Analytical monitoring of personalized drugs containing monoclonal antibodies: An alliance of patient and personnel safety

Dissertation

zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften
– Dr. rer. nat. –

vorgelegt von

Lars Maximilian Heinrich Reinders

geboren in
Duisburg

Fakultät für Chemie
der
Universität Duisburg-Essen

2023

Die vorliegende Arbeit wurde im Zeitraum von Juli 2018 bis Juli 2022 im Arbeitskreis von Prof. Dr. Torsten C. Schmidt in der Fakultät für Chemie im Bereich Instrumentelle Analytische Chemie der Universität Duisburg-Essen durchgeführt. Die praktischen Arbeiten erfolgten am Institut für Energie- und Umwelttechnik e.V. in Duisburg.

Tag der Disputation: 05.05.2023

Gutachter: Prof. Dr. Torsten C. Schmidt

Prof. Dr. Martin Jäger

Vorsitzender: Prof. Dr. Stephan Barcikowski

DOI: 10.17185/duepublico/78419
URN: urn:nbn:de:hbz:465-20230522-103545-4

Danksagung

Der schwierigste Teil der Dissertation ist die Danksagung. Eigentlich muss man sich bei allen Menschen bedanken, welche einem in seinem Leben begegnet sind. Dies schließt sowohl positive, negative und scheinbar flüchtige Begegnungen ein. Denn all diese Momente haben einen zu dem Menschen gemacht, welcher man ist. Nur so war es möglich den Weg von der Geburt, über die Schule, Ausbildung und das Studium hin zur Promotion einzuschlagen. Auch wenn ich euch nicht alle namentlich erwähnen kann, möchte ich euch danken.

Nichtsdestotrotz haben einige Personen einen intensiveren Einfluss auf die Promotion ausgeübt. Ich möchte mich daher namentlich bei meinem Doktorvater Prof. Dr. Torsten C. Schmidt und Prof. Dr. Martin Jäger bedanken. Ihr habt mir beide gezeigt, dass der Weg zur wissenschaftlichen Erkenntnis nicht immer geradlinig ist und es in der Praxis doch mehr Einflussfaktoren gibt, als es die Theorie vermuten lässt.

Dies bedeutet auch dass nicht alle Experimente bei der ersten Versuchsdurchführung funktioniert haben und das der Weg in das Tal der Tränen deutlich einfacher ist als aus diesem hinaus. Ich möchte mich daher auch bei meinen Mitdoktoranden Tobias Werres und Kjell Kochale bedanken, welche mir eindrucksvoll gezeigt haben, dass es Anderen ähnlich geht.

Weiterhin möchte ich mich bei Dr. Thorsten Teutenberg, Dr. Jochen Türk und Martin Klaßen bedanken. Ihr habt euch immer Zeit für fachlichen Austausch genommen und mir gezeigt nicht das große Ganze aus den Augen zu verlieren.

Neben der fachlichen Komponente kann eine Promotion jedoch nur gelingen, sofern auch emotionale Unterstützung und Verständnis für den benötigten Zeitaufwand im privaten Umfeld vorhanden ist. Hier möchte ich daher ein besonderes Dankeschön an meine Eltern Sandra Schmidt-Reinders und Dirk Reinders, sowie meine Geschwister Emilie-Lieselotte Süsselbeck und Stella Brandes aussprechen. Gleichwertig möchte ich Markus Schmidt danken, welcher mich wie ein Vater großgezogen hat. Euch allen einen vielen Dank für die Möglichkeit des Jammerns und der Versorgung mit Süßigkeiten und Gebäck.

Ebenfalls möchte ich mich bei meinen Mitbewohnern Hubert und Heidrun bedanken. Das Zusammenleben mit euch verläuft reibungslos und ihr seid zu jeder Tageszeit für mich da.

Summary

In modern cancer therapy, both the drug and the dose administered are adapted to the patient. As a result, the required preparations are not manufactured on a large scale, but individually by pharmaceutical specialists. The pharmaceutical active substances are usually cytostatics and monoclonal antibodies with carcinogenic, mutagenic or reproduction-toxic (CMR) properties. This leads to an area of tension between patient safety and occupational safety.

For the quality assurance of personalised preparations, a sampling concept was developed under the aspect of occupational safety. With this concept, five cytostatic drugs and three monoclonal antibodies in preparations were tested for identity and content. It was shown that 96% of the cytostatic preparations (n=136) and 100% of the monoclonal antibody preparations (n=10) met the legal requirements. The quality of the preparations can thus be assessed as very good.

For further characterisation of the monoclonal antibodies, affinity chromatography with immobilised FcγRIIIa was performed. To enable coupling of affinity chromatography with high-resolution mass spectrometry (HRMS), 2D-HPLC was used for online desalting. It was shown that monoclonal antibodies with glycan modifications with higher galactose or lower fucose content have a higher affinity for the receptor.

Furthermore, it was demonstrated that monoclonal antibodies can not only be characterised by means of affinity chromatography, 2D-HPLC and HRMS, but also distinguished from their biosimilars.

As part of the work on occupational safety, a method was developed for quantifying airborne monoclonal antibodies in the trace range. Using this method, it was possible to show that there is a potential for release during patient-specific production. This is triggered by the piercing of the drug containers and the occurrence of a pressure equalisation process. The release could be quantified to 15 ng. By using pressure equalisation systems (spikes), an unintentional release can be effectively prevented. As part of the method developed, the antibodies are quantified at the peptide level, which requires enzymatic digestion. Since it is time-consuming and may take several hours, the digestion step was automated using immobilised enzymes and coupled online with mass spectrometric detection. This reduced the required digestion time by 98%.

Zusammenfassung

In der modernen Krebstherapie werden sowohl das Medikament als auch die verabreichte Dosis an den Patienten angepasst. Dies hat zur Folge, dass die benötigten Präparate nicht großtechnisch, sondern individuell durch pharmazeutisches Fachpersonal hergestellt werden. Bei den Wirkstoffen handelt es sich in der Regel um Zytostatika und monoklonale Antikörper mit kanzerogenen, mutagenen oder reproduktionstoxischen (CMR) Eigenschaften. Dies führt zu einem Spannungsfeld zwischen Patientensicherheit und Arbeitssicherheit.

Zur Qualitätssicherung personalisierter Zubereitungen wurde unter dem Aspekt des Arbeitsschutzes ein Probenahmekonzept entwickelt. Mit diesem wurden fünf Zytostatika und drei monoklonale Antikörper in Zubereitungen auf Identität und Gehalt untersucht. Es konnte gezeigt werden, dass 96% der Zytostatikazubereitungen (n=136) und 100% der monoklonalen Antikörperzubereitungen (n=10) den gesetzlichen Anforderungen entsprechen. Die Qualität der Präparate kann somit als sehr gut bewertet werden.

Zur weiteren Charakterisierung der monoklonalen Antikörper wurde eine Affinitätschromatographie mit immobilisiertem FcγRIIIa durchgeführt. Um eine Kopplung der Affinitätschromatographie mit hochauflösender Massenspektrometrie (HRMS) zu ermöglichen, wurde die 2D-HPLC zur Online-Entsalzung eingesetzt. Es konnte gezeigt werden, dass monoklonale Antikörper mit Glykanmodifikationen mit höherem Galaktose- bzw. niedrigerem Fucoseanteil eine höhere Affinität zum Rezeptor besitzen.

Weiterhin konnte gezeigt werden, dass monoklonale Antikörper mittels Affinitätschromatographie, 2D-HPLC und HRMS nicht nur charakterisiert, sondern auch von ihren Biosimilars unterschieden werden können.

Im Rahmen der Arbeiten zur Arbeitssicherheit wurde eine Methode zur Quantifizierung von luftgetragenen monoklonalen Antikörpern im Spurenbereich entwickelt. Mit dieser Methode konnte gezeigt werden, dass bei der patientenindividuellen Herstellung ein Freisetzungspotenzial besteht. Dieses entsteht beim Durchstechen der Medikamentenbehälter und des Eintretens eines Druckausgleichprozesses. Die Freisetzung konnte auf 15 ng quantifiziert werden. Durch den Einsatz von Druckausgleichssystemen (Spikes) kann eine unbeabsichtigte Freisetzung wirksam verhindert werden. Bei der entwickelten Methode werden die Antikörper auf Peptidebene quantifiziert, was einen enzymatischen Verdau voraussetzt. Da dieser zeitaufwändig ist und mehrere Stunden dauert, wurde er mit Hilfe immobilisierter Enzyme automatisiert und online mit der massenspektrometrischen Detektion gekoppelt. Dadurch konnte die benötigte Verdauungszeit um 98% reduziert werden.

Content

Chapter 1 General Introduction

1.1 The history of cancer and its treatment

Cancer is often referred to as the disease of the twentieth century both in the scientific literature and in the popular science press [1-3]. This is probably due to the fact that the chance of developing cancer in industrialised countries is 1:2, which can be attributed to the steadily increasing life expectancy [4]. However, this statement reduces cancer to a modern problem. In fact, there are historical records from 3000 BC describing breast cancer and its surgical treatment [1,5]. There are also findings of cancer in dinosaur fossils [6,7], so cancer cannot be limited to either modernity or humanity.

Although the disease had been known for so long, it took until 400 BC to attribute it to biological causes rather than the powers of gods [8-12]. Nevertheless, treatments continued to be limited to surgical procedures, from which patients often died. Modern oncology therefore began from the 17th century, where the disease was systematically studied and triggering factors such as tobacco consumption were identified [1].

The first non-surgical treatments became possible with the discovery of X-rays and their application to cancer, so that modern radiotherapy began from 1920 [1,13,14]. The first breakthroughs in the drug treatment of cancer were accidentally achieved during the Second World War, when a ship loaded with mustard gas was sunk and the gas was released. The survivors showed bone marrow aplasia, so they produced fewer red and white blood cells [15]. The mustard gas-derived mechlorethamine from the nitrogen mustard substance class was eventually the first cytostatic used to treat cancer [16,17]. Even today, substances of the nitrogen mustard class such as cyclophosphamide are used as cytostatic agents [18-21].

Although progress continued to be made in the development of cytostatic drugs, these all had a systemic effect and thus not only damaged the cancer but also healthy cell tissue [22]. From 1975 on, it became possible to produce monoclonal antibodies through the work of Köhler and Milstein [23]. The antibodies specifically bind to antigens that are found in larger numbers on cancer cells, thus achieving high specificity and low systemic toxicity. Köhler and Milstein achieved this by producing hybridoma cells by fusing mouse B lymphocytes and human myeloma cells. The hybridoma cells can produce large amounts of antibodies [23].

However, the use of monoclonal antibodies as therapeutic agents from 1980 onwards was not successful, due to their mouse origin. Patients showed defensive reactions, which ended in neutralisation of the drug, allergic and anaphylactic reactions [24-27]. Only further research

and the reduction of the mouse protein content finally led to the approval of rituximab, the first monoclonal antibody with a cancer indication, in 1997 [1]. Due to the good drug profile described above, more than 100 therapeutic antibodies are now approved, half of which are used for cancer treatment [28]. In comparison, about 50 small molecule cytostatic drugs are used for cancer treatment as of 2022 [29]. Thus, the number of cytostatics approximately equals that of monoclonal antibodies for cancer therapy. This illustrates the high relevance of monoclonal antibodies in modern medicine.

1.2 Workplace safety for CMR drugs

Cytostatic drugs and monoclonal antibodies are of crucial importance in the treatment of cancer patients, but they are therefore also highly potent active substances with properties relevant to occupational safety. For cytostatics, this specifically means carcinogenic, mutagenic or reprotoxic properties (CMR substances) [30]. The classification of monoclonal antibodies, on the other hand, is not straightforward, as testing for CMR properties is not mandatory for these compounds, which is why only few data are available. As a result, the classifications and recommendations for action vary. These range from assessment as harmless to CMR properties. However, a common consensus is the suspicion of a sensitisation potential [31-36] coming from protein-based drugs and a case report [37]. Due to this high degree of uncertainty, monoclonal antibodies are therefore often prepared in specialised pharmacies in accordance with the precautionary principle analogous to cytostatics. This means, for example, processing in safety cabinets or isolators and wearing suitable protective clothing [31].

The dose needed to treat the patient is determined individually. The calculated body surface area of the patient is often used to this purpose, considering body height and body weight. In addition, the results of examinations and the patient's state of health must be considered [38]. This high degree of individualisation means that the pharmaceutical industry does not provide drug solutions that can be applied directly, but only provides concentrates and lyophilisates. Their processing is shown in Figure 1-1.

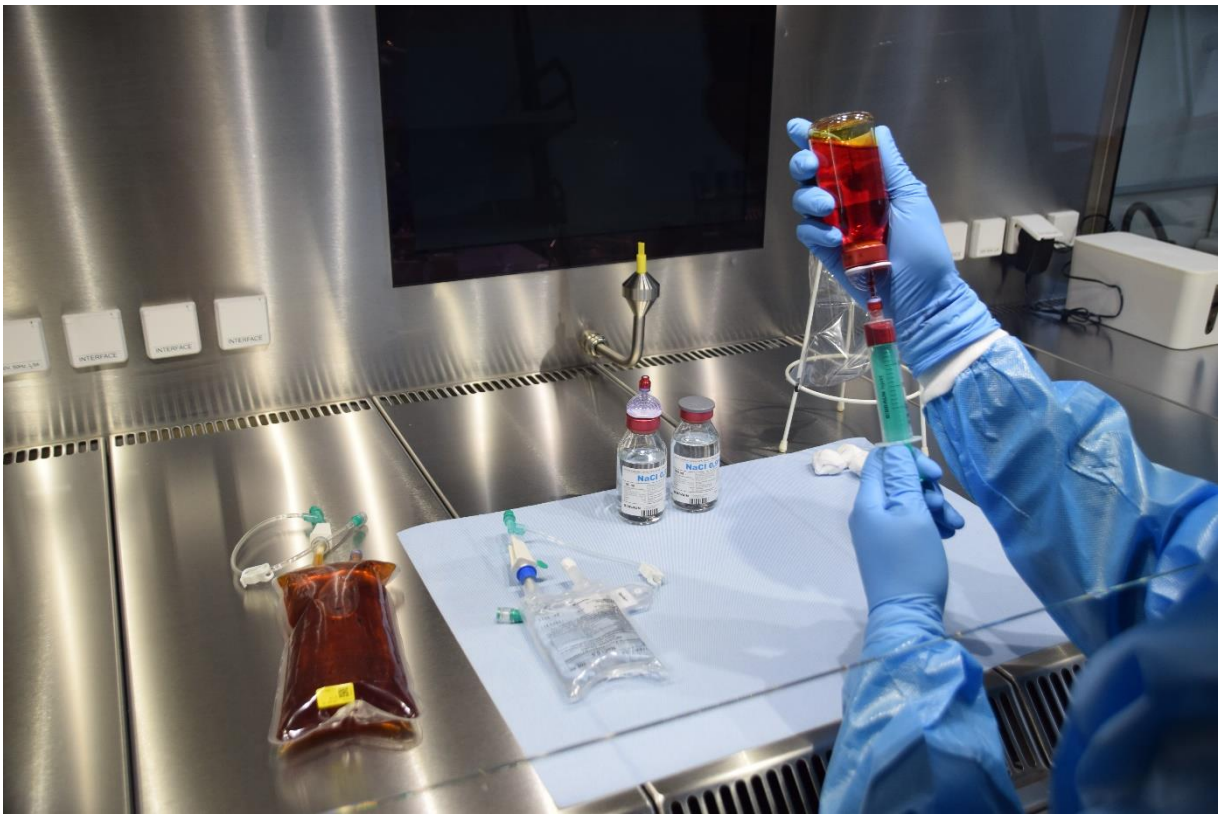


Figure 1-1: Exemplary representation of a workplace to produce personalised drugs. The concentrates from the drugs are aliquoted and transferred to the application bag.

During preparation, lyophilisates and highly concentrated liquids are brought to the required dose and dosage form by means of dissolution, dilution, and transfer steps [30,39,40].

Due to the manual processing of highly potent CMR drugs there is a potential for release and thus also for contamination. This results from spillages, releases during pressure equalisation processes such as the piercing of medicine containers or external contamination on these that occurs during production. Contamination is not limited to surfaces and air but can also be detected in the urine of personnel [41-51]. Although the data situation for cytostatic drugs is clear due to the large number of studies, there are currently no data on the contamination situation with monoclonal antibodies. Here, there is a need for the development of appropriate analytical methods and their application as occupational safety investigations.

1.3 Patient safety measures

In addition to the occupational health and safety risk for pharmaceutical staff, there is a risk for patients that individual preparations may be unintentionally underdosed or overdosed due to processing or communication errors. Depending on the dosage, this can endanger the success of the therapy or the patient's life [52-56]. Consequently, various measures are taken to ensure quality. These include, for example, working according to the four-eyes principle, in which a

visual check is carried out by a second person, camera or video recording, or gravimetric control of all individual steps. Furthermore, organisational measures are taken, for example, to exclude the mix-up of medicines by ensuring that only one medicine is being manufactured at a time [38,57-59].

Although these measures are usually sufficient to ensure quality, they can be circumvented by deliberate manipulation. This happened, for example, in a very popular case in Germany. In a court case, it was proven that more than 14,000 patient-specific preparations were not insignificantly reduced in quality (underdosed), although the full amount of active ingredient was billed [60]. As a result, public confidence in patient-specific preparations was shaken and questions arose as to whether there were other pharmacies that under-dosed preparations [61]. The regulatory agency therefore carried out unannounced inspections of pharmacies in its territory and took samples containing cytostatic drugs for analysis. Of the 123 samples examined, only one was objectionable [62]. This demonstrates that it is an isolated case and not a systematic problem.

1.4 Analysis of personalized drug products

The analysis to determine the active ingredient content was carried out by the agency in accordance with the European Pharmacopoeia. In order to determine the active ingredient content, the volume and the active ingredient concentration were determined. According to the European Pharmacopoeia, the volume of infusion preparations is determined by opening and transferring them to a dry measuring cylinder [63]. However, due to the CMR properties described above, this procedure is questionable from the point of view of occupational safety, which is why the development of alternative procedures for CMR substances should be striven for. Methods for determining the concentrations of active substances can be taken from the individual monographs for cytostatic drugs and are based, for example, on the coupling of high-performance liquid chromatography (HPLC) and diode array detection (DAD). For monoclonal antibodies, on the other hand, no analytical methods are described in the European Pharmacopoeia, which is why these need to be established and validated independently [64].

However, HPLC-DAD coupling is only suitable for monoclonal antibodies to a limited extent. It is possible to separate a variety of monoclonal antibodies using hydrophobic interaction chromatography (HIC), while some elude. This means that an unambiguous substance verification via retention time is not possible [65]. With regard to the absorption spectrum, no differentiation is possible, either [66]. In the case of capillary electrophoresis analysis, the situation is analogous to HIC [67]. Analytical techniques based on Fourier transform infrared

spectroscopy (FTIR) or Raman do not lead to reliable identity verification, either, as the spectra of monoclonal antibodies differ only slightly [66]. A suitable analytical method, on the other hand, is mass spectrometry, where substance verification is carried out via the mass of the analyte [68].

It is thus possible to determine the identity and content of the active ingredient of patient-specific preparations with cytostatics and monoclonal antibodies, so that unannounced sampling by authorities can prevent further attempts at deception. Since the manipulation scandal, however, it is not only the authorities who are trying to uncover attempts at deception, but also pharmacies are trying to demonstrate that they prepare according to pharmaceutical rules. To this end, some pharmacies have introduced voluntary self-regulation. However, this focuses on cytostatics, so that low-cost analytical methods such as HPLC-DAD or combinations of absorption and Raman spectroscopy can be used. Nevertheless, even these analytical methods are considered too expensive for the majority of pharmacies [38], which is why they occasionally send preparations to external analytical service providers.

To avoid the specific production of preparations for analytics, returns can be used. Returns are preparations that were intended for a patient but were not administered to him. They are therefore particularly informative samples, since it cannot be foreseen in advance which preparations will be analysed. Another advantage of the analysis of returned samples by an external provider is that more complex parameters such as structural changes and their effects on the therapeutic effect can also be investigated.

1.5 Structure and mechanisms of monoclonal antibodies

Such modifications can be induced, for example, by exposure to light, incorrect storage temperatures and mechanical stress during preparation or transport. The induced modifications cannot only reduce the therapeutic efficacy but can also increase immunogenicity and thus additionally endanger the patient [69].

One analytical approach is to determine the effects without determining the responsible modification or sum of modifications. For example, cell-based assays can be used to determine antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), two modes of action of monoclonal antibodies [70]. Alternatively, the receptor relevant for the mode of action can be immobilised and used for affinity chromatography [71].

For affinity chromatography of therapeutic monoclonal antibodies, Fc-gamma receptor IIIa (FcγRIII) is of particular importance, as it occurs on natural killer cells and thus plays a role in ADCC. The binding strength between antibody and receptor correlates with the retention time in affinity chromatography, so that changes in the chromatogram indicate modifications. The receptor binds near the hinge region of monoclonal antibodies near to asparagine 297 on the CH2 domain to which the glycans are bound. In case of modifications, these can thus not only be detected, but also their position can be narrowed down, which facilitates an optional chemical characterisation [71].

To illustrate the location of the binding site, the structure of a monoclonal antibody is shown in Figure 1-2. A monoclonal antibody consists of two light chains with a molecular weight of 25 kDa each and two heavy chains with 50 kDa each. This results in a molecular weight of about 150 kDa. The heavy and light chains are connected by disulphide bonds. The glycans are located on the heavy chain.

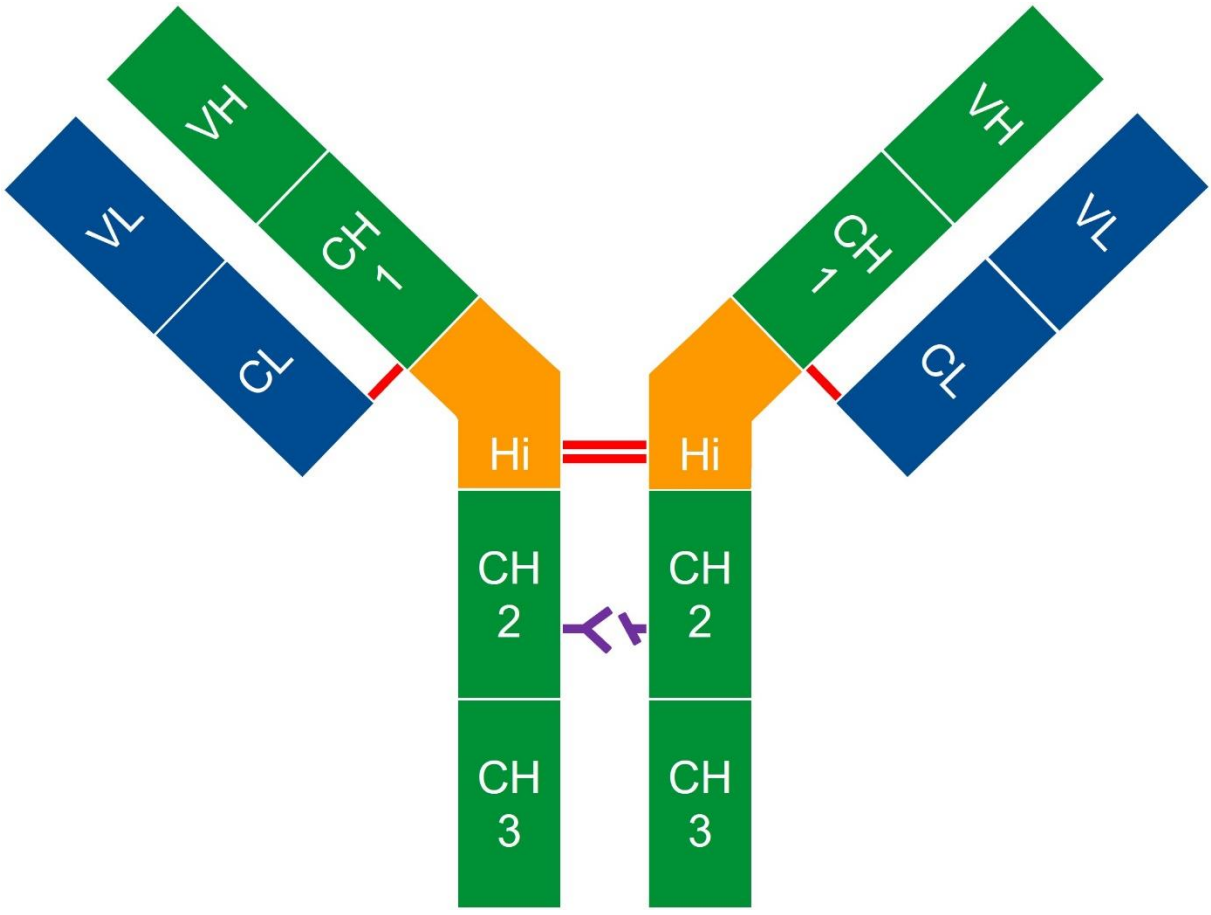


Figure 1-2: Schematic structure of a monoclonal antibody. The heavy chain is shown in green, the light chain in blue and the hinge region in orange. In addition, the disulphide bonds (red) and glycans (purple) are shown. VH: Variable region of the heavy chain. CH: Constant region of the heavy chain. VL: Variable region of the light chain. CL: Constant part of the light chain. Hi: Hinge region.

The second approach is to characterise the structure of the monoclonal antibody and assess whether any changes detected have an effect. However, a comprehensive characterisation requires various complementary analytical techniques such as multidimensional liquid chromatography, mass spectrometry or spectroscopic methods [72-75].

For a complete characterisation, both analytical approaches are needed, which is why combination will be necessary in the future.

1.6 Production of monoclonal antibodies and biosimilars

Independently of this, however, there is also a need for evaluation criteria for the analytical result, as this is subject to a certain range of variation in the case of monoclonal antibodies. The variation is due to the manufacturing process. Due to their complexity, which is expressed, for example, in the high molecular weight of 150,000 Da, monoclonal antibodies are not chemically synthesised but biotechnologically produced. For this purpose, animal cell cultures such as Chinese hamster ovary (CHO) cells or mouse myeloma cells (NS0 and SP2/0) are used, which express the monoclonal antibodies [76]. The exact structure thus depends, among other things, on the cell line used, its age or the environmental conditions, which is why individual batches of a monoclonal antibody fluctuate in an analytically detectable but therapeutically irrelevant range [69].

Once the patent protection of the monoclonal antibody is expired, the complexity of the assessment is further increased, as biosimilars are approved. Biosimilars are the same monoclonal antibody produced by a different company. Since different production conditions are therefore used, the reference product (originator) and the follow-up product (biosimilar) differ slightly [69]. According to the definition of the European Medicines Agency (EMA), the biosimilar must be very similar to the originator in terms of structure, biological activity, efficacy, safety and immunogenicity profile [77]. Due to this microvariability, the assessment must always critically examine whether it is a permissible variation or a process-related change. Subsequently, it can be checked whether there is a risk for the patient.

1.7 Routes of entry and significance for occupational safety and health

However, in patient-specific preparation, not only the safety of the patient but also that of the preparing staff must be ensured. As already mentioned, there are no data on workplace exposure to monoclonal antibodies. However, from the measures taken for patient protection and theoretical considerations, the risk of exposure as well as the required analytical procedures can be estimated.

The entry routes in occupational health and safety are ingestion, absorption via the skin and inhalation [31,33,34]. The ingestion route is very unlikely, as it is generally not allowed to eat and drink during preparation and in the cleanroom [31]. Should this unlikely event nevertheless occur, monoclonal antibodies are denatured and digested in the gastrointestinal tract [78]. For product protection, gloves are worn during preparation, which also prevent dermal contact with monoclonal antibodies [31]. When the glove is damaged, dermal uptake of monoclonal antibodies may occur. However, for significant amounts of substance to be absorbed through the skin, the molecular weight should not exceed 500 Da [79]. Monoclonal antibodies are significantly heavier, being about 150,000 Da, hence resorption is very unlikely [34]. In order to enable aseptic conditions and a low-particle environment, work is carried out in a safety cabinet or isolator during preparation. This significantly reduces the risk of inhalation exposure [31]. Nevertheless, pressure equalisation processes, such as those that occur during the piercing of drug vials, can lead to liquid droplets containing monoclonal antibodies being ejected and penetrating the air barrier of a safety cabinet [34]. Inhalation is thus theoretically possible, which is why the focus of occupational safety investigations should be on airborne monoclonal antibodies.

According to Halsen et al., aerosol concentrations of several hundred micrograms per cubic metre of air can occur in the short term. Taking into account the active ingredient concentration and data of other drugs, an uptake of monoclonal antibodies in the range of several micrograms per work shift is therefore to be expected [34].

1.8 Quantification of airborne monoclonal antibodies

Although the expected daily intake is in the range of several micrograms, corresponding analytical methods have to be much more sensitive. This is because sampling for air samples usually does not take the entire work shift, but only 15 min. Therefore, analytical methods are needed that can detect a few nanograms [80]. This can be achieved with immunoassays [81]. However, these require that the antibody is still capable of binding. The aerosolisation process, exposes the monoclonal antibodies to high stress and may therefore lead to agglomeration [82,83]. It can thus not be excluded that the antibodies are no longer capable of binding and thus an underestimation would result. Agglomerates are of particular importance in occupational health and safety because they are more immunogenic than intact monoclonal antibodies [82-84]. It is therefore crucial that the analytical procedure includes them.

Mass spectrometry can be used to detect intact monoclonal antibodies and their agglomerates. However, in mass spectrometry analysis of monoclonal antibodies, a large number of charge

states is present, resulting in the formation of a charge distribution. This leads to a signal intensity distributed over many charge states. Hence, sensitivity decreases. To overcome this challenge, the monoclonal antibody can be digested by enzymes such as trypsin to form peptides, that represent smaller fragments of the monoclonal antibody.

Quantification can then be achieved indirectly using specific peptides referred to as marker or signature peptides. This process is illustrated in Figure 1-3. The advantage of this approach is that peptides do not have a broad charge distribution and can therefore be quantified sensitively [85-93].

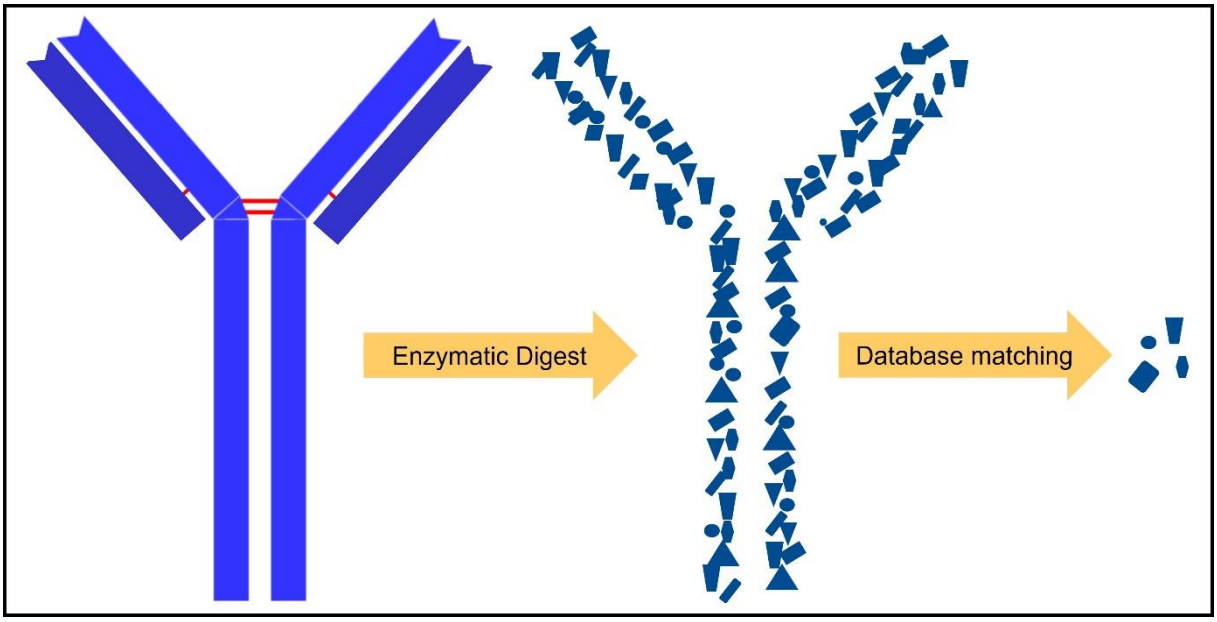


Figure 1-3: For indirect quantification of monoclonal antibodies, they are digested with an enzyme such as trypsin, forming peptides. A specific peptide can then be analysed as a proxy for the monoclonal antibody. To determine a specific peptide, the monoclonal antibody is digested in silico and the peptides formed are compared with a database. Specific peptides are characterised by the fact that they do not occur in the database.

However, the required enzymatic digest for sample preparation is time-consuming and takes up to 24 hours, depending on the protocol. Since the digestion is carried out in solution, the amount of enzyme cannot be increased to reduce the required duration, as increased autodigestion may occur. Nevertheless, when the enzyme is immobilised on a solid phase, the autolysis process can be prevented, since a collision of enzymes with each other is excluded. In this way, it is possible to use several milligrams of enzyme instead of only a few micrograms compared to digestion in solution. In this way, a digestion time of 10 minutes was achieved for myoglobin, for example [94-98]. It is therefore desirable to extend approaches from immobilised enzymes to monoclonal antibodies and to couple these online with a mass spectrometer.

1.9 References

1. Falzone L, Salomone S, Libra M (2018) Evolution of Cancer Pharmacological Treatments at the Turn of the Third Millennium. Front Pharmacol 9:1300. doi:10.3389/fphar.2018.01300

2. Arnold-Forster A (2016) A pathology of progress? Locating the historiography of cancer. Br J Hist Sci 49 (4):627-634. doi:10.1017/S0007087416001175

3. Spiegel D (1953) Die Krankheit der Epoche. https://www.spiegel.de/politik/die-krankheit-der-epoche-a-8621cce4-0002-0001-0000-000025656655. Accessed 20.01.2023

4. Newell H, Sausville E (2016) Cytotoxic drugs: past, present and future. Cancer Chemother Pharmacol 77 (1):1. doi:10.1007/s00280-015-2917-2

5. Breasted JH (1930) The Edwin Smith surgical papyrus. Chicago, Ill. : The University of Chicago Press, 1930.,

6. Rothschild BM, Tanke DH, Helbling M, 2nd, Martin LD (2003) Epidemiologic study of tumors in dinosaurs. Naturwissenschaften 90 (11):495-500. doi:10.1007/s00114-003-0473-9

7. Dumbrava MD, Rothschild BM, Weishampel DB, Csiki-Sava Z, Andrei RA, Acheson KA, Codrea VA (2016) A dinosaurian facial deformity and the first occurrence of ameloblastoma in the fossil record. Sci Rep 6:29271. doi:10.1038/srep29271

8. Englemann (1875) Ebers Papyrus.

9. Bryan CP, Smith GE (1930) The Papyrus Ebers.

10. Kelly J, Mahalingam S (2015) Surgical treatment of head and neck cancers in the ancient world. J Laryngol Otol 129 (6):535-539. doi:10.1017/S0022215115001218

11. Karpozilos A, Pavlidis N (2004) The treatment of cancer in Greek antiquity. Eur J Cancer 40 (14):2033-2040. doi:10.1016/j.ejca.2004.04.036

12. Tsoucalas G, Sgantzos M (2016) Hippocrates (ca 460-370 BC) on nasal cancer. Journal of BUON : official journal of the Balkan Union of Oncology 21:1031-1034

13. Grubbe EH (1933) Priority in the Therapeutic Use of X-rays. Radiology 21:156-162

14. Nakayama DK, Bonasso PC (2016) The History of Multimodal Treatment of Wilms' Tumor. Am Surg 82 (6):487-492

15. Brookes P (1990) The early history of the biological alkylating agents, 1918-1968. Mutat Res 233 (1-2):3-14. doi:10.1016/0027-5107(90)90145-t

16. Kieler J (1951) Nitrogen mustard therapy in Hodgkin's disease, lympho-reticulosarcomatosis and lymphatic leukaemia. Acta radiol 36 (6):461-468. doi:10.3109/00016925109176998

17. Goodwin WE, Mims MM, Young HH, 2nd (1967) Rhabdomyosarcoma of the prostate in a child; first 5-year survival. (Combined treatment by preoperative, local irradiation; actinomycin D; intra-arterial nitrogen mustard and hypothermia; radical surgery and ureterosigmoidostomy). Trans Am Assoc Genitourin Surg 59:186-191

18. Lane M (1959) Some effects of cyclophosphamide (cytoxan) on normal mice and mice with L1210 leukemia. J Natl Cancer Inst 23:1347-1359

19. Emadi A, Jones RJ, Brodsky RA (2009) Cyclophosphamide and cancer: golden anniversary. Nat Rev Clin Oncol 6 (11):638-647. doi:10.1038/nrclinonc.2009.146

20. Brummaier T, Pohanka E, Studnicka-Benke A, Pieringer H (2013) Using cyclophosphamide in inflammatory rheumatic diseases. Eur J Intern Med 24 (7):590-596. doi:10.1016/j.ejim.2013.02.008

21. Kim J, Chan JJ (2017) Cyclophosphamide in dermatology. Australas J Dermatol 58 (1):5-17. doi:10.1111/ajd.12406

22. Tsimberidou AM (2015) Targeted therapy in cancer. Cancer Chemother Pharmacol 76 (6):1113-1132. doi:10.1007/s00280-015-2861-1

23. Kohler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256 (5517):495-497. doi:10.1038/256495a0

24. Nadler LM, Stashenko P, Hardy R, Kaplan WD, Button LN, Kufe DW, Antman KH, Schlossman SF (1980) Serotherapy of a patient with a monoclonal antibody directed against a human lymphoma-associated antigen. Cancer Res 40 (9):3147-3154

25. Miller RA, Maloney DG, Warnke R, Levy R (1982) Treatment of B-cell lymphoma with monoclonal anti-idiotype antibody. N Engl J Med 306 (9):517-522. doi:10.1056/NEJM198203043060906

26. Dillman RO, Beauregard JC, Halpern SE, Clutter M (1986) Toxicities and side effects associated with intravenous infusions of murine monoclonal antibodies. J Biol Response Mod 5 (1):73-84

27. Kornbrot B, Kobayashi RH, Singer A, Tempero MA, Heiner DC (1994) Hypersensitivity to therapeutic murine monoclonal antibodies. Nebr Med J 79 (12):393-398

28. Kaplon H, Chenoweth A, Crescioli S, Reichert JM (2022) Antibodies to watch in 2022. MAbs 14 (1):2014296. doi:10.1080/19420862.2021.2014296

29. Maucher IV (2022) Zytostatika. https://www.gelbe-liste.de/wirkstoffgruppen/zytostatika#:~:text=Aktuell%20gibt%20es%20rund%2050,der%20spezifischen%20Krebsart%20zu%20finden. Accessed 20.01.2023

30. Sessink PJM, Sewell G, Vandenbroucke J Preventing occupational exposure to cytotoxic and other hazardous drugs.

31. ISOPP Standards for the Safe Handling of Cytotoxics (2022). J Oncol Pharm Pract 28 (3_suppl):S1-S126. doi:10.1177/10781552211070933

32. Alexander M, King J, Bajel A, Doecke C, Fox P, Lingaratnam S, Mellor JD, Nicholson L, Roos I, Saunders T, Wilkes J, Zielinski R, Byrne J, MacMillan K, Mollo A, Kirsa S, Green M (2014) Australian consensus guidelines for the safe handling of monoclonal antibodies for cancer treatment by healthcare personnel. Intern Med J 44 (10):1018-1026. doi:10.1111/imj.12564

33. King J, Alexander M, Byrne J, MacMillan K, Mollo A, Kirsa S, Green M (2016) A review of the evidence for occupational exposure risks to novel anticancer agents - A focus on monoclonal antibodies. J Oncol Pharm Pract 22 (1):121-134. doi:10.1177/1078155214550729

34. Halsen G, Kramer I (2011) Assessing the risk to health care staff from long-term exposure to anticancer drugs--the case of monoclonal antibodies. J Oncol Pharm Pract 17 (1):68-80. doi:10.1177/1078155210376847

35. Langford SA, Fradgley S, Evans M, Blanks C Assessing the risk of handling monoclonal antibodies. In, 2008.

36. Bauters T, Vandenbroucke J (2019) Development of a flowchart for risk assessment and allocation of preparation of monoclonal antibodies. J Oncol Pharm Pract 25 (1):187-191. doi:10.1177/1078155217743095

37. Barrott L, Foreman E (2020) Staff sensitisation to therapeutic monoclonal antibodies resulting from workplace exposure: A case report. J Oncol Pharm Pract 26 (5):1282-1284. doi:10.1177/1078155220901750

38. Lagarce F (2017) Centrally Prepared Cytotoxic Drugs: What Is the Purpose of Their Quality Control? Pharmaceutical Technology in Hospital Pharmacy 2 (1):29-33. doi:doi:10.1515/pthp-2017-0006

39. Sendra-Garcia A, Martinez-Gomez MA, Albert-Mari A, Jimenez-Torres NV, Climente-Marti M (2019) Quantitative and qualitative control of antineoplastic preparations: Gravimetry versus HPLC. J Oncol Pharm Pract 25 (5):1204-1216. doi:10.1177/1078155219834999

40. Jochums M, Reinders LMH, Tuerk J, Teutenberg T (2022) Flexible Digitization of Highly Individualized Workflows Demonstrated Through the Quality Control of Patient-Specific Cytostatic Application Bags: Digitization from the Perspective of Small and Medium-Sized Laboratories. Adv Biochem Eng Biotechnol 182:115-129. doi:10.1007/10_2021_190

41. Friese CR, McArdle C, Zhao T, Sun D, Spasojevic I, Polovich M, McCullagh MC (2015) Antineoplastic Drug Exposure in an Ambulatory Setting: A Pilot Study. Cancer Nursing 38 (2):111-117. doi:10.1097/ncc.0000000000000143

42. Kiffmeyer T, Kube C, Opiolka S, Schmidt K, Sessink P (2002) Vapour pressures, evaporation behaviour and airborne concentrations of hazardous drugs: Implications for occupational safety. Pharm J 268

43. Connor TH, Shults M, Fraser MP (2000) Determination of the vaporization of solutions of mutagenic antineoplastic agents at 23 and 37 degrees C using a desiccator technique. Mutat Res 470 (1):85-92. doi:10.1016/s1383-5718(00)00105-4

44. Sessink PJM, Boer KA, Scheefhals APH, Anzion RBM, Bos RP (1992) Occupational Exposure to Antineoplastic Agents at Several Departments in a Hospital - Environmental Contamination and Excretion of Cyclophosphamide and Ifosfamide in Urine of Exposed Workers. Int Arch Occ Env Hea 64 (2):105-112. doi:Doi 10.1007/Bf00381477

45. Nygren O, Gustavsson B, Ström L, Friberg A (2002) Cisplatin contamination observed on the outside of drug vials. Ann Occup Hyg 46 (6):555-557. doi:10.1093/annhyg/mef074

46. Favier B, Gilles L, Ardiet C, Latour JF (2003) External contamination of vials containing cytotoxic agents supplied by pharmaceutical manufacturers. Journal of Oncology Pharmacy Practice 9 (1):15-20. doi:10.1191/1078155203jp102oa

