Automation in drug product development – efficiency and quality

Elisa Sophie Haßler

Degree Project in Analytical Chemistry, 2024
Department of Chemistry
Lund University
Sweden

MSc, 30 hp

LUND
UNIVERSITY

Automation in drug product development – efficiency and quality

Elisa Sophie Haßler

LUND UNIVERSITY

Degree Project in Analytical Chemistry
2024
MSc, 30 hp

Supervisor:
Margareta Sandahl
Lund University

Examiner:
Peter Spégel
Lund University

Assistant supervisor:
Alexandra Chukharkina
AstraZeneca

Assistant supervisor:
Magnus Klarqvist
AstraZeneca

Lund University
Department of Chemistry
Centre for Analysis and Synthesis
P.O. Box 124
SE-221 00 Lund, Sweden

Title in popular language

Discovering and developing new medicines is crucial for public health, and verifying their quality at every step of this process is equally important. This involves analysing drugs in detail, often using advanced techniques that require meticulous preparation of the drug samples, ensuring that, for example, a tablet preparation can be effectively analysed using specific instruments. While automated systems have improved this process, the pharmaceutical industry has been slow to fully adopt them, leading to the continued use of manual sample preparation methods, which can be time consuming and resource intensive.

Several studies suggest that automated sample preparation could offer equivalent results to manual methods while saving time, costs, and resources. However, understanding the precision (how close measurement results in a series are to each other) and accuracy (how close measurement results are to the true value) of automated approaches compared to manual ones remains an area of research.

This work aims to address this gap by establishing an automated sample preparation workflow and comparing it with the manual procedure. This includes an evaluation of both methods for their suitability in drug analysis, investigating various parameters, including accuracy and precision, according to regulatory validation guidelines. Additionally, the measurement uncertainty, describing the level of doubt associated with the exact value of a result due to factors like equipment limitations or variations in conditions, for both the manual and automated sample preparation are assessed to judge the quality and reliability of the measurements.

The study has shown that both methods generally yielded similar results, with the automated workflow proving suitable for drug sample preparation and analysis. However, the manual method demonstrated slightly higher accuracy and a lower uncertainty, indicating higher quality. Automation, on the other hand, reduced the hands-on analyst time and solvent usage, leading to significant gains in efficiency, environmental friendliness, and safety of the work environment.

While moving forward, the choice between manual and automated sample preparation should be made on a case-by-case basis, considering the advantages and drawbacks of both approaches, the work also highlights the potential for additional improvement in the automated sample preparation workflow to enhance both its efficiency and quality further.

Abstract

Introduction: This work evaluates the efficiency and quality of drug substance and product automated sample preparation for liquid chromatography analysis in comparison to the manual preparation technique.

Background: Despite the potential advantages of process automation in improving efficiency, accuracy, and precision while reducing time and resources, sample preparation in the early phases of drug development in the pharmaceutical industry is predominantly performed manually. Moreover, recent literature comparing manual and automated methods in this context is limited. Additionally, assessing the uncertainty inherent in analytical measurements is often overlooked, yet crucial for ensuring data quality and reliability.

Aim(s): This work aims to evaluate the equivalence of manual and automated sample preparation methods and to assess the associated measurement uncertainties, with the goal to improve efficiency and quality in analytical workflows by implementing automated systems.

Methods: An automated workflow for sample preparation of drug substance and product was established using the Tecan Fluent liquid handling system. A comprehensive method validation, including specificity, accuracy, precision, linearity, limit of quantification, and stability in solution, was conducted for both manual and automated sample preparation of drug substance and drug product. Furthermore, a thorough uncertainty analysis was performed to judge reliability of the manual and automated method.

Results: The manual and automated sample preparation and analysis were both successfully validated, yielding equivalent results, although the manual procedure exhibited higher accuracy. Implementation of the automated workflow resulted in a 72% reduction in hands-on analyst time, and a 69% reduction in required solvent volume. The uncertainty analysis revealed a higher uncertainty for the automated approach compared to the manual sample preparation.

Conclusion: While the manual method delivers results with better quality, the automated procedure demonstrated superior efficiency and additional optimization strategies could further enhance the quality and efficiency of the automated workflow.

Keywords: Automation, method validation, pharmaceutical analysis, sample preparation, uncertainty analysis

Acknowledgement

First of all, I am grateful to AstraZeneca for providing me with the opportunity and resources to conduct this work within their organization. I want to extend my deepest appreciation to my industrial supervisors at AstraZeneca, Alexandra Chukharkina and Magnus Klarqvist, for their invaluable support, mentorship, and expertise. Their guidance and insightful feedback have been instrumental in refining the scope of this research and in completing this degree project. Special thanks are also extended to Johan Hulthe, whose collaboration and assistance have been indispensable throughout the entire project.

Furthermore, I want to express my appreciation to my academic supervisor and examiner at Lund University, Margareta Sandahl and Peter Spégel, for their constructive criticism and guidance, which have provided invaluable perspectives that greatly enriched this work.

I would also like to acknowledge the unwavering support of my family and friends, whose encouragement has been a constant source of motivation throughout this journey.

In conclusion, I am deeply grateful to everyone mentioned above and all others who have played a role, however small, in the completion of this degree project.

Table of contents

1 List of abbreviations

(s)..... *standard*
(u) ..... *sample (the unknown)*
A ..... *peak area*
API ..... *active pharmaceutical ingredient*
c ..... *concentration*
d ..... *dilution*
Eq ..... *Equation*
FR..... *fridge*
HPLC..... *high performance liquid chromatography*
ICH ..... *International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use*
LC..... *liquid chromatography*
LOD..... *limit of detection*
LOQ..... *limit of quantification*
m ..... *mass*
PDA..... *photodiode array*
PTFE..... *polytetrafluoroethylene*
rep(x) ..... *repeatability of x*
RSD ..... *relative standard deviation*
RT..... *room temperature*
SOP..... *standard operating procedure*
TPW ..... *tablet processing workstation by manufacturer Sotax*
u(x) ..... *uncertainty of x*
UHPLC..... *ultra high performance liquid chromatography*
UV ..... *ultraviolet*
v ..... *volume*
WC ..... *working concentration*

2 Introduction

After discovering a promising therapeutic substance, it takes many years to develop and test a potential new drug before a finished product eventually reaches the market[1]. During the early phases of clinical development, flexibility is key as various formulation compositions and strengths are evaluated to turn the active pharmaceutical ingredient (API) into an ideal drug product. Throughout this process, adherence to quality standards is essential, focusing on aspects such as the identity, content, and purity of a new preparation[2]. Consequently, the analysis of drug substances and products through techniques like high performance liquid chromatography (HPLC) is a crucial and routinely performed task. Equally important as the method for determination is the sample preparation process, which typically involves dispersion, dissolution, and dilution of the sample to obtain the desired working concentration (WC). For drug products, such as tablets or capsules, that contain insoluble excipients alongside the API, an additional filtration step may be necessary before the sample is ready for injection into the HPLC instrument[3].

A comparative analysis of surveys conducted between 1991 and 2023, assessing sample preparation for chromatographic analysis in various industries, underscores a significant reduction in both the time required for sample preparation and the associated number of errors over the past three decades[4]. This decline is largely attributed to the increased use of automated systems, which offer higher throughput, efficiency, and precision, alongside a standardized and completely traceable generation of data. The resulting reduced human interference minimizes human error, reduces exposure of workers to dangerous substances, and lowers operational costs[5, 6].

While publications from about 20 years ago, such as Han and Munro describing the transfer of a manual method to an automated system[7], Toro et al. developing and validating an automated method for tablet content uniformity testing[8], and Shamrock et al. discussing method transfer and validation of an automated tablet sample preparation[9], indicate early interest in automation, recent publications on this topic are scarce. Examples from the last two years include a study by Liu et al., focusing on method development for automated sample preparation[6], and a publication by Fileš and Andersson, which describes the first use of an automated sample preparation system for protein tablet preparations[10]. This indicates that in spite of noticeable advancements, the pharmaceutical industry is progressing slower than other sectors in transitioning towards an autonomous working environment independent of

human intervention[11]. As of today, sample preparation for subsequent analysis remains predominantly a manual task, demanding significant time and resources[5]. This is despite the comparative studies by Han and Munro[7], Toro et al.[8], Shamrock et al.[9], and Fileš and Andersson[10], all of which indicated higher efficiency with the automated approach while achieving equivalent results compared to the manual methods. However, Fileš and Andersson also observed that, contrary to expectations, the use of automated systems did not enhance method repeatability[10]. This emphasizes the need for additional research, particularly in comparing manual and up-to-date automated methods, with a specific focus on assessing precision and accuracy, alongside method efficiency. Addressing this knowledge gap is a primary objective of the present work.

The equipment for automated sample preparation can be tailored to a specific task or more flexible, ranging from simple systems specialized solely in the addition of liquid to highly complex systems capable of automatically weighing, transferring, diluting, agitating, homogenizing, filtrating, and directly injecting samples into liquid chromatography (LC) instruments[3]. The most common complex system found in literature and in the pharmaceutical industry, is the automated tablet processing workstation (TPW) from Sotax[12]. The TPW is characterized by its ability to process 100 samples sequentially and employs tube-based liquid transfers[12]. This configuration requires thorough washing after each sample to minimize carryover. Moreover, the TPW integrates specialized operations like wet grinding[12], setting it apart from conventional manual methods. Alternative automation solutions to the TPW include the Accroma system from Archer Science[13] or the Fluent workstation manufactured by Tecan[14]. The Fluent liquid handling robot, also utilized in this work, offers parallel sample processing capabilities, and can be customized with features such as magnetic stirring, thereby facilitating transfer of a manual method to the automated system[14].

After method transfer to a different system, modification to fit a different application, or the development of a completely new method, the entire procedure must undergo validation to ensure its suitability for the intended use and compliance with the quality requirements[15, 16]. Several guidelines on validation are provided by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), proposing various parameters to assess the performance of a method and suggesting acceptance criteria that need to be met during validation[16]. These parameters generally include specificity, accuracy, linearity, and precision of the method. Precision can further be divided into repeatability

(assessing the same conditions in a short time), intermediate precision (including variations within the same laboratory), and reproducibility (evaluating variations between different laboratories). Additional method characteristics that are usually investigated include the limit of detection (LOD), limit of quantification (LOQ), and the robustness of the method[15]. To avoid continuous validation activities during early drug product development, upon for example changes in tablet strength, analysts can use the bracketing approach for method validation, which involves evaluating only the lowest and highest strength products, claiming that if they meet the specified criteria, the intermediate strengths are covered as well[17].

A successfully validated method leads to an increased confidence in the reliability of results acquired during sample analysis. However, it is crucial to acknowledge the presence of random errors, leading to variation in the obtained results[18]. These variations contribute to the overall uncertainty of the measurement and arise from various sources. In sample preparation, weighing[19] and dispensing of diluent[20] are the error sources with the greatest influence[3], but the instrumental method itself also contributes to the overall uncertainty[21]. Therefore, it is essential not only to report a result but also to specify the range of values which can be attributed to the measurand with a certain level of confidence[18]. However, despite being an essential part of every measurement and crucial to ensure data reliability and quality, uncertainty often remains overlooked. This work aims to close this gap in knowledge by evaluating the uncertainty in the performed measurements.

The EURACHEM/CITAC guide[18] and the NORDTEST handbook[22] provide examples of how to conduct an uncertainty analysis in an analytical laboratory. According to these guidelines, uncertainties can be quantitated using either the “top-down” or the “bottom-up” approach. The top-down approach aims to directly quantify the overall uncertainty by using data from intermediate precision studies, while the bottom-up approach tries to quantify each individual uncertainty separately and then sum up all the contributions[23]. Few comparisons exist between these approaches, and while some publications claim both yield similar results[23], others describe the top-down approach as more accurate[22].

In this thesis, validation is performed to assess method equivalency through a comparative analysis of manual and automated sample preparation, aiming to enhance efficiency and precision in the analytical workflow by using automated systems. Additionally, the work includes a comprehensive uncertainty assessment to evaluate data reliability and quality.

3 Materials and Methods

3.1 Chemicals

HPLC grade water and HPLC grade acetonitrile purchased from Merck KGaA (Darmstadt, Germany) were used for the preparation of the diluent and mobile phases. Molecular biology grade ammonium acetate solution (7.5 M) purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany) was used for the preparation of the mobile phases.

The analysed drug substance, API in powder form with a specified purity of 99.8%, was manufactured by AstraZeneca (Macclesfield, UK). The used excipients to mimic the drug product, a tablet preparation, were mannitol, manufactured by Roquette Fréres (Lestrem, France), microcrystalline cellulose and croscarmellose sodium, manufactured by DuPont (Cork, Ireland), hydroxypropyl cellulose, manufactured by Ashland Aqualon Functional Ingredients (Hopewell, USA), magnesium stearate, manufactured by Peter Greven (Venlo, Netherlands), and silicon dioxide, manufactured by W. R. Grace (Curtis Bay, USA).

3.2 Automation equipment

The automated sample preparation was run on a customized Tecan Fluent 1080 automated workstation, shown in Figure 1.

