose sprays and eye drops. The used raw material 1-naphthylacetonitrile is commonly synthesized from the potential genotoxic impurity 1-chloromethylnaphthalene. In order to guarantee, that the amount of the potential genotoxic impurity is at or below the acceptable limit of 48 ppm in the product, a gas chromatography-mass spectrometry (GCMS) method was developed at TU Wien, which allows the absolute quantitation of 1-chloromethylnaphthalene in the raw material 1-naphthylacetonitrile. A five-point standard addition procedure was used for the quantitation. The analytes of interest cover a broad range of polarity, which is why a non-polar stationary phase had to be combined with a polar solvent. Since 1-chloromethylnaphthalene reacts in the presence of methanol and ethanol, isopropyl alcohol was chosen as the solvent. In order to compare different individual sample measurements and to compensate for all possible volume errors and variations in the function of the instrument, naphthalene-d_8 was implemented as internal standard. The used raw material 1-naphthylacetonitrile contains several non-volatile impurities, which create active sites in the inlet liner and the beginning of the column. Those active sites must be saturated prior to each standard addition procedure to establish comparable measurement conditions. The developed method was transferred to and validated at the receiving laboratory. A quantitation limit of 0.97 ppm for 1-chloromethylnaphthalene was determined and the linearity of the standard addition curve was shown in the range of 2 - 10 ppm, with a coefficient of determination (R^2) ≥ 0.9939 (Acceptance criteria: $R^2 \geq 0.98$). The obtained relative standard deviation (RSD) of the repeatability experiments was 6.13 % (Acceptance criteria: RSD ≤ 20 %) and recovery rates between 92.5 % - 100.5 % were determined in the course of the accuracy experiments (Acceptance criteria: recovery rate between 80 % - 120 %). Specificity and robustness of the method were also successfully shown in the course of the validation.

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List of abbreviations

CID	Collision Induced Fragmentation
EC	European Comission
ET	Elution temperature
GC	Gas chromatography
GCMS	Gas chromatograpy-mass spectrometry
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IPA	Isopropyl alcohol
H.E.T.P.	high equivalent of the theoretical plates
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
min	Minutes
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MRM	Multiple reaction monitoring
m/z	Mass to charge ratio
n.a.	Not applicable
NIST	National Institute of Standards and Technology
P/N	Part number
ppm	Parts per million
RSD	Relative standard deviation
RT	Retention time
SST	System suitability test

List of symbols

a	y-intercept of the standard addition curve
α	Significance level (type I error)
$AB_{(g)}$	Gas phase analyte
$[AB]^{+\bullet}_{(g)}$	Radical cation of the molecular species
b	Slope of the standard addition curve
e^-	electron
d_i	Inner diameter of the inlet liner [mm]
ρ	Solvent density [g/mL at 20°C]
1/k	relative result uncertainty for the determination of the quantitation limit
L	Length of the inlet liner [mm]
M	Molecular weight [g/moL]
m	Repeats of the calibration
n	Total number of measurements within a calibration
p_{inlet}	Inlet pressure [Pa] (sum of head pressure and atmospheric pressure)
Q_x	Sum of squared differences of the individual calibration levels from the mean calibration level
R	Gas constant [(Pa*mL)/(moL*K)]
s_{X0}	process standard deviation
$s_{y,x}$	Standard error of the regression
T_{inj}	Inlet temperature/ Injection temperature [K]
$t_{n-2,\alpha}$	Value of t-distribution for given significance level (one-tailed)
$t_{n-2,\alpha/2}$	Value of t-distribution for given significance level (two-tailed)
V_{exp}	Expansion volume [µL]
V_{inj}	Injection volume [µL]

V_{Liner}	Volume of the inlet liner [µL]
$V_{Liner,eff}$	Actual available volume of the inlet liner [µL]
$\overline{x}$	arithmetic mean of the concentrations of all calibration samples
x_{BG}	Limit of quantitation
x_{NG}	Limit of Detection
y_k	critical value for the measure

1 Introduction

1.1 Motivation

The chemical synthesis of pharmaceutical substances involves the use of different materials such as reagents, catalysts, solvents, and processing aids. As a consequence, those substances remain, to a greater or lesser extent, as impurities in the final product [1].

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) provides instructions for the qualification and control of those impurities. Mutagenic impurities in drug substances and associated drug products are assessed and controlled according to the multidisciplinary guideline ICH M7(R1) [1].

The company LOBA Feinchemie GmbH is focused on the production of fine chemicals and active pharmaceutical ingredients. One of the products of LOBA Feinchemie GmbH is naphazoline (Figure 1, **c**) or in more particular its salts naphazoline hydrochloride and naphazoline nitrate. Both of them are used as active pharmaceutical ingredients in nose sprays and eye drops. The raw material used for the production of naphazoline at LOBA Feinchemie GmbH is 1-naphthylacetonitrile (Figure 1, **b**), purchased from Changzhou United Chemical Co., LTD. However, 1-naphthylacetonitrile is commonly produced by using 1-chloromethylnaphthalene (Figure 1, **a**) as a starting material.

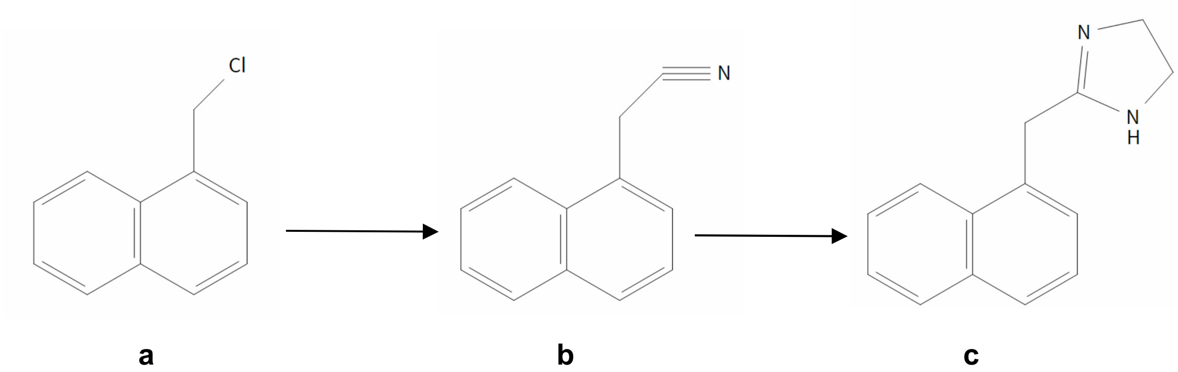


Figure 1: Scheme of the production process of naphazoline. a: 1-chloromethylnaphthalene. b: 1-naphthylacetonitrile. c: naphazoline.

According to Ashby et al. [2], 1-chloromethylnaphthalene has an alerting structure due to its configuration as aromatic substituted primary alkyl halide. Since there are no mutagenicity data of 1-chloromethylnaphthalene available, it is treated as class II impurity according to ICH M7(R1) [1]. Therefore the amount of 1-chloromethylnaphthalene has to be controlled at or below acceptable limits. The company LOBA Feinchemie GmbH has defined the limit for class II impurities in its products with a threshold of 48 ppm.

Since the production process, as well as the raw material 1-naphthylacetonitrile, are expensive, it makes sense to analyze the amount of the impurity 1-chloromethylnaphthalene in the raw material 1-naphthylacetonitrile to decide, whether an acquired batch can be used for the production of naphazoline or not. Quantitation of impurities can be done either with a quantitative test or a limit test. Where a limit test only allows the statement whether the amount of the impurity is below or above the predefined limit, a quantitative test enables the absolute quantitation of the impurity in the investigated substance.

Conventional methods for the separation, identification, and quantitation of organic substances in the range of ppm are the chromatographic techniques gas chromatography (GC) and high-performance liquid chromatography (HPLC) in combination with mass spectrometry (MS).

Which technique suits best, depends primarily on the analyte and the matrix, but also the intent of the analysis and the available equipment is essential. GC is limited to thermally stable, volatile and therefore low molecular mass analytes with boiling points below 500°C or vapor pressures of several kPa below this temperature. HPLC on the other side has no limitations regarding volatility, molecular mass, and polarity of the analyte [3].

However, in case of GC a higher peak capacity due to the much higher number of theoretical plates can be obtained compared to HPLC, which is a significant advantage in fields of trace analysis and analysis of complex samples [4].

According to the properties of the investigated substances, GC, as well as LC, are likely to be applicable for the successful separation of the investigated analytes.

1.2 Aim

The quality control of Loba Feinchemie is equipped with a GCMS-QP2020 from Shimadzu Corporation, which allows the quantitation of suitable substances in the range of ppm. The aim of this work was to develop an analytical method based on gas chromatography-mass spectrometry (GCMS), which allows the absolute quantitation of the potential genotoxic impurity 1-chloromethylnaphthalene in the raw material 1-naphthylacetonitrile at TU Wien, and to transfer and validate the method at the receiving laboratory of Loba Feinchemie GmbH.

The research group *Mass Spectrometric Bio and Polymer Analysis* of TU Wien is equipped with a GCMS-TQ8040 from Shimadzu which is capable of multiple reation monitoring (MRM). MRM enables higher sensitivity compared to the commonly used selected or multiple ion monitoring mode (SIM). Since the device of Loba Feinchemie GmbH (GCMS-QP2020) is not able to perform MRM, this mode was not used for method development.

2 Principles

GCMS combines the separation power of GC with the detection power of MS [5]. In the following, those techniques are explained in more detail with a special focus on their use as a hyphenated technique.

2.1 Gas Chromatography

GC is a separation technique based on the distribution of the sample components between the gaseous mobile phase and the stationary phase, which is either a solid or an immobilized liquid. GC is commonly used for thermally stable and volatile analytes. Substances with poor volatility and/or poor thermal stability may also be applicable to GC after appropriate derivatization. Therefore, it can be used for a wide range of applications, such as the separation of petroleum products, oligosaccharides, lipids or permanent gases [6].

The typical model of a gas chromatograph is shown in Figure 2.

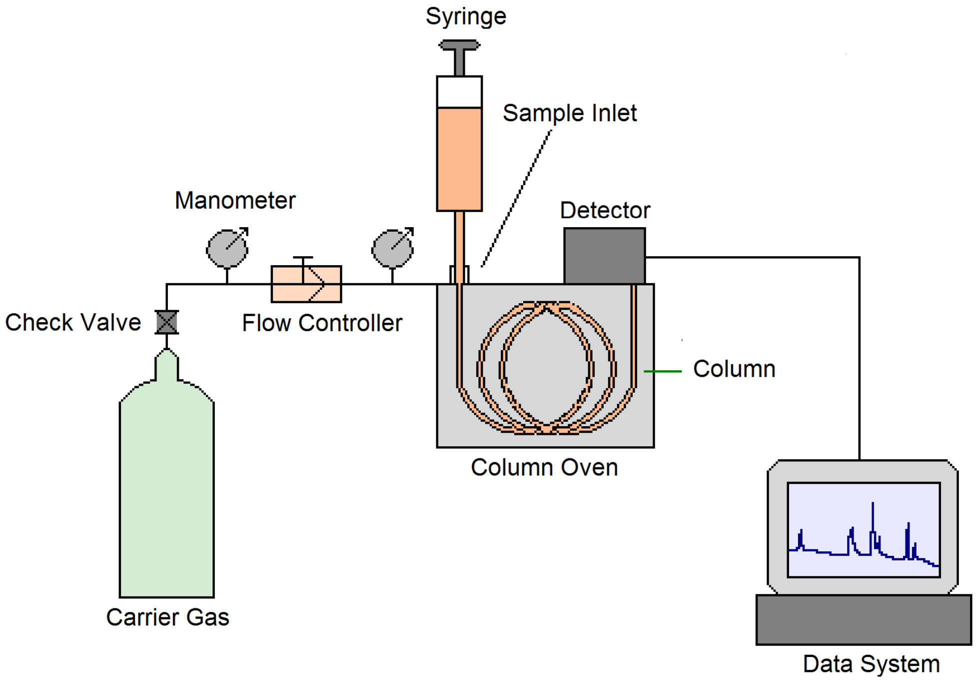


Figure 2: Scheme of a universal GC. Figure adapted from [6]

In the following chapters, the main parts and functions of a gas chromatograph are explained in more detail.

2.1.1 Mobile Phase

The mobile phase, also termed carrier gas, is an inert gas which is used for the transfer of the analytes through the analytical column. The most commonly used mobile phase in GC is helium; hydrogen and nitrogen are used for special applications only [6]. The efficiency of a chromatographic separation can be described by the number of theoretical plates or the high equivalent of the theoretical plates (H.E.T.P.) for a given length of the analytical column.
The lower the H.E.T.P., the better the separation efficiency of the chromatographic separation. The Van Deemter equation reveals the relationship between the average linear velocity of the mobile phase and the high equivalent of the theoretical plates. The Van Deemter curves of commonly used carrier gases in GC are shown in Figure 3.

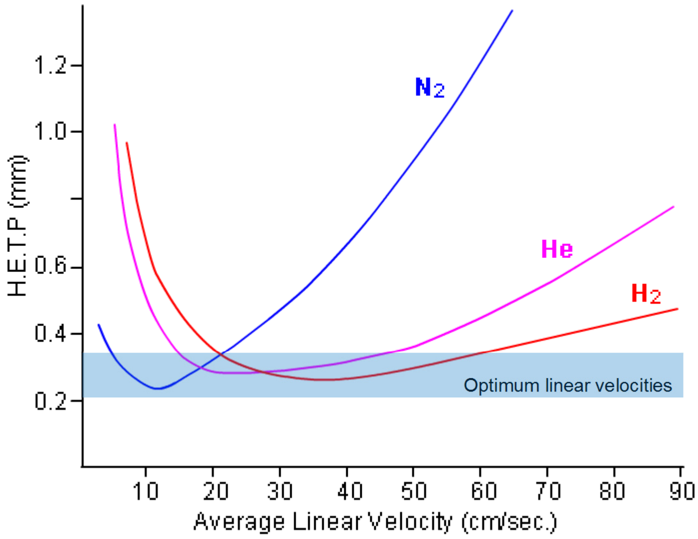


Figure 3: Van Deemter curves of commonly used carrier gases (Nitrogen, Helium and Hydrogen) in GC by using a stationary phase of cross-linked methyl silicone (length: 25 m; inner diameter: 0.25 mm; retention factor: 4.95). Figure adapted from [8]

As shown in Figure 3, the use of nitrogen allows the lowest possible H.E.T.P. and, therefore, the best efficiency. However, this minimum can only be achieved at low average linear velocities, which is associated with a longer analysis time. Hydrogen is the preferred gas in fast and efficient analysis because of the small slope at increasing velocity in the corresponding Van Deemter curve, but special care must be taken due to the risk of spontaneous explosion [9]. Helium is a good compromise, combining high efficiency at high linear velocities without special risks.

2.1.2 Sample Inlet

The main task of the sample inlet is the volatilization of the sample and its transfer onto the analytical column with the carrier gas. If the sample is already gaseous, it can be directly applied by using for instance headspace injection or on-column injection. Liquid samples are injected with a syringe, either manually or automatically, and have to be volatilized subsequently. Depending on the properties of the sample components, different types of sample introduction/sample inlets are used. The most common method of sample introduction is a split/splitless sample inlet. The typical scheme of a split/splitless sample inlet is shown in Figure 4.

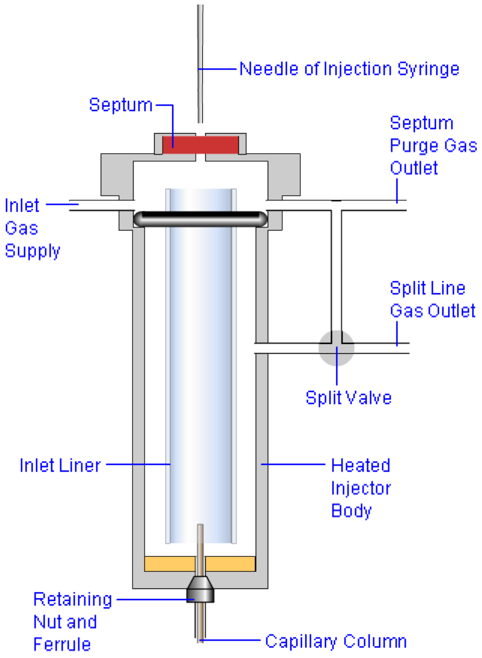


Figure 4: Split/Splitless Inlet. Figure adapted from [10]

The split/splitless inlet consists of a heatable metal block and a fused silica tube, also called inlet liner, which protects the sample from possible decomposition due to the direct contact with the hot metal (temperatures up to 350°C are used) [6]. In split injection mode, the split valve is opened during sample injection. Depending on the split ratio only a small, defined fraction of the sample enters the column. The rest of the sample flows past the column entrance and out of the inlet via the split line. Split injection is mainly used in cases of high sample concentrations to avoid column overload. A disadvantage of split injection is the sample discrimination in cases of samples with wide ranges of boiling points [10]. If it is necessary that the whole sample is loaded onto the column, as for trace analysis or to avoid sample discrimination, the split valve is closed during sample injection. This type of injection is called splitless injection.

2.1.2.1 Optimizing the injection volume

As described, a liquid sample volatilizes and expands into the gas phase after its injection into the sample inlet. If the total volume of the generated gas exceeds the volume of the inlet liner, the excess gas spills over into the inlet gas supply and septum purge lines. Since the temperature in those lines is usually much lower than in the sample inlet, recondensation and depositing of the analytes onto the inner walls of the tubing are possible. In case of the next sample injection, those deposited analytes are introduced in the sample inlet again, leading to reduced quantitative accuracy and reproducibility [10].

In order to avoid a so-called backflash, the expansion volume has to be determined for a defined injection volume and must be compared with the available volume of the sample inlet. The main part of the introduced sample is the sample solvent. Therefore, the expansion volume of the injected sample depends mainly on the properties of the solvent. Further, the inlet temperature, as well as the inlet pressure have to be considered in the calculation.

$$V_{exp} = \frac{n \cdot R \cdot T_{inj}}{p_{inlet}} \qquad \text{(Equation 1)}$$

$$n = \frac{\rho}{M} \cdot V_{inj} \qquad \text{(Equation 2)}$$

$$V_{exp} = \frac{V_{inj} \cdot \rho \cdot R \cdot T_{inj}}{M \cdot p_{inlet}} \qquad \text{(Equation 3)}$$

The volume of the cylindrical inlet liner can be calculated as shown in equation 4.

$$V_{Liner} = (\frac{d_i}{2})^2 \cdot \pi \cdot L \qquad \text{(Equation 4)}$$

The presence of carrier gas in the inlet liner diminishes the available volume by approximately half [23].

$$V_{Liner,eff} = \frac{(\frac{d_i}{2})^2 \cdot \pi \cdot L}{2} \qquad \text{(Equation 5)}$$

In case of split injection, the split valve is opened during the sample injection. Therefore the expanded gas can escape through the split valve which decreases the risk of a backflash.

2.1.3 Chromatographic separation

As already mentioned in chapter 2.1, the separation of the analytes in GC is based on the distribution of the sample components between the gaseous mobile phase and the stationary phase. Since there is no chemical interaction between the analytes and the carrier gas, the retention of the analytes depends only on their vapor pressure and their interaction with the stationary phase [6]. Therefore, the chemical nature of the stationary phase, as well as the column temperature, are the most critical variables influencing the distribution coefficients of the analytes and further their separation [7]. As described in 2.1.1 also the choice of the carrier gas and its adjusted linear velocity influence the separation efficiency.

2.1.4 Column

There are two types of columns applied in GC: the packed column and the capillary column. Packed columns were mainly used in the beginnings of GC. They are usually glass- or steel tubes, packed with inert support material which is coated with a thin film of a high-boiling liquid as stationary phase. Packed columns have usually an inner diameter of 3-8 mm with a length of 1-3 m [6].

With the invention of capillary columns in the 1970s, packed columns lost their primacy in the application of GC. Capillary columns are long narrow tubes of fused silica, coated with the stationary phase on the inner surface and a polyimide polymer on the outside. The polymer on the outside improves the strength and flexibility of the capillary column. The open tubular construction of capillary columns leads to a low drop in pressure across the column, which is why they can be manufactured up to 100 m. Typical dimensions of capillary columns are 30 - 50 m of length with inner diameters of 0.1 - 0.75 mm. The higher length of capillary columns compared to packed columns allows significantly higher numbers of theoretical plates and further higher separation efficiency [6,7]. Stationary phases of capillary columns are mainly polymeric liquids based on siloxanes. Those siloxanes are often cross-linked to improve chemical and thermal stability [6].

In the course of this work, a non-polar stationary phase with a composition of 5 % Phenyl and 95 % Dimethyl-Arylenesiloxane (Column: DB-5MS UI from Agilent) was used. The inclusion of an arylene moiety in the siloxane polymer, which includes phenyl groups, increases stability against degradation of the stationary phase (also called column bleed). These modifications lead to improved sensitivity, which is needed especially in case of GCMS [11].

2.1.5 Detector

After passing the analytical column, the separated gas phase analytes are detected and transformed into a suitable signal with the detector. Depending on the task, there is a huge variety of detectors available. Detectors in GC are usually classified according to the following criteria:

1. Concentration or Mass Flow Detectors
2. Selective or Universal Detectors
3. Destructive or Non-Destructive Detectors

The most important detector in GC is the flame ionization detector because of the high sensitivity, the wide range of linearity and its applicability to hydrocarbons. Besides the high sensitivity (detection limit: 10 pg), the flame ionization detector is a universal detector, which means that all present hydrocarbons in a sample are detected. This can lead to overlapping peaks and thus, loss of information, which can be a problem if complex samples are analyzed [6].

The detection system MS and its use in combination with GC are explained in more detail in chapter 2.2 and 2.3.

2.2 Mass Spectrometry

Mass spectrometers are analytical instruments used for the ionization of the analytes and the subsequent separation of the generated gas phase ions according to their mass to charge ratio (m/z).

The typical scheme of a mass spectrometer is shown in Figure 5.

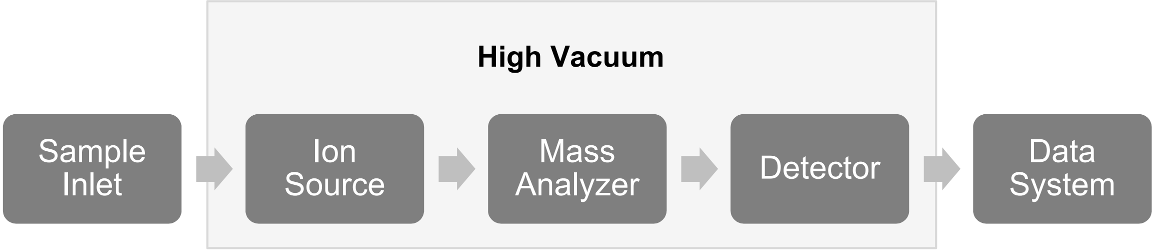


Figure 5: Typical scheme of a mass spectrometer

2.2.1 Ionization

The first step of mass spectrometry is the ionization of the analytes. There are several methods, used for the ionization. The most popular ionization techniques are listed below:

- Electron Impact Ionization
- Chemical Ionization
- Field Ionization
- Fast Atom Bombardment
- Electrospray Ionization
- Matrix Assisted Laser Desorption Ionization
- Atmospheric Pressure Photoionization

In GCMS the separated gas phase analytes are ionized by using either electron impact ionization or chemical ionization. The applied ionization technique in this work was electron impact ionization. The typical scheme of the electron impact ionization process is shown in Figure 6.

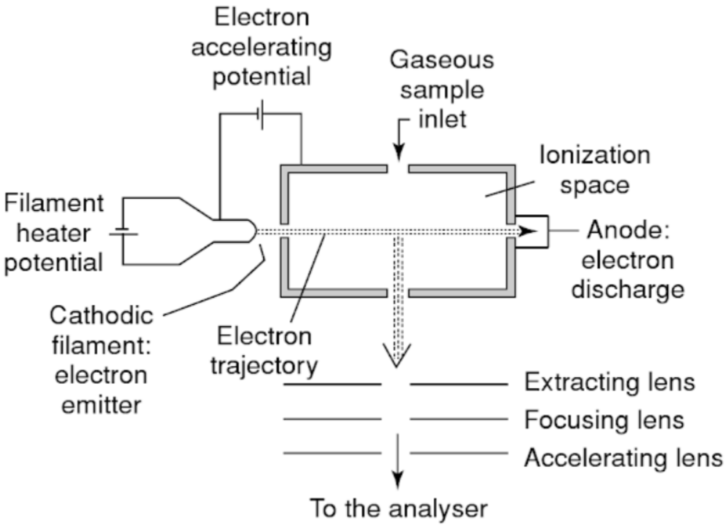


Figure 6: Diagram of an electron impact ionization source [13]

Electrons are emitted from a heated filament and are accelerated by using an appropriate potential (5-100 eV). The beam of electrons collides with the entering gas phase analytes, leading to the following interactions [28]:

$$AB_{(g)} + e^- \rightarrow [AB]^{+\bullet}_{(g)} + 2e^- \qquad \text{(Equation 6)}$$

$$AB_{(g)} + e^- \rightarrow [AB]^{2+}_{(g)} + 2e^- \qquad \text{(Equation 7)}$$

$$AB_{(g)} + e^- \rightarrow A^{+}_{(g)} + B^{\bullet}_{(g)} + 2e^- \qquad \text{(Equation 8)}$$

$$AB_{(g)} + e^- \rightarrow A^{+}_{(g)} + B^{-}_{(g)} + 2e^- \qquad \text{(Equation 9)}$$

$$AB_{(g)} + e^- \rightarrow [AB]^{-\bullet}_{(g)} \qquad \text{(Equation 10)}$$

$$AB_{(g)} + e^- \rightarrow A^{-\bullet}_{(g)} + B_{(g)} \qquad \text{(Equation 11)}$$

The most important interaction leads to the ionization of the analyte and the formation of a radical cation of the molecular species (see equation 7). Depending on the stability of the molecule and the used ionization energy, the generated parent ion often fragments into specific fragment ions. Standardizing the used ionization energy leads to a good reproducibility regarding the type of the generated fragment ions, as well as their abundance. This reproducibility is an important tool for qualitative analysis and allows fast identification of samples with unknown composition by comparing the results with reference mass spectra of comprehensive databases like those of NIST or Wiley. An ionization energy of 70 eV is currently used in all commercial applications, leading to a good fragmentation of most analytes and, therefore, comprehensive structural information [6]. An ionization energy of 20 eV was used in former GCMS devices, resulting in less fragmentation and, therefore, less structural information [31].

2.2.2 Mass Analyzer

After ionization, the gas phase ions are transferred into the mass analyzer where they are separated according to their m/z value. In case of GCMS the following mass analyzers are usually applied [5]:

- Quadrupole mass analyzer
- Ion trap mass analyzer
- Time of flight mass analyzer
- Magnetic sector mass analyzer

In the course of this work, the quadrupole mass analyzer was used (see Figure 7).

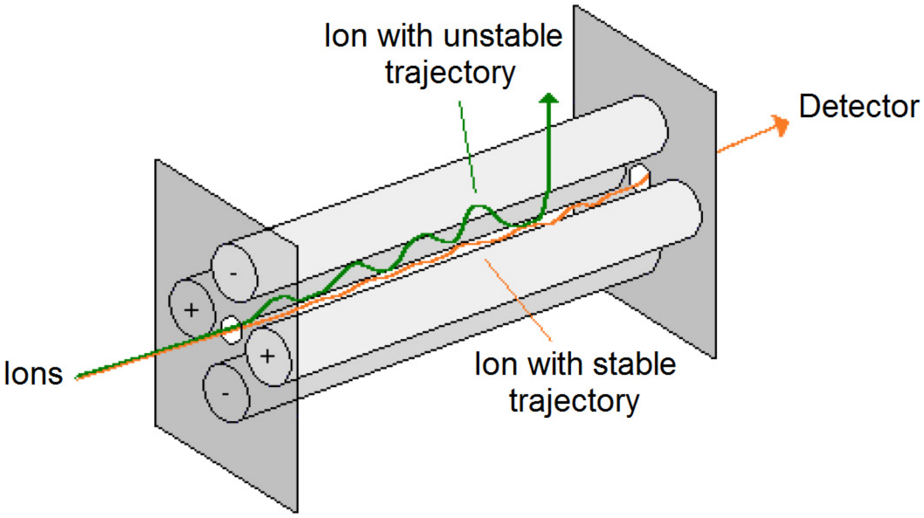


Figure 7: Scheme of a quadrupole mass analyzer. Figure adapted from [6]

A quadrupole mass analyzer consists of 4 hyperbolic rods, acting as electrodes. The quadrupole mass analyzer can be used either as selective or universal detector: By adjusting a specific combination of direct current and alternating current, only ions with specific m/z values are allowed to pass the mass analyzer. Ions with m/z beyond the defined values are not able to pass since they collide with the rod and get discharged [6]. This monitoring mode is called selected ion monitoring (SIM). A total ion current chromatogram (TIC) is obtained if ions of all possible m/z ratios are allowed to pass the mass analyzer. The universal detection of all m/z values is associated with a high background signal and thus lower sensitivity.