47. Connor TH, Sessink PJM, Harrison BR, Pretty JR, Peters BG, Alfaro RM, Bilos A, Beckmann G, Bing MR, Anderson LM, Dechristoforo R (2005) Surface contamination of chemotherapy drug vials and evaluation of new vial-cleaning techniques: results of three studies. American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists 62 5:475-484

48. McDevitt JJ, Lees PS, McDiarmid MA (1993) Exposure of hospital pharmacists and nurses to antineoplastic agents. J Occup Med 35 (1):57-60

49. Minoia C, Turci R, Sottani C, Schiavi A, Perbellini L, Angeleri S, Draicchio F, Apostoli P (1998) Application of high performance liquid chromatography/tandem mass spectrometry in the environmental and biological monitoring of health care personnel occupationally exposed to cyclophosphamide and ifosfamide. Rapid Commun Mass Spectrom 12 (20):1485-1493. doi:10.1002/(sici)1097-0231(19981030)12:20<1485::Aid-rcm333>3.0.Co;2-n

50. Schmaus G, Schierl R, Funck S (2002) Monitoring surface contamination by antineoplastic drugs using gas chromatography-mass spectrometry and voltammetry. Am J Health Syst Pharm 59 (10):956-961. doi:10.1093/ajhp/59.10.956

51. Baker ES, Connor TH (1996) Monitoring occupational exposure to cancer chemotherapy drugs. Am J Health Syst Pharm 53 (22):2713-2723. doi:10.1093/ajhp/53.22.2713

52. Muller T (2003) Typical medication errors in oncology: analysis and prevention strategies. Onkologie 26 (6):539-544. doi:10.1159/000074148

53. Lipczak H, Knudsen JL, Nissen A (2011) Safety hazards in cancer care: findings using three different methods. BMJ Qual Saf 20 (12):1052-1056. doi:10.1136/bmjqs.2010.050856

54. Car LT, Papachristou N, Urch C, Majeed A, Atun R, Car J, Vincent C (2017) Prioritizing medication safety in care of people with cancer: clinicians' views on main problems and solutions. J Glob Health 7 (1):011001. doi:10.7189/jogh.07.011001

55. Gilbar PJ (2014) Intrathecal chemotherapy: potential for medication error. Cancer Nurs 37 (4):299-309. doi:10.1097/ncc.0000000000000108

56. Letarte N, Gabay MP, Bressler LR, Long KE, Stachnik JM, Villano JL (2014) Analyzing temozolomide medication errors: potentially fatal. J Neuro-Oncol 120 (1):111-115. doi:10.1007/s11060-014-1523-1

57. Lecordier J, Heluin Y, Plivard C, Bureau A, Mouawad C, Chaillot B, Lahet JJ (2011) Safety in the preparation of cytotoxic drugs: How to integrate gravimetric control in the quality assurance policy? Biomed Pharmacother 65 (1):17-21. doi:10.1016/j.biopha.2010.12.014

58. Lebel D, Thibault M, Bussières JF (2010) Asynchronous validation and documentation of sterile compounding in a hospital pharmacy. Can J Hosp Pharm 63 (4):323-327. doi:10.4212/cjhp.v63i4.937

59. Benizri F, Dalifard B, Zemmour C, Henriquet M, Fougereau E, Le Franc B (2016) DrugCam®-An intelligent video camera system to make safe cytotoxic drug preparations. Int J Pharm 502 (1-2):198-207. doi:10.1016/j.ijpharm.2016.02.028

60. Beschluss des Bundesgerichtshofs 4 StR 503/19 vom 10. Juni 2020.

61. Deutsche Apotheker Zeitung (2018) Der Fall Bottrop – ein Albtraum. https://www.deutsche-apotheker-zeitung.de/daz-az/2018/daz-13-2018/der-fall-bottrop-ein-albtraum. Accessed 20.01.2023

62. Ministerium für Arbeit, Gesundheit und Soziales des Landes Nordrhein-Westfalen (2018) Ergebnisse von Apothekenkontrollen liegen vor. https://www.mags.nrw/pressemitteilung/ergebnisse-von-apothekenkontrollen-liegen-vor. Accessed 20.01.2023

63. Europäisches Arzneibuch 2.9.17 Bestimmung des entnehmbaren Volumens von Parenteralia.

64. Europäisches Arzneibuch Version 10

65. Fekete S, Veuthey JL, Beck A, Guillarme D (2016) Hydrophobic interaction chromatography for the characterization of monoclonal antibodies and related products. J Pharm Biomed Anal 130:3-18. doi:10.1016/j.jpba.2016.04.004

66. Bazin C, Cassard B, Caudron E, Prognon P, Havard L (2015) Comparative analysis of methods for real-time analytical control of chemotherapies preparations. Int J Pharm 494 (1):329-336. doi:10.1016/j.ijpharm.2015.08.041

67. Jaccoulet E, Smadja C, Prognon P, Taverna M (2015) Capillary electrophoresis for rapid identification of monoclonal antibodies for routine application in hospital. Electrophoresis 36 (17):2050-2056. doi:10.1002/elps.201400603

68. Ladwig PM, Barnidge DR, Willrich MAV (2017) Mass Spectrometry Approaches for Identification and Quantitation of Therapeutic Monoclonal Antibodies in the Clinical Laboratory. Clin Vaccine Immunol 24 (5). doi:10.1128/CVI.00545-16

69. Le Basle Y, Chennell P, Tokhadze N, Astier A, Sautou V (2020) Physicochemical Stability of Monoclonal Antibodies: A Review. J Pharm Sci 109 (1):169-190. doi:10.1016/j.xphs.2019.08.009

70. Bansal R, Dash R, Rathore AS (2020) Impact of mAb Aggregation on Its Biological Activity: Rituximab as a Case Study. J Pharm Sci 109 (9):2684-2698. doi:10.1016/j.xphs.2020.05.015

71. Atis C, Jukka K, Egbert M, Toru T, Kazuaki M (2020) Analytical Characterization of Monoclonal Antibodies with Novel Fc Receptor-Based Chromatography Technique. In: Nima R (ed) Monoclonal Antibodies. IntechOpen, Rijeka, p Ch. 4. doi:10.5772/intechopen.95356

72. Gstottner C, Klemm D, Haberger M, Bathke A, Wegele H, Bell C, Kopf R (2018) Fast and Automated Characterization of Antibody Variants with 4D HPLC/MS. Anal Chem 90 (3):2119-2125. doi:10.1021/acs.analchem.7b04372

73. Camperi J, Dai L, Guillarme D, Stella C (2020) Fast and Automated Characterization of Monoclonal Antibody Minor Variants from Cell Cultures by Combined Protein-A and Multidimensional LC/MS Methodologies. Anal Chem 92 (12):8506-8513. doi:10.1021/acs.analchem.0c01250

74. Derenne A, Derfoufi KM, Cowper B, Delporte C, Goormaghtigh E (2020) FTIR spectroscopy as an analytical tool to compare glycosylation in therapeutic monoclonal antibodies. Anal Chim Acta 1112:62-71. doi:10.1016/j.aca.2020.03.038

75. Joshi V, Shivach T, Yadav N, Rathore AS (2014) Circular dichroism spectroscopy as a tool for monitoring aggregation in monoclonal antibody therapeutics. Anal Chem 86 (23):11606-11613. doi:10.1021/ac503140j

76. Beck A, Goetsch L, Dumontet C, Corvaia N (2017) Strategies and challenges for the next generation of antibody drug conjugates. Nature Reviews Drug Discovery 16 (5):315-337. doi:10.1038/nrd.2016.268

77. European Medicines Agency Biosimilar medicines: overview. https://www.ema.europa.eu/en/human-regulatory/overview/biosimilarmedicines-overview. Accessed 05.11.2022

78. Ferri N, Bellosta S, Baldessin L, Boccia D, Racagni G, Corsini A (2016) Pharmacokinetics interactions of monoclonal antibodies. Pharmacol Res 111:592-599. doi:10.1016/j.phrs.2016.07.015

79. Bos JD, Meinardi MM (2000) The 500 Dalton rule for the skin penetration of chemical compounds and drugs. Exp Dermatol 9 (3):165-169. doi:10.1034/j.1600-0625.2000.009003165.x

80. Engineers ISoP (2012) Assessing the particulate containment performance of pharmaceutical equipment.

81. Liu XF, Wang X, Weaver RJ, Calliste L, Xia C, He YJ, Chen L (2012) Validation of a Gyrolab assay for quantification of rituximab in human serum. J Pharmacol Toxicol Methods 65 (3):107-114. doi:10.1016/j.vascn.2012.03.001

82. Carter PJ, Lazar GA (2018) Next generation antibody drugs: pursuit of the 'high-hanging fruit'. Nat Rev Drug Discov 17 (3):197-223. doi:10.1038/nrd.2017.227

83. Respaud R, Vecellio L, Diot P, Heuze-Vourc'h N (2015) Nebulization as a delivery method for mAbs in respiratory diseases. Expert Opin Drug Deliv 12 (6):1027-1039. doi:10.1517/17425247.2015.999039

84. Fellner RC, Terryah ST, Tarran R (2016) Inhaled protein/peptide-based therapies for respiratory disease. Mol Cell Pediatr 3 (1):16. doi:10.1186/s40348-016-0044-8

85. Zhang H, Cui W, Gross ML (2014) Mass spectrometry for the biophysical characterization of therapeutic monoclonal antibodies. FEBS Lett 588 (2):308-317. doi:10.1016/j.febslet.2013.11.027

86. Rosati S, Yang Y, Barendregt A, Heck AJ (2014) Detailed mass analysis of structural heterogeneity in monoclonal antibodies using native mass spectrometry. Nat Protoc 9 (4):967-976. doi:10.1038/nprot.2014.057

87. Heudi O, Barteau S, Zimmer D, Schmidt J, Bill K, Lehmann N, Bauer C, Kretz O (2008) Towards absolute quantification of therapeutic monoclonal antibody in serum by LC-MS/MS using isotope-labeled antibody standard and protein cleavage isotope dilution mass spectrometry. Anal Chem 80 (11):4200-4207. doi:10.1021/ac800205s

88. Hagman C, Ricke D, Ewert S, Bek S, Falchetto R, Bitsch F (2008) Absolute quantification of monoclonal antibodies in biofluids by liquid chromatography-tandem mass spectrometry. Anal Chem 80 (4):1290-1296. doi:10.1021/ac702115b

89. Bee JS, Davis M, Freund E, Carpenter JF, Randolph TW (2010) Aggregation of a monoclonal antibody induced by adsorption to stainless steel. Biotechnol Bioeng 105 (1):121-129. doi:10.1002/bit.22525

90. Putz S, Reinders J, Reinders Y, Sickmann A (2005) Mass spectrometry-based peptide quantification: applications and limitations. Expert Rev Proteomics 2 (3):381-392. doi:10.1586/14789450.2.3.381

91. John H, Walden M, Schafer S, Genz S, Forssmann WG (2004) Analytical procedures for quantification of peptides in pharmaceutical research by liquid chromatography-mass spectrometry. Anal Bioanal Chem 378 (4):883-897. doi:10.1007/s00216-003-2298-y

92. Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B (2007) Quantitative mass spectrometry in proteomics: a critical review. Anal Bioanal Chem 389 (4):1017-1031. doi:10.1007/s00216-007-1486-6

93. Hoofnagle AN, Whiteaker JR, Carr SA, Kuhn E, Liu T, Massoni SA, Thomas SN, Townsend RR, Zimmerman LJ, Boja E, Chen J, Crimmins DL, Davies SR, Gao Y, Hiltke TR, Ketchum KA, Kinsinger CR, Mesri M, Meyer MR, Qian WJ, Schoenherr RM, Scott MG, Shi T, Whiteley GR, Wrobel JA, Wu C, Ackermann BL, Aebersold R, Barnidge DR, Bunk DM, Clarke N, Fishman JB, Grant RP, Kusebauch U, Kushnir MM, Lowenthal MS, Moritz RL, Neubert H, Patterson SD, Rockwood AL, Rogers J, Singh RJ, Van Eyk JE, Wong SH, Zhang S, Chan DW, Chen X, Ellis MJ, Liebler DC, Rodland KD, Rodriguez H, Smith RD, Zhang Z, Zhang H, Paulovich AG (2016) Recommendations for the Generation, Quantification, Storage, and Handling of Peptides Used for Mass Spectrometry-Based Assays. Clin Chem 62 (1):48-69. doi:10.1373/clinchem.2015.250563

94. Ma J, Zhang L, Liang Z, Zhang W, Zhang Y (2007) Monolith-based immobilized enzyme reactors: recent developments and applications for proteome analysis. J Sep Sci 30 (17):3050-3059. doi:10.1002/jssc.200700362

95. Slechtova T, Gilar M, Kalikova K, Moore SM, Jorgenson JW, Tesarova E (2017) Performance comparison of three trypsin columns used in liquid chromatography. J Chromatogr A 1490:126-132. doi:10.1016/j.chroma.2017.02.024

96. Moore S, Hess S, Jorgenson J (2016) Characterization of an immobilized enzyme reactor for on-line protein digestion. J Chromatogr A 1476:1-8. doi:10.1016/j.chroma.2016.11.021

97. Safdar M, Spross J, Janis J (2014) Microscale immobilized enzyme reactors in proteomics: latest developments. J Chromatogr A 1324:1-10. doi:10.1016/j.chroma.2013.11.045

98. Freije JR, Mulder PP, Werkman W, Rieux L, Niederlander HA, Verpoorte E, Bischoff R (2005) Chemically modified, immobilized trypsin reactor with improved digestion efficiency. J Proteome Res 4 (5):1805-1813. doi:10.1021/pr050142y

Chapter 2 Aims and Scope

Since the first therapeutic monoclonal antibody with a cancer indication was approved in 1997, the market share of this substance class has increased significantly. Today, they are an important component in many therapeutic regimens.

Nevertheless, there are still many unanswered questions regarding handling, processing and application to the patient, which can only be answered with the assistance of instrumental analytics. The aim of this work was to develop methods to collect data that answer critical questions in the area of patient safety and occupational health. The interaction of both aspects can be seen in Figure 2-1 (Blue).

In the area of patient safety, it is of utmost importance to ensure that the patient receives the dose of medication that has been prescribed. However, as these are aseptic personalised medicines, not every production can be analysed. Therefore, a sampling and analysis strategy needed to be developed that not only minimises measurement uncertainty, but also takes into account occupational health and safety. The final methods for cytostatic drugs and monoclonal antibodies were applied to real samples in order to evaluate the preparation quality (Chapters 3 and 4; Figure 2-1, Brown).

Based thereon, monoclonal antibodies as drug agents were characterised in greater depth in Chapters 5 and 6 (Figure 2-1, Green). This is of particular importance for evaluating the quality of biosimilars. Since monoclonal antibodies are very complex molecules, affinity chromatography, high-resolution mass spectrometry and multidimensional chromatography with various separation mechanisms were required.

During the production, application or analysis of monoclonal antibodies, an unintentional release may occur. For occupational safety, airborne monoclonal antibodies are a particular challenge. Therefore, a method was developed to determine the release during routine work. In addition, measures to reduce the release potential were evaluated to derive a recommendation for action (Chapter 7; Figure 2-1, Purple).

In order to detect the low concentrations in occupational safety and health, the analytical methods are based on indirect quantification at the peptide level. For this purpose, an enzymatic digestion is required. This is time-consuming and carries the risk of release when handling the monoclonal antibodies. The enzymatic digestion process was therefore automated in chapter 8 and coupled online with the mass spectrometric analysis (Figure 2-1, Purple).

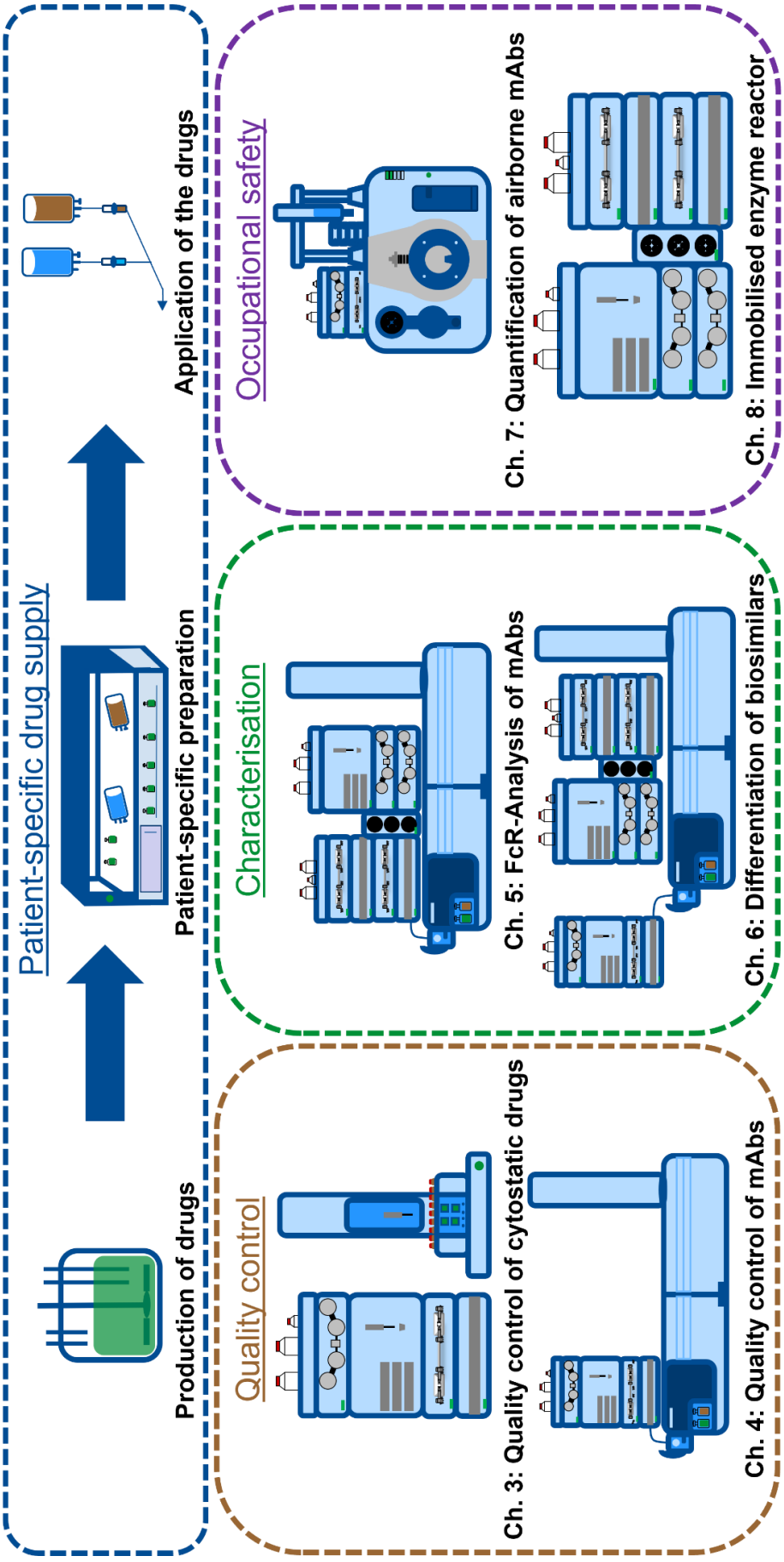


Figure 2-1: Graphical overview of the thesis scope.

Chapter 3 Quality control of cytostatic drug preparations - Comparison of workflow and performance of Raman/UV and high performance liquid chromatography coupled with diode array detection (HPLC-DAD)

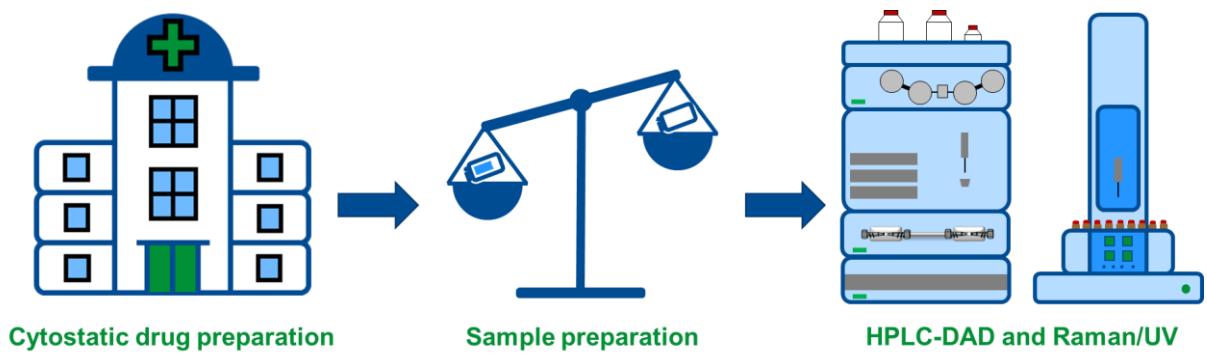
This chapter was adapted from: Reinders, L.M.H., Klassen, M.D., vom Eyser, C., Teutenberg, T., Jaeger, M., Schmidt, T.C., Tuerk, T., Quality control of cytostatic drug preparations—comparison of workflow and performance of Raman/UV and high-performance liquid chromatography coupled with diode array detection (HPLC-DAD). Anal Bioanal Chem 413, 2587–2596 (2021). https://doi.org/10.1007/s00216-021-03223-9

Abstract: The drugs used for treatment during chemotherapy are manufactured individually for each patient in specialised pharmacies. Thorough quality control to confirm the identity of the delivered active pharmaceutical ingredient and the final concentration of the prepared application solution is not standardized yet except for optical or gravimetric testing. However, solution stability problems, counterfeit drugs, and erroneous or deliberate underdosage may occur and negatively influence the quality of the product and could cause severe health risks for the patient. To take a step towards analytical quality control, an on-site analytical instrument using Raman and UV absorption spectroscopy was employed and the results were compared to high-performance liquid chromatography coupled to diode array detection.

Within the scope of the technology evaluation, the uncertainty of measurement was determined for the analysis of the five frequently used cytostatic drugs 5-fluorouracil, cyclophosphamide, gemcitabine, irinotecan and paclitaxel. The Raman/UV technique (2.0-3.2% uncertainty of measurement; Level of confidence: 95%) achieves a combined uncertainty of measurement comparable to HPLC-DAD (1.7-3.2% uncertainty of measurement; Level of confidence: 95%) for the substances 5-fluorouracil, cyclophosphamide and gemcitabine. However, the uncertainty of measurement for the substances irinotecan and paclitaxel is three times higher when the Raman/UV technique is used. This is due to the fact that the Raman/UV technique analyses the undiluted sample, therefore the sample has a higher viscosity and tendency to foam.

Out of 136 patient-specific preparations analysed within this study, 96% had a deviation of less than 10% from the target content.

Graphical Abstract:



3.1 Introduction

Modern cancer therapy makes use of the personalized medicine concept, i.e. the treatment is adapted to the individual patient. The prescribed drug products are prepared in special pharmacies as are found in hospitals. For application purposes, the pharmacist processes the drug substance provided by the pharmaceutical industry. In general, a dissolution is the major preparation step. However, errors or fraud can occur during formulation. Typical unintentional preparation errors comprise the erroneous exchange of single and total dose as well as the miscalculation of dilution factors. Fraudulent activities may include the processing of counterfeit drug substance and deliberate underdosing. While, overdosing may cause severe side effects, underdosage endangers the success of the therapy [1,2].

As manifold the reasons for medication errors are, as numerous are the interventions taken to prevent them. In general, volumetric control is exercised by the four-eye principle for the preparation of cytostatic drug solutions. This is carried out either through a second person or video support [3]. Further control techniques in use are gravimetric and instrumental analysis of the final drug product. In the field of instrumental quality control, combinations of Raman spectroscopy and UV-absorption (Raman/UV), as well as hyphenation of high-performance liquid chromatography and diode array detection (HPLC-DAD) are most frequently used. Other common methods include high-performance thin-layer chromatography, capillary electrophoresis, infrared spectroscopy, flow injection analysis coupled with a DAD or UV analysis without chromatographic separation methods [4-10, 23, 25]. Furthermore, there are approaches to perform the analysis non-invasively through the sample container using near-infrared or Raman spectroscopy [11,12].

Quality control using HPLC-DAD offers many advantages. With this technique it is possible to separate the formulation substances from the active pharmaceutical ingredient (API). The separation minimizes the influence of the formulation substances on the analytical result. Furthermore, the specific UV detection allows the use of only one calibration assay for each API, independent of the manufacturer and the formulation substances used. In addition to quantification, substance identity can be confirmed via the whole UV spectrum and retention time. Yet, some substances have indistinguishable UV spectra such as cyclophosphamide (CP) and ifosfamide (IF). In such cases, a good chromatographic separation together with a highly precisely measured retention time is necessary. Nevertheless, the development and validation of an HPLC-DAD method can be challenging and requires highly qualified personnel.

In contrast to the highly versatile HPLC-DAD instruments, special Raman/UV spectrometers for the quality control of patient-specific preparations are available. These devices do not use chromatography, which means that formulation substances are not separated. This can lead to different calibrations being required for the same API. However, no special analytical expertise is required from the user. Due to their ease of operation and short analysis times in the range of a few minutes, these combined Raman scattered light and adsorption measurement instruments are suitable for on-site use in pharmacies or hospitals as a real-time quality control system.

Independent of the chosen analytical method, it is a prerequisite to define suitable quality criteria such as API identity and amount or maximum allowed concentration of impurities. In terms of the deviation of the drug amount from the target value, the European Pharmacopoeia (Ph. Eur.) does not specify exact limits [13]. The United States Pharmacopeia (USP) and Pharmacopoea Helvetica (Ph. Helv.) allow deviations of up to 10%, unless otherwise defined in the respective monograph [14,15]. In Germany, the decision is a federal state issue. In a request to the state of North Rhine-Westphalia in Germany, the Ministry of Labour, Health and Social Affairs of the state of North Rhine-Westphalia (MAGS) explained the composition of the tolerated deviation of up to 10% for patient-specific parenteralia, which are prepared from drugs [16]. It was pointed out that the drugs used to produce the patient-specific preparations might have a deviation in concentration of up to 5% from the target value [17]. Furthermore, a manufacturing accuracy of 5% was assumed, providing that the pharmaceutical rules for the quality of medicinal products are respected, so it would add up to a permitted deviation of up to 10% [16]. A uniform German regulation does not yet exist, which is why other federal states may deviate from it.

In this study HPLC-DAD and Raman/UV analysis methods for identification and quantification of the commonly used cytostatic drugs 5-fluorouracil (5-FU), cyclophosphamide (CP), gemcitabine (Gem), irinotecan (Irino) and paclitaxel (Pac) in patient-individual application preparations were developed and validated. Subsequently, both analytical techniques were compared in terms of measurement uncertainty and compound identification. In addition, 136 patient-specific preparations were analysed and the quality evaluated. In order to evaluate the quality, the influence of sample preparation was examined. Of course, the influence of sample stability was also evaluated. In this context the maximum tolerated deviation of 10% is discussed. The aim of these investigations was to examine the currently prevailing manufacturing quality and to evaluate whether an analytical on-site control would be a useful supplement to current practice.

3.2 Materials and Methods

3.2.1 Chemicals and reagents

The cytostatic drugs 5-FU, Irino and Pac were purchased as 5-FU medac 5000 mg, Irinomedac 100 mg and Taxomedac 100 mg from Medac GmbH (Wedel, Germany). CP was purchased as Endoxan (500 mg) from Baxter (Herford, Germany). Gem was acquired as Gemcitabin Hexal (2000 mg) from Hexal AG (Holzkirchen, Germany). A list of all formulation substances contained in the drugs can be found in the supplementary information. Th. Geyer GmbH & Co. KG (Renningen, Germany) supplied acetonitrile and water with the purity LC-MS grade (Chemsolute). Formic acid (FA; HiPerSolv CHROMANORM) was purchased from VWR (Radnor, USA) and 0.9% sodium chloride solution from B. Braun (Melsungen, Germany). A consumables set for the Raman/UV system contained the rinsing, hellmanex®, reference (mix of caffeine and isopropanol) and background (TritonTM X-100) solution and was obtained from Icônes Services, Sucé-sur-Erdre, France. All drugs, chemicals and reagents were stored according to the recommendations of the manufacturer.

3.2.2 Gravimetric determination of the amount of liquid in preparations

The following procedure was applied to all samples and is called gravimetric correction in the main text. Therefore, the application bag including application equipment was weighed with a Sartorius MSE4202S (Göttingen, Germany), emptied and weighed again. By calculating the difference between the weight of the full and the empty bag, the weight of the solution was obtained. The weight of the solution was then multiplied by its temperature corrected density to calculate the actual liquid volume.

All data to determine the errors for the methods “no volumetric correction” and for the use of a “correction factor” were collected according to the following workflow. Ten FreeFlex (LOT: 14MM7336 EXP: November 2021) application bags (Fresenius Kabi Deutschland, Bad Homburg vor der Höhe, Germany) pre-filled with 0.9% NaCl solution were weighed separately. The bags were emptied, rinsed with water, dried in the air for 48 h and weighed again. Further data are compiled in Table 3-6.

3.2.3 Determination of the uncertainty of measurement

The uncertainty of measurement was calculated as the root of the sum of the squared errors. The same approach was chosen for the combined uncertainty of measurement. The calculation of the extended combined uncertainty of measurement was performed with a confidence level of 95%, which means that the combined uncertainty of measurement was multiplied by a factor of 1.96 [18].

3.2.4 Raman/UV

The Raman/UV analyses were performed, inside a lab room, using an i-QCRx system (B&W Tek Europe GmbH, Lübeck, Germany). This system consisted of an autosampler, fluidic control and main detection module and was controlled by i-QCRx Analysis software package Version 4.0 - Revision 0. The settings were set to default. An identification library was created with QL_Analyst Version 4.0 - Revision 0. The library contained spectra of the five cytostatic compounds 5-FU, CP, Gem, Irino and Pac at ten different concentration levels. The ten calibration points were distributed equidistantly over the calibration range. The calibration ranges for the individual substances are: 1.0-20 mg mL^{-1} (5-FU), 0.50-10 mg mL^{-1} (CP), 1.0-30 mg mL^{-1} (Gem), 0.20-2.0 mg mL^{-1} (Irino) and 0.10-1.2 mg mL^{-1} (Pac). The dilutions were carried out with 0.9% NaCl solution. The wavelengths used for quantification were 319 nm (5-FU), 205 nm (CP), 302 nm (Gem), 267 nm (Irino) and 254 nm (Pac). An explanation of the selection of quantification wavelengths can be found in the supplementary information.

3.2.5 HPLC-DAD

The HPLC system was acquired from Agilent Technologies (Santa Clara, USA) and consisted of a 1200 series binary pump, column oven, DAD and 1260 Infinity II autosampler. The temperature of the autosampler is not controlled. However, the device is located in a room that has been thermostatted to 23°C. For instrument control, Chromeleon Version 6.80 SR13 Build 3967 (Dionex Corporation, Sunnyvale, USA part of Thermo Fisher Scientific, Waltham, USA) was used. The mobile phase was A: water + 0.1% FA and B: acetonitrile + 0.1% FA. Further method parameters are given in Table 3-1. All calibration points and quality control samples were prepared with 0.9% NaCl solution.

Table 3-1: Description of the method parameters for HPLC-DAD analysis. An explanation of the selection of quantification wavelengths can be found in the supplementary information.

Analyte	5-Fluorouracil		Cyclophosphamide		Gemcitabine		Irinotecan		Paclitaxel	
Column	Hypercarb 5 µm; 2.1 x 50 mm (Thermo Fisher Scientific, Waltham, USA)		ShimPack XR-ODS 2.2 µm; 3.0 x 50 mm (Shimadzu, Kyoto, Japan)		SunShell RP-Aqua 2.6 µm; 2.1 x 50 mm (dichrom GmbH, Germany)		ShimPack XR-ODS 2.2 µm; 3.0 x 50 mm (Shimadzu, Kyoto, Japan)		ShimPack XR-ODS 2.2 µm; 3.0 x 50 mm (Shimadzu, Kyoto, Japan)	
Column temperature (°C)	40		40		30		40		40	
Injection volume (µL)	2		10		2		9		5	
Flow rate (mL min^{-1})	0.5		0.4		0.2		0.5		0.3	
Gradient (min // %B)	0.0 – 1.0	5 – 95	0.0 – 3.0	20 – 90	0.0 – 2.0	5 – 5	0.0 – 2.8	5 – 95	0.0 – 0.5	30 – 95
	1.0 – 1.5	95 – 95	3.0 – 3.01	90 – 20			2.8 – 3.0	95 – 95	0.5 – 1.5	95 – 95
	1.5 – 1.6	95 – 5	3.01 – 5.0	20 - 20			3.0 – 3.01	95 – 5	1.5 – 3.0	95 – 30
	1.6 – 3.0	5 – 5					3.01 – 5.0	5 – 5	3.0 – 4.0	30 – 30
Wavelength (nm)	265		195		268		255		227	
Calibration range (mg mL^{-1})	0.1-0.9		0.1-0.9		0.1-0.9		0.001-0.050		0.001-0.050	

3.2.6 Determination of storage stability

For the determination of the storage stability, drug solutions with a content of the active pharmaceutical ingredient of 10 mg mL^{-1} for 5-fluorouracil, 2 mg mL^{-1} for cyclophosphamide, 6 mg mL^{-1} for gemcitabine, 1 mg mL^{-1} for irinotecan and 0.3 mg mL^{-1} for paclitaxel were prepared and aliquoted. The dilutions were prepared with 0.9% NaCl solution. To minimise the influence of atmospheric oxygen, the vessels were always sealed when stored. Each aliquot was stored in the dark at 6°C ± 2, at room temperature (22°C ± 3) and at 37 °C ± 1. For storage at 6°C ± 2, the refrigerator model KBS402U (KBS Gastrotechnik GmbH, Mainz) and for storage at 37°C ± 1, the heating cabinet IPP55 Plus (Memmert GmbH + Co. KG, Büchenbach) was used. The concentration of the API in the aliquots was determined in triplicate 0, 3, 7, 14, 21 and 42 days after preparation.

3.2.7 Patient-specific preparations

Within the scope of the study, a total of 136 patient-specific cytostatic preparations were analysed. These were prepared in 45 different pharmacies or hospitals located in Germany. All preparations were compounded in a medium of 0.9% NaCl solution. The samples analysed were returns. These are preparations which, for various reasons, could not be administered to the patient. The sampled pharmacies or hospitals therefore had no knowledge of which preparations were being analysed. The number of preparations, as well as the concentration range to be expected, is distributed among the individual substances as follows: 26x 5-FU (1.5-14.5 mg mL^{-1}), 33x CP (0.69-8.2 mg mL^{-1}), 17x Gem (2.8-7.2 mg mL^{-1}), 52x Pac (0.11-0.72 mg mL^{-1}), 8x Irino (0.27-0.64 mg mL^{-1}). The preparations were in application bags from different manufacturers, with the bag sizes distributed as follows: 6x 50 mL, 16x 100 mL, 69x 250 mL, 42x 500 mL, 3x 1000 mL. Samples were diluted to the calibration range in 0.9% NaCl solution prior to analysis by HPLC-DAD. A dilution step was not necessary for analysis with Raman/UV.

3.2.8 Validation study

As part of the validation, an intra-day assay, an inter-day assay, the carry-over and a linearity test were determined. For the HPLC-DAD measurements the calibration solutions described in chapter 3.2.5 were analysed. To determine the carry-over, a 0.9% NaCl solution was analysed after the highest calibration point. For the intra-day assay and inter-day assay, independent quality control (QC) samples were analysed. The QC samples had a concentration of 0.4 mg mL^{-1} for the intra-day assay for the substances 5-FU, CP and Gem, whereas they had a concentration of 0.015 mg mL^{-1} for the substances Irino and Pac. The concentrations for the inter-day assay were 0.5 mg mL^{-1} and 0.020 mg mL^{-1}, respectively. For the intra-day assay, the QC sample was analysed ten times on the same day and for the inter-day assay four times on each of three different days. The test for linearity was carried out according to Mandel, with a statistical safety of 95% [26]. The experimental procedure of the validation study for the Raman/UV instrument is identical to the HPLC-DAD, although the concentrations and number of replicate measurements differ. The respective calibration ranges can be found in chapter 3.2.4. The concentrations of the QC samples were identical for the inter-day assay and intra-day assay and were as follows: 10 mg mL^{-1} (5-FU), 5 mg mL^{-1} (CP), 2 mg mL^{-1} (Gem), 1 mg mL^{-1} (Irino) and 0.6 mg mL^{-1} (Pac). The number of replicate measurements in the intra-day assay is 4 (5-FU), 10 (CP), 4 (Gem), 9 (Irino) and 4 (Pac), respectively, whereas in the inter-day assay it is 12 (5-FU), 30 (CP), 11 (Gem), 17 (Irino) and 9 (Pac), respectively.

When samples were analysed later, a QC sample was also analysed using both techniques. A maximum deviation of 5% was defined for this QC sample.

3.3 General Considerations

Each analytical result is only an estimate of the true value since the analytical result is afflicted with an uncertainty. The factors influencing the uncertainty depend on the chosen workflow, but can be described and quantified by a random and systematic component. The task of the analyst is to identify and minimize these errors [18].

The main influencing parameters for quality control of patient-specific application solutions are shown in the Ishikawa diagram in Figure 3-1 and are related to organisational workflows and human issues, as well as technical issues of, e.g., materials, sample preparation and the chosen analysis technique.

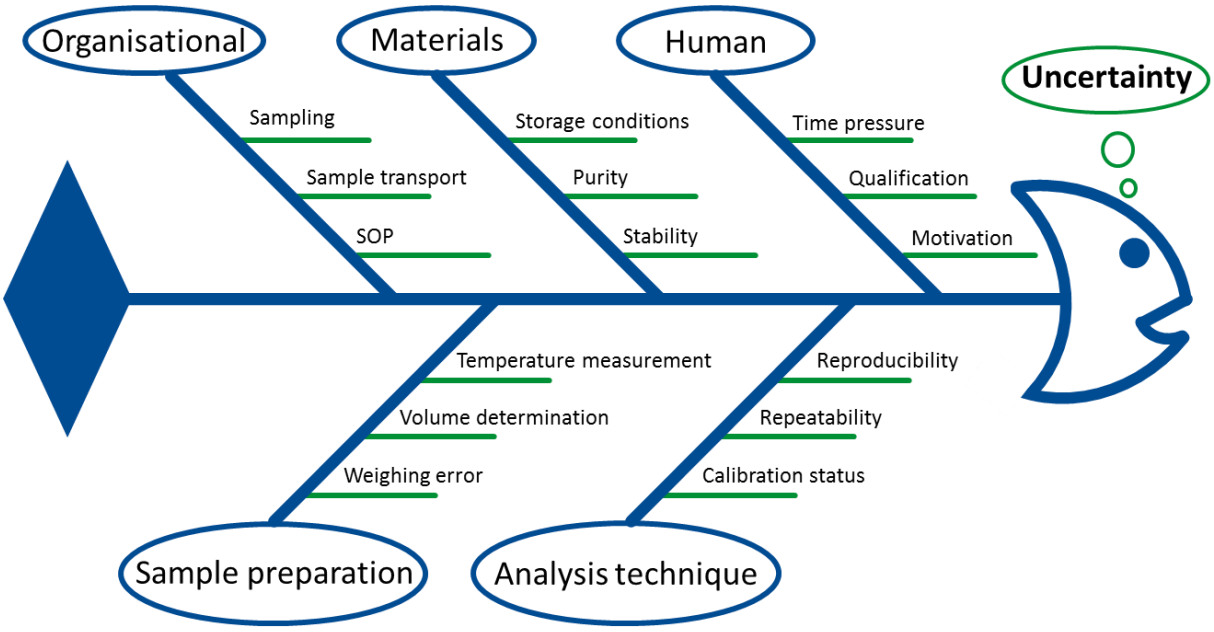


Figure 3-1: Ishikawa diagram for the visualisation of the main variables that exert an influence on the uncertainty of measurement of patient specific cytostatic drug application solutions. SOP: Standard operating procedure.

The workflow starts with the sampling, whereby no error occurs because the entire application bag is sent to the analysis laboratory. However, the stability of the sample must be known, otherwise degradation of the analyte may occur during transport. Since sample degradation is dependent on time and temperature, corresponding stability data must be obtained (See Chapter 3.4.1). Furthermore, the preparation must be checked on arrival for damage and whether light-sensitive substances have been transported darkened.

After arrival of the sample at the analysis laboratory, an appropriate sample preparation is carried out. As the analytical methods used measure the concentration of the active substance, but the relevant information is the amount of the active pharmaceutical ingredient, the volume of the application solution must be determined. The European Pharmacopoeia prescribes a method for the volumetric determination of patient-specific infusion solutions [19]. Here, the bag for the cytostatic drug preparation is to be opened and the volume of the liquid is transferred completely into a measuring cylinder.

However, this method has two major disadvantages. Firstly, the application bags are opened and the entire volume is removed, which means that the preparation solution can no longer be administered to the patient. Secondly, the laboratory personnel must deal openly with substances that are carcinogenic, mutagenic or toxic to reproduction (CMR).

Due to the second disadvantage, the procedure is in conflict with Council Directive 90/394/EEC [17], which describes the STOP principle in handling carcinogens. STOP stands for substitution, technical measures, organisational measures, personal protective equipment and indicates the order in which measures should be taken to improve the occupational safety. Substitution is not possible because the analyte owns the carcinogenic properties. However, it is possible to take technical measures and work in a closed system, which significantly reduces the occupational safety risk. In order to improve occupational safety, three methods which do not require the open handling of CMR substances were therefore compared with the pharmacopoeia method and compared in terms of uncertainty (See Chapter 3.4.2).

After the sample preparation has been carried out, the analysis can be performed. Quality control samples are repeatedly analysed to determine the uncertainty contribution of the analytical system.

3.4 Results and Discussion

3.4.1 Sample transport and stability testing

In order to assess the quality of the preparation, it is necessary to ensure that no changes in analyte concentration and identity occur during transport from the production site to the analytical laboratory and during storage in the laboratory until analysis.

These analyses were carried out using HPLC-DAD as triplicate. The stability data were acquired at low (6°C ± 2) and high temperature (37°C ± 1) as well as at ambient temperature (22°C ± 3). A sample is considered to be stable until degradation of more than 5% of the API was observed.

The tested active ingredients 5-FU, Gem and Irino had a stability of at least 42 days, regardless of temperature. Pac showed a temperature-independent behaviour, but the degradation rate exceeds 5% after 14 days. In contrast, the recovery for CP depends on temperature which is shown in Table 3-2.

Table 3-2: Degradation rates of cyclophosphamide in % at different temperatures after 0, 3, 7, 14, 21 and 42 days. The analysis was performed as triplicate.

	Day 0	Day 3	Day 7	Day 14	Day 21	Day 42
6°C ± 2	0.0 ± 0.3	-2 ± 1	1 ± 0.6	-1 ± 0.2	-1 ± 0.2	0 ± 0.3
22°C ± 3	0.0 ± 0.1	0.0 ± 0.2	4 ± 0.4	10 ± 1	17 ± 0.3	36 ± 0.4
37°C ± 1	0.0 ± 0.3	25 ± 0.9	55 ± 0.4	80 ± 0.3	92 ± 0.04	100 ± n/a

Cyclophosphamide had a stability of up to 42 days when refrigerated. However, transport as well as analysis had to be completed after 7 days, when carried out at ambient temperature. The data showed strong substance degradation as soon as the temperature rose (25% after three days). Due to the temperature-dependent stability, cyclophosphamide preparations should be shipped refrigerated. The APIs 5-FU, Gem, Irino and Pac on the other hand can be shipped at room temperature or refrigerated. Although all tested solutions were stable when refrigerated, the sample should be checked for precipitation after refrigerated transport or storage. This applies in particular to highly concentrated solutions.