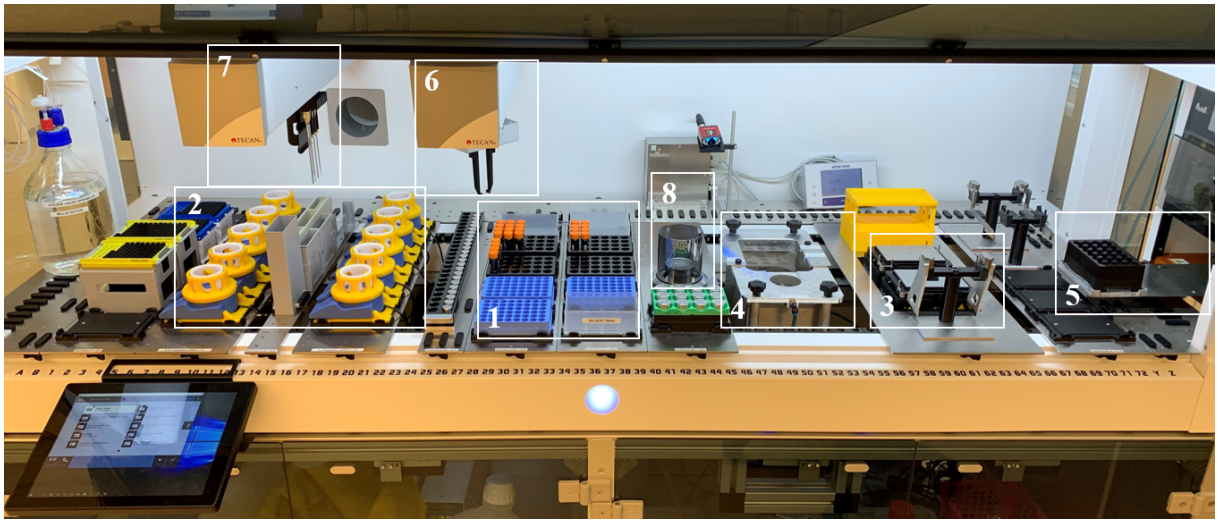


Figure 1: Worktable of the Tecan Fluent 1080 automated workstation with racks for different vials and diluent reservoirs (1), magnetic stirrers (2), heated shakers (3), an ultrasonic bath (4), an integrated decapper (5), one robotic arm for labware transfer (6) and one for liquid handling (7), and an integrated weighing module (8).

Figure 1 illustrates the worktable of the Fluent 1080, which accommodates various vials and corresponding racks, as well as diluent-filled reservoirs (1). It features up to ten magnetic

stirrers (2), two heated shakers (3), and an ultrasonic bath for tablet and powder dissolution (4), alongside an integrated decapper for vial de- and recapping (5). The robotic arms facilitate sample transfer and liquid handling tasks of the automated workstation. One arm transports labware across different positions (6), while the second arm comprises eight pipetting channels with fixed steel or disposable plastic tips for liquid handling of volumes between 50 and 5000 µl (7). Liquid additions and sample dilutions are controlled either volumetrically through internal calibration, or gravimetrically via the integrated weighing module (WXS204) from Mettler Toledo (8). The setup and methods of the Fluent 1080, controlled by the FluentControl software, are highly customizable to fit a variation of applications. All essential process parameters including weights and volumes are recorded automatically[14].

3.3 Methods

3.3.1 Ultra high performance liquid chromatography

All samples prepared in this work were analysed using a Waters ACQUITY ultra high performance liquid chromatography (UHPLC) H-class system, equipped with an autosampler and a photodiode array (PDA) detector. The analyses were conducted on a Waters ACQUITY ethylene bridged hybrid C18 column (2.1 x 50 mm) with a particle size of 1.7 µm. Method parameters included an injection volume of 2 µl, a flow rate of 0.6 ml/min, a column temperature maintained at 40 °C, and a detection wavelength set at 220 nm. The mobile phase A consisted of 12.5 mM ammonium acetate in water, and mobile phase B consisted of 12.5 mM ammonium acetate in a 90:10 (v/v) acetonitrile to water mixture. Specific details regarding the gradient are provided in appendix 8.1. Instrument control, data collection, processing, and reporting were managed using the Empower software from Waters.

3.3.2 Method validation

Method validation aimed to demonstrate the suitability of the automated sample preparation method for the analysis of the investigated drug substance and drug product. Additionally, it was conducted to assess method equivalency between the manual and automated sample preparation through a comparative analysis. The parameters investigated include specificity, defined as the capability of the method to determine the amount of analyte in the sample in the presence of components likely to be present[15], accuracy, defined in this work as the closeness of the measurement result to an accepted true value[15], precision, defined as the

closeness between the results of a number of measurements[15], linearity, LOQ, and stability in solution. The method validation approach and acceptance criteria were established in accordance with internal standard operating procedures (SOPs) and relevant ICH guidelines.

Table 1 shows the plan for the drug substance method validation, including acceptance criteria, information on the number of samples prepared for each test, their API content relative to the WC, and the number of injections into the chromatographic instrument. The WC is the desired concentration of the sample when injected into the HPLC instrument to achieve an optimal signal, depending on the specific compound and method of analysis.

Table 1: Parameter and acceptance criteria for the drug substance validation, as well as how many samples at which API concentration relative to the working concentration were prepared, and the number of injections. Precision is also assessed at 0.1% WC, which covers the internally defined drug substance specification limit.

Parameter	**% API of WC**	**No. of samples**	**No. of injections**	**Acceptance criteria**
Specificity	0	1	1	No peak interference when comparing sample to blank
	100	1	1	
Accuracy	100	3	3	Recovery between 99.0% and 101.0%
Precision	100	6	1	Relative standard deviation ≤ 3%
	0.1	6	1	Relative standard deviation < 10%
LOQ	0.05	1	6	Signal-to-noise ratio > 10 Relative standard deviation < 20%
Linearity	150	2	1	No visible curvature after linear regression R^2 >0.99 y-axis intercept not greater than 2% of peak area of 100% sample
	120	2	1	
	100	2	1	
	80	2	1	
	50	2	1	
	30	2	1	
	10	2	1	
	1	2	1	

Parameter	% API of WC	No. of samples	No. of injections	Acceptance criteria
	0.5	2	1	
	0.05	2	1	
Stability in solution	100	1	1	Value after storage: ± 2.0% of initial
	0.05	1	1	Value after storage: ± 99% of initial

The samples for stability in solution were initially analysed immediately after their preparation. Subsequently, three LC vials for each concentration were stored at room temperature (RT), while three were stored in the fridge (FR). These samples where then analysed after 24 hours, 48 hours, and one week of storage.

To assess the suitability of the method for analysing the drug product, the bracketing approach was adopted, focusing solely on the highest strength (300 mg API in a 500 mg tablet) and the lowest strength (1 mg API in a 100 mg tablet) product. To simulate a tablet, all excipients were weighed precisely according to their proportions in the 500 mg respectively 100 mg tablets, and the API was added at 80 %, 100 %, or 120 % of the specified strength. Specificity, accuracy, and stability in solution were investigated as outlined in Table 2.

Table 2: Parameter and acceptance criteria for the drug product validation, as well as how many samples at which API concentration relative to the strength were prepared, and the number of injections. Stability in solution was only assessed for the low strength drug product samples.

Parameter	Excipients % of tablet	API % of strength	No. of samples	No. of injections	Acceptance criteria
Specificity	100	0	1	1	No peak interference when comparing sample to blank
	100	100	1	1	
Accuracy	100	80	3	3	Recovery between 97.0% and 103.0%
	100	100	3	3	
	100	120	3	3	
Stability in solution	100	100	1	1	Value after storage: ± 2.0% of initial

The stability in solution samples, assessed only for the low strength drug product, were stored at the same conditions as those for the drug substance and analysed at 0, 24, and 48 hours.

3.3.3 Manual sample preparation

All samples were prepared and volumetrically diluted in a 50:50 (v/v) mixture of acetonitrile and water, which also served as the blank. The working concentration of 100% was defined as 0.15 mg/ml. API and excipients were weighed using an analytical microbalance (XPR6UD5) from Mettler Toledo.

For the standards and all 100% drug substance samples, 7.5 mg of API was sonicated for 10 minutes in 25 ml of diluent, then brought to a final volume of 50 ml with diluent. Six of the 100% samples were further diluted to 0.1% WC. The linearity samples were prepared from two stock solutions at 150% WC and 30% WC. For the two 150% stock solutions, 11.25 mg of API was sonicated for 10 minutes in 25 ml of diluent, followed by an addition of 25 ml of diluent to reach a final volume of 50 ml. These stock solutions were then diluted to 120%, 100%, 80%, and 50% of the WC. For the two 30% stock solutions, 9 mg of API was sonicated for 10 minutes in 100 ml of diluent, followed by an addition of 100 ml of diluent to reach a final volume of 200 ml. These stock solutions were diluted to 10% WC. The 10% solutions were further diluted to 0.5% WC and 1% WC, and the 1% solutions were further diluted to 0.05% WC. All samples were transferred into LC vials for analysis.

For the analysis of the high and low strength drug product, excipients were weighed according to Table 3 in ten replicates.

Table 3: Excipient composition of the investigated high strength and low strength tablets.

Excipient	**High strength tablet (500 mg containing 300 mg API)**	**Low strength tablet (100 mg containing 1 mg API)**
Mannitol	110 mg	63 mg
Microcrystalline cellulose	45 mg	27 mg
Croscarmellose sodium	20 mg	4 mg
Hydroxypropyl cellulose	15 mg	3 mg
Magnesium stearate	5 mg	1 mg
Silicon dioxide	5 mg	1 mg

Additionally, for the high strength samples, 240 mg of API was weighed for the three 80% samples, 300 mg for the three 100% samples, and 360 mg for the three 120% samples. For the

low strength samples, 0.8 mg of API was weighed for the three 80% samples, 1.0 mg for the three 100% samples, and 1.2 mg for the three 120% samples. One sample in each case was left without API addition. To each high strength sample, 80 ml of diluent was added, and to each low strength sample, 4 ml of diluent was added. The samples were mixed for 1 hour at 190 rpm in an orbital shaker. Subsequently, 40 ml of diluent was added to the high strength samples, and 2 ml of diluent was added to the low strength samples. The samples were then sonicated for 20 minutes. The ten high strength samples were brought to a total volume of 200 ml with diluent and then diluted at a ratio of 1:10 to obtain the working concentration of 0.15 mg/ml for the 100% sample. The ten low strength samples were filled to a total volume of 10 ml, resulting in a working concentration of 0.1 mg/ml for the 100% sample. All samples were finally filtered through a 0.45 µm PTFE filter (Pall Acrodisc One) into LC vials for analysis.

3.3.4 Automated sample preparation

All samples were prepared using the Tecan Fluent 1080 automated workstation, employing a 50:50 (v/v) mixture of acetonitrile and water. The working concentration of 100% was defined as 0.15 mg/ml.

Both the API and excipients were weighed using an automated balance (XPR226Q) from Mettler Toledo, equipped with an automatic sample changer. API, hydroxypropyl cellulose and silicon dioxide were dispensed using QH008-BNMP powder dosing heads from Mettler Toledo. Mannitol, microcrystalline cellulose, croscarmellose sodium, and magnesium stearate were dispensed using QH002-CNMW powder dosing heads from Mettler Toledo.

As the automated balance is calibrated for a minimum weight of 5 mg, the API for the low strength drug product samples in quantities of 0.8 mg, 1.0 mg, and 1.2 mg was weighed manually. Additionally, when preparing the high strength drug product samples, there were several malfunctions with the balance. Consequently, only the API for the 80% samples and two of the 100% samples was weighed using the automated balance. The API for the remaining 100% sample and all 120% samples was weighed manually.

For standards and drug substance samples, 5 mg of API was weighed into twelve sample tubes, which were then placed in designated racks on the Fluent worktable (see Figure 1). For the drug product, excipients were weighed according to Table 3, and API was added as for the manual method outlined in chapter 3.3.3, into ten glass bottles which were then positioned on

magnetic stirrers on the Fluent worktable (see Figure 1). All subsequent steps were executed automatically by the robotic system, with all dilutions performed in 6 ml sample tubes.

For the drug substance samples, 5 ml of diluent was added in two steps, with a sonication period of 600 seconds following each liquid addition, resulting in the generation of twelve stock solutions. Liquid addition steps were controlled gravimetrically, with the final concentration in each tube automatically calculated based on the diluent density (determined as shown in appendix 8.7) and sample weight, which were pre-entered into the FluentControl software. Eight of the stock solutions were subsequently diluted to 100% of the WC, which was also pre-entered. Six of the 100% samples underwent further dilution to 0.1% WC. Two of the stock solutions were diluted to 150% WC, with subsequent dilutions to 120%, 80% and 50% WC. After handling the 150% samples, the system conducted two blank preparations in LC vials for carryover assessment. The remaining two stock solutions were diluted to 30% WC and further diluted to 10% and 1% WC. The 10% samples were further diluted to 0.5% WC, while the 1% samples were diluted to 0.05% WC. All samples were automatically transferred to designated LC vials for subsequent offline analysis.

For the drug product samples, 10 ml of diluent was added for the low strength, and 100 ml was added for the high strength, both in two steps with a magnetic stirring duration of 10 minutes following each gravimetrically controlled liquid addition. Low strength drug product samples with a WC of 0.1 mg/ml for the 100% sample were directly transferred to designated LC vials. High strength drug product samples were diluted in sample tubes to obtain a WC of 0.15 mg/ml for the 100% sample before being transferred to LC vials. Upon completion of the Fluent run and before analysis, all drug product samples in LC vials were manually filtered using 0.45 µm syringeless PTFE filters (Whatman Mini-UniPrep G2) and a hand compressor.

3.3.5 Uncertainty analysis

The uncertainty analysis aimed to evaluate the reliability of the methods and obtained results. It was performed following the guidelines outlined in the EURACHEM/CITAC guide.

Initially, the process steps, as well as the measurand and all related parameters were defined. Subsequently, all relevant sources of uncertainty were identified and visually represented in a cause-and-effect diagram[18]. In the third step, the uncertainties were quantified.