The quadrupole mass analyzer is a continuous mass analyzer, which means that a constant flow of ions is transferred into the mass analyzer. Compared to other mass analyzers like the Fourier Transform Ion Cyclotron Resonance (FTICR) or the Orbitrap (Mass range: 30 000 - 50 000 u; Mass resolution: 100 000 - 500 000), the quadrupole mass analyzer covers only a low mass range (4000 u) and offers a low mass resolution (4000). The constant bandwidth across the whole mass range, results in lower resolution at lower m/z values. However, the low costs, the high sensitivity, as well as the good reproducibility make the quadrupole mass analyzer very popular. Due to the fast scanning speed (µsec), it is often used in combination with chromatography [31].

Even higher sensitivity and selectivity can be obtained by using tandem mass spectrometry (MS/MS). MS/MS analysis can be achieved by applying a triple quadrupole mass analyzer, which uses a combination of two quadrupoles with a collision cell between them [5]. A precursor ion is selectively filtered by the first quadrupole mass analyzer and enters the collision cell, where it is further fragmented by collision induced dissociation (CID). The specific built fragments are then separated by the second quadrupole analyzer. Typical applications of MS/MS are the product ion scan, the precursor scan, the neutral loss scan and the selected reaction monitoring [31].

2.2.3 Detector

Detection of the (fragment) ions in mass spectrometers is usually done by converting the ions into electrons, then multiplying the electrons and finally counting them. Direct detection of the (fragment) ions is also possible, but results in lower sensitivity [6]. The detector used in this work was an electron multiplier. The principle of an electron multiplier is shown in Figure 8.

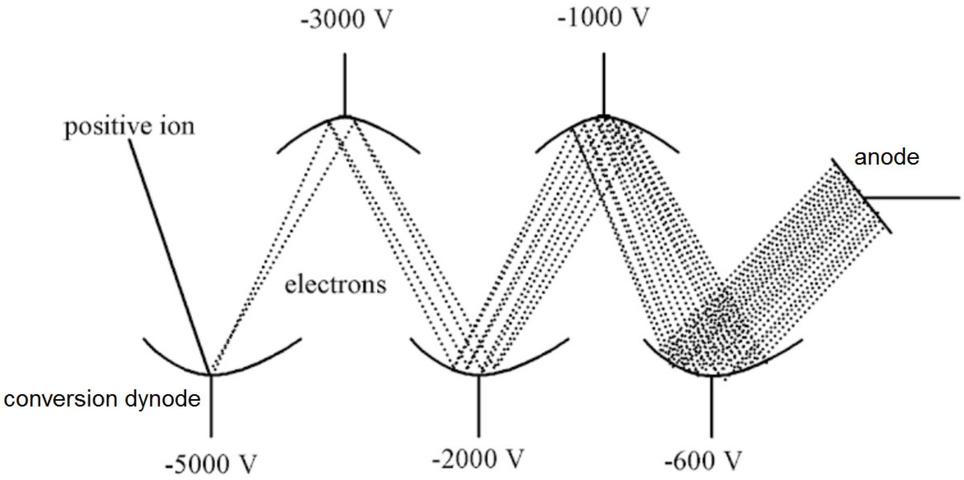


Figure 8: Principle of an electron multiplier. Figure adapted from [14]

The investigated ion is accelerated towards the conversion dynode where one or more electrons are emitted due to the collision. Those electrons are then accelerated to the next electrode where again 1 - 3 electrons are emitted by hitting the surface. This process is repeated several times, leading to a measurable current proportional to the amplification of the electrons and, therefore, the initial amount of entering ions. By adjusting the detector voltage, the power of electron amplification and further the signal intensity can be controlled [6].

2.3 Use of MS in combination with GC

The mass spectrometer is one of the most flexible and powerful detectors used in combination with GC [6]. Besides high sensitivity detection (Limit of Detection: SIM: 1 - 10 pg; MRM: 5 - 10 times lower than those achieved by SIM), it allows specific compound identification by structural elucidation via spectral interpretation [5,6,29]. Mass spectrometers are used under high vacuum conditions to avoid collisions of ions with other ions or particles, to increase the sensitivity and to allow the generation of free electrons in the gas phase [15].
The transition of the atmospheric pressure from GC to the high vacuum system of MS was a big obstacle in times when packed columns were used primarily. The high volume (up to 20 mL/min) of gas emerging from the GC had to be vented by “carrier gas splitting” to maintain the vacuum under which the analyzing device must operate. With the implementation of capillary columns also the gas flow rates decreased (approx. 1 mL/min), which allowed the direct injection from the column into the mass spectrometer [5,6].

2.4 Validation

Chemical products are offered by guaranteeing specific quality standards. Depending on the type of product, the requirements can vary rigorously. Especially in case of pharmaceuticals, the quality of the product has crucial importance. To ensure that those requirements are met for each batch, appropriate analytical procedures have to be performed. The validation of an analytical procedure aims to show, that the procedure is suitable for its intended purpose. Validations based on the quality guideline ICH Q2(R1) [24] are part of registration applications submitted within the EC, Japan, and USA and ensure that the quality of a product is measured in a proper and well-defined way [12].

2.5 Quantitation strategies

Quantitation in GCMS is commonly done by external calibration or the standard addition procedure.

2.5.1 External calibration

The substance to be determined is used to prepare standard solutions with defined concentrations. A calibration curve is generated by plotting the measured peak areas against the corresponding concentrations of the different calibration levels [16].

External calibration is the easiest way of quantitation, but can only be applied if the sample matrix is known and reproducible or the investigated substance is extractable by solid phase extraction. The non-consideration of matrix effects would lead to inaccurate results. Solid phase extraction is a commonly used method for sample preparation in the field of GCMS. However, an internal standard is needed to check the efficiency of the extraction procedure. Commonly a substance which is similar to the investigated substance, or the deuterated version of the substance itself, is applied as an internal standard for solid phase extraction.

2.5.2 Standard addition procedure

If external calibration is not applicable, the standard addition procedure is a possible solution for quantitation. If for example the composition of the sample is unknown and no appropriate internal standard is available for solid phase extraction, external calibration is no option.

The method of standard addition involves the addition of known quantities of the substance to be determined, to a constant, but unknown quantity of the substance in the sample. To one sample no standard is added. The analysis leads to a calibration function, which intercepts the y-axis at the peak area of the untreated sample. The concentration of the substance in the sample can be determined by extrapolating the calibration curve and locating the point where it intercepts the x-axis [16].

2.5.3 Improvement of the external calibration and the standard addition procedure by internal standardization

Even by using highly reproducible injection techniques like an autosampler, standard deviations of 5 - 10 % are obtained in GCMS due to injection errors and small changes in the electronics and the applied potentials in the mass spectrometer [16]. The internal standardization (also termed normalization) is an improvement of the external calibration and the standard addition procedure, which overcomes those problems. As in the procedure of solid phase extraction, a substance which is similar to the investigated substance in terms of properties and structure, or the isotopically labeled version of the substance itself, is applied as internal standard. Besides the similarity to the investigated analyte, the internal standard itself must not be present in the original sample. Further it must be as inactive as possible. The internal standard is added to each calibration level in the same concentration. By calculating the peak area of the substance being determined relative to the peak area of the internal standard, all possible volume errors and variations in the function of the instrument are compensated [16].

$$Corrected\ Area\ [AU] = \frac{Peak\ Area\ of\ Target\ Analyte\ [AU]}{Peak\ Area\ of\ Internal\ Standard\ [AU]} \qquad \text{(Equation 12)}$$

Commonly, the internal standard is added at a concentration of one third to one half of the expected maximum target analyte concentration. This is especially important in case of MS-detectors to prevent the interference with the ionization of the analyte molecule [20].

3 Experimental

3.1 Materials

3.1.1 Chemical and reagents

The chemicals and reagents used in this thesis are shown in Table 1.

Table 1: Used chemicals and reagents

Substance	Product Number	Supplier	Purity/Quality
1-Chloromethylnaphthalene (Lot# A0387978)	10945	ACROS Organics, Geel, Belgium	96.3 %
Naphthalene-d_8 (Lot# MBBC3287)	176044	Sigma Aldrich, St. Louis USA	≥ 98 %
Isopropyl alcohol	1.01040	Merck, Darmstadt, Germany	Gradient grade for liquid chromatography
Methanol	1.06007	Merck, Darmstadt, Germany	Hypergrade for LC-MS
1-Naphthylacetonitrile (Internal Lot# #0000011533)	-	Changzhou United Chemical Co., LTD, Changzhou China	-
Naphazoline (Internal Lot# #0731900001)	-	LOBA Feinchemie GmbH, Fischamend, Austria	-
Ethanol	111727	Merck, Darmstadt, Germany	Gradient grade for liquid chromatography

3.1.2 Equipment/Instruments

3.1.2.1 Method development

- Pipettes (1 - 5000 µl) and tips from Eppendorf, Hamburg, Germany
- Inlet Liner #1: Liner for split/splitless injection (P/N: 221-75193) from Shimadzu, Kyoto, Japan
- Inlet Liner #2: Liner for split/splitless injection (P/N: AG0-4683) from Phenomenex, Torrance, USA
- Inlet Liner #3: Liner for splitless injection (P/N: 221-48876-02) from Shimadzu, Kyoto, Japan
- Analytical Column DB-5MS UI: 30 m x 0.25 mm x 0.25 µm (P/N: 122-5532UI) from Agilent, Santa Clara, USA
- Pre-Column Fused Silica, Ultimate Plus Deactivated: 10 m x 0.25 mm (P/N: CP802510) from Agilent, Santa Clara, USA
- Enduro Blue Septum (P/N: 221-75180) from Shimadzu, Kyoto, Japan
- Centrifuge tube 50 mL with screw cap (P/N: 525-0224) from VWR, Radnor, USA
- Safe-Lock Tubes 2.0 mL (P/N: 0030 120.094) from Eppendorf, Hamburg, Germany

- GCMS TQ-8040 from Shimadzu, Kyoto, Japan
    - Software:
        - GCMS Real-Time Analysis Version 4.20
        - GCMS Postrun Analysis Version 4.20
        - GCMS Analysis Editor Version 4.20
        - GCMS Browser Version 4.20
    - Databases:
        - Wiley9
        - NIST11

3.1.2.2 Validation

- Multipipettes and tips from Eppendorf, Hamburg, Germany
- Inlet Liner #3: Liner for splitless injection (P/N: 221-48876-02) from Shimadzu, Kyoto, Japan
- Analytical Column DB-5MS UI: 30 m x 0.25 mm x 0.25 µm (P/N: 122-5532UI) from Agilent, Santa Clara, California
- Pre-Column Fused Silica, Ultimate Plus Deactivated: 10 m x 0.25 mm (P/N: CP802510) from Agilent, Santa Clara, California
- Enduro Blue Septum (P/N: 221-75180) from Shimadzu, Kyoto, Japan
- Centrifuge tube 50 mL with screw cap (P/N: 525-0224) from VWR, Radnor, USA
- Safe-Lock Tubes 2.0 mL (P/N: 0030 120.094) from Eppendorf, Hamburg, Germany
- GCMS QP-2020 from Shimadzu, Kyoto, Japan
    - Software:
        - GCMS Real-Time Analysis Version 4.45
        - GCMS Postrun Analysis Version 4.45
        - GCMS Analysis Editor Version 4.45
        - GCMS Browser Version 4.45
    - Databases:
        - NIST11
- Statistics program: DataLab from Epina GmbH (Version 3.530, Evaluation Copy)

3.2 Gas Chromatography

The method development was carried out on a GCMS-TQ8040 from Shimadzu at the research group *Mass Spectrometric Bio and Polymer Analysis* at TU Wien. Helium was used as carrier gas and a DB-5MS UI column was used as stationary phase. The main part of the method development was done without a connected pre-column. However, a pre-column (Fused Silica, Ultimate Plus Deactivated: 10 m x 0.25 mm, details see chapter 3.1.2.1) was implemented in the end of the method development.

The validation of the method was carried out on a GCMS-QP2020 from Shimadzu at the receiving laboratory of LOBA Feinchemie GmbH. Helium was used as carrier gas and a DB-5MS UI column, connected with a pre-column (same pre-column as in method development), was used as stationary phase.

3.2.1 Optimizing Injection Volume

In order to avoid reduced quantitative accuracy and reproducibility because of a backflash (explanation see chapter 2.1.2.1), the injection volume was optimized for quantitative experiments. Table 2 lists the actual expansion volumes in relation to the inlet liner volume under the used conditions.

Table 2: Comparison of the expansion volume and the available inlet liner volume under the used settings and conditions

				Inlet Liner	d_i	L [mm]	$V_{\text{Liner,eff}}$ [µL]
					3.4	95	431.18
	ρ [g/mL]	M [g/moL]	p_{inlet} [kPa]	V_{inj} [mL]	T_{inj} [K]	R [(Pa*mL)/(moL*K)]	V_{exp} [µL]
Ethanol	0.789	46.07	213525	0.001	573.15	8.31E+06	382.34
IPA	0.786	60.10	213525	0.001	573.15	8.31E+06	291.86
			145025	0.0005	573.15	8.31E+06	214.86

3.2.2 Sample preparation

The method of standard addition was chosen for the quantitation of the impurity 1-chloromethylnaphthalene in the raw material 1-naphthylacetonitrile (argumentation see chapter 4.1.4). First experiments revealed that the expected amount of 1-chloromethylnaphthalene in the raw material was approximately 5 ppm. Therefore, a range between 2 and 10 ppm of 1-chloromethylnaphthalene in the raw material was investigated. The general workflow of the standard addition procedure is described below:

1. A solution of 10 mg raw material 1-naphthylacetonitrile/mL isopropyl alcohol (IPA) is prepared (Solution 1)
2. A solution of 0.1 µg 1-chloromethylnaphthalene/mL Solution 1 is prepared (Solution 2)

   Composition of Solution 2: 0.1 µg 1-chloromethylnaphthalene/mL IPA
   10 mg raw material 1-naphthylacetonitrile/mL IPA

   0.1 µg are equal to 10 ppm of 1-chloromethylnaphthalene relative to 10 mg of the raw material 1-naphthylacetonitrile
3. Solution 1 and Solution 2 are mixed according to Table 3, to cover a range of 2 – 10 ppm of 1-chloromethylnaphthalene in the raw material

Table 3: Preparation of the spiking levels for the standard addition procedure

Spiking Level	Volume [µL]		spiked concentration
	Solution 1	Solution 2	[ppm 1-chloromethylnaphthalene]
0	1000	0	0
1	800	200	2
2	600	400	4
3	400	600	6
4	200	800	8
5	0	1000	10

4. The purity grade of 96.3 % of 1-chloromethylnaphtalene has to be considered to obtain the actual concentrations of the spiking levels (see Table 4)

Table 4: Actual concentrations of the spiking levels for the standard addition procedure

Spiking Level	Spiked concentration [ppm 1-chloromethylnaphthalene] Assumed purity: 100 %	Spiked concentration [ppm 1-chloromethylnaphthalene] Actual purity: 96.3 %
0	0	0
1	2	1.93
2	4	3.85
3	6	5.78
4	8	7.70
5	10	9.63

3.2.3 Settings of GCMS

Since the aim of this work was the development of an analytical method which allows the absolute quantitation of the potential genotoxic impurity 1-chloromethylnaphthalene in the raw material 1-naphthylacetonitrile, a variety of different settings was used in the course of this work. Additional issues also demanded the use of different, appropriate methods. All used methods are, therefore, listed in the respective chapters. However, the settings of the final method for the quantiation of 1-chloromethylnaphthalene in the raw material 1-naphthylacetonitrile for the device GCMS-TQ8040 are shown in Table 5.

Table 5: Settings of GC, MS and Sampler of the final method for the quantitation of 1-chloromethylnaphthalene in the raw material 1-naphthylacetonitrile for the device GCMS-TQ8040.

<table>
<tr><th colspan="3">Gas Chromatograph</th><th colspan="2">Mass Spectrometer:</th></tr>
<tr><td>Column</td><td colspan="2">Agilent DB-5MS UI</td><td>Interface temperature</td><td>325 °C</td></tr>
<tr><td>Length:</td><td colspan="2">30 m</td><td>Ion source temperature:</td><td>250 °C</td></tr>
<tr><td>Thickness:</td><td colspan="2">0.25 µm</td><td>Solvent cut time:</td><td>4.5 min</td></tr>
<tr><td>Diameter:</td><td colspan="2">0.25 mm</td><td>Event time:</td><td>0.1 sec</td></tr>
<tr><td>Pre-Column:</td><td colspan="2">Yes (see 3.1.2)</td><td>Start time data acquisition:</td><td>5 min</td></tr>
<tr><td>Liner:</td><td colspan="2">Inlet Liner #3</td><td>End time data acquisition:</td><td>8.4 min</td></tr>
<tr><td>Injection volume:</td><td colspan="2">0.5 µL</td><td>Acquisition mode:</td><td>Q3-SIM</td></tr>
<tr><td>Injection mode:</td><td colspan="2">Splitless</td><td>Detector voltage relative to the tuning result:</td><td>0 kV</td></tr>
<tr><td>Sampling time:</td><td colspan="2">1 min</td><td rowspan="6">Recorded m/z values:</td><td>115</td></tr>
<tr><td>Injection temperature:</td><td colspan="2">300 °C</td><td>136</td></tr>
<tr><td>Head pressure:</td><td colspan="2">43.7 kPa</td><td>139</td></tr>
<tr><td>Linear velocity:</td><td colspan="2">25 cm/s</td><td>141</td></tr>
<tr><td>Column flow:</td><td colspan="2">0.63 mL/min</td><td>176</td></tr>
<tr><td>Purge flow:</td><td colspan="2">3 mL/min</td><td>178</td></tr>
<tr><td>Split ratio after sampling time:</td><td colspan="2">158.5</td><th colspan="2">Sampler</th></tr>
<tr><td rowspan="2">Carrier gas saver:</td><td colspan="2" rowspan="2">Yes
(Split ratio: 1:50 after 8 min)</td><td># of rinses with methanol (Pre-run):</td><td>12</td></tr>
<tr><td># of rinses with methanol (Post-run):</td><td>12</td></tr>
<tr><td colspan="3">Oven temperature program</td><td># of rinses with sample:</td><td>1</td></tr>
<tr><td>Rate (°C/min)</td><td>Final temperature (°C)</td><td>Hold time [min]</td><td>Washing volume:</td><td>8 µL</td></tr>
<tr><td>-</td><td>60</td><td>1</td><td colspan="2" rowspan="4"></td></tr>
<tr><td>30</td><td>190</td><td>0</td></tr>
<tr><td>10</td><td>225</td><td>0</td></tr>
<tr><td>250</td><td>325</td><td>15</td></tr>
</table>

The settings of the final method for the quantiation of 1-chloromethylnaphthalene in the raw material 1-naphthylacetonitrile for the device GCMS-QP2040 are shown in Table 6.

Table 6: Settings of GC, MS and Sampler of the final method for the quantitation of 1-chloromethylnaphthalene in the raw material 1-naphthylacetonitrile for the device GCMS-QP2020.

Gas Chromatograph	
Column	Agilent DB-5MS UI
Length:	30 m
Thickness:	0.25 µm
Diameter:	0.25 mm
Pre-Column:	Yes (see 3.1.2)
Liner:	Inlet Liner #3
Injection volume:	0.5 µL
Injection mode:	Splitless
Sampling time:	1 min
Injection temperature:	300 °C
Head pressure:	43.7 kPa
Linear velocity:	25 cm/s
Column flow:	0.63 mL/min
Purge flow:	3 mL/min
Split ratio after sampling time:	158.5
Carrier gas saver:	Yes (Split ratio: 1:50 after 8 min)

Oven temperature program		
Rate (°C/min)	Final temperature (°C)	Hold time [min]
-	60	1
30	190	0
10	225	0
250	325	15

Mass Spectrometer:	
Interface temperature	325 °C
Ion source temperature:	250 °C
Solvent cut time:	4.5 min
Event time:	0.1 sec
Start time data acquisition:	5 min
End time data acquisition:	8.4 min
Acquisition mode:	SIM
Detector voltage relative to the tuning result:	0.05 kV
Recorded m/z values:	115
	136
	139
	176
	178

Sampler	
# of rinses with methanol (Pre-run):	12
# of rinses with methanol (Post-run):	12
# of rinses with sample:	1
Washing volume:	8 µL

4 Results and discussion

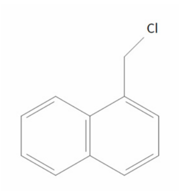
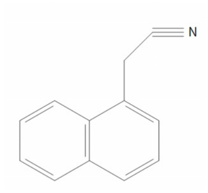
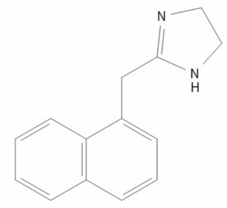
4.1 Qualitative Method Development

The aim was to develop a method, which allows the absolute quantitation of the impurity 1-chloromethylnaphthalene in the raw material 1-naphthylacetonitrile. The matrix naphazoline was considered in fundamental decisions to provide a good starting point for further method development.

4.1.1 Applicability of GC and choice of the stationary phase

To get an idea if the investigated analytes are applicable for GC and, if so, which stationary phase should be used for the method development, the analytes of interest were investigated in more detail regarding their properties. GC relevant properties of the investigated analytes are shown in Table 7.

Table 7: Relevant properties of the analytes of interest

			
	impurity	raw material	product
Substance	1-Chloromethylnaphthalene	1-Naphthylacetonitrile	Naphazoline
CAS registry number	86-52-2 [27]	132-75-2 [27]	835-31-4 [27]
Boiling point at 1 bar [°C]	291.5 [27]	324 (predicted) [27]	440.5 (predicted) [27]
Volatility	semivolatile [17]	semivolatile [17]	n.a.
Melting point [°C]	32 [27]	32.5 [27]	119 [27]
Polarity	non polar	moderate polar	polar

The analyte of interest, 1-chloromethylnaphthalene, was the deciding reason in the choice of the stationary phase. Since the way of quantitation (external calibration or method of standard addition) was not clear at the beginning, the interactions between the stationary phase and the raw material 1-naphthylacetonitrile, as well as the final product naphazoline, were also considered in the decision making process.

1-chloromethynaphthalene and 1-naphthylacetonitrile can be classified as semi-volatile substances due to their "low" boiling points [17]. Therefore, it is most likely that they are compatible with gas chromatography. The upper temperature limit for conventional GC columns is around 350 °C, which is the reason that analyte boiling points rarely exceed 400 °C [5]. Since the boiling point of naphazoline is above 400 °C, it had to be tested if GC is applicable.

Literature search was performed to see, if the investigated analytes were already examined by GCMS and, if so, which stationary phase was used. The search led to no clear results concerning the choice of the stationary phase since those analytes were not investigated by GC in the past.

Since the investigated analytes cover a broad range of polarity (see Table 7), the choice of the stationary phase was made in favor of 1-chloromethylnaphthalene, the substance to be quantified. DB-5MS UI columns are commonly used for semivolatiles and halogenated compounds [18]. Therefore a non-polar DB-5MS UI column from Agilent was chosen for the method development (details see chapter 2.1.4).

4.1.2 Determination and optimization of conditions for qualitative separation

In order to check if the investigated analytes (1-chloromethylnaphthalene, 1-naphthylacetonitrile, and naphazoline) can be separated and detected by GCMS, and to obtain a starting point for advanced temperature programming, a scouting gradient was carried out, measuring a sample with the following composition:

<u>Sample:</u>

Solvent:	Methanol
Concentration:	100 µg 1-Chloromethylnaphthalene/mL 100 µg 1-Naphtylacetonitrile (raw material)/mL 1000 µg Naphazoline (product)/mL

A scouting gradient is commonly used to investigate the nature of the sample (volatility range, number and separation of components, suitability of stationary phase, etc.). The initial oven temperature is set as low as practically possible and a ramp rate of 10°C/min is used until the maximum temperature of the column is reached. A hold time of 10 minutes at the maximum temperature gives higher boiling point analytes an opportunity to elute from the column [30].

Details regarding the used parameters of the scouting gradient are shown in Table 8.

Table 8: Settings of GC, MS and Sampler: Scouting gradient

Gas Chromatograph	
Column	Agilent DB-5MS UI
Length:	30 m
Thickness:	0.25 µm
Diameter:	0.25 mm
Pre-Column:	None
Liner:	Inlet Liner #1
Injection volume:	1 µL
Injection mode:	Split
Split ratio:	1:50
Injection temperature:	300 °C
Head Pressure:	49.5 kPa
Linear velocity:	36.1 cm/s
Column Flow:	1 mL/min
Purge Flow:	3 mL/min
Carrier Gas Saver:	No

Oven temperature program		
Rate (°C/min)	Final temperature (°C)	Hold time [min]
-	40	1
10	325	10

Mass Spectrometer	
Interface temperature	310 °C
Ion Source temperature:	200 °C
Solvent Cut Time:	2 min
Event Time:	0.1 sec
Start Time Data Acquisition:	4 min
End Time Data Acquisition:	39.5 min
Acquisition Mode:	Q3-Scan
Recorded m/z values:	50 – 1090
Detector voltage relative to the tuning result:	0 kV

Sampler	
# of Rinses with Solvent (Pre-run):	1
# of Rinses with Solvent (Post-run):	2
# of Rinses with Sample:	0
Washing Volume:	6 µL

The obtained TIC-chromatogram is shown in Figure 9.

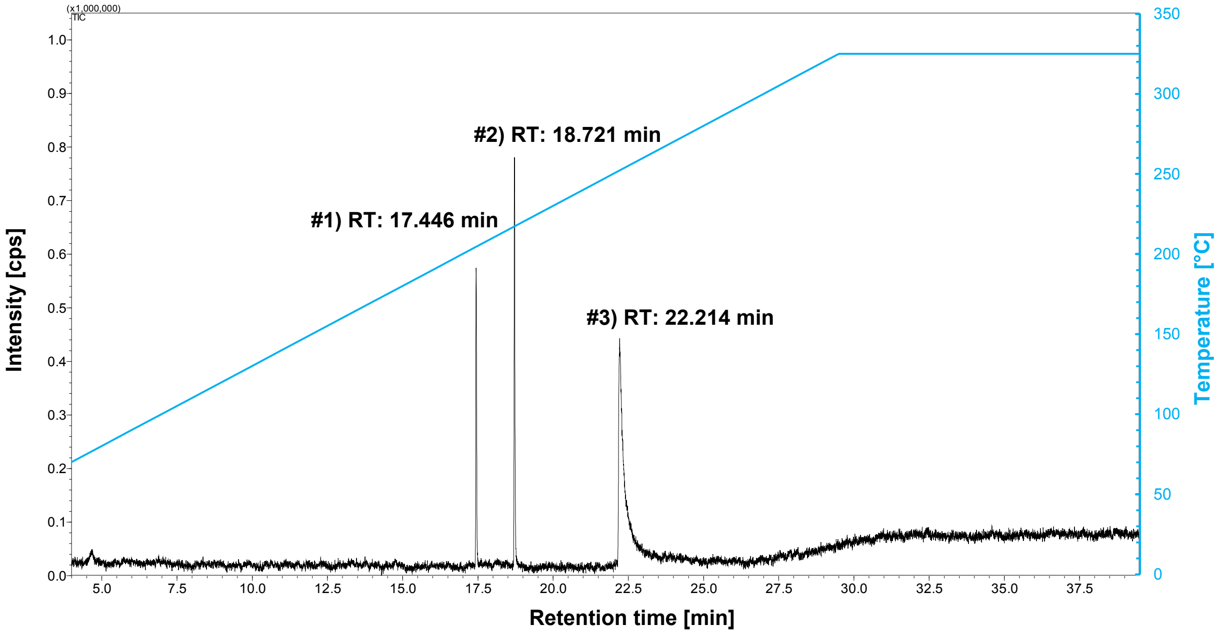


Figure 9: TIC-chromatogram of the performed scouting gradient of a sample with a concentration of 100 µg 1-chloromethylnaphthalene, 100 µg 1-naphthylacetonitrile and 1000 µg naphazoline/mL methanol. Peak #1: 1-chloromethylnaphthalene; Peak #2: 1-naphthylacetonitrile; Peak #3: naphazoline

In case of 1-chloromethylnaphthalene (peak # 1 in Figure 9) and 1-naphthylacetonitrile (peak #2 in Figure 9), a satisfying peak shape could be observed. Despite its high boiling point, also naphazoline (see peak #3 in Figure 9) could be successfully separated and detected by GCMS. The tailing of the corresponding peak is probably due to the bad interaction of the non-polar stationary phase and the polar analyte, or the slow evaporation of naphazoline.