3.4.2 Development of a method for sample preparation

HPLC-DAD and Raman/UV are both analytical techniques that can be used to determine the concentration of the API. However, in the context of quality control of cytostatic drug preparations, the absolute amount is important, which is why the volume of the preparation must also be determined. For this purpose, three methods that meet the requirements of a closed system were compared with the Ph. Eur. method (See Chapter 3.3).

Following the first method (No Correction) no experimental determination of the volume is performed and the injected drug volume is added to the volume of the carrier solution e.g. 0.9% NaCl or 5% glucose. However, this method does not take into account that the application bags already prefilled with carrier solution do not contain the specified volume but are always overfilled. The infusion solutions are overfilled to ensure that the specified volume of liquid can be withdrawn over the entire runtime specification. This is because solvents evaporate continuously, depending on the bag material and storage conditions. The total amount of overfilling is manufacturer and product specific [20,21].

The tested bag batch showed an overfilling of 4.8%, which corresponds to the systematic error of the "No Correction" method. The overfilling of this specific batch was found very constant with a random error of 0.30% so that it may be taken into account with a correction factor. However, this would only correct the error caused by overfilling and would not take into account volume errors when injecting the drug.

In order to take these variations into account, the correction factor should be based on the bag weight, as in the second tested method (Correction factor). Therefore, ten bags of a batch were emptied, cleaned, dried and weighed. For cytostatic drug applications to be analysed, the entire application bag was weighed and the previously determined mean batch empty weight was subtracted.

Compared to the first method (No Correction), all volumes were taken into account and the bag did not need to be opened. It should be noted that this method can only determine the weight of the solution and not its volume. To convert weight into volume, the density of the solution needs to be known. Unless an explicit density determination is carried out and the density of a 0.9% saline solution (1.00506 g mL^{-1} at 20°C) is used as an approximation, an error of 0.35% will occur. Together with the error from the standard deviation of the tare weight determination, this method yielded an error of 0.38% and is listed as “Correction factor” in Table 3-3. Thus it is possible to determine the volume of the preparation bag with a small error without destroying it or exposing the laboratory staff to highly toxic cytostatic drugs.

Table 3-3: Comparison of three different methods for volume determination of patient specific application solutions. For the determination of the errors ten application bags were used, as well as the density determinations of 136 samples. For the methods "Correction factor" and "Gravimetric correction" two different values were obtained, depending on whether (a) no density determination was carried out or (b) an experimental density determination was carried out. $q_{Systematic\_sp}$: Systematic error of sample preparation. q_{Random_sp}: Random error of sample preparation. $q_{Density}$: Error of the density determination. u_{csp}: combined uncertainty of sample preparation.

Method	No Correction	Correction factor	Gravimetric correction
$q_{Systematic\_sp}$ **(%)**	4.8	0.00	0.15
q_{Random_sp} **(%)**	0.30	0.15	0.03
$q_{Density}$ **(%)**	n/a	0.35[a] / 0.10[b]	0.35[a] / 0.10[b]
u_{csp} **(%)**	4.8	0.38[a] / 0.18[b]	0.38[a] / 0.18[b]

The described method “Correction factor” can only be used if the application bag and application equipment used are known in advance and a correction factor for the empty bag and application equipment combination has been determined. Due to the large number of manufacturers and possible combinations of application bags and application equipment, this method is mainly suitable for self-control. An external laboratory service provider that performs quality control for different pharmacies and hospitals would otherwise have to collect too many correction factors and of course update them for each new batch. A flexible and broadly applicable method is therefore needed, which can be carried out with a small amount of prior knowledge.

In order to maintain this flexibility while at the same time taking into account occupational health and safety aspects, the method from the European Pharmacopoeia was modified and is called “Gravimetric correction”. The cytostatic drug application solutions are connected to an empty application bag, which allows the transfer of the liquid to be carried out in a closed system. The bag containing the cytostatic drug preparation solution is weighed before and after the transfer of the liquid. As with the correction factor method, the density is needed to calculate the volume. For this, either the approximation can be used again or an experimental determination of the density takes place. The second procedure (experimental determination) leads to a reduction of the error for the density determination from 0.35% to 0.10%. As not the entire bag volume can be transferred and very small amounts of liquid are still remaining, a

systematic error of 0.15% occurs. However, the chosen method of operation is highly reproducible, resulting in a low random error of only 0.03%. This leads to a combined uncertainty of measurement of 0.18%, if the density is determined.

When comparing the uncertainties, the methods "correction factor" and "gravimetric correction" are equivalent with an error of 0.38% (without density determination) and 0.18% (with density determination). The uncertainty of the method from the European Pharmacopoeia depends on the accuracy of the measuring cylinder used and is therefore in the range 0.50-1.0%. Thus, methods are available which have a lower uncertainty and simultaneously consider the aspects of occupational health and safety.

3.4.3 Comparison of Raman/UV and HPLC-DAD workflows

Before samples were measured using the Raman/UV and HPLC-DAD analysis systems, the methods were validated by intra-day, inter-day assays, check for carry-over and test for linearity. The complete validation results are given in the supplementary information, whereas the resulting errors and uncertainties are presented in Table 3-4.

Table 3-4: Errors derived from the validation for the substances 5-fluorouracil (5-FU), cyclophosphamide (CP), gemcitabine (Gem), irinotecan (Irino) and paclitaxel (Pac). The errors are divided into systematic ($q_{Systematic}$) and random (q_{Random}). Furthermore, the uncertainty (u) of the analytical method was calculated.

Substance	5-FU	CP	Gem	Irino	Pac
$q_{Systematic\_HPLC\text{-}DAD}$ (%)	0.50	1.5	0.90	0.86	0.35
$q_{Systematic\_Raman/UV}$ (%)	0.38	1.5	0.19	2.2	1.9
$q_{Random\_HPLC\text{-}DAD}$ (%)	0.69	0.60	0.36	0.57	0.25
$q_{Random\_Raman/UV}$ (%)	1.5	0.69	1.0	2.5	2.2
$u_{HPLC\text{-}DAD}$ (%)	0.95	1.6	0.97	1.0	0.43
$u_{Raman/UV}$ (%)	1.6	1.6	1.0	3.3	2.8

The uncertainties are comparable for the substances 5-fluorouracil, cyclophosphamide and gemcitabine, whereas there are differences for irinotecan and paclitaxel. Highly concentrated solutions of these two APIs showed a higher viscosity and a clear tendency to foam, which can lead to problems during the injection process by the autosampler. It should be noted that these properties may also result from the formulation substances.

The samples used for HPLC-DAD analysis were diluted, so there was no interference during the injection process. The samples for Raman/UV analysis could not be further diluted because the Raman intensity was no longer sufficient for substance identification (See Supplementary information). However, if a lower uncertainty is required for Raman/UV analysis, two measurements can be performed, one undiluted for substance identification and one diluted for quantification. It is considered that this can reduce the level of errors.

After the uncertainty of the sample preparation and of the analytical systems is known, the combined uncertainty of measurement can be calculated. For the calculation, the method of gravimetric correction with experimental density determination (u_{csp}=0.18%) was chosen for the uncertainty of the sample preparation. Furthermore, a coverage factor of 1.96 was used to increase the level of confidence to 95%. The expanded combined uncertainties of measurement for the Raman/UV and HPLC-DAD workflows determined using this approach are shown in Table 3-5.

Table 3-5: Summary of the extended combined uncertainties of measurement for the substances 5-fluorouracil (5-FU), cyclophosphamide (CP), gemcitabine (Gem), irinotecan (Irino) and paclitaxel (Pac). Sample preparation was performed by the gravimetric approach with experimental density determination. The values were calculated for a level of confidence of 95% with k = 1.96.

Substance	5-FU	CP	Gem	Irino	Pac
$U_{HPLC\text{-}DAD}$ (%)	1.7	3.2	1.9	2.1	0.91
$U_{Raman/UV}$ (%)	3.1	3.2	2.0	6.5	5.6

The calculated expanded combined uncertainties of measurement range from 0.91%-3.2% for HPLC DAD and 2.0-6.5% for the Raman/UV technique. Against the background that it should be checked whether the content of an application solution deviates by more than 10% from the target value, the uncertainties of measurement are sufficiently low for both techniques.

3.4.4 Analysis of cytostatic drug application solutions

After method validation was completed, 136 cytostatic drug preparations were analysed as triplicate using HPLC-DAD and Raman/UV. The cytostatic drug preparations examined were returns. Returns are cytostatic drug preparations which could not be administered to the patient for various reasons. In the study, care was taken to ensure that only returns which were not opened have been analysed.

For the evaluation of the preparation quality, a maximum permissible deviation from the target content was set to 10%. According to DIN EN ISO/IEC 17025:2018 [22], analytical testing laboratories have to identify and consider all significant contributions to uncertainty of measurement during their evaluation including those arising from sampling. Some working groups choose a substance independent approach and therefore increase the acceptance criterion to 15% [23,8,24]. In this study, the expanded combined uncertainty of measurement (Table 3-5) was added to the 10% depending on the substance and analysis method. This means, for example, that a 5-FU sample analysed by HPLC-DAD may differ from the theoretical content by up to 11.7%. In contrast, a cyclophosphamide sample, which was analysed using Raman/UV, may differ up to 13.2%.

When quantifying with HPLC-DAD, 96% of the returns had a deviation of less than 10% plus uncertainty of measurement from the target value. In comparison, only 84% of the preparation solutions fulfilled the quality criterion when the analysis was performed using Raman/UV. As can be seen in Figure 3-2, not only the recovery differs overall, but there are also mayor differences in the distribution of the results when using the Raman/UV technique.

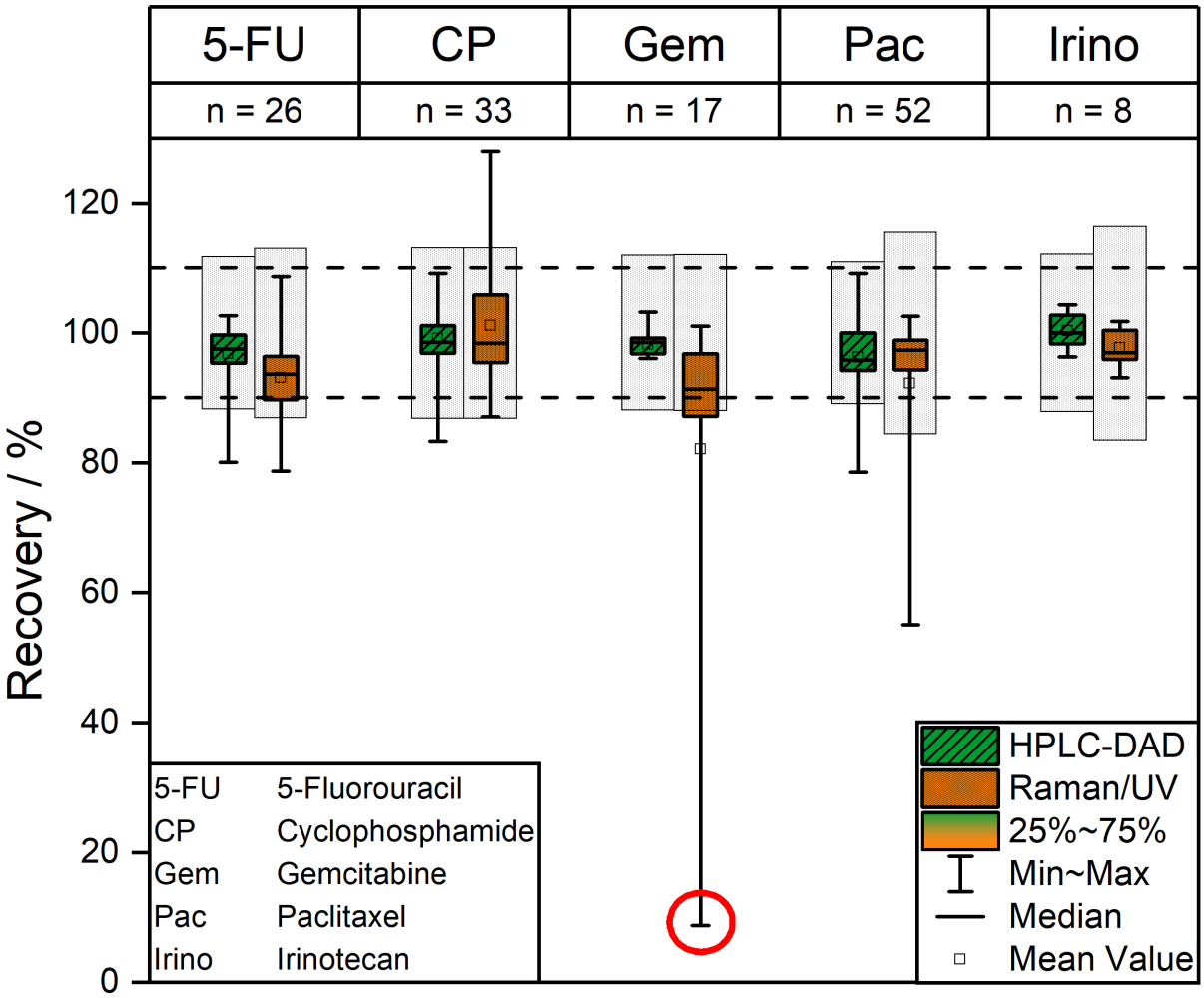


Figure 3-2: Comparison of the recovery of cytostatic drugs in application solutions using HPLC-DAD (left bar / green) and Raman/UV (right bar / orange). The dotted lines mark a deviation of 10% from the target value. In addition, grey boxes are shown, which extend the 10% deviation by the compound-specific uncertainty of measurement. The value circled in red highlights a significantly lower recovery using Raman/UV (9%) compared to HPLC-DAD (97%).

The sample circled in red in Figure 3-2 had a recovery of 97% for the API when analysed using HPLC-DAD, but only a recovery of 9% when analysed using Raman/UV. Figure 3-3A shows the Raman-spectra of the sample (orange line, gemcitabine containing drug solution of manufacturer B) and of gemcitabine containing drug solution of manufacturer A, which was used as calibrant (green line). Correspondingly, the UV-spectra of the gemcitabine containing drug solution of manufacturer A and B are shown in Figure 3-3B. The Raman-spectra in Figure 3-3A differed significantly, although they both contained the same API (gemcitabine), which indicated the presence of different formulation ingredients.

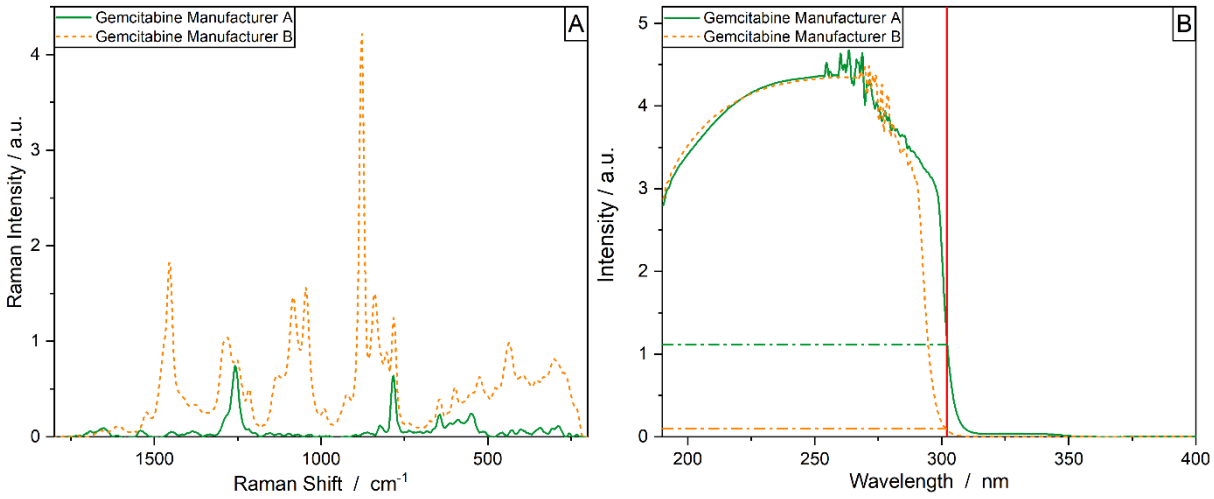


Figure 3-3: Analysis of two gemcitabine-containing drug solutions using Raman/UV. Manufacturer A does not use any formulation substances, whereas manufacturer B adds ethanol, propylene glycol and macroglycol 300. Figure 3-3A shows the Raman spectrum used for identification. Figure 3-3B shows the UV spectrum used for quantification. The wavelength of 302 nm which was used for quantification is marked by the red vertical line.

In this study, only the API was known but not the drug solution and formulation. The comparison of the sample with a database showed that the sample was most likely prepared from a drug containing gemcitabine, which contained ethanol, propylene glycol and macroglycol 300 as additional formulation substances. Figure 3-4 allows the comparison of the sample spectrum with the corresponding drug spectrum listed in the database.

The Raman spectrum only serves for formulation identification, which is why deviations in this spectrum cannot explain the significantly lower recovery. The quantification of gemcitabine was performed at a wavelength of 302 nm. As can be seen in Figure 3-3B, the formulation substances used by manufacturer B caused a UV shift into the short-wavelength range. This shift had the extinction drop by more than 90%.

The sample was then re-evaluated with the drug identified during the database comparison. Using a calibration that took the formulation substances into account, the Raman/UV technique yielded a recovery of 92% for the deviating sample, which corresponds to a deviation of less than 10% plus uncertainty of measurement. The Raman/UV technique therefore is able to provide comparable results to HPLC-DAD, as long as the calibration takes formulation constituents sufficiently into account.

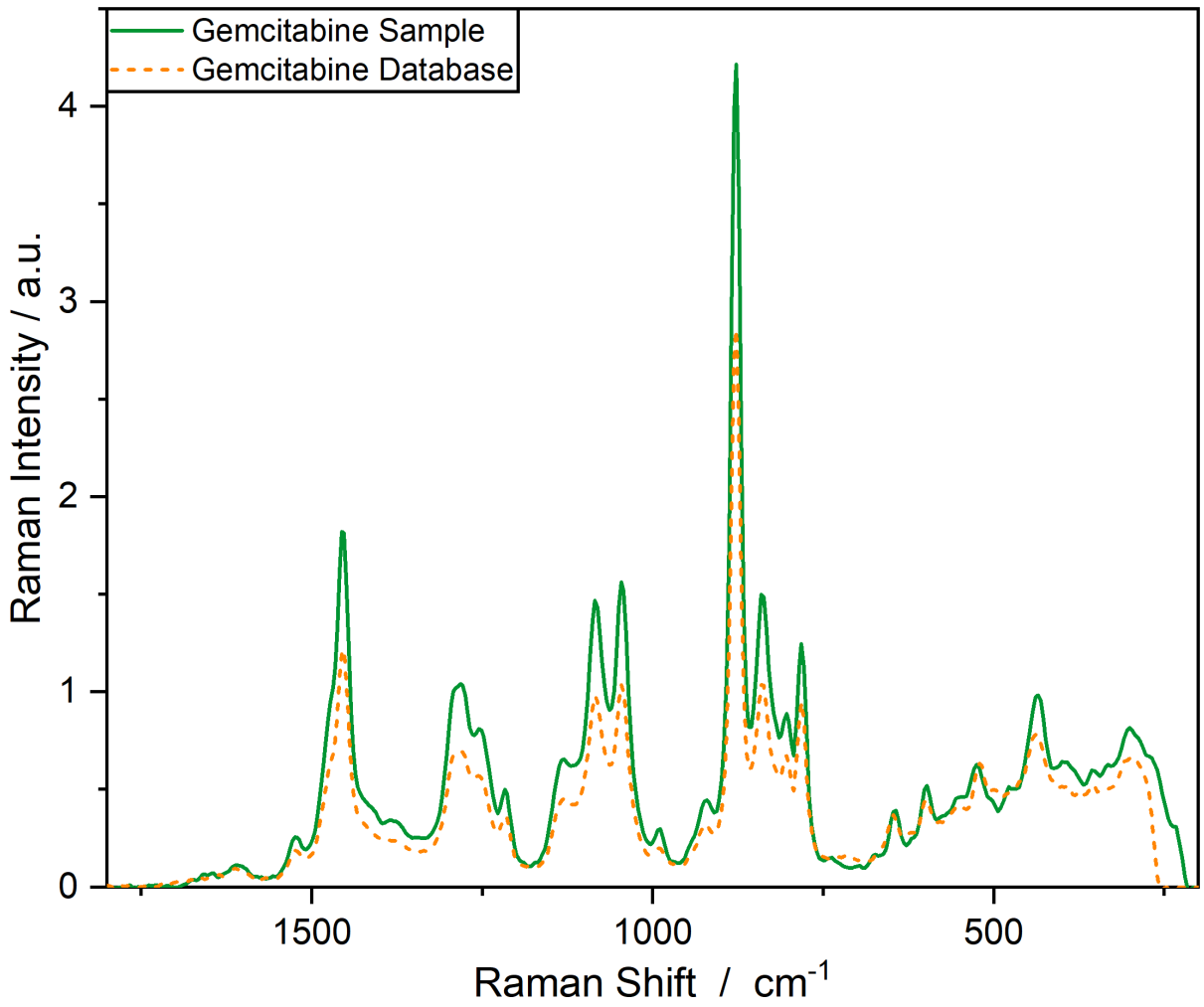


Figure 3-4: Comparison of the gemcitabine sample (green) with a gemcitabine containing drug that contains ethanol, propylene glycol and macroglycol 300 as additional formulation substances (orange).

The Raman/UV technique is therefore suitable for samples where sufficient knowledge about the drug substance and product under investigation is available. When the knowledge about the sample is insufficient, HPLC-DAD is recommended since formulation substances have a minor influence on the analysis due to the chromatographic separation.

3.5 Conclusion

The compared analytical techniques HPLC-DAD and Raman/UV both proved suitable for quality control of patient-specific preparations of cytostatic drugs. Since the Raman/UV technique does not include chromatographic separations, formulation substances were found to exercise an influence on the analysis, which must be taken into account by a corresponding calibration. The technique is therefore well suited for voluntary self-monitoring of the formulating pharmacies and hospitals. In consequence, quality control progresses. HPLC-DAD, on the other hand, allows for separation of formulation substances, requiring much less knowledge of the sample composition, which is dependent on the manufacturer, is required. This renders the technique suitable for analysis in external or remote laboratories.

3.6 Supplementary Information

3.6.1 Volume determination

Table 3-6 shows the complete data on bag emptying of application solutions pre-filled with isotonic saline solution. It is noticeable that the bags are heavily overfilled on average by 4.8%.

Table 3-6: Data for volume determination of application bags pre-filled with 0.9% NaCl solution.

	Full (g)	Empty (g)	Clean and dry (g)	Content (g)	Filling (%)	Remaining volume (mL)	Remaining volume (%)
Bag 1	539.32	14.20	13.47	525.85	105.2	0.73	0.14
Bag 2	535.93	14.38	13.46	522.47	104.5	0.92	0.18
Bag 3	539.04	14.14	13.46	525.58	105.1	0.68	0.13
Bag 4	538.75	14.37	13.43	525.32	105.1	0.94	0.18
Bag 5	536.01	13.99	13.47	522.54	104.5	0.52	0.10
Bag 6	539.30	14.35	13.44	525.86	105.2	0.91	0.17
Bag 7	535.27	14.36	13.43	521.84	104.4	0.93	0.18
Bag 8	537.17	14.07	13.42	523.75	104.8	0.65	0.12
Bag 9	537.41	14.47	13.47	523.94	104.8	1.00	0.19
Bag 10	536.76	14.11	13.47	523.29	104.7	0.64	0.12
Mean	537.50	14.24	13.45	524.04	104.8	0.79	0.15
SD	1.52	0.16	0.02	1.52	0.30	0.17	0.03

3.6.2 Validation data

Table 3-7 and Table 3-8 contain the validation data for HPLC-DAD and Table 3-9 and Table 3-10 for Raman/UV. No carry-over was observed for any API with both analytical techniques. The linearity is given over the entire calibration range.

Table 3-7: Intra-day-assay of HPLC-DAD methods of the five investigated substances 5-fluorouracil (5-FU), cyclophosphamide (CP), gemcitabine (Gem), irinotecan (Irino) and paclitaxel (Pac). The intra-day-assay was performed with n=10. The examined parameters are recovery (R), the relative standard deviation of the recovery (RSD), the average retention time (RT) and its standard deviation (SD).

Substance	5-FU	CP	Gem	Irino	Pac
R (%)	100.9	101.2	100.3	104.0	99.0
RSD (%)	0.1	0.2	0.4	0.3	0.6
RT (min)	0.627	2.530	0.771	2.260	2.660
SD (min)	0.005	0.001	0.003	0.002	0.001

Table 3-8: Inter-day-assay of HPLC-DAD methods of the five investigated substances 5-fluorouracil (5-FU), cyclophosphamide (CP), gemcitabine (Gem), irinotecan (Irino) and paclitaxel (Pac). The examined parameters are the recovery rate (R), the relative standard deviation of the recovery rate (RSD), the average retention time (RT) and its standard deviation (SD). The R and RSD are evaluated with n=12 on three different days and the RT and SD are evaluated with n=4 on four different days.

Substance	5-FU	CP	Gem	Irino	Pac
R (%)	100.5	101.5	100.9	99.1	99.7
RSD (%)	0.7	0.6	0.4	0.6	0.3
RT (min)	0.635	2.520	0.775	2.250	2.660
SD (min)	0.001	0.011	0.008	0.009	0.005

Table 3-9: Intra-day-assay of Raman/UV of the five investigated substances 5-fluorouracil (5-FU), cyclophosphamide (CP), gemcitabine (Gem), irinotecan (Irino) and paclitaxel (Pac). The examined parameters are recovery (R) and the relative standard deviation of the recovery rate (RSD).

Substance	5-FU	CP	Gem	Irino	Pac
n	4	10	4	9	4
R (%)	101.1	98.0	99.8	101.5	97.8
RSD (%)	0.1	0.3	0.6	0.4	1

Table 3-10: Inter-day-assay of Raman/UV of the five investigated substances 5-fluorouracil (5-FU), cyclophosphamide (CP), gemcitabine (Gem), irinotecan (Irino) and paclitaxel (Pac). The examined parameters are recovery (R) and the relative standard deviation of the recovery (RSD). The R and RSD are evaluated at three different days.

Substance	5-FU	CP	Gem	Irino	Pac
n	12	30	11	17	9
R (%)	100.4	98.5	99.8	102.2	98.1
RSD (%)	1.5	0.7	1.0	2.5	2.2

3.6.3 Concentration dependent Raman intensity

Figure 3-5 shows the Raman spectra of the drug Irinomedac with the API irinotecan at 1 mg mL^{-1} (green) and 0.1 mg mL^{-1} (orange). Substance identification is only possible at a concentration of 1 mg mL^{-1} because the intensity of the Raman signals is too low at 0.1 mg mL^{-1}.

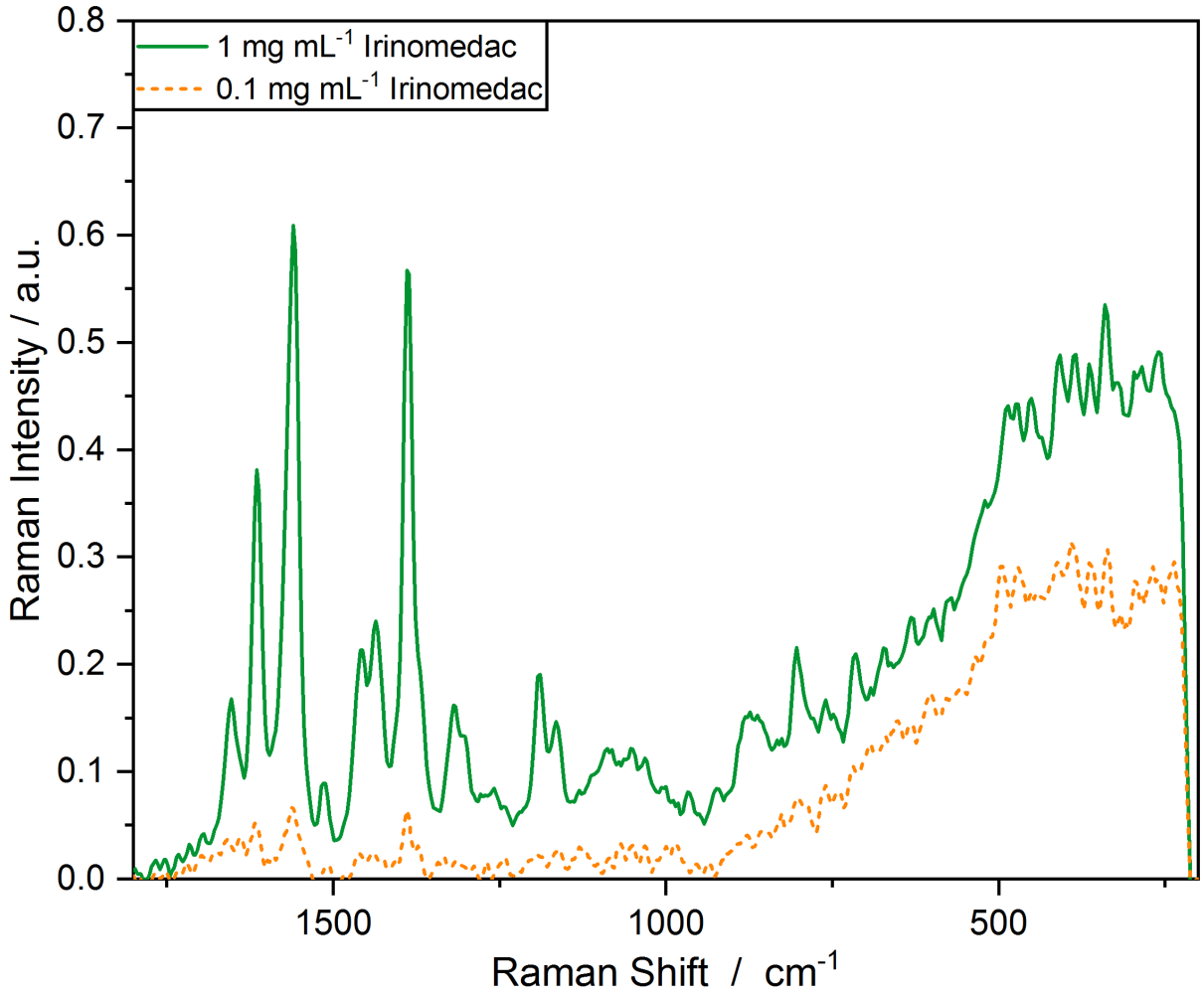


Figure 3-5: Comparison of the Raman spectra of the drug Irinomedac with the API irinotecan at a concentration of 1 mg mL^{-1} (green) and 0.1 mg mL^{-1} (orange).

3.6.4 Selection of the quantification wavelength

As part of the method validation, UV spectra of the individual substances were recorded, which are shown in Figure 3-6. Based on these UV spectra, the wavelengths for quantification were selected. If low concentrations have to be detected in an HPLC-DAD analysis, it is recommended to select the wavelength that represents the global maximum. However, when analysing patient-specific cytostatic solutions, the concentration of the API is very high, and further considerations should be taken into account when selecting the quantification wavelength.

In order to avoid diluting the sample too much and thus increasing the uncertainty of measurement due to the additional sample preparation steps, it may make sense to switch from the global maximum to a local maximum, as it was done in the case of irinotecan, for example. Furthermore, it must be checked whether known impurities coelute and cause absorption on the same wavelength. Here, changing the quantification wavelength can improve robustness. Therefore, considering the above points, the wavelengths 265 nm (5-FU), 195 nm (CP), 268 nm (Gem), 255 nm (Irino), and 227 nm (Pac) were selected for the HPLC-DAD analyses. The selected wavelengths were plotted in green in Figure 3-6.

In the case of Raman/UV analysis, however, the choice of quantitation wavelength is based on different considerations. The samples are injected undiluted so that the Raman intensity is sufficiently high for substance identification (See Figure 3-5). However, for UV quantification wavelengths at the global or local maximum lead to a saturation effect, making quantification impossible. Therefore, to determine the optimum wavelength, a complete UV spectrum is recorded for each calibration point. Using this data set, the software, provided by the manufacturer, determines the correlation between the absorbance and the concentration, as well as the deviation from the target value, for each wavelength. The result is output as a performance/deviation curve. The optimum quantification wavelength corresponds to the global maximum of the curve determined in this way. The quantification wavelengths determined for the Raman/UV technique are 319 nm (5-FU), 205 nm (CP), 302 nm (Gem), 267 nm (Irino), and 254 nm (Pac). They are shown in orange in Figure 3-6.

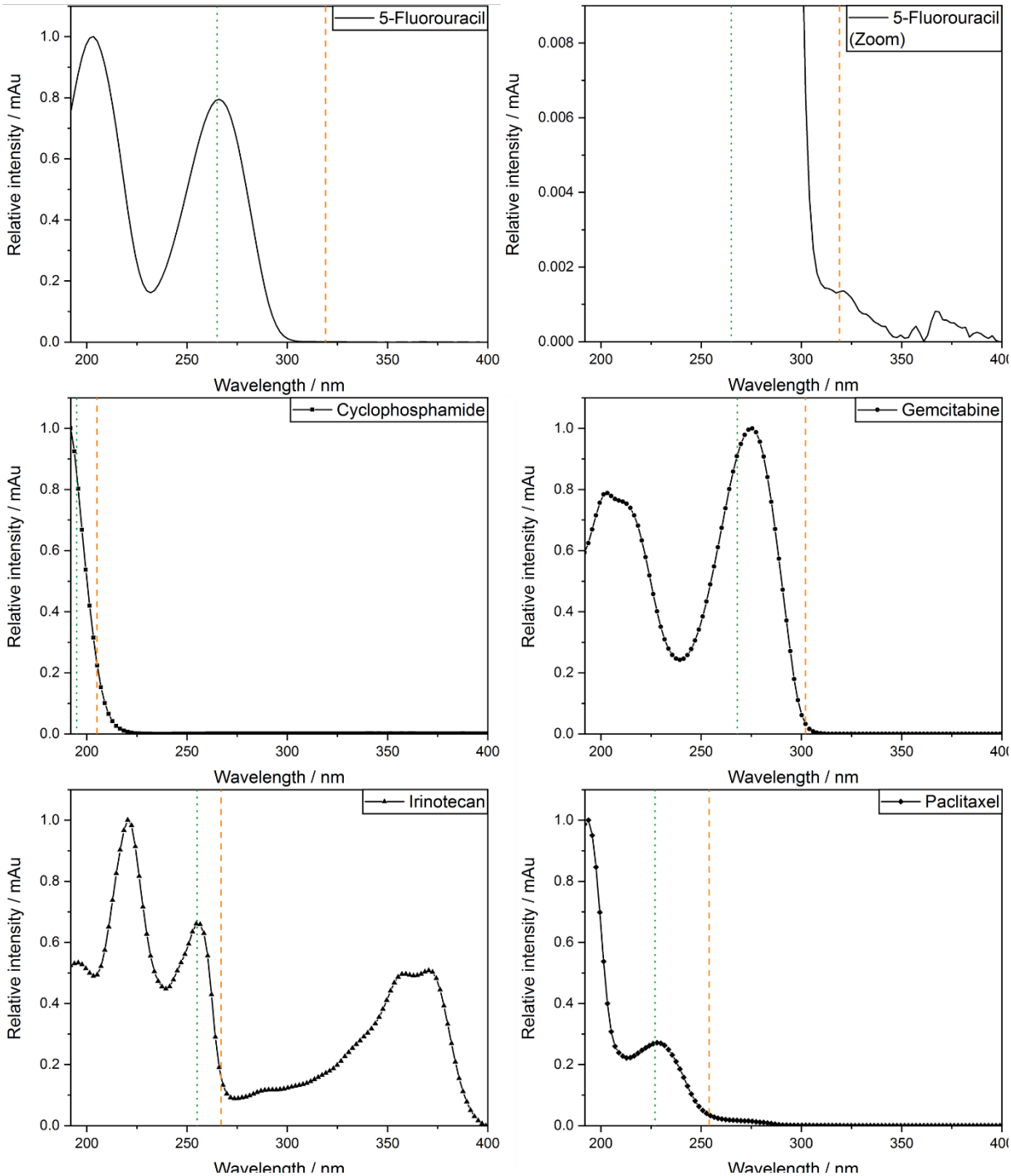


Figure 3-6: UV spectra of the five commonly used cytostatics 5-fluorouracil, cyclophosphamide, gemcitabine, irinotecan and paclitaxel. The quantification wavelength of the HPLC-DAD technique is shown as green dotted line and that of the Raman/UV technique is shown as orange dashed line.

3.6.5 Formulation substances of the drugs

Table 3-11 contains the composition of all drugs used in the study.

Table 3-11: Overview of the ingredients of all drugs used.

Drug	Formulation Substances
5-FU medac	5-Fluorouracil, sodium hydroxide, water for injection
Irinomedac	Irinotecanhydrochlorid-Trihydrat, sorbitol, lactic acid, sodium hydroxide, water for injection
Taxomedac	Paclitaxel, macrogolglycerol ricinoleate, ethanol, citric acid
Endoxan	Cyclophosphamide
Gemcitabin Hexal	Gemcitabine hydrochloride, water for injection, hydrochloric acid 10 % (for pH adjustment)

3.7 References

1. Muller T (2003) Typical medication errors in oncology: Analysis and prevention strategies. Onkologie 26 (6):539-544. doi:10.1159/000074148

2. Sendra-Garcia A, Martinez-Gomez MA, Albert-Mari A, Jimenez-Torres NV, Climente-Marti M (2019) Quantitative and qualitative control of antineoplastic preparations: Gravimetry versus HPLC. J Oncol Pharm Pract 25 (5):1204-1216. doi:10.1177/1078155219834999

3. Benizri F, Dalifard B, Zemmour C, Henriquet M, Fougereau E, Le Franc B (2016) DrugCam (R)-An intelligent video camera system to make safe cytotoxic drug preparations. Int J Pharmaceut 502 (1-2):198-207. doi:10.1016/j.ijpharm.2016.02.028

4. Bazin C, Cassard B, Caudron E, Prognon P, Havard L (2015) Comparative analysis of methods for real-time analytical control of chemotherapies preparations. Int J Pharmaceut 494 (1):329-336. doi:10.1016/j.ijpharm.2015.08.041

5. Lagarce F (2017) Centrally Prepared Cytotoxic Drugs: What Is the Purpose of Their Quality Control? Pharm Technol Hosp Pharm 2 (1):29-33. doi:10.1515/pthp-2017-0006

6. Bouligand J, Paci A, Mercier L, Vassal G, Bourget P (2004) High-performance thin-layer chromatography with a derivatization procedure, a suitable method for the identification and

the quantitation of busulfan in various pharmaceutical products. J Pharmaceut Biomed 34 (3):525-530. doi:10.1016/S0731-7085(03)00630-7

7. Paci A, Mercier L, Bourget P (2003) Identification and quantitation of antineoplastic compounds in chemotherapeutic infusion bags by use of HPTLC: application to the vinca-alkaloids. J Pharmaceut Biomed 30 (5):1603-1610. doi 10.1016/S0731-7085(02)00541-1

8. Delmas A, Gordien JB, Bernadou JM, Roudaut M, Gresser A, Malki L, Saux MC, Breilh D (2009) Quantitative and qualitative control of cytotoxic preparations by HPLC-UV in a centralized parenteral preparations unit. J Pharmaceut Biomed 49 (5):1213-1220. doi:10.1016/j.jpba.2009.03.007

9. Jaccoulet E, Schweitzer-Chaput A, Toussaint B, Prognon P, Caudron E (2018) Simple and ultra-fast recognition and quantitation of compounded monoclonal antibodies: Application to flow injection analysis combined to UV spectroscopy and matching method. Talanta 187:279-286. doi:10.1016/j.talanta.2018.05.042

10. Jaccoulet E, Smadja C, Prognon P, Taverna M (2015) Capillary electrophoresis for rapid identification of monoclonal antibodies for routine application in hospital. Electrophoresis 36 (17):2050-2056. doi:10.1002/elps.201400603

11. Le LMM, Caudron E, Baillet-Guffroy A, Eyeleigh L (2014) Non-invasive quantification of 5-fluorouracil and gemcitabine in aqueous matrix by direct measurement through glass vials using near-infrared spectroscopy. Talanta 119:361-366. doi:10.1016/j.talanta.2013.10.060

12. Le L, Berge M, Tfayli A, Guffroy AB, Prognon P, Dowek A, Caudron E (2019) Quantification of gemcitabine intravenous drugs by direct measurement in chemotherapy plastic bags using a handheld Raman spectrometer. Talanta 196:376-380. doi:10.1016/j.talanta.2018.11.062

13. Deutscher Apotheker Verlag, (2018) European Pharmacopoeia, 9th Ed.

14. Schmiedel DA-VDR (2018) USP 42 - NF 37 The United States Pharmacopeia and National Formulary 2019: Main Edition Plus Supplements 1 and 2.

15. Swissmedic (2019) Pharmacopoea Helvetica, 11th Ed.

16. Busch A (2019) Ergebnisse von Apothekenkontrollen Aktenzeichen IV B 5 - G.0611.

17. Council Directive (1990) Council Directive 90/394/EEC of 28 June 1990 on the protection of workers from the risks related to exposure to carcinogens at work (Sixth individual Directive within the meaning of Article 16 (1) of Directive 89/391/EEC).

18. Working Group 1 of the Joint Committee for Guides in Metrology (2008) Evaluation of measurement data — Guide to the expression of uncertainty in measurement.

19. Pharmacopoea Europaea (2018) Test for Extractable Volume of Parenteral Preparations.

20. Cohen MR, Smetzer JL (2014) Understanding and Managing Intravenous Container Overfill; Potential Dose Confusion. Hospital Pharmacy 49 (3):221-226. doi:10.1310/hpj4903-221

21. Thiessen JJ (2013) A review of the oncology under-dosing incident. URL: http://www.health.gov.on.ca/en/public/programs/cancer/drugsupply/docs/report_thiessen_oncology_under-dosing.pdf Last accessed: 07.09.2020

22. DIN EN ISO/IEC 17025:2018 General requirements for the competence of testing and calibration laboratories (2017).

23. Nardella F, Beck M, Collart-Dutilleul P, Becker G, Boulanger C, Perello L, Gairard-Dory A, Gourieux B, Ubeaud-Sequier G (2016) A UV-Raman spectrometry method for quality control of anticancer preparations: Results after 18 months of implementation in hospital pharmacy. Int J Pharmaceut 499 (1-2):343-350. doi:10.1016/j.ijpharm.2016.01.002

24. Dziopa F, Galy G, Bauler S, Vincent B, Crochon S, Tall ML, Pirot F, Pivot C (2013) A quantitative and qualitative method to control chemotherapeutic preparations by Fourier transform infrared–ultraviolet spectrophotometry. J Oncol Pharm Pract 19 (2):121-129. doi:10.1177/1078155212457963

25. Chouquet T, Benoit G, Morand K (2017) Analytical Control of Pediatric Chemotherapy Preparations with a UV-Raman Automaton: Results After 18 Months of Implementation and Development of A Suitable Method for Low Volume Preparations. Pharm Technol Hosp Pharm 2(3): 117–129. doi: 10.1515/pthp-2017-0021

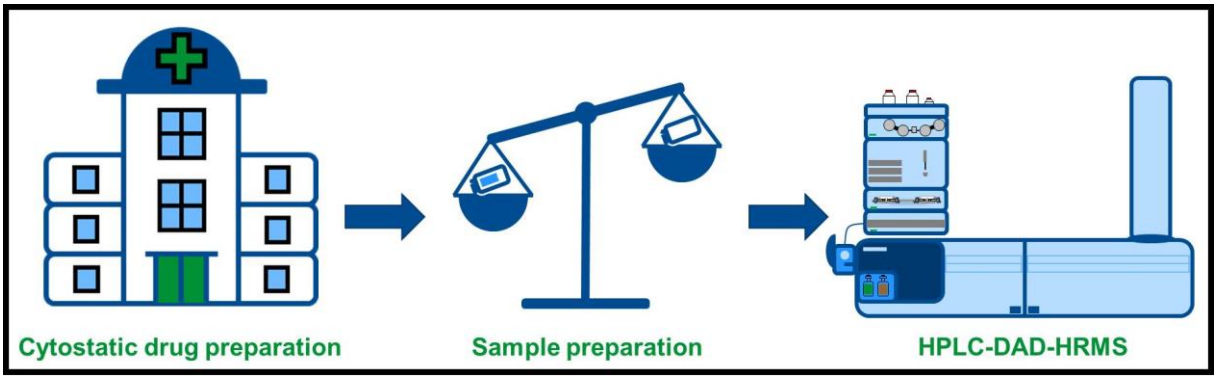
26. Mandel J. The statistical analysis of experimental data. Washington: Wiley; 1964.