For the top-down approach, it is recommended to assess the intermediate precision over 60 preparations and a time period of at least one year[22]. Due to the limited timeframe of this work, it was not possible to fulfil these criteria. Consequently, the decision was made to use the bottom-up approach in this study.

The bottom-up uncertainty quantification aimed to assess individual uncertainty contributions for manual and automated preparation and analysis of samples at a high (0.15 mg/ml) and low (0.075 µg/ml) concentration separately and combine them. This involved utilizing information from previously performed repeatability studies, calibration certificates, and instrument specifications. The uncertainty associated with the UHPLC injection volume, separation, detection, and peak integration was treated as a single contribution. It was determined by assessing the repeatability of the chromatographic process through 11 consecutive injections of the same sample at 0.15 mg/ml and 0.075 µg/ml.

Finally, the combined relative standard uncertainty and the expanded uncertainty, expressing the result in a 95% confidence interval and using a coverage factor of k=2, were calculated for both the manual and the automated method.

4 Results and discussions

4.1 Optimization of the automated sample preparation

The automated workflow underwent optimization in several steps, after an initial drug substance sample preparation demonstrated inadequate accuracy. This optimization is illustrated in Figure 2.

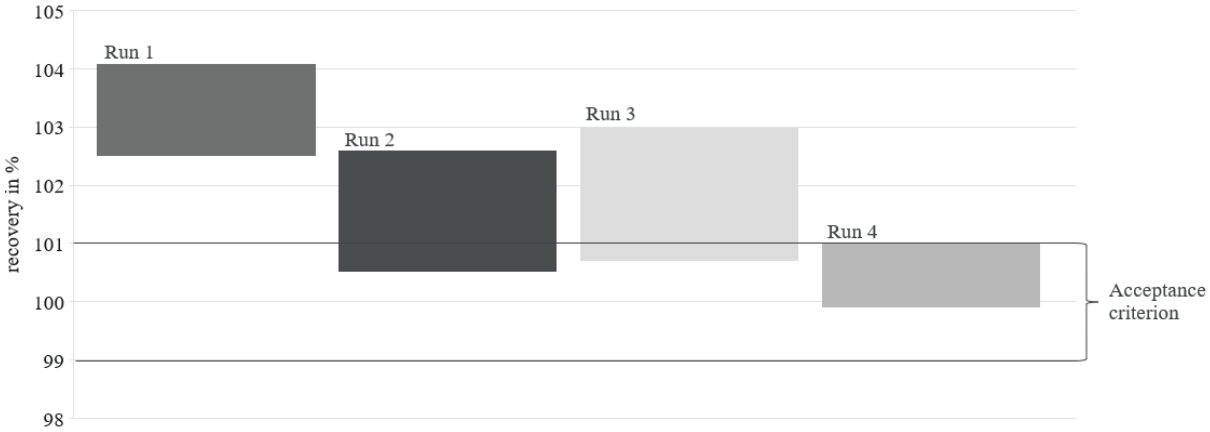


Figure 2: Recovery (in %) of the runs of the automated sample preparation optimization, including the recovery interval specified as the accuracy acceptance criterion. Run 1: initial workflow. Run 2: increased mixing and shaking of stock solutions. Run 3: further mixing of samples before transfer to LC vials. Run 4: immediate closing of LC vials. The corresponding raw data can be found in appendix 8.2.

After a first run (Run 1) which did not comply with the acceptance criteria, 20 minutes of shaking all stock solutions before their dilution was introduced, and the number of mixing cycles in the sample tubes was increased, resulting in an improved accuracy (Run 2). Subsequently, additional mixing steps were incorporated just before transferring the sample to LC vials (Run 3). However, this adjustment did not yield further improvement. Due to consistently higher-than-expected recovery rates, a potential evaporation issue from the LC vials, which remain open on the Tecan Fluent worktable until the end of the run, was investigated. Closing every vial immediately after liquid transfer finally resulted in a significant improvement in accuracy, aligning with the acceptance criteria (Run 4).

To further investigate the evaporation phenomenon, a small experiment was designed, involving the transfer of two samples into LC vials, which were then closed after 0, 1, 2, 5 and 10 minutes, and analysed. The findings of this investigation are depicted in Figure 3.

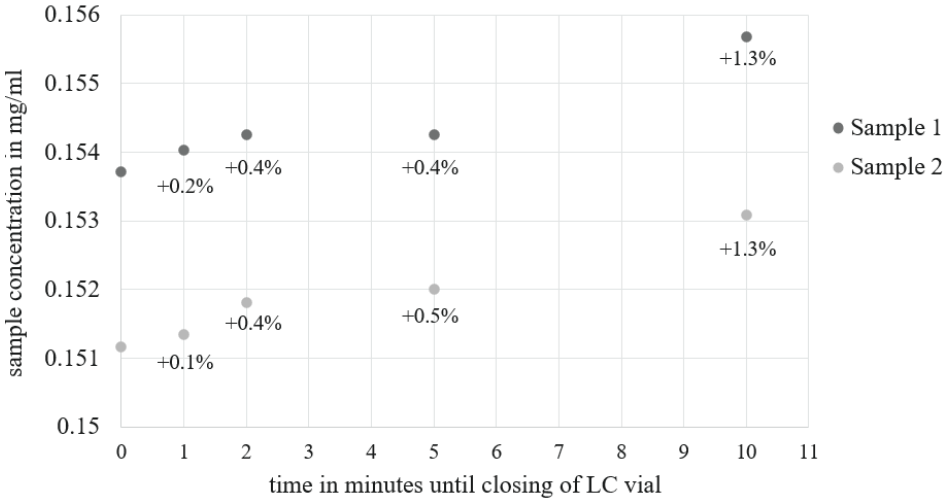


Figure 3: Sample concentration depending on the time between filling and closing of the LC vials, including concentration change in % in comparison to the sample concentration at 0 minutes. The corresponding raw data can be found in appendix 8.2.

As anticipated, Figure 3 shows an increase in sample concentration with increasing duration of open vial exposure on the worktable. In a drug substance validation run, the total time from the transfer of the first sample to its LC vial until the closure of all vials at the end of the run is approximately 1 hour. However, after just 10 minutes, both samples displayed a change in concentration of +1.3%, explaining the challenge of meeting the accuracy acceptance criterion, defined as a recovery of 100% ± 1%, when the vials are not immediately closed.

Based on the findings in Figure 3, it is recommended to close each vial within one minute after liquid transfer. However, this approach requires careful observation and frequent pausing towards the end of the automated run, thereby increasing the workload for the analyst. Potential further improvements of the workflow, such as incorporating a feature on the Tecan Fluent to automatically close the LC vials after liquid transfer or implementing cooling for the LC vial racks to mitigate evaporation, should thus be explored in subsequent work.

4.2 Method validation

Method validation was conducted to demonstrate the suitability of both manual and automated sample preparation for analysing the investigated drug substance and drug product, as well as to assess method equivalency. The subsequent tables

Table 4 and

Table 5 present the validation results of the manual and automated drug substance and low strength drug product preparations, considering the parameters and acceptance criteria described in Table 2.

Table 4: Validation results of the drug substance validation for the manual and automated method, including acceptance criteria for every parameter. Results that did not fulfil the acceptance criteria are highlighted in grey. The corresponding raw data can be found in appendix 8.3.

Parameter	**Acceptance criterion**	**Manual method**	**Automated method**
Specificity	No peak interference	No peak interference	No peak interference
Accuracy	Recovery between 99.0% and 101.0%	Recovery between 100.0% and 100.4%	Recovery between 99.9% and 101.0%
Precision at 100% WC	Relative standard deviation ≤ 3%	Relative standard deviation of 1.6%	Relative standard deviation of 0.7%
Precision at 0.1% WC	Relative standard deviation < 10%	Relative standard deviation of 3.1%	Relative standard deviation of 1.8%
LOQ	Signal-to-noise ratio >10 Relative standard deviation < 20%	Signal-to-noise ratio 18.4 Relative standard deviation of 2.7%	Signal-to-noise ratio 10.3 Relative standard deviation of 3.8%
Linearity	No visible curvature after linear regression R^2 >0.99 y-axis intercept not greater than 2% of peak area of 100% sample	No visible curvature after linear regression R^2 of 0.999 y-axis intercept equals 0.3% of peak area of 100% sample	No visible curvature after linear regression R^2 of 0.999 y-axis intercept equals 0.008% of peak area of 100% sample
Stability at 100% WC at RT	Value after storage: ± 2.0% of initial	+0.5% of initial conc. after 24 hours	+1.5% of initial conc. after 24 hours
		+0.9% of initial conc. after 48 hours	+1.6% of initial conc. after 48 hours
		+4.5% of initial conc. after 1 week	+1.8% of initial conc. after 1 week
Stability at 100% WC in FR	Value after storage: ± 2.0% of initial	–0.1% of initial conc. after 24 hours	+1.0% of initial conc. after 24 hours
		+0.4% of initial conc. after 48 hours	+0.4% of initial conc. after 48 hours

Parameter	Acceptance criterion	Manual method	Automated method
		+1.3% of initial conc. after 1 week	-0.2% of initial conc. after 1 week
Stability at 0.05% WC at RT	Value after storage: ± 99% of initial	+20.5% of initial conc. after 24 hours	+2.9% of initial conc. after 24 hours
		+6.8% of initial conc. after 48 hours	-14.5% of initial conc. after 48 hours
		+35.6% of initial conc. after 1 week	+37.7% of initial conc. after 1 week
Stability at 0.05% WC in FR	Value after storage: ± 99% of initial	+6.8% of initial conc. after 24 hours	-5.5% of initial conc. after 24 hours
		-1.4% of initial conc. after 48 hours	-12.3% of initial conc. after 48 hours
		+23.3% of initial conc. after 1 week	+28.8% of initial conc. after 1 week

Table 5: Validation results of the drug product low strength validation for the manual and automated method, including acceptance criteria for every parameter. The low strength drug product samples of the manual method were analysed after 67 hours of storage due to a power outage preventing analysis after 48 hours. Results that did not fulfil the acceptance criteria are highlighted in grey and the raw data can be found in appendix 8.48.4.

Parameter	Acceptance criterion	Manual method	Automated method
Specificity	No peak interference	No peak interference	No peak interference
Accuracy at 80%	Recovery between 97.0% and 103.0%	Recovery between 100.6% and 101.5%	Recovery between 101.2% and 102.4%
Accuracy at 100%	Recovery between 97.0% and 103.0%	Recovery between 100.0% and 101.7%	Recovery between 97.9% and 99.0%
Accuracy at 120%	Recovery between 97.0% and 103.0%	Recovery between 99.8% and 101.9%	Recovery between 98.6% and 100.0%
Stability 100% at RT	Value after storage: ± 2.0% of initial	+0.9% of initial conc. after 24 hours	+2.9% of initial conc. after 24 hours
		+1.6% of initial conc. after 67 hours	-3.1% of initial conc. after 48 hours
Stability 100% in FR	Value after storage: ± 2.0% of initial	+0.3% of initial conc. after 24 hours	+1.0% of initial conc. after 24 hours

Parameter	Acceptance criterion	Manual method	Automated method
		+0.7% of initial conc. after 67 hours	-0.9% of initial conc. after 48 hours

The results presented in tables

Table 4 and

Table 5 demonstrate the successful validation of the manual and automated method for drug substance and low strength drug product preparation and analysis, complying with all acceptance criteria, apart from the manual method drug substance stability after one week at RT, and the automated method drug product stability after 24 and 48 hours at RT. In general, to ensure optimal reliability of the results, it is recommended to analyse the samples immediately after preparation or store them in a refrigerator.

Both methods achieved equivalent results in terms of their specificity, linearity, LOQ and stability in solution. The automated method demonstrated better precision at 100% and 0.1% WC. However, a two-tailed Welch's t-test with a significance level of 0.05 revealed no statistically significant difference in the means between the manual and automated sample preparations (see appendix 8.6). On the other hand, the validation results indicate a slightly better average accuracy for the manual drug substance sample preparation compared to the automated approach, as demonstrated by a two-tailed Welch's t-test (see appendix 8.6).

For the high strength drug product samples, several error messages occurred when weighing API with the automatic balance. Therefore, some samples were weighed manually also for the automated method. The validation results are presented in Table 6.

Table 6: Validation results of the drug product high strength validation for the manual and automated method, including acceptance criteria for every parameter and the way of weighing each sample for the automated method. The corresponding raw data can be found in appendix 8.5. Results that did not fulfil the acceptance criteria are highlighted in grey.

Parameter	Acceptance criterion	Manual method	Automated method
Specificity	No peak interference	No peak interference	No peak interference
			Sample 1: automated weighing

Parameter	Acceptance criterion	Manual method	Automated method
Accuracy at 80%	Recovery between 97.0% and 103.0%	Recovery between 100.9% and 101.8%	Recovery between 95.2% and 95.5%
			Sample 2: automated weighing Recovery between 95.7% and 95.8%
			Sample 3: automated weighing Recovery between 96.8% and 96.9%
Accuracy at 100%	Recovery between 97.0% and 103.0%	Recovery between 100.2% and 100.9%	Sample 1: automated weighing Recovery between 96.8% and 96.9%
			Sample 2: automated weighing Recovery between 97.5% and 97.6%
			Sample 3: manual weighing Recovery between 99.2% and 99.3%
Accuracy at 120%	Recovery between 97.0% and 103.0%	Recovery between 100.0% and 100.5%	Sample 1: manual weighing Recovery of 98.9%
			Sample 2: manual weighing Recovery of 102.7%
			Sample 3: manual weighing Recovery between 102.3% and 102.5%

The results in Table 6 highlight that, for the automated method, 4 out of the 5 samples weighed with the automatic balance did not meet the acceptance criteria. On the other hand, all manually prepared samples and all samples that were manually weighed and automatically prepared met the acceptance criteria. This suggests that the source of error lies not in the automated sample preparation using the Tecan Fluent, but rather in the weighing process using the automatic balance, which also showed several error messages during weighing. It is therefore assumed that if the validation were to be repeated with a functional balance, the automated sample preparation would meet the acceptance criteria. However, due to the limited timeframe of this work, it was not possible to conduct this additional validation test.