The corresponding mass spectra of the detected peaks, as well as their interpretation are shown in Figure 10 - Figure 12. The average masses of the investigated analytes are listed below:

$M_{avg.}$ 1-chloromethylnaphthalene:	176.64 g/moL
$M_{avg.}$ 1-naphthylacetonitrile:	167.21 g/moL
$M_{avg.}$ naphazoline:	210.27 g/moL

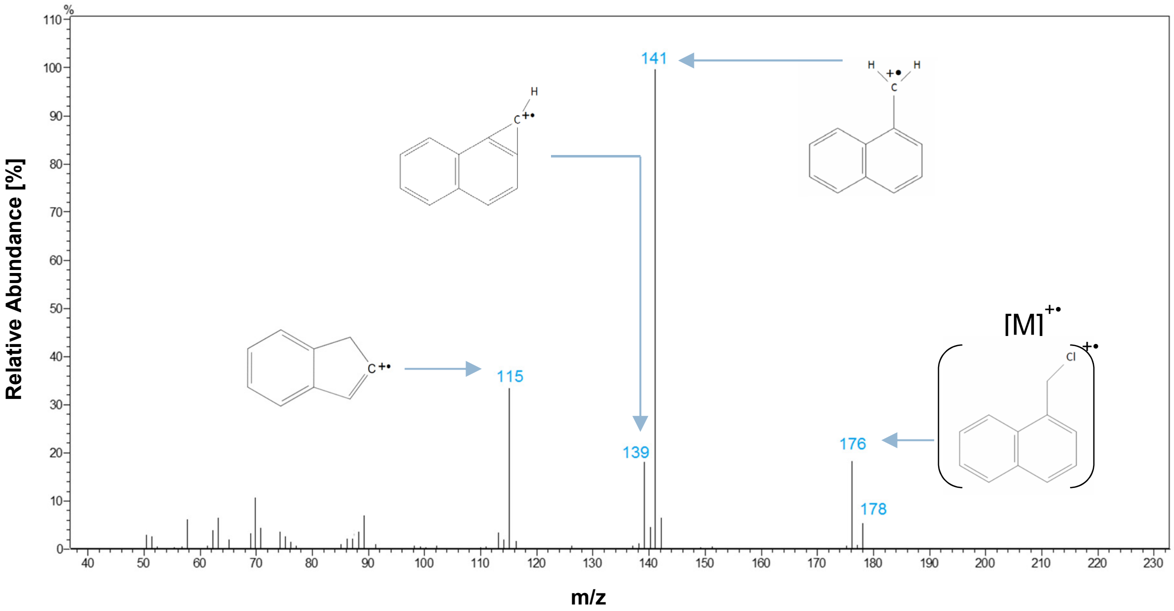


Figure 10: Mass spectrum of peak #1 in Figure 9. The most abundant ions are labeled and evaluated. Similarity to 1-chloromethylnaphthalene: 93 % according to the NIST database.

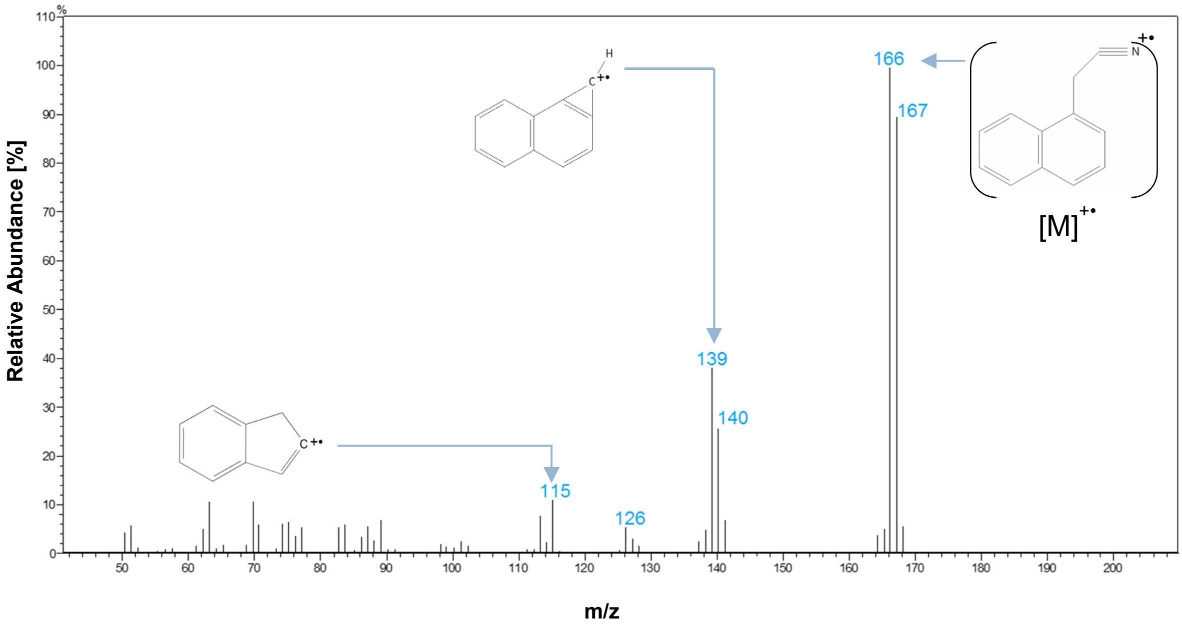


Figure 11: Mass spectrum of peak #2 in Figure 9. The most abundant ions are labeled and evaluated. Similarity to 1-naphthylacetonitrile: 91 % according to the NIST database.

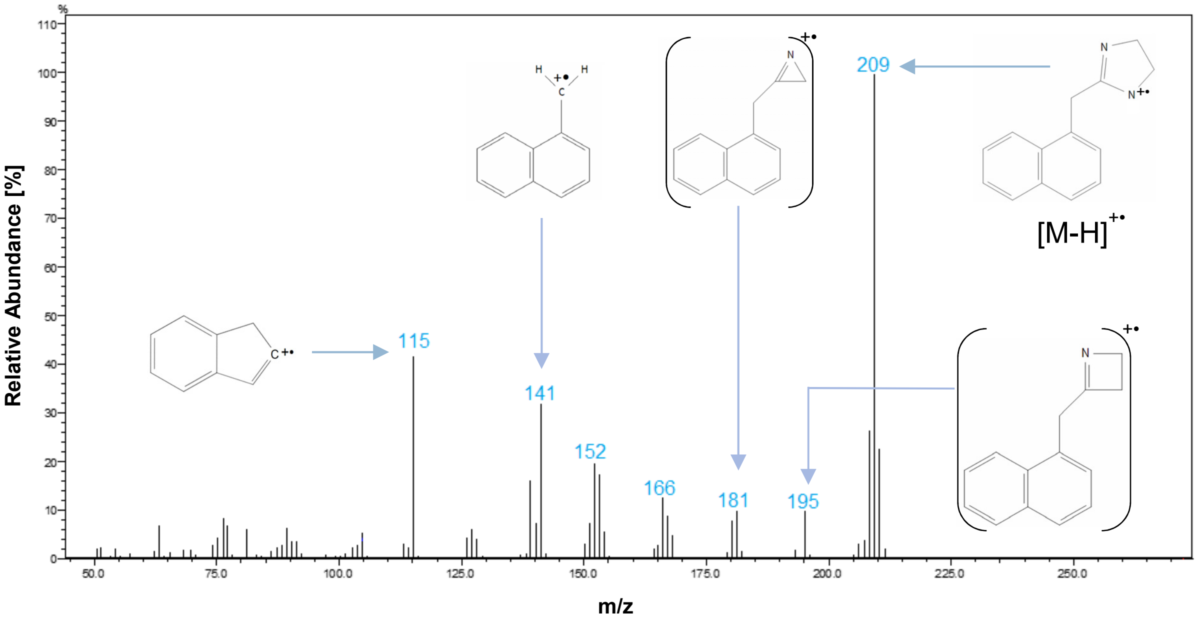


Figure 12: Mass spectrum of peak #3 in Figure 9. The most abundant ions are labeled and evaluated. Similarity to naphazoline: 89 % according to the NIST database.

The comparison of the obtained mass spectra with the NIST database resulted in good similarities:

1-chloromethylnaphthalene:	Similarity of 93 %
1-naphthylacetonitrile:	Similarity of 91 %
naphazoline:	Similarity of 89 %

Deviations compared to the mass spectra in the database are mainly due to background signals (detected m/z values: 50 - 1090).

4.1.2.1 Method optimization for qualitative separation

Based on the obtained results of the scouting gradient (see chapter 4.1.2), the method was optimized regarding signal intensity, peak shape and run time by increasing the linear velocity, changing the initial oven temperature and the heating rates, and increasing the temperature of the ion source. The optimized settings are listed in Table 9.

Table 9: Settings of GC, MS and Sampler: Optimized method for qualitative separation of the investigated analytes

Gas Chromatograph	
Column	Agilent DB-5MS UI
Length:	30 m
Thickness:	0.25 µm
Diameter:	0.25 mm
Pre-Column:	None
Liner:	Inlet Liner #1
Injection volume:	1 µL
Injection mode:	Split
Split ratio:	1:50
Injection temperature:	300 °C
Head pressure:	112.2 kPa
Linear velocity:	45.6 cm/s
Column flow:	1.5 mL/min
Purge flow:	3 mL/min
Carrier gas saver:	No

Oven temperature program		
Rate (°C/min)	Final temperature (°C)	Hold time [min]
-	100	1
30	190	0
10	225	0
25	270	0
30	325	5

Mass Spectrometer	
Interface temperature	310 °C
Ion source temperature:	250 °C
Solvent cut time:	2 min
Event time:	0.3 sec
Start time data acquisition:	2 min
End time data acquisition:	16.13 min
Acquisition mode:	Q3-Scan
Recorded m/z values:	50 – 1090
Detector voltage relative to the tuning result:	0 kV

Sampler	
# of rinses with solvent (Pre-run):	1
# of rinses with solvent (Post-run):	2
# of rinses with sample:	0
Washing volume:	6 µL

The results of the optimized method for qualitative separation (pink labeled chromatogram) are compared to those of the scouting gradient (black labeled chromatogram) in Figure 13. Further, the retention times (RT) and the elution temperatures (ET) of the investigated analytes are labeled in Figure 13.

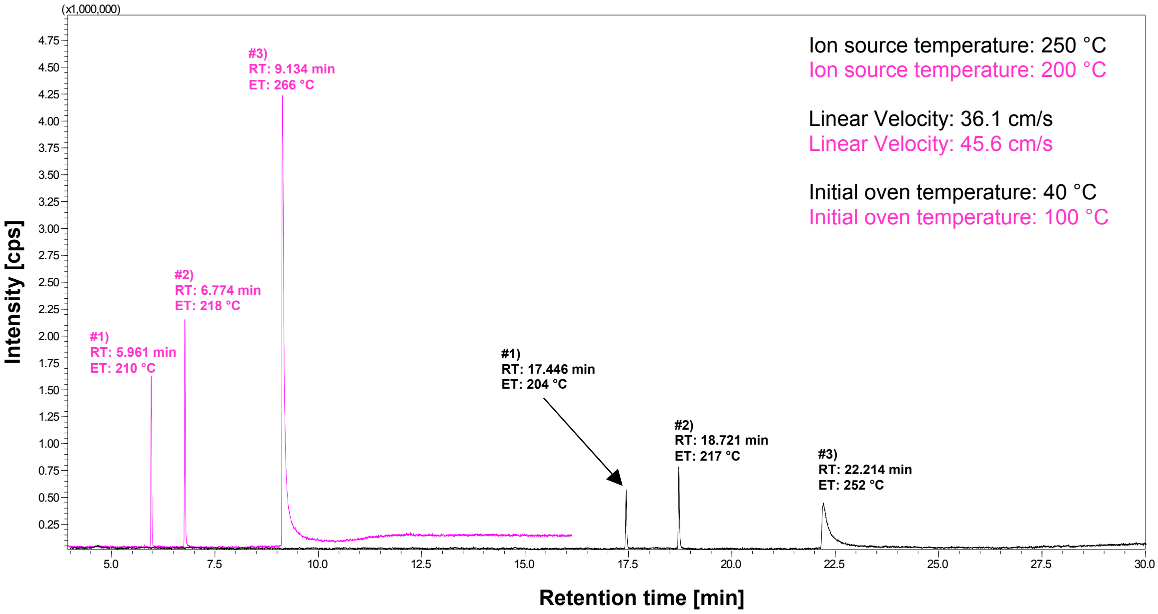


Figure 13: Zoom: Comparison of the results of the scouting gradient (black labeled chromatogram) to those of the optimized method (pink labeled chromatogram) in scan acquisition mode. Concentration of the measured sample in both cases: 100 µg 1-chloromethylnaphthalene, 100 µg 1-naphthylacetonitrile and 1000 µg naphazoline/mL methanol. Peak #1: 1-chloromethylnaphthalene; Peak #2: 1-naphthylacetonitrile; Peak #3: naphazoline.

As can be seen in Figure 13, the signal intensities of all investigated analytes could be improved by increasing the temperature of the ion source from 200°C to 250°C. Further, the analysis time was shortened by increasing the linear velocity from 36.1 cm/s to 45.6 cm/s and changing the heating rate, as well as the initial oven temperature. Despite faster analysis, the peak shapes, as well as the separation efficiency, were maintained.

4.1.3 Choice of the solvent

Since all investigated analytes (1-chloromethylnaphthalene, 1-naphthylacetonitrile and naphazoline) are solid at room temperature (see Table 7), a single solvent had to be chosen which is suitable for all of them. SIM allows detection limits as low as 1 - 10 pg. With the typical injection volume of 1 µL, 1 ppm of a solution with a concentration of 10 mg/mL is equivalent to the absolute amount of 10 pg loaded onto the column. Therefore a solubility of at least 10 mg substance/mL solvent has to be achieved.

Due to the low limit of detection, only solvents with appropriate purity grades can be used for GCMS. The following solvents were tested:

- Ethanol
- Methanol
- Isopropyl alcohol
- n-Hexane
- Dichloromethane

The results regarding the solubility of the tested solvents are shown in Table 10.

Table 10: Results regarding the solubility of the investigated solvents

Solvent	10 mg 1-chloromethylnaphthalene/mL solvent		10 mg 1-naphthylacetonitrile/mL solvent		10 mg naphazoline/mL solvent	
	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble
Methanol	x		x		x	
Ethanol	x		x		x	
Isopropanol	x		x		x	
Dichloromethane	x			x		x
n-Hexane	x			x		x

As expected, only 1-chloromethylnaphthalene was soluble in the non-polar solvents n-hexane and dichloromethane. However, all investigated analytes were completely soluble in the polar solvents methanol, ethanol and IPA. The best solubility was observed in case of methanol (completely dissolved after 5 seconds of vortexing), followed by ethanol (completely dissolved after 10 seconds of vortexing) and IPA (completely dissolved after 15 seconds of vortexing).

Therefore, a polar solvent had to be chosen, although the combination of a polar solvent and a non-polar stationary phase is not optimal and can lead to broad, split, fronting or tailing peaks of early eluting substances. A possibility to circumvent this problem would be the introduction of a retention gap at the beginning of the column. A retention gap is a short piece of a capillary column, which is coated using a phase which matches the sample polarity [10]. Since none of those problems were observed in case of the investigated analytes, a retention gap was not used.

4.1.3.1 Reactions of 1-chloromethylnaphthalene with the solvent methanol

By measuring a sample with a concentration of 20 µg 1-chloromethylnaphthalene/mL methanol, using the settings described in Table 14, the SIM-chromatogram in Figure 14 was obtained.

As can be seen in Figure 14, two more substances (labeled with #1 and #2) were detected in relatively high concentrations besides the target analyte 1-chloromethylnaphthalene (labeled with #3 in Figure 14). Since the purity grade of the used 1-chloromethylnaphthalene was 96.3 %, and the signal intensities reflect approximately equal concentrations, a reaction of 1-chloromethylnaphthalene in the presence of methanol was assumed.

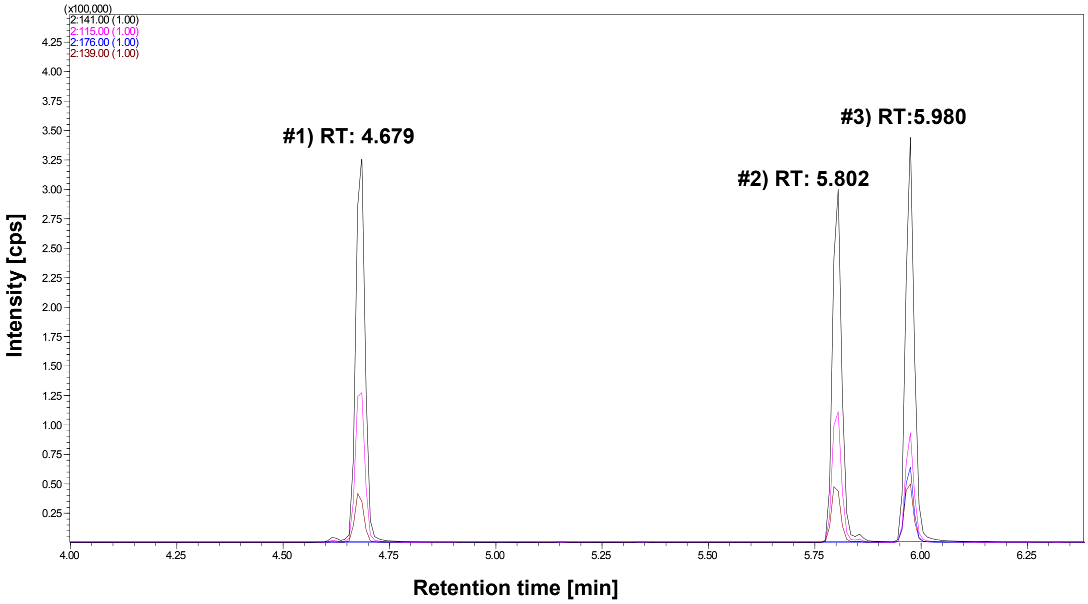


Figure 14: SIM-chromatogram of a sample with a concentration of 20 µg 1-chloromethylnaphthalene/mL methanol using splitless injection. Peak #1: 1-methylnaphthalene. Peak #2: 1-methoxymethylnaphthalene. Peak #3: 1-chloromethylnaphthalene.

The identity of those substances was determined by recording a total ion chromatogram of a sample with 10 µg 1-chloromethylnaphthalene/mL methanol by using the splitless injection technique, and comparing the corresponding mass spectra to the NIST database (see appendix, chapter 6.1). The substance labeled with #1 in Figure 14 was identified as 1-methylnaphthalene, and the substance labeled with #2 in Figure 14 as 1-methoxymethylnaphthalene.

Further literature search revealed that 1-chloromethylnaphthalene reacts in the presence of methanol in a photolytic reaction to 1-methoxymethylnaphthalene [19]. A reaction of 1-chloromethylnaphthalene to 1-methylnaphthalene in the presence of methanol is not known, but it is highly likely, that 1-methylnaphthalene is the result of a side reaction.

4.1.3.2 Investigation of reactions between 1-chloromethylnaphthalene and the solvents ethanol and isopropyl alcohol

Due to the reaction of 1-chloromethylnaphthalene with the solvent methanol, the applicability of the solvents ethanol and isopropyl alcohol was investigated in more detail. Usually a TIC-Scan is performed to identify any possible reactions between the investigated analyte and the solvent. Since the only possible reaction products between 1-chloromethylnaphthalene and the solvents ethanol and IPA are 1-methylnaphthalene and the ether derivatives of 1-methylnaphthalene, respectively a SIM-Scan of the expected fragment ions of 1-methylnaphthalene (m/z 115, 139 and 141) was chosen to check for any reaction products. Therefore, samples with 20 µg 1-chloromethylnaphthelene/mL solvent (Ethanol and IPA) were measured using the settings described in Table 14 to check if any unwanted side products occur.

The results are shown in Figure 15 and Figure 16.

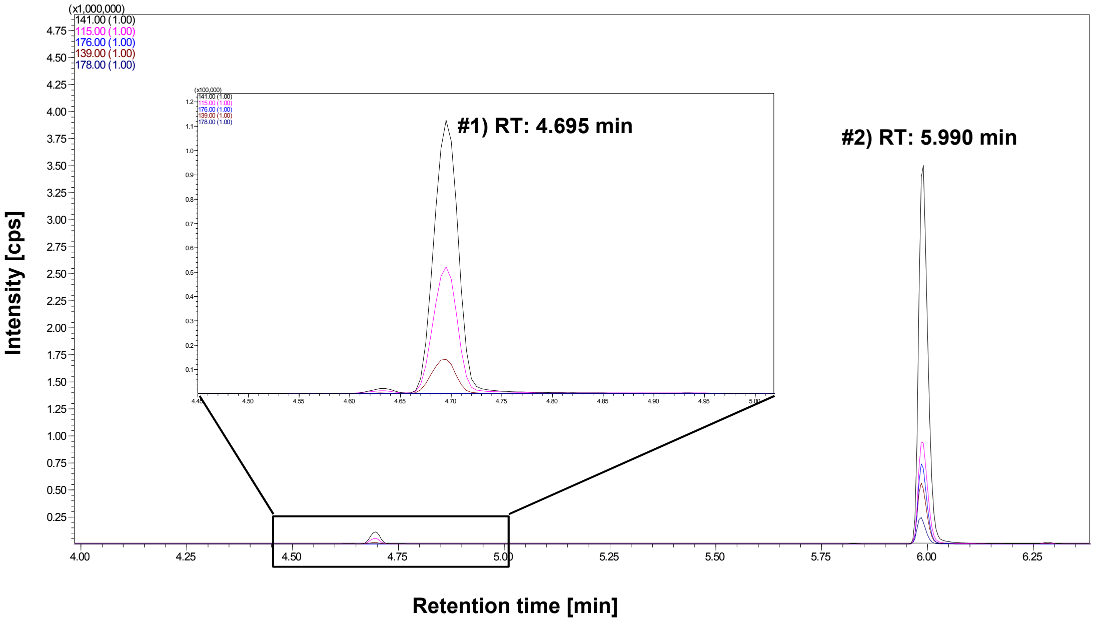


Figure 15: SIM-chromatogram of a sample with a concentration of 20 µg 1-chloromethylnaphthalene/mL ethanol using splitless injection. Peak #1: 1-methylnaphthalene. Peak #2: 1-chloromethylnaphthalene

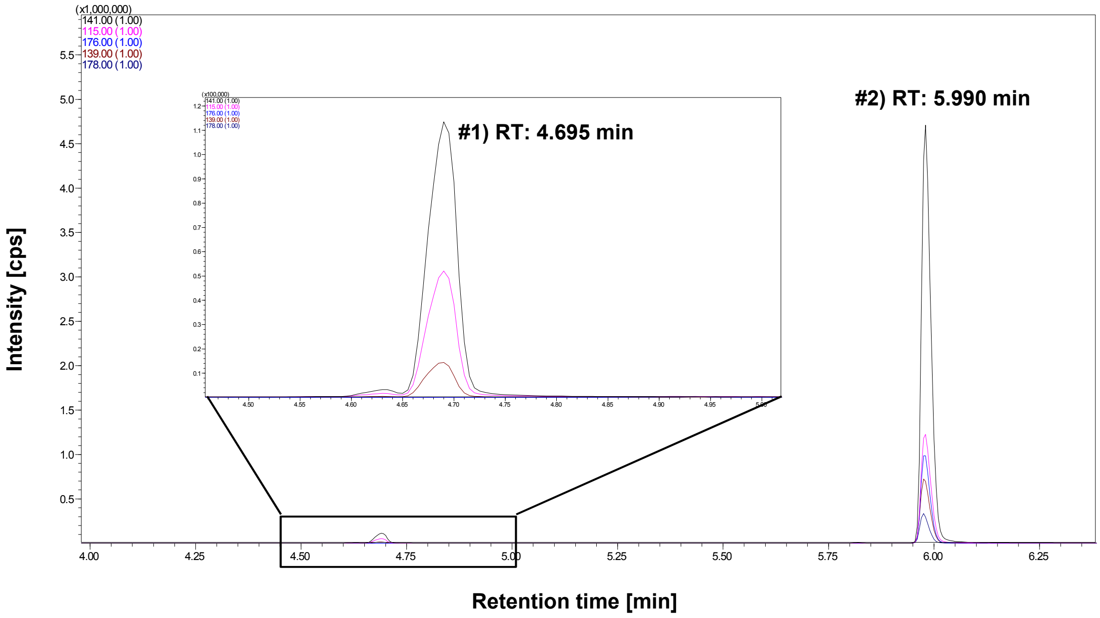


Figure 16: SIM-chromatogram of a sample with a concentration of 20 µg 1-chloromethylnaphthalene/mL IPA using splitless injection. Peak #1: 1-methylnaphthalene. Peak #2: 1-chloromethylnaphthalene

At first sight, both chromatograms seem identical: In both cases, small amounts of a substance with a retention time of 4.695 min were detected besides the main component 1-chloromethylnpahthalene (see Figure 15 and Figure 16). As the retention time and the fragment ion distribution are equal to those of 1-methylnaphthalene (see Figure 14), it is most likely that this substance is 1-methylnaphthalene. Since the purity grade of 1-chloromethylnaphthalene is 96.3 %, the presence of impurities in such low amounts is a reasonable explanation. The fact that the peak height of 1-methylnaphthalene is similar in both cases is also a clue that the low amount of 1-methylnaphthalene is rather an enclosed impurity than a reaction product.

If we take a closer look at the SIM-chromatogram in Figure 15 (used solvent: ethanol), another small peak can be detected at a retention time of 6.285 min (see Figure 17, **A**). Since this peak did not occur when IPA was used as solvent (see Figure 17, **B**), a reaction of 1-chloromethylnaphthalene in the presence of ethanol cannot be ruled out.

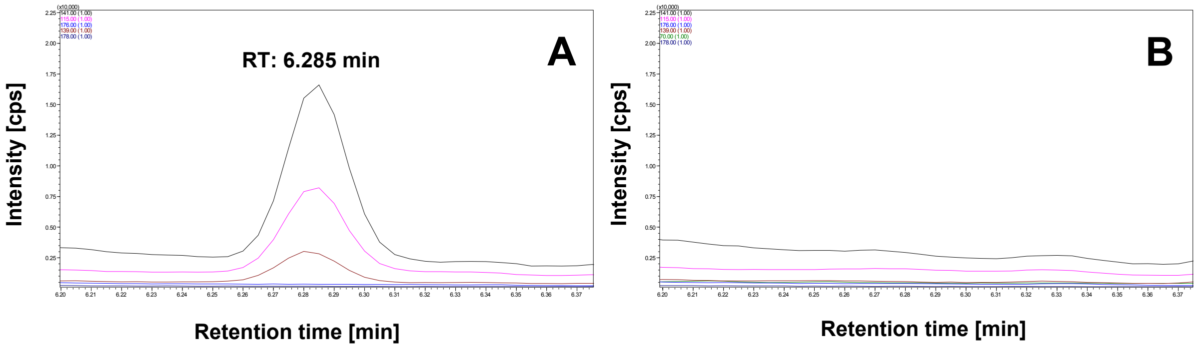


Figure 17: Zoom of the SIM-chromatograms between a retention time of 6.2 and 6.375 min by using ethanol (A) and IPA (B) as solvents

The small peak at a retention time of 6.285 min was not detected immediately, which is why some qualitative experiments were performed with ethanol as solvent. Since there are no significant deviations in separation, retention time and peak shape of 1-chloromethylnaphthalene by using either ethanol or IPA (compare Figure 15 and Figure 16), the results can be seen as generally valid. However, the standard addition experiments for the quantitation of 1-chloromethylnaphthalene in the raw material 1-naphthylacetonitrile were performed with the solvent IPA.

4.1.3.3 Investigation of reactions between 1-naphthylacetonitrile and the solvent ethanol

Since the reaction of 1-chloromethylnaphthalene with ethanol was not discovered immediately (see chapter 4.1.3.2), some experiments of 1-naphthylacetonitrile were performed with the solvent ethanol. In order to check if there are any relevant reactions of the raw material 1-naphthylacetonitrile with ethanol, a sample of 10 mg 1-naphthylacetonitrile/mL ethanol was measured seven times and the peak pattern was compared. Further, a TIC-chromatogram of the pure solvent was recorded between each sample injection to check if there any remaining contaminants in the system left. Details regarding the used settings are listed in Table 11.

Table 11: Settings of GC, MS and Sampler: Investigation of possible reactions between 1-naphthylacetonitrile and ethanol

Gas Chromatograph	
Column	Agilent DB-5MS UI
Length:	30 m
Thickness:	0.25 µm
Diameter:	0.25 mm
Pre-Column:	None
Liner:	Inlet Liner #1
Injection volume:	1 µl
Injection mode:	Splitless
Sampling time:	1 min
Injection temperature:	300 °C
Head Pressure:	112.2 kPa
Linear velocity:	45.6 cm/s
Column flow:	1.5 mL/min
Purge flow:	30 mL/min
Split ratio after sampling time:	1:10
Carrier gas saver:	No

Oven temperature program		
Rate [°C/min]	Final temperature [°C]	Hold time [min]
-	100	1
30	190	0
10	225	0
250	325	5

Mass Spectrometer	
Interface temperature	325 °C
Ion source temperature:	250 °C
Solvent cut time:	3 min
Event Time:	0.3 sec
Start time data acquisition:	3.5 min
End time data acquisition:	6.39 min
Acquisition mode:	Q3-SIM
Detector voltage relative to the tuning result:	0 kV
Recorded m/z values:	115
	139
	141
	176
	178

Sampler	
# of rinses with solvent (Pre-run):	8
# of rinses with solvent (Post-run):	8
# of rinses with sample:	1
Washing volume:	6 µl

Since an overlay of all chromatograms would be too confusing, only the first and the last measurement are compared in more detail (period of 5 hours in between). Figure 18 shows the chromatograms of the first (black labeled) and the seventh (pink labeled) measurement (overlay of all seven chromatograms is shown in the appendix, see chapter 6.3). Each chromatogram represents the sum of the detected ions with m/z values of 115, 139, 141, 176 and 178. The two main peaks in Figure 18 were identified in the course of the experiments described in chapter 4.1.5. In order to determine possible deviations for low abundant substances, a closer look was taken by zooming into the chromatogram shown in Figure 18 (zoom see Figure 19).