Chapter 4 Quality control of personalized drug products – Identity and quantity of monoclonal antibodies as active pharmaceutical ingredient

This chapter was adapted from: Reinders, L.M.H., Klassen, M.D., Jaeger, M., Schmidt, T.C., Teutenberg, T., Tuerk, J., Quality control of personalized drug products – Identity and quantity of monoclonal antibodies as active pharmaceutical ingredient. J Pharm Sci (2023) Submitted

Abstract: Deliberate underdosing occurred in personalized preparations of drugs such as monoclonal antibodies as the active pharmaceutical ingredient in the past. To ensure the required quality standard and to prevent future fraud attempts at an early stage, a HPLC-DAD-HRMS method was established. Thereby, identity and quantity of the active ingredients bevacizumab, rituximab and trastuzumab were determined. The analysis of ten samples from seven pharmacies fulfilled the quality criteria and were therefore not objectionable.

Graphical Abstract:



4.1 Introduction

In tumor treatment, the use of personalized medicine is common. Hence, drug and dose are adapted to the patient´s constitution. For this purpose, concentrates are provided by the pharmaceutical industry and diluted to the required dose by trained pharmaceutical personnel [1,2]. This manual step is controlled by quality assurance measures such as working according to the four-eyes principle. In this measure, a second person visually inspects the utensils and volumes used by the preparer. Alternatively, the control can be performed by a camera system. Another approach is to monitor the preparation process gravimetrically [3].

Furthermore, the quality control may be carried out analytically. For this purpose, the volume and concentration of the preparation need be determined. Yet, when determining the volume, the application can no longer be administered to the patient. As a result, preparations sampled by supervisory agencies must be produced twice. Quality control samples need to be produced especially for the analytical determination, but a simpler and less expensive solution is the analysis of returns. These are preparations that were intended for a patient but were not administered e.g., due to the patient's state of health. The informative value of the analysis of returns is therefore very high, since the application bag was prepared for a patient and not only for quality control.

For additional instrumental analysis, a deviation from the target content must not exceed 10% after preparation [7]. Studies on cytostatic drugs could demonstrate that very high compliance rates of 96% (n=136) [4] and 98% (n=622) [5] were achieved through controlling analytical quality by HPLC-DAD. These results imply that current measures are sufficient to ensure high patient safety.

Nevertheless, a German pharmacist achieved sad notoriety for intentionally underdosing over 14,000 preparations. Mainly high-cost monoclonal antibodies were concerned, resulting in a financial loss of 13.6 million euros [6]. In contrast to the above mentioned cytostatics, quality control for the analysis of immune therapeutics such as mAb is not straight forward. One reason for this is the high molecular weight of mAbs, which is about 150,000 Da. This results in a high degree of complexity.

To enable analytical quality control for monoclonal antibodies, a high-performance-liquid chromatography diode array detector high-resolution mass spectrometer (HPLC-DAD-HRMS) method was developed to identify and quantify bevacizumab, rituximab, and trastuzumab in personalized preparations. The developed analytical method was subsequently validated, and returned preparations were analyzed.

4.2 Materials and methods

4.2.1 Chemicals and reagents

The monoclonal antibodies bevacizumab, rituximab and trastuzumab were purchased as Avastin, MabThera and Herceptin from Hoffmann-La Roche (Basel, Switzerland). Water and acetonitrile were purchased from Merck KgaA (Darmstadt, Germany). Physiologic salt solution (0.9% NaCl) was received from B. Braun (Melsungen, Germany). Formic acid (FA) was purchased from Sigma-Aldrich (St-Louis, USA) and isopropanol from Th. Geyer (Renningen, Germany).

4.2.2 HPLC-DAD-HRMS analysis method

The analytical system consisted of a 1260 Infinity II bio-inert HPLC (Agilent Technologies, Waldbronn, Germany) which was coupled to a 6560 IM QTOF HRMS (Agilent Technologies, Waldbronn, Germany). Both systems were controlled via Masshunter Workstation software (build 9.0.9044.1 SP 1) from Agilent Technologies. The analytical method used a Zorbax 300SB-C8 2.1 x 150 mm 5 µm including a Zorbax 300SB-C8; 2.1 x 12.5 mm 5 µm precolumn as stationary phases (Agilent Technologies, Waldbronn, Germany). The column temperature was set at 80°C. The injection volume was 0.1 µL and the detection wavelength of the DAD was 214 nm. The mobile phases used were (A) water + 0.1% FA and (B) acetonitrile / isopropanol / water (70:20:10 *v/v/v*) + 0.1% FA. The gradient program was set as 0.0-2.0 min 5-5%B; 2.0-3.0 min 5-50%B; 3.0-6.0 min 50-50%B; 6.0-6.5 min 50-90%B; 6.5-9.5 min 90-90%B; 9.5-10.0 min 90-5%B; 10.0-14.0 min 5-5%B. Ionization on the high-resolution mass spectrometer was ESI positive. The other settings were: 350°C gas temperature, 12 L min^{-1} gas flow, 60 psig nebulizer gas, 400°C sheath gas temperature, 11 L min^{-1} sheath gas flow, 5500 V capillary voltage (VCap), 2000 V nozzle voltage, 380 V fragmentor voltage, 750 V octupole rod repel voltage (OctopoleRPPeak). Data processing was performed using Masshunter BioConfirm (build 10.0.10136.0) from Agilent Technologies (Waldbronn, Germany). The deconvolution algorithm was Maximum Entropy (MaxEnt) and processed signals *m/z* 2000-3500 at a retention time of 5.1-5.8 min. The signals had at least a signal to noise ratio of 30, with the baseline corrected by a factor of 7. The calculated mass range was 140,000-160,000 Da with a resolution of 0.05 Da. The individual charge states were considered as proton adducts. The sample preparation method of Reinders et al. [4] was used to analyze the returned samples. The theoretical concentration was calculated using the volume determined according to Reinders et al. [4] and dose indicated on the preparation.

4.2.3 Validation of the HPLC-DAD-HRMS method

Linearity was checked in the working range of 0.5-5.0 mg mL^{-1} by equidistant ten-point calibration. Carry-over was determined by analyzing a 0.9% NaCl solution after the 5.0 mg mL^{-1} standard. For the intra-day assay, ten mAb-solutions with a concentration of 2.5 mg mL^{-1} were analyzed (n = 10). For the inter-day assay, four mAb-solutions each with a concentration of 2.5 mg mL^{-1} were analyzed on three different days (n = 12). All solutions were prepared in 0.9% NaCl, corresponding to the sample matrix.

4.3 Results and Discussion

4.3.1 Validation and application of the HPLC-DAD-HRMS analysis method

For quality control of personalized preparations, it is not only necessary to quantify the content, but also to confirm the substance identity. However, reversed phase chromatography is not suitable to separate monoclonal antibodies from each other, since the retention factor is insufficient to distinguish mAbs. Since only one mAb occurs in each of the personalized preparations, DAD can still be used for quantification. High-resolution mass spectrometry (HRMS) can be used for intact mAbs identification. As can be seen in Figure 4-1, the monoclonal antibodies can be distinguished according to their mass after deconvolution of the high-resolution mass spectra.

Therefore, in the validation, the DAD data was used for quantification of the concentration. For identification, the mass of glycan modifications labeled in Figure 4-1 was determined. The glycomodifications G1F:G1F and G0F:G2F are isobaric and cannot therefore be resolved. The summary of the validation can be found in Table 4-1.

The validation results of the three monoclonal antibodies showed recoveries near 100% (Deviation < 5%), a linear calibration model (regression coefficient >0.995) and a high mass accuracy (standard deviation <10 Da). The method is therefore suitable for analyzing mAbs at personalized pharmaceutical preparations.

A total of 10 returned preparations from 7 different pharmacies were analyzed. Returns are personalized preparations that were manufactured by pharmacies for a patient but could not be administered to him. The returns included six preparations of bevacizumab, two preparations of trastuzumab, and two preparations of rituximab. The results can be seen in Table 4-2.

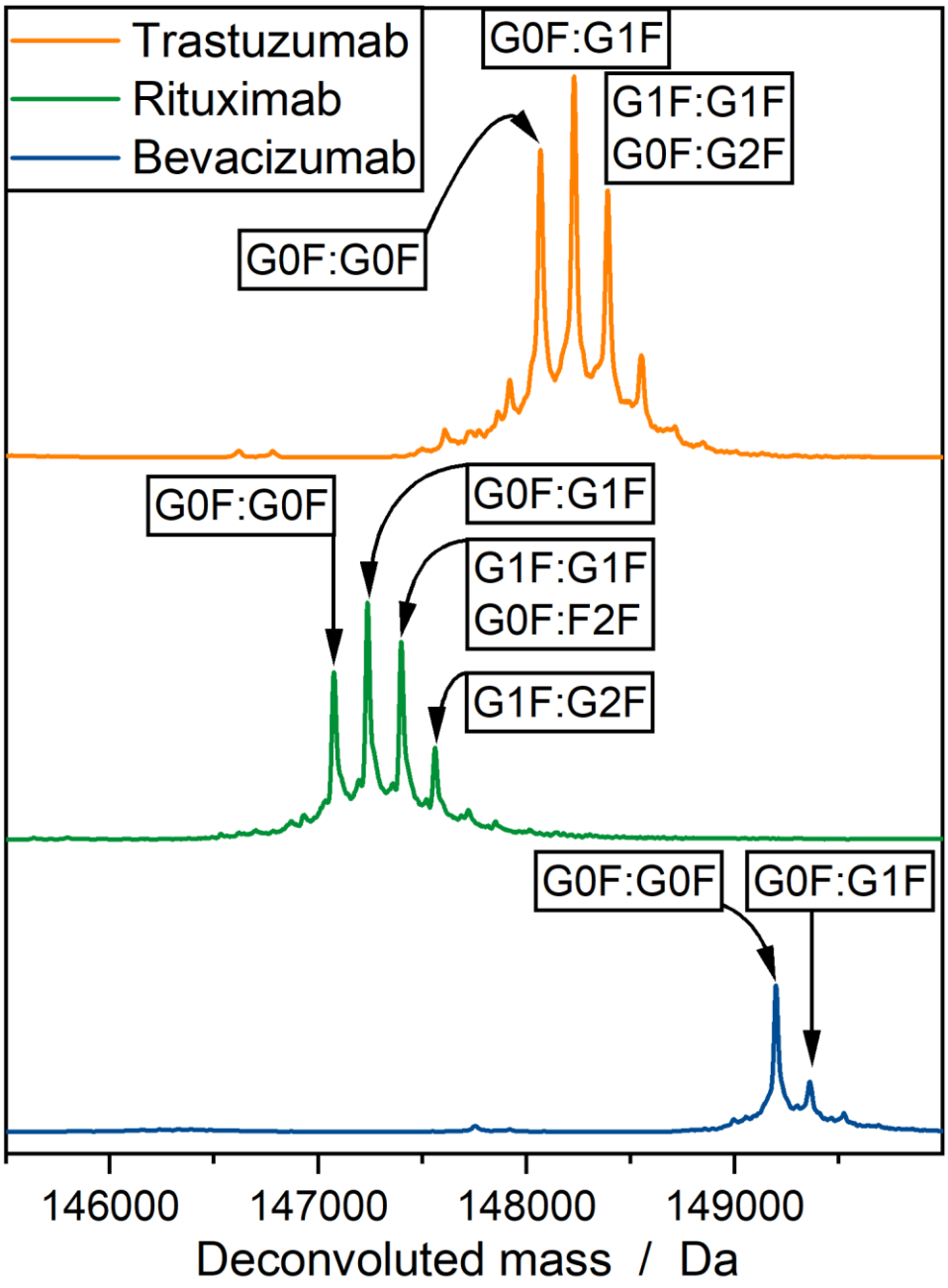


Figure 4-1: Deconvoluted mass spectra of the monoclonal antibodies trastuzumab (orange), rituximab (green), and bevacizumab (blue). The sugar modifications are labelled (details see text).

Table 4-1: Method validation performance parameters for the monoclonal antibodies bevacizumab, rituximab, and trastuzumab. Linearity was assessed over the working range of 0.5 - 5.0 mg mL^{-1} and carry-over by analysis of a blank sample following the 5.0 mg mL^{-1} standard. The intra-day assay variation test was performed by analyzing ten standards in sequence (n = 10). The inter-day assay variation test was performed by analyzing four samples on three different days (n = 12).

		Bevacizumab	Rituximab	Trastuzumab
	Linearity (R^2)	0.996	0.998	0.997
	Carry-Over (%)	< 0.1%	0.7	2
Intraday-Assay	Recovery (%)	101 ± 0.4	102 ± 3	100 ± 2
	G0F:G0F (Da)	149,199 ± 1	147,078 ± 3	148,067 ± 1
	G0F:G1F (Da)	149,361 ± 1	147,402 ± 3	148,228 ± 1
	G1F:G1F G0F:G2F (Da)	-	147,563 ± 3	148,390 ± 1
	G1F:G2F (Da)	-	147,563 ± 3	-
Inter-Day-Assay	Recovery (%)	102 ± 0.5	102 ± 4	101 ± 4
	G0F:G0F (Da)	149,201 ± 5	147,079 ± 3	148,061 ± 5
	G0F:G1F (Da)	149,362 ± 6	147,241 ± 3	148,222 ± 5
	G1F:G1F G0F:G2F (Da)	-	147,403 ± 3	148,383 ± 5
	G1F:G2F (Da)	-	147,564 ± 3	-

Table 4-2: Overview of the analyzed samples. The concentration calculated from the sample preparation, the measured concentration and the deviation of both concentrations from each other are indicated. The analysis was performed as a triplicate determination (n=3).

Analyt	Calculated concentration (mg mL^{-1})	Measured concentration (mg mL^{-1})	Deviation (%)
Bevacizumab	2.86	2.84 ± 0.14	1
Bevacizumab	1.52	1.52 ± 0.05	0.2
Bevacizumab	2.78	2.95 ± 0.04	6
Bevacizumab	2.85	3.12 ± 0.04	10
Bevacizumab	3.10	2.80 ± 0.01	10
Bevacizumab	2.37	2.43 ± 0.08	3
Rituximab	2.02	1.88 ± 0.02	7
Rituximab	2.06	1.87 ± 0.09	9
Trastuzumab	1.29	1.27 ± 0.04	2
Trastuzumab	1.30	1.41± 0.03	2

To ensure that preparations are not objectionable, a maximum mass deviation in identification of 10 Da and a content deviation of 10% are specified. The mean value of the triple determination serves as the basis for evaluation. Of all samples tested, the stated active pharmaceutical ingredient content and the active pharmaceutical ingredient identity could be confirmed.

This means that the preparation quality is very high, analogous to cytostatics.

4.4 Conclusion and outlook

An HPLC-DAD-HRMS analytical method was developed and validated to identify and quantify monoclonal antibodies in personalized drug products. The analysis of ten personalized preparations found none objectionable. This result is comparable to studies on cytostatic drugs.

Furthermore, the developed method may serve as a starting point for stability investigations of monoclonal antibodies. For this purpose, the workflow needs to be expanded to other analytical methods such as ion exchange or size exclusion chromatography.

4.5 References

1. Pharmacy ESoO (2018) QUAPOS 6 – quality standard for the oncology pharmacy service.

2. Sendra-Garcia A, Martinez-Gomez MA, Albert-Mari A, Jimenez-Torres NV, Climente-Marti M (2019) Quantitative and qualitative control of antineoplastic preparations: Gravimetry versus HPLC. J Oncol Pharm Pract 25 (5):1204-1216. doi:10.1177/1078155219834999

3. Benizri F, Dalifard B, Zemmour C, Henriquet M, Fougereau E, Le Franc B (2016) DrugCam(R)-An intelligent video camera system to make safe cytotoxic drug preparations. Int J Pharm 502 (1-2):198-207. doi:10.1016/j.ijpharm.2016.02.028

4. Reinders LMH, Klassen MD, Vom Eyser C, Teutenberg T, Jaeger M, Schmidt TC, Tuerk J (2021) Quality control of cytostatic drug preparations-comparison of workflow and performance of Raman/UV and high-performance liquid chromatography coupled with diode array detection (HPLC-DAD). Anal Bioanal Chem 413 (9):2587-2596. doi:10.1007/s00216-021-03223-9

5. Jochums M, Reinders LMH, Tuerk J, Teutenberg T (2022) Flexible Digitization of Highly Individualized Workflows Demonstrated Through the Quality Control of Patient-Specific Cytostatic Application Bags: Digitization from the Perspective of Small and Medium-Sized Laboratories. Adv Biochem Eng Biotechnol. doi:10.1007/10_2021_190

6. German Federal Supreme Court (2020) Urteil im sogenannten ‘Apotheker’-Verfahren wegen Verstoßes u.a. gegen das Arzneimittelgesetz rechtskräftig. https://www.bundesgerichtshof.de/SharedDocs/Pressemitteilungen/DE/2020/2020088.html.

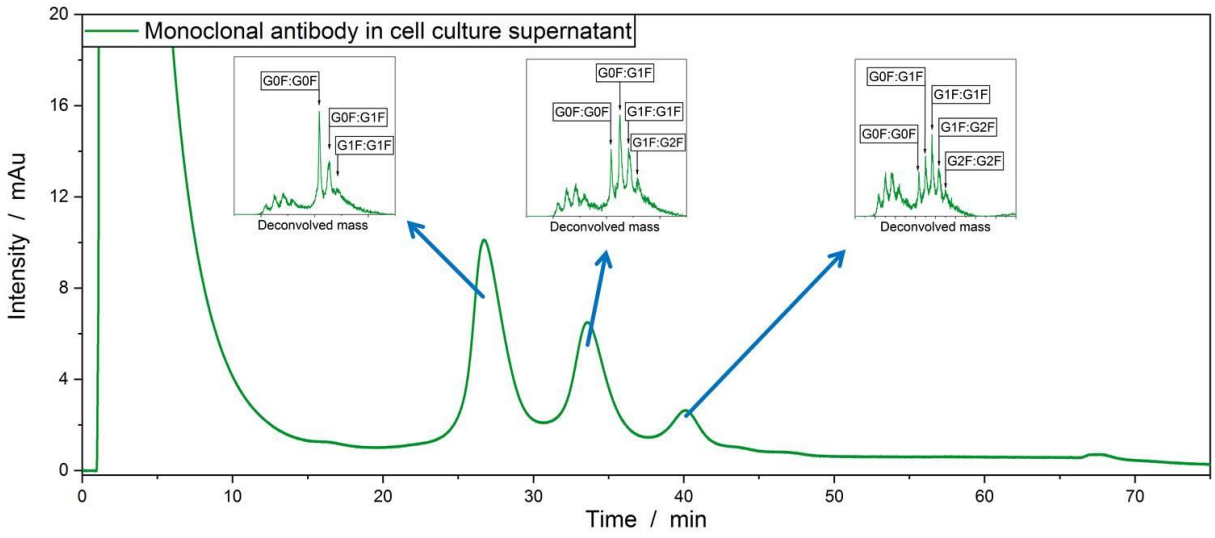
7. Personal communication between Lars Reinders (Institute for Energy and Environmental Technology) and Dominique-André Busch (Ministry of Labor, Health and Social Affairs of North Rhine-Westphalia) dated May 10, 2019 Reference IV B 5 - G.0611

Chapter 5 Development of a two-dimensional liquid chromatography high resolution mass spectrometry method for the characterization of monoclonal antibodies in cell-free culture supernatant via FcR affinity chromatography

This chapter was adapted from: Reinders, L.M.H., Klassen, M.D., Endres, P., Krumm, A., Jaeger, M., Schmidt, T.C. Teutenberg, T., Development of a two-dimensional liquid chromatography high resolution mass spectrometry method for the characterization of monoclonal antibodies in cell-free culture supernatant via FcR affinity chromatography. Chromatographia 86, 79-85 (2023) https://doi.org/10.1007/s10337-022-04228-x

Abstract: A monoclonal antibody‘s (mAb) glycosylation pattern has a significant impact on its binding affinity to the FcγRIIIa receptor and thus on antibody-dependent cell-mediated cytotoxicity. However, to analyse the relation in cell cultures, complex sample preparation is required. In this study, we demonstrate the analytical capability of a two-dimensional liquid chromatography-mass spectrometry (2D-HPLC-HRMS) method to analyze the affinity of mAbs to the FcγRIIIa receptor while simultaneously characterizing the glycoforms involved. Results were comparable in purified samples and cell culture-free supernatants. This was demonstrated with the monoclonal antibodies adalimumab, pertuzumab, ustekinumab, tocilizumab and omalizumab. Our method hence renders extensive purification of mAb samples superfluous.

Graphical Abstract:



5.1 Introduction

The majority of clinically approved biotherapeutics are monoclonal antibodies (mAbs), primarily belonging to the immunoglobulin G subclass 1 (IgG1) [1]. Glycosylation leads to post translational heterogeneity of mAbs. The glycosylation pattern is regarded as a critical quality attribute and thus requires monitoring during development, upstream and downstream processing as well as in pharmaceutical quality control [2]. Antibody glycosylation has a major impact on drug product stability, serum half-life, secretion, immunogenicity and function [3,4].

Functional regulation is achieved by binding of an IgG to cellular fragment crystallisable receptors (FcR), which depends on both the Fc domain glycosylation pattern and on the IgG subclass [5]. One such interaction is that of IgGs and the specific receptor FcγRIIIa. If the receptor binds with high affinity, the antibody-dependent cellular cytotoxicity (ADCC) will be increased, constituting a potential mode of action of antibodies [6, 14].

The highly conserved glycan moiety at position Asn-297 induces structural changes to the Fc region required for binding to the FcγRIIIa. Non-glycosylated antibodies have no affinity to the Fc receptor. The glycosylated part consists of a well-defined biantennary structure of a hepta-saccharide, made of N-acetylglucosamine (GlcNAc) and mannose and followed by variable additions of fucose, galactose and sialic acid [7]. Subtle distinctions in this glycan composition can affect the conformational rigidity of the Fc structure and may also alter the interaction with the Fc receptor by direct contact. In fucosylated and non-galactosylated mAbs, the strength of interaction between mAb and Fc receptor is diminished or non-existent, whereas the affinity increases in non-fucosylated and galactosylated mAbs [6].

Current methodologies to analyze Fc glycosylation and its impact on FcR binding rely on purified antibodies and multi-step processes. These include hydrophilic-interaction chromatography of released and labelled glycans for glycoprofiling [8] and surface-plasmon resonance (SPR) analysis for FcR-affinity analysis [9].

In this work, we demonstrate a novel method for analyzing mAbs by two-dimensional high-performance-liquid-chromatography high-resolution mass spectrometry (2D-HPLC-HRMS) in cell-free culture supernatant and purified samples. This method allows for a detailed screening of glycosylation pattern and ADCC of mAbs in upstream and downstream processing. It can be used for relative quantitation of glycoforms, especially when aiming at distinguishing changes in glycoform distribution. The applicability and strength of the method will be shown for adalimumab (ADLM), pertuzumab (PTZ), ustekinumab (UTKM), tocilizumab (TCLZ) and omalizumab (OMLZ).

5.2 Materials and methods

5.2.1 Chemicals and reagents

The cell-free culture supernatants of adalimumab, omalizumab, pertuzumab, tocilizumab and ustekinumab were purchased from Bioceros B. V. (rebranded to Polpharma Biologics Utrecht B.V. (Utrecht, Netherlands)). The cells of the cell cultures consisted of Chinese Hamster Ovary (CHO) cells. Acetonitrile (ACN) and water (H_2O) were provided in hypergrade (LiChrosolv) from Merck KGaA (Darmstadt, Germany). Furthermore, tris(hydroxymethyl)aminomethane (TRIS), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium acetate, sodium dihydrogen phosphate dihydrate and di-sodium hydrogen phosphate were purchased from Merck KGaA. Hydrochloric acid (HCl) was purchased from Fluka (St. Louis, USA) and sodium hydroxide (NaOH) from AppliChem (St. Louis, USA). Formic acid (FA) was obtained from Sigma-Aldrich (St. Louis, USA) and isopropanol from Th. Geyer (Renningen, Germany). Carl Roth (Karlsruhe, Germany) provided tri-sodium citrate dihydrate.

5.2.2 Cell fluid purification using protein A

Sodium phosphate buffer (0.1 M) with pH 7.0 was prepared. The pH was adjusted to 7.0 with 4 M sodium hydroxide solution using an inoLab pH 720 from WTW (Weilheim, Germany). A SkillPak AF-rProtein A HC-650F 5 mL column (Tosoh Bioscience GmbH, Griesheim, Germany), was equilibrated with three column volumes (CV) of 0.1 M sodium phosphate buffer at pH 7.0. Different mAb containing cell-free culture supernatants (10 mL) were loaded onto the column using a sample loop. The column was washed with three CV 0.1 M sodium phosphate buffer (sufficient to return baseline to zero). Afterwards, the bound mAb was eluted using three CV 0.1 M sodium acetate pH 3.0. Fractions of 1 mL were collected during elution. The column was cleaned with five CV of 0.2 M sodium hydroxide solution and re-equilibrated with three CV of sodium phosphate buffer. The fractions exhibiting a UV signal at 280 nm of > 20 mAU were pooled and the pH of the eluate was increased to 6.5 (± 0.25) using 1 M TRIS solution. The protein concentration for each eluate pool was measured offline using a NanoDrop 2000c photometer from ThermoFisher Scientific (Waltham, USA). An extinction coefficient of 1.4 mL mg^{-1} cm^{-1} was assumed (value for IgG in software version 1.6.198 of the NanoDrop 2000c) for all mAbs.

5.2.3 Online 2D-HPLC-HRMS

All analyses were performed using a 1290 Infinity II 2D-LC system (Agilent Technologies, Waldbronn, Germany) coupled to a 6560 Ion Mobility LC/Q-TOF mass spectrometer (Agilent Technologies, Waldbronn, Germany). The 2D-LC system consisted of two pumps, two column ovens, two diode array detectors, a multisampler and three valves. The 2D-LC system was controlled via OpenLab CDS Chemstation Edition Rev. C.01.09 [144] and the HRMS system via Masshunter Workstation software Build 9.0.9044.1 SP1 from Agilent Technologies (Waldbronn, Germany). For separation, a TSKgel FcR-IIIA-NPR 4.6 x 75 mm column (Tosoh Bioscience GmbH, Griesheim, Germany) was used in the first dimension at 15°C at a flow rate of 0.4 mL min^{-1}. The injection volume was 15 µL. Samples were diluted to 1 mg mL^{-1} with 0.9% NaCl before injection. The mobile phase was 50 mM tri-sodium citrate dihydrate + 150 mM NaCl at a pH of 6.7 for mobile phase A and at a pH of 4.7 for mobile phase B. The pH value was adjusted with HCl or NaOH and measured with the pH meter CG 840B (Schott, Ratingen, Germany). The gradient programme was: 0-10 min 1-1% B; 10-55 min 1-99% B; 55-65 min 99-99% B; 65-65.5 min 99-1% B; 65.5-75 min 1-1% B. The detection of the first dimension was done at a wavelength of 280 nm with a reference wavelength of 390 nm. The heart-cut mode was used to transfer the sample from the first to the second separation dimension, whereas the signals were transferred at peak maximum. The sampling time was 0.1 min which corresponds to 40 µL. On the second separation dimension, a TSKgel Protein C4-300, 3 µm, 2 x 50 mm column (Tosoh Bioscience GmbH, Griesheim, Germany) was used at 50°C and at a flow rate of 0.3 mL min^{-1}. As mobile phases (A) H_2O + 0.1% FA and (B) IPA/ACN/H_2O (70:20:10 *v/v/v*) + 0.1% FA were used. The IPA content in mobile phase B serves to increase the elution strength, compared to pure ACN. Furthermore, IPA leads to better unfolding and solvation of the unfolded protein. This leads to minimization of secondary interactions, resulting in a sharper peak. At the same time, recovery is improved. In addition to chromatographic properties, IPA improves ESI ionization by lowering the surface tension of the solution [15]. The ACN content in mobile phase B also leads to unfolding of the protein. However, following a different mechanism, allowing the protein to stabilize more charges during ionization and to be detected at lower mass-to-charge ratios [16]. The gradient programme was: 0-1 min 5-5% B; 1-1.1 min 5-40% B; 1.1-4 min 40-40% B; 4-4.1 min 40-5% B; 4.1-6 min 5-5% B. For detection in the second dimension, HRMS was used. The settings of the HRMS instrument were: Instrument mode: High (*m/z* 20000); Slicer Mode: High Resolution; Ion Source: Dual AJS ESI; Ion polarity: Positive; Acquisition mode: QTOF-only; LC-Stream: First minute per cut to waste; Gas temperature: 350°C; Drying gas: 12 L min^{-1};

Nebulizer: 60 psig; Sheath gas temperature: 400°C; Sheath gas flow: 11 L min^{-1}; capillary voltage (VCap): 5500 V; Nozzle voltage: 2000 V; Fragmentor voltage: 380 V; Octopole rod repel voltage (Oct 1 RF Vpp): 750 V; Mass range: *m/z* 500-5000; Acquisition Rate: 1 spectrum s^{-1}. Deconvolution was performed using MassHunter workstation software BioConfirm version 10.0 build 10.0.10136.0 (Agilent Technologies, Waldbronn, Germany).

5.3 Results and Discussion

5.3.1 FcR chromatography using 1D-HPLC-DAD

Matrix influences can negatively affect chromatographic procedures and falsify the analytical result. To verify the absence of matrix influence, cell-free culture supernatants and purified samples were analyzed. The matrix content differed by a factor of 175 (evaluation by height; see Supplementary information).

As can be seen from Figure 5-1, the matrix eluted in the range of 0-15 min, whereas the monoclonal antibodies were in the retention time range of 20-45 min. This means that all five monoclonal antibodies tested possessed affinity to the immobilized FcγRIIIa ligand. Due to this effective matrix separation, there was no significant difference in the results of the determination of peak areas (Table 5-1) and retention times (Table 5-2). The difference of the results of peak areas was 0.4% on average (min-max: 0.0-1.2%) and was 0.20 min (min-max: 0.02-0.71 min) in retention times. The individual values of the results can be taken from the Supplementary information.

This means that time- and resource-consuming purification steps were not needed, which makes quality control results available more quickly during production and allows prompt process control.

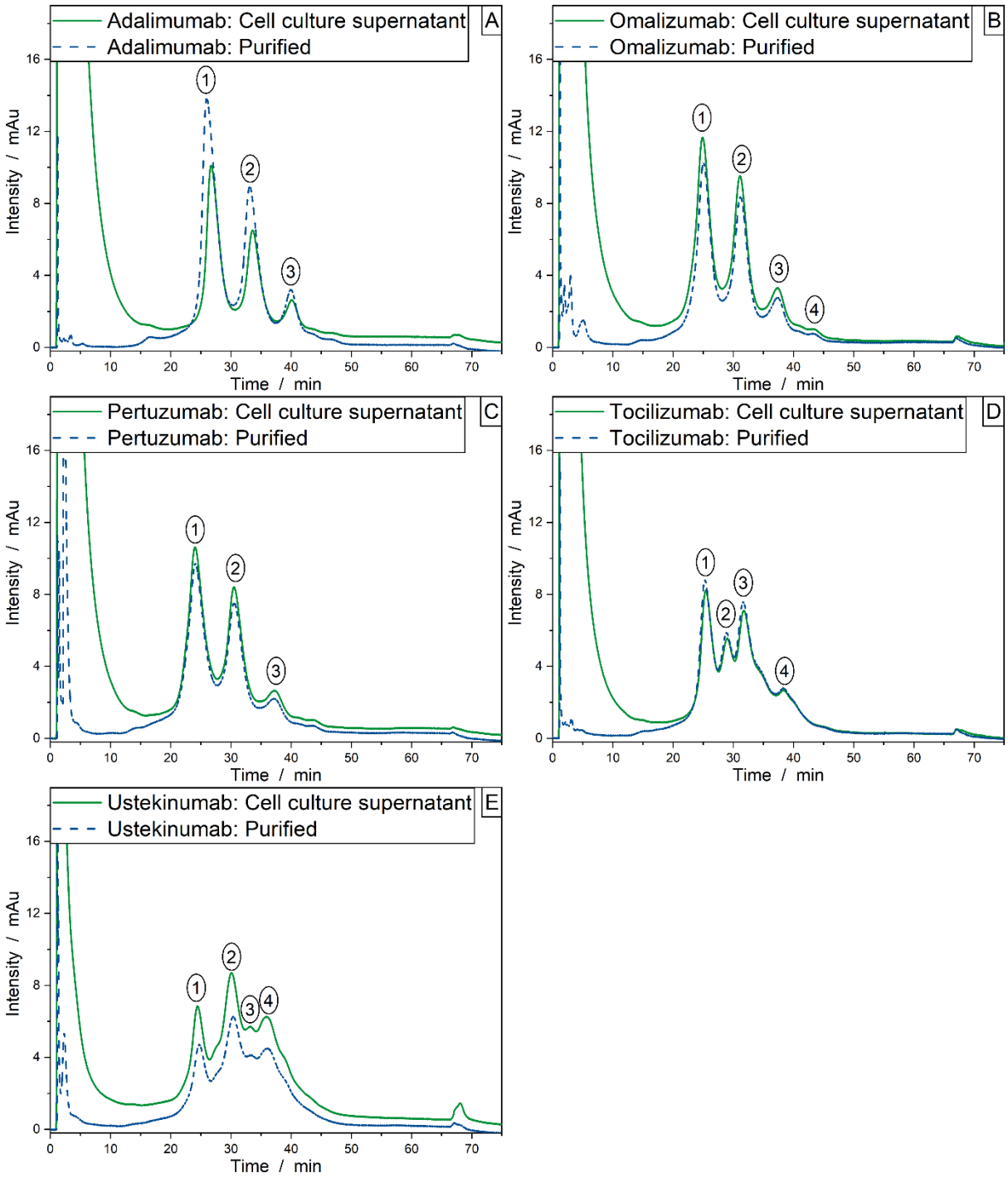


Figure 5-1: Comparison of chromatograms of five monoclonal antibodies in cell culture-free supernatant (green, solid) and purified samples (blue, dashed).

Table 5-1: Relative area percentages and standard deviation of the relevant peaks of the FcR affinity chromatography. The relevant signals correspond to the fraction numbers in Figure 5-1. All analyses were performed as triplicates (n=3). ccs: cell-free culture supernatant; pur: purified; ADLM: adalimumab; PTZ: pertuzumab; UTKM: ustekinumab; TCLZ: tocilizumab; OMLZ: omalizumab; n. d.: not detected.

	Peak no. 1 (Area %)	Peak no. 2 (Area %)	Peak no. 3 (Area %)	Peak no. 4 (Area %)
ADLM ccs	58.7 ± 0.2	32.2 ± 0.5	9.1 ± 0.4	n. d.
ADLM pur	59.8 ± 0.4	31.9 ± 0.3	8.3 ± 0.4	n. d.
PTZ ccs	57.9 ± 0.5	35.7 ± 0.4	6.4 ± 0.6	n. d.
PTZ pur	57.8 ± 0.3	36.0 ± 0.1	6.3 ± 0.2	n. d.
UTKM ccs	39.0 ± 0.1	41.8 ± 0.4	1.6 ± 0.3	17.6 ± 0.8
UTKM pur	38.8 ± 0.4	41.9 ± 0.9	1.1 ± 0.1	18.2 ± 0.1
TCLZ ccs	56.7 ± 1.0	11.4 ± 0.5	28.0 ± 0.1	3.9 ± 0.8
TCLZ pur	56.6 ± 0.5	11.6 ± 0.2	28.7 ± 0.4	3.1 ± 0.1
OMLZ ccs	53.5 ± 0.5	36.8 ± 0.8	8.7 ± 0.3	1.0 ± 0.1
OMLZ pur	52.3 ± 0.5	38.0 ± 0.5	8.7 ± 0.4	1.0 ± 0.3

Table 5-2: Retention times and Standard deviation of relevant signals of the FcR affinity chromatography via retention time. The evaluated signals are marked by numbers in Figure 5-1. All analyses were performed as triplicate (n=3). ccs: cell-free culture supernatant; pur: purified; ADLM: adalimumab; PTZ: pertuzumab; UTKM: ustekinumab; TCLZ: tocilizumab; OMLZ: omalizumab; n. d.: not detected.

	Peak no. 1 (min)	Peak no. 2 (min)	Peak no. 3 (min)	Peak no. 4 (min)
ADLM ccs	26.71 ± 0.04	33.59 ± 0.05	40.12 ± 0.07	n. d.
ADLM pur	25.97 ± 0.03	33.11 ± 0.01	39.94 ± 0.01	n. d.
PTZ ccs	24.00 ± 0.01	30.50 ± 0.04	37.39 ± 0.01	n. d.
PTZ pur	24.13 ± 0.02	30.57 ± 0.03	37.37 ± 0.09	n. d.
UTKM ccs	24.45 ± 0.04	30.09 ± 0.04	33.23 ± 0.03	36.13 ± 0.09
UTKM pur	24.75 ± 0.03	30.35 ± 0.10	33.47 ± 0.00	36.43 ± 0.09
TCLZ ccs	25.40 ± 0.05	28.99 ± 0.05	31.75 ± 0.09	38.51 ± 0.00
TCLZ pur	25.26 ± 0.01	28.87 ± 0.02	31.62 ± 0.03	38.39 ± 0.11
OMLZ ccs	24.89 ± 0.03	31.12 ± 0.04	37.42 ± 0.02	43.64 ± 0.06
OMLZ pur	25.04 ± 0.02	31.21 ± 0.01	37.39 ± 0.02	43.60 ± 0.06

5.3.2 FcR chromatography using 2D-HPLC-HRMS

The chromatographic method although robust to matrix effects used non-volatile salts in the mobile phase. This means that direct coupling to high-resolution mass spectrometry (HRMS) was not desirable. To enable detection by HRMS, 2D-HPLC was used, with the second dimension serving solely for desalting. This makes it possible to couple an efficient chromatographic method with a highly informative detection system.

Interestingly, high-resolution mass spectrometry for adalimumab and pertuzumab (See Figure 5-2) revealed additional signals with a lower mass. These signals were not observed for the other antibody samples. The mass differences were 1,004 Da ± 8 Da for adalimumab and 1,034 Da ± 6 Da for pertuzumab. The mass difference was not sufficiently large as due to the absence of a whole heavy or light chain or a glycan, such as G0, G0F, G1F etc. It may be interpreted as caused by the presence of proteases in the cell-free culture supernatant, leading to the cleavage of amino acids [10-13]. The retention mechanism used for chromatography was based on the interaction with the Fc region of a mAb. Since the putative digestion product co-eluted with the intact mAb in the same fraction, it can be assumed that the degradation did not occur in the Fc region but in the fragment antigen binding (Fab) region. As one possible explanation may be the loss of amino acids at the light chain, the sequence DIQMTQSPS (1,005 Da) at the N-terminal end would explain the difference for adalimumab. The peptide TKSFNRGEC (1,040 Da) at the C-terminal end would match the difference in the case of pertuzumab. Yet, the HRMS-spectral pattern might also originate from a superposition of signals from unrelated or partially related modifications. Hence, no unequivocal statement can be made without further investigations.

Independent of those signals, the glycoforms for all five investigated mAbs were examined. A summary of the results can be found in Table 5-3. The results show that glycomodifications with a higher galactose content such as G2F:G2F were eluted later than modifications with a lower content. Hence higher galactose content leads to an increased affinity to the immobilised Fc receptor. Furthermore, a partially non-fucosylated glycomodification (G0:G0F) was identified in the samples containing pertuzumab and omalizumab from the second fraction onwards, which suggests a lower affinity for fucose. The data also show that complete baseline separation of the glycan modifications is not achieved with FcR affinity chromatography, since later eluting fractions (fraction 3 and 4) also contained glycomodifications found in fraction 1, e. g. G0F:G0F.

Nevertheless, it can be concluded, allowing not only quality control, but also cell culture screening and optimization towards increased receptor affinity.

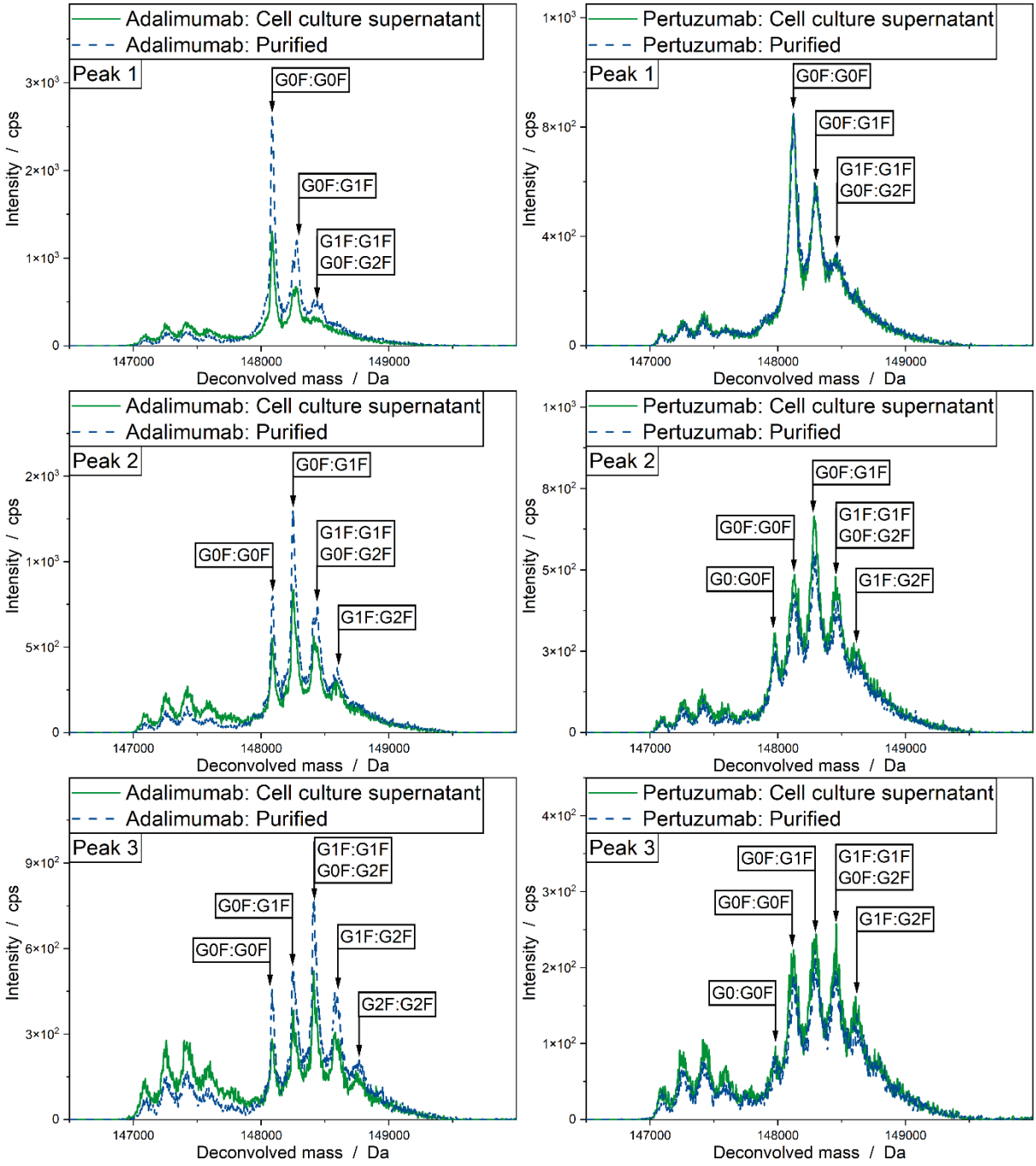


Figure 5-2: Deconvolved HRMS spectra of signals from Figure 5-1A and Figure 5-1C for adlimumab and pertuzumab in cell-free supernatant (green, solid) and purified samples (blue, dashed).