4.3 Comparison of the manual and automated method

Compared to the manual procedure, five of the nine process steps were automated in the automated workflow, as depicted in Figure 4 a). This automation reduces the workload for the analyst and results in a decrease in hands-on analyst time by 72%, as illustrated in Figure 4 b). These results align with other studies, for example, Fileš and Andersson reported a 71% reduction in required time upon automation of the sample preparation[10].

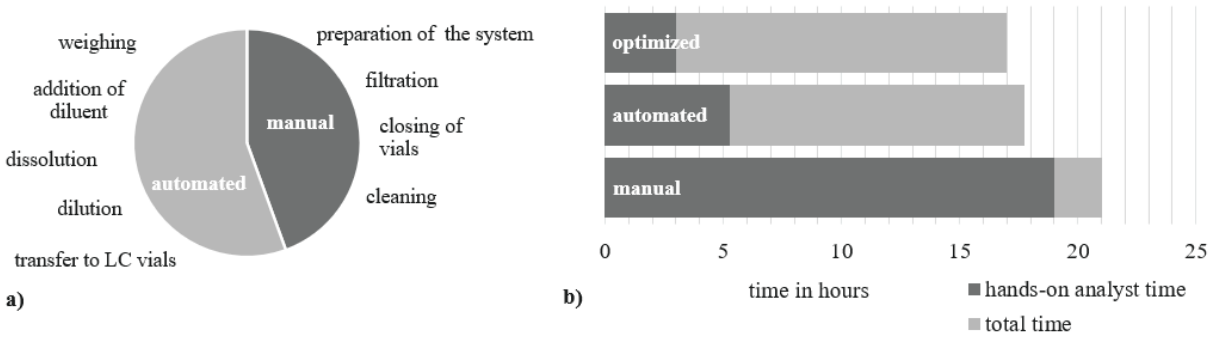
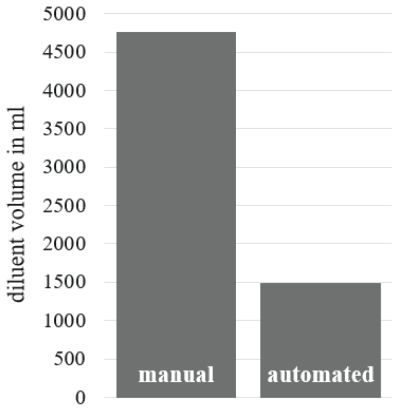


Figure 4: a) Automated and manually performed process steps in the automated method. b) Total and hands-on analyst time for the preparation of all validation samples using the manual method, the automated method, and a further optimized automated method resolving issues with LC vial evaporation and automatic weighing.

However, for the automated workflow in this work, issues such as evaporation and the immediate closing of LC vials (as discussed in section 4.1), as well as problems with the automatic balance necessitating manual weighing of API for the automated drug product preparations, contribute to the hands-on analyst time. Further optimization of the method to address these issues holds the potential to decrease the hands-on analyst time even more, achieving an 84% reduction compared to the manual approach, as depicted in Figure 4 b).

Another contributing factor to the workload reduction is the automated recording and standardized reporting of all process data, including weights and volumes, by the Tecan Fluent, also enhancing data traceability.



Additionally, the decreased working volume in the automated workflow results in a 69% reduction in required solvent volume, as illustrated in figure 5. This reduction in diluent consumption positions the automated workflow as a greener and more environmentally friendly alternative to the manual method. Moreover, automation, by reducing direct worker exposure to substances, further enhances safety of the work environment.

In general, the significant reduction of required time and solvent achieved through automation leads to increased process efficiency and holds the potential to notably decrease operational costs for sample preparation.

Figure 5: Required volume for the preparation of validation samples using the manual and automated method.

4.4 Uncertainty analysis

The uncertainty analysis was performed to assess method reliability by quantifying the uncertainty in both manual and automated preparation and chromatographic analysis, exemplary for a sample at a high (0.15 mg/ml) and low (0.075 µg/ml) concentration.

4.4.1 Identification of process steps and specification of the measurand

The process steps involved in the manual and automated preparation and analysis of a high and low concentration sample were identified as outlined in Table 7.

Table 7: Identified process steps for the preparation and analysis of a high (0.15 mg/ml) and a low (0.075 µg/ml) concentration sample with the manual and automated method.

Process step	**Manual method**		**Automated method**	
	High conc.	**Low conc.**	**High conc.**	**Low conc.**
1)	Weighing of 7.5 mg API	Weighing of 7.5 mg API	Weighing of 5 mg API	Weighing of 5 mg API
2)	Dissolving in 50 ml volumetric flask	Dissolving in 50 ml volumetric flask Pipetting of 1 ml into new flask Dilution in 100 ml volumetric flask Pipetting of 2.5 ml into new flask Dilution in 100 ml volumetric flask	Addition of 5 ml to dissolve Pipetting of 0.75 ml into new vial Addition of 4.25 ml	Addition of 5 ml to dissolve Pipetting of 0.75 ml into new vial Addition of 4.25 ml Pipetting of 0.1 ml into new vial Addition of 4.9 ml Pipetting of 0.1 ml into new vial Addition of 3.9 ml

Process step	Manual method		Automated method	
	High conc.	**Low conc.**	**High conc.**	**Low conc.**
3)	Transfer into LC vial	Transfer into LC vial	Transfer into LC vial	Transfer into LC vial
4)	Injection of 2 µl UHPLC-UV Data processing	Injection of 2 µl UHPLC-UV Data processing	Injection of 2 µl UHPLC-UV Data processing	Injection of 2 µl UHPLC-UV Data processing

The obtained result after the chromatographic separation and UV detection, and subsequent data processing using the Empower software yields the concentration of the sample. This concentration is derived by comparing the peak area of the sample with the peak area of a standard with known concentration, prepared in the same way as a high concentration sample. The measurand was thus specified as follows in equation 1:

$$c(u) = \frac{A(u)}{A(s)} * c(s) = \frac{A(u)}{A(s)} * \frac{m(s)}{d(s)} \quad (Eq.\ 1).$$

In this work, (u) denotes the sample (the unknown), while (s) denotes the standard. The variable ‘c’ represents the concentration, ‘A’ the peak area, ‘m’ the mass, and ‘d’ the dilution volume. Since both the standard and sample are prepared from the same batch of API, they share the same purity, which was therefore omitted in the specification of the measurand.

4.4.2 Identification of uncertainties

As outlined in the measurand specification, the concentration of the sample is dependent on the peak area of the sample, the peak area of the standard, the amount of API weighed for the standard, and the dilution of the standard. To systematically assess the sources of uncertainty affecting the determination of the sample concentration, a cause-and-effect diagram was created. Figure 6 illustrates this diagram, depicting a selection of the most significant but not all potential sources of uncertainty, underscoring the complexity of the uncertainty analysis.

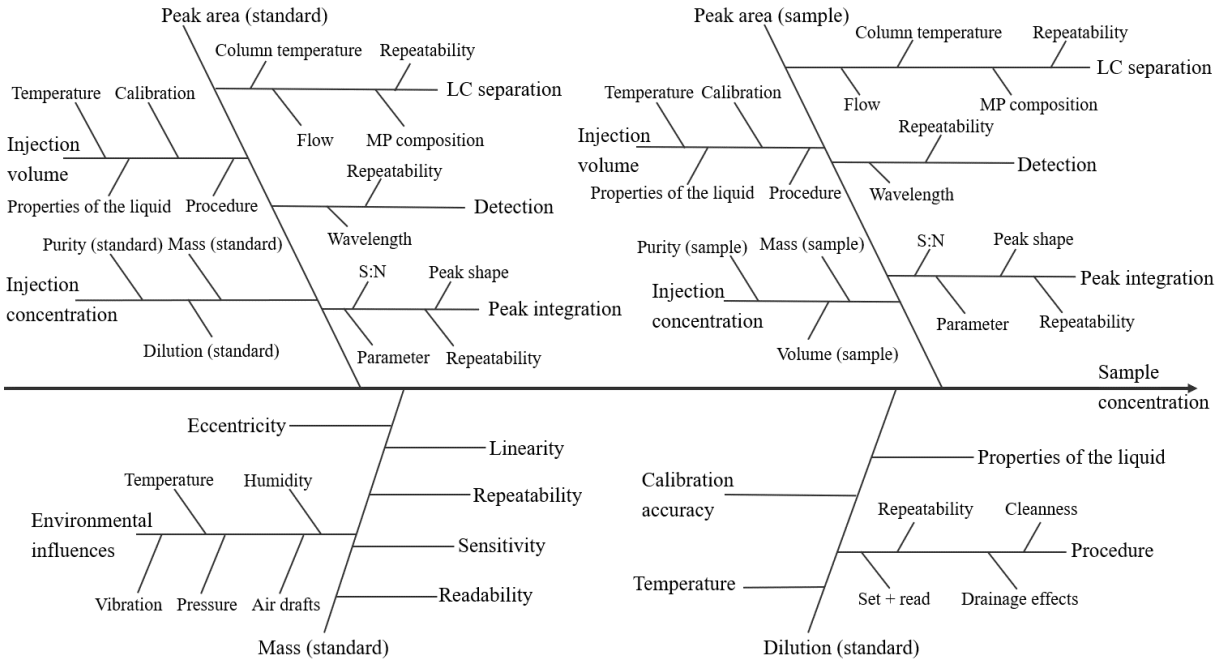


Figure 6: Cause-and-effect diagram including a selection of the most significant uncertainty influences in the determination of the sample concentration.

As depicted in Figure 6, the determination of mass is predominantly influenced by factors such as the linearity, readability, sensitivity, and eccentricity of the balance, along with the repeatability of the procedure and environmental conditions including temperature, humidity, pressure, air drafts, or vibration[19]. Gravimetrically controlled volumetric operations face similar uncertainties, with the additional consideration of the uncertainty in the density of the liquid. In this work, most liquid additions are volumetrically controlled. These operations rely on the calibration accuracy of the pipette or volumetric flask, the temperature, properties of the liquid, and the procedure itself, such as the method of setting and reading a meniscus in a volumetric flask, or the speed of aspiration when using a pipette[20]. The most complex uncertainty contribution in this analysis arises from the peak areas, primarily affected by factors related to LC separation, detection, peak integration, and injection volume and concentration, each with several additional influences[21].

4.4.3 Quantification of uncertainties using the bottom-up approach

Based on the measurand specification, equation 2 was derived to calculate the relative standard uncertainty of the sample concentration:

$$\frac{u(c(u))}{c(u)} = \sqrt{\left(\frac{u(A(s))}{A(s)}\right)^2 + \left(\frac{u(A(u))}{A(u)}\right)^2 + \left(\frac{u(m(s))}{m(s)}\right)^2 + \left(\frac{u(d(s))}{d(s)}\right)^2} \quad \textit{(Eq. 2).}$$

In this work, all uncertainties are denoted with the variable 'u'. For the quantification of uncertainties, it was assumed that there are no systematic errors in the procedures, making the bias of both methods negligible.

The aim with the bottom-up approach was to quantify each uncertainty contribution illustrated in Figure 6. However, due to the complexity of the analysis, a few simplifications were made. The LC injection volume, separation, detection, and peak integration were treated as a single uncertainty influence, determined by the repeatability of the LC-UV analysis[24]. The rationale for this approach in the present work was the emphasis on the sample preparation rather than the analysis itself. Consequently, the uncertainty of the peak area was calculated using equation 3, considering the LC-UV repeatability (rep(LC-UV)) plus the injection concentration of the sample, namely its mass, dilution, and purity:

$$\frac{u(A)}{A} = \sqrt{(\frac{u(m)}{m})^2 + (\frac{u(d)}{d})^2 + (\frac{u(Purity)}{Purity})^2 + rep(LC-UV)^2} \quad (Eq.\ 3).$$

In the following subsections, the stepwise quantification of the parameters of equations 2 and 3, including their influences shown in Figure 6 will be explained in detail.

4.4.3.1 Quantification of the uncertainty of the mass

All balances used in this work are operated and calibrated in a controlled environment. Therefore, the environmental influences were assumed to be negligible. This assumption enables the calculation of the uncertainty for every mass using the following equation:

$$u(m) = \sqrt{u(repeatability)^2 + u(linearity)^2 + u(readability)^2 + u(sensitivity)^2 + u(eccentricity)^2} \ (Eq.\ 4).$$

For the weighing with the automatic balance, the eccentricity was assumed to be negligible, as it is expected for the automated system to always dispense the API in the centre of the weighing pan.

The standard deviations and uncertainties for all influences in equation 4 were obtained from instrument specifications and calculated assuming a rectangular probability distribution. The rectangular probability distribution considers all values to be equally probable and aligns with the lack of precise knowledge on how the standard deviations in the specifications were derived[18]. Further details on the calculation of all mass uncertainties can be found in appendix 8.9.

4.4.3.2 Quantification of the uncertainty of the dilution

The uncertainty of the total dilution is determined by the sum of the relative uncertainties of all liquid addition steps of a certain volume (v) in the method, as described by equation 5:

$$\frac{u(d)}{d} = \sqrt{\sum_{i=1}^{n} \left(\frac{u(v_i)}{v_i}\right)^2} \quad \textit{(Eq. 5).}$$

For all liquid addition steps, it was assumed that the influence of the properties of the liquid is negligible[20].