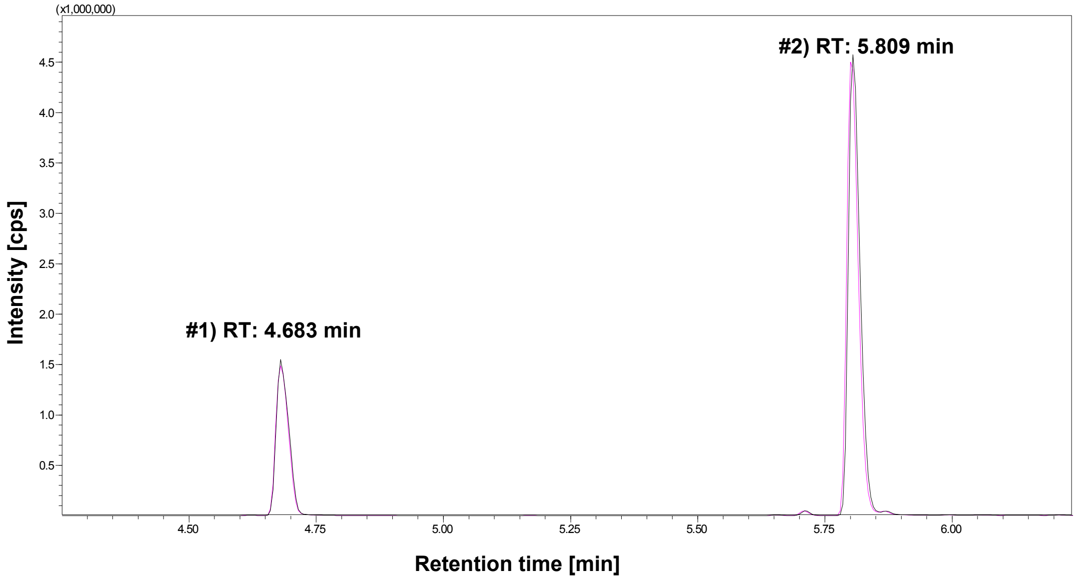


Figure 18: Overlay of the chromatograms of the first (black labeled) and the seventh (pink labeled) measurement. Sample containing 10 mg 1-naphthylacetonitrile/mL ethanol. Each chromatogram represents the sum of the detected ions with m/z values of 115, 139, 141, 176 and 178. Peak #1: 1-methylnaphthalene. Peak #2: 1-methoxymethylnaphthalene

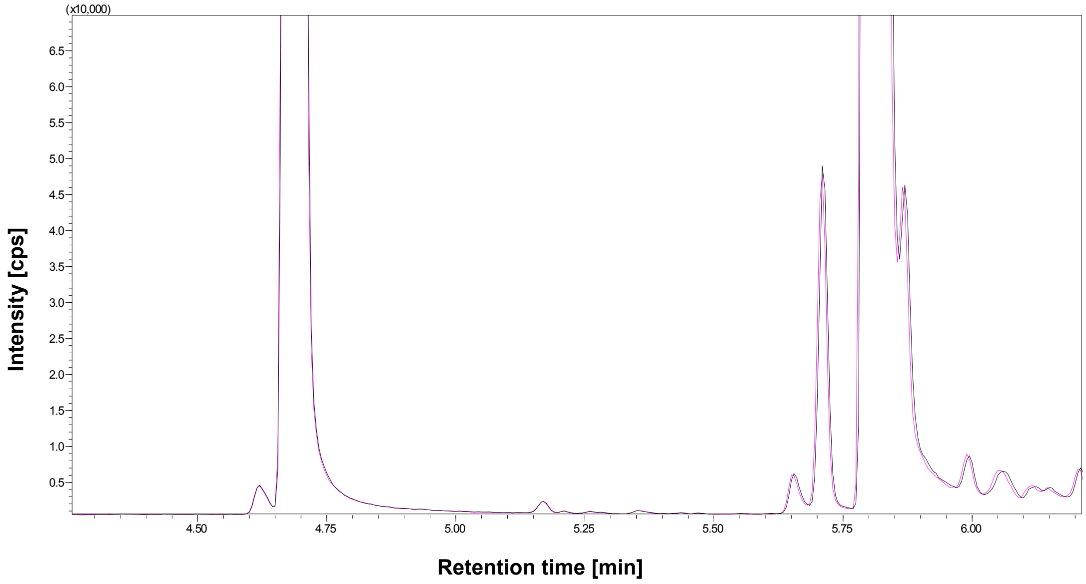


Figure 19: Zoom: Overlay of the chromatograms of the first (black labeled) and the seventh (pink labeled) measurement. Sample containing 10 mg 1-naphthylacetonitrile/mL ethanol. Each chromatogram represents the sum of the detected ions with m/z values of 115, 139, 141, 176 and 178.

Figure 18 and Figure 19 show no significant deviations in the peak pattern. In order to show this in an exemplary way, the peak areas of m/z 141 of 1-methylnaphthalene (Peak #1 in Figure 18) and 1-methoxymethylnaphthalene (Peak #2 in Figure 18) were compared throughout the seven runs.

Table 12: Investigation of reactions between 1-naphthylacetonitrile and the solvent ethanol

	# of Analysis	Peak area m/z 141 1-methylnaphthalene (Peak #1 in Figure 18) [AU]	Peak area m/z 141 1-methoxymethylnaphthalene (Peak #2 in Figure 18) [AU]
Sample: 10 mg 1-naphthylacetonitrile/mL Ethanol	1	1666708	4789129
	2	1706417	4942528
	3	1711861	4965291
	4	1704655	4898798
	5	1551800	4451858
	6	1602201	4670906
	7	1635728	4720795
Average		1654196	4777044
Standard Deviation		60943	181644
RSD [%]		3.7	3.8

As can be seen in Table 12, there is no significant change in the investigated peak areas. Further, no increasing or decreasing trend of the peak areas could be observed. Relevant reactions of the raw material 1-naphthylacetonitrile with the solvent ethanol (within 5 hours) can, therefore, be excluded. Further, the TIC-chromatograms of the pure solvent between each sample injection showed no contamination in the investigated range.

4.1.3.4 Investigation of reactions between 1-naphthylacetonitrile and the solvent isopropyl alcohol

Due to the observed reactions of 1-chloromethylnaphthalene with methanol and ethanol, it had to be checked, if there are any relevant reactions of the raw material 1-naphthylacetonitrile in the presence of the solvent IPA. This was done by measuring a sample of 10 mg raw material 1-naphthylacetonitrile/mL IPA eleven times as described in chapter 4.2.1.1 and Table 21 and comparing the obtained chromatograms. Compared to the procedure described in 4.1.3.3, the following adaptions were made: methanol was used as washing solution for the syringe and the number of rinses was increased; bake out of the column was extended; sample inlet was purged with higher volumes of carrier gas; interface temperature was increased (further details see chapter 4.2.1.1).

Only the first and the eleventh measurement are compared in more detail (period of twelve hours in between). Figure 20 shows the chromatograms of the first (black labeled) and the eleventh (pink labeled) measurement (overlay of all chromatograms is shown in the appendix, see chapter 6.3). Each chromatogram represents the sum of the detected ions with m/z values of 115, 139, 141, 176 and 178. The two main peaks in Figure 20 were identified in the course of the experiments described in chapter 4.1.5. In order to determine possible deviations for low abundant substances, a closer look was taken by zooming into the chromatogram shown in Figure 20 (zoom see Figure 21).

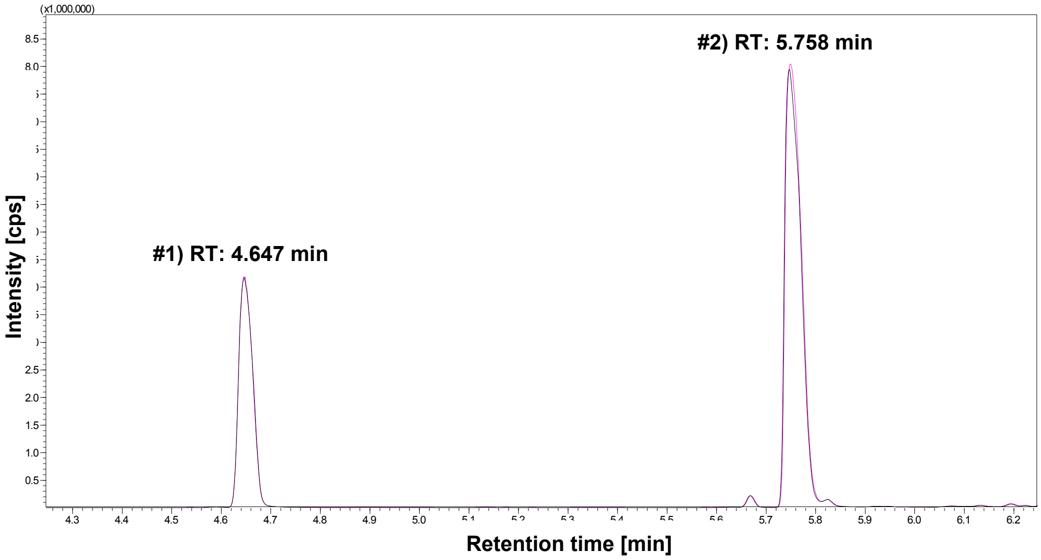


Figure 20: Overlay of the chromatograms of the first (black labeled) and the eleventh (pink labeled) measurement. Sample containing 10 mg 1-naphthylacetonitrile/mL IPA. Each chromatogram represents the sum of the detected ions with m/z values of 115, 139, 141, 176 and 178. Peak #1: 1-methylnaphthalene. Peak #2: 1-methoxymethylnaphthalene

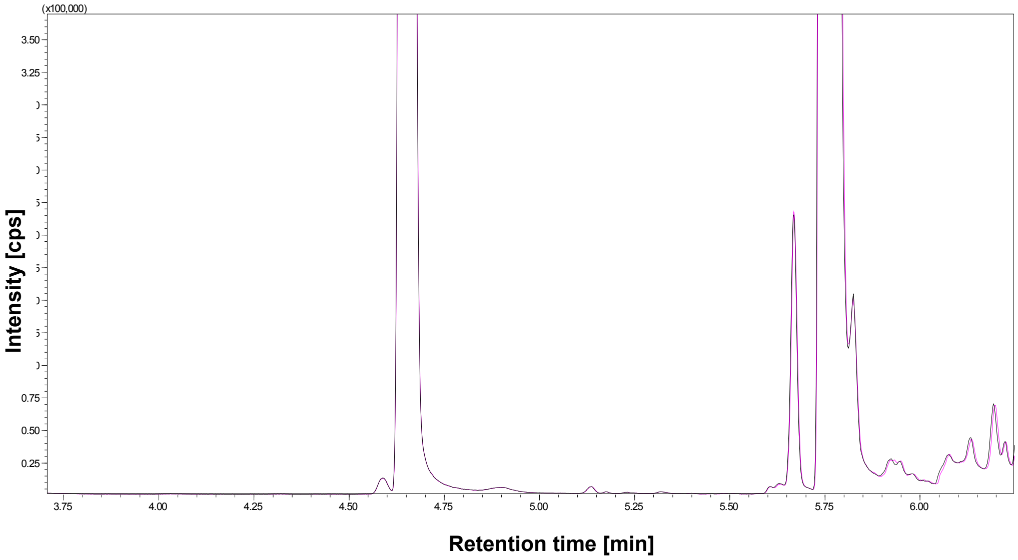


Figure 21: Zoom: Overlay of the chromatograms of the first (black labeled) and the eleventh (pink labeled) measurement. Sample containing 10 mg 1-naphthylacetonitrile/mL IPA. Each chromatogram represents the sum of the detected ions with m/z values of 115, 139, 141, 176 and 178.

Figure 20 and Figure 21 show no significant deviations in the peak pattern and the corresponding peak areas. In order to show this in an exemplary way, the peak areas of m/z 141 of 1-methylnaphthalene (Peak #1 in Figure 20) and 1-methoxymethylnaphthalene (Peak #2 in Figure 20) were compared throughout the eleven runs.

Table 13: Investigation of reactions between 1-naphthylacetonitrile and the solvent IPA

	# of Analysis	Peak area m/z 141 1-methylnaphthalene (Peak #1 in Figure 20) [AU]	Peak area m/z 141 1-methoxymethylnaphthalene (Peak #2 in Figure 20) [AU]
Sample: 10 mg 1-naphthylacetonitrile/mL IPA	1	5427822	12256978
	2	5506272	12406590
	3	5574443	12571385
	4	5579242	12784401
	5	5576464	12713701
	6	5634125	12712487
	7	5559330	12595352
	8	5619250	12703729
	9	5576970	12780308
	10	5582597	12671973
	11	5544057	12678742
Average		5561870	12625059
Standard Deviation		55882	162247
RSD [%]		1.00	1.29

As can be seen in Table 13, there is no significant change in the investigated peak areas. Further, no increasing or decreasing trend of the peak areas could be observed. Relevant reactions of the raw material 1-naphthylacetonitrile with the solvent IPA (within twelve hours) can, therefore, be excluded. The measurements of the pure solvent between each sample injection showed no contamination in the investigated range.

4.1.4 Detection and separation of 1-chloromethylnaphthalene in the raw material 1-naphthylacetonitrile

To check if the target analyte 1-chloromethylnaphthalene can be detected in the raw material 1-naphthylacetonitrile in the range of ppm, a sample of 10 mg raw material 1-naphthylacetonitrile/mL ethanol was spiked with 10 ppm 1-chloromethylnaphthalene and measured by SIM.

Sample:

Solvent:	Ethanol
Concentration:	10 mg 1-Naphtylacetonitrile (raw material)/mL 0.1 µg 1-Chloromethylnaphthalene/mL (=10 ppm related to 1-naphthylacetonitrile)

Due to the same core structure, 1-chloromethylnaphthalene shares some of its most intense, specific fragment ions with 1-naphthylacetonitrile (m/z: 115, 139, 141; compare Figure 10 and Figure 11). To avoid saturation and further damage of the detector through the high concentration of 1-naphthylacetonitrile (10 mg/mL), the scan time was adjusted.

For time-saving reasons, the temperature program of Table 9 was modified by increasing the heating rate after the complete elution of 1-chloromethylnaphthalene. Details regarding the used settings are listed in Table 14.

Table 14: Settings of GC, MS and Sampler: Detection and Separation of 1-Chloromethylnaphthalene in the raw material

Gas Chromatograph	
Column	Agilent DB-5MS UI
Length	30 m
Thickness	0.25 µm
Diameter	0.25 mm
Pre-Column:	None
Liner:	Inlet Liner #2
Injection volume:	1 µL
Injection mode:	Splitless
Sampling time:	1 min
Injection temperature:	300 °C
Head pressure:	112.2 kPa
Linear velocity:	45.6 cm/s
Column flow:	1.5 mL/min
Purge flow:	3 mL/min
Split ratio after sampling time:	1:10
Carrier gas saver:	No

Oven temperature program		
Rate [°C/min]	Final temperature [°C]	Hold time [min]
-	100	1
30	190	0
10	225	0
250	325	5

Mass Spectrometer	
Interface temperature	310 °C
Ion source temperature:	250 °C
Solvent cut time:	3 min
Event time:	0.1 sec
Start time data acquisition:	3 min
End time data acquisition:	6.39 min
Acquisition mode:	Q3-SIM
Detector voltage relative to the tuning result:	0 kV
Recorded m/z values:	115
	139
	141
	176
	178

Sampler	
# of rinses with solvent (Pre-run):	1
# of rinses with solvent (Post-run):	2
# of rinses with sample:	0
Washing volume:	6 µL

The obtained SIM-chromatogram is shown in Figure 22.

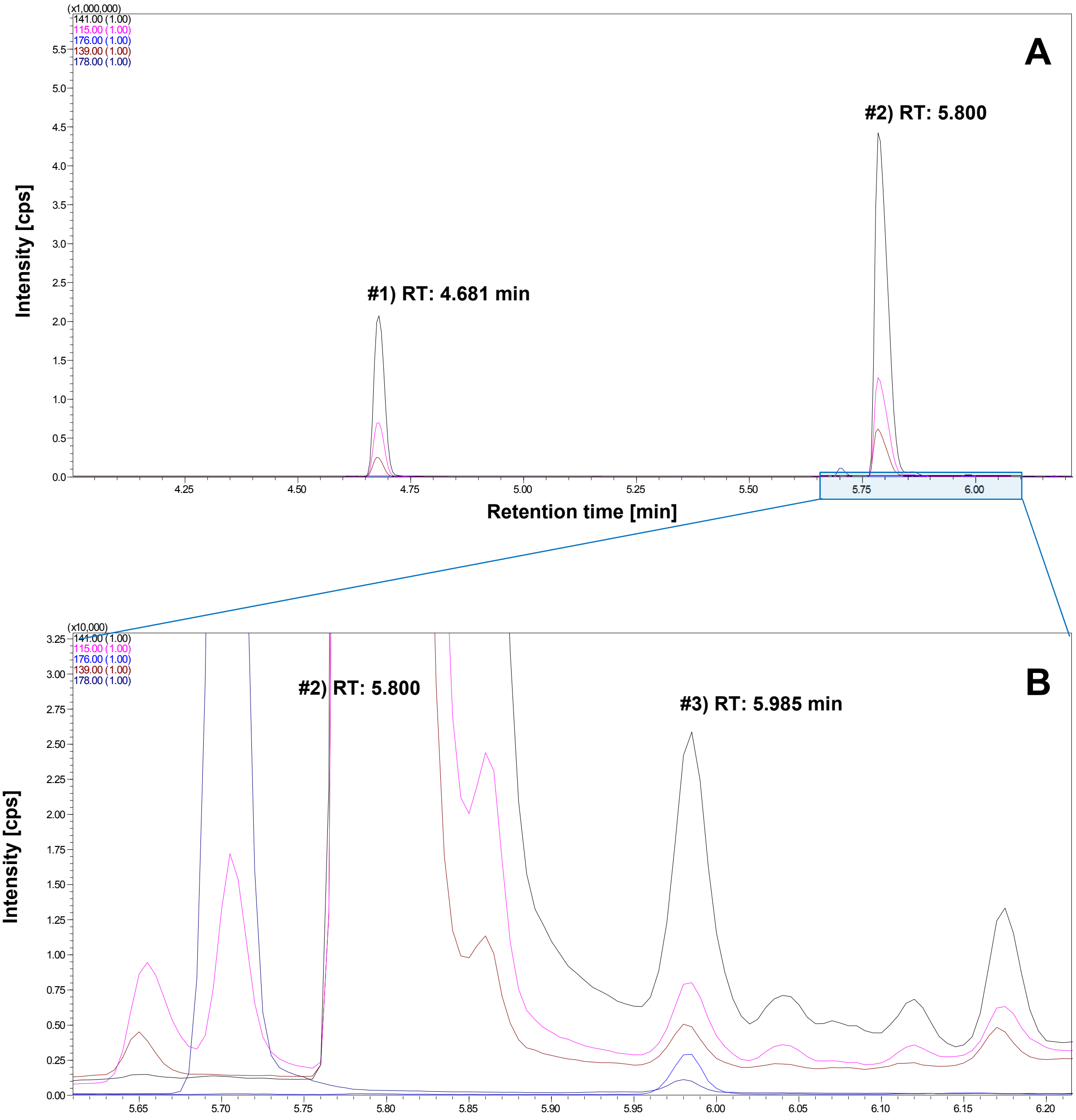


Figure 22: A: SIM-chromatogram of a sample with a concentration of 10 mg 1-naphthylacetonitrile/mL ethanol spiked with 10 ppm 1-chloromethylnaphthalene. Most abundant impurities of the raw material: Peak#1: 1-methylnaphthalene; Peak#2: 1-methoxymethylnaphthalene. B: Zoom of the blue box in A. Peak#3: 1-Chloromethynaphthalene

1-chloromethylnaphthalene (peak #3 in Figure 22 (B)) could be identified by its retention time and the presence of its specific fragment ions. Several other impurities, which share some of the investigated m/z ratios, were also detected. As can be seen in Figure 22 (B), some of them overlap with the most intense fragment ions of 1-chloromethynaphthalene. The most abundant impurities could be identified as 1-methylnaphthalene (peak #1 in Figure 22 (A)) and 1-methoxymethylnaphthalene (peak #2 in Figure 22 (A)) in the course of the experiments described in chapter 4.1.5.

A closer look at the peak of 1-chloromethylnaphthalene, peak #3 in Figure 22 (B), reveals that the molecule ion with a mass to charge ratio of 176 is baseline separated. Therefore, the standard addition procedure is an option for the quantitation of 1-chloromethylnaphthalene in the raw material 1-naphthylacetonitrile in the range of ppm.

4.1.5 Screening for Impurities

4.1.5.1 Impurities in the raw material 1-naphthylacetonitrile

Qualitative analysis showed that there are several other impurities in the raw material besides 1-chloromethylnaphthalene (see chapter 4.1.4). To obtain an impurity profile of the raw material, a TIC of a sample with 10 mg raw material 1-naphthylacetonitrile/mL ethanol was recorded. To avoid saturation and further damage of the detector through the high concentration of 1-naphthylacetonitrile, the scan time was adjusted. Therefore, only impurities with lower boiling points than 1-naphthylacetonitrile could be detected. Further, the split injection technique was used to improve the quality of the chromatography by applying only a very narrow zone of the sample onto the column [21]. Since the aim of this experiment was to obtain qualitative information regarding present impurities, sample discrimination was no problem. Details regarding the used settings are listed in Table 15.

Table 15: Settings of GC, MS and Sampler: Screening for impurities in the raw material 1-naphthylacetonitrile

Gas Chromatograph	
Column	Agilent DB-5MS UI
Length:	30 m
Thickness:	0.25 µm
Diameter:	0.25 mm
Pre-Column:	None
Liner:	Inlet Liner #2
Injection volume:	0.5 µL
Injection mode:	Split
Injection temperature:	300 °C
Head pressure:	112.2 kPa
Linear velocity:	45.6 cm/s
Column flow:	1.5 mL/min
Purge flow:	3 mL/min
Split ratio:	1:10
Carrier gas saver:	No

Oven temperature program		
Rate (°C/min)	Final temperature (°C)	hold Time [min]
-	100	1
30	190	0
10	225	0
250	325	15

Mass Spectrometer	
Interface temperature	325 °C
Ion source temperature:	250 °C
Solvent cut time:	2 min
Event time:	0.3 sec
Start time data acquisition:	2 min
End time data acquisition:	6.5 min
Acquisition mode:	Q3-Scan
Recorded m/z values:	50 – 1090
Detector voltage relative to the tuning result:	0 kV

Sampler	
# of rinses with solvent (Pre-run):	3
# of rinses with solvent (Post-run):	3
# of rinses with sample:	1
Washing volume:	6 µL

The obtained TIC-chromatogram is shown in Figure 23. The labeled peaks in Figure 23 are described in Table 16 in more detail. The identification of those peaks was done by comparing the corresponding mass spectra with the NIST database. In some cases, several structurally related compounds were listed as potential candidates. In these instances the substance which was reasonable to be present and additionally had a high similarity with the spectrum of the database was chosen. A definite confirmation regarding the determined identities in Table 16 is at this time not possible. Further investigations can be performed but were not of interest for this thesis.

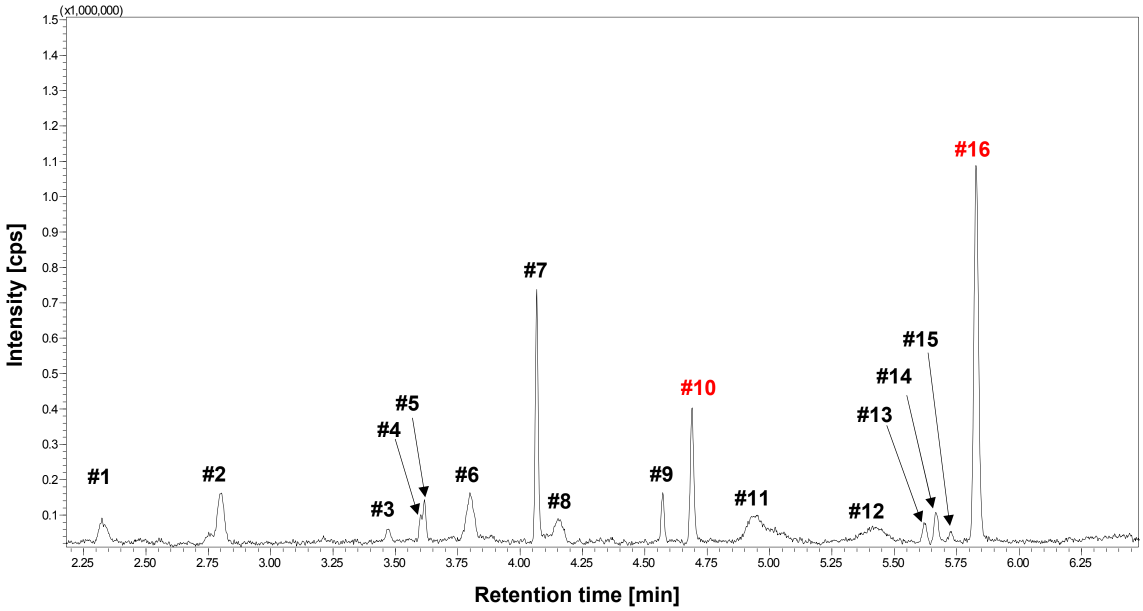


Figure 23: Screening for impurities in the raw material: TIC-chromatogram of a sample with a concentration of 10 mg 1-naphthylacetonitrile/mL ethanol using split injection (split ratio: 1:10)

Table 16: Screening for impurities: Identified impurities in the raw material 1-naphthylacetonitrile. The red labeled substances feature some of the most intense fragment ions of 1-chloromethylnaphthalene and were also observed in the SIM-chromatograms of 1-naphthylacetonitrile (see chapter 4.1.4)

Peak #	Substance	Retention time	Similarity [%]
1	2,3-Dimethylhexane	2.324	92
2	2,4-Dimethyl-1-Heptene	2.800	93
3	3-Ethylheptane	3.471	83
4	o-Tolualdehyde	3.502	89
5	Undecane	3.616	91
6	3,3-Dimethyloctane	3.802	90
7	Naphthalene	4.066	96
8	4,5-Dimethyl-2-Undecene	4.147	86
9	n-Dodecane	4.566	93
10	1-Methylnaphthalene	4.688	92
11	1,3-Di-tert-butylbenzene	4.717	86
12	Oxalic acid-6-ethyloct-3-yl-hexylester	5.416	82
13	Tridecane	5.623	85
14	1-Naphthalenecarboxaldehyde	5.664	88
14	2-Propyl-Benzothiophene	5.724	65
16	1-Methoxymethylnaphthalene	5.826	94

The red labeled substances in Table 16 and Figure 23 feature some of the most intense fragment ions of 1-chloromethylnaphthalene. Therefore, they were also observed in the SIM-chromatograms of 1-naphthylacetonitrile (see chapter 4.1.4). The corresponding mass spectra of those two substances are shown in the appendix (chapter 6.2). 1-chloromethylnaphthalene itself was not detected in the course of this experiment because of its low concentration in the raw material.

4.1.5.2 Impurities in the product naphazoline

As a matter of interest, the experiment described in 4.1.5.1 was also carried out analogously for a sample of 10 mg naphazoline/mL IPA. Since split injection led to no signals, the sample was measured with the splitless injection technique. Details regarding the used setting are listed in Table 17.

Table 17: Settings of GC, MS and Sampler: Screening for impurities in the product naphazoline

Gas Chromatograph		**Mass Spectrometer**	
Column	Agilent DB-5MS UI	Interface temperature	325 °C
Length	30 m	Ion Source temperature:	250 °C
Thickness	0.25 µm	Solvent cut time:	2 min
Diameter	0.25 mm	Event time:	0.1 sec
Pre-Column:	None	Start time data acquisition:	2 min
Liner:	Inlet Liner #2	End time data acquisition:	8 min
Injection volume:	0.5 µL	Acquisition mode:	Q3-Scan
Injection mode:	Splitless	Recorded m/z values:	50 – 1090
Sampling time:	1 min	Detector voltage relative to the tuning result:	0 kV
Injection temperature:	300 °C	**Sampler**	
Head pressure:	112.2 kPa	# of rinses with solvent (Pre-run):	1
Linear velocity:	45.6 cm/s	# of rinses with solvent (Post-run):	6
Column flow:	1.5 mL/min	# of rinses with sample:	1
Purge flow:	3 mL/min	Washing volume:	6 µL
Split ratio after sampling time:	1:10		
Carrier gas saver:	No		

Oven temperature program		
Rate (°C/min)	Final temperature (°C)	Hold time [min]
-	100	1
30	190	0
10	225	0
250	325	15

The obtained TIC-chromatogram is shown in Figure 24. The labeled peak in Figure 24 is described in Table 18 in more detail. The identification of this peak was done by comparing the corresponding mass spectra with the NIST database.