Table 5-3: Assignment of glycan modifications as a function of the peaks shown in Figure 5-1 for the monoclonal antibodies. D: Detected sugar modification within a peak. M: Most intensive glycan modification within a peak. P: Peak in which the glycan modification is most intense. ADLM: adalimumab; PTZ: pertuzumab; UTKM: ustekinumab; TCLZ: tocilizumab; OMLZ: omalizumab.

mAb	Peak (no.)	G0:G0F	G0F:G0F	G0F:G1F	G1F:G1F	G1F:G2F	G2F:G2F
ADLM	1	-	D, M, P	D	D	-	-
	2	-	D	D, M, P	D	D	-
	3	-	D	D	D, M, P	D, P	D, P
PTZ	1	-	D, M, P	D	D	-	-
	2	D, P	D	D, M, P	D, P	D, P	-
	3	D	D	D	D, M	D	-
UTKM	1	-	D, P	D, M, P	D, P	-	-
	2	-	D	D	D, M	-	-
	3	-	D	D	D, M	-	-
	4	-	D	D	D	D, M, P	-
TCLZ	1	-	D, P	D, M, P	D	-	-
	2	-	D	D, M	D	D	-
	3	-	D	D	D, M, P	D, P	-
	4	-	D	D	D	D, M	-
OMLZ	1	-	D, M, P	D, P	D, P	-	-
	2	D, P	D	D, M	D	-	-
	3	D	D	D	D, M	D, P	D, P
	4	D	D	D	D, M	D	D

5.4 Conclusion

The data demonstrate that FcR chromatography provides comparable peak areas, retention times and masses regardless of whether the samples are heavily matrix loaded (cell culture supernatant) or purified. This was shown when using a 1D-HPLC-DAD and a 2D-HPLC-HRMS coupling.

Via the obtained data, it could be shown that glycan modifications with a higher galactose or lower fucose content have a higher affinity for FcγRIIIa.

5.5 Supplementary Information

5.5.1 Illustration of the matrix signal in the cell-free culture supernatant

The matrix signal in the cell-free supernatant has a height of 2100 mAu, whereas it has a height of 12 mAu in the purified sample. Thus, it differs by a factor of 175. For clarification, both chromatograms for adalimumab are shown in Figure 5-3.

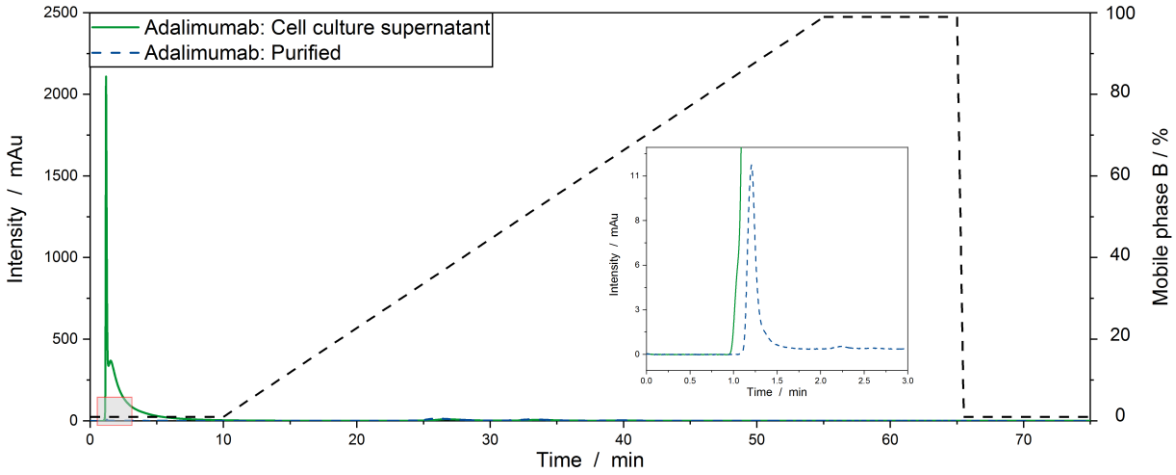


Figure 5-3: Comparison of chromatograms from cell-free supernatant and purified sample for adalimumab. For better visualization of the matrix signal of the purified sample, the section was enlarged in the range of 0-3 min. In addition, the solvent gradient is shown in black dashed.

5.5.2 Deviation of relative peak areas between cell-free culture supernatant and purified sample

The difference in the determination of peak areas is 0.4% on average (min-max: 0.0-1.2%) and is 0.20 min (min-max: 0.02-0.71 min) in the determination of retention times. The individual values can be taken from Table 5-4.

Table 5-4: Comparison of differences in peak areas and retention times in cell culture free supernatants and purified samples. ADLM: adalimumab; PTZ: pertuzumab; UTKM: ustekinumab; TCLZ: tocilizumab; OMLZ: omalizumab; n/a.: not applicable.

	Peak no. 1		**Peak no. 2**		**Peak no. 3**		**Peak no. 4**	
	(Area %)	(min)	(Area %)	(min)	(Area %)	(min)	(Area %)	(min)
ADLM	1.1	0.7	0.2	0.5	0.8	0.2	n/a	n/a
PTZ	0.1	0.1	0.2	0.1	0.1	0.0	n/a	n/a
UTKM	0.2	0.3	0.1	0.3	0.5	0.2	0.6	0.3
TCLZ	0.1	0.1	0.2	0.1	0.6	0.1	0.8	0.1
OMLZ	1.2	0.1	0.1	0.1	0.0	0.0	0.0	0.1

5.6 References

1. Lu RM, Hwang YC, Liu IJ, Lee CC, Tsai HZ, Li HJ, Wu HC (2020) Development of therapeutic antibodies for the treatment of diseases. J Biomed Sci 27 (1):1. doi:10.1186/s12929-019-0592-z

2. Reusch D, Tejada ML (2015) Fc glycans of therapeutic antibodies as critical quality attributes. Glycobiology 25 (12):1325-1334. doi:10.1093/glycob/cwv065

3. Arnold JN, Wormald MR, Sim RB, Rudd PM, Dwek RA (2007) The impact of glycosylation on the biological function and structure of human immunoglobulins. Annu Rev Immunol 25:21-50. doi:10.1146/annurev.immunol.25.022106.141702

4. Pincetic A, Bournazos S, DiLillo DJ, Maamary J, Wang TT, Dahan R, Fiebiger BM, Ravetch JV (2014) Type I and type II Fc receptors regulate innate and adaptive immunity. Nat Immunol 15 (8):707-716. doi:10.1038/ni.2939

5. Bournazos S, Gupta A, Ravetch JV (2020) The role of IgG Fc receptors in antibody-dependent enhancement. Nat Rev Immunol 20 (10):633-643. doi:10.1038/s41577-020-00410-0

6. Ferrara C, Grau S, Jager C, Sondermann P, Brunker P, Waldhauer I, Hennig M, Ruf A, Rufer AC, Stihle M, Umana P, Benz J (2011) Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcgammaRIII and antibodies lacking core fucose. Proc Natl Acad Sci U S A 108 (31):12669-12674. doi:10.1073/pnas.1108455108

7. Dekkers G, Plomp R, Koeleman CA, Visser R, von Horsten HH, Sandig V, Rispens T, Wuhrer M, Vidarsson G (2016) Multi-level glyco-engineering techniques to generate IgG with defined Fc-glycans. Sci Rep 6:36964. doi:10.1038/srep36964

8. Zhang L, Luo S, Zhang B (2016) Glycan analysis of therapeutic glycoproteins. MAbs 8 (2):205-215. doi:10.1080/19420862.2015.1117719

9. Geuijen KPM, Oppers-Tiemissen C, Egging DF, Simons PJ, Boon L, Schasfoort RBM, Eppink MHM (2017) Rapid screening of IgG quality attributes - effects on Fc receptor binding. FEBS Open Bio 7 (10):1557-1574. doi:10.1002/2211-5463.12283

10. Robert F, Bierau H, Rossi M, Agugiaro D, Soranzo T, Broly H, Mitchell-Logean C (2009) Degradation of an Fc-fusion recombinant protein by host cell proteases: Identification of a CHO cathepsin D protease. Biotechnol Bioeng 104 (6):1132-1141. doi:10.1002/bit.22494

11. Laux H, Romand S, Nuciforo S, Farady CJ, Tapparel J, Buechmann-Moeller S, Sommer B, Oakeley EJ, Bodendorf U (2018) Degradation of recombinant proteins by Chinese hamster ovary host cell proteases is prevented by matriptase-1 knockout. Biotechnol Bioeng 115 (10):2530-2540. doi:10.1002/bit.26731

12. Dorai H, Santiago A, Campbell M, Tang QM, Lewis MJ, Wang Y, Lu QZ, Wu SL, Hancock W (2011) Characterization of the proteases involved in the N-terminal clipping of glucagon-like-peptide-1-antibody fusion proteins. Biotechnol Prog 27 (1):220-231. doi:10.1002/btpr.537

13. Luo H, Tie L, Cao M, Hunter AK, Pabst TM, Du J, Field R, Li Y, Wang WK (2019) Cathepsin L Causes Proteolytic Cleavage of Chinese-Hamster-Ovary Cell Expressed Proteins During Processing and Storage: Identification, Characterization, and Mitigation. Biotechnol Prog 35 (1):e2732. doi:10.1002/btpr.2732

14. Reinders, L.M.H., Klassen, M.D., Teutenberg, T. et al. Comparison of originator and biosimilar monoclonal antibodies using HRMS, Fc affinity chromatography, and 2D-HPLC. Anal Bioanal Chem (2022). https://doi.org/10.1007/s00216-022-04236-8

15. Dillon TM, Bondarenko PV, Rehder DS, Pipes GD, Kleemann GR, Ricci MS. Optimization of a reversed-phase high-performance liquid chromatography/mass spectrometry method for characterizing recombinant antibody heterogeneity and stability. J Chromatogr A. (2006) 7; 1120 1-2: 112-20. doi: 10.1016/j.chroma.2006.01.016.

16. Bondarenko PV, Second TP, Zabrouskov V, Makarov AA, Zhang Z. Mass measurement and top-down HPLC/MS analysis of intact monoclonal antibodies on a hybrid linear quadrupole ion trap-Orbitrap mass spectrometer. J Am Soc Mass Spectrom. (2009) 8:1415-24. doi: 10.1016/j.jasms.2009.03.020.

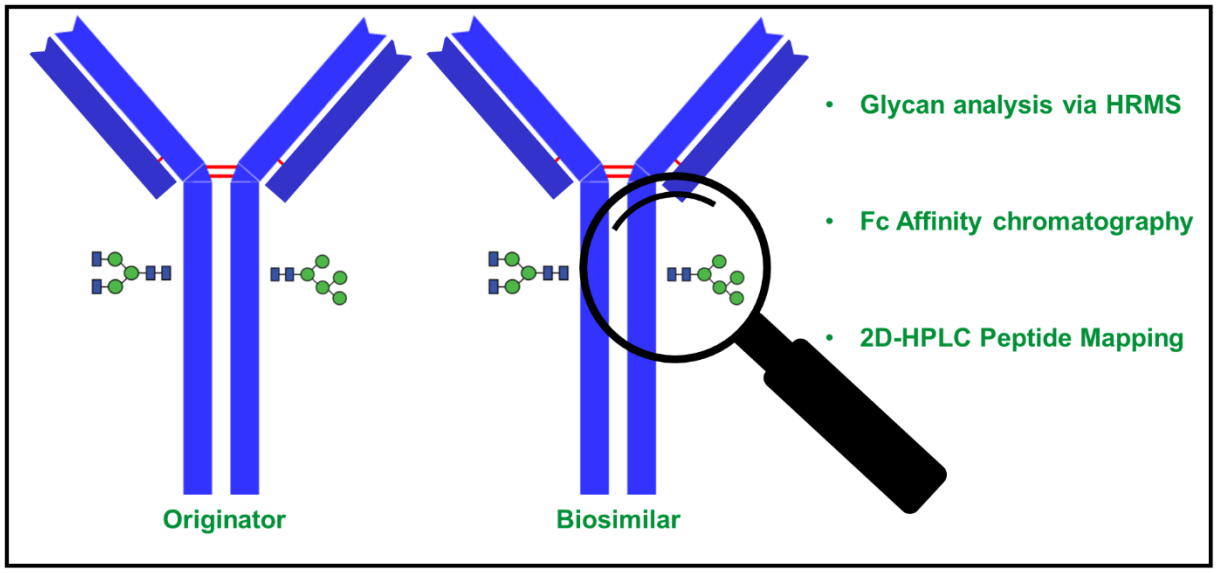
Chapter 6 Comparison of originator and biosimilar monoclonal antibodies using HRMS, Fc affinity chromatography and 2D-HPLC

This chapter was adapted from: Reinders, L.M.H., Klassen, M.D., Teutenberg, T., Jaeger, M., Schmidt, T.C., Comparison of originator and biosimilar monoclonal antibodies using HRMS, Fc affinity chromatography, and 2D-HPLC. Anal Bioanal Chem 414, 6761–6769 (2022). https://doi.org/10.1007/s00216-022-04236-8

Abstract: Due to the complex manufacturing process of therapeutic monoclonal antibodies, it is hardly possible to produce an identical copy of the original product (originator). Consequently, follow-on products (biosimilars) must demonstrate their efficacy being similar to the originator in terms of structure and function. During this process, a variety of analytical methods are required for this purpose. This study focuses on three particularly relevant analytical techniques: High-resolution mass spectrometry, fragment crystallisable (Fc) affinity chromatography and two-dimensional peptide mapping. Each analytical method proved able to identify specific differences between originator and biosimilar.

High resolution mass spectrometry was used to characterize the glycan pattern. It was shown that a trastuzumab biosimilar did not have the G0:G0F sugar modification identified in the originator. The application of affinity chromatography to rituximab showed that originator and biosimilar interacted differently with the immobilized Fc receptor. Furthermore, 2D-HPLC peptide mapping demonstrated the influence of orthogonality of separation dimensions, leading to differentiation of a rituximab originator and biosimilar.

Graphical Abstract:



6.1 Introduction

Therapeutic monoclonal antibodies (mAbs) represent a group of drugs with significant market growth [1]. They are proteins with a molecular weight of about 150,000 Da. Each antibody is divided into two light (25,000 Da) and two heavy chains (50,000 Da) [2,3]. Their production is usually carried out in animal cell cultures such as chinese hamster ovary cells (CHO) or mouse myeloma cells (NS0 and SP2/0) [1,3]. These mammalian expression systems lead to a characteristic glycosylation pattern ensuring therapeutic efficacy [1].

The highly complex production process also has an impact on the generation of post-translational modifications, which is the reason for it being impossible to produce an exact copy of the original substance (originator). The follow-on products are therefore called biosimilars and not generics. However, in order to ensure the efficacy and safety of the biosimilar, the regulatory authorities only accept minor modifications from the originator [3].

The European Medicines Agency (EMA) [4] has therefore published a guideline to assess the similarity of biological medicinal products containing monoclonal antibodies. Herein, a stepwise approach consisting of non-clinical and clinical studies is recommended. Step one of the non-clinical studies includes in vitro assays such as determination of binding to antigens or to the respective isoforms of the relevant fragment crystallisable (Fc) gamma receptors. In step two, it is to be assessed whether additional in vivo studies are necessary. Among other things, the presence of additional quality attributes (e.g. new post-translational modifications) that were not present in the originator must be taken into account. On the basis of the analytical evaluation, a decision must be made whether in vivo studies are required or clinical trials should be initiated. The scope of the clinical studies needed will depend on the results of the non-clinical studies, and a stepwise approach will be taken for these as well [4].

However, the characterization of monoclonal antibodies does not end with the approval of the drug, as a continuous quality monitoring of the active pharmaceutical ingredient (API) has to be performed. This is because fluctuations in the production process or up-scaling can lead to modifications such as an altered glycosylation pattern [5-7].

Modern analytical methods are required for the detection of biosimilarity and subsequent analytical quality control. An elegant approach is the characterization of the glycosylation pattern by high-resolution mass spectrometry (HRMS). The glycosylation pattern of mAbs has an impact on safety and efficacy, so that its characterization is mandatory [8]. The determination of molecular weight (MW) by LC-HRMS is useful for this purpose, which has the advantage that no additional sample preparation steps are required [3,9]. However, it should be noted that

it is not the MW that is determined, but rather the most frequent isotopic mass, which nevertheless approximates the MW in the case of large macromolecules such as mAbs [20]. However, ionization in the mass spectrometer of intact monoclonal antibodies results in a charge pattern which is superimposed by the isotope pattern. Such superposition exists for all glycosylation species. These complex patterns can be converted by deconvolution algorithms into the most frequent isotope mass or, approximately, the MW [9].

Although an HRMS approach can provide information about the presence or absence of post-translational modifications, it is difficult to link them to a therapeutic effect. For the correlation, appropriate binding assays or affinity chromatography need to be used. For example, retention time in affinity chromatography can be correlated with antibody-dependent cell-mediated cytotoxicity (ADCC). Furthermore, affinity chromatography offers the possibility to use low-cost spectroscopic detection methods without the need for HRMS measurements. Despite the use of low-cost detection techniques, the robustness of the method is not compromised since other matrix components interact only slightly with the stationary phase and can therefore be chromatographically separated from the analyte [12].

In addition to the analysis of intact monoclonal antibodies, peptide mapping needs to be performed for complete characterisation, as some modifications would otherwise remain undetected. An example of this is deamidation, which leads to a mass shift of only 1 Da [2]. However, one-dimensional liquid chromatography usually does not have the necessary peak capacity to allow complete sequence recovery [15]. To increase peak capacity, comprehensive two-dimensional liquid chromatography (LC x LC) can be used. Here, a sample mixture is first chromatographically separated and the eluate of the column is continuously transferred to a second separation phase with different separation mechanism. How much the separation mechanisms differ with respect to the analyte can be indicated by calculation the orthogonality [16-19]. In simplified terms, this describes how well the substances are distributed across the two-dimensional separation space.

The aim of this study therefore was to demonstrate that different analytical methods can be used for the characterization of the originator and biosimilar. Specifically, the advantages and disadvantages of glycan analysis by LC-HRMS, Fc chromatography, and 2D HPLC will be discussed and demonstrated using analysis examples of originator and biosimilar.

6.2 Materials and methods

6.2.1 Chemicals and reagents

The monoclonal antibodies bevacizumab, cetuximab, daratumumab, omalizumab, rituximab and trastuzumab were sourced as originator, which includes the drugs Avastin (Hoffmann-La Roche, Basel, Switzerland), Erbitux (Merck KGaA, Darmstadt, Germany), Darzalex (Janssen-Cilag GmbH, Neuss, Germany), Xolair (Novartis Pharma, Basel, Switzerland), MabThera (Hoffmann-La Roche, Basel, Switzerland) and Herceptin (Hoffmann-La Roche, Basel, Switzerland). Furthermore, the biosimilars Truxima (Celltrion Healthcare Hungary Kft., Budapest, Hungary), Rixathon (Sandoz, Holzkirchen, Germany) and Herzuma (Celltrion Healthcare Hungary Kft., Budapest, Hungary) were used. In addition, a rituximab research sample was provided by Tosoh Bioscience GmbH, Darmstadt, Germany. Water, acetonitrile and sodium chloride were purchased from Merck KGaA (Darmstadt, Germany). Formic acid (FA), PBS buffer, sodium acetate, guanidine hydrochloride (GuHCl), dithiothreitol (DTT), ammonium bicarbonate (NH_4HCO_3) and tris(hydroxymethyl)aminomethane hydrochloride (TrisHCl) were purchased from Sigma-Aldrich (St- Louis, USA). Sequencing grade modified trypsin was purchased from Promega (Madison, USA). Isopropanol (IPA) was obtained from Th. Geyer (Renningen, Germany), hydrochloric acid from Fluka (St. Louis, USA), sodium hydroxide and ammonia from AppliChem (Darmstadt, Germany).

6.2.2 Analysis conditions and detector settings for the determination of the glycan pattern by LC-HRMS

The analytical system consists of a 1260 Infinity II (Agilent Technologies, Waldbronn, Germany) liquid chromatography system coupled to a 6560 Ion Mobility LC/Q-TOF (Agilent Technologies, Waldbronn, Germany). The liquid chromatography system includes pump, autosampler, column thermostat, and diode array detector. The whole system was controlled via Masshunter Workstation software (Build 9.0.9044.1 SP1) from Agilent Technologies (Waldbronn, Germany). Separation was performed at 80°C. The mobile phase consisted of water + 0.1% formic acid (mobile phase A) and acetonitrile/isopropanol/water (70:20:10 *v/v/v*) + 0.1% formic acid (mobile phase B). A Zorbax 300 SB-C8 2.1 x 150 mm, 5 µm (Agilent Technologies, Waldbronn, Germany) stationary phase was installed. A Zorbax 300 SB-C8 2.1 x 12.5 mm, 5 µm (Agilent Technologies, Waldbronn, Germany) was used as precolumn. The injection volume was 0.1 µL and the flow rate was set to 0.5 mL min^{-1}. The concentration of the API was 2.5 mg mL^{-1}. The gradient program was: 0.0-2.0 min 5-5% B; 2.0-3.0 min 5-50% B; 3.0-6.0 min 50-50% B; 6.0-6.5 min 50-90% B; 6.5-9.5 min 90-90% B; 9.5-10.5 min 90-5%

B; 10.0-14.0 min 5-5% B. The mass spectrometer recorded all signals in the range of *m/z* 500-5000. ESI positive was selected as ionization mode. The other settings were: 350°C gas temperature, 12 L min^{-1} gas flow, 60 psig nebulizer gas, 400°C sheat gas temperature, 11 L min^{-1} sheat gas flow, 5500 V capillary voltage (VCap), 2000 V nozzle voltage, 380 V fragmentor voltage, 750 V octopole rod repel voltage (OctopoleRPPeak).

The signals of the antibody were evaluated using Masshunter BioConfirm (Build 10.0.10136.0) from Agilent Technologies (Waldbronn, Germany). For deconvolution using the Maximum Entropy (MaxEnt) algorithm, the signals from *m/z* 2000 - 3500 with a retention time of 5.1 - 5.8 min are used, which have a signal-to-noise ratio of at least 30. The baseline is adjusted by a factor of 7. The mass range to be calculated is 140,000 - 160,000 Da with a mass resolution of 0.05 Da. The charge states are considered to be proton adducts.

6.2.3 2D-HPLC and the FC affinity chromatography method

A 1290 Infinity II 2D-LC system (Agilent Technologies, Waldbronn, Germany) was used. It consisted of two HPLC pumps, two column ovens, two diode array detectors, a multisampler and three valve drives, yet only the flow path of the first dimension was used. The system was controlled via OpenLab CDS Chemstation Edition Rev. C.01.09 [144] (Agilent Technologies, Waldbronn, Germany). A TSKgel FcR-3A-NPR 4.6 x 75 mm stationary phase (Tosoh Bioscience GmbH, Darmstadt, Germany) at 15°C was selected for analysis. A 50 mM citrate buffer was used as mobile phase. Mobile phase A was adjusted to a pH of 5.9 and mobile phase B to 4.3. The flow rate was set to 0.5 mL min^{-1}, and the injection volume to 5 µL. The concentration of the API was 5 mg mL^{-1}. The gradient program was 0-20 min 0-62.5% B, 20-20.1 min 62.5-100% B, 20.1-40 min 100-100% B, 40-40.1 min 100-0% B, 40.1-50 min 0-0% B. Detection was performed at 280 nm.

6.2.4 Selectivity screening for 2D-HPLC

Selectivity screening was performed using the 2D HPLC system described above. However, the injection volume was increased to 20 µL. The preparation of the samples, as well as their concentration, are described below. The modulation time was 1 min for RPC methods (pH 2.7) in the second dimension and 1.5 min for HILIC methods. An active solvent modulation (ASM) factor of 3 was used. The importance and use of an ASM valve is explained in the supplementary information. The combinations of stationary phases and pH tested were RP(pH 10)/RP(pH 2.7), RP(pH 10)/HILIC, RP(pH 2.7)/HILIC, SCX/RP(pH 2.7), SEC/RP(pH 2.7),

HIC/RP(pH 2.7), SCX/HILIC, SEC/HILIC, and HIC/HILIC. The detailed analysis conditions can be found in Table 6-1.

Table 6-1: Summary of analytical conditions for selectivity screening by 2D-HPLC.

Dimension	First	First	First	First	First	Second	Second
Selectivity	RP (pH 2.7)	RP (pH 10)	SCX	SEC	HIC	RP (pH 2.7)	HILIC
Column	Zorbax Eclipse Plus C18 RRHD 50 x 2.1 mm 1.8 µm (Agilent Technologies, Waldbronn, Germany)	Zorbax Extend-C18 50 x 2.1 mm 1.8 µm (Agilent Technologies, Waldbronn, Germany)	Agilent Bio SCX NP3 SS 50 x 4.6 mm 3 µm (Agilent Technologies, Waldbronn, Germany)	YMC-SEC MAB 150 x 0.3 mm 3 µm (YMC Europe GmbH, Dinslaken, Germany)	TSKgel Butyl-NPR 35 x 4.6 mm 2.5 µm (Tosoh Bioscience GmbH, Darmstadt, Germany)	Zorbax Eclipse Plus C18 RRHD 50 x 2.1 mm 1.8 µm (Agilent Technologies, Waldbronn, Germany)	HALO Penta-Hilic 50 x 2.1 mm 2.7 µm (Advanced Materials Technology, Wilmington, USA)
Temperature (°C)	60	30	40	40	40	60	60
Flow rate (µL min^{-1})	20 for 70 min; afterwards 300	20 for 70 min; afterwards 300	20 for 90 min; afterwards 300	5	20 for 90 min; afterwards 300	2000	2000
Mobile phase	A: H_2O+0.1%FA B: ACN+0.1%FA	A: NH_4FA-Buffer (pH 10) B: ACN	A: H_2O+0.1%FA B: H_2O+0.1%FA + 1M NaCl	A: 150 mM PBS-Buffer B: /	A: 150 mM PBS-Buffer B: 150 mM PBS-Buffer + 3.3 M Sodium acetate	A: H_2O+0.1%FA B: ACN+0.1%FA	A: 10 mM ammonium acetate + 0.1% acetic acid (pH 4.6) in H_2O B: 10 mM ammonium acetate + 0.1% acetic acid in 90/10 ACN/H_2O
Gradient	0-60 5-95	0-60 5-95	0-60 0-80	0-120 0-0	0-60 90-0	0.0-0.18 5-5	0.0-0.18 95-95
(min // %B)	60-70 95-95	60-70 95-95	60-90 80-80		60-90 0-0	0.18-0.68 5-95	0.18-0.68 95-60
	70-72 95-95	70-72 95-95	90-100 80-80		90-100 0-0	0.68-0.80 95-95	0.68-0.70 60-95
	72-72.1 95-5	72-72.1 95-5	100-101 80-0		100-101 0-90	0.80-0.81 95-5	0.70-1.5 95-95
	72.1-75 5-5	72.1-75 5-5	101-120 0-0		101-119 90-90	0.81-1.0 5-5	

6.2.5 Tryptic digest

In order to generate the peptides for selectivity screening, a mixture of the monoclonal antibodies bevacizumab, cetuximab, daratumumab, omalizumab, rituximab and trastuzumab at a concentration of 1 mg mL^{-1} was tryptically digested. For this purpose, 50 µL of sample were mixed with 50 µL of denaturation buffer. The denaturation buffer consisted of 3 M GuHCl, 100 mM TrisHCl, and 8 mM DTT. The mixture was heated to 95°C for 20 min using a WiseTherm HB-48P heating block (Witteg Labortechnik GmbH, Wertheim, Germany). After cooling to room temperature, 575 µL of a 50 mM NH4HCO3 solution was added first, followed by 50 µL of trypsin solution (1 µg absolute). Tryptic digestion was performed after mixing for 21 h at 37°C in an IPP55 Plus incubator (Memmert GmbH + Co. KG, Büchenbach, Germany). The reaction was quenched by adding 25 µL of formic acid.

6.3 Results and Discussion

6.3.1 Intact analysis performed on mAbs: Innovator and Biosimilar

Before the evaluation of the mass spectrum of a mAb can be performed, the charge distribution obtained must be deconvoluted. The result of such a deconvolution is shown in Figure 6-1 for trastuzumab, where the drug product Herceptin is the originator and Herzuma the biosimilar. Furthermore, results for the monoclonal antibody rituximab can be obtained from the supplementary information.

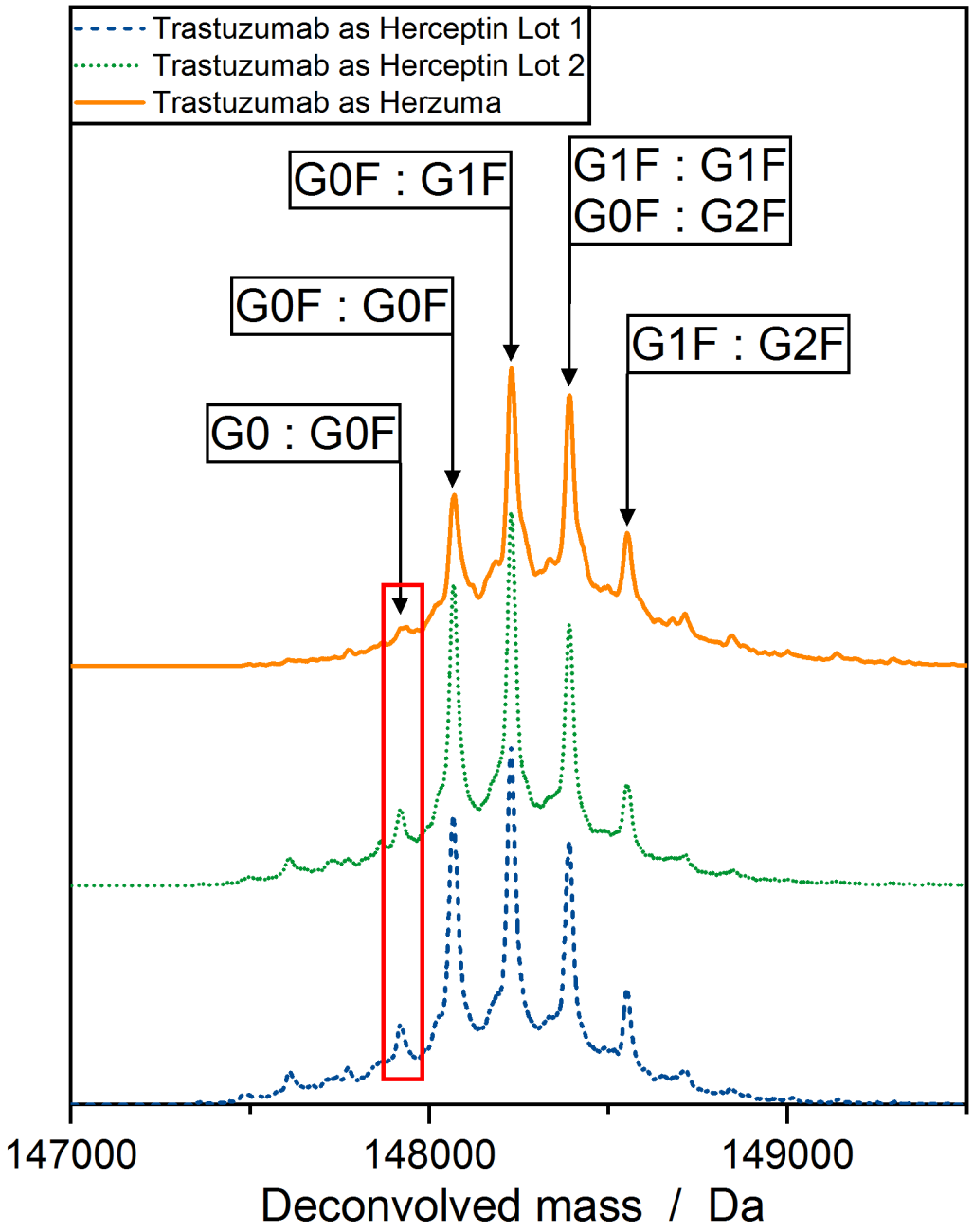


Figure 6-1: Deconvolved mass spectra of trastuzumab as Herceptin (green dotted and blue dashed) and Herzuma (orange solid). The sugar modifications were assigned to individual signals. Furthermore, the G0:G0F modification is highlighted by a red box.

The data in Figure 6-1 show the glycan pattern of trastuzumab, with the originator giving rise to five signals. The biosimilar, in contrast, had only four clearly prominent signals. This is because the G0:G0F modification could not be detected in the biosimilar. A more detailed evaluation of the signals can be found in Table 6-2.

Table 6-2: Deconvolved masses at charge z=1 of trastuzumab as Herceptin (originator) and Herzuma (biosimilar). The masses were assigned to the sugar modifications. Four analyses were performed per sample on three different days (n=12).

Substance	**G0:G0F**	**G0F:G0F**	**G0F:G1F**	**G1F:G1F G0F:G2F**	**G1F:G2F**
	(Da)	**(Da)**	**(Da)**	**(Da)**	**(Da)**
Herceptin Lot 1	147,914 ± 5	148,061 ± 5	148,222 ± 5	148,383 ± 5	148,545 ± 5
Herceptin Lot 2	147,914 ± 6	148,061 ± 5	148,222 ± 5	148,384 ± 5	148,544 ± 5
Herzuma	n/a	148,062 ± 5	148,222 ± 5	148,384 ± 5	148,546 ± 5

The deconvolved masses of the individual signals are consistent with theory and analyses of other research groups [2,10]. The masses for Herceptin and Herzuma were found identical, which was to be expected since the latter is an approved biosimilar. For the G0:G0F modification, it should be noted that it is the sugar modification with the lowest intensity. Although the G0:G0F modification was not detected, Lee et al. demonstrated that the G0 glycan is present in Herzuma to a similar extent as in Herceptin [21]. Since the proportion of the individual sugar modifications are known to vary with the production batch, these variations can be recognized using LC-HRMS [11]. This means that it is difficult to determine the cause of such differences, as molecular differences can be masked by batch variations. In order for the differences to be traceable to the analyte, various batches must be examined.

Regardless of the reason for the absence of the modification, these minor differences between originator and biosimilar could be successfully detected. Hence, LC-HRMS of intact mAbs is a suitable tool to characterize the glycosylation pattern.

6.3.2 Quantification of affinity for an Fc gamma receptor IIIA ligand

Although HRMS proved well suited for glycan analysis, it is difficult to predict the effects to the API from the sugar modifications detected. Thereby, the glycan structure has an influence on the binding affinity to the Fc receptor, which is involved in antibody-dependent cell-mediated cytotoxicity (ADCC). Using affinity chromatography with an immobilized Fc receptor, it is thus possible to generate a correlation to ADCC and at the same time make a statement about the sugar modifications responsible for this [12,7].

Figure 6-2 shows the application of Fc affinity chromatography to different rituximab drugs, where MabThera is the originator and Truxima as well as Rixathon are the biosimilars. Furthermore, a rituximab biosimilar candidate currently in development (R&D sample) was investigated.

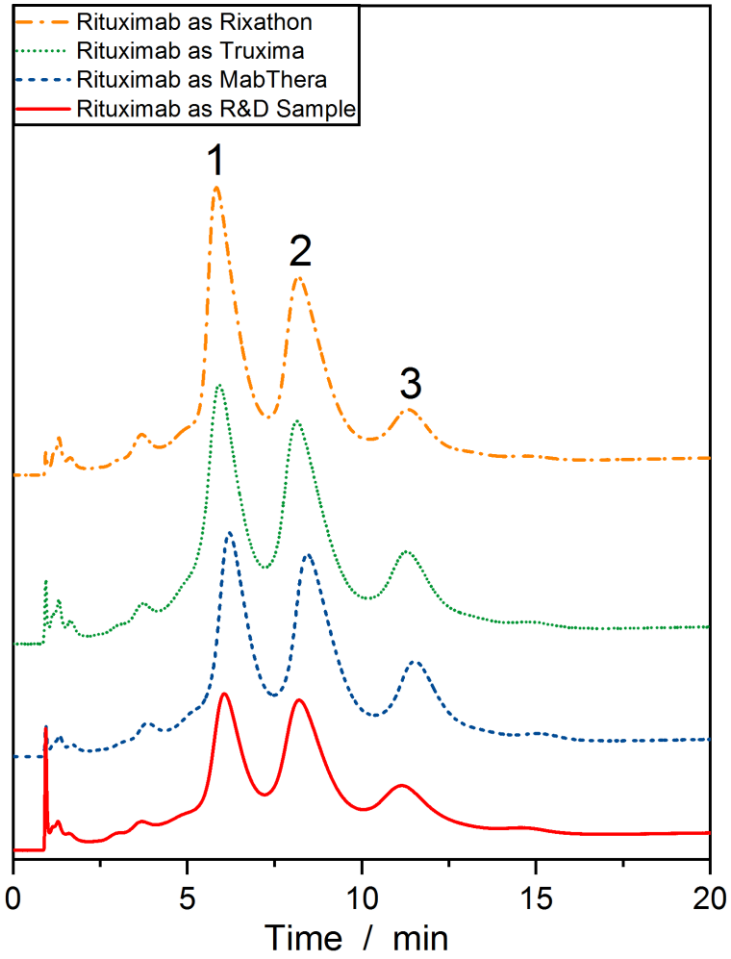


Figure 6-2: Chromatogram of the analysis of rituximab by FcR affinity chromatography. The originator MabThera (blue dashed) was compared with the biosimilars Truxima (green dotted) and Rixathon (orange dotted and dashed), as well as a research sample (red solid). All samples showed three signals, whereby increasing retention time is associated with increasing affinity for the Fc receptor and thus increased ADCC activity.

The analysis gives three main signals for all samples, which is in agreement with the literature [12]. It should be noted that longer retention leads to increased ADCC activity. This relationship was confirmed by Chakrabarti et al. for rituximab by correlating FcR chromatography analysis results with an ADCC binding assay. They were able to show that ADCC activity was highest from the last eluting compound (peak 3) and lowest for the first eluting compound (peak 1). The activity from the unseparated monoclonal antibody represented a mixture and is intermediate between that of peak 1 and peak 2 [12]. The correlation between ADCC activity and retention time in FcR chromatography was also shown for other mAbs [7]. A quantitative evaluation of Figure 6-2 can be found in Table 6-3.

Table 6-3: Normalized areas of Fc affinity chromatography shown in Figure 6-2. The analyses were performed as duplicate (n=2).

	Normalized area (%)		
Drug	**Peak 1**	**Peak 2**	**Peak 3**
Rixathon	50.4 ± 0.4	40.4 ± 0.02	9.2 ± 0.3
Truxima	41.8 ± 0.2	43.3 ± 0.3	14.8 ± 0.5
MabThera	38.4 ± 0.2	43.5 ± 0.3	18.1 ± 0.1
R&D Sample	38.1 ± 0.4	46.4 ± 0.6	15.5 ± 0.9

The comparison of the R&D sample with MabThera shows comparable results for all three signals, making this also a promising biosimilar candidate.

Interestingly, the normalized area of peak 1 and 3 of the biosimilar Rixathon differed significantly from the originator MabThera (50.4% vs. 38.4% and 9.2% vs 18.1%, respectively), suggesting lower ADCC activity. However, studies demonstrated comparable ADCC activity between Rixathon and MabThera [13,14]. Considering the influence of sugar modifications on the Fc affinity chromatography, it could be shown that more terminal galactose as well as a higher proportion of afucosylated glycans led to a higher retention [12]. Studies have also shown that the glycan structure of Rixathon was within the range of the batch variability of MabThera. Nevertheless, small differences were found in sugar modifications with a low intensity [13,14]. The results of our own HRMS analysis are shown in Table 6-4.

Table 6-4: Relative areas of sugar modifications of rituximab as MabThera (originator) and Rixathon (biosimilar) according to HRMS analysis. The analysis was performed as a quadruple determination (n=4).

Substance	G0F:G0F (%)	G0F:G1F (%)	G1F:G1F G0F:G2F (%)	G1F:G2F (%)
MabThera	20.1 ± 3.5	28.5 ± 5.0	30.6 ± 5.8	20.9 ± 3.9
Rixathon	27.6 ± 2.3	30.0 ± 1.2	24.7 ± 0.8	17.8 ± 0.7

The results of HRMS analysis show that the sugar modification G0F:G0F has a relative area of 27.6% ± 2.3% for the biosimilar Rixathon and 20.1% ± 3.5 for the originator MabThera. Since this modification elutes mainly at peak 1 in affinity chromatography, the increased area of peak 1 for Rixathon can thus be explained. Similarly, the difference of peak 3 in affinity chromatography can be explained. This can be attributed to the lower proportion of modifications with a higher proportion of terminal galactose (See Table 6-4).

Fc affinity chromatography can thus be used to characterize monoclonal antibodies and estimate their ADCC properties. Due to its sensitivity to changes in glycan composition, it is a suitable tool to detect differences in manufacturing batches and biosimilars. Furthermore, this technique can be used to quickly check whether a biosimilar candidate is promising and should be investigated further.

6.3.3 Comparison of biosimilar and originator by 2D-HPLC

The use of comprehensive 2D HPLC, where the two separation dimensions are orthogonal, increases peak capacity and hence chromatographic separation. In this study, we applied a highly orthogonal method to samples from tryptic digest yielding results that proved equal to mass spectrometer utilization.

Figure 6-3 shows the determination of orthogonality for a tryptic digest of a mixture containing six monoclonal antibodies, using different separation mechanisms. Each spot represents at least one peptide.