For volumetric flasks, if operated correctly, their procedure and calibration accuracy are covered by the specified tolerance[20], allowing the calculation of their uncertainty as shown in equation 6:

$$u(v) = \sqrt{u(calibration)^2 + u(procedure)^2 + u(temperature)^2} = \sqrt{u(tolerance)^2 + u(temperature)^2} \quad \textit{(Eq. 6).}$$

For the pipettes in the automated system, a previous study had determined the repeatability using a 50:50 (v/v) mixture of acetonitrile and water, while for the pipettes used in the manual method, a previous study had determined the repeatability using water (see appendix 8.7). These experiments cover variation in calibration and procedure. Consequently, the uncertainty of all volumetric additions using the automated system and of all volumetric additions through pipetting in the manual procedure was calculated using equation 7:

$$u(v) = \sqrt{u(calibration)^2 + u(procedure)^2 + u(temperature)^2} = \sqrt{rep(pipette)^2 + u(temperature)^2} \quad \textit{(Eq. 7).}$$

The uncertainty in temperature for all volumetric operations was quantified using equation 8, assuming a triangular probability distribution:

$$u(temperature) = \frac{V * \alpha * \Delta T}{\sqrt{6}} \quad \textit{(Eq. 8)}\ ^{20}$$

where V is the volume, ΔT is the temperature variation around the working temperature, and α is the cubical thermal volumetric expansion coefficient[20]. In this work, all calculations were based on a ΔT value of 4 K and an α value of 0.001108 K^{-1}, equivalent to the cubical thermal volumetric expansion coefficient of a 50:50 (v/v) mixture of acetonitrile and water at 25 °C [25].

The uncertainty of the first liquid addition step in the automated method, which is gravimetrically controlled, was calculated using equation 9, considering the uncertainty in the mass (calculated using equation 4) plus the uncertainty in the density of the liquid:

$$\frac{u(v)}{v} = \sqrt{\left(\frac{u(m)}{m}\right)^2 + \left(\frac{u(density)}{density}\right)^2} \quad (Eq.\ 9).$$

A repeatability study had previously been conducted, determining the relative standard deviation in the mass when pipetting 50 ml of a 50:50 (v/v) mixture of acetonitrile and water (see appendix 8.7). This data, along with the specification of the used 50 ml volumetric pipette, was utilized to calculate the uncertainty in the liquid density using equation 10:

$$\frac{u(density)}{density} = \sqrt{\left(\frac{u(m)}{m}\right)^2 + \left(\frac{u(v)}{v}\right)^2} = \sqrt{rep(mass)^2 + \left(\frac{u(v)}{v}\right)^2} \quad (Eq.\ 10).$$

For all volumetric flasks and the volumetric pipette used in the uncertainty determination of the density, the tolerance was obtained from the specifications, and the uncertainty was calculated assuming a rectangular probability distribution. For all data obtained through traceable repeatability studies, such as the pipetting repeatability in the manual and automated method, or the weighing repeatability of the mass for the density determination, a normal probability distribution was assumed[18]. Further details on the calculation of all dilution uncertainties can be found in appendix 8.9.

4.4.3.3 Quantification of the uncertainty of the purity

The uncertainty in the purity was quantified using equation 11, adapted from an uncertainty determination by Meyer[24], assuming a rectangular probability distribution:

$$u(Purity) = \frac{(100 - Purity)}{2\sqrt{3}} \quad (Eq.\ 11)\ [24].$$

4.4.3.4 Quantification of the LC-UV repeatability

The liquid chromatography and UV detection repeatability was determined through 11 consecutive injections of the same sample at a high and low concentration with the results shown in Table 8. The uncertainty was calculated assuming a normal probability distribution.

Table 8: Results of the LC-UV repeatability study of 11 consecutive injections of the same sample at a high (0.15 mg/ml) and low (0.075µg/ml) concentration. The raw data can be found in appendix 8.8.

	LC-UV repeatability at 0.15 mg/ml	**LC-UV repeatability at 0.075 µg/ml**
RSD	$0.\ 051*10^{-2}$	$4.8*10^{-2}$

As anticipated, the results in Table 8 demonstrate that with an RSD of 0.05% the method is highly suitable for analysing samples at 0.15 mg/ml, for which the procedure is optimised. However, at a very low concentration of 0.075 µg/ml, the variation increases with an RSD of 4.8%, and the method becomes less precise.

4.4.4 Results of the bottom-up uncertainty quantification

The results of the bottom-up uncertainty quantification according to the procedure defined in the previous subchapters are summed up in the following Table 9 and Figure 7, which show the expanded relative standard uncertainty, covering a 95% confidence interval. Applying the bottom-up approach, this uncertainty was identified to be 0.015 for the manual preparation of a high concentration sample, 0.097 for the manual preparation of a low concentration sample, 0.030 for the automated preparation of a high concentration sample, and 0.10 for the automated preparation of a low concentration sample.

Table 9: Results of the relative standard uncertainty determination for samples at a high (0.15 mg/ml) and low (0.075 µg/ml) concentration, prepared with the manual and automated method, including the uncertainty as a percentage. The expanded uncertainty was calculated by multiplication with a coverage factor of k=2. Details on the calculations can be found in appendix 8.9.

Relative uncertainty	**Manual high concentration**	**Manual low concentration**	**Automated high concentration**	**Automated low concentration**
$u(c(u))/c(u)$	$7.3*10^{-3}$	$49*10^{-3}$	$15*10^{-3}$	$51*10^{-3}$
Expanded	$15*10^{-3}$	$97*10^{-3}$	$30*10^{-3}$	$100*10^{-3}$

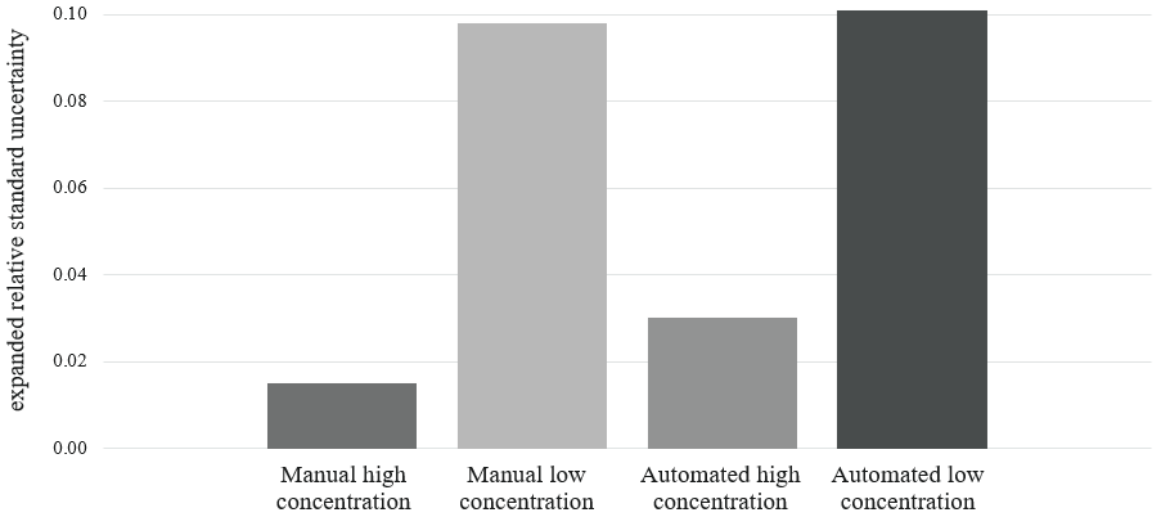


Figure 7: Expanded relative standard uncertainty for the preparation and analysis of a high (0.15 mg/ml) and low (0.075 µg/ml) concentration sample with the manual and automated method.

In general, the uncertainty analysis results align with the method validation findings, which indicated a better accuracy for the manual method in preparing drug substance samples at 0.15 mg/ml. However, the results in Table 9 also show that the uncertainty intervals for both approaches, ±3.0% for the automated and ±1.5% for the manual procedure, exceed the specified accuracy acceptance interval, defined as a recovery of 100% ±1%. Therefore, it may be necessary to reconsider the validity of the acceptance criteria and initiate a discussion about adjusting the accuracy acceptance interval defined in the internal SOPs.

4.4.5 Comparison of the manual and automated method

The bottom-up uncertainty determination results in Table 9 and Figure 7 indicate that the manual method has a lower uncertainty compared to the automated method when preparing a sample at 0.15 mg/ml. The uncertainty influences contributing to the combined relative standard uncertainty are shown in Figure 8.

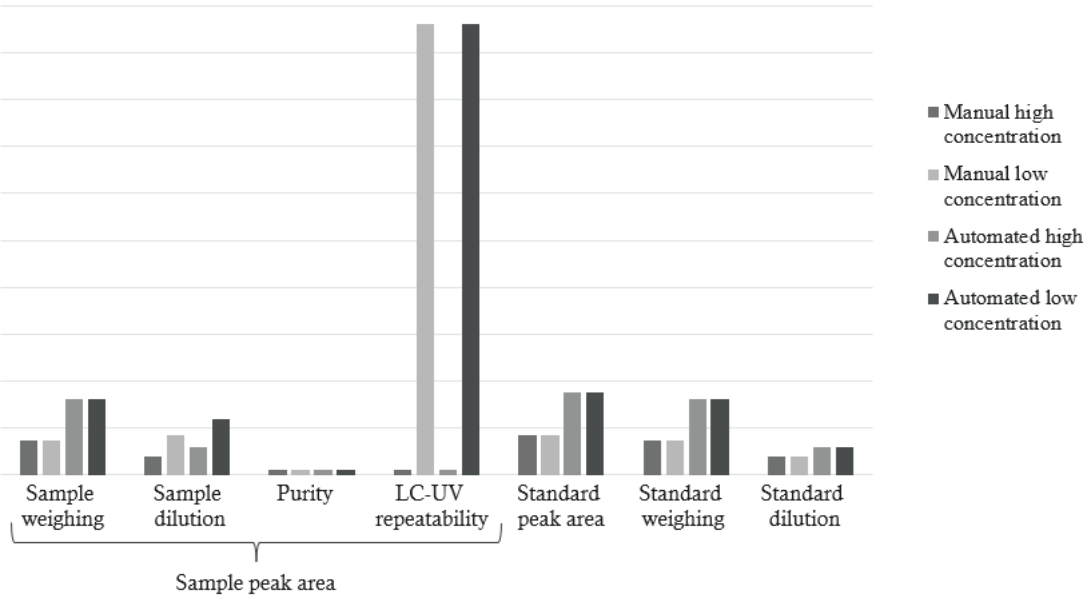


Figure 8: Contribution of the sample peak area, the standard peak area, standard weighing and standard dilution to the expanded relative standard uncertainty in the preparation of a high (0.15 mg/ml) and low (0.075 µg/ml) concentration sample with the manual and automated method. Details can be found in appendix 8.9.

As shown in Figure 7, both methods exhibit similar uncertainties when preparing a low concentration sample. This similarity is attributed to the significant influence of the LC-UV repeatability, which is dominant at lower concentrations, as shown in Figure 8. Consequently, differences in weighing or dilution play a minor role compared to the high concentration sample, for which, as shown in Figure 8, the difference in the uncertainty of the preparation and analysis with the manual and automated method primarily arises from the weighing and dilution. The manual method operates with larger volumes, requiring fewer dilution steps than the automated approach, thereby resulting in a lower uncertainty in the dilution process. Furthermore, the microbalance used in the manual procedure exhibits higher repeatability and sensitivity, and better readability and linearity compared to the balance on the automatic weighing robot. The expanded relative standard uncertainty for a high concentration sample which is manually weighed with the microbalance and then automatically prepared by the Tecan Fluent was identified to be 0.022 (see appendix 8.9), and therefore closer to the uncertainty identified for the manually prepared sample than to the uncertainty determined for the automatically prepared sample weighed by the automatic weighing robot. This underscores the potential for improving the automated workflow by replacing the automatic balance, which has also posed functional challenges during method validation.

5 Conclusions

The validation of both manual and automated approaches for preparing drug product and drug substance samples for UHPLC analysis was successful and showed the methods to be overall equivalent. However, the manual approach exhibited slightly higher accuracy, further confirmed by the lower uncertainty associated with the measurement results. The automated procedure, on the other hand, demonstrated significant savings in time, cost, and resources, along with notable improvements in safety and data handling.

The choice between the two sample preparation approaches going forward should be evaluated on a case-by-case basis, considering factors such as efficiency gains through automation versus potential quality differences. Moreover, the automated workflow shows potential for additional improvement to enhance efficiency and quality further, for example, using a better automatic balance could address the functional issues observed in this work and lower the weighing contribution to the relative standard uncertainty.

6 Future aspects

Based on the results of this work, the high strength tablet validation using the automated approach should be repeated, either with a functional automatic balance or by manually weighing all samples. This step is crucial to confirm the successful validation of the automated sample preparation workflow using the Tecan Fluent for drug products, according to the bracketing approach.

Additionally, to fully exploit the potential of the automated workflow, it is essential to investigate solutions to minimize evaporation. This could involve implementing features on the Tecan Fluent to close or cool LC vials. Furthermore, the results raise questions regarding the need to replace the automatic weighing robot with a reliably functioning system. Ideally, such a system would be calibrated for weighing amounts as small as 0.8 mg and possess better repeatability, sensitivity, readability, and linearity to achieve higher quality weighing results.

Moreover, the work highlighted the necessity of engaging in discussions on how to define accuracy acceptance criteria for future work. Therefore, it would be beneficial to assess the intermediate precision of both the automated and manual workflows over the next year and calculate the uncertainty using the top-down approach. This will enable a more thorough evaluation of the quality of the results.