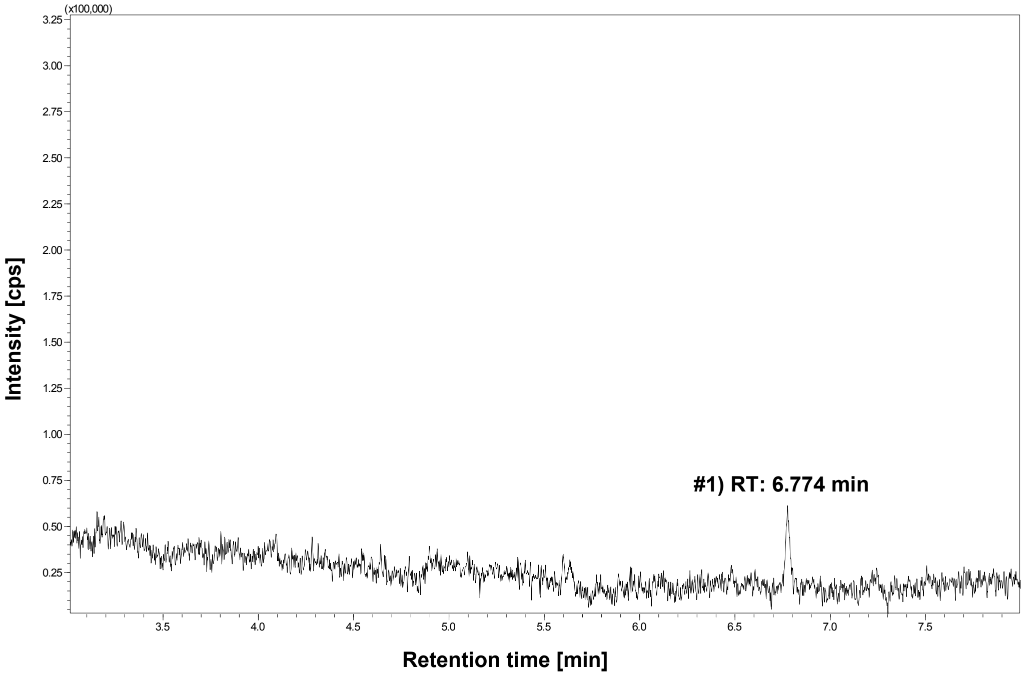


Figure 24: Screening for impurities in the final product: TIC-chromatogram of a sample with 10 mg naphazoline/mL IPA using splitless injection

Table 18: Screening for impurities: Identified impurities in the product naphazoline

Peak #	Substance	Retention time	Similarity [%]
1	1-Naphthylacetonitrile	6.774	80

As can be seen in Figure 24 and Table 18, the raw material 1-naphthylacetonitrile was the only impurity which could be detected in the final product. The similarity of only 80 % is due to the low signal of the detected substance and the rather high background signals (recorded m/z values: 50 - 1090) giving also a noisy mass spectrum, resulting in worse spectral similarities.

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4.2 Quantitative method development

4.2.1 Peak area reproducibility within a batch

The method described in Table 14 allows the baseline-separated detection of the ion m/z 176 of 1-chloromethylnaphthalene in the raw material 1-naphthylacetonitrile in the ppm-range (compare to chapter 4.1.4). To make sure that this method delivers reproducible results, six samples with 10 mg raw material 1-naphthylacetonitrile/mL ethanol were prepared individually and measured one after another with the settings described in Table 14. Details of the sample preparation are listed in Table 19. This approach was repeated with the same samples, 1.5 hours after the first measurement cycle was finished.

Table 19: Investigation of peak area reproducibility within a batch: Sample Preparation

#	Weighed portion of raw material [mg]	Volume ethanol [mL]	Concentration [mg/mL]
1	179.7	17.97	10
2	189.1	18.91	
3	174.1	17.41	
4	191.4	19.14	
5	189.2	18.92	
6	183.6	18.36	

The results are shown in Figure 25 and Table 20.

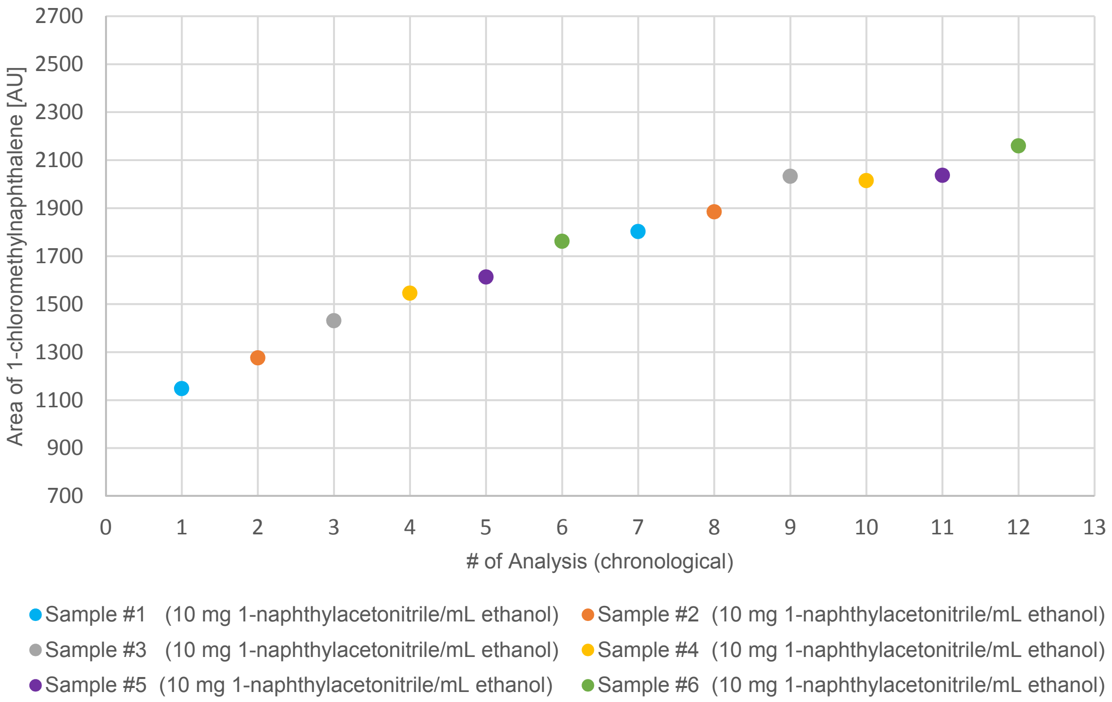


Figure 25: Peak areas for each sample measured in two batches

Table 20: Peak areas for each sample measured in two batches

	# of Analysis	Sample #	Composition	Area m/z 176 1-chloromethylnaphthalene [AU]
Batch #1	1	Sample #1	10 mg 1-naphthylacetonitrile/mL ethanol	1148
	2	Sample #2		1276
	3	Sample #3		1431
	4	Sample #4		1546
	5	Sample #5		1613
	6	Sample #6		1762
Batch #2	7	Sample #1		1803
	8	Sample #2		1885
	9	Sample #3		2033
	10	Sample #4		2015
	11	Sample #5		2037
	12	Sample #6		2160

As shown in Figure 25 and Table 20, the peak area of 1-chloromethylnaphthalene increased significantly with ongoing measurements of the six individual samples, although the sample concentration remained the same. The trend of increasing peak areas continued by measuring the same samples again. Therefore, an accumulation of the sample in the system was assumed.

4.2.1.1 Removing of/screening for remaining contaminants

Due to the results of the experiments regarding the peak area reproducibility within a batch, it was assumed that some of the sample remained in the system after injection. In order to remove remaining sample residues and to make sure that there are no contaminations left after each sample injection, the procedure (settings see Table 14) was adapted in the following way:

1. Methanol was used as washing solution for the syringe due to its increased solubility and the number of rinses (Post-run) was increased from 2 (see Table 14) to 6
2. Bake out of the column was extended from 5 min (see Table 14) to 15 min
3. Sample inlet was purged with high volumes of carrier gas after each sample measurement to remove possible remaining contaminants through the split valve (Split ratio after sampling time increased from 1:10 to 1:50; total flow increased from 46.5 mL/min to 81.5 mL/min)
4. Pure solvent was measured between individual sample injections to screen for remaining contaminants in the system by recording a
    - TIC-Scan
    - SIM-Scan

In order to avoid condensation of high boiling point analytes in the interface, the interface temperature was increased from 310°C (see Table 14) to the maximum column temperature of 325°C. The adapted settings are listed in Table 21.

Table 21: Settings of GC, MS and Sampler: Removing of/Screening for remaining contaminants

Gas Chromatograph	
Column	Agilent DB-5MS UI
Length:	30 m
Thickness:	0.25 µm
Diameter:	0.25 mm
Pre-Column:	None
Liner:	Inlet Liner #2
Injection volume:	1 µL
Injection mode:	Splitless
Sampling time:	1 min
Injection temperature:	300 °C
Head pressure:	112.2 kPa
Linear velocity:	45.6 cm/s
Column flow:	1.5 mL/min
Purge flow:	5 mL/min
Split ratio after sampling time:	50
Carrier gas saver:	Yes (Split ratio 1:10 after 6 min)

Oven temperature program		
Rate (°C/min)	Final temperature (°C)	Hold time [min]
-	100	1
30	190	0
10	225	0
250	325	15

Mass Spectrometer:	
Interface temperature	325 °C
Ion source temperature:	250 °C
Solvent cut time:	3 min
Event time:	0.05 sec
Start time data acquisition:	3.5 min
End time data acquisition:	6.39 min
Acquisition mode:	Q3-SIM
Detector voltage relative to the tuning result:	0 kV
Recorded m/z values:	115
	139
	141
	176
	178

Sampler	
# of rinses with methanol (Pre-run):	1
# of rinses with methanol (Post-run):	6
# of rinses with sample:	1
Washing volume:	6 µL

The effectiveness of the applied adaptions was checked in the course of the standard addition experiments described in 4.2.3. To make sure that the obtained signals are reproducible, the untreated sample was measured three times before and three times after the standard addition experiments, carried out on the following test dates:

- 10.08.2018
- 12.08.2018
- 19.08.2018

The results of the experiments described above are shown in Table 22.

Table 22: Results of the applied actions to remove remaining contaminants

		Test date: 10.08.2018	Test date: 12.08.2018	Test date: 19.08.2018
Sample	# of Analysis	Area m/z 176 1-chloromethylnaphthalene [AU]	Area m/z 176 1-chloromethylnaphthalene [AU]	Area m/z 176 1-chloromethylnaphthalene [AU]
10 mg 1-naphthylacetonitrile/mL IPA	1	1457	1372	1678
	2	1387	1399	1557
	3	1481	1395	1527
	4	1416	1317	1602
	5	1430	1314	1648
	6	1393	1382	1527
	Average	**1427**	**1363**	**1590**
	RSD [%]	**2.56**	**2.80**	**4.01**

As shown in Table 22, no trend of increasing peak areas, as well as a good peak area reproducibility of the unspiked sample, were observed with ongoing measurements.

4.2.2 Internal Standardization

In order to compensate for all possible volume errors and variations in the function of the instrument, naphthalene-d_8 was chosen as internal standard.

4.2.2.1 Internal standard: 1-Methoxymethylnaphthalene

The impurity 1-methoxymethylnaphthalene (see chapter 4.1.5) of the raw material 1-naphthylacetonitrile was temporarily used as internal standard (m/z 141 was used for internal standardization) to set up the method. Since all spiking levels of a sample contain the same amount of the impurity 1-methoxymethylnaphthalene, no further sample preparation was necessary.

The improvement of using the internal standardization is shown by correcting the peak areas of 1-chloromethylnaphthalene listed in Table 22. The results are summed up in Table 23 (corresponding values for the calculation are shown in the appendix, chapter 6.4). The comparison of the relative standard deviations in Table 22 and Table 23 shows, that the internal standardization leads to an improvement of the peak area reproducibility.

Table 23: Results of the applied actions to remove remaining contaminants corrected with the internal standard 1-methoxymethylnaphthalene (m/z 141 of 1-methoxymethylnaphthalene was used for internal standardization)

		Test date: 10.08.2018	Test date: 12.08.2018	Test date: 19.08.2018
Sample	# of Analysis	Corrected Area m/z 176 1-chloromethylnaphthalene [AU]	Corrected Area m/z 176 1-chloromethylnaphthalene [AU]	Corrected Area m/z 176 1-chloromethylnaphthalene [AU]
10 mg 1-naphthylacetonitrile/mL IPA	1	1.49E-04	1.39E-04	1.31E-04
	2	1.42E-04	1.41E-04	1.24E-04
	3	1.51E-04	1.40E-04	1.21E-04
	4	1.47E-04	1.37E-04	1.28E-04
	5	1.48E-04	1.43E-04	1.31E-04
	6	1.46E-04	1.47E-04	1.24E-04
	Average	**1.47E-04**	**1.41E-04**	**1.27E-04**
	RSD [%]	**2.03**	**2.37**	**3.23**

Although the internal standardization using the impurity 1-methoxymethylnaphthalene leads to an improvement of the results, there are several reasons why this practice is not suitable for the routine: The amount of the impurity 1-methoxymethylnaphthalene can vary from sample to sample and it is also likely that no 1-methoxymethylnaphthalene is present in the raw material 1-naphthylacetonitrile. The comparison of different samples, as well as the check of the system performance are therefore not possible by using the incorporated impurity 1-methoxymethylnaphthalene of the raw material. Thus, the implementation of naphthalene-d_8 (or another suitable substance) as internal standard was indispensable.

4.2.2.2 Internal standard: Naphthalene-d_8

A sample of 10 mg raw material 1-naphthylacetonitrile/mL IPA spiked with 10 ppm 1-chloromethylnaphthalene and 5 ppm naphthalene-d_8 was prepared and measured with the settings given in Table 27 (see chapter 4.2.4.1). Since naphthalene-d_8 is a quite stable compound, electron impact ionization mainly leads to the formation of the corresponding radical cation (m/z 136). Fragment ions of naphthalene-d_8 only have small intensities, which is the reason why only the radical cation with m/z 136 was measured for the detection and analysis of naphthalene-d_8. Figure 26 shows the results of the experiment.

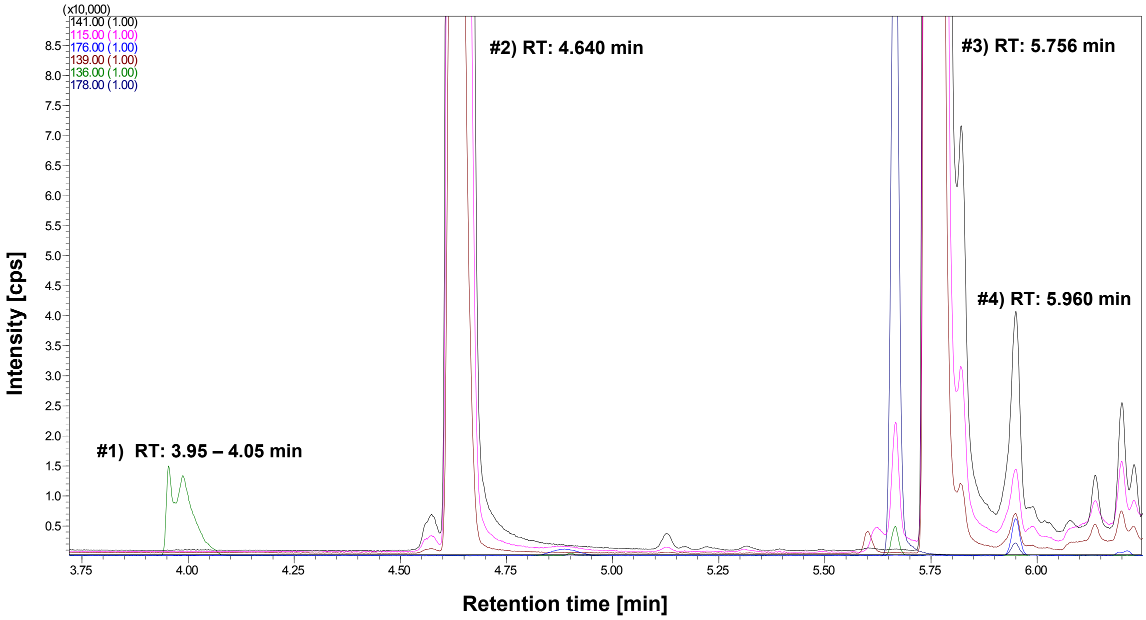


Figure 26: SIM-Chromatogram of a sample with a concentration of 10 mg 1-naphthylacetonitrile/mL IPA spiked with 10 ppm 1-chloromethylnaphthalene and 5 ppm naphthalene-d_8. Settings of Table 27 were used for the measurement. Peak #1) naphthalene-d_8; Peak #2) 1-methylnaphthalene; Peak #3) 1-methoxymethylnaphthalene; Peak #4) 1-chloromethylnaphthalene

As can be seen in Figure 26, the analysis of naphthalene-d_8 (base peak m/z: 136) resulted in a split peak by using the settings of Table 27. The combination of a polar solvent and a non-polar stationary phase is not optimal and can lead to broad, split, fronting or tailing peaks of early eluting substances [10]. A possibility to circumvent the application of a retention gap could be the use of the solvent effect: The initial oven temperature is set 10 to 20°C below the boiling point of the solvent, which allows the analytes to condense and further to focus on the head of the column. In turn, the analytes bandwidth decreases and the risk of split peaks can be reduced [22].

In addition to the adaption of the solvent effect, a pre-column was introduced to protect the analytical column against non-volatile impurities (details see chapter 4.2.6). In order to check the effectivity of these two adaptions, a sample with a composition of 10 mg raw material 1-naphthylacetonitrile/mL IPA was spiked with 10 ppm 1-chloromethylnaphthalene and 5 ppm naphthalene-d_8 and was measured with the settings described in Table 32. The results are shown in Figure 27.

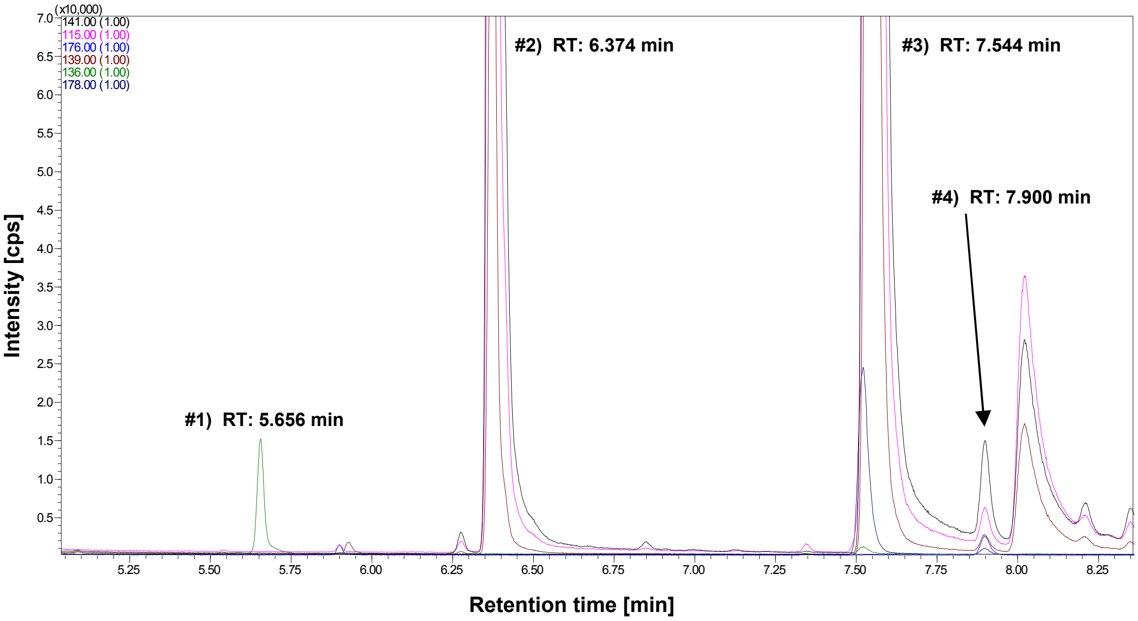


Figure 27: SIM-chromatogram of a sample with a concentration of 10 mg 1-naphthylacetonitrile/mL IPA spiked with 10 ppm 1-chloromethylnaphthalene and 5 ppm naphthalene-d8. A pre-column, as well as the settings described in Table 32, were used for the measurement. Peak #1) naphthalene-d_8; Peak #2) 1-methylnaphthalene; Peak #3) 1-methoxymethylnaphthalene; Peak #4) 1-chloromethylnaphthalene

As shown in Figure 27, a good peak shape was observed for naphthalene-d_8 (base peak m/z 136). Although the retention times shifted significantly (compare Figure 26 and Figure 27), the separation of m/z 176 of 1-chloromethylnaphthalene was not affected.

4.2.3 Investigation of the applicability of the standard addition procedure

Individual approaches of the standard addition procedure (preparation see chapter 3.2.2) were performed as described in chapter 4.2.1.1. The internal standardization was done by the use of the impurity 1-methoxymethylnaphthalene (fragment ion with a mass to charge ratio of 141 was used) of the raw material. To make sure that the obtained signals are reproducible, the untreated sample was measured in the beginning and at the end of the standard addition procedure.

The results of the experiments are shown in Figure 28 and Table 24. The corresponding values for the calculations are shown in the appendix (see chapter 6.4).

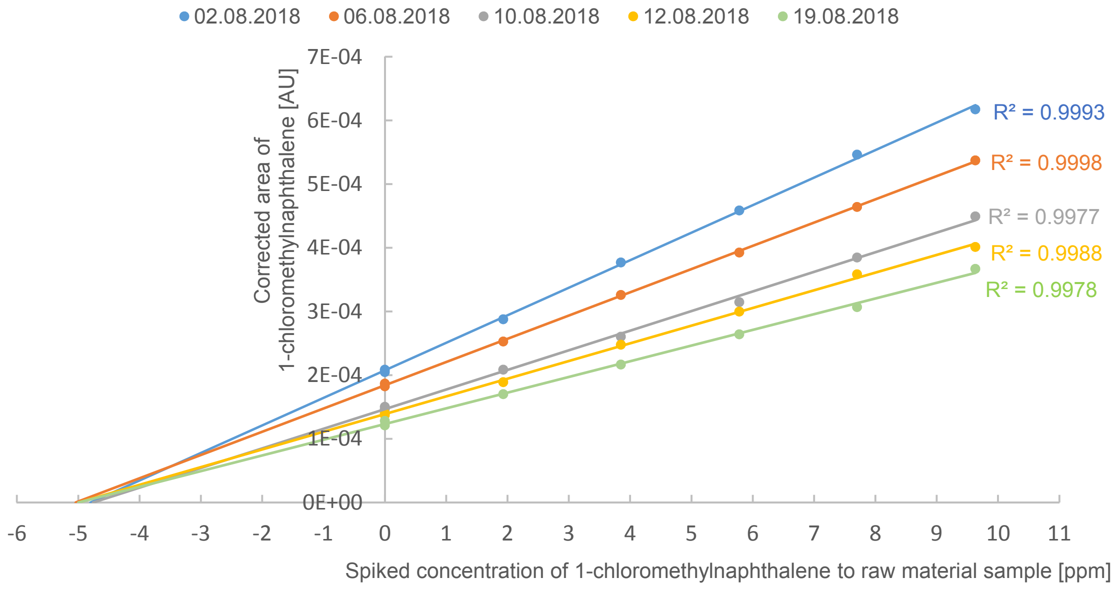


Figure 28: Results of five individual standard addition procedures of the same sample. The experiments were performed as described in chapter 4.2.1.1.

Table 24: Results of five individual standard addition procedures of the same sample. The experiments were performed as described in chapter 4.2.1.1.

Sample: 10 mg 1-naphthylacetonitrile/mL IPA	Spiked concentration of 1-chloromethylnaphthalene [ppm 1-Chloromethylnaphthalene]	Corrected area m/z 176 1-chloromethylnaphthalene [AU]					Average corrected area m/z 176 of 1-chloromethylnaphthalene [AU]	Standard deviation	RSD [%]
		Test date: 02.08.2018	Test date: 06.08.2018	Test date: 10.08.2018	Test date: 12.08.2018	Test date: 19.08.2018			
	0	2.09E-04	1.87E-04	1.51E-04	1.40E-04	1.21E-04	1.62E-04	3.56E-05	**22.0**
	1.93	2.88E-04	2.53E-04	2.09E-04	1.89E-04	1.70E-04	2.22E-04	4.80E-05	**21.6**
	3.85	3.77E-04	3.26E-04	2.60E-04	2.48E-04	2.17E-04	2.86E-04	6.48E-05	**22.7**
	5.78	4.59E-04	3.93E-04	3.15E-04	3.00E-04	2.64E-04	3.46E-04	7.85E-05	**22.7**
	7.70	5.46E-04	4.64E-04	3.85E-04	3.58E-04	3.07E-04	4.12E-04	9.41E-05	**22.8**
	9.63	6.18E-04	5.37E-04	4.50E-04	4.01E-04	3.67E-04	4.75E-04	1.02E-04	**21.6**
	0	2.05E-04	1.83E-04	1.47E-04	1.37E-04	1.28E-04	1.60E-04	3.25E-05	**20.3**

As can be seen in Figure 28, a coefficient of determination higher than 0.9977 was observed for each individual standard addition experiment. A good peak area reproducibility of the untreated sample, measured in the beginning and at the end of each individual standard addition experiment, confirms comparable measuring conditions throughout the single experiments (see Table 25).

Table 25: Results of the untreated sample measured in the beginning and at the end of the standard addition experiments as described in chapter 4.2.1.1

	Corrected area m/z 176 1-chloromethylnaphthalene [AU]				
Untreated sample: 10 mg 1-naphthylacetonitrile/mL IPA	Test date: 02.08.2018	Test date: 06.08.2018	Test date: 10.08.2018	Test date: 12.08.2018	Test date: 19.08.2018
Measured in the beginning	2.09E-04	1.87E-04	1.51E-04	1.40E-04	1.21E-04
Measured in the end	2.05E-04	1.83E-04	1.47E-04	1.37E-04	1.28E-04
Average	2.07E-04	1.85E-04	1.49E-04	1.39E-04	1.25E-04
Standard Deviation	2.68E-06	3.03E-06	2.57E-06	2.24E-06	4.97E-06
RSD [%]	1.3	1.6	1.7	1.6	4.0

Apart from that, the peak area reproducibility of the different spiking levels between the individual approaches was rather poor, yet constant in terms of percentage over the whole calibration (see Table 24 and Figure 29).

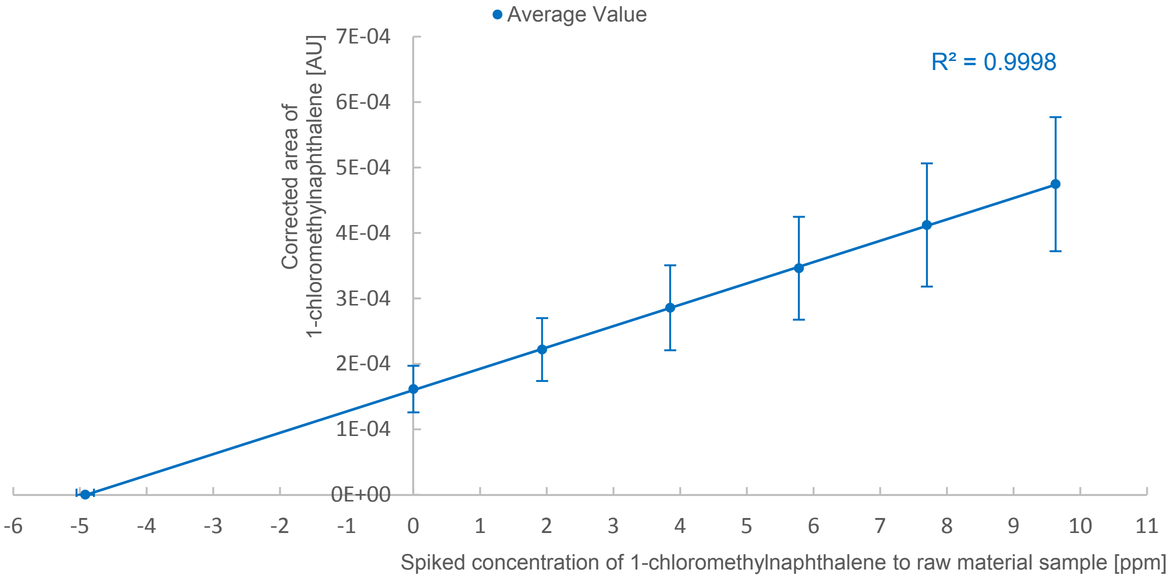


Figure 29: Averaged results of five individual standard addition procedures of the same sample and the corresponding absolute standard deviations. The experiments were performed as described in chapter 4.2.1.1.

However, also the slopes of the different standard addition curves differed significantly from each other, which led to a very good reproducibility of the final results (see Table 26). The observed relative standard deviation of 2.65 % would be more than satisfying for validation on the industrial scale.

Table 26: Final results of five individual standard addition procedures of the same sample. The experiments were performed as described in chapter 4.2.1.1

Sample	Test Date	Equation of the standard addition curve	Calculated concentratiotion [ppm 1-chloromethylnaphthalene]
10 mg 1-naphthylacetonitrile/mL IPA	02.08.2018	y = 4.3242E-05x + 2.0744E-04	4.80
	06.08.2018	y = 3.6504E-05x + 1.8399E-04	5.04
	10.08.2018	y = 3.0806E-05x + 1.4653E-04	4.76
	12.08.2018	y = 2.7786E-05x + 1.3880E-04	4.99
	19.08.2018	y = 2.4656E-05x + 1.2322E-04	5.00
		Average	4.92
		Standard Deviation	0.13
		RSD [%]	2.65

A closer look at Figure 28 reveals that the signals of the different spiking levels became lower with continuous testing date, indicating decreasing sensitivity of the method. The screening for impurities (see chapter 4.1.5) showed that the raw material 1-naphthylacetonitrile contains various impurities. Besides the detected volatile impurities, it is very likely, that the raw material also contains some non-volatile substances just like metals, inorganic acids, or other non-volatile substances. Such contaminants adsorb on the inlet liner and the beginning of the column and create active sites, which might irreversibly bind the target analyte 1-chloromethylnaphthalene, making it not accessible for the measurement. With ongoing injections, more and more non-volatile substances are introduced into the system and hence, more active sites are generated. The more active sites, the lower the relative amount of 1-chloromethylnaphthalene which reaches the detector.