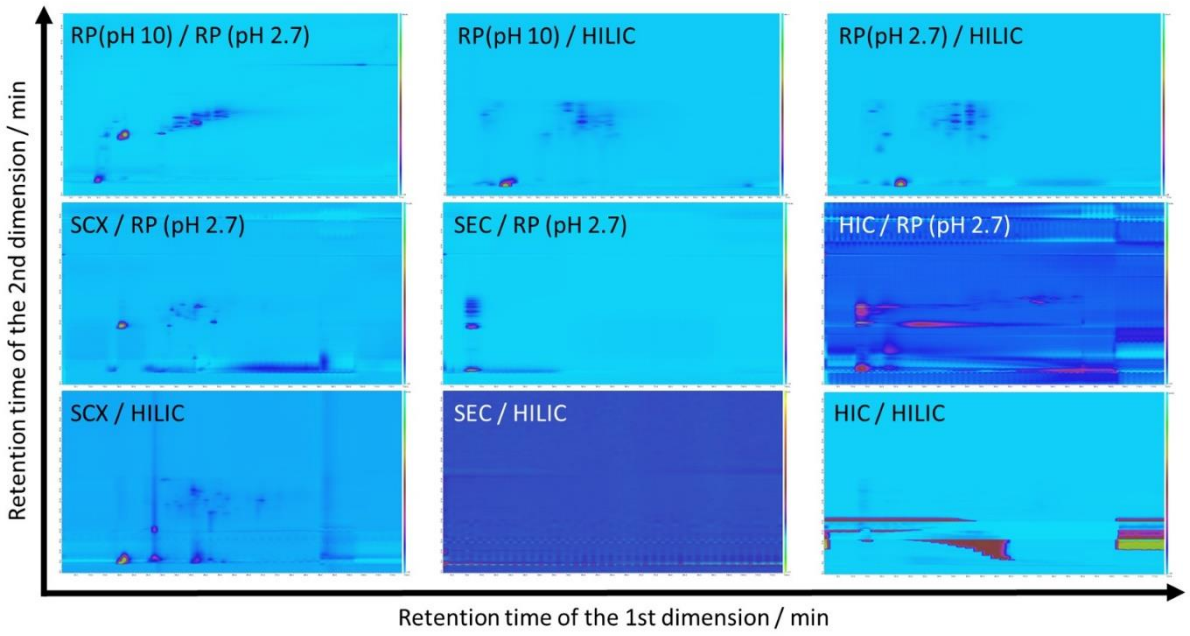


Figure 6-3: Illustration of comprehensive 2D-HPLC chromatograms for the determination of orthogonality for a peptide mixture. The peptide mixture was generated by tryptic digestion of a mixture of 1 mg mL^{-1} each of cetuximab, daratumumab, rituximab, bevacizumab, omalizumab and trastuzumab.

The combinations of SEC/HILIC and HIC/HILIC cannot be recommended due to solvent incompatibilities. The combination of SEC/RP and HIC/RP did not provide good orthogonality, which can be seen from the fact that the substances were not distributed across the separation space, but rather formed clusters that were not well resolved. The other five combinations used led to a distribution of the substances across the separation space.

Although our analytical data show that there are potentially several suitable combinations (RP pH 10 / RP pH 2.7; RP pH 10 / HILIC; RP pH 2.7 / HILIC; SCX / RP pH 2.7; SCX / HILIC), in practice it may not be appropriate to use the combination with the highest peak capacity. For example, Vanhoenacker et al. showed that a combination of RP/RP had a higher peak capacity, but some oxidative changes of trastuzumab could only be detected with the SCX/RP combination [15]. This means that regardless of the maximum peak capacity, diverse combinations of separation mechanisms should be used for complete characterisation.

Figure 6-4 shows an example of the application of a possible combination (RP pH 2.7/HILIC) to distinguish originator and biosimilar monoclonal antibodies.

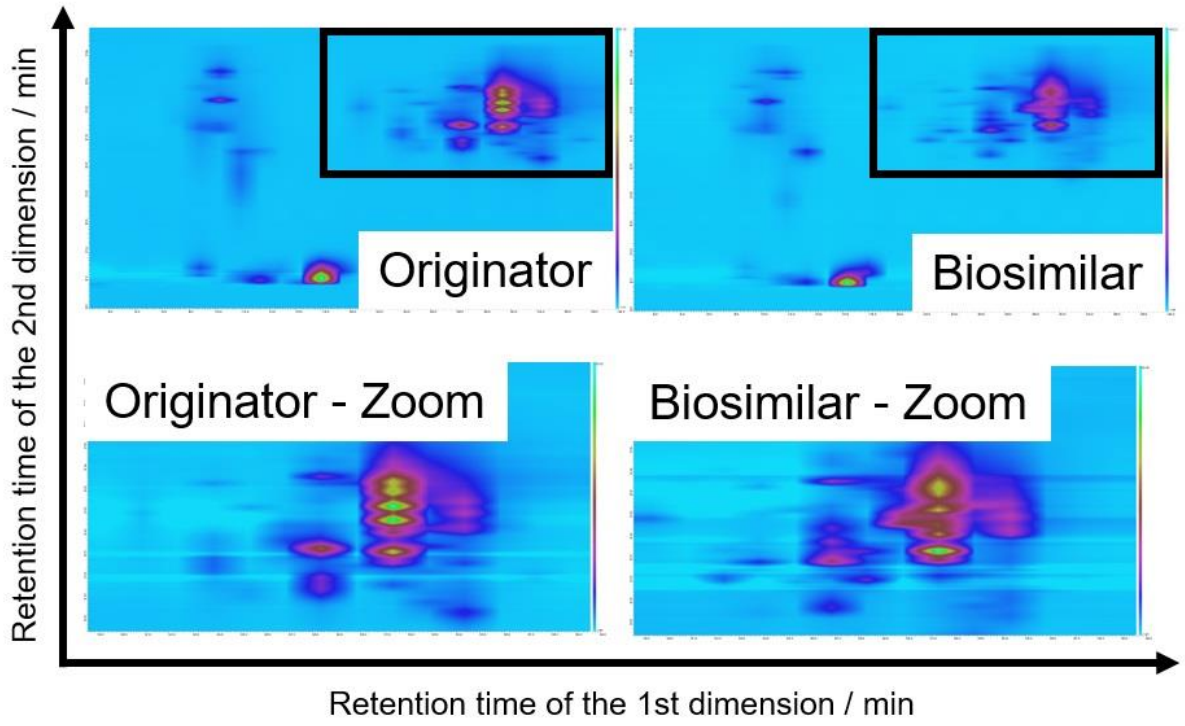


Figure 6-4: RP (pH 2.7)-HILIC separation of a rituximab biosimilar (right) with the originator (left). The enlarged area has been marked by a black frame.

The enlarged areas of Figure 6-4 show a different distribution of the substance spots. Although no mass spectrometer was used to identify the peptides, a different peak pattern of the biosimilar peptides indicates that they were peptides that differed from the originator, which means that biosimilar and originator can be distinguished by using a UV detector.

Therefore, our data show that it is possible to detect differences in mAb samples (innovator vs. biosimilar) with a UV detector due to the specific peak pattern across the two-dimensional separation space.

6.4 Conclusion

Characterization of monoclonal antibodies and evaluation of biosimilar and originator similarity make great demands on analytical techniques. A wide variety of instrumental analytical techniques was needed to obtain sufficient structural and functional information. However, by choosing and combining LC-HRMS, Fc affinity chromatography, and 2D-HPLC, we could show that analytical techniques are becoming sufficiently sophisticated to greatly reduce sample preparation, allow structural characterization, distinguish originator and biosimilar functionally and offers the possibility to quickly identify new biosimilar candidates.

Although a large amount of information can be obtained by combining the above analytical methods, they also have certain limitations when considered individually. High-resolution mass

spectrometry of intact mAbs is an excellent tool to characterize the glycan structure, but it does not provide information about its effects on ADCC. Fc affinity chromatography, on the other hand, can be used to determine the ADCC properties of a mAb, but cannot be used to accurately characterize the glycan structure. If additional information about the primary sequence is required, characterization should be performed at the peptide level. Here, 2D-HPLC coupled to UV detection offers the possibility to distinguish a biosimilar from its originator if the separation mechanisms have a high orthogonality.

6.5 Supplementary Information

6.5.1 Function and benefit of the ASM valve

In 2D-HPLC experiments, a valve is used to transfer one or more fractions online from the first to the second separation dimension. Thereby, not only the sample fraction is transferred as a substance plug, but also the solvent in which it is carried. Mixing with the solvent of the second separation dimension is not provided. This could lead to solvent incompatibilities. Specifically, this will be explained using the example of the RP-HILIC coupling used in the study. Using RP chromatography, water is the weakest eluent while using HILIC it is the strongest eluent. This can lead to substances eluting with the void time or forming split peaks during the direct transfer from the first to the second separation dimension. One way to avoid this is to use an active solvent modulation (ASM) capillary. The experimental setup for this is shown in Figure 6-5.

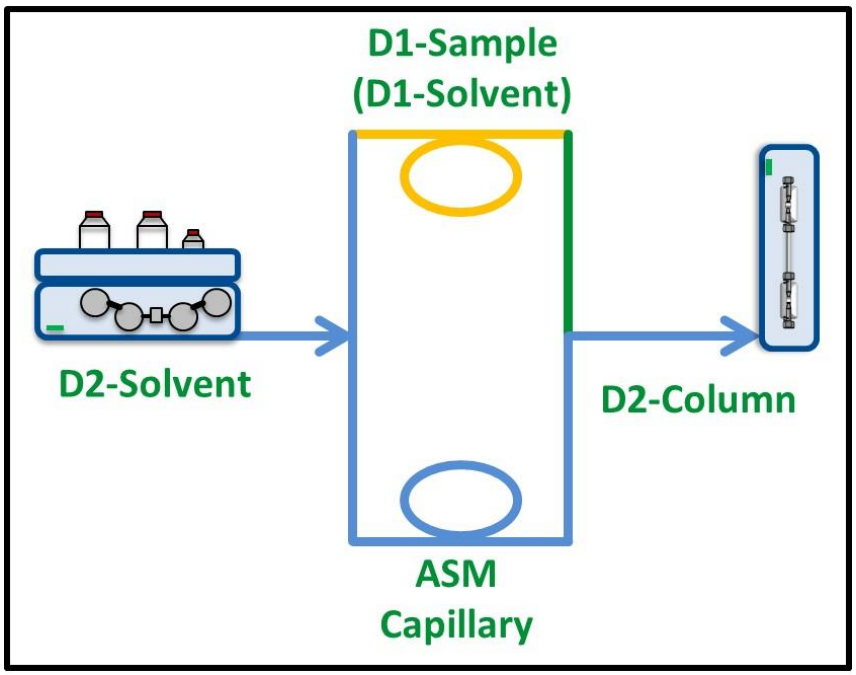


Figure 6-5: Illustration of the operation of the ASM capillaries. The principle is explained in the text.

The flow of the second separation dimension is divided and directed partly through the sample loop, in which a fraction of the first dimension eluate is located, and partly through the ASM capillary. After flowing through both capillaries, the mobile phase is merged before being applied to the second separation dimension. Depending on the length of the ASM capillary and the resulting pressure, the dilution factor can be varied. For very high dilution factors it is recommended to use an additional HPLC pump instead of a capillary.

The impact of the ASM capillary is shown in Figure 6-6.

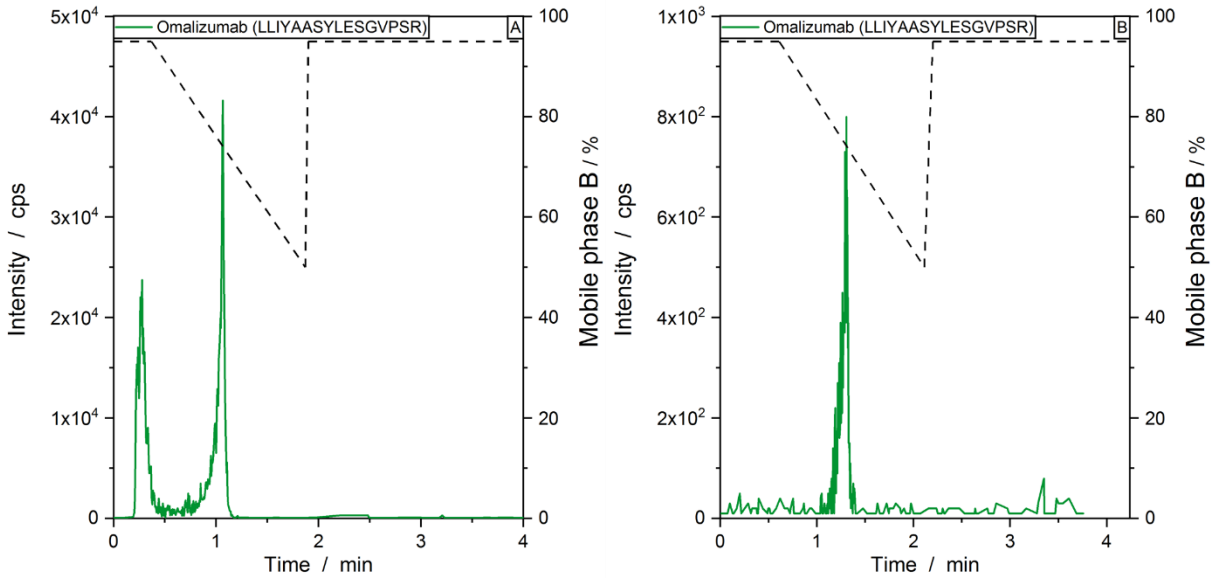


Figure 6-6: Illustration of the second dimension of an RP-HILIC coupling for the analysis of a peptide from the monoclonal antibody omalizumab. The analysis was performed (A) without ASM capillary or (B) with an ASM factor of 3.

On the one hand analysis of the LLIYAASYLESGVPSR peptide of omalizumab by RP-HILIC coupling resulted in a double peak (Figure 6-6A) without the use of online dilution, where the first signal was due to partial elution during the void time. The installation of an ASM capillary, on the other hand, has led to sufficient dilution of the stronger solvent, allowing elution in one signal (Figure 6-6B).

6.5.2 Analysis of rituximab by HRMS

Table 6-5: Deconvolved masses of rituximab as MabThera (originator) and Rixathon (biosimilar). The masses were assigned to the sugar modifications. The analysis was performed as a quadruple determination (n=4).

Substance	G0F:G0F (Da)	G0F:G1F (Da)	G1F:G1F G0F:G2F (Da)	G1F:G2F (Da)
MabThera	147,082 ± 3	147,247 ± 2	147,409 ± 2	147,575 ± 4
Rixathon	147,084 ± 3	147,248 ± 4	147,410 ± 3	147,566 ± 3

6.6 References

1. Grilo AL, Mantalaris A (2019) The Increasingly Human and Profitable Monoclonal Antibody Market. Trends Biotechnol 37 (1):9-16. doi:10.1016/j.tibtech.2018.05.014

2. Beck A, Wagner-Rousset E, Ayoub D, Van Dorsselaer A, Sanglier-Cianferani S (2013) Characterization of Therapeutic Antibodies and Related Products. Anal Chem 85 (2):715-736. doi:10.1021/ac3032355

3. Beck A, Sanglier-Cianferani S, Van Dorsselaer A (2012) Biosimilar, Biobetter, and Next Generation Antibody Characterization by Mass Spectrometry. Anal Chem 84 (11):4637-4646. doi:10.1021/ac3002885

4. Agency EM (2012) Guideline on similar biological medicinal products containing monoclonal antibodies – non-clinical and clinical issues. London

5. Shukla AA, Thommes J (2010) Recent advances in large-scale production of monoclonal antibodies and related proteins. Trends Biotechnol 28 (5):253-261. doi:10.1016/j.tibtech.2010.02.001

6. Hossler P, Khattak SF, Li ZJ (2009) Optimal and consistent protein glycosylation in mammalian cell culture. Glycobiology 19 (9):936-949. doi:10.1093/glycob/cwp079

7. Xie LQ, Zhang EH, Xu YP, Gao WY, Wang LL, Xie MHW, Qin PL, Lu LH, Li SP, Shen PC, Jiang WD, Liu S (2020) Demonstrating Analytical Similarity of Trastuzumab Biosimilar HLX02 to Herceptin (R) with a Panel of Sensitive and Orthogonal Methods Including a Novel Fc gamma RIIIa Affinity Chromatography Technology. Biodrugs 34 (3):363-379. doi:10.1007/s40259-020-00407-0

8. Sandra K, Vandenheede I, Sandra P (2014) Modern chromatographic and mass spectrometric techniques for protein biopharmaceutical characterization. J Chromatogr A 1335:81-103. doi:10.1016/j.chroma.2013.11.057

9. Reinders LMH, Klassen MD, Jaeger M, Teutenberg T, Tuerk J (2018) Development of an analytical method to assess the occupational health risk of therapeutic monoclonal antibodies using LC-HRMS. Anal Bioanal Chem 410 (11):2829-2836. doi:10.1007/s00216-018-0966-1

10. Delobel A, Cantais F, Catrain A, Dereux E, Van Vyncht G (2013) Therapeutic antibody glycosylation analysis: a contract research organization perspective in the frame of batch release or comparability support. Methods Mol Biol 988:115-143. doi:10.1007/978-1-62703-327-5_8

11. Damen CWN, Chen WB, Chakraborty AB, van Oosterhout M, Mazzeo JR, Gebler JC, Schellens JHM, Rosing H, Beijnen JH (2009) Electrospray Ionization Quadrupole Ion-Mobility Time-of-Flight Mass Spectrometry as a Tool to Distinguish the Lot-to-Lot Heterogeneity in N-Glycosylation Profile of the Therapeutic Monoclonal Antibody Trastuzumab. J Am Soc Mass Spectr 20 (11):2021-2033. doi:10.1016/j.jasms.2009.07.017

12. Chakrabarti A, Kervinen J, Müller E, Tanaka T, Muranaka K (2020) Analytical Characterization of Monoclonal Antibodies with Novel Fc Receptor-Based Chromatography Technique, Monoclonal Antibodies. In: Monoclonal Antibodies. doi:http://dx.doi.org/10.5772/intechopen.95356

13. Visser J, Feuerstein I, Stangler T, Schmiederer T, Fritsch C, Schiestl M (2013) Physicochemical and Functional Comparability Between the Proposed Biosimilar Rituximab GP2013 and Originator Rituximab. Biodrugs 27 (5):495-507. doi:10.1007/s40259-013-0036-3

14. Jurczak W, Cohen S, Illidge TM, da Silva A, Amersdorffer J (2019) Scientific rationale underpinning the development of biosimilar rituximab in hematological cancers and inflammatory diseases. Future Oncol 15 (36):4223-4234. doi:10.2217/fon-2019-0430

15. Vanhoenacker G, Vandenheede I, David F, Sandra P, Sandra K (2015) Comprehensive two-dimensional liquid chromatography of therapeutic monoclonal antibody digests. Anal Bioanal Chem 407 (1):355-366. doi:10.1007/s00216-014-8299-1

16. Semard G, Peulon-Agasse V, Bruchet A, Bouillon JP, Cardinael P (2010) Convex hull: A new method to determine the separation space used and to optimize operating conditions for comprehensive two-dimensional gas chromatography. J Chromatogr A 1217 (33):5449-5454. doi:10.1016/j.chroma.2010.06.048

17. Gilar M, Olivova P, Daly AE, Gebler JC (2005) Orthogonality of separation in two-dimensional liquid chromatography. Anal Chem 77 (19):6426-6434. doi:10.1021/ac050923i

18. Camenzuli M, Schoenmakers PJ (2014) A new measure of orthogonality for multi-dimensional chromatography. Anal Chim Acta 838:93-101. doi:10.1016/j.aca.2014.05.048

19. Leonhardt J, Teutenberg T, Buschmann G, Gassner O, Schmidt TC (2016) A new method for the determination of peak distribution across a two-dimensional separation space for the identification of optimal column combinations. Anal Bioanal Chem 408 (28):8079-8088. doi:10.1007/s00216-016-9911-3

20. Hesse M, Meier H, Zeeh B (2012) Spektroskopische Methoden in der organischen Chemie; 8. Edition, Georg Thieme Verlag KG, Stuttgart, ISBN 978-3-13-576108-4

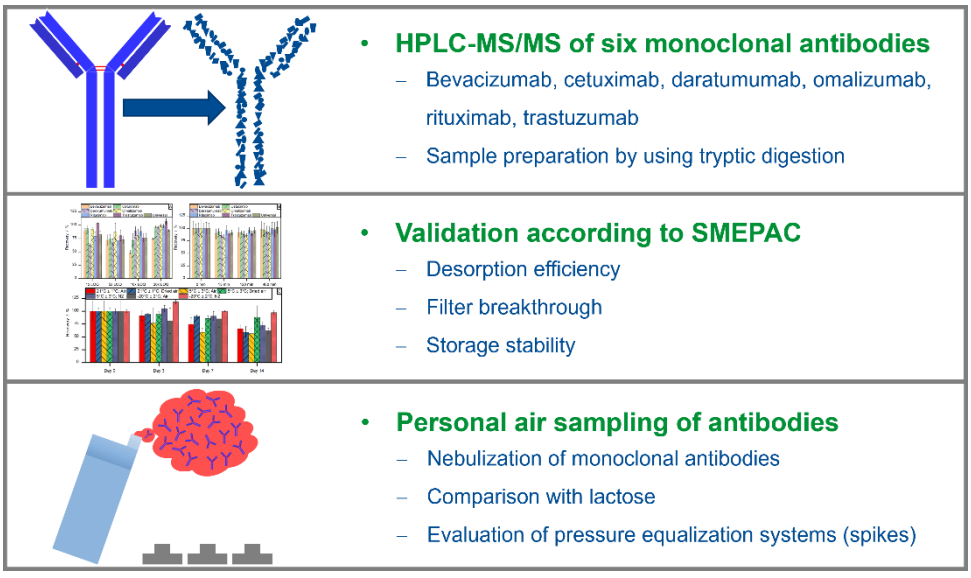
21. Lee J, Kang HA, Bae JS, Kim KD, Lee KH, Lim KH, Choo MJ, Chang SJ (2018) Evaluation of analytical similarity between trastuzumab biosimilar CT-P6 and reference product using statistical analyses, mAbs, 10:4, 547-571, DOI: 10.1080/19420862.2018.1440170

Chapter 7 Development and validation of a method for airborne monoclonal antibodies to quantify workplace exposure

This chapter was adapted from: Reinders, L.M.H., Noelle, D., Klassen, T., Jaeger, M., Schmidt, T.C., Tuerk, J., Teutenberg, T. Development and validation of a method for airborne monoclonal antibodies to quantify workplace exposure. J Pharm Biomed Anal 221, 115046 (2022) https://doi.org/10.1016/j.jpba.2022.115046

Abstract: Modern therapy strategies are based on patient-specific treatment where the drug and dose are optimal adapted to the patient's needs. In recent drugs, monoclonal antibodies (mAbs) are increasingly used as active ingredients. Their patient-specific formulations are not part of the pharmaceutical industry's manufacturing process but are prepared from concentrates by pharmaceutical personnel. During the manufacturing process, however, active pharmaceutical ingredients are released in trace amounts or, in the case of accidents and spills, also in high concentrations. Regardless of the source of entry, mAbs can become airborne, be inhaled, and cause undesirable side-effects such as sensitization. To assess the risk for pharmaceutical personnel, a personal air sampling method was developed and validated for bevacizumab, cetuximab, daratumumab, omalizumab, rituximab and trastuzumab. The method is based on the combination of high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS). The analytical method achieves a limit of detection of 0.30-8.8 ng mL^{-1}, recoveries of 83-96% (intra-day assay) and 75-89% (inter-day assay), with no detectable carry-over. A polycarbonate filter proved suitable for sampling airborne monoclonal antibodies, as it achieved 80-104% recovery across all mAbs. It also showed concentration-independent desorption efficiency. The sampling duration can be up to 480 min without negatively affecting the recovery. MAbs are stable on the polycarbonate filter at 5°C for 3 days (recovery: 94% ± 5%) and at 20°C for 14 days (recovery: 97% ± 4%). Our method demonstrated that there is a potential for release when handling monoclonal antibodies. However, this can be reduced below the limit of detection by using pressure equalization systems (spikes).

Graphical Abstract:



7.1 Introduction

In the last years the number of antibody therapeutics undergoing initial regulatory review has reached a new high level. As a result, 131 antibody therapeutics are already approved or under regulatory review as of November 2021. Interestingly, nearly half (45%) of all antibody therapeutics are used to treat cancer [1]. Thereby, the major route of administration is parenteral administration [2-4].

Due to the systemic route of administration, an insufficient dose of therapeutic agent might reach the tumor site, resulting in a reduced effect. Therefore, inhalative administration of monoclonal antibodies (mAbs), such as cetuximab, has been studied to provide local treatment of certain tumor diseases [5, 6]. This route represents a promising opportunity for patients. Yet, it poses an increased risk for occupational health and safety, as pharmaceutical personnel may also be unintentionally exposed.

Irrespective of future trends, active pharmaceutical ingredients (API) may be released during pressure equalization processes [7]. Regardless of whether aerosolization is intentional or unintentional, especially mAbs can in consequence denature and agglomerate, increasing immunogenicity [8]. Furthermore, in the field of occupational health and safety, personnel are likely to be exposed to low doses of mAbs over a longer period of time. It is assumed that these long-term low doses cause sensitization [7]. Besides the extrapolation of side effects at parenteral doses [7], this effect is also derived by other case studies [9].

The other routes of entry, ingestion and dermal contact, are of minor relevance to occupational health and safety, because mAbs are denatured in the gastrointestinal tract due to the low pH and digested enzymatically [10]. Furthermore, dermal absorption decreases rapidly when the molecular weight of the API exceeds 500 Da. The average molecular weight of mAbs is about 150,000 Da [11-14].

There is a need for analytical methods to detect airborne mAbs, as they pose a potential risk to occupational health and safety. Reinders et al. [13] have therefore developed a method for air sampling of three mAbs (daratumumab, rituximab and trastuzumab) for manufacturing process validation. However, this method had a detection limit in the range of µg per sample and is not suitable for short-term sampling, as it is common in personal air sampling. Furthermore, the study did not include aerosolization experiments and a broader comparison with common methods such as lactose measurements according to the standardized measurement of equipment particulate airborne concentration (SMEPAC).

Therefore, in this study, we present an analytical method and its validation based on personal air sampling of the mAbs bevacizumab, cetuximab, daratumumab, omalizumab, rituximab, trastuzumab. Furthermore, we present validation data following SMEPAC guidelines and results from aerosolization experiments. Comparative studies were also performed in the processing of monoclonal antibodies with and without pressure equalization systems.

7.2 Materials and methods

7.2.1 Chemicals and reagents

Bevacizumab (BVCZ), rituximab (RTX), and trastuzumab (TTZ) were purchased as Avastin 25 mg mL^{-1}, MabThera 10 mg mL^{-1} and Herceptin 150 mg from Roche Pharma AG (Basel, Switzerland). Cetuximab (CTX) was purchased as Erbitux 5 mg mL^{-1} from Merck KGaA (Darmstadt, Germany), daratumumab (DRTM) as Darzalex 20 mg mL^{-1} from Janssen-Cilag AG (Neuss, Germany) and omalizumab (OMLZ) as Xolair 150 mg mL^{-1} from Novartis AG (Basel, Switzerland). Guanidine hydrochloride (GuHCl), dithiothreitol (DTT), ammonium bicarbonate (NH_4HCO_3), tris(hydroxymethyl)aminomethane hydrochloride (TrisHCl), phosphate-buffered saline (PBS), lactose and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, USA). Hypergrade acetonitrile and water (LiChrosolv) were acquired from Merck KGaA (Darmstadt, Germany). Sequencing grade modified trypsin was received from Promega Corporation (Madison, USA), and 0.9% sodium chloride solution (0.9%-NaCl) from B. Braun Melsungen AG (Melsungen, Germany).

7.2.2 Tryptic digest

50 µL of antibody-containing sample solution were mixed with 50 µL of denaturation buffer in a 1.5 mL glass vial (Chromatographie Zubehör Trott, Kriftel, Germany) and heated after mixing to 95°C in the WiseTherm HB-48P heating block (Witteg Labortechnik GmbH, Wertheim, Germany) for 20 min. The denaturing buffer consisted of 3 M GuHCl, 100 mM TrisHCl and 8 mM DTT. After cooling to room temperature, 575 µL of a 50 mM NH_4HCO_3 solution was added to the mixture. Then, 50 µL of a 20 mg mL^{-1} trypsin solution was added and tryptic digestion was performed at 37°C in an IPP55 Plus incubator (Memmert GmbH + Co. KG, Büchenbach, Germany) for 21 h. At the end of the reaction, 25 µL FA were added.

7.2.3 Chromatography and mass spectrometry of peptides

The analytical system consisted of a binary 1260 pump (Agilent Technologies, Waldbronn, Germany), an HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), a Mistral 886 (Spark Holland, Emmen, Netherlands) column oven, and a QTRAP 6500 mass spectrometer (AB Sciex Instruments, Darmstadt, Germany). Control of the overall system and data analysis was performed with Analyst 1.6.3 Build 5095 (AB Sciex, Darmstadt, Germany). The temperature of the autosampler was set to 6°C. The mobile phases used were (A) water + 0.1% FA and (B) acetonitrile + 0.1% FA. The other method settings are shown in Table 7-1.

Table 7-1: Summary of method parameters used for the quantification of peptides.

Stationary phase	Eclipse Plus C18 RRHD 1.8 µm; 2.1 x 50 mm; 95 Å (Agilent Technologies, Waldbronn, Germany)	
Column temperature (°C)	60	
Injection volume (µL)	50	
Flow rate (mL min^{-1})	0.5	
Gradient (min // %B)	0.0-1.0	10-10
	1.0-9.0	10-27.8
	9.0-9.1	27.8-95
	9.1-10.5	95-95
	10.5-10.6	95-10
	10.6-12.0	10-10
Gas temperature (°C)	550	
Gas counterflow (L min^{-1})	30	
Nebulizer gas (L min^{-1})	50	
Heater gas	60	
Entry potential (V)	10	
IonSpray potential (V)	5500	
Dwell time (ms)	200	
Ionization mode	Electrospray Ionization	

Detection of the monoclonal antibodies (mAbs) was indirect via specific peptides formed from the mAbs during tryptic digestion. The peptide sequences used are VLIYFTSSLHSGVPSR (bevacizumab), YASESISGIPSR (cetuximab), LLIYDASNR (daratumumab), LLIYAASYLESGVPSR (omalizumab), FSGSGSGTSYSLTISR (rituximab), LLIYSASFLYSGVPSR (trastuzumab) and DSTYSLSSTLTLSK (universal). The tandem mass spectrometry (MS/MS) transitions can be found in Table 7-2. The parameters specific to each mass transfer and the time schedule can be found in the Supplementary information.

Table 7-2: Overview of the MS/MS transitions used. The mAbs were detected indirectly via selected peptides.

	Quantifier		**Qualifier**	
Transition for	Q1 *m/z*	Q3 *m/z*	Q1 *m/z*	Q3 *m/z*
Bevacizumab	588.3	359.2	588.3	602.3
Cetuximab	633.8	359.2	633.8	816.4
Daratumumab	532.8	725.3	532.8	562.3
Omalizumab	870.0	359.2	580.3	359.2
Rituximab	803.9	926.5	803.9	839.5
Trastuzumab	887.0	359.2	591.7	359.2
Universal	751.9	1036.6	751.9	836.5

To validate the method, a calibration curve with ten equidistant interpolation points was analysed. The concentration range was 20-200 ng mL^{-1} bevacizumab (BVCZ), 3-30 ng mL^{-1} cetuximab (CTX), 2-20 ng mL^{-1} daratumumab (DRTM), 10-100 ng mL^{-1} omalizumab (OMLZ), 20-200 ng mL^{-1} rituximab (RTX), and 30-300 ng mL^{-1} trastuzumab (TTZ). Furthermore, a quality control (QC) sample was prepared from independent batches in the middle working range. The limit of detection (LOD) was defined as a signal-to-noise (S/N) ratio of 3 and the limit of quantitation (LOQ) as a S/N of 10. For the determination of the intra-day assay, the QC was analyzed ten times on the same day (n=10). To determine the inter-day assay, QC was analyzed four times on each of three different days (n=12). Carry-over was determined by measuring a blank sample after the highest calibration point. In the absence of signal in the blank sample, the detection limit was used for carry-over calculation.

7.2.4 Selection of the syringe filter

To determine the adsorption behavior of mAbs on syringe filters, a mAb solution was prepared and filtered using a syringe filter. For this purpose, the syringe filter was first conditioned with 300 µL mAb mixture, which was subsequently wasted. 500 µL of the mAb solution was filtered and tryptically digested. The experiments were performed as triplicate determinations. The concentration of the mAb mixture was 2 mg mL^{-1} BVCZ, 0.3 mg mL^{-1} CTX, 0.2 mg mL^{-1} DRTM, 1 mg mL^{-1} OMLZ, 1 mg mL^{-1} RTX, and 3 mg mL^{-1} TTZ. Chromafil RC (25 mm, 0.45 µm), Chromafil CA (25 mm, 0.45 µm; CA1), Chromafil XTRA PET (25 mm, 0.45 µm), Chromafil PTFE (25 mm, 0.45 µm) syringe filters from Macherey-Nagel GmbH & Co. KG (Düren, Germany), membrane filter CA (4 mm, 0.45 µm; CA2) and membrane filter Nylon (4 mm, 0.20 µm) from Chromatographie Zubehör Trott (Kriftel, Germany) were tested.

7.2.5 Air filter selection

Air filters were doped with 15 µL mAb solution. After drying at room temperature, the filters were transferred to a 120 mL extraction beaker (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and extracted with 5 mL PBS buffer by manual shaking for 30 s. The extract was then tryptically digested. Experiments were performed as triplicate determinations (n=3). The concentration of the doping solution was 2 mg mL^{-1} BVCZ, 0.3 mg mL^{-1} CTX, 0.2 mg mL^{-1} DRTM, 1 mg mL^{-1} OMLZ, 1 mg mL^{-1} RTX, and 3 mg mL^{-1} TTZ. The filters tested consisted of polycarbonate (Nuclepore, Whatman plc, Maidstone, United Kingdom), polyvinylidene fluoride (15229, RCT Reichelt Chemietechnik GmbH + Co., Heidelberg, Germany), polytetrafluoroethylene (TE38, Whatman plc, Maidstone, United Kingdom), glass fiber (GF-3, Macherey-Nagel, Düren, Germany), and quartz fiber (QF-10, Macherey-Nagel, Düren, Germany). As the glass fiber and quartz fiber filters disintegrated during extraction, they were filtered prior to tryptic digestion using membrane filter CA (4 mm, 0.45 µm) from Chromatographie Zubehör Trott (Kriftel, Germany).

7.2.6 Validation of the air filter

To validate the air filter material, the polycarbonate filter (Nuclepore, Whatman plc, Maidstone, United Kingdom) was investigated for desorption efficiency, breakthrough stability and storage stability as a triple determination.

To determine the desorption efficiency, polycarbonate filters were doped with a solution that equaled 1x, 5x, 10x and 20x the LOQ. After drying at room temperature, the air filters were processed as described in section Air filter selection.

For breakthrough stability, the air filters were doped with a solution that equaled five times the LOQ and dried at room temperature. The filters were then placed in the filter holders, which were connected to the SG10-2A sampling pump (GSA Messgerätebau GmbH, Ratingen, Germany). A flow rate of 3.5 L min^{-1} was applied for duration of 0, 15, 120, and 480 min. Subsequently, the filters were extracted as described in section Air filter selection.

For storage stability, the air filters were doped with a solution that equaled five times the LOQ and stored after drying at room temperature at -20°C ± 2°C, 6°C ± 2°C, and 21°C ± 1°C under nitrogen atmosphere, air atmosphere, and dried air atmosphere. The nitrogen atmosphere was ensured by working in a GP concept isolator (Jacomex SAS, Dagneux, France) and the dry air atmosphere was ensured by storage in a desiccator.

7.2.7 Quantitation of lactose

The chromatographic separation system was a Shimadzu Prominence LC 20 (Duisburg, Germany), which consists of a binary pump (LC-20AD), a column oven (CTO-20AC), a degasser (DGU-20A3), and an autosampler (SIL-20AC) that was set to 6°C. The system was coupled to a QTRAP 3200 (AB Sciex Instruments, Darmstadt, Germany). Control of the overall system and data analysis were performed using Analyst 1.6.3 Build 5095 software from AB Sciex (Darmstadt, Germany). The mobile phases used were (A) water + 0.1% FA and (B) acetonitrile + 0.1% FA. The other method settings are given in Table 7-3.

Table 7-3: Summary of method parameters for the quantitation of lactose.

Stationary phase	Thermo HyperCarb 3.0 µm; 2.1 x 50 mm; (Thermo Fisher Scientific Inc., Meerbusch, Germany)	
Column temperature (°C)	40	
Injection volume (µL)	100	
Flow rate (mL min^{-1})	0.4	
Gradient (min // %B)	0.0-2.5	2-100
	2.5-3.0	100-100
	3.0-3.1	100-2
	3.1-5.0	2-2
Gas temperature (°C)	250	
Gas counterflow (L min^{-1})	10	
Nebulizer gas (L min^{-1})	30	
Heater gas (L min^{-1})	60	
Entry potential (V)	7	
IonSpray potential (V)	5000	
Dwell time (ms)	150	
Ionization mode	Electrospray Ionization Positive	
Quantifier, *m/z*	343 → 163	
Qualifier, *m/z*	343 → 85	

7.2.8 Performance of the aerosolization experiments

The nebulization experiments were performed in a sealed 1 m³ room cube. The aerosol was generated using a U22 Pocket Nebulizer (Omron K.K., Mannheim, Germany). The nebulizer was placed at a height of 30 cm. In order to achieve the required inclination of 30°, a corresponding fixture was manufactured using 3D printing. The experimental setup is shown graphically in the Supplementary information. During the experiments, the SG10-2A sampling pump (GSA Messgerätebau GmbH, Ratingen, Germany) was set to a flow rate of 3.5 L min^{-1} and a sampling time of 15 min.

To determine the sampling location with the highest recovery, the distribution of the surrogate lactose in the room cube was determined. For this purpose, the sampling location was varied in 10 cm steps to the entry source. The nebulizing solution used was 1 mL of an aqueous lactose

solution with a concentration of 50 µg mL^{-1}. Lactose was sampled using glass fiber filters (GF-3, Macherey-Nagel, Düren, Germany) in accordance on the ISPE Good Practice Guide: Assessing the Particulate Containment Performance of Pharmaceutical Equipment [15]. The experiments were performed as triplicate determinations and are presented in the Supplementary information. In experiments with lactose in matrix, the matrix solution consisted of 0.9 mg glacial acetic acid, 127.5 mg mannitol, 8 mg polysorbate 20, 14.8 mg sodium acetate trihydrate, and 17.5 mg sodium chloride in 5 mL water. In experiments with daratumumab, this was used at a concentration of 750 µg mL^{-1}. The air filter for sampling daratumumab was made of polycarbonate (Nuclepore, Whatman plc, Maidstone, United Kingdom).

7.2.9 Application of the developed sampling method

To prepare the trastuzumab preparations, the drug Kanjinti 420 mg (Amgen, Thousand Oaks, USA) was dissolved in 20 mL water for injection according to the manufacturer's recommendation. A 20 mL BD Plastipak syringe with Luer-Lock connector (Becton Dickinson, New Jersey, USA) and a Mini-Spike Chemo Micro-Tip (B. Braun, Melsungen, Germany) were used for this purpose. The Mini-Spike Chemo Micro-Tip has a 0.2 µm venting filter, but no particle filter. A total of 680 mg trastuzumab was transferred into a 500 mL Easyflex+ application bag (Carelide GmbH, Langen, Germany) pre-filled with isotonic saline. The preparation was carried out in a safety cabinet type H130 (Berner International GmbH, Elmshorn, Germany), which was operated switched on or off depending on the experimental procedure. During preparation, the preparer carried a sampling head in the breathing zone. The tests were carried out as single determinations. The preparations were discarded after the experiments and not administered to any patient.

7.3 Results and Discussion

7.3.1 Development and validation of a multi-analyte method for the quantification of peptides

Monoclonal antibodies (mAbs) carry multiple charges after electrospray ionization due to size, shape, and physico-chemical properties. A distribution of charges results in a decrease in sensitivity in ESI-MS (see Supplementary Part for more information). Peptides, on the other hand, have a much narrower charge distribution compared to intact proteins, thereby profiting from higher sensitivity [12, 13]. However, to avoid analyzing all peptides formed during enzymatic digestion, signature peptides specific for a certain protein or mAb can be selected, allowing indirect quantitation of intact proteins. Furthermore, one peptide with a conserved sequence was found present in all mAbs. This specific peptide can be used as a sum parameter to detect mAbs.

For this purpose, signature peptides for daratumumab (DRTM), rituximab (RTX) and trastuzumab (TTZ), as well as the universal peptide were taken from our previous study [13]. To identify the signature peptides of bevacizumab (BVCZ), cetuximab (CTX) and omalizumab (OMLZ), their primary sequences were digested in silico and the peptides formed were compared with those of all other therapeutic antibodies, whose primary sequences were available. A list of all therapeutic antibodies used for matching, as well as an evaluation of the selected signature peptides can be found in the Supplementary information. The identified peptides were VLIYFTSSLHSGVPSR (BVCZ), YASESISGIPSR (CTX), LLIYDASNR (DRTM), LLIYAASYLESGVPSR (OMLZ), FSGSGSGTSYSLTISR (RTX), LLIYSASFLYSGVPSR (TTZ) and DSTYSLSSTLTLSK (universal).

The chromatographic method was optimized to achieve baseline separation of the selected peptides. Independent of co-eluting matrix, this allows the use of a scheduled MS/MS setting, maximizing dwell time and thus sensitivity.

A representative chromatogram obtained is shown in Figure 7-1. The critical peak pair consists of the signature peptides for DRTM and RTX. A chromatographic resolution of 1.6 demonstrates that a baseline separation was achieved for all target-peptides.

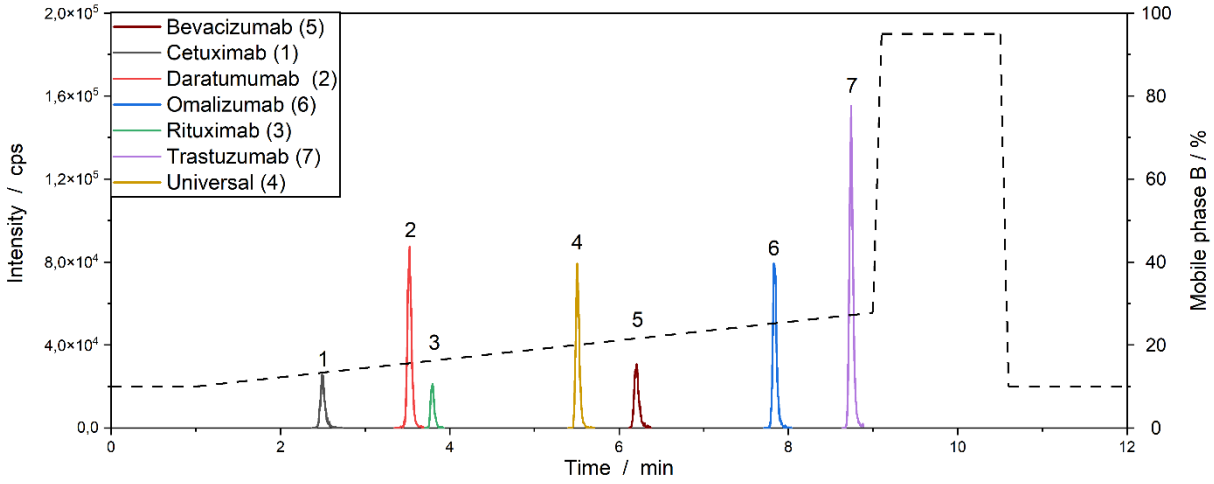


Figure 7-1: Chromatogram of the six signature peptides for the monoclonal antibodies bevacizumab, cetuximab, daratumumab, omalizumab, rituximab, and trastuzumab. Peak number 4 is the universal peptide that can be found in all selected mAbs. The concentration of all substances used is 1 mg mL^{-1}. The dashed line represents the gradient program. For further chromatographic and mass spectrometric settings, see material and methods.