7 References

1. N Singh, P.V., S Tanwar, J-L Poyet, K Tsaioun, B O Villoutreix Drug discovery and development: introduction to the general public and patient groups. *Frontiers in Drug Discovery* **3** (2023).

2. ICH Q8(R2) Guideline on pharmaceutical development. (2009).

3. (Ed), B.N. Sample Preparation of Pharmaceutical Dosage Forms. (Springer, 2011).

4. Raynie, D.E. Trends in Sample Preparation, Part I: Current State of the Field. *LCGC North America* **41**, 374-380,393 (2023).

5. Thurow, K. Strategies for automating analytical and bioanalytical laboratories. *Analytical and Bioanalytical Chemistry* **415**, 5057-5066 (2023).

6. L S Liu, A.M., K P Xiao Method development on automated sample preparation system for content uniformity and assay testing in a product with multiple APIs and new strategy for quick development and application. *Journal of Pharmaceutical and Biomedical Analysis* **208** (2022).

7. S M Han, A.M. Transfer from manual to automated sample preparation: a case study. *Journal of Pharmaceutical and Biomedical Analysis* **20**, 785-790 (1999).

8. I Toro, J.F.D., J L Fabregas, J Claramunt Development and validation of a fully automated method for the chromatographic determination of content uniformity of drug tablets. *Journal of Pharmaceutical and Biomedical Analysis* **36**, 57-63 (2004).

9. W F Shamrock, K.R., D K Lloyd Automated sample preparation of Roxifiban tablets: transfer of a manual method to an automated workstation. *Journal of Pharmaceutical and Biomedical Analysis* **21**, 1225-1232 (2000).

10. I Files, V.A. Automated sample preparation of protein solid dosage forms: Novel application for the tablet processing workstation. *SLAS Technology* **28**, 258-263 (2023).

11. N S Arden, A.C.F., K Tyner, l X Yu, S L Lee Industry 4.0 for pharmaceutical manufacturing: Preparing for the smart factories of the future. *International Journal of Pharmaceutics* **602** (2021).

12. Sotax TPW - Automated Sample Preparation Workstation. *https://www.sotax.com/sample_preparation/tpw* (Accessed: 07/03/2024).

13. Science, A. ACCROMA 2.0. *https://www.archerscience.com/accroma* (Accessed: 14/03/2024).

14. Tecan Fluent Automation Workstation. *https://lifesciences.tecan.com/fluent-laboratory-automation-workstation* (Accessed: 07/03/2024).

15. ICH Q2(R1) Validation of Analytical Procedures: Text and Methodology Q2(R1). (2005).

16. ICH Q2(R2) Guideline on validation of analytical procedures. (2023).

17. EMA Guideline on process validation for finished products - information and data to eb provided in regulatory submissions. (2016).

18. EURACHEM, C. EURACHEM / CITAC Guide CG4: Quantifying Uncertainty in Analytical Measurement. (2012).

19. M. Salahinejad, F.A. Uncertainty Measurement of Weighing results from an Electronic Analytical Balance. *Measurement Science Review* **7** (2007).

20. Kadis, R. Evaluation of measurement uncertainty in volumetric operations: the tolerance-based approach and the actual performance-based approach. *Talanta* **64**, 167-173 (2004).

21. Barwick, V.J. Sources of uncertainty in gas chromatography and high-performance liquid chromatography. *Journal of Chromatography A* **849**, 13-33 (1999).

22. NORDTEST NORDTEST NT TR537: Handbook for Calculation of Measurement Uncertainty in Environmental Laboratories. (2017).

23. F Martinello, N.S., M Skitek, A Jerin The top-down approach to measurement uncertainty: which formula should we use in laboratory medicine. *Biochemia Media* **30** (2020).

24. Meyer, V.R. Measurement Uncertainty of Liquid Chromatographic Analyses Visualized by Ishikawa Diagrams. *Journal of Chromatographic Science* **41**, 439-443 (2003).

25. Marı´a del Carmen Grande, J.A.J., Carmen R. Barrero, Carlos M. Marschoff , Hugo L. Bianchi The (water + acetonitrile) mixture revisited: A new approach for calculating partial molar volumes. *The Journal of Chemical Thermodynamics* **38**, 760-768 (2006).

8 Appendix

8.1 Gradient details of the UHPLC method

Table 10: Gradient details of the UHPLC method.

Time	% Mobile phase A	% Mobile phase B
0 min	95	5
3.6 min	10	90
3.9 min	10	90
4.0 min	95	5
5.5 min	95	5

8.2 Raw data of the automated workflow optimization

8.2.1 Drug substance recovery

Table 11: Raw data of the accuracy determination of the automated method during the several runs of workflow optimization. The initial concentration was calculated based on the weighed amount of API and added volume of diluent by the Tecan Fluent. The concentration was obtained after data evaluation using the Empower software.

			Run 1		Run 2		Run 3		Run 4	
Sample	**No.**	**Inj.**	**Initial concentration (mg/ml)**	**Concentration (mg/ml)**	**Initial concentration (mg/ml)**	**Concentration (mg/ml)**	**Initial concentration (mg/ml)**	**Concentration (mg/ml)**	**Initial concentration (mg/ml)**	**Concentration (mg/ml)**
100 %	1	1	0.1522	0.15687	0.1525	0.15640	0.1524	0.15372	0.1520	0.15272
		2		0.15686		0.15626		0.15350		0.15264
		3		0.15674		0.15638		0.15356		0.15254
100 %	2	1	0.1532	0.15703	0.1520	0.15283	0.1524	0.16797	0.1535	0.15351
		2		0.15730		0.15282		0.16791		0.15364
		3		0.15731		0.15279		0.16766		0.15359
100 %	3	1	0.1527	0.15895	0.1522	0.15405	0.1522	0.15682	0.1554	0.15551
		2		0.15873		0.15401		0.15657		0.15546

		3		0.15867		0.15407		0.15656		0.15551

8.2.2 Evaporation test

Table 12: Sample concentration obtained after data evaluation using the Empower software, depending on the waiting time between liquid transfer to the LC vial and analysis.

Time in minutes	**Sample concentration (mg/ml)**	
	Sample 1	**Sample 2**
0	0.153711	0.151172
1	0.154028	0.151353
2	0.154259	0.151818
5	0.154250	0.152001
10	0.155682	0.153083

8.3 Raw data of the drug substance method validation

8.3.1 Specificity

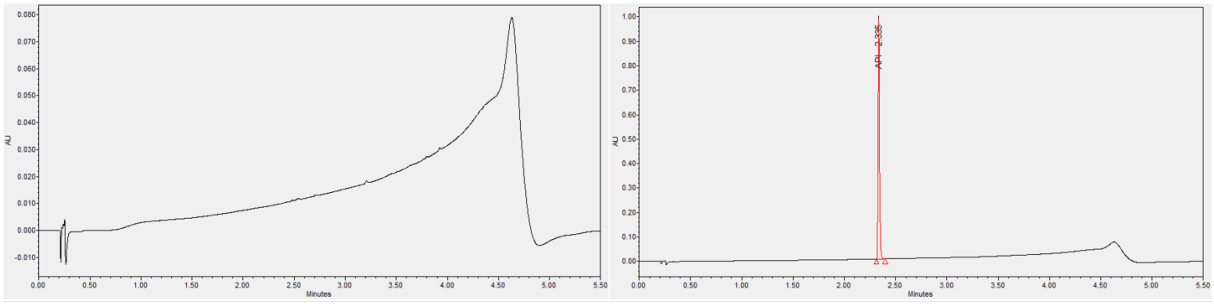


Figure 9: Specificity determination of the manual method: comparison of the blank (left) and 100% WC sample (right).

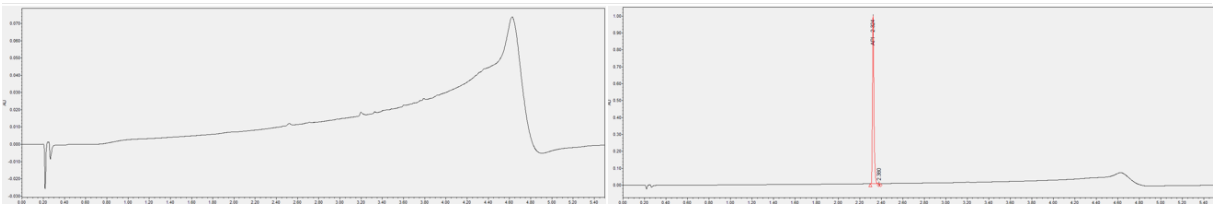


Figure 10: Specificity determination of the automated method: comparison of the blank (left) and 100% WC sample (right).

8.3.2 Accuracy

Table 13: Raw data of the accuracy determination of the manual and automated method. The initial concentration was calculated based on the weighed amount of API and added volume of diluent, either manually

or automatically by the Tecan Fluent. The concentration was obtained after data evaluation using the Empower software.

			Manual method		Automated method	
Sample	**No.**	**Inj.**	**Initial concentration (mg/ml)**	**Concentration (mg/ml)**	**Initial concentration (mg/ml)**	**Concentration (mg/ml)**
100%	1	1	0.1520	0.152720	0.1523	0.152596
		2		0.152644		0.152383
		3		0.152544		0.152204
100%	2	1	0.1535	0.153507	0.1523	0.153748
		2		0.153638		0.153844
		3		0.153594		0.153808
100%	3	1	0.1554	0.155514	0.1526	0.153664
		2		0.155457		0.153261
		3		0.155510		0.153431

8.3.3 Precision

Table 14: Raw data of the precision determination of the manual and automated method. The concentration was obtained after data evaluation using the Empower software.

			Manual method	Automated method
Sample	**No.**	**Inj.**	**Concentration (mg/ml)**	**Concentration (mg/ml)**
100%	1	1	0.153022	0.152452
	2	1	0.153882	0.154018
	3	1	0.155535	0.153483
	4	1	0.155639	0.156030
	5	1	0.152071	0.153735
	6	1	0.148460	0.154636
0.1%	1	1	0.000151	0.000164
	2	1	0.000153	0.000161

	3	1	0.000163	0.000158
	4	1	0.000153	0.000159
	5	1	0.000160	0.000155
	6	1	0.000150	0.000157

8.3.4 LOQ

Table 15: Raw data of the LOQ determination of the manual and automated method. The S:N ratio was calculated according to the European and US Pharmacopoeia by the Empower software, and the concentration was obtained after data evaluation using the Empower software.

		Manual method	**Automated method**
Sample	**Inj.**	**Concentration (mg/ml)**	**Concentration (mg/ml)**
0.05%	1	0.000074	0.000090
	2	0.000070	0.000088
	3	0.000074	0.000085
	4	0.000070	0.000081
	5	0.000075	0.000083
	6	0.000073	0.000082
Sample	**Inj.**	**Average S:N ratio**	**Average S:N ratio**
0.05%	1-6	18.4	10.3

8.3.5 Linearity

Table 16: Raw data of the linearity determination of the manual method. The initial concentration was calculated based on the weighed amount of API and added volume of diluent.. The peak area was obtained after data evaluation using the Empower software.

Sample	**No.**	**Inj.**	**Initial concentration (mg/ml)**	**Peak area (µV*s)**	**Sample**	**No.**	**Inj.**	**Initial concentration (mg/ml)**	**Peak area (µV*s)**
0.05%	1	1	$7.570*10^{-5}$	429	50%	1	1	0.07422	453154
		2		411			2		453031
		3		414			3		453441

0.05%	2	1	$7.602*10^{-5}$	408	50%	2	1	0.07504	452133
		2		429			2		451804
		3		379			3		452523
0.5%	1	1	$7.570*10^{-4}$	4474	80%	1	1	0.1188	716939
		2		4533			2		717042
		3		4535			3		718095
0.5%	2	1	$7.602*10^{-4}$	4608	80%	2	1	0.1201	725496
		2		4547			2		725698
		3		4522			3		724767
1%	1	1	0.001514	9004	100%	1	1	0.1484	889130
		2		8980			2		889127
		3		9018			3		889538
1%	2	1	0.001520	8965	100%	2	1	0.1501	895491
		2		8973			2		895514
		3		8999			3		896844
10%	1	1	0.01514	91429	120%	1	1	0.1781	1062570
		2		91398			2		1063329
		3		91415			3		1062954
10%	2	1	0.01520	92339	120%	2	1	0.1801	1068091
		2		92333			2		1067427
		3		92334			3		1066888
30%	1	1	0.04542	273470	150%	1	1	0.2227	1317593
		2		273283			2		1316425
		3		273250			3		1317832

30%	2	1	0.04561	274761	150%	2	1	0.2251	1329810
		2		274642			2		1329339
		3		274820			3		1329557

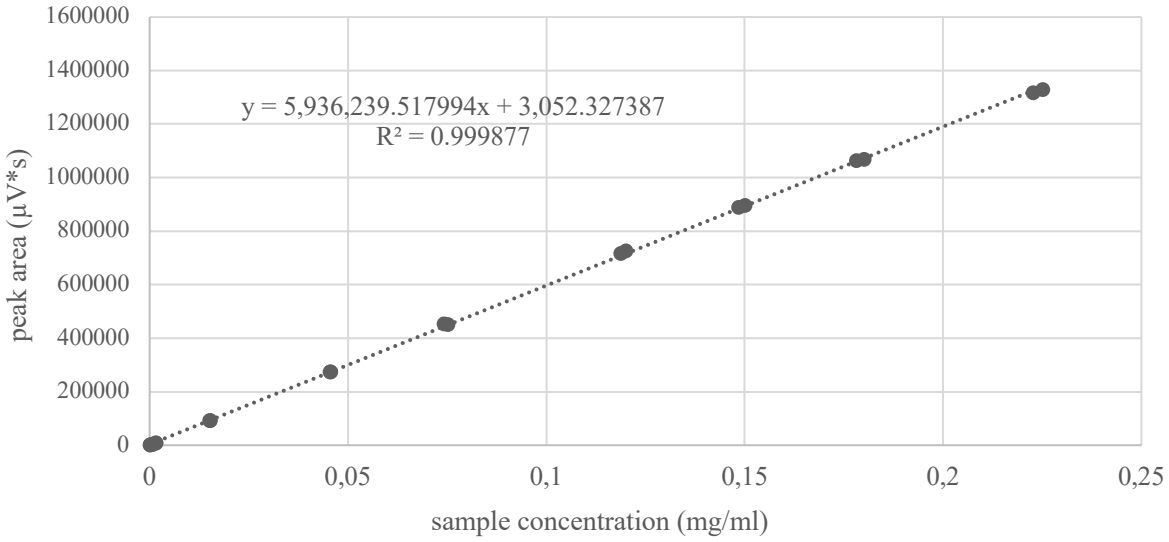


*Figure 11: Linearity determination of the manual method: sample concentration in mg/ml plotted versus the peak area in µV*s obtained after data evaluation using the Empower software, as well as linear regression of the curve.*

Table 17: Raw data of the linearity determination of the automated method. The initial concentration was calculated automatically by the Tecan Fluent, based on the weighed amount of API and added volume of diluent. The peak area was obtained after data evaluation using the Empower software.