As described in chapter 4.2.1.1, two blank injections (pure solvent) were performed between each sample injection in order to check if there is any remaining sample in the system left. As can be seen on the stable results of the untreated sample (see Table 25), the measurement of the pure solvent obviously leads to a balanced saturation level of the active sites during an approach of the standard addition procedure.

If no further sample, or in more particular 1-chloromethylnaphthaele, is introduced into the system over a longer period, the saturated sites release the bound 1-chloromethylnaphthalene and become active again. This leads to different saturation levels between individual approaches of the standard addition procedure and hence, to poor peak area reproducibility of the different spiking levels between those approaches. Since no relevant contaminations were observed by measuring the pure solvent between the sample injections, those measurements are not necessary and can be omitted.

4.2.4 Investigation of active sites

4.2.4.1 Saturation of active sites by several priming injections

The experiments described in 4.2.3 led to a good reproducibility of the determined concentration of 1-chloromethylnaphthalene in the sample of the raw material 1-naphthylacetonitrile. However, the peak area reproducibility of the different spiking levels between the individual approaches of the standard addition procedure was rather poor. To check if active sites on the inlet liner or the column are the reason for the poor peak area reproducibility, several priming injections were performed to saturate them. Therefore, a sample of 10 mg 1-naphthylacetonitrile/mL IPA spiked with 10 ppm 1-chloromethylnaphthalene and 5 ppm naphthalene-d_8 (internal standard; base peak m/z: 136) was measured consecutively eight times. As described in chapter 4.2.3, the measurement of the pure solvent between each injection is not necessary and was omitted to maintain the saturation of the active sites. However, to ensure the removal of relevant contaminations, the number of rinses with methanol and the total volume of carrier gas purging the inlet were increased compared to the settings described in Table 21. The used settings are listed in Table 27.

Table 27: Settings of GC, MS and Sampler: Investigation of active sites

Gas Chromatograph	
Column	Agilent DB-5MS UI
Length:	30 m
Thickness:	0.25 µm
Diameter:	0.25 mm
Pre-Column:	None
Liner:	Inlet Liner #2
Injection volume:	1 µL
Injection mode:	Splitless
Sampling time:	1 min
Injection temperature:	300 °C
Head pressure:	112.2 kPa
Linear velocity:	45.6 cm/s
Column flow:	1.5 mL/min
Purge flow:	3 mL/min
Split ratio after sampling time:	1:66
Carrier gas saver:	Yes Split ratio 1:10 after 8 min

Oven temperature program		
Rate (°C/min)	Final temperature (°C)	Hold time [min]
-	100	1
30	190	0
10	225	0
250	325	15

Mass Spectrometer:	
Interface temperature	325 °C
Ion source temperature:	250 °C
Solvent cut time:	3.2 min
Event time:	0.1 sec
Start time data acquisition:	3.7 min
End time data acquisition:	6.25 min
Acquisition mode:	Q3-SIM
Detector voltage relative to the tuning result:	0 kV
Recorded m/z values:	115
	136
	139
	141
	176
	178

Sampler	
# of rinses with methanol (Pre-run):	12
# of rinses with methanol (Post-run):	12
# of rinses with sample:	1
Washing volume:	8 µL

The results of the experiments are shown in Figure 30. Since the added naphthalene-d_8 resulted in a split peak under the used parameters (see chapter 4.2.2), the internal standardization was done by using the impurity 1-methoxymethylnaphthalene (fragment ion m/z 141 was used) of the raw material 1-naphthylacetonitrile. The corresponding values for the calculations are shown in the appendix (see chapter 6.4).

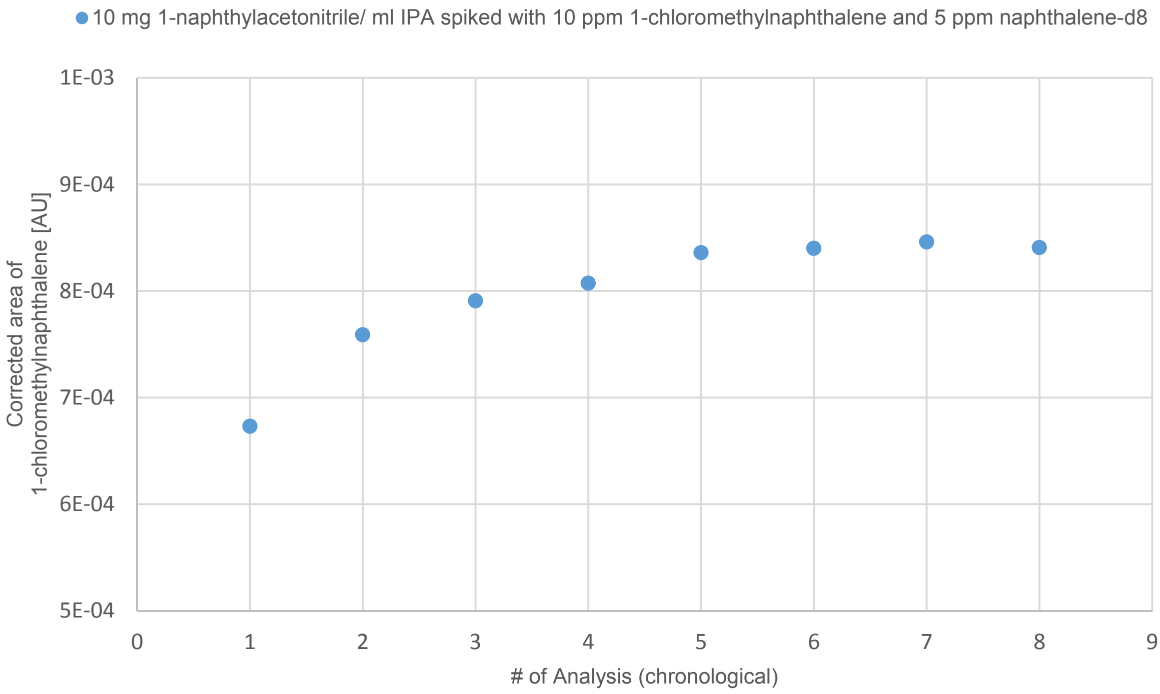


Figure 30: Results to the investigation of active sites

As shown in Figure 30, the corrected area of 1-chloromethylnaphthalene increased with ongoing measurements until the peak area stabilized after four injections. The observed trend of the peak area verifies the hypothesis of active sites: After the first four injections, all active sites were saturated. From then on the whole amount of introduced 1-chloromethylnaphthalene was able to reach the detector, which in turn led to stable peak areas in case of the last four measurements. In order to establish comparable conditions for each individual approach, the active sites must, therefore, be saturated by appropriate priming. If an internal standard is added to the samples, similar actions have to be performed to saturate active sites which bind the internal standard. The saturation of active sites had to be introduced into the method to maintain the sensitivity of the method and to make individual measurements of the same sample comparable concerning the peak areas (RSD of approximately 20 % obtained for each spiking level of the standard addition procedure without saturation).

4.2.4.2 Saturation of active sites at once by injecting a highly concentrated priming sample

Since the saturation of active sites as described in chapter 4.2.4.1 is pretty time-consuming, the approach of saturating all active sites at once by injecting a highly concentrated sample was considered.

Therefore a sample of

10 µg 1-Chloromethylnaphthalene/mL IPA
10 µg Naphthalene-d_8/mL IPA

was prepared and injected two times before consecutively measuring a sample of 10 mg 1-naphthylacetonitrile/mL IPA, spiked with 10 ppm 1-chloromethylnaphthalene and 5 ppm naphthalene-d_8 eight times, as described in 4.2.6 (pre-column implemented and method optimized for naphthalene-d_8). The internal standardization was done by naphthalene-d_8 (molecular ion with m/z of 136 was used).

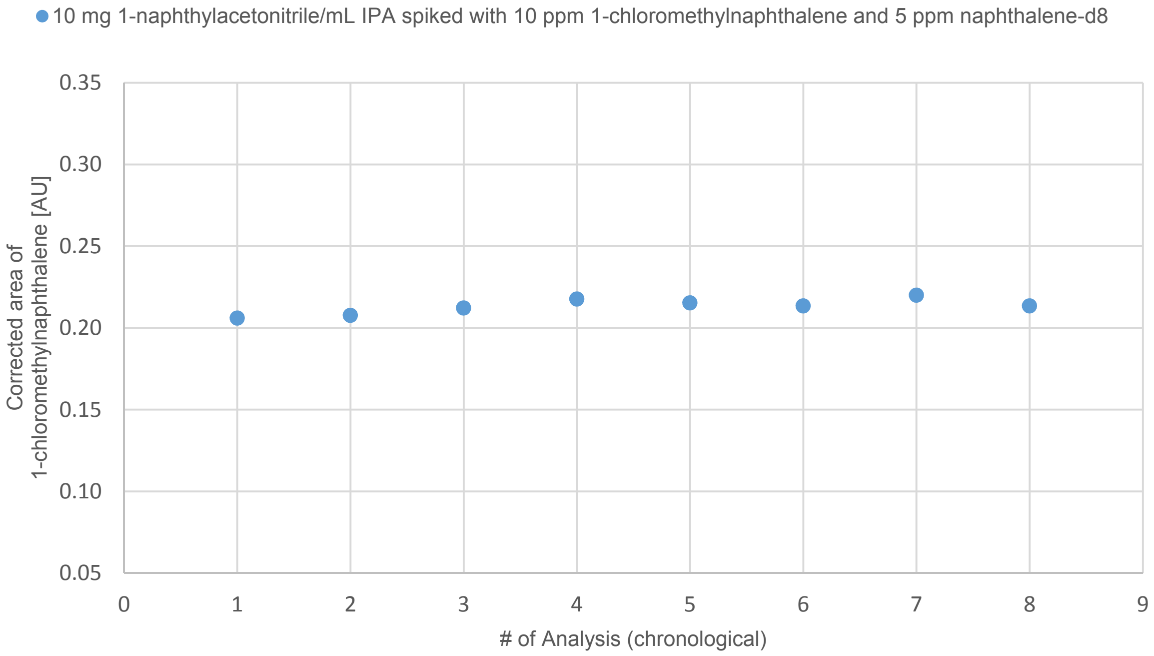


Figure 31: Results of eight consecutive measurements of a sample containing 10 mg 1-naphthylacetonitrile/mL IPA spiked with 10 ppm 1-chloromethylnaphthalene and 5 ppm naphthalene-d_8 after saturating the active sites of the inlet liner and the beginning of the column by injecting two times a priming sample with a concentration of 10 µg 1-chloromethylnaphthalene and 10 µg naphthalene-d_8/mL IPA.

Table 28: Results of eight consecutive measurements of a sample containing 10 mg 1-naphthylacetonitrile/mL IPA spiked with 10 ppm 1-chloromethylnaphthalene and 5 ppm naphthalene-d_8 after saturating the active sites of the inlet liner and the beginning of the column by injecting two times a priming sample with a concentration of 10 µg 1-chloromethylnaphthalene and 10 µg naphthalene-d_8/mL IPA.

Sample	# of Analysis	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 136 Naphthalene-d_8 [AU]	Corrected peak area of 1-chloromethylnaphthalene [AU]
10 mg 1-naphthylacetonitrile/mL IPA spiked with 10 ppm 1-chloromethylnaphthalene and 5 ppm naphthalene-d_8	1	3940	19123	0.206
	2	4109	19782	0.208
	3	4298	20256	0.212
	4	4571	21003	0.218
	5	4680	21732	0.215
	6	4557	21344	0.214
	7	4628	21033	0.220
	8	4597	21536	0.213
Average				0.213
Standard Deviation				0.005
RSD [%]				2.20

As can be seen in Figure 31 and Table 28, the signal of 1-chloromethylnaphthalene in the sample remained stable after two injections of a priming sample with a concentration of 10 µg 1-chloromethylnaphthalene and 10 µg naphthalene-d_8/mL IPA. The saturation of active sites is therefore also possible by injecting a highly concentrated sample of the investigated analytes.

4.2.5 Peak area reproducibility between different individual approaches of the standard addition procedure

Individual approaches of the standard addition procedure (sample preparation see chapter 3.2.2) were performed on different days by using the settings of Table 27. To saturate the active sites and establish comparable conditions for each individual approach, a priming sample (10 mg 1-naphthylacetonitrile/mL isopropyl alcohol spiked with 10 ppm 1-chloromethylnaphthalene) had been injected eight times before the standard addition experiments were started. As described in chapter 4.2.4.1, the analysis of the pure solvent between the samples was omitted to maintain the saturation of the active sites. To make sure that the obtained signals are reproducible, the untreated sample was measured in the beginning and at the end of the standard addition procedure. Since naphthalene-d_8 resulted in a split peak under the used parameters (see chapter 4.2.2.2), the internal standardization was done by using the impurity 1-methoxymethylnaphthalene (fragment ion with a mass to charge ratio of 141 was used) of the raw material 1-naphthylacetonitrile. The results of the experiments are shown in Figure 32 and Table 29.

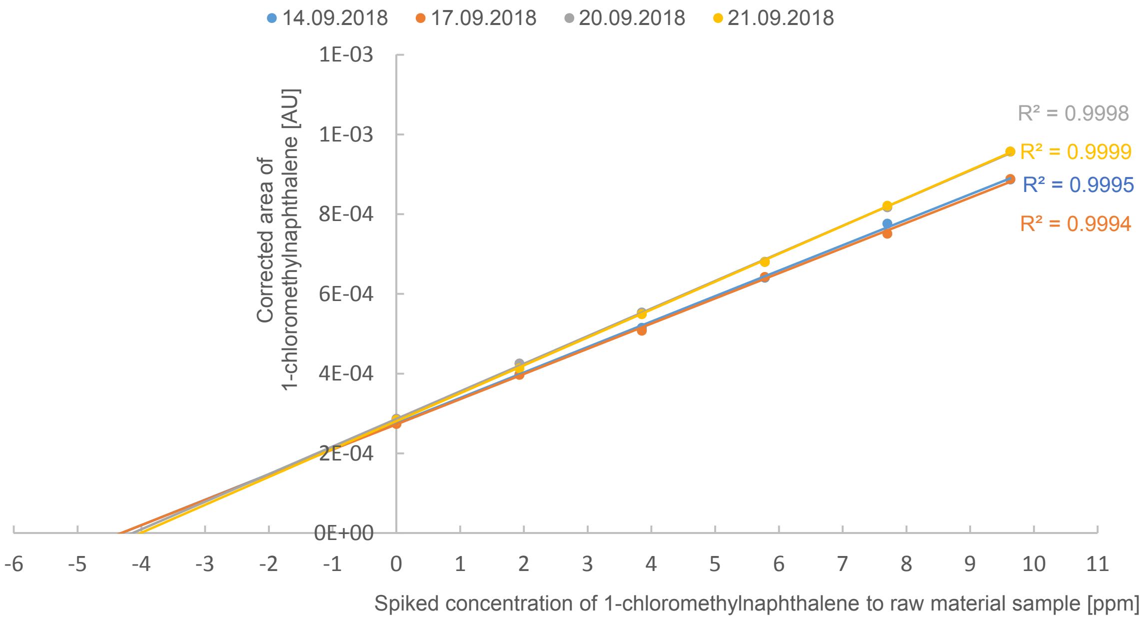


Figure 32: Results of four individual standard addition procedures of the same sample. The experiments were performed as described in chapter 4.2.5

Table 29: Results of four individual standard addition procedures of the same sample. The experiments were performed as described in chapter 4.2.5

Sample: 10 mg 1-naphthylacetonitrile/mL IPA	Spiked concentration of 1-chloromethylnaphthalene [ppm 1-Chloromethylnaphthalene]	Corrected area m/z 176 1-chloromethylnaphthalene [AU]				Average corrected area m/z 176 1-chloromethylnaphthalene [AU]	Standard deviation	RSD [%]
		Test date: 14.09.2018	Test date: 17.09.2018	Test date: 20.09.2018	Test date: 21.09.2018			
Sample: 10 mg 1-naphthylacetonitrile/mL IPA	0	2.75E-04	2.74E-04	2.87E-04	2.84E-04	2.80E-04	6.49E-06	**2.3**
	1.93	3.98E-04	3.97E-04	4.25E-04	4.13E-04	4.08E-04	1.36E-05	**3.3**
	3.85	5.15E-04	5.08E-04	5.53E-04	5.49E-04	5.31E-04	2.34E-05	**4.4**
	5.78	6.41E-04	6.42E-04	6.81E-04	6.80E-04	6.61E-04	2.24E-05	**3.4**
	7.70	7.76E-04	7.51E-04	8.17E-04	8.21E-04	7.91E-04	3.37E-05	**4.3**
	9.63	8.87E-04	8.88E-04	9.57E-04	9.57E-04	9.22E-04	4.00E-05	**4.3**
	0	2.80E-04	2.75E-04	2.84E-04	2.82E-04	2.80E-04	3.82E-06	**1.4**

As can be seen in Figure 32, a coefficient of determination higher than 0.9994 was observed for each individual standard addition experiment. A good peak area reproducibility of the untreated sample, measured in the beginning and at the end of each standard addition procedure, confirms comparable measuring conditions throughout the single experiments (see Table 30).

Table 30: Results of the untreated sample measured in the beginning and at the end of the standard addition experiments described in chapter 4.2.5

	Corrected area m/z 176 1-chloromethylnaphthalene [AU]			
Untreated sample: 10 mg 1-naphthylacetonitrile/mL IPA	Test date: 14.09.2018	Test date: 17.09.2018	Test date: 20.09.2018	Test date: 21.09.2018
Measured in the beginning	2.75E-04	2.74E-04	2.87E-04	2.84E-04
Measured in the end	2.80E-04	2.75E-04	2.84E-04	2.82E-04
Average	2.77E-04	2.74E-04	2.85E-04	2.83E-04
Standard Deviation	3.55E-06	8.79E-07	2.00E-06	1.27E-06
RSD [%]	1.3	0.3	0.7	0.4

In addition, a good peak area reproducibility of the different spiking levels between the individual approaches was observed (see Table 29 and Figure 33). The good reproducibility of the peak areas of the different spiking levels between the individual approaches also led to a good reproducibility of the final results (see Table 31).

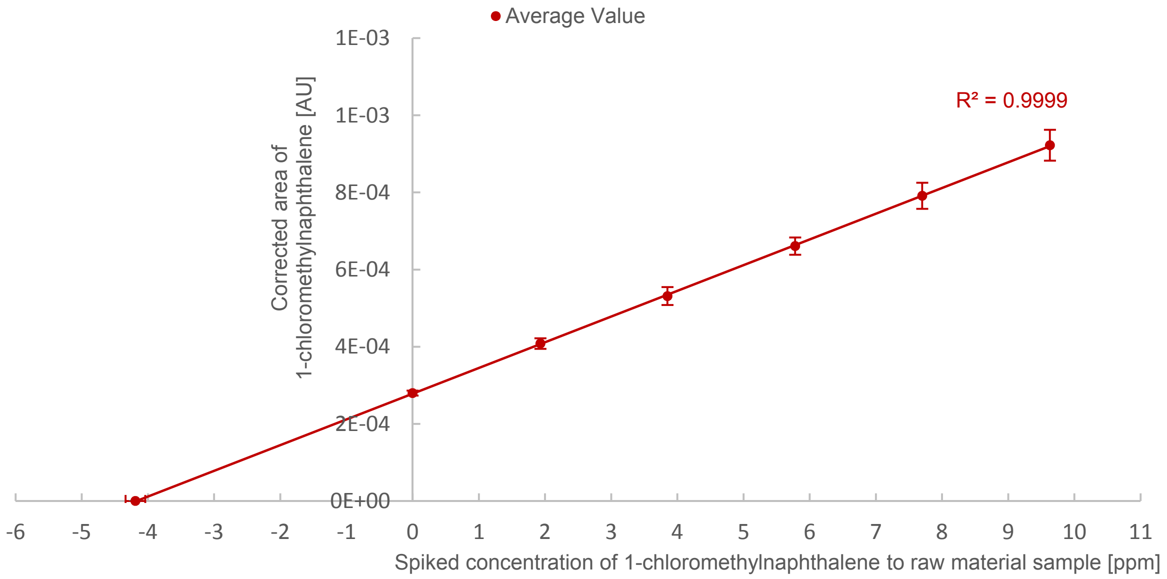


Figure 33: Averaged results of four individual standard addition procedure of the same sample and the corresponding absolute standard deviations. The experiments were performed as described in chapter 4.2.5.

Table 31: Final results of four individual standard addition procedures of the same sample. The experiments were performed as described in chapter 4.2.5

Sample	Test Date	Equation of the standard addition curve	Calculated concentratiotion [ppm 1-chloromethylnaphthalene]
10 mg 1-naphthylacetonitrile/mL IPA	14.09.2018	y = 6.3829E-05x + 2.7525E-04	4.31
	17.09.2018	y = 6.3180E-05x + 2.7275E-04	4.32
	20.09.2018	y = 6.9220E-05x + 2.8626E-04	4.14
	21.09.2018	y = 6,9971E-05x + 2.8068E-04	4.01
		Average	4.19
		Standard deviation	0.15
		RSD [%]	3.53

However, the obtained results are slightly lower than those of the experiments performed without priming injections.

Concentration of 1-chloromethylnaphthalene in the raw material 1-naphtylacetonitrile

without priming injections:	4.92 ppm
with priming injections:	4.19 ppm

This could be caused by several reasons such as slightly different measuring conditions due to the active sites or different calibration functions.

4.2.6 Implementation of the pre-column

The main part of the method development was done with a stored and already used DB-5MS UI column without a connected pre-column.

Due to the content of known and also unknown impurities in the raw material 1-naphthylacetonitrile (see chapter 4.1.5 and 4.2.4) and the high amount of raw material which is loaded onto the analytical column with each sample injection, an uncoated pre-column was installed to increase the lifetime of the analytical column. According to the extended length, the corresponding parameters of the settings used in chapter 4.2.5 (see Table 27) were adjusted to maintain, and in case of naphthalene-d_8, optimize separation and peak shapes:

- Solvent focusing: The initial oven temperature was set to 60°C (20°C below the boiling point of isopropyl alcohol), which allows the condensation and further the focusing of the analytes on the head of the pre-column. The analyte bandwidth decreases and the risk of split peaks can be reduced [22].
- In order to increase the number of theoretical plates and, therefore, the separation efficiency, the linear velocity of the carrier gas helium was changed from 46.5 cm/s to 25 cm/s (compare to Figure 3)

Details regarding the final settings are listed in Table 32.

Table 32: Settings of GC, MS and Sampler: Implementation of the pre-column

Gas Chromatograph	
Column	Agilent DB-5MS UI
Length:	30 m
Thickness:	0.25 µm
Diameter:	0.25 mm
Pre-Column:	Yes (see 3.1.2)
Liner:	Inlet Liner #3
Injection volume:	0.5 µL
Injection mode:	Splitless
Sampling time:	1 min
Injection temperature:	300 °C
Head pressure:	43.7 kPa
Linear velocity:	25 cm/s
Column flow:	0.63 mL/min
Purge flow:	3 mL/min
Split ratio after sampling time:	158.5
Carrier gas saver:	Yes Split ratio: 1:50 after 8 min

Oven temperature program		
Rate (°C/min)	Final temperature (°C)	Hold time [min]
-	60	1
30	190	0
10	225	0
250	325	15

Mass Spectrometer:	
Interface temperature	325 °C
Ion source temperature:	250 °C
Solvent cut time:	4.5 min
Event time:	0.1 sec
Start time data acquisition:	5 min
End time data acquisition:	8.4 min
Acquisition mode:	Q3-SIM
Detector voltage relative to the tuning result:	0 kV
Recorded m/z values:	115
	136
	139
	141
	176
	178

Sampler	
# of rinses with methanol (Pre-run):	12
# of rinses with methanol (Post-run):	12
# of rinses with sample:	1
Washing volume:	8 µL

4.2.6.1 Applicability of the final method

In order to check if the adaptions described in chapter 4.2.6 (implementation of the pre-column and optimization of the method for the internal standard naphthalene-d$_8$) lead to reproducible and comparable results, the standard addition procedure was performed as described in 3.2.2. Naphthalene-d$_8$ was used as internal standard. Therefore, a stock solution of 10 µg naphthalene-d$_8$/mL isopropyl alcohol was prepared. 5 µL of this solution were added to 1 mL of the untreated sample, as well as to 1 mL of the various spiking levels of the standard addition procedure, leading to a final concentration of approx. 0.05 µg naphthalene-d$_8$/10 mg raw material 1-naphthylacetonitrile (equivalent to approx. 5 ppm naphthalene-d_8) in each spiking level.

To saturate the active sites and establish comparable conditions for each individual approach, a priming sample (10 mg 1-naphthylacetonitrile/mL isopropyl alcohol spiked with 10 ppm 1-chloromethylnaphthalene and 5 ppm naphthalene-d_8) had been injected eight times before the standard addition experiment was started. Again, the analysis of the pure solvent between the samples was omitted to maintain the saturation of the active sites. To make sure that the obtained signals are reproducible, the untreated sample was measured in the beginning and at the end of the standard addition procedure. For time reasons, only two measurements were made to verify the applicability of the procedure described above.

The results of the experiments are shown in Figure 34 and Table 33.

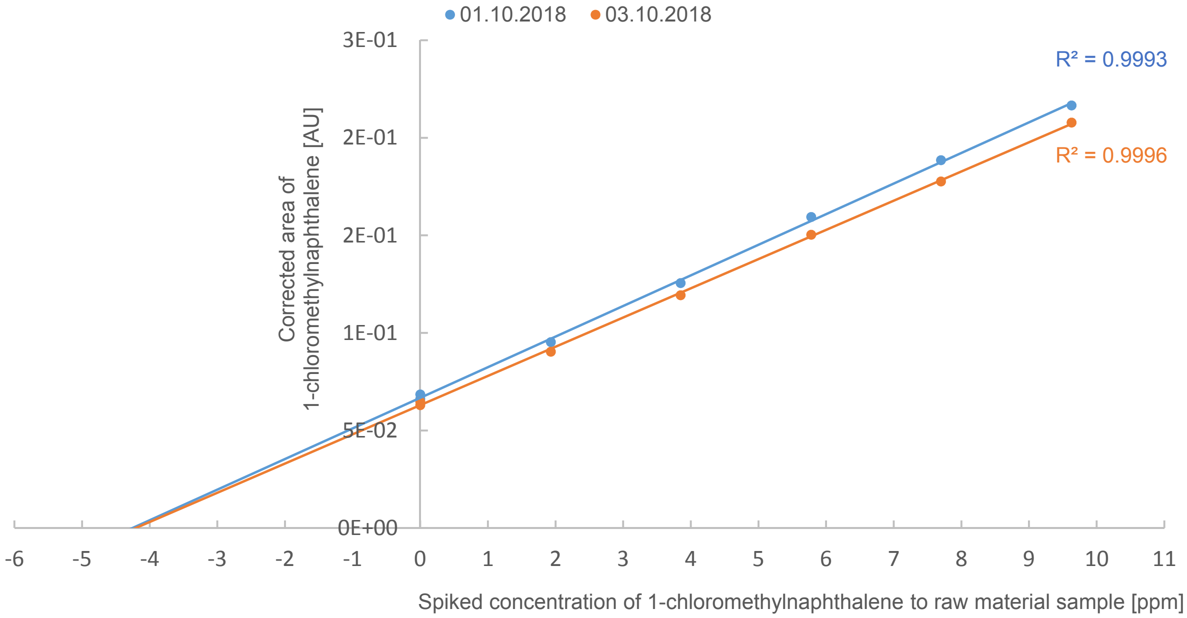


Figure 34: Results of two individual standard addition procedures of the same sample. The experiments were performed as described in chapter 4.2.6

Table 33: Results of two individual standard addition procedures of the same sample. The experiments were performed as described in chapter 4.2.6

Sample: 10 mg 1-naphthylacetonitrile/mL IPA	Spiked concentration of 1-chloromethylnaphthalene [ppm 1-Chloromethylnaphthalene]	Corrected area m/z 176 1-chloromethylnaphthalene [AU]		Average corrected area m/z 176 of1-chloromethylnaphthalene [AU]	Standard deviation	RSD [%]
		Test date: 01.10.2018	Test date: 03.10.2018			
	0	6.62E-02	6.47E-02	6.54E-02	1.03E-03	**1.6**
	1.93	9.53E-02	9.04E-02	9.28E-02	3.50E-03	**3.8**
	3.85	1.26E-01	1.19E-01	1.22E-01	4.39E-03	**3.6**
	5.78	1.59E-01	1.50E-01	1.55E-01	6.43E-03	**4.2**
	7.70	1.89E-01	1.78E-01	1.83E-01	7.71E-03	**4.2**
	9.63	2.17E-01	2.08E-01	2.12E-01	6.20E-03	**2.9**
	0	6.85E-02	6.30E-02	6.57E-02	3.90E-03	**5.9**

As can be seen in Figure 34, a coefficient of determination higher than 0.9993 was observed for each individual standard addition experiment. A good peak area reproducibility of the untreated sample, measured in the beginning and at the end of each individual standard addition procedure, confirms comparable measuring conditions throughout the single experiments (see Table 34).