Validation was performed by intra-day assay, inter-day assay and determination of carry-over. In addition, the limit of detection (LOD) and the limit of quantification (LOQ) of the method were calculated. The results of the method validation are shown in Table 7-4.

Table 7-4: Summary of method validation. Limit of detection (LOD) is defined as signal-to-noise (S/N) ratio of 3 and limit of quantification (LOQ) as S/N of 10. In the intra-day assay an independent quality control (QC) sample was analyzed ten times (n=10). The inter-day assay was performed by analyzing one QC four times on three different days (n=12). *Since no carry-over was detected, this was calculated from the LOD.

Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Recovery (Intra-Day-Assay) (%)	Recovery (Inter-Day-Assay) (%)	Carry-Over (%)
Bevacizumab	3.4	11	96 ± 6	79 ± 5	< 1.7*
Cetuximab	1.7	5.7	85 ± 3	84 ± 11	< 5.6*
Daratumumab	0.30	1.0	83 ± 3	89 ± 10	< 1.5*
Omalizumab	1.7	5.7	92 ± 5	75 ± 3	< 1.7*
Rituximab	3.9	13	86 ± 4	83 ± 11	< 2.0*
Trastuzumab	8.8	29	91 ± 4	79 ± 2	< 2.9*
Universal	2.1	7.0	95 ± 3	76 ± 3	< 0.2*

If the validation is evaluated according to the Food and Drug Administration (FDA) guideline "Bioanalytical Method Validation" [16], it can be rated as successful. The intra-day assay and inter-day assay values are in the range of 83-96% and 75-89%, respectively, with standard deviations of 3-6% and 3-11%, respectively. In accordance with this guideline, approved analytical values are in the range of 75-115% with a standard deviation of up to 15%. No carry-over was observed during method validation.

7.3.2 Selection and validation of suitable filter materials

In addition to the development and validation of the analytical method, a sampling method was developed. For this purpose, the approach chosen, based on SMEPAC, was to draw air over a filter in order to enrich airborne mAbs on it. However, the subsequent extraction process may cause certain air filters (glass and quartz fibre) to degrade, requiring the suspension to be filtered before further use. Therefore, syringe filters were tested for their adsorption behavior with respect to mAb-containing solutions. The results of the recovery of the filtered antibody solution are shown in Figure 7-2.

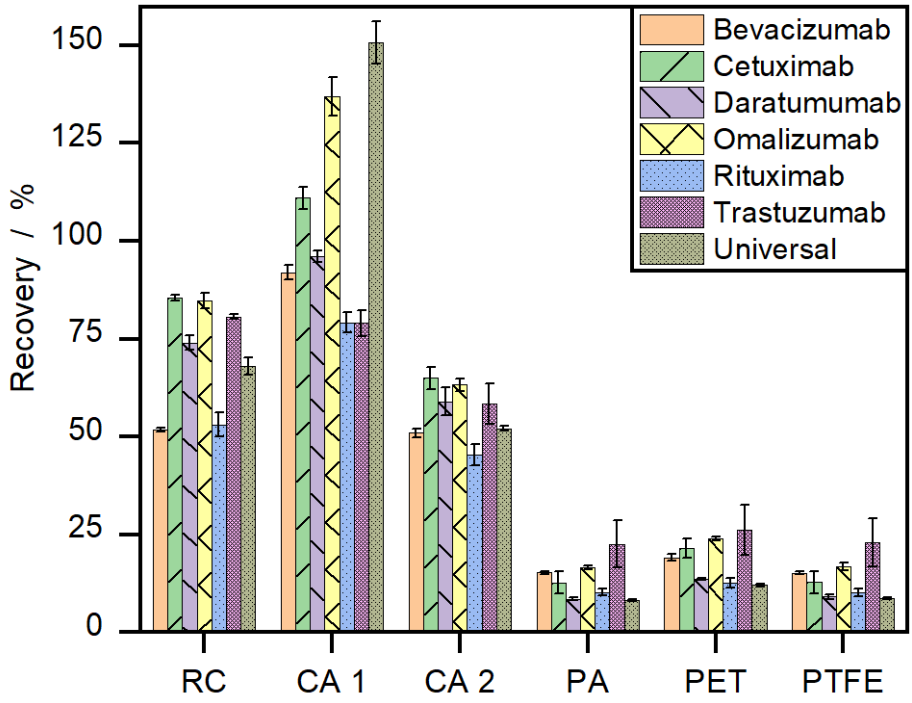


Figure 7-2: Results for the selection of a material for use as syringe filter. For this purpose, a solution containing the monoclonal antibodies was filtered. The filtered solution was analysed. The antibodies were detected via signature peptides. The total protein concentration of the solution was 7.5 mg mL^{-1}. The experimental details are described in Material and Methods. The experiments were performed in triplicates (n=3). RC: regenerated cellulose; CA: cellulose acetate; PA: polyamide; PET: polyethylene terephthalate; PTFE: polytetrafluoroethylene.

Syringe filters made of regenerated cellulose (RC) resulted in recoveries in the range of 52-85%. Interestingly, the two syringe filters made of cellulose acetate (CA) showed significantly different recoveries. The CA syringe filter from manufacturer 1 showed recoveries in the range of 79-151%, whereas that from manufacturer 2 had a recovery of 45-63%. The different recoveries (over-finding for CA1; under-finding for CA2) could be due to a different degree of acetylation [17]. In the evaluation of the blank samples, no signal was detected in the CA1 filter, which means that recoveries greater than 100% cannot be explained. The syringe filters made of polyamide (PA), polyethylene terephthalate (PET) and polytetrafluoroethylene (PTFE) had recoveries below 30%, indicating high protein binding capacity. Thus, these syringe filters were unsuitable for the filtration of antibody-containing solutions.

In summary, it was determined that filtration of solutions containing monoclonal antibodies should be avoided because losses occurred. However, if filtration is required, filters made of RC or CA2 should be used. This would be the case if particles are released during the subsequent air filter extraction. CA2 filters were used for the subsequent experiments in this study, as they are smaller in size and can therefore be used with lower volumes.

To test air filters suitability, five different filter materials were doped with antibody solutions and extracted after the solution had dried. Polycarbonate (PC), polyvinylidene fluoride (PVDF) and polytetrafluoroethylene (PTFE) are filters that do not decompose during processing, while glass fiber (GF) and quartz fiber (QF) are filter materials that decompose during processing. The filter materials that decompose during extraction (GF and QF) were additionally filtered using CA2 filters to separate the filter components after extraction. The results of the tests are shown in Figure 7-3.

Air filters made of PC yielded the best results (80-104%), with PVDF (75-128%) and PTFE (60-89%) also giving high recoveries. However, filters made of GF and QF only led to recoveries of 15-39% and 17-37%, respectively, and were therefore unsuitable. Since the GF and QF filters decompose during extraction, the resulting suspensions were filtered. When the losses due to the filtration step (see Figure 7-2) were considered, the corrected recovery increased to 25-62% for GF and 29-58% for QF. This was still well below the values for the other materials. The adsorption of monoclonal antibodies on glass has already been described in the literature [18]. The decomposition of GF and QF filters increases the accessible surface for the protein solution during extraction, such that the adsorption process of mAbs on the filter material is intensified.

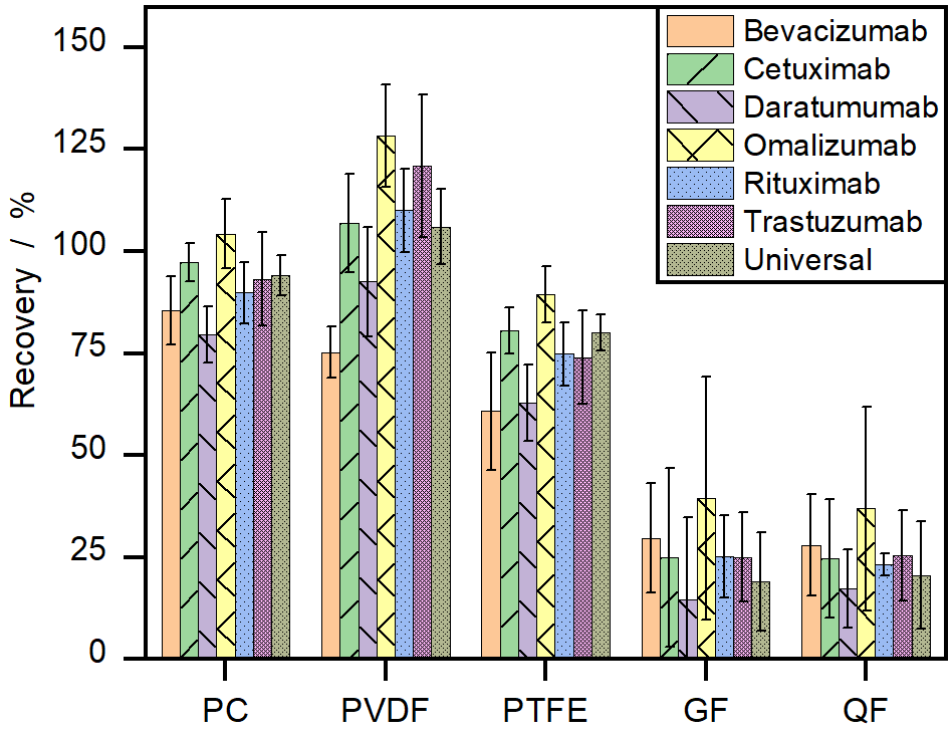


Figure 7-3: Results for the selection of a material for use as air filter. For this purpose, the filters were doped with a mixture of the antibodies. After drying the doped filters, these were extracted with PBS buffer. The antibodies were detected via their signature peptides. The total protein concentration of the solution was 7.5 mg mL^{-1}. The experimental details are described in Material and Methods. The experiments were performed in triplicates (n=3). PC: polycarbonate; PVDF: polyvinylidene fluoride; PTFE: polytetrafluoroethylene; GF: glass fibre; QF: quartz fibre.

Since the PC air filter gave the best results, it was used for all further experiments and validated in terms of desorption efficiency, retention capacity and storage stability. These experiments were performed in accordance with the standardised measurement of equipment particulate airborne concentration (SMEPAC) guideline [15] and can be found in Figure 7-4.

To determine the desorption efficiency (Figure 7-4A), polycarbonate filters were doped with a solution that equals 1x, 5x, 10x and 20x the limit of quantification (LOQ) to check whether a concentration-dependent behaviour is observed. The average desorption efficiency is 83% ± 13% and is independent of the doped concentration.

For testing the breakthrough (Figure 7-4B) of the filters, in addition to doping with mAb solution (5x LOQ), they were clamped in a filter holder and exposed to a volume flow of 3.5 L min^{-1} for 0, 15, 120 and 480 min. The recovery after 480 min (8 h) was 97% on average for all analytes and concentration ranges from 92-100%, which is in the measurement precision of the recovery for the universal peptide (102% ± 12%). This means that the personal air sampling method can be used over an entire work shift without significant losses during longer sampling times.

The storage stability tests were carried out at different temperatures (21 °C, 5 °C and 20 °C) and at different storage conditions (air, dried air and N_2). Based on this test design, the influence of temperature, atmospheric oxygen and humidity can be determined. The results are shown in Figure 7-4C.

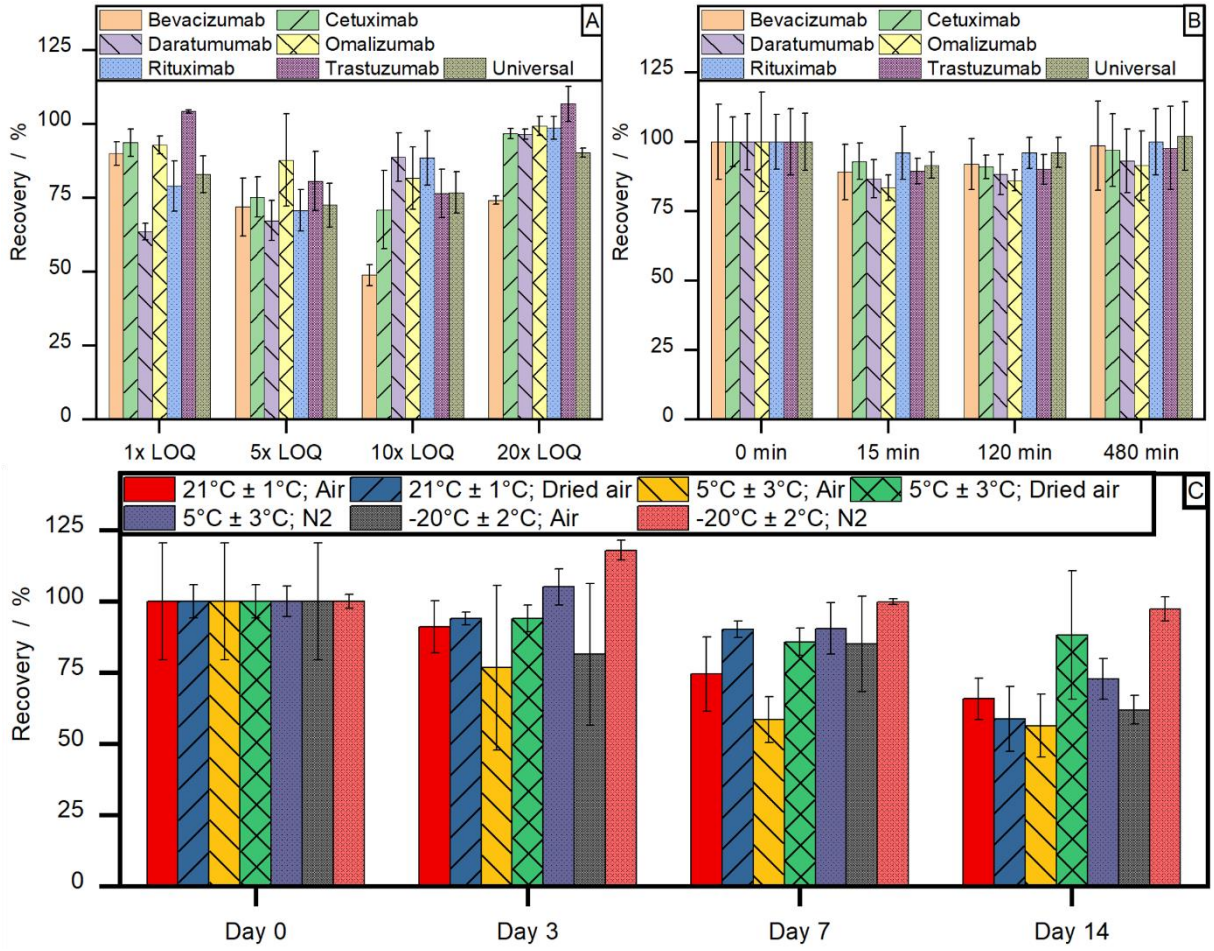


Figure 7-4: Results of the validation of the polycarbonate filter. The tests included (A) the desorption efficiency as a function of the quantity applied, (B) the filter breakthrough as a function of time and (C) the storage stability at different temperatures, conditions and times. The analyses are triplicate determinations (n=3). The antibodies were detected indirectly via their peptides. The experimental details are described in Material and Methods.

The highest recovery after 14 days and thus the highest stability was achieved with storage at -20 °C (97% ± 4%) and N_2. In general, a comparison of the data after 14 days shows that a lower storage temperature leads to a higher stability if humidity is excluded. In this case, the recovery increases from 59% ± 11% (21 °C ± 1 °C; dried air) to 88% ± 23% (5 °C ± 3 °C; dried air) to 97% ± 4% (-20 °C ± 2 °C; N_2). The exclusion of humidity is of particular importance here, because during the experiment, condensation was observed in the storage vessels of the cooled samples (5 °C and 20 °C). This leads to a dilution effect and can favour microbial degradation

of the monoclonal antibodies. The effect of this is reflected in the analytical data by a lower recovery of the samples stored under air with their counterparts stored under dried air or N_2.

The analytical results of the samples at 5°C ± 3°C under dried air and N_2 are comparable to each other; therefore additional degradation by atmospheric oxygen does not occur.

In addition to the conditions for long-term storage (14 days), the conditions for sample shipment must be defined. For this purpose, the stability data after 3 days can be used, as this corresponds to a usual shipping time. This shows that refrigerated samples (5°C ± 3°C; dried air) do not experience significant substance degradation during the short storage period (recovery: 94% ± 5%).

In summary, a PC filter fulfils all requirements, as the mAbs do not decompose on the filter during sampling. Furthermore, it is possible to store PC filters contaminated with mAbs in a refrigerator for a period of 3 days at a temperature of 5°C or for 14 days at a temperature of -20°C. In this case, care should be taken to ensure that there is no condensation of humidity. The data show that lower temperatures and avoidance of humidity improve storage stability. Both findings are consistent with the literature [19].

7.3.3 Performance of nebulization experiments

To test the suitability of the developed method, daratumumab (Darzalex) was aerosolized using mesh nebulizer and sampling was performed simultaneously. To simulate a sampling experiment close to reality, the sampling was performed at a defined distance from the emission source. This allows the aerosol to spread in space and subsequently prevents a complete recovery. However, in order to evaluate the recovery achieved by the method, it is compared with the recovery from a sampling method for lactose accepted by SMEPAC. In order to exclude the influence of external factors such as temperature fluctuations or air flow, the experiments were carried out in a closed, temperature-controlled 1 m^3 cubic chamber. The results are shown in Figure 7-5.

First, the dispersion of the aerosol with lactose in the experimental room was characterised to determine the sampling points with the highest possible recovery (see Supplementary information). These experiments showed that the solvent has a high influence on the aerosolization. As shown in Figure 7-5A, the recovery of lactose in an aqueous matrix differs on average by a factor of 23 compared to lactose in a matrix consisting of the formulants of Darzalex (glacial acetic acid, mannitol, polysorbate 20, sodium acetate trihydrate and sodium

chloride). This significant difference is probably due to the surfactant polysorbate 20, which reduces the surface tension, thus changing conditions of aerosolization.

Regarding the influence of the matrix, lactose in matrix was used for the comparison with daratumumab as Darzalex. This showed that, regardless of whether the active pharmaceutical ingredient daratumumab or lactose were nebulized separately (Figure 7-5B) or mixed together (Figure 7-5C), the recoveries of daratumumab were only 28% ± 5% and 20% ± 2% of those of lactose, respectively.

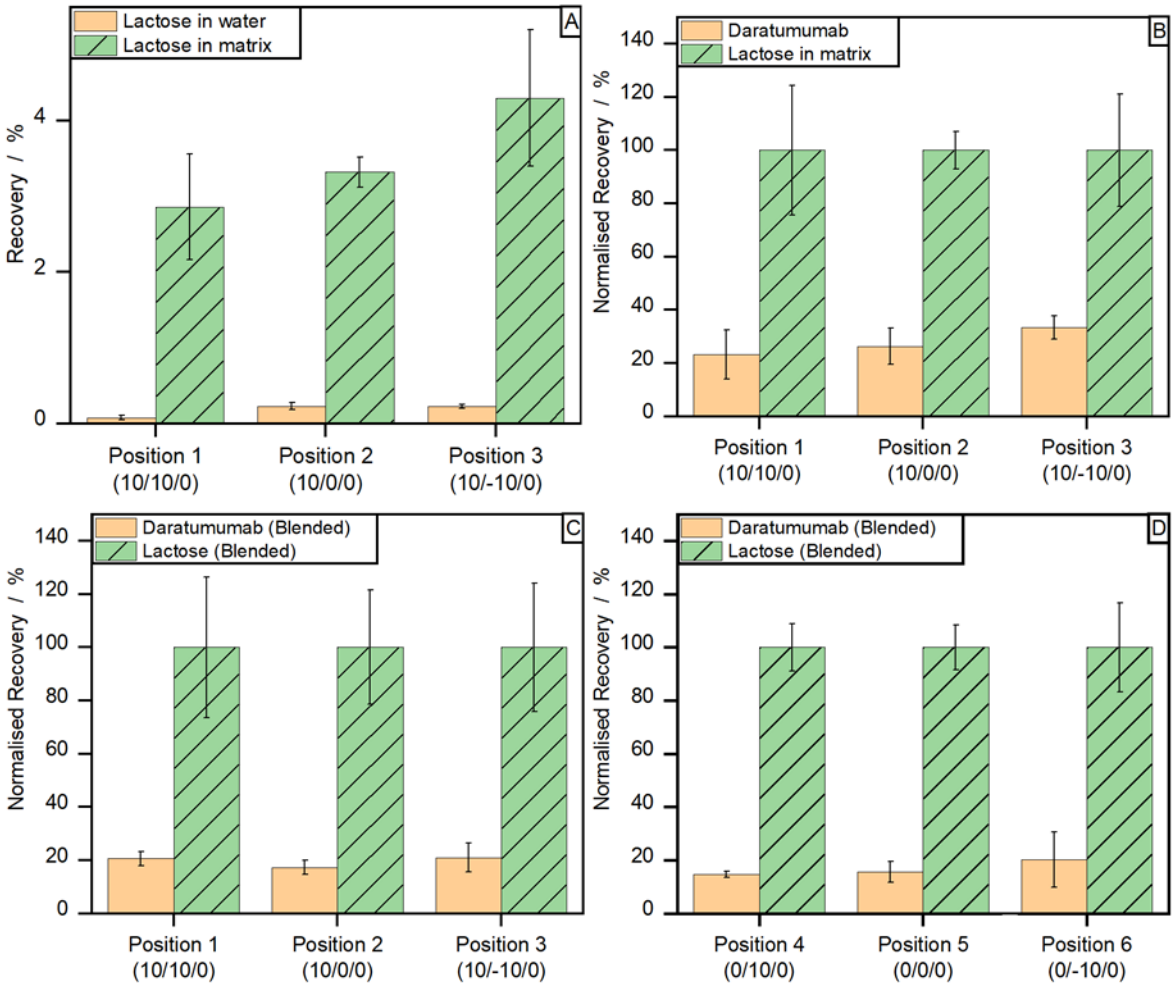


Figure 7-5: Results of the aerosolization experiments. Analysed were (A) lactose in water and lactose in matrix, (B) daratumumab as Darzalex and lactose in matrix, (C) daratumumab as Darzalex and lactose mixed and (D) daratumumab as Darzalex and lactose mixed with sampling points closer to the source of entry. The matrix consists of the formulation substances of Darzalex (see Material and methods). The analyses are triplicate determinations (n=3). A more detailed explanation of the positions including graphical representation can be found in the Supplementary information.

To check whether daratumumab sampling depends significantly on distance to the aerosolization device, the position of the sampling point was varied (Figure 7-5D). A recovery of 18% ± 3% for daratumumab was achieved, which is comparable to the results from the previous sampling points (Figure 7-5C), where the recovery is 20% ± 2%. Therefore, it can be assumed that the spatial distribution of lactose and daratumumab is the same. This means that the under finding is a systematic error.

Respaud et al [1] were able to show that aggregates are formed when antibody solutions are nebulised. The explanation for this is that the antibodies are subjected to degradative stress during the formation of an air-liquid interface. The maximum number of antibody molecules that can attach to the interface depends on the properties of the droplet. This means that at higher concentrations a higher proportion of antibody molecules are not present at the interface and are thus protected from degradative stress. Interestingly, too high antibody concentrations also lead to aggregation, as they increase the probability of protein-protein interactions. Since Respaud et al [1] have already observed this behavior across a concentration range of 2-40 mg mL^{-1}, it is expected that this trend will continue at lower concentrations and thus be particularly noticeable at the 50 µg mL^{-1} concentration used in this study. This process could be an explanation for the systematic undersampling. Due to the extractive processing of the samples, only monoclonal antibodies and soluble aggregates are detected. Non-soluble aggregates are separated during the extraction process.

In addition, Hertel et al [2,3] demonstrated that the liquid reservoir of a mesh nebulizer heats up during nebulization, creating thermal stress on the mAb. This would also be a potential source for aggregate formation. Switching from a mesh nebulizer to a jet nebulizer or ultrasonic nebulizer would not correct the systematic error but would probably intensify it. With the latter two technologies, the liquid is nebulized at least 10-15 times before the aerosol leaves the device. This means a repeated occurrence of degradative forces due to the air-liquid interface. Furthermore, with both technologies there is an increased heat emission [2,3].

Based on the good results of the validation of the filter material (See chapter Selection and validation of suitable filter materials) and the results of a reproducible sampling, the lower mAb recovery compared to lactose is considered a systematic error. This means that a reproducible sampling method has been developed which can be used to determine mAb contamination in a pharmaceutical environment.

7.3.4 Application of the developed sampling method

The developed method for personal air sampling of mAbs is suitable to answer relevant questions in the field of occupational health and safety. In particular, potential release and minimization of such release are of importance as well as what quantity of active pharmaceutical ingredient may arrive at the person handling the substance.

Our previously published method for process analysis was used to determine the release potential [13]. Here, a new drug container Darzalex (API: daratumumab) was taken from the refrigerator and penetrated with a cannula. The graphical illustration of the experiment can be found in the Supplementary information. Furthermore, 1 mL of liquid was taken and transferred into a second empty vial. During this procedure, a released amount of daratumumab of 15 ng absolute was detected (data not shown). Hence, there is a release potential when handling monoclonal antibodies. Halsen et al [7] described an expected aerosol release during pressurization in the range of several hundred micrograms of liquid per m3. The drug Darzalex has an active ingredient content of 2% (w/w). Based on a sampling time of 1 min (3.5 L) and the drug content, the expected amount of detected daratumumab is 7-70 ng absolute. The detailed calculation can be found in the Supplementary Part. Thus, the determined content of 15 ng is plausible.

Since the risk of unintentional releases has been demonstrated, it was examined which measures would allow effective reduction. For this purpose, trastuzumab preparations were made with a pressure equalisation system (spike with 0.2 µm vent filter). During preparation, the preparer carried the sampling head in inhalation zone and the method developed in this study was applied. A description of the construction of a spike, as well as how to wear the sampling head are shown in the Supplementary information. Regardless of whether the safety cabinet was turned on or off, no monoclonal antibody could be detected.

This means that although there is a potential for release, it can be significantly reduced by reduction measures.

7.4 Conclusion and outlook

For the first time, a method for personal air sampling of monoclonal antibodies was established. For this purpose, an analytical method based on HPLC-MS/MS was developed, as well as a sampling procedure for the monoclonal antibodies bevacizumab, cetuximab, daratumumab, omalizumab, rituximab and trastuzumab. The sampling procedure uses a polycarbonate filter that meets the requirements of SMEPAC. Furthermore, the developed method was tested under simulated conditions by targeted nebulization of an antibody-containing solution and was evaluated as suitable for use. When performing activities with monoclonal antibodies, it could be shown that there is a release potential of active pharmaceutical ingredient. However, this can be reduced by using pressure equalisation systems such as spikes.

7.5 Supplementary Information

7.5.1 Experimental setup for the nebulization experiments

The nebulization experiments were performed in a closed room cube with a volume of 1 m^3. Sampling was performed at the positions marked in Figure 7-6. These vary in their distance along all three spatial directions. The spatial directions are defined as X (abscissa axis; horizontal coordinate axis), Y (ordinate axis, vertical coordinate axis) and Z (applicate axis; height). The distance was determined from the outlet of the input source to the inlet of the sampling head, as plotted. The holder used for the input source (U22 Pocket Nebulizer; Omron K.K., Mannheim, Germany) was self-designed using a 3D printer and ensures a tilt angle of 30° over the duration of the experiment. The required angle of 30° is based on the manufacturer's instructions to ensure uniform nebulisation. To prevent the nebulizer from falling forward due to the tilt, the holder was additionally fixed.

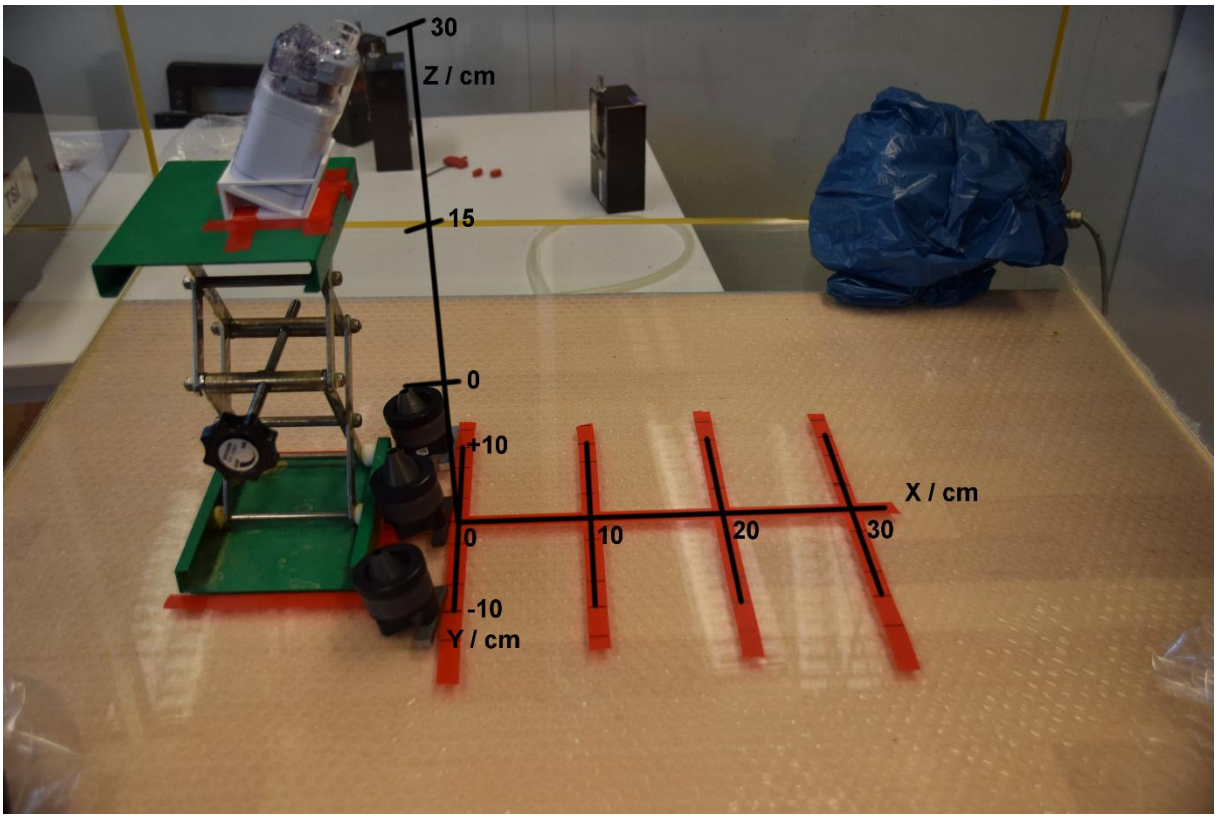


Figure 7-6: Illustration of the experimental setup for the nebulization experiments in the room cube. The details of the experimental procedure can be found in the material and methods section.

7.5.2 Distribution of lactose in the experimental room

The determined recovery for lactose depends on the distance of the sampling point to the input source, with more distant sampling points leading to a lower recovery. This behavior is shown in Figure 7-7. This is particularly evident at the closest sampling point 0/0/15 (X/Y/Z), which has the highest recovery. Interestingly, for the sampling points at a height of 15 cm along the X-axis, no recovery could be determined for a Y-value of 10 cm, but it could be determined for a height of 0 cm. This is because the nebulizer produced a systematic error and ejected the aerosol formed rather in the direction of Y=-10 cm. The fact that a recovery at Y=10 cm was nevertheless achieved at a height of 0 cm is due to the fact that the aerosol is distributed evenly in the spatial directions when it hits the ground because of the resistance. The analysis values obtained are thus in conformity with the optically observed aerosol dispersion.

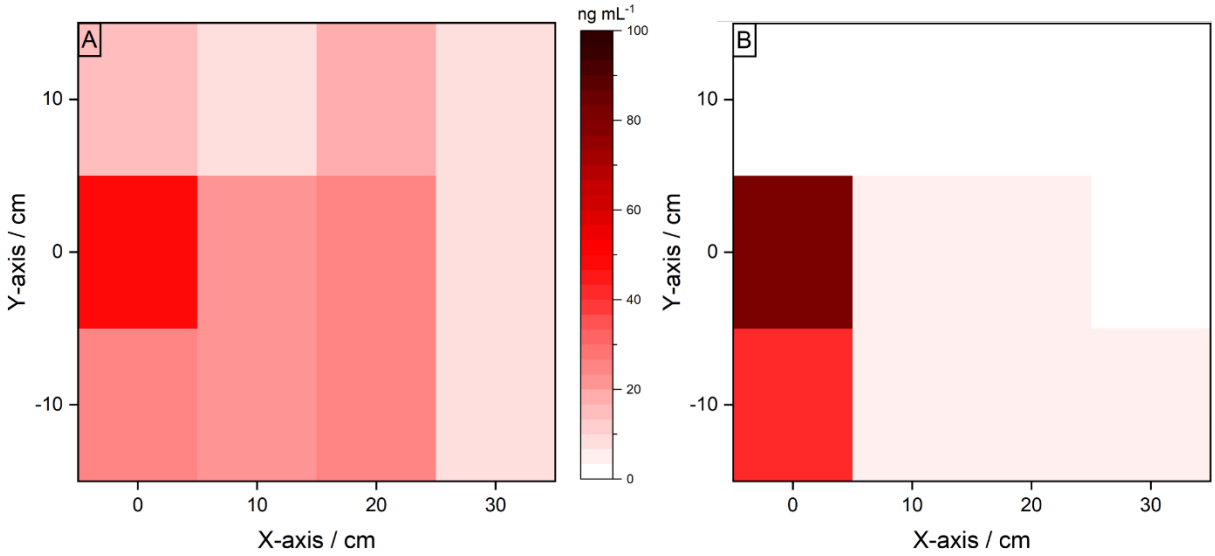


Figure 7-7: Distribution of lactose in the experimental space at a height of (A) 0 cm and (B) 15 cm. A photo of the experimental room is shown in Figure 7-6.

7.5.3 Analysis of intact monoclonal antibodies to demonstrate charge formation

Intact monoclonal antibodies form a distinct charge distribution during electrospray ionization under denaturing conditions. This is shown in Figure 7-8A for rituximab as MabThera at a concentration of 2.5 mg mL^{-1}.

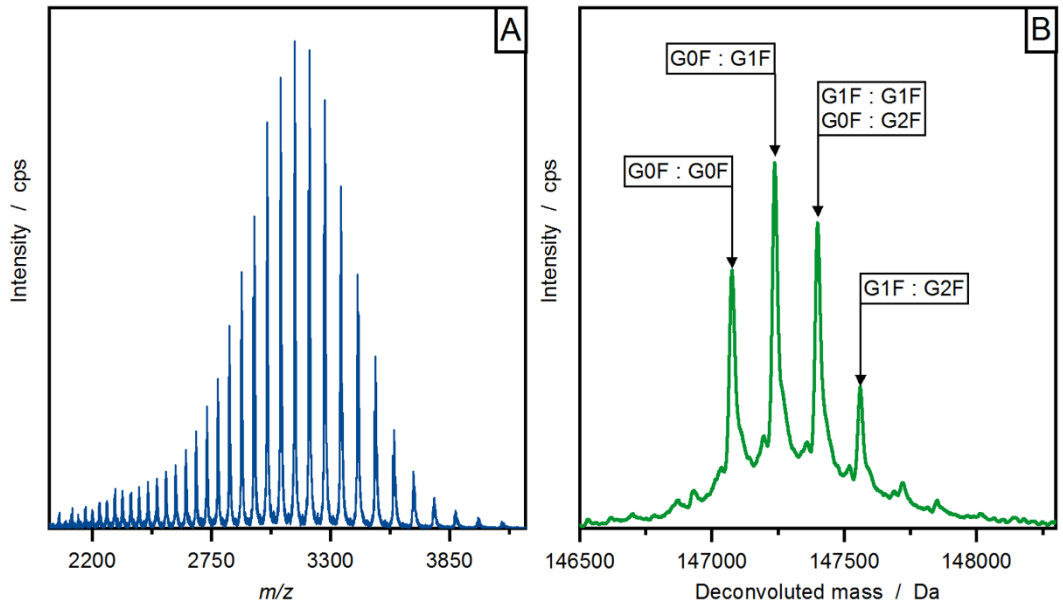


Figure 7-8: of rituximab as MabThera by high-resolution mass spectrometry. Figure A shows the raw data obtained (charge distribution), whereas Figure B shows the analysis data processed via the maximum entropy algorithm.

As the analyte concentration of 2.5 mg mL^{-1} illustrates, it is not possible to analyze sensitively due to the charge distribution that forms. Nevertheless, the analysis of monoclonal antibodies by high-resolution mass spectrometry can be used to characterize them. This is illustrated in Figure 7-8B. For this purpose, the deconvoluted mass was calculated from the charge distribution using a mathematical algorithm (in this case maximum entropy). This can be used to characterize modifications such as the sugar modifications of a monoclonal antibody.

7.5.4 Listing and evaluation of the monoclonal antibodies and peptides used

Only peptides with a length of 5-25 amino acids were used, as peptides that are too short are non-specific and peptides that are too long lead to a charge pattern. Furthermore, peptides with the amino acid methionine were excluded, as this tends to oxidise. With this approach, only peptides specific for omalizumab (LLIYAASYLESGVPSR) and cetuximab (YASESISGIPSR) could be found without an occurrence in other monoclonal antibodies. Therefore, the peptides with the least overlap were selected. The peptide for bevacizumab (VLIYFTSSLHSGVPSR) is also found in ranibizumab. The peptide for trastuzumab (LLIYSASFLYSGVPSR) in atezolizumab and the peptide for rituximab (FSGSGSGTSYSLTISR) in ibritumomab tiuxetan, siltuximab and tositumomab. For daratumumab (LLIYDASNR), there is shared commonality with golimumab, necitumumab, nivolumab, ofatumumab and olaratumab. Furthermore, a universal peptide (DSTYSLSSTLTLSK) was selected. This is found in all mAbs with a kappa-light chain. This is true for 69 of 81 mAbs.

All monoclonal antibodies that have been used for sequence matching are listed below.

Abciximab, Adalimumab, Alemtuzumab, Alirocumab, Atezolizumab, Avelumab, Basiliximab, Benralizumab, Bezlotoxumab, Blinatumomab, Brentuximab vedotin, Brodalumab, Brolucizumab, Burosumab , Canakinumab, Caplacizumab, Cemiplimab, Certolizumab pegol, Crizanlizumab, Daclizumab, Denosumab, Dinutuximab, Dupilumab, Durvalumab, Eculizumab, Edrecolomab, Efalizumab, Elotuzumab, Emapalumab , Emicizumab, Eptinezumab, Erenumab, Evolocumab, Fremanzezumab, Galcanezumab, Gemtuzumab ozogamicin, Golimumab, Guselkumab, Ibalizumab, Ibritumomab tiuxetan, Idarucizumab, Infliximab, Ipilimumab, Isatuximab, Ixekizumab, Lanadelumab, Leronlimab, Mepolizumab, Mogamulizumab, Moxetumomab pasudotox, Muromonab- CD3, Narsoplimab, Natalizumab, Necitumumab, Nivolumab, Obiltoxaximab, Obinutuzumab, Ocrelizumab, Ofaturmumab, Olaratumab, Palivizumab, Panitumumab, Pembrolizumab, Pertuzumab, Ramucirumab, Ranibizumab, Ravulizumab-ALXN1210, Riskanizumab, Romosozumab, Sacituzumab

govitecan, Sarilumab, Satralizumab, Secukinumab, Siltuximab, Tafasitamab, Teprotumumab, Tildrakizumab, Tocilizumab, Tositumomab, Ustekinumab, Vedolizumab.

7.5.5 Specific parameters for the analysis of peptides

For the detection of the peptides, the settings used in Table 7-5 were made on the mass spectrometer (MS). Electrospray ionisation was chosen for ionisation. In this method, the mobile phase is ionised and nebulised in the ion source. As the nebulised droplets evaporate, the charge density increases to the Rayleigh limit and discharges in a Coloumb explosion, forming smaller droplets. This process repeats until the analyte is ionised. When co-eluting the analyte with other sample components, substances with a higher ionisation efficiency are preferentially ionised, leaving fewer free charges available for the remaining substances and decreasing the probability of ionisation. Ion suppression can thus occur. However, even after successful ionisation, there may be a discharge of the analyte through proton exchange reactions in the gas phase. In addition, some substances, such as salts, can prevent the Rayleigh limit from being reached, which means that no ionisation occurs in the first place.

High performance liquid chromatography (HPLC) can be used to prevent co-elution of analytes from matrix components. Also. A sufficient retention is required to separate salts eluting at the void time causing severe ion suppression. Furthermore, by coupling HPLC and MS (HPLC-MS), the MS can be used in scheduled mode. In this mode, the set MS/MS transitions are not recorded over the entire chromatographic run, but only at certain time periods. This makes it possible to reduce the number of simultaneous MS/MS transitions, thus increasing the dwell time. This improves the sensitivity and thus the limit of quantification.

Table 7-5: Specific parameters for the analysis of peptides. DP: Declustering potential. CE: Collision energy. CXP: Collision cell exit potential. The MS/MS detection window is 60 s in total, of which 30 s are recorded before and 30 s after the scheduled time.

Q1 *m/z*	Q3 *m/z*	Scheduled time (min)	DP	CE	CXP
532.8	862.3	3.80	77	25	15
532.8	725.3	3.80	72	26	35
803.9	926.5	4.05	73	40	14
803.9	839.5	4.05	60	41	15
887.0	359.2	8.92	50	31	16
591.7	359.2	8.92	40	44	22
751.9	1036.6	5.73	63	31	45
751.9	836.5	5.73	60	25	18
588.3	602.3	6.42	50	30	15
588.3	359.2	6.42	50	30	15
633.8	359.2	2.79	50	30	15
633.8	816.4	2.79	50	30	15
870.0	359.2	8.06	50	30	15
580.3	359.2	8.06	50	30	15

7.5.6 Calculation of the expected daratumumab release

The expected recovered daratumumab content (D_c) depends on the amount of active pharmaceutical ingredient released per volume unit, the sampling duration (t) and the volume flow rate (Q) used during sampling. Multiplying the sampling duration and volume flow rate gives the sampling volume. The amount of active pharmaceutical ingredient released per volume unit can be calculated from the amount of liquid released per volume unit (V) and the active pharmaceutical ingredient content (w). Equation 1 is thus obtained.

Equation 1: $D_c = V * w * t * Q$

Halsen et al. describe the amount of liquid released per volume unit as several hundred micrograms per m³. A range of V = 100-1000 µg m^{-3} is therefore calculated. The drug has an active pharmaceutical ingredient content of w = 2% (20 mg per 1000 mg). The sampling time was t = 1 min, at a volume flow rate Q = 3.5 L min^{-1} (0.0035 m^3 min^{-1}).