Sample	**No.**	**Inj.**	**Initial concentration (mg/ml)**	**Peak area (µV*s)**	**Sample**	**No.**	**Inj.**	**Initial concentration (mg/ml)**	**Peak area (µV*s)**
0.05%	1	1	0.0000761	409	50%	1	1	0.07603	448495
		2		371			2		448613
		3		403			3		449277
0.05%	2	1	0.0000761	335	50%	2	1	0.07613	449428
		2		362			2		450002
		3		374			3		450240
0.5%	1	1	0.000761	4274	80%	1	1	0.1216	719666
		2		4308			2		719587

		3		4300			3		720622
0.5%	2	1	0.000761	4397	80%	2	1	0.1218	721655
		2		4383			2		720975
		3		4385			3		721293
1%	1	1	0.001522	8767	100%	1	1	0.1522	922668
		2		8796			2		921446
		3		8758			3		922521
1%	2	1	0.001523	8826	100%	2	1	0.1523	924998
		2		8858			2		924561
		3		8816			3		924637
10%	1	1	0.01522	90152	120%	1	1	0.1825	1097569
		2		90115			2		1097092
		3		90083			3		1096986
10%	2	1	0.01523	91516	120%	2	1	0.1827	1094282
		2		91575			2		1094841
		3		91367			3		1094130
30%	1	1	0.04566	277767	150%	1	1	0.2281	1362500
		2		276858			2		1361594
		3		276759			3		1361868
30%	2	1	0.04568	280390	150%	2	1	0.2284	1362041
		2		281034			2		1359629
		3		281081			3		1360343

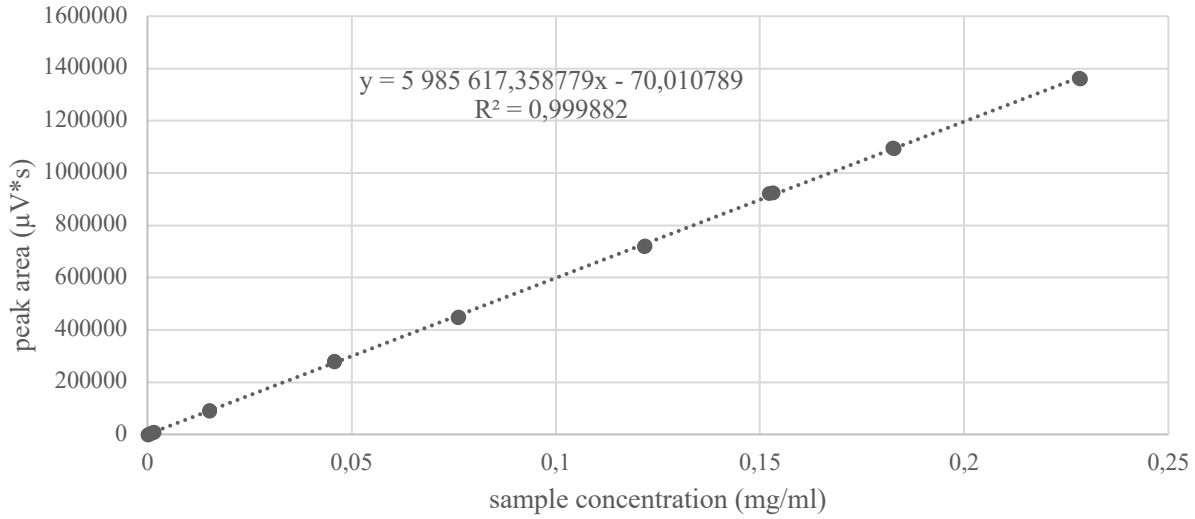


*Figure 12: Linearity determination of the automated method: sample concentration in mg/ml plotted versus the peak area in µV*s obtained after data evaluation using the Empower software, as well as linear regression of the curve.*

8.3.6 Stability

Table 18: Raw data of the stability determination of the manual and automated method. The concentration was obtained after data evaluation using the Empower software.

			Manual method	**Automated method**
Sample	**Time**	**Inj.**	**Concentration (mg/ml)**	**Concentration (mg/ml)**
100%	0h	1	0.155980	RT: 0.149997 FR: 0.15075
	24h RT	1	0.156687	0.152204
	24h FR	1	0.155747	0.152321
	48h RT	1	0.157368	0.152451
	48h FR	1	0.156528	0.151374
	1 week RT	1	0.163041	0.152652
	1 week FR	1	0.157956	0.150387
0.05%	0h	1	0.000073	RT: 0.000069 FR: 0.000073
	24h RT	1	0.000088	0.000071
	24h FR	1	0.000078	0.000069

	48h RT	1	0.000078	0.000059
	48h FR	1	0.000072	0.000064
	1 week RT	1	0.000099	0.000095
	1 week FR	1	0.000090	0.000094

8.4 Raw data of the drug product (low strength) method validation

8.4.1 Specificity

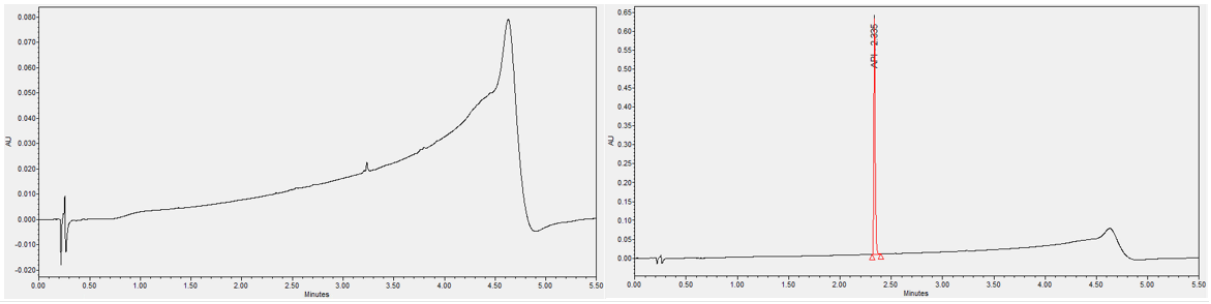


Figure 13: Specificity determination of the manual method: comparison of the blank (left) and 100% strength sample (right).

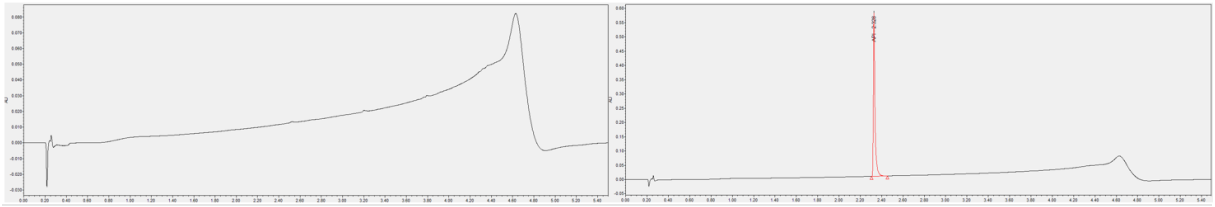


Figure 14: Specificity determination of the automated method: comparison of the blank (left) and 100% strength sample (right).

8.4.2 Accuracy

Table 19: Raw data of the accuracy determination of the manual and automated method. The initial concentration was calculated based on the weighed amount of API and added volume of diluent, either manually or automatically by the Tecan Fluent. The concentration was obtained after data evaluation using the Empower software.

			Manual method		**Automated method**	
Sample	**No.**	**Inj.**	**Initial concentration (mg/ml)**	**Concentration (mg/ml)**	**Initial concentration (mg/ml)**	**Concentration (mg/ml)**
80%	1	1	0.07750	0.078644	0.072151	0.073027
		2		0.078589		0.073048
		3		0.078559		0.073048

80%	2	1	0.07525	0.075940	0.085996	0.088061
		2		0.075866		0.087893
		3		0.075819		0.087874
80%	3	1	0.07730	0.077811	0.086871	0.088735
		2		0.077765		0.088691
		3		0.077732		0.088654
100%	1	1	0.10450	0.106290	0.097544	0.096362
		2		0.106168		0.096281
		3		0.106017		0.096367
100%	2	1	0.09825	0.098542	0.094613	0.093731
		2		0.098280		0.093567
		3		0.098377		0.093676
100%	3	1	0.11145	0.111620	0.100589	0.098555
		2		0.111775		0.098497
		3		0.111897		0.098528
120%	1	1	0.11930	0.121552	0.117693	0.116156
		2		0.121537		0.116077
		3		0.121421		0.116247
120%	2	1	0.12115	0.123006	0.114691	0.114420
		2		0.123259		0.114384
		3		0.123107		0.114744
120%	3	1	0.12145	0.121278	0.124633	0.123917
		2		0.121465		0.123587
		3		0.121151		0.123728

8.4.3 Stability

Table 20: Raw data of the stability determination of the manual and automated method. The concentration was obtained after data evaluation using the Empower software.

			Manual method	**Automated method**
Sample	**Time**	**Inj.**	**Concentration (mg/ml)**	**Concentration (mg/ml)**
100%	0h	1	0.106259	RT: 0.117537 FR: 0.115730
	24h RT	1	0.107232	0.114161
	24h FR	1	0.106596	0.114612
	67h RT	1	0.107968	0.121177
	67h FR	1	0.106973	0.116813

8.5 Raw data of the drug product (high strength) method validation

8.5.1 Specificity

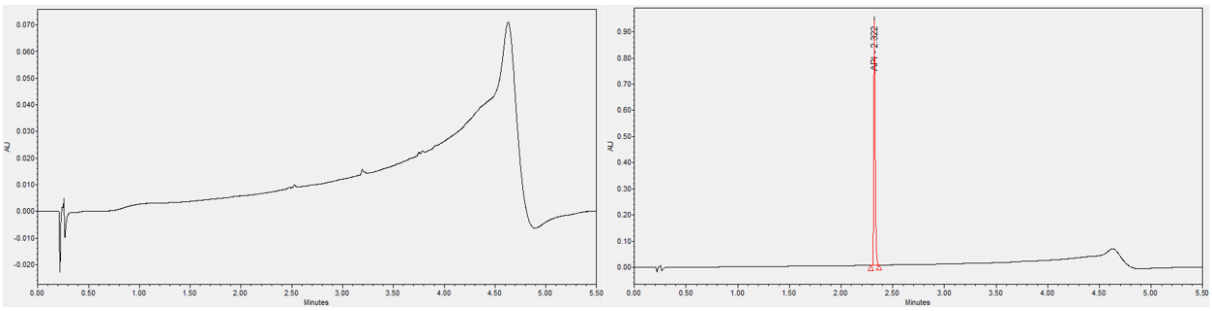


Figure 15: Specificity determination of the manual method: comparison of the blank (left) and 100% strength sample (right).

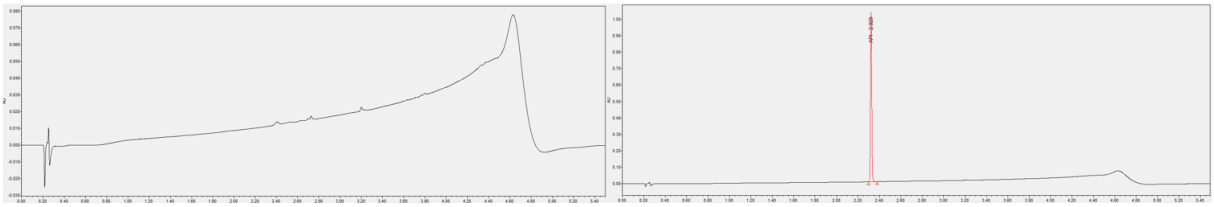


Figure 16: Specificity determination of the automated method: comparison of the blank (left) and 100% strength sample (right).

8.5.2 Accuracy

Table 21: Raw data of the accuracy determination of the manual and automated method. The initial concentration was calculated based on the weighed amount of API and added volume of diluent, either manually

or automatically by the Tecan Fluent. The concentration was obtained after data evaluation using the Empower software.