Table 34: Results of the untreated sample measured in the beginning and at the end of the standard addition experiments described in chapter 4.2.6

	Corrected area m/z 176 1-chloromethylnaphthalene [AU]	
Untreated sample: 10 mg 1-naphthylacetonitrile/mL IPA	Test date: 14.09.2018	Test date: 17.09.2018
Measured in the beginning	6.62E-02	6.47E-02
Measured in the end	6.85E-02	6.30E-02
Average	6.73E-02	6.38E-02
Standard Deviation	1.62E-03	1.25E-03
RSD [%]	2.4	2.0

Further, a good peak area reproducibility of the different spiking levels between the two individual approaches was observed (see Table 33). The good reproducibility of the peak areas of the different spiking levels between the individual approaches also led to a good reproducibility of the final results (see Table 35).

Table 35: Results of two individual approaches of the standard addition procedure. The experiments were performed as described in chapter 4.2.6

Sample	Test Date	Equation of the standard addition curve	Calculated concentration [ppm 1-chloromethylnaphthalene]
10 mg 1-naphthylacetonitrile/mL IPA	01.10.2018	y = 1.5685E-02x + 6.6709E-02	4.21
	03.10.2018	y = 1.4968E-02x + 6.2971E-02	4.25
		Average	4.23
		Standard Deviation	0.03
		RSD [%]	0.77

Average concentration of 1-chloromethylnaphthalene in the raw material 1-naphtylacetonitrile after eight priming injections:

- without pre-column: 4.19 ppm
- with pre-column: 4.23 ppm

4.3 Method Transfer

The final GCMS method (see Table 32, chapter 4.2.6) was developed at the research group *Mass Spectrometric Bio and Polymer Analysis* at TU Wien and had to be validated at the receiving laboratory of LOBA Feinchemie GmbH. The device at the development laboratory (GCMS-TQ8040) and the device at the receiving laboratory (GCMS-QP2020) differ in the construction of the mass spectrometer, which can lead to differences in the results regarding the signal intensities. In order to obtain a proper signal of 1-chloromethylnaphthalene on the device of the receiving laboratory, the detector voltage had to be adjusted 0.05 kV relative to the tuning results (at the development laboratory no adjustment of the detector voltage was necessary after tuning). Due to the increased detector voltage, the ion with a mass to charge ratio of 141 was excluded from monitoring to avoid detector saturation. All other settings remained as described in Table 32.

4.4 Validation at the receiving laboratory

The classification of the analytical method was done according to ICH Q2 (R1) [24]:

☐ Identification
☒ Test for impurities (☒ Quantitative or ☐ Limit)
☐ Assay
☐ Microbiological method (☐ quantitative or ☐ presence/absence or ☐ Identification)

☐ not classifiable

Table 36: Overview of the validation parameters for different analytical procedures according to ICH Q2 (R1) [24]

Type of analytical procedure	IDENTIFICATION	TESTING FOR IMPURITIES		ASSAY - dissolution (measurement only) - content/potency
characteristics		quantitat.	limit	
Accuracy	-	+	-	+
Precision				+
Repeatability	-	+	-	+ (1)
Intermediate Precision	-	+ (1)	-	
Specificity (2)	+	+	+	+
Detection Limit	-	- (3)	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- signifies that this characteristic is not normally evaluated
+ signifies that this characteristic is normally evaluated
(1) in cases where reproducibility has been performed, intermediate precision is not needed
(2) lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s)
(3) may be needed in some cases

According to Table 36, the following parameters had to be investigated:

- Accuracy
- Repeatability
- Intermediate Precision
- Specificity
- Quantitation Limit
- Linearity
- Range

4.4.1 General Workflow

4.4.1.1 Preparation of the internal standard solution

A solution of 10 µg naphthalene-d_8/mL IPA is prepared and stored at +2°C - +8°C.

4.4.1.2 Standard addition experiment

1. A solution of 10 mg raw material 1-naphthylacetonitrile/mL IPA is prepared (Solution 1)
2. A solution of 0.1 µg 1-chloromethylnaphthalene/mL Solution 1 is prepared (Solution 2)
   Composition of Solution 2:
   0.1 µg 1-chloromethylnaphthalene/mL IPA
   10 mg raw material 1-naphthylacetonitrile/mL IPA
   0.1 µg are equal to 10 ppm of 1-chloromethylnaphthalene relative to 10 mg of the raw material 1-naphthylacetonitrile
3. Solution 1 and Solution 2 are mixed according to Table 37, to cover a range of 2 - 10 ppm of 1-chloromethylnaphthalene in the raw material

Table 37: Preparation of the spiking levels for the standard addition procedure

Spiking Level	Volume [µL]		spiked concentration
	Solution 1	Solution 2	[ppm 1-chloromethylnaphthalene]
0	1000	0	0
1	800	200	2
2	600	400	4
3	400	600	6
4	200	800	8
5	0	1000	10

4. The purity grade of 96.3 % of 1-chloromethylnaphtalene has to be considered to obtain the actual concentrations of the spiking levels (see Table 38)

Table 38: Actual concentrations of the spiking levels for the standard addition procedure

Spiking Level	Spiked concentration [ppm 1-chloromethylnaphthalene]	
	Assumed purity grade of 100 %	Actual purity grade of 96.3 %
0	0	0
1	2	1.93
2	4	3.85
3	6	5.78
4	8	7.70
5	10	9.63

5 µL of the internal standard solution (concentration: 10 µg naphthalene-d_8/mL IPA) are added to 1 mL of the untreated sample, as well as to 1 mL of the various spiking levels, leading to a final concentration of approx. 0.05 µg naphthalene-d_8/10 mg 1-naphthylacetonitrile (equivalent to approx. 5 ppm naphthalene-d_8).

As described in the experiments of the robustness (see chapter 4.4.5.8), the unspiked sample was measured three times before and three times after the spiked samples were measured. Therefore, the average corrected area of these six measurements was used for the calculation of the standard addition curve.

4.4.1.3 Preparation of the priming solutions

In order to saturate active sites, a priming sample with the following concentration is prepared:

Priming Sample: 10 µg 1-Chloromethylnaphthalene/mL IPA
10 µg Naphthalene-d_8/mL IPA

4.4.1.4 Preparation of the System Suitability Test (SST)

The SST is used to show that the equipment, the electronics and the analytical operations constitute an integral system that can be evaluated as such [24]. The SST-sample is prepared equivalently to spiking level #5 as described in chapter 4.4.1.2.

4.4.2 Batch table

Table 39 represents the batch table which was used during the validation. Priming injections were necessary to saturate active sites in the liner and the pre-column. If more than one individual sample was measured within one batch, the saturation had to be done only for the first sample.

Table 39: Exemplary batch table for the validation experiments

# of Measurement	# Vial	Sample Name
1	1	IPA
2	2	Blank_001 (0.05 µg naphthalene-d$_8$/mL IPA)
3	3	Priming injection_001 (=Priming Sample)
4	3	Priming injection_002 (=Priming Sample)
5	4	SST_001
6	4	SST_002
7	4	SST_003
8	4	SST_004
11	4	SST_005
12	4	SST_006
13	4	SST_007
14	5	Sample_0 ppm_001
15	6	Sample_0 ppm_002
16	7	Sample_0 ppm_003
17	8	Sample_1.926 ppm
18	9	Sample_3.852 ppm
19	10	Sample_5.778 ppm
20	11	Sample_7.704 ppm
21	12	Sample_9.63 ppm
22	13	Sample_0 ppm_004
23	14	Sample_0 ppm_005
24	15	Sample_0 ppm_006
25	16	Blank_002 (0.05 µg naphthalene-d$_8$/mL IPA)

4.4.3 Settings of GC, MS and Sampler

The used settings in the course of the validation are shown in Table 40 - Table 42.

Table 40: Settings of GC, MS and Sampler: Method file for spiked and unspiked samples

<table>
<tr><th colspan="3">Gas Chromatograph</th><th colspan="2">Mass Spectrometer:</th></tr>
<tr><td>Column</td><td colspan="2">Agilent DB-5MS UI</td><td>Interface temperature</td><td>325 °C</td></tr>
<tr><td>Length:</td><td colspan="2">30 m</td><td>Ion source temperature:</td><td>250 °C</td></tr>
<tr><td>Thickness:</td><td colspan="2">0.25 µm</td><td>Solvent cut time:</td><td>4.5 min</td></tr>
<tr><td>Diameter:</td><td colspan="2">0.25 mm</td><td>Event time:</td><td>0.1 sec</td></tr>
<tr><td>Pre-Column:</td><td colspan="2">Yes (see 3.1.2)</td><td>Start time data acquisition:</td><td>5 min</td></tr>
<tr><td>Liner:</td><td colspan="2">Inlet Liner #3</td><td>End time data acquisition:</td><td>8.4 min</td></tr>
<tr><td>Injection volume:</td><td colspan="2">0.5 µL</td><td>Acquisition mode:</td><td>SIM</td></tr>
<tr><td>Injection mode:</td><td colspan="2">Splitless</td><td>Detector voltage relative to the tuning result</td><td>0.05 kV</td></tr>
<tr><td>Sampling time:</td><td colspan="2">1 min</td><td rowspan="5">Recorded m/z values:</td><td>115</td></tr>
<tr><td>Injection temperature:</td><td colspan="2">300 °C</td><td>136</td></tr>
<tr><td>Head pressure:</td><td colspan="2">43.7 kPa</td><td>139</td></tr>
<tr><td>Linear velocity:</td><td colspan="2">25 cm/s</td><td>176</td></tr>
<tr><td>Column flow:</td><td colspan="2">0.63 mL/min</td><td>178</td></tr>
<tr><td>Purge flow:</td><td colspan="2">3 mL/min</td><th colspan="2">Sampler</th></tr>
<tr><td>Split ratio after sampling time:</td><td colspan="2">158.5</td><td># of rinses with methanol (Pre-run):</td><td>12</td></tr>
<tr><td>Carrier gas saver:</td><td colspan="2">Yes (Split ratio: 50 after 8 min)</td><td># of rinses with methanol (Post-run):</td><td>12</td></tr>
<tr><td colspan="3">Oven temperature program</td><td># of rinses with sample:</td><td>1</td></tr>
<tr><td>Rate (°C/min)</td><td>Final temperature (°C)</td><td>Hold time [min]</td><td>Washing volume:</td><td>8 µL</td></tr>
<tr><td>-</td><td>60</td><td>1</td><td colspan="2" rowspan="4"></td></tr>
<tr><td>30</td><td>190</td><td>0</td></tr>
<tr><td>10</td><td>225</td><td>0</td></tr>
<tr><td>250</td><td>325</td><td>15</td></tr>
</table>

Table 41: Settings of GC, MS and Sampler: Method file for blank

Gas Chromatograph			**Mass Spectrometer:**	
Column	Agilent DB-5MS UI		Interface temperature	325 °C
Length:	30 m		Ion source temperature:	250 °C
Thickness:	0.25 µm		Solvent cut time:	4.5 min
Diameter:	0.25 mm		Event time:	0.1 sec
Pre-Column:	Yes (see 3.1.2)		Start time data acquisition:	5 min
Liner:	Inlet Liner #3		End time data acquisition:	12 min
Injection volume:	0.5 µL		Acquisition mode:	SIM
Injection mode:	Splitless		Detector voltage relative to the tuning result	0.05 kV
Sampling time:	1 min		Recorded m/z values:	115
Injection temperature:	300 °C			136
Head pressure:	43.7 kPa			139
Linear velocity:	25 cm/s			141
Column flow:	0.63 mL/min			166
Purge flow:	3 mL/min			176
Split ratio after sampling time:	158.5			178
Carrier gas saver:	Yes (Split ratio: 50 after 8 min)		**Sampler**	
Oven temperature program			# of rinses with methanol (Pre-run):	12
Rate (°C/min)	Final temperature (°C)	Hold time [min]	# of rinses with methanol (Post-run):	12
-	60	1	# of rinses with sample:	1
30	190	0	Washing volume:	8 µL
10	225	0		
250	325	3		

Table 42: Settings of GC, MS and Sampler: Method file for priming sample

Gas Chromatograph	
Column	Agilent DB-5MS UI
Length	30 m
Thickness	0.25 µm
Diameter	0.25 mm
Pre-Column:	Yes (see 3.1.2)
Liner:	Inlet Liner #3
Injection volume:	0.5 µL
Injection mode:	Splitless
Sampling time:	1 min
Injection temperature:	300 °C
Head pressure:	43.7 kPa
Linear velocity:	25 cm/s
Column flow:	0.63 mL/min
Purge flow:	3 mL/min
Split ratio after sampling time:	158.5
Carrier gas saver:	Yes (Split ratio: 50 after 8 min)

Oven temperature program		
Rate (°C/min)	Final temperature (°C)	Hold time [min]
-	100	1
30	190	0
10	225	0
250	325	15

Mass Spectrometer	
Interface temperature	325 °C
Ion Source temperature:	250 °C
Solvent cut time:	4.5 min
Event time:	0.1 sec
Start time data acquisition:	5 min
End time data acquisition:	9 min
Acquisition mode:	Scan
Recorded m/z values:	50 – 1090
Detector voltage relative to the tuning result	0 kV

Sampler	
# of rinses with methanol (Pre-run):	1
# of rinses with methanol (Post-run):	6
# of rinses with sample:	1
Washing volume:	6 µL

4.4.4 Test design

The analytical performance characteristics were investigated in the course of the following test:

- Test-ID: 698501_181019_A_GK
  - Validation sample: 1-naphthylacetonitrile (Internal Lot# #0000011533)
  - Three consecutive tests

Table 43: Test design of the validation

Parameter	Test-ID: 698501_181019_A_GK
Accuracy	Shown by the approach with the lowest coefficient of determination
Repeatability	Shown by three consecutive approaches of the standard addition procedure
Intermediate Precision	n.a.
Specificity	Shown by the first of the three consecutive approaches of the standard addition experiments
Quantitation Limit	Determined by the results of the three consecutive approaches of the standard addition experiments
Linearity	Shown by three consecutive approaches of the standard addition procedure
Range	See chapter 4.4.5.6
Robustness	Shown by three consecutive approaches of the standard addition procedure
SST	See chapter 4.4.5.9

4.4.5 Investigation of the analytical performance characteristics

The results of the investigated analytical performance characteristics are described in the following chapters. The corresponding values for the calculations are shown in the appendix (see appendix, chapter 6.4).

4.4.5.1 Repeatability

The repeatability was shown by measuring the validation sample in 3 independent approaches in the course of the same batch-file.

Procedure:

Every approach was performed according to 4.4.1.

Acceptance Criteria:

The RSD between the calculated concentrations of the validation sample must not be higher than 20 %.

Results:

The results regarding the repeatability are shown in Table 44.

Table 44: Validation Results: Repeatability

Test-ID	# Approach	Determined concentration of 1-chloromethylnaphthalene [ppm]
698501_181019_A_GK	1	3.58
	2	3.91
	3	3.48
	Average	3.66
	Standard deviation	0.22
	RSD [%]	**6.13**

Assessment:

The acceptance criterion regarding the repeatability was ☒ met
☐ not met

Remark: RSD: **6.13 %**

4.4.5.2 Linearity

The linearity was shown within the scope of the repeatability.

Acceptance Criteria:

The coefficient of determination had to be ≥ 0.98 for each independent approach.

The residuals of the regression line had to be normally distributed for each approach.

The residuals must not be serially correlated.

Results:

The results regarding the linearity are shown in Table 45.

Table 45: Validation Results: Linearity I

Test-ID	# Approach	Coefficient of Determination
698501_181019_A_GK	1	0.9939
	2	0.9959
	3	0.9971

Table 46: Validation Results: Linearity II. The analysis of the residuals is shown in the appendix (chapter 6.5)

Test-ID	# Approach	Residuals normally distributed	Residuals serial correlated	Residuals variances are homogenous
698501_181019_A_GK	1	Yes	No	Yes
	2	Yes	No	Yes
	3	Yes	No	Yes

Assessment:

The acceptance criterion regarding the linearity was

☒ met

☐ not met

4.4.5.3 Accuracy

The accuracy was shown within the scope of the repeatability and the linearity.

Procedure:

The standard calibration curve was generated in different ways, each time omitting one concentration (1.926, 3.852, 5.778 and 7.704 ppm) of the spiked samples. The spiked concentration of the omitted sample was then calculated by inserting the corresponding signal into the appropriate linear equation. The approach with the lowest coefficient of determination was used to verify the accuracy.

Acceptance Criteria:

The coefficient of determination had to be ≥ 0.98 for each linear equation.

The recovery rate of the calculated concentration of the particular spiking levels had to be between 80 % - 120 %.

Results:

The approach with the lowest coefficient of determination was approach #1 of 698501_181019_A_GK (see Table 45). The results are shown in Table 47.

Table 47: Validation Results: Accuracy

Actual concentration [ppm 1-CMN]	Calculated concentration [ppm 1-CMN]	Coefficient of Determination	Recovery rate [%]
1.926	1.815	0.9929	94.3
3.852	3.688	0.9941	95.8
5.778	5.804	0.9938	100.5
7.704	7.126	0.9974	92.5

Assessment:

The acceptance criterion regarding the accuracy was

☒ met

☐ not met

Remark: Recovery rate: **92.5 – 100.5 %**

4.4.5.4 Quantitation Limit

Based on the results of the repeatability, the quantitation limit (x_{BG}) was determined according to DIN 32645 [25] by using the calibration curve method. The prerequisites of the linearity (see chapter 4.4.5.2) must be met.

Results:

Predefined parameters:

α	0.01
n	11
m	3
k	3
$t_{n,\alpha}$	2.821
$t_{n,\alpha/2}$	3.250

Calculated parameters based on the average results of the repeatability experiments:

a	0.03493
b	0.00905
$s_{y,x}$	0.00134
$\overline{x}$	2.6264
Q_x	128.11
s_{x0}	0.14800
Δa	0.00261
y_k	0.03754
x_{NG} [ppm]	0.289
x_{BG} [ppm]	**0.966**

Assessment:
A quantitation limit of 0.97 ppm was determined for the used method.

4.4.5.5 Intermediate Precision

For reasons of time, the investigation of the intermediate precision was postponed and is not part of this diploma thesis.

4.4.5.6 Range

The expected amount of 1-chloromethylnaphthalene is in the range of 0.02 - 0.1 µg 1-chloromethylnaphthalene/10 mg 1-naphthylacetonitrile (= 2 - 10 ppm). The applicability of the range was shown within the scope of the linearity and the accuracy.

4.4.5.7 Specificity

The specificity of the method was shown within the scope of the repeatability.

Acceptance Criteria:

The RSD between the average retention times of m/z 176 of 1-chloromethylnaphthalene in

- the priming sample
- and the samples of the standard addition procedure

must not differ more than 1 % within an approach.

The characteristic ion m/z 176 of 1-chloromethylnaphthalene in the samples of the standard addition procedure has to be baseline-separated. The presence of the corresponding reference ions (m/z 115, m/z 139 and m/z 178) must verify the identification of 1-chloromethylnaphthalene.

Results:

The results regarding the repeatability are shown in Table 48.

Table 48: Validation Results: Specificity

Sample	Retention time [min]
Average: priming sample	7.892
Average: standard addition procedure	7.897
Average	7.895
Standard deviation	0.0037
RSD [%]	0.046

The ion m/z 176 of 1-chloromethylnaphthalene in the samples of the standard addition procedure was baseline-separated in each case. The corresponding reference ions m/z 115, m/z 139 and m/z 178 were present in each sample of the standard addition procedure.

Assessment:

The acceptance criterion regarding the specificity was ☒ met
☐ not met

Remark: RSD: **0.046 %**

4.4.5.8 Robustness: Stability of the Samples in the Autosampler

Procedure

To confirm that there is no variation in sample composition during an approach of the standard addition procedure, the unspiked sample was measured three times before and three times after the spiked samples were measured.

Acceptance Criteria:

The RSD of the corrected areas (internal standardization) of the fragment ion m/z 176 of 1-chloromethylnaphthalene in the unspiked sample must not be higher than 15 % within one approach.

The RSD of the average corrected areas of the fragment ion m/z 176 of 1-chloromethylnaphthalene in the unspiked sample must not be higher than 15 % between the three approaches performed in the course of the repeatability.

Results:

The results regarding the robustness are shown in Table 49.

Table 49: Validation Results: Robustness

Test-ID	# Approach	RSD [%]
698501_181019_A_GK	1	2.88
	2	3.08
	3	4.99
	Average	1.42

Assessment:

The acceptance criterion regarding the stability of samples in the autosampler was

☒ met
☐ not met

4.4.5.9 System Suitability Test

The SST was carried out according to chapter <621> of USP 40-NF 35 [26].

Procedure

Since no suitable sample (similar matrix as actual samples, concentration of target analyte in the expected range) was available, the validation sample (spiking level #5) was used as SST-sample (preparation see chapter 4.4.1.4). The SST-sample was consecutively measured seven times after saturation of the active sites and prior to the standard addition procedure. In case of success, the validation sample can be implemented as SST for the current method.

Acceptance Criteria:

The RSD of the corrected areas of the fragment ion m/z 176 of 1-chloromethylnaphthalene in the sample of the SST must not be higher than 20 % between the seven replicate injections.

Results:

The results regarding the SST are shown in Table 50.

Table 50: Validation Results: System Suitability Test

Test-ID	RSD [%]
698501_181019_A_GK	3.73

Assessment:

The acceptance criterion regarding the SST was

☒ met
☐ not met

4.4.6 Assessment of the validation

The validation was

☒ successful
☐ not successful

5 Conclusion

In the course of this thesis, a method was developed which allows the absolute quantitation of the potential genotoxic impurity 1-chloromethylnaphthalene in 1-naphthylacetonitrile, the raw material of naphazoline hydrochloride and naphazoline nitrate. Further, the method was successfully transferred to and validated at the receiving laboratory according to ICH Q2 (R1) [24]. It was shown that the purchased raw material 1-naphthylacetonitrile contains several volatile and non-volatile impurities. To confirm their nature and their concentration in the raw material 1-naphthylaceetonitrile, further investigations are necessary. By using the standard addition procedure for the quantitation of the potential genotoxic impurity, all possible matrix effects and variations in the sample composition are considered. The investigated range of 2 - 10 ppm 1-chloromethylnaphthalene can be adjusted to lower or higher concentrations by adapting the spiking concentrations and the detector voltage. In this case a revalidation regarding the new defined range has to be performed. Fundamental decisions in the method development were made by considering the matrix of naphazoline, the precursor of naphazoline hydrochloride and naphazoline nitrate. Thus, the existing method is a good starting point for further method development.

6 Appendix

6.1 Investigation of reaction between 1-chloromethylnaphthalene and methanol

As described in 4.1.3.1, two substances were detected besides 1-chloromethylnaphthalene by using methanol as solvent. The identity of those substances was determined by recording a total ion chromatogram of a sample with 10 µg 1-chloromethylnaphthalene/mL methanol by using the splitless injection technique and comparing the corresponding mass spectra with the NIST database. Details regarding the used settings are listed in Table 51.

Table 51: Settings of GC, MS and Sampler: Investigation of reaction between 1-chloromethylnaphthalene and methanol

Gas Chromatograph		Mass Spectrometer	
Column	Agilent DB-5MS UI	Interface temperature	310 °C
Length:	30 m	Ion source temperature:	250 °C
Thickness:	0.25 µm	Solvent cut time:	2 min
Diameter:	0.25 mm	Event time:	0.3 sec
Pre-Column:	None	Start time data acquisition:	2.5 min
Liner:	Inlet Liner #1	End time data acquisition:	6.39 min
Injection volume:	1 µl	Acquisition mode:	Q3-Scan
Injection mode:	Split	Recorded m/z values:	50 – 400
Injection temperature:	300 °C	Detector voltage relative to the tuning result:	0 kV
Head pressure:	112.2 kPa	**Sampler**	
Linear velocity:	45.6 cm/s	# of rinses with solvent (Pre-run):	1
Column flow:	1.5 mL/min	# of rinses with solvent (Post-run):	2
Purge flow:	3 mL/min	# of rinses with sample:	0
Split ratio:	30	Washing volume:	6 µl
Carrier gas saver:	No		

Oven temperature program		
Rate (°C/min)	Final temperature (°C)	Hold time [min]
-	100	1
30	190	0
10	225	0
25	270	0
30	325	5

The obtained TIC is shown in Figure 35.

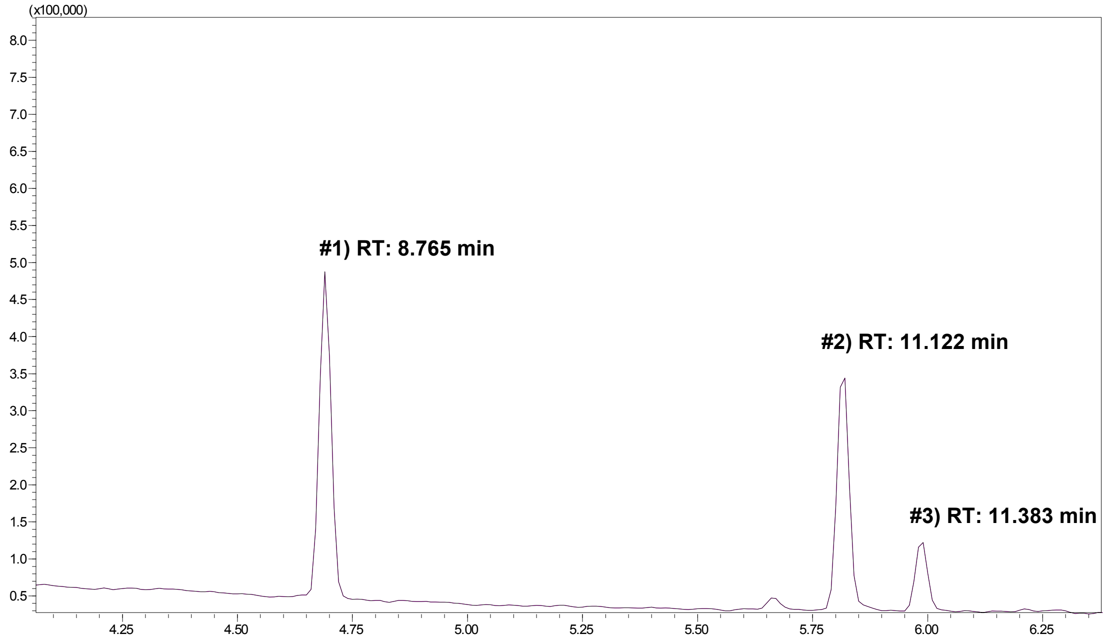


Figure 35: TIC-chromatogram of a sample with a concentration of 10 µg 1-chloromethylnaphthalene/mL methanol using splitless injection. Peak #1: 1-methylnaphthalene. Peak #2: 1-methoxymethylnaphthalene. Peak #3: 1-chloromethylnaphthalene.

The corresponding mass spectra of the initially unknown detected peaks (peak #1 and peak #2) were compared to those of the NIST database which are most likely (see Figure 36 and Figure 37).

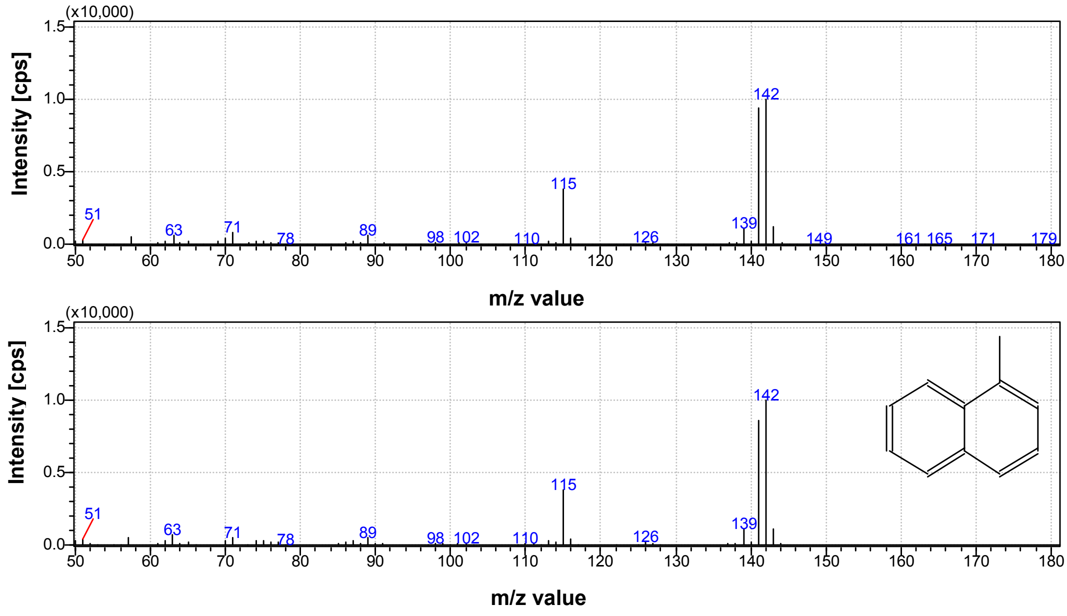


Figure 36: Upper pictue: Mass spectrum of peak#1 of Figure 35. Lower Picture: Mass spectrum of 1-methylnaphthalene from the NIST database. Similarity: 97 %

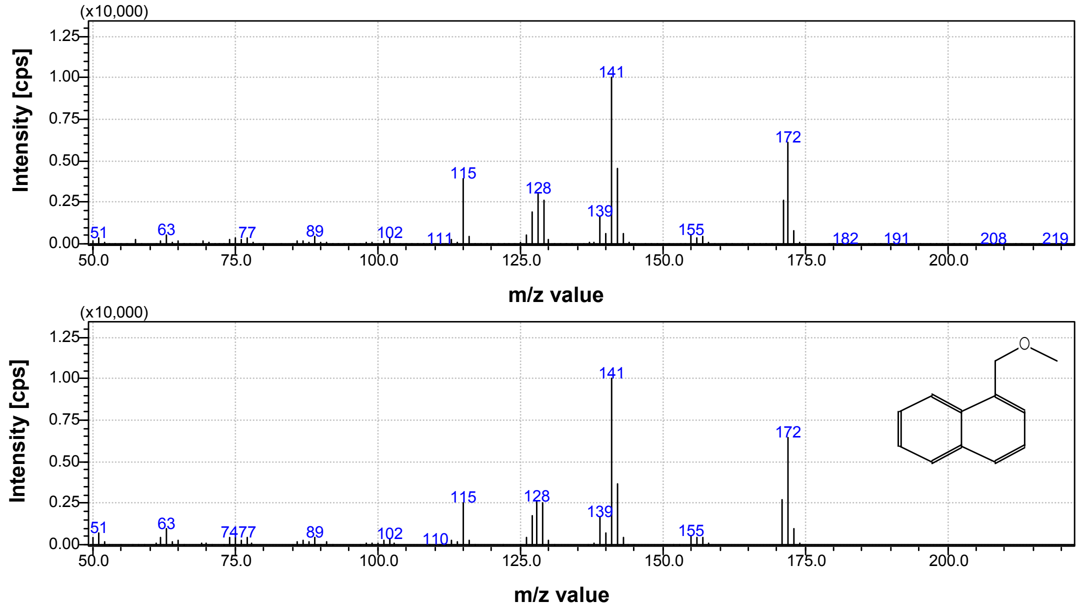


Figure 37: Upper pictue: Mass spectrum of peak#2 of Figure 35. Lower Picture: Mass spectrum of 1-methoxymethylnaphthalene from the NIST database. Similarity: 95 %

Differences to the mass spectra of the database are due to background signals (detected m/z values: 50 -400).