Provided these values are substituted into Equation 1, the minimum (Dmin) or maximum (Dmax) expected recovered daratumumab content is obtained.

Equation 2: D_{min} = 100 µg m^{-3} * 0.02 * 1 min * 0.0035 m^3 min^{-1} = 0.007 µg = 7 ng

Equation 3: Dmax = 1000 µg m^{-3} * 0.02 * 1 min * 0.0035 m^3 min^{-1} = 0.07 µg = 70 ng

Thus, daratumumab recovery is expected to be in the range of 7-70 ng.

7.5.7 Application of the developed sampling method

A fresh drug container was pierced with a cannula in the same way as shown in Figure 7-9. The piercing was performed before the container was turned upside down. During the piercing process, pressure equalization occurs between the drug container and the ambient air. This can cause liquid to be entrained and aerosolized. This aerosol can be detected analytically.

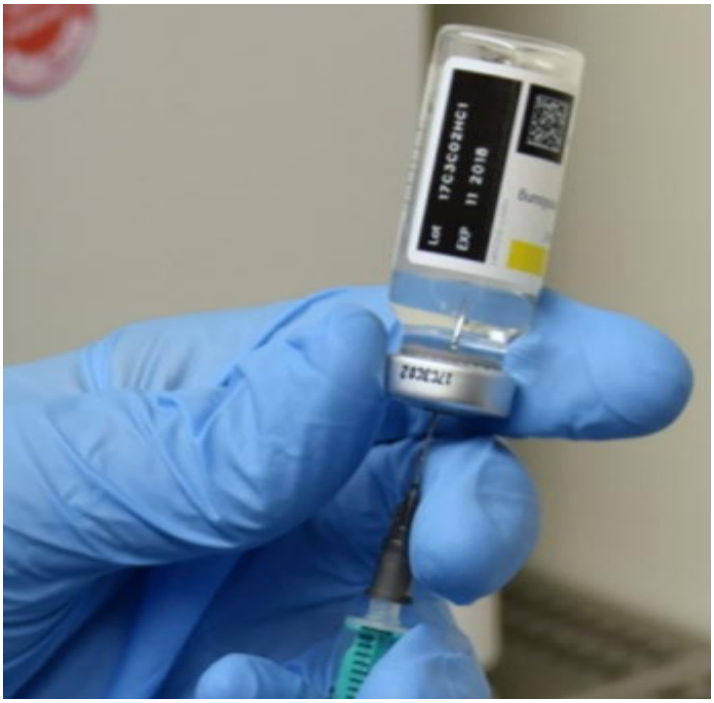


Figure 7-9: Piercing a drug container with a cannula.

A pressure equalization system such as a spike (Figure 7-10A) can be used to prevent unwanted aerosol release. The spike used in the experiments is shown as a cross-section in Figure 7-10B. Here it can be seen that it consists of two chambers. The left chamber contains the 0.2 µm venting filter, which prevents the unwanted release of aerosols. The liquid flows through the right chamber. To minimize adsorption effects and shear forces, there is no filter in the liquid passage.

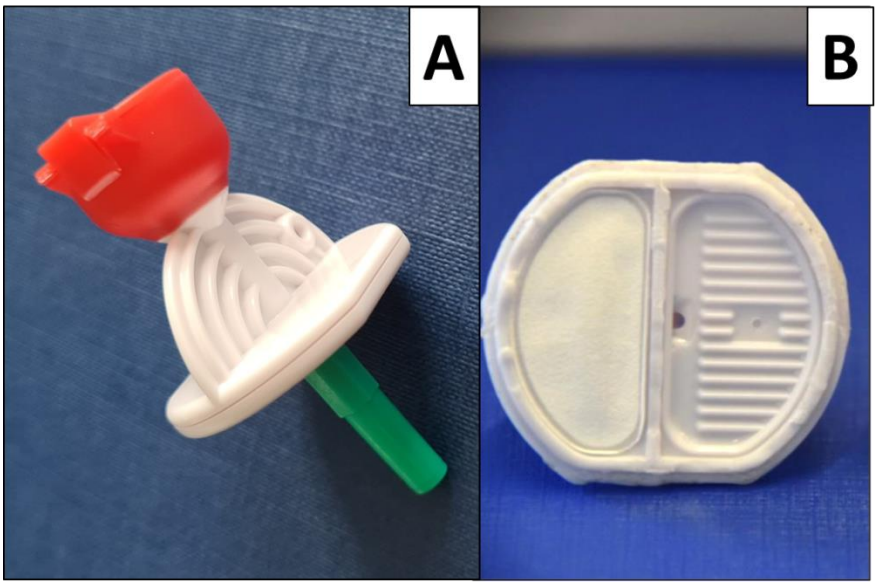


Figure 7-10: Illustration of the spike used in the experiments as (A) intact spike and as (B) cross-section.

To check whether a person is exposed to a released aerosol, a sampling head can be attached to the person's breathing zone. This is exemplified in Figure 7-11. The sampling head contains the filter on which the monoclonal antibodies are deposited.

Figure 7-11: Depiction of a person wearing the personal air-sampling pump. The sampling head is highlighted by a red circle.

7.6 References

[1] H. Kaplon, A. Chenoweth, S. Crescioli, J.M. Reichert, Antibodies to watch in 2022, Mabs-Austin 14(1) (2022).

[2] J.T. Ryman, B. Meibohm, Pharmacokinetics of Monoclonal Antibodies, Cpt-Pharmacomet Syst 6(9) (2017) 576-588.

[3] R.J. Keizer, A.D. Huitema, J.H. Schellens, J.H. Beijnen, Clinical pharmacokinetics of therapeutic monoclonal antibodies, Clin Pharmacokinet 49(8) (2010) 493-507.

[4] L. Liu, Pharmacokinetics of monoclonal antibodies and Fc-fusion proteins, Protein Cell 9(1) (2018) 15-32.

[5] A. Maillet, N. Congy-Jolivet, S. Le Guellec, L. Vecellio, S. Hamard, Y. Courty, A. Courtois, F. Gauthier, P. Diot, G. Thibault, E. Lemarie, N. Heuze-Vourc'h, Aerodynamical, immunological and pharmacological properties of the anticancer antibody cetuximab, Pharm Res-Dordr 25(6) (2008) 1318-1326.

[6] A. Maillet, L. Guilleminault, E. Lemarie, S. Lerondel, N. Azzopardi, J. Montharu, N. Congy-Jolivet, P. Reverdiau, B. Legrain, C. Parent, D.H. Douvin, J. Hureaux, Y. Courty, M. De Monte, P. Diot, G. Paintaud, A. Le Pape, H. Watier, N. Heuze-Vourc'h, The airways, a novel route for delivering monoclonal antibodies to treat lung tumors, Pharm Res 28(9) (2011) 2147-56.

[7] G. Halsen, I. Kramer, Assessing the risk to health care staff from long-term exposure to anticancer drugs--the case of monoclonal antibodies, J Oncol Pharm Pract 17(1) (2011) 68-80.

[8] P.J. Carter, G.A. Lazar, Next generation antibody drugs: pursuit of the 'high-hanging fruit', Nat Rev Drug Discov 17(3) (2018) 197-223.

[9] L. Barrott, E. Foreman, Staff sensitisation to therapeutic monoclonal antibodies resulting from workplace exposure: A case report, J Oncol Pharm Pract 26(5) (2020) 1282-1284.

[10] N. Ferri, S. Bellosta, L. Baldessin, D. Boccia, G. Racagni, A. Corsini, Pharmacokinetics interactions of monoclonal antibodies, Pharmacol Res 111 (2016) 592-599.

[11] L.M.H. Reinders, M.D. Klassen, T. Teutenberg, M. Jaeger, T.C. Schmidt, Development of a multidimensional online method for the characterization and quantification of monoclonal antibodies using immobilized flow-through enzyme reactors, Anal Bioanal Chem 413(28) (2021) 7119-7128.

[12] A. Beck, E. Wagner-Rousset, D. Ayoub, A. Van Dorsselaer, S. Sanglier-Cianferani, Characterization of therapeutic antibodies and related products, Anal Chem 85(2) (2013) 715-36.

[13] L.M.H. Reinders, M.D. Klassen, M. Jaeger, T. Teutenberg, J. Tuerk, Development of an analytical method to assess the occupational health risk of therapeutic monoclonal antibodies using LC-HRMS, Anal Bioanal Chem 410(11) (2018) 2829-2836.

[14] J.D. Bos, M.M. Meinardi, The 500 Dalton rule for the skin penetration of chemical compounds and drugs, Exp Dermatol 9(3) (2000) 165-9.

[15] International Society of Pharmaceutical Engineers, Assessing the particulate containment performance of pharmaceutical equipment, Second edition. ed., ISPE, Tampa, Florida, USA, 2012.

[16] Bioanalytical method validation guidance for industry. https://www.regulations.gov/document/FDA-2013-D-1020-0039, 2018 (accessed 21.04.2022.).

[17] M. Zenker, Nebenbestandteile von Chemiezellstoffen und ihre Veränderung im Herstellungsprozess von Celluloseacetat und Filtertow, Fakultät fur Mathematik, Informatik und Naturwissenschaften, Universität Hamburg, Hamburg, 2010.

[18] J.M. Mathes, Protein Adsorption to Vial Surfaces – Quantification, Structural and Mechanistic Studies Fakultät für Chemie und Pharmazie, Ludwig-Maximilians-Universität München, München, 2010.

[19] Y.Q. Lin, Y. Zhang, C. Li, L. Li, K. Zhang, S. Li, Evaluation of dry blood spot technique for quantification of an Anti-CD20 monoclonal antibody drug in human blood samples, J Pharmacol Toxicol Methods 65(1) (2012) 44-8.

[20] R. Respaud, D. Marchand, C. Parent, T. Pelat, P. Thullier, J.F. Tournamille, M.C. Viaud-Massuard, P. Diot, M. Si-Tahar, L. Vecellio, N. Heuze-Vourc'h, Effect of formulation on the stability and aerosol performance of a nebulized antibody, Mabs-Austin 6(5) (2014) 1347-55.

[21] S. Hertel, T. Pohl, W. Friess, G. Winter, Prediction of protein degradation during vibrating mesh nebulization via a high throughput screening method, Eur J Pharm Biopharm 87(2) (2014) 386-394.

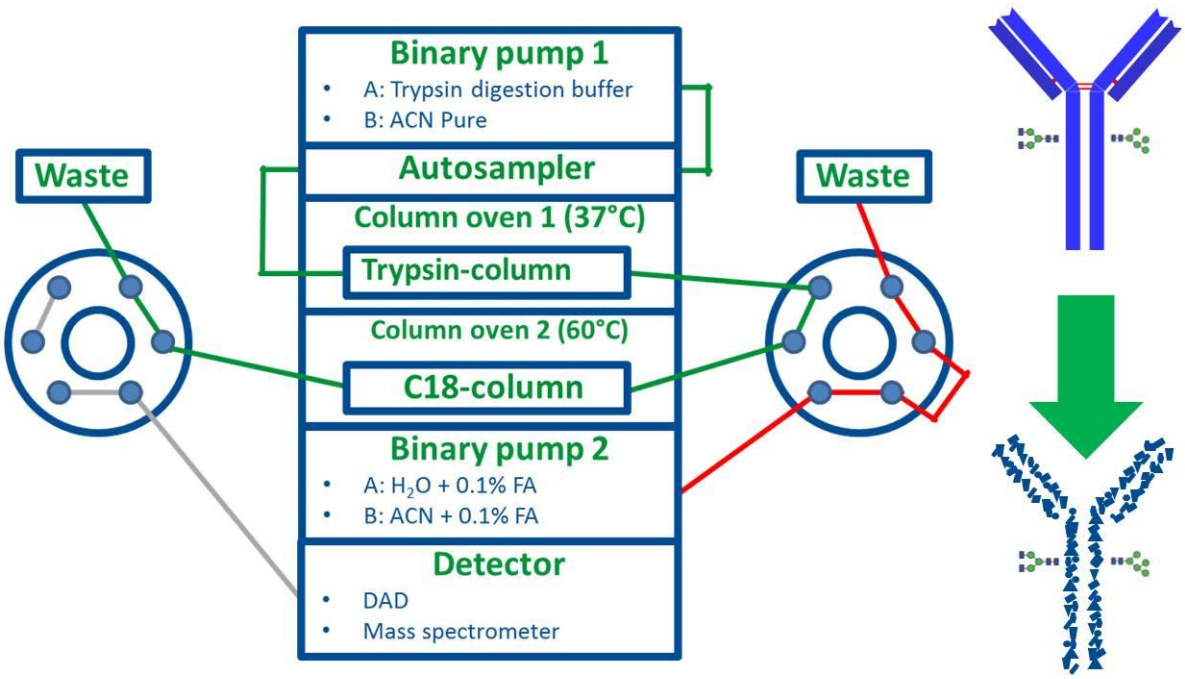
[22] S. Hertel, T. Pohl, W. Friess, G. Winter, That's cool! - Nebulization of thermolabile proteins with a cooled vibrating mesh nebulizer, Eur J Pharm Biopharm 87(2) (2014) 357-365.

Chapter 8 Development of a multidimensional online method for the characterisation and quantification of monoclonal antibodies using immobilized flow-through enzyme reactors

This chapter was adapted from: Reinders, L.M.H., Klassen, M.D., Teutenberg, T., Jaeger, M., Schmidt, T.C., Development of a multidimensional online method for the characterization and quantification of monoclonal antibodies using immobilized flow-through enzyme reactors. Anal Bioanal Chem 413, 7119–7128 (2021). https://doi.org/10.1007/s00216-021-03683-z

Abstract: Complete characterisation and quantification of monoclonal antibodies often relies on enzymatic digestion with trypsin. In order to accelerate and automate this frequently performed sample preparation step, immobilized enzyme reactors (IMER) compatible with standard HPLC systems were used. This allows an automated online approach in all analytical laboratories. We were able to demonstrate that the required digestion time for the model monoclonal antibody rituximab could be reduced to 20 min. Nevertheless, a previous denaturation of the protein is required, which also needs 20 min. Recoveries were determined at various concentrations and were 100% ± 1% at 100 ng on column, 96% ± 7% at 250 ng on column, and 98% ± 2% at 450 ng on column. Despite these good recoveries, complete digestion was not achieved, resulting in a poorer limit of quantification. This is 50 ng on column under optimized IMER conditions, whereas an offline digest on the same system achieved 0.3 ng on column. Furthermore, our work revealed that TRIS buffers, when used with an IMER system, led to alteration of the peptides and induced modifications in the peptides. Therefore, the addition of TRIS should be avoided when working at elevated temperatures of about 60°C. Nevertheless, our results have shown that the recovery is not significantly influenced whether TRIS is used or not (recovery: 96 ± 7% with TRIS vs. 100 ± 9% without TRIS).

Graphical Abstract:



8.1 Introduction

Monoclonal antibodies (mAbs) are complex biomolecules with a molecular weight of approximately 150,000 Da [1]. They are often produced with the use of mammalian cell lines. Thereby, variabilities in production conditions affect the posttranslational modification, which can be observed i.e. as changes in the antibody`s glycosylation pattern or in the amount of deamidated and oxidized peptides. The influence on the mAb is not only exercised by the culture medium. It also depends on the production conditions in general, such as the performance of an upscaling. In order to guarantee a constant product quality, immediate process monitoring and control is therefore indispensable [2]. In this context, peptide analysis can provide valuable information.

Peptide analysis is often combined with mass spectrometry for sensitive detection, but also to identify modifications via occurring mass differences. Nevertheless, peptides must first be generated from the mAb, which is achieved through proteases. These are enzymes that often have a defined and specific cleavage pattern, thereby yielding reliable and reproducible peptide sequences [1,3].

Enzymatic digestion is carried out in solution, resulting in very long incubation times of several hours, which may cause self-digestion of the enzyme. However, a long incubation time prohibits the use of peptide analysis for real-time process control. One way to circumvent this issue is to use immobilized enzymes [4]. Due to the immobilization, the likelihood of autolysis is reduced. This allows the use of an increased amount of enzymes compared to digestion in solution and thus the reduction of the required digestion time [5], down to a few minutes to seconds [6-10]. The use of high enzyme amounts in an in-solution digestion, instead, would reduce the specificity of trypsin. This can be explained by the fact that the probability of trypsin encountering itself instead of the protein increases. This would favour autolysis and lead to pseudotrypsin activity. This correlation was also demonstrated for modified trypsin [11].

The reaction of mAbs with enzymes immobilized on magnetic beads has already been demonstrated [12]. Although this approach works well, it is labour intensive due to various pipetting steps. Immobilized enzyme reactors are suitable for reducing the required effort. When immobilized enzymes are packed in HPLC columns the enzymatic digestion takes place in flow-through, which offers new coupling and automation opportunities. For example, Gstöttner et al. have presented a system to separate, reduce and digest the charge variants of mAbs and carried out peptide mapping. Their system proved well suited for characterization. Yet, a complex setup was required including four HPLC pumps, three analytical separation

columns, the immobilized enzyme reactor (IMER), various valves and a self-written macro to harmonize the individual software packages. Furthermore, they report to have had difficulties by analysing small and hydrophilic peptides [13].

In this publication, we demonstrate the online digestion of mAbs, not only for characterisation but also for quantification. For this purpose, rituximab and trypsin were selected as model analyte and model enzyme. Furthermore, the complexity of the system was reduced by employing only two HPLC pumps. When selecting the method parameters, care was taken to ensure that the overall system was able to detect all peptides formed. Furthermore, the system should require a maximum of one hour for digestion and analysis of the peptides formed. In addition, results are compared with those obtained from digestion in solution. Also studied were the effects of TRIS as a buffer additive.

8.2 Materials and methods

8.2.1 Chemicals and reagents

The monoclonal antibody rituximab was purchased as MabThera 100 mg from Hoffmann-La Roche (Basel, Switzerland). Guanidine hydrochloride (GuHCl), dithiothreitol (DTT), ammonium bicarbonate (NH_4HCO_3), tris(hydroxymethyl)aminomethane hydrochloride (TrisHCl), tris(hydroxymethyl)aminomethane (TRIS) and formic acid (FA) were purchased from Sigma-Aldrich (St- Louis, USA). Acetonitrile and water were provided in hypergrade (LiChrosolv) from Merck KGaA (Darmstadt, Germany). Furthermore, sodium chloride (NaCl) was purchased from Merck KGaA. Sequencing grade modified trypsin was purchased from Promega (Madison, USA) and calcium chloride dihydrate ($CaCl_2$) from Riedel-de Haën AG (Seelze, Germany). Hydrochloric acid (HCl) was purchased from Fluka (St. Louis, USA) and sodium hydroxide (NaOH) from AppliChem (Darmstadt, Germany). Pepscan (Lelystad, The Netherlands) has synthesized the isotope-labelled peptide PVRFSGSGSGTSYSLTISR*VEA, where the asterisk denotes the labelled amino acid.

8.2.2 Offline tryptic digest

For offline tryptic digestion, 50 µL of antibody solution was mixed with 50 µL of denaturation buffer in a 1.5 mL glass vial (Chromatographie Zubehör Trott, Kriftel, Germany). The denaturation buffer consisted of 3 M GuHCl, 100 mM TrisHCl, and 8 mM DTT. The mixture was heated to 95°C for 20 min in a WiseTherm HB-48P heating block (Witteg Labortechnik GmbH, Wertheim, Germany). After cooling to room temperature, 575 µL of a 50 mM NH_4HCO_3 solution was added. Furthermore, 50 µL of trypsin solution (1 µg absolute) was added, mixed, and the resulting solution incubated for 21 h at 37°C in an IPP55 Plus incubator (Memmert GmbH + Co. KG, Büchenbach, Germany). After completion of the reaction, 25 µL of FA was added. Completeness of tryptic digestion was demonstrated prior to use of the protocol, with data presented in the Supplementary information.

8.2.3 Tryptic digest using an enzyme reactor

Tryptic digestion by an immobilized enzyme reactor (IMER) was performed using a Perfinity trypsin column 2.1 x 33 mm; 20 µm (Perfinity Biosciences Inc, West Lafayette, USA) and a 1290 Infinity II 2D-LC system (Agilent Technologies, Waldbronn, Germany). The 2D HPLC system was operated in the standard configuration and consisted of a multisampler, two HPLC pumps, two column ovens, two diode array detectors, and three valve drives. The system setup is shown graphically in the Supplementary information. All components were from the 1290 Infinity II series. The whole system was controlled by the OpenLab CDS Chemstation Edition Rev. C.01.09 [144] software. The IMER was operated isocratically with a digestion buffer in the first dimension. The digestion buffer (mobile phase A) consisted of 20 mM TRIS, 280 mM NaCl, 5 mM $CaCl_2$ and was adjusted to pH 8. For experiments without TRIS, it was omitted without replacement. In this case, the pH value was adjusted with NaOH and HCl. The conditions for the second dimension are given below. The flow rate defined the contact time of IMER and protein. The following flow rates and resulting contact times were set to 50 µL min^{-1} (2 min), 25 µL min^{-1} (4 min), 10 µL min^{-1} (10 min), and 5 µL min^{-1} (20 min). For a contact time of 0 min, a flow rate of 50 µL min^{-1} was used after detaching the IMER. The column temperature of the IMER was set to 37°C and the injection volume was adjusted to 10 µL. For transferring protein and peptides to the second dimension, the comprehensive mode was used with a modulation time of 0.8 min. During the analysis of the peptides formed by the IMER or remaining protein amount, the IMER was flushed. During the rinsing method, the amount of mobile phase B (acetonitrile) was increased from 0% to 20% and held for 10 min. Subsequently,

equilibration was performed with 0% B for 6 min. The flow rate was 0.5 mL min^{-1}. All measurements in our study were carried out with the same IMER.

8.2.4 Analysis of intact and denatured rituximab

Detection of the protein signal from intact or denatured rituximab was done at a wavelength of 280 nm and a bandwidth of 4 nm. As reference wavelength, 390 nm with a bandwidth of 10 nm was chosen. The mobile phases in the second dimension consisted of A: water + 0.1% FA and B: acetonitrile + 0.1% FA, at a flow rate of 0.5 mL min^{-1}. The analysis of the first dimension was performed using IMER, cf. above. Further conditions for the analysis are summarized in Table 8-1.

Table 8-1: Overview of method parameters for analysis of intact and denaturised rituximab, after flow through of an IMER.

Method	**Intact**		**Denaturised**	
Column	PLRP-S; 1000 Å; 5 µm; 50 x 2.1 mm (Agilent Technologies GmbH & Co. KG, Waldbronn, Germany)		YMC-Triart Bio C4; 300 Å; 3 µm; 100 x 2.1 mm (YMC Europe GmbH, Dinslaken, Germany)	
Column temperature (°C)	60		50	
Gradient (min / %B)	0 – 2	5 – 5	0 – 3	20 – 20
	2 – 12	5 – 95	3 – 13	20 – 95
	12 – 14	95 – 95	13 – 16	95 – 95
	14 – 14.1	95 – 5	16 – 16.1	95 – 20
	14.1 – 17	5 – 5	16.1 – 19	20 – 20

8.2.5 Peptide monitoring

For peptide monitoring, either an LC/MSD XT (Agilent Technologies, Waldbronn, Germany) or a 6560 IMS-QTOF (Agilent Technologies, Waldbronn, Germany) was coupled to the system setup described above. The LC/MSD XT was controlled via OpenLab CDS Chemstation Edition Rev. C.01.09 [144] and the 6560 IMS QTOF was controlled via MassHunter Workstation Software Version B.09.00 Build 9.0.9044.1 SP1 (Agilent Technologies, Waldbronn, Germany). Regardless of the detection system, the peptides were separated and analysed on a Poroshell 120 SB-Aq; 120 Å; 2.7 µm; 100 x 2.1 mm (Agilent Technologies, Waldbronn, Germany) stationary phase. The separation was performed at 60°C and at a flow rate of 0.5 mL min^{-1} with eluent A: water + 0.1% FA and B: acetonitrile + 0.1% FA. The gradient was: 0-2 min 0-0 %B; 2-12 min 0-95 %B; 12-15 min 95-95 %B; 15-15.1 min 95-0 %B; 15.1-18 min 0-0 %B. The settings of the mass spectrometer are summarised in Table 8-2.

Table 8-2: Mass spectrometer settings for peptide analysis. The LC/MSD XT was adjusted depending on the peptide under investigation. Index a: Settings for the peptide GAGTTVSAASTK. Index b: Settings for the peptide FSGSGSGTSYSLTISR.

System	LC/MSD XT	6560 IMS-QTOF
Drying gas flow (L min^{-1})	11	8
Nebulizer pressure (psig)	35	35
Drying gas temperature (°C)	325	320
Sheath gas temperature (°C)	325	350
Sheath gas flow (L min^{-1})	10	11
Capillary voltage (V)	4000	3500
Nozzle voltage (V)	0	1000
Fragmentor (V)	139[a], 147[b]	350
Octopole RF peak (V)	n/a	750
Dwell time (ms)	290	200
Mass range (*m/z*)	625.8[a], 803.9[b]	100-3200

8.2.6 Characterization of the tryptic digest

First, the working range was estimated by a dilution series ranging from 0.1 ng to 500 ng on column over 20 calibration points. After working range estimation, a dilution series was analysed in the expectation range using ten equidistantly distributed calibration points. Using the calibration points, a calibration line was created (R^2>0.96). The calibration model varied depending on the experiment and is described in Table 8-5. Furthermore, to determine concentration dependence, a tenfold injection was performed at 100 and 450 ng on column without TRIS addition. The lowest calibration point was defined as the quantitation limit. A tenfold injection of the standard solution in the middle of the working range, recovery and standard deviation were calculated. Carry-over was determined once as area percent by analysing a blank following the calibration standard with highest concentration. For antibody sequence recovery, the sequence of rituximab was digested in silico using Skyline version 4.1.0.18169 (MacCoss Lab, Seattle, USA). Trypsin or trypsin/chymotrypsin were selected as enzymes. Only peptides with a minimum of three amino acids were considered. The formation of the predicted peptides was verified by high-resolution mass spectrometry (6560 IMS-QTOF).

8.3 Results and Discussion

8.3.1 Implementation of the immobilized enzyme reactor

Using the selected digestion buffer (TRIS buffer pH 8; See Supplementary Part for more informations), the optimal contact time and necessary sample preparation steps were determined. The contact time indicates how long the protein (rituximab) remains in the IMER and is a function of the flow rate. For this purpose, five different contact times were chosen, whereby the contact time of 0 min was obtained by removing the IMER. As can be seen in Figure 8-1A, complete degradation of rituximab did not occur even at the longest contact time (20 min). This result was expected as monoclonal antibodies are stable entities and a denaturation and reduction step is usually carried out before the actual tryptic digestion [14,15].

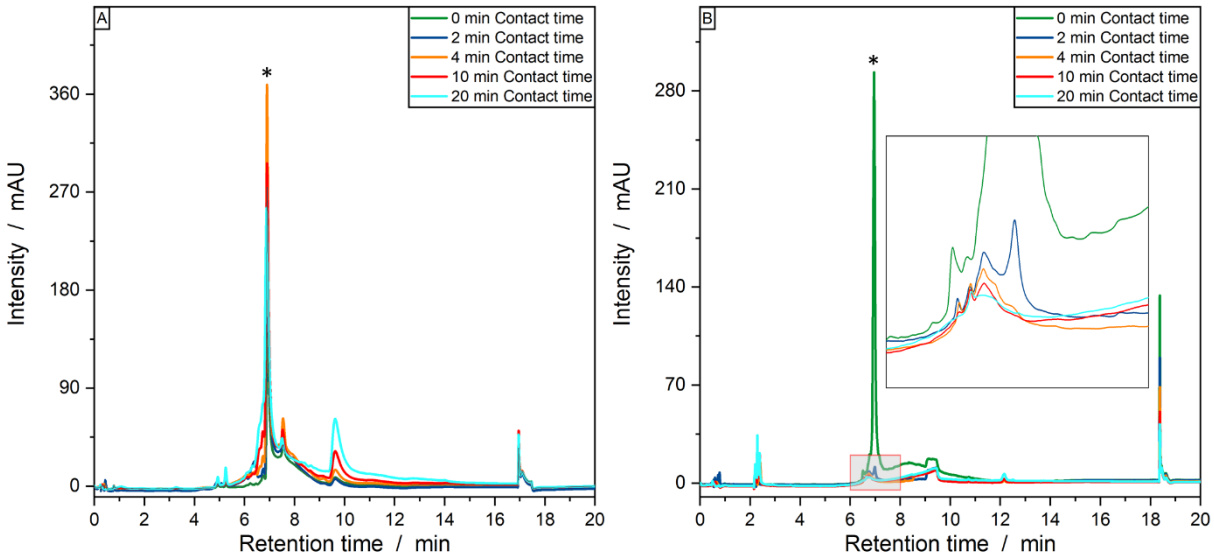


Figure 8-1: Chromatogram of rituximab (A) and denaturised rituximab (B) after passing an immobilized enzyme reactor at different contact times. The asterisk marks the antibody signal. In addition, the area framed red in Figure B shows a zoom. For analysis conditions, please refer to materials and methods.

It was therefore tested whether sufficient denaturation of the model protein rituximab could be achieved by adding 20% acetonitrile to the digestion buffer without negatively affecting the activity of the IMER. The addition of acetonitrile is intended to destabilize the hydrogen bonds of rituximab, resulting in denaturation. However, in contrast to offline denaturation, acetonitrile does not cleave any disulfide bonds. The analyses have shown that an increased degradation of the protein takes place, but degradation is still not complete (data are shown in the Supplementary information). Therefore, as in other studies [16,17], denaturation and reduction were performed offline and thereafter rituximab was injected onto the IMER. The majority (98.5%) of the denaturised rituximab was already converted at a contact time of 2 min, as shown

in Figure 8-1B. By increasing the contact time to 4 min only a small improvement was achieved (99.6% vs. 98.5%), whereas the protein signal remained almost constant when the contact time was increased further. Although this result already indicates very fast digestion, addition of organic solvent is known to increase the activity of enzyme reactors even further [18,19]. Therefore, the combination of an offline denaturation and reduction step and addition of 20% acetonitrile to the digestion buffer was investigated. The results (see Supplementary information) show that the digestion leads to a similar result when compared to denaturation alone, with additional signals appearing in the chromatogram as the contact time increases. These signals may be peptides that would have been adsorbed on the IMER without the addition of acetonitrile or an indication that the IMER is degraded by the high organic solvent content.

Furthermore, it should be considered that the aim of the coupling is to investigate the peptides formed and not the antibody signal. This means that the use of organic solvents would hinder peptide focusing on the analytical column so that information will be lost. This was also the problem for Gstöttner et al., which is why hydrophilic peptides could not be analysed [12]. Therefore, for further experiments no organic solvent was added to the denaturing buffer.

The experiments have thus shown that stable analytes such as rituximab have to undergo an initial denaturation step before they can be applied to an IMER. If this is carried out, a contact time of only 2 min is sufficient to degrade rituximab almost completely.

8.3.2 Monitoring of the peptides formed in the enzyme reactor

Although the data shown in Figure 8-1B indicate that the protein is nearly completely degraded after a contact time of 2 min, this does not mean that complete conversion to peptides has occurred. This is, however, the basic prerequisite for a quantitation method with maximum sensitivity. Furthermore, the most complete digestion ensures that no protein residues irreversibly adsorb on the head of the analytical separation column.

Peptide formation was therefore monitored by measuring the intensity of a peptide specific for rituximab. The selected peptide FSGSGSGTSYSLTISR was shown to be stable during offline tryptic digestion in previous studies [20]. Due to this information it was expected that the intensity of the peptide would initially increase with increasing contact time and remain constant once complete digestion was achieved. However, a decreasing intensity with increasing contact time was observed, which contradicts the expectation (see Figure 8-2).

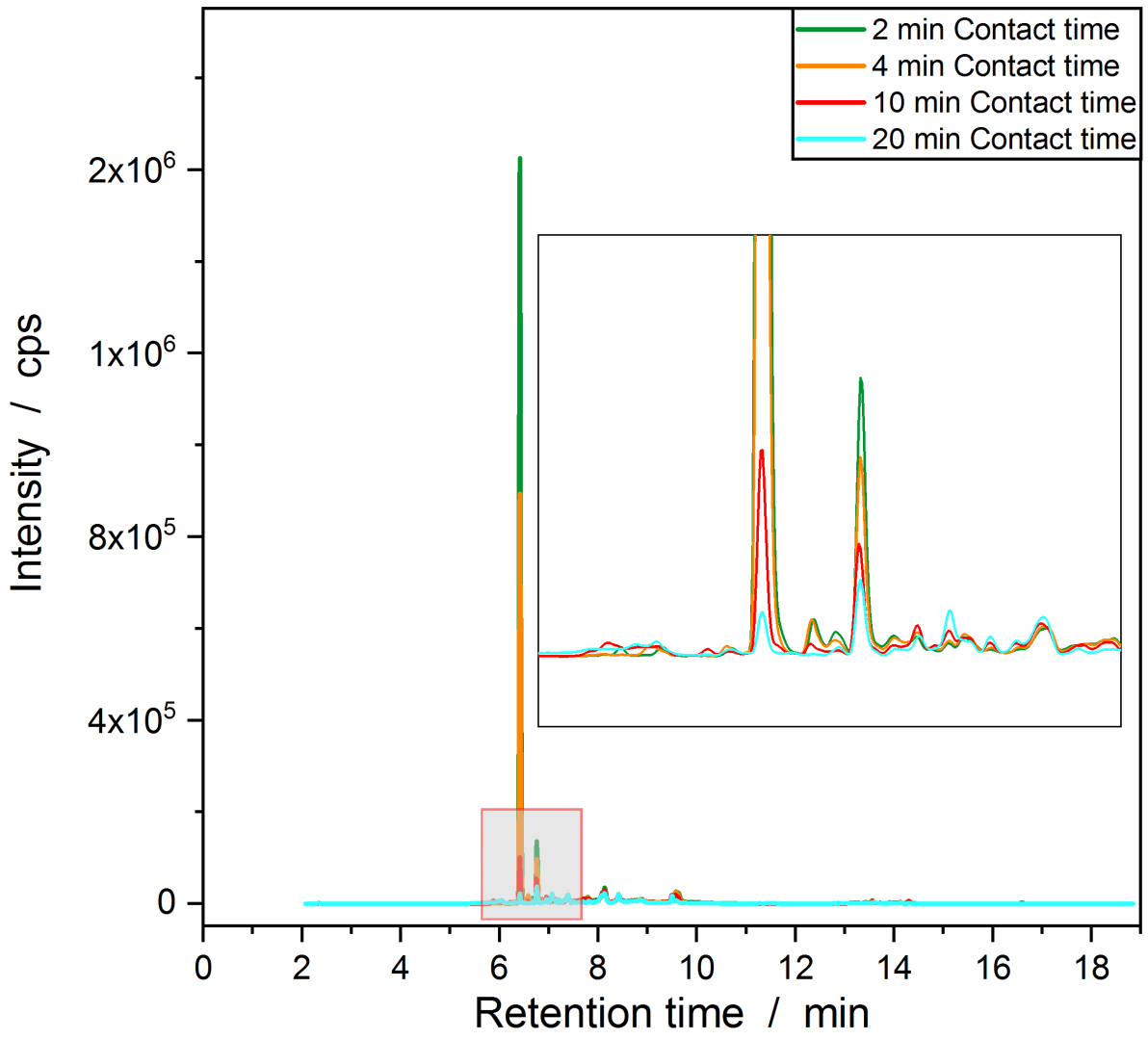


Figure 8-2: Monitoring of the specific peptide for rituximab (FSGSGSGTSYSLTISR) at different contact times. Contrary to expectations, the signal intensity decreases with increasing contact time. The area framed in red shows the zoom area. The analysis conditions can be found in Table 8-2.

The analysis of the high-resolution mass spectrometry data shows a deviation of -0.37 ppm from the theoretical *m/z* ratio. This deviation is within the range of analytical uncertainty, which strongly supports the structure assignment. For further verification of substance identity, an isotopically labelled extended peptide standard was used. The retention time difference between the peptide formed from the antibody and the internal standard is only 0.02 min, which further corroborates correct assignment of the substance. Since it is highly likely to be the peptide and previous work has shown that the peptide studied is stable [20], the degradation seems to be triggered by the use of the IMER.

For a better understanding of this effect, the sequence coverage of the IMER was compared with that of a manual tryptic digest. The sequence coverage of the manual tryptic digestion of rituximab is 76% (38/50), whereas the IMER leads to a sequence coverage of 44% (22/50) with

a contact time of 20 min. The sequence coverage at a contact time of 2 min is 52% (26/50). This means that more peptides are affected by possible degradation. In total, 18 peptides recovered in the manual tryptic digestion were not found when using the enzyme reactor. The peptides stemmed from both light and heavy chain with an approximately even distribution. The low sequence recovery prevents its use for universal peptide mapping, but does not preclude quantification of individual peptides or critical modifications. Reasons for low sequence recovery are discussed below.

The possible reasons for not recovering the 18 peptides from enzyme reactor digestion may be (specific) non-enzymatic cleavage, further enzymatic activity or induction of chemical modifications. Non-enzymatic cleavage may occur, for example, due to the prevailing slightly basic conditions (pH 8) in combination with the elevated temperature (60°C) on the analytical separation column during focusing [21]. Formic acid (FA) was reported to induce cleavage [21], which is used during chromatographic separation. Yet, the separation conditions for manual and enzyme reactor digestion were identical, which renders the explanation questionable. Furthermore, only the cleavage of peptides with aspartic acid may be explained this way, which only occurs in 6 of the 18 peptides [22].

Chemical modification is more likely than induced cleavage. Song et al. reported that the peptide they analysed underwent a mass shift of 12 Da after being stored at elevated temperature (70°C) in a TRIS buffer. Although they could not elucidate the exact reaction mechanism, they could show that TRIS was partially converted to formaldehyde (FALD), which reacts with the peptide [23]. The decay of TRIS to FALD and subsequent reaction with the peptides may thus be a possible explanation for the absence of the peptides. Fortunately, the reaction mechanism is now better understood. FALD can induce three modifications in peptides: Methylene bridge (+12 Da), dimethylene bridge (+24 Da) and methylol (+30 Da) [24,25]. On the basis of these modifications, the theoretical mass of the peptides was calculated and compared with the results of the analysis from high-resolution mass spectrometry. The results are summarized in Table 8-3. A total of 8 out of 18 peptides could be assigned to a modification.

Table 8-3: List of peptides that were experimentally identified in an in-solution tryptic digest of rituximab but did not occur in a tryptic digest by an IMER at a contact time of 20 min. Furthermore, possible occurring modifications are assigned to the peptides. The ppm error of the modification detected in the tryptic digest obtained by IMER in the presence of TRIS is indicated. Additionally, the peptides were marked (superscript a), which could be recovered in an IMER digest without TRIS and the ppm error of the modifications, respectively.

Peptide	Methylen (ppm error)	Dimethylen (ppm error)	Methylol (ppm error)
EAK	-29	16	
VDK	15		
EYK	30 / -7[a]		
LEIK	10		
SFNR	-44	24	
QTPGR[a]			20 / 18[a]
VQWK			
VTMTCR			
ATLTADK			
SLSLSPGK			
DSTYSLSSTLTLSK[a]			-7
FSGSGSGTSYSLTISR[a]			
QIVLSQSPAILSASPGEK[a]			
SSSTAYMQLSSLTSEDSAVYYCAR	16		
THTCPPCPAPELLGGPSVFLFPPKPK			
WQQGNVFSCSVMHEALHNHYTQK			
VEAEDAATYYCQQWTSNPPTFGGGTK			
STYYGGDWYFNVWGAGTTVTVSAASTK			

In order to further verify this hypothesis, the analysis was repeated without TRIS in the digestion buffer at the same contact time (20 min). This analysis showed that four of the 18 peptides could be found which could not be detected in the presence of TRIS. Furthermore, only two modified peptides were observed during the analysis without TRIS (marked with a superscript a in Table 8-3), whereas the number was ten when TRIS was added. This means that the majority of the modifications were induced by TRIS. However, the origin of the two remaining modifications cannot be clearly determined. As an alternative to omitting TRIS, it should be possible to perform the focusing of the peptides on the second separation dimension at a lower temperature, since the decay of TRIS to FALD is only described at higher temperatures. However, this would lead to additional cooling and heating steps during the analysis, which would prolong the analysis.

Although the induction of chemical modifications is plausible, this cannot explain the absence of all peptides, so an additional degradation effect must be present, such as a more extensive

enzymatic cleavage. Indeed, the process of immobilization has an effect not only on the stability of the enzyme, but also on its activity and specificity [26]. This means that the cleavage pattern of immobilized enzymes may differ from those in solution. Furthermore, the IMER that has been used in this study may also be contaminated with alternative enzymes. The occurrence of an altered cleavage pattern due to chymotrypsin impurities has already been shown in trypsin in-solution digestions [29]. Here, it was shown that the effect appears particularly at high enzyme concentrations, which are present when using an IMER.

Both cases could also explain the observed degradation of peptides. Indeed, peptides could be identified that are indicative of chymotrypsin-like cleavage, such as SSSTAY. Nevertheless, assuming trypsin/chymotrypsin modification, only 36% (28/77) sequence recovery is obtained with rituximab using the IMER. The low sequence recovery indicates only a low amount of chymotrypsin in the form of impurities. Alternatively, other effects are present that are partially leading to the same peptides.

In summary, our peptide monitoring revealed that using TRIS buffer with the IMER produced a divergent peptide pattern compared to the one obtained in solution digestion.

In addition to possible modifications induced by FALD, the effects of IMER on asparagine deamidation were investigated. Here, it was observed that the peptides VVSVLTVLHQDWLNGK and GLEWIGAIYPGNGDTSYNQK possessed a significantly increased proportion of deamidated peptide when IMER was used (43.2% vs 96.6% and 28.6% vs 64.1%, respectively). However, there are also peptides such as VDNALQSGNSQESVTEQDSK in which the deamidated fraction is higher in offline digestion (4.6% vs 0.8%). A complete overview can be found in Table 8-4. In addition, it should be noted that ammonium bicarbonate was used in the offline tryptic digestion. This provides a basic pH value and can induce deamidation [27].

Table 8-4: Data on the percentage of deamidated peptide in comparison between offline and IMER digestion. IMER digestion was performed without TRIS addition and at a contact time of 20 min.

Sequence	Offline (%)	IMER (%)
NQVSLTCLVK	2.4	0.0
FNWYVDGVEVHNAK	1.3	5.7
SGTASVVCLLNNFYPR	2.0	0.0
ASGYTFTSYNMHWVK	4.6	1.8
VVSVLTVLHQDWLNGK	43.2	96.6
VDNALQSGNSQESVTEQDSK	4.6	0.8
GLEWIGAIYPGNGDTSYNQK	28.6	64.1
GFYPSDIAVEWESNGQPENNYK	1.6	0.0
STYYGGDWYFNVWGAGTTVTVSAASTK	0.0	0.0

The results show that, depending on the peptide, IMER digestion offers the possibility to investigate the modification behavior of peptides in more detail. However, it can be assumed that, as shown above with the example of the TRIS buffer, the analysis conditions used and in particular the digestion buffer selected have an influence on this behavior.

8.3.3 Selection of a stable peptide and determination of the optimal contact times

The data above showed that most of the protein is digested after 2 min, whereas some peptides continue to degrade even with longer contact times if a TRIS buffer is used. This means that for indirect quantification, either a different peptide than selected in manual tryptic digestion must be chosen, or the