			Manual method		Automated method	
Sample	**No.**	**Inj.**	**Initial concentration (mg/ml)**	**Concentration (mg/ml)**	**Initial concentration (mg/ml)**	**Concentration (mg/ml)**
80%	1	1	0.1202	0.121460	0.1197	0.114338
		2		0.121481		0.113970
		3		0.121400		0.114030
80%	2	1	0.1200	0.122149	0.1199	0.114822
		2		0.121500		0.114743
		3		0.122105		0.114782
80%	3	1	0.1201	0.121209	0.1207	0.116925
		2		0.121107		0.116852
		3		0.121201		0.117034
100%	1	1	0.1502	0.151536	0.1505	0.145823
		2		0.151408		0.145615
		3		0.151549		0.145658
100%	2	1	0.1504	0.151069	0.1504	0.146601
		2		0.151063		0.146700
		3		0.151088		0.146603
100%	3	1	0.1504	0.150781	0.1503	0.149237
		2		0.150959		0.149260
		3		0.150862		0.149140
120%	1	1	0.1802	0.180766	0.1789	0.177054
		2		0.180815		0.176920
		3		0.180720		0.177021

120%	2	1	0.1799	0.180593	0.1798	0.184666
		2		0.180737		0.184712
		3		0.180777		0.184639
120%	3	1	0.1782	0.178240	0.1767	0.181118
		2		0.178185		0.180716
		3		0.178177		0.180679

8.6 Statistical comparison of the manual and automated method validation

Table 22: Statistical comparison of the manual and automated method precision and accuracy through a two-tailed Welch's t-test with a significance level of 0.05.

	Manual method		**Automated method**		**t**	**Degrees of freedom**	**Critical value**
	Average	**Stdev**	**Average**	**Stdev**			
Precision 100%	0.153 mg/ml	0.000244	0.154 mg/ml	0.000110	0.8779	7	2.365
Precision 0.1%	0.000155 mg/ml	$4.8*10^{-6}$	0.000159 mg/ml	$2.9*10^{-6}$	1.7504	5	2.571
Accuracy Recovery	100.17%	0.1491	100.53%	0.3944	2.6089	10	2.228

8.7 Results of previously performed studies

Table 23: Results of the previously performed pipetting repeatability determination of the fixed steel and disposable tip pipettes of the Tecan Fluent 1080 of pipetting 4.9 ml, 0.75 ml, and 0.1 ml of a 50:50 mixture of acetonitrile and water.

	Fixed steel pipette	**Disposable tip pipette**	
	4.9 ml	**0.75 ml**	**0.1 ml**
Determination 1	4442.0 mg	679.6 mg	90.8 mg
Determination 2	4444.2 mg	679.4 mg	91.1 mg
Determination 3	4438.4 mg	678.8 mg	90.9 mg
Determination 4	4442.3 mg	679.1 mg	91.0 mg
Determination 5	4442.9 mg	679.0 mg	90.8 mg

Determination 6	4437.8 mg	679.3 mg	90.8 mg
Determination 7	4440.3 mg	679.7 mg	91.5 mg
Determination 8	4441.0 mg	680.8 mg	90.8 mg
Determination 9	4440.4 mg	680.0 mg	90.6 mg
Determination 10	4443.4 mg	679.6 mg	90.7 mg
Determination 11	4437.7 mg	681.3 mg	90.7 mg
Density	0.90652 mg/ml		
Standard deviation	0.002449	0.00112	0.000261

Table 24: Results of the previously performed repeatability determination of manually pipetting 10 ml and 1 ml of water.

Nominal volume	**10 ml**	**1000 µl**
Determination 1	9.97036 g	0.99792 g
Determination 2	9.97017 g	0.99773 g
Determination 3	9.96990 g	0.99808 g
Determination 4	9.96987 g	0.99783 g
Determination 5	9.97007 g	0.99794 g
Determination 6	9.97018 g	0.99798 g
Standard deviation	0.00017	0.00011

Table 25: Results of the previously performed repeatability determination of weighing 50 ml of a 50:50 mixture of acetonitrile and water.

Weight determination	**Mass**	**Average**	**RSD**
1	45.3160 g	45.3259 g	0.011698
2	45.3423 g		
3	45.3193 g		

8.8 Raw data of the liquid chromatography repeatability study

Table 26: Raw data of the liquid chromatography repeatability study through 11 consecutive injections of the same sample at 0.15 mg/ml and 0.075 µg/ml.

High concentration (0.15 mg/ml) sample				**Low concentration (0.075 µg/ml) sample**			
Inj.	**Peak area (µV*s)**	**Inj.**	**Peak area (µV*s)**	**Inj.**	**Peak area (µV*s)**	**Inj.**	**Peak area (µV*s)**
1	824982	7	825207	1	444	7	406
2	825785	8	825801	2	373	8	400
3	825990	9	826217	3	424	9	398
4	826249	10	825960	4	394	10	436
5	825942	11	825502	5	395	11	408
6	826451	**RSD**	0.000514	6	420	**RSD**	0.047950

8.9 Raw data of the bottom-up uncertainty quantification

Table 27: Uncertainty quantification of the mass determination for the manual and automated method. The specifications are derived from calibration certificates of the microbalance (manual), respectively the automatic balance. U(x) was calculated assuming a rectangular probability distribution. U(m) was calculated using equation 4.

	Manual method		**Automated method**	
	Specification	**u(x)**	**Specification**	**u(x)**
Repeatability	0.0005 mg	0.000289 mg	0.004 mg	0.002309 mg
Linearity	0.004 mg	0.002309 mg	0.005 mg	0.002887 mg
Readability	0.0005 mg	0.000289 mg	0.005 mg	0.002887 mg
Sensitivity	0.048 mg	0.027713 mg	0.07 mg	0.040415 mg
Eccentricity	0.003 mg	0.001732 mg	n/a	n/a
u(m)	0.027866 mg		0.040686 mg	
m	7.5 mg		5 mg	

Table 28: Uncertainty quantification for the manual dilution. V denotes the nominal capacity of the pipette or volumetric flask. The tolerance was obtained from instrument specifications, and u(x was calculated assuming a rectangular probability distribution. The pipetting repeatability was obtained from a previously performed study (see table Table 24: Results of the previously performed repeatability determination of manually pipetting 10 ml

and 1 ml of water. The uncertainty of the temperature was calculated using equation 8 with a ΔT value of 4K and an α value of 0.001108 K^{-1}, assuming a triangular probability distribution. U(v) was calculated using equation 7. U(d)/d was calculated using equation 5.

Liquid addition step: volume		**High concentration sample**				**Low concentration sample**			
		V	**Spec.**	**u(x)**	**u(v)**	**V**	**Spec.**	**u(x)**	**u(v)**
1: 50 ml	Tol.	50 ml	0.06	0.0346	0.0969	50 ml	0.06	0.0346	0.0969
	Temp.		0.2216	0.0905			0.2216	0.0905	
2: 1 ml	Rep	n/a				1 ml	0.00011	0.00012	0.0018
	Temp.						0.0044	0.0018	
3: 100 ml	Tol.	n/a				100 ml	0.1	0.0577	0.1899
	Temp.						0.4432	0.1809	
4: 2.5 ml	Rep.	n/a				10 ml	0.00017	0.00017	0.0045
	Temp.						0.01108	0.00452	
5: 50 ml	Tol.	n/a				50 ml	0.06	0.0346	0.0969
	Temp.						0.2216	0.0905	
u(d)/d		0.001937				0.004205			

Table 29: Uncertainty quantification of the first gravimetrically controlled automated dilution step. The specifications for the mass determination are derived from the balance incorporated into the Fluent worktable. U(x) was calculated assuming a rectangular probability distribution. U(m) was calculated using equation 4. The repeatability of the mass was obtained from a previously performed study (see table Table 25). V denotes the nominal capacity of the pipette. The tolerance was obtained from instrument specifications, and u(x) was calculated assuming a rectangular probability distribution. The uncertainty of the temperature was calculated using equation 8 with a ΔT value of 4K and an α value of 0.001108 K^{-1}, assuming a triangular probability distribution. U(dens)/dens was calculated using equation 11. U(v)/v was calculated using equation 9.

Gravimetric addition of 5 ml					
	m	**Specification**	**u(x)**	**u(m)**	**u(m/m)**
Repeatability	45325 mg	0.1 mg	0.057735	0.196103	$4.33*10^{-6}$
Linearity		0.25 mg	0.144338		
Readability		0.1 mg	0.057735		
Sensitivity		0.1813 mg	0.104674		

Density, ρ= 9065 mg/ml					
	V/m	**Specification**	**u(x)**	**u(m)/u(v)**	**u(dens)/dens**
rep(mass)	45325.9 mg	11.69796	11.69796	11.69796	$2.11*10^{-7}$
Tolerance	50 ml	0.05	0.028868	0.094962	
Temperature		0.2216	0.090468		
u(v)/v	$8.66*10^{-7}$				

Table 30: Uncertainty quantification for the automated dilution steps 2-7. V denotes the nominal capacity of the pipette or volumetric flask. The repeatability was obtained from a previously performed study (see table Table 23). U(x) was calculated assuming a rectangular probability distribution. The uncertainty of the temperature was calculated using equation 8 with a ΔT value of 4K and an α value of 0.001108 K^{-1}, assuming a triangular probability distribution. U(v) was calculated using equation 6. U(d)/d was calculated using equation 5.

Liquid addition step: volume		**High concentration sample**				**Low concentration sample**			
		V	**Spec.**	**u(x)**	**u(v)**	**V**	**Spec.**	**u(x)**	**u(v)**
2: 0.75 ml	Rep.	0.75 ml	0.00112	0.00112	0.002346	0.75 ml	0.00112	0.00112	0.002346
	Temp.		0.003324	0.001357			0.003324	0.001357	
3: 4.25 ml	Rep.	4.9 ml	0.002449	0.002449	0.001899	4.9 ml	0.002449	0.002449	0.001899
	Temp.		0.018836	0.00769			0.018836	0.00769	
4: 0.1 ml	Rep.	n/a				0.1 ml	0.000261	0.000261	0.000318
	Temp.						0.000443	0.000181	
5: 4.9 ml	Rep.	n/a				4.9 ml	0.002449	0.002449	0.009198
	Temp.						0.021717	0.008866	
6: 0.1 ml	Rep.	n/a				0.1 ml	0.000261	0.000261	0.000318

	Temp.			0.000443	0.000181	
7: 3.9 ml	Rep.	n/a	3.9 ml	0.002449	0.002449	0.007469
	Temp.			0.017285	0.007056	
u(d)/d		0.003018		0.006039		

Table 31: Results of the peak area uncertainty determination for samples at a high (0.15 mg/ml) and low (0.075 µg/ml) concentration, prepared with the manual and automated method. The relative uncertainty of the peak area was calculated using equation 3. The relative uncertainty of the purity was calculated using equation 11 and the specified purity of 99.8%.

Relative uncertainty	Manual high	Manual low	Automated high	Automated low
$u(m)/m$	$3.7*10^{-3}$	$3.7*10^{-3}$	$8.1*10^{-3}$	$8.1*10^{-3}$
$u(d)/d$	$1.9*10^{-3}$	$4.2*10^{-3}$	$3.0*10^{-3}$	$6.0*10^{-2}$
$u(Purity)/Purity$	$0.58*10^{-3}$	$0.58*10^{-3}$	$0.58*10^{-3}$	$0.58*10^{-3}$
$rep(LC)$	$0.51*10^{-3}$	$48*10^{-3}$	$0.51*10^{-3}$	$48*10^{-3}$
$u(A)/A$	$4.3*10^{-3}$	$48*10^{-3}$	$8.8*10^{-3}$	$49*10^{-3}$

Table 32: Results of the relative standard uncertainty determination for samples at a high (0.15 mg/ml) and low (0.075 µg/ml) concentration, prepared with the manual and automated method. The relative standard uncertainty was calculated using equation 2. The expanded uncertainty was calculated by multiplication with a coverage factor of k=2.

Relative uncertainty	Manual high	Manual low	Automated high	Automated low
$u(A(s))/A(s)$	$4.3*10^{-3}$	$4.3*10^{-3}$	$8.8*10^{-3}$	$8.8*10^{-3}$
$u(A(u))/A(u)$	$4.3*10^{-3}$	$48*10^{-3}$	$8.8*10^{-3}$	$49*10^{-3}$
$u(m(s))/m(s)$	$3.7*10^{-3}$	$3.7*10^{-3}$	$8.1*10^{-3}$	$8.1*10^{-3}$
$u(d(s))/d(s)$	$1.9*10^{-3}$	$1.9*10^{-3}$	$3.0*10^{-3}$	$3.0*10^{-3}$
$u(c(u))/c(u)$	$7.3*10^{-3}$	$49*10^{-3}$	$15*10^{-3}$	$51*10^{-3}$
Expanded	$15*10^{-3}$	$97*10^{-3}$	$30*10^{-3}$	$100*10^{-3}$

Table 33: Results of the uncertainty determination for a sample at a high concentration (0.15 mg/ml) which is manually weighed using the microbalance and automatically prepared using the Tecan Fluent. The relative

standard uncertainty was calculated using equation 2. The expanded uncertainty was calculated by multiplication with a coverage factor of k=2.

Relative uncertainty	**Sample high**	**Relative uncertainty**	**Sample high**
$u(m)/m$	5.6*10^{-3}	$u(A(s))/A(s)\ and\ u(A(u))/A(u)$	6.4*10^{-3}
$u(d)/d$	3.0*10^{-3}	$u(m(s))/m(s)$	5.6*10^{-3}
$u(Purity)/Purity$	0.58*10^{-3}	$u(d(s))/d(s)$	3.0*10^{-3}
$rep(LC)$	0. 51*10^{-3}	$u(c(u))/c(u)$	11*10^{-3}
$u(A)/A$	6.4*10^{-3}	Expanded	22*10^{-3}