6.2 Screening for Impurities in the raw material 1-naphthylacetonitrile

The corresponding mass spectra of the red labeled peaks (peak #10 and peak #16) in Figure 23 were compared to those of the NIST database which are most likely (see Figure 38 and Figure 39).

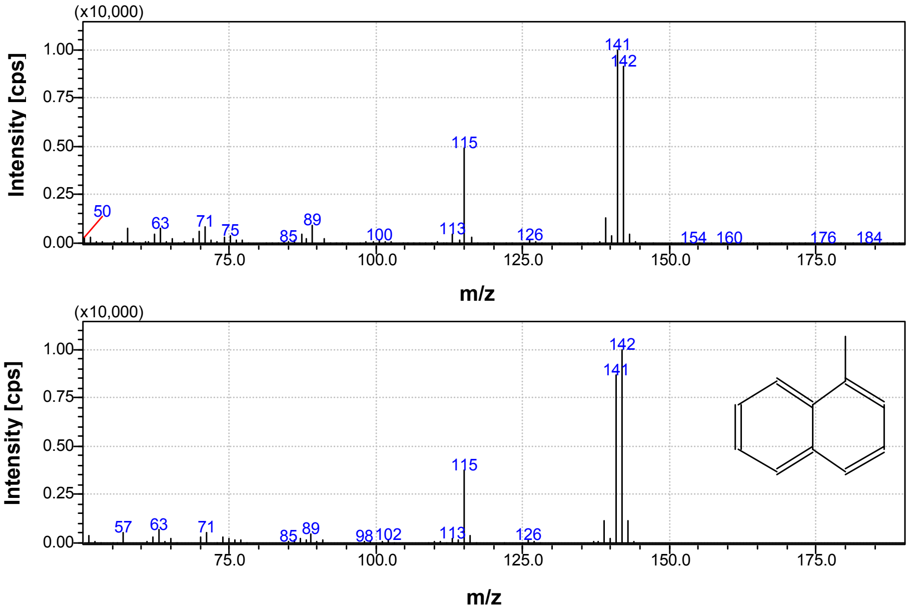


Figure 38: Upper picture: Mass spectrum of peak#10 of Figure 23. Lower Picture: Mass spectrum of 1-methylnaphthalene from the NIST database. Similarity: 93 %

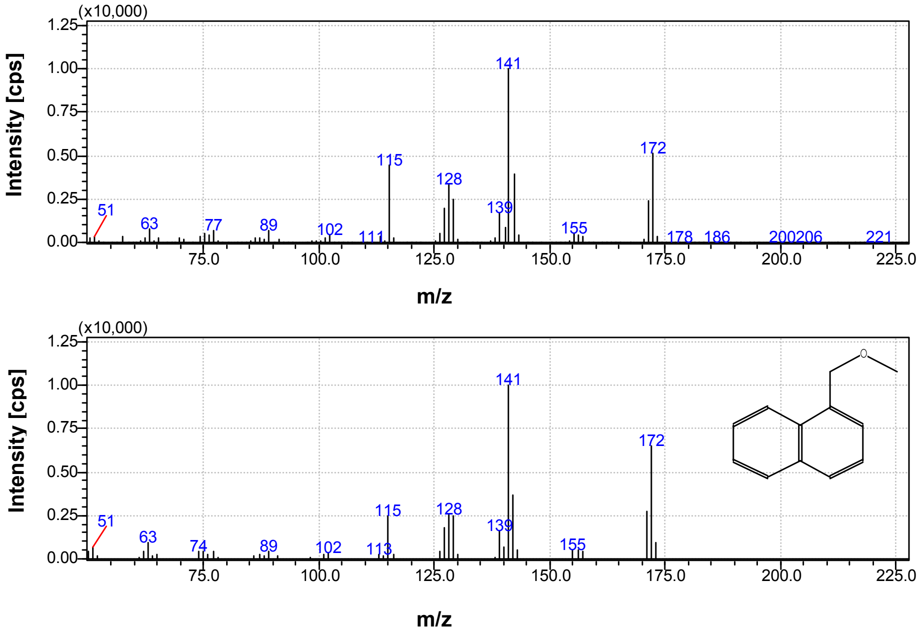


Figure 39: Upper picture: Mass spectrum of peak#16 of Figure 23. Lower Picture: Mass spectrum of 1-methoxymethylnaphthalene from the NIST database. Similarity: 94 %

Differences to the mass spectra of the database are due to background signals (recorded m/z values: 50 -1050).

6.3 Investigation of reactions between 1-naphthylacetonitrile and the solvents ethanol and isopropyl alcohol

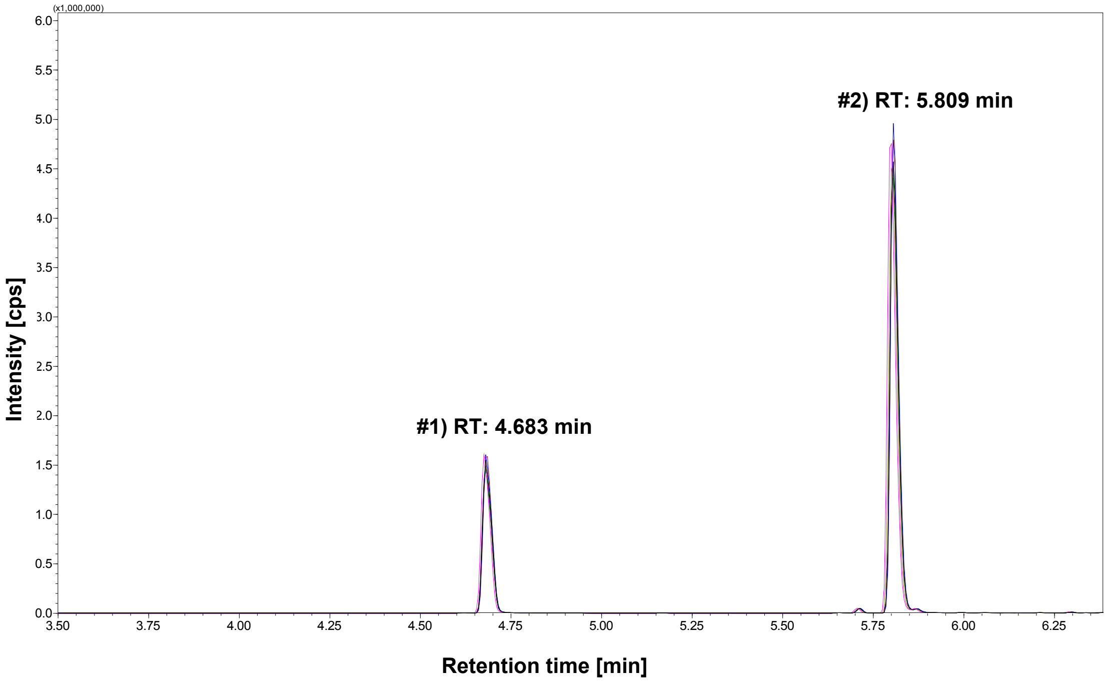


Figure 40: Overlay of all seven chromatograms of the measurements described in chapter 4.1.3.3. Sample containing 10 mg 1-naphthylacetonitrile/mL ethanol. Each chromatogram represents the sum of the detected ions with m/z values of 115, 139, 141, 176 and 178. Peak #1: 1-methylnaphthalene. Peak #2: 1-methoxymethylnaphthalene

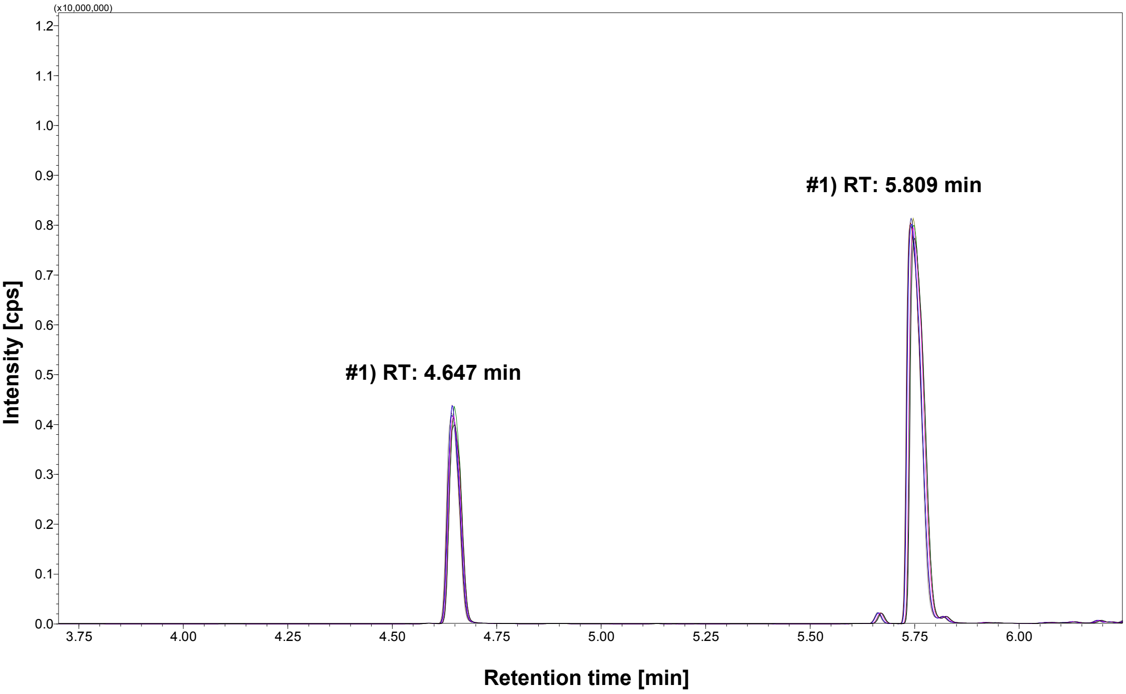


Figure 41: Overlay of the first eight chromatograms of the eleven measurements described in chapter 4.1.3.4 (maximum of eight chromatograms for data comparison applicable). Sample containing 10 mg 1-naphthylacetonitrile/mL IPA. Each chromatogram represents the sum of the detected ions with m/z values of 115, 139, 141, 176 and 178. Peak #1: 1-methylnaphthalene. Peak #2: 1-methoxymethylnaphthalene

6.4 Measured data

Table 52: Raw data of the standard addition experiments described in chapter 4.2.3

Test date:		02.08.2018		06.08.2018		10.08.2018		12.08.2018		19.08.2018	
Sample: 10 mg 1-naphthylacetonitrile/mL IPA	Spiked concentration of 1-chloromethylnaphthalene [ppm 1-Chloromethylnaphthalene]	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 141 1-methoxymethylnaphthalene [AU]	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 141 1-methoxymethylnaphthalene [AU]	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 141 1-methoxymethylnaphthalene [AU]	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 141 1-methoxymethylnaphthalene [AU]	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 141 1-methoxymethylnaphthalene [AU]
	0	-	-	-	-	1457	9750442	1372	9874305	1678	12812316
	0	-	-	-	-	1387	9754288	1399	9891297	1557	12516472
	0	1975	9461226	1780	9526583	1481	9840259	1395	9936578	1527	12593496
	1.93	2834	9848814	2471	9766085	2067	9900047	1868	9880646	2186	12834757
	3.85	3715	9849958	3173	9734279	2586	9928568	2463	9931926	2575	11886189
	5.78	4522	9857128	3534	9002360	2889	9182141	2761	9205258	3180	12034050
	7.70	5365	9819811	4315	9293741	3630	9431880	3377	9423573	3722	12123266
	9.63	6031	9765103	5063	9422551	4307	9580364	3858	9612206	4481	12206990
	0	2026	9885124	1727	9460149	1416	9640774	1317	9597114	1602	12488673
	0	-	-	-	-	1430	9635287	1314	9168074	1648	12557566
	0	-	-	-	-	1393	9554860	1382	9422115	1527	12320320

Table 53: Raw data of the standard addition experiments described in chapter 4.2.5

Test date:		14.09.2018		17.09.2018		20.09.2018		21.09.2018	
Sample: 10 mg 1-naphthylacetonitrile/mL IPA	Spiked concentration of 1-chloromethylnaphthalene [ppm 1-Chloromethylnaphthalene]	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 141 1-methoxymethylnaphthalene [AU]	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 141 1-methoxymethylnaphthalene [AU]	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 141 1-methoxymethylnaphthalene [AU]	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 141 1-methoxymethylnaphthalene [AU]
	0	2634	9590943	2804	10242472	2811	9805270	2395	8439820
	1.93	3779	9505047	3772	9505908	4087	9612168	3495	8459802
	3.85	4907	9527721	4851	9557567	5290	9558425	4698	8555088
	5.78	6262	9774640	6130	9543433	6507	9558589	5865	8626130
	7.70	7469	9622319	7226	9622823	7743	9477471	7116	8666211
	9.63	8527	9612919	8441	9504320	9125	9538184	8301	8672149
	0	2759	9865721	2901	10548893	2756	9709131	2478	8787827

Table 54: Investigation of active sites: Priming injections (chapter 4.2.4.1)

Test date:		17.09.2018		
Sample: 10 mg 1-naphthylacetonitrile/mL IPA	Spiked concentration of 1-chloromethylnaphthalene [ppm 1-Chloromethylnaphthalene]	# of Analysis	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 141 1-methoxymethylnaphthalene [AU]
	9.63	1	6268	9311678
		2	7109	9367419
		3	7446	9415386
		4	7634	9455540
		5	7983	9549692
		6	7971	9488999
		7	8066	9533641
		8	8123	9661617

Table 55: Raw data of the standard addition experiments described in chapter 4.2.6.1

Test date:		01.10.2018		03.10.2018	
Sample: 10 mg 1-naphthylacetonitrile/mL IPA	Spiked concentration of 1-chloromethylnaphthalene [ppm 1-Chloromethylnaphthalene]	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 136 naphthalene-d_8 [AU]	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 136 naphthalene-d_8 [AU]
	0	1594	24088	1449	22391
	1.93	2349	24645	2065	22853
	3.85	2908	23165	2731	22886
	5.78	3868	24257	3470	23078
	7.70	4362	23135	4192	23598
	9.63	5206	24033	5081	24445
	0	1651	24113	1486	23605

Table 56: Raw data of the validation experiments / SST (chapter 4.4.5.9)

Test-ID:	20181019_User_GK	
Sample: 10 mg 1-naphthylacetonitrile/mL IPA	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 136 naphthalene-d_8 [AU]
Blank_001	0	34769
SST_001	5235	47617
SST_002	7355	66809
SST_003	8286	72349
SST_004	8546	74423
SST_005	9192	75248
SST_006	9331	79177
SST_007	9408	81044
Blank_002	0	77779

Table 57: Raw data of the validation experiments / Standard addition procedure (chapter 4.4)

Test-ID:		20181019_User_GK					
# Approach		1		2		3	
Sample: 10 mg 1-naphthylacetonitrile/mL IPA	Spiked concentration of 1-chloromethylnaphthalene [ppm 1-Chloromethylnaphthalene]	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 136 naphthalene-d_8 [AU]	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 136 naphthalene-d_8 [AU]	Peak area m/z 176 1-chloromethylnaphthalene [AU]	Peak area m/z 136 naphthalene-d_8 [AU]
	0	2079	58249	3098	87164	3291	88651
	0	2461	71332	3138	86286	3259	92239
	0	2539	76552	3280	89111	3019	91307
	1.93	3938	79094	4589	86242	4520	90849
	3.85	5191	77548	5829	88927	6525	93507
	5.78	7026	81670	7746	88674	8020	92280
	7.70	8060	80870	9315	88111	9867	92925
	9.63	10493	84112	10732	89286	11777	92452
	0	2971	83281	3284	90297	3325	93294
	0	2906	84485	3057	88566	3587	93906
	0	2998	84355	3079	90431	3200	90481

6.5 Validation: Evaluation of residuals for linearity

The residuals of the regressions lines of the linearity experiments (see chapter 4.4.5.2) were evaluated with DataLab. The results are shown below.

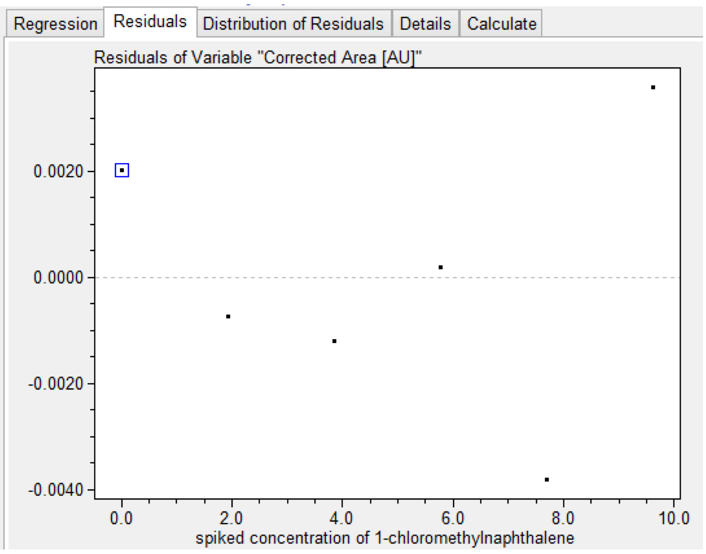
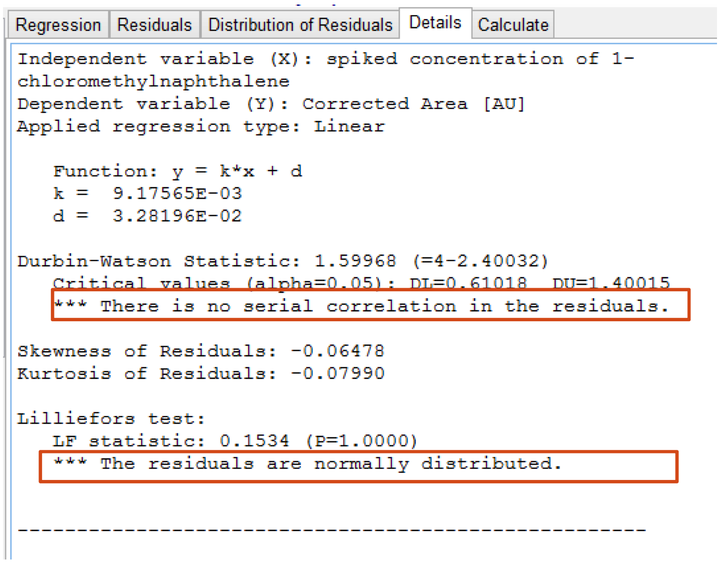


Figure 42: Test-ID: 20181019_User_GK: Approach #1. Evaluation of the residuals of the regression line with DataLab: Residual variances are homogenous



```
Independent variable (X): spiked concentration of 1-
chloromethylnaphthalene
Dependent variable (Y): Corrected Area [AU]
Applied regression type: Linear

   Function: y = k*x + d
   k =  9.17565E-03
   d =  3.28196E-02

Durbin-Watson Statistic: 1.59968  (=4-2.40032)
   Critical values (alpha=0.05): DL=0.61018  DU=1.40015
   *** There is no serial correlation in the residuals.

Skewness of Residuals: -0.06478
Kurtosis of Residuals: -0.07990

Lilliefors test:
   LF statistic: 0.1534  (P=1.0000)
   *** The residuals are normally distributed.


-----------------------------------------------------
```

Figure 43: Test-ID: 20181019_User_GK: Approach #1. Evaluation of the residuals of the regression line with DataLab

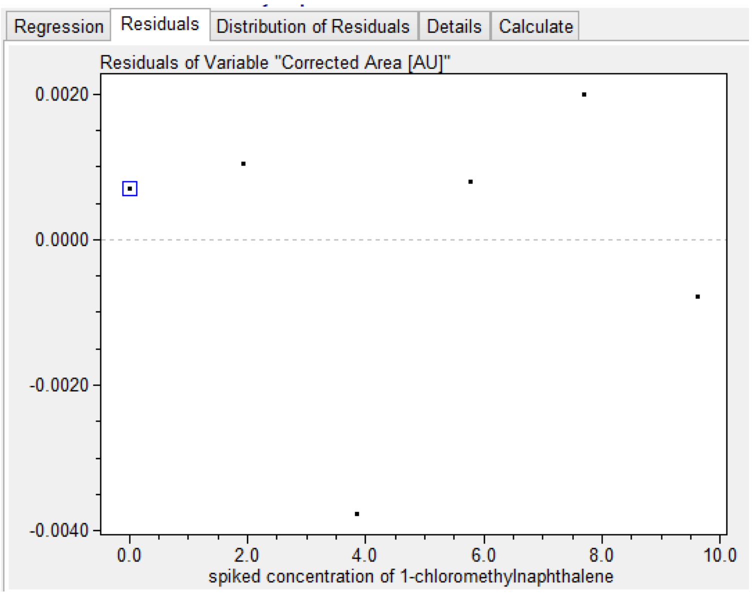
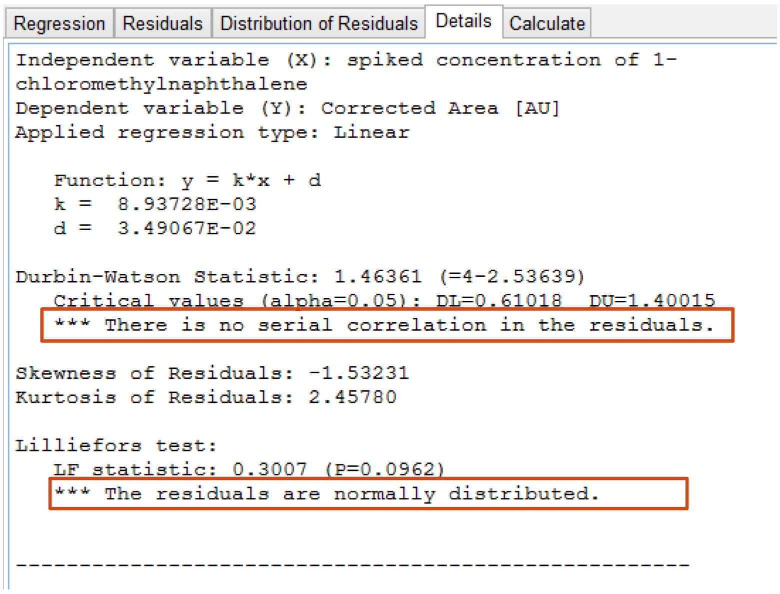


Figure 44: Test-ID: 20181019_User_GK: Approach #2. Evaluation of the residuals of the regression line with DataLab: Residual variances are homogenous



```
Independent variable (X): spiked concentration of 1-
chloromethylnaphthalene
Dependent variable (Y): Corrected Area [AU]
Applied regression type: Linear

   Function: y = k*x + d
   k =  8.93728E-03
   d =  3.49067E-02

Durbin-Watson Statistic: 1.46361 (=4-2.53639)
   Critical values (alpha=0.05): DL=0.61018  DU=1.40015
   *** There is no serial correlation in the residuals.

Skewness of Residuals: -1.53231
Kurtosis of Residuals: 2.45780

Lilliefors test:
   LF statistic: 0.3007 (P=0.0962)
   *** The residuals are normally distributed.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```

Figure 45: Test-ID: 20181019_User_GK: Approach #2. Evaluation of the residuals of the regression line with DataLab

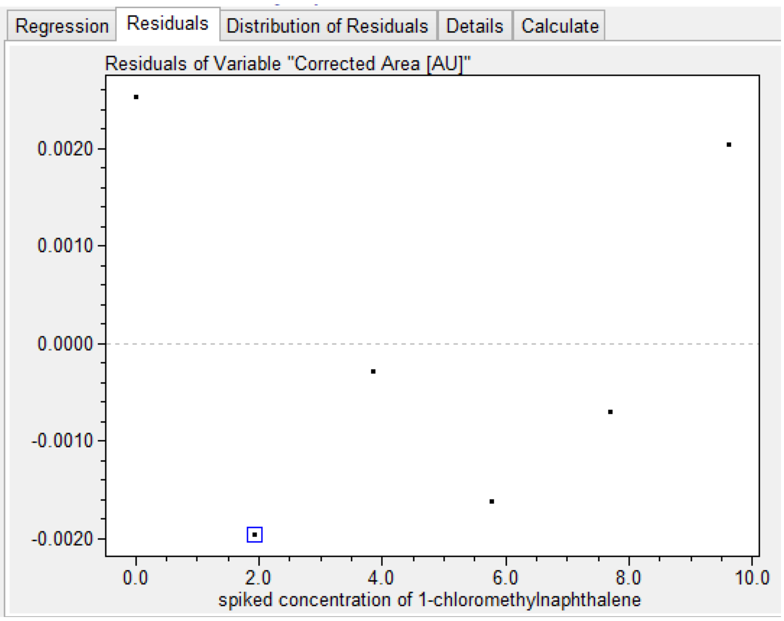
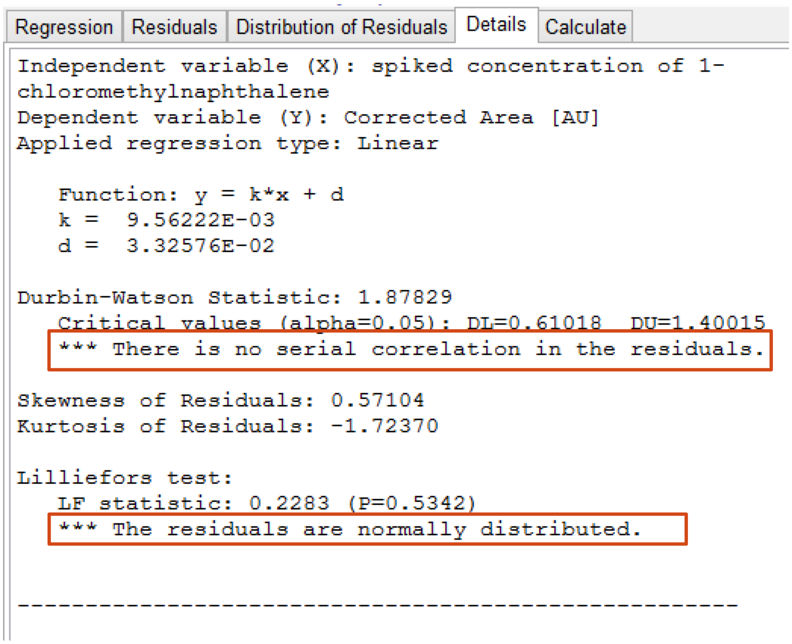


Figure 46: Test-ID: 20181019_User_GK: Approach #3. Evaluation of the residuals of the regression line with DataLab: Residual variances are homogenous



```
Independent variable (X): spiked concentration of 1-
chloromethylnaphthalene
Dependent variable (Y): Corrected Area [AU]
Applied regression type: Linear

   Function: y = k*x + d
   k =  9.56222E-03
   d =  3.32576E-02

Durbin-Watson Statistic: 1.87829
   Critical values (alpha=0.05): DL=0.61018  DU=1.40015
   *** There is no serial correlation in the residuals.

Skewness of Residuals: 0.57104
Kurtosis of Residuals: -1.72370

Lilliefors test:
   LF statistic: 0.2283 (P=0.5342)
   *** The residuals are normally distributed.


-----------------------------------------------------
```

Figure 47: Test-ID: 20181019_User_GK: Approach #3. Evaluation of the residuals of the regression line with DataLab: Residual variances are homogenous

7 References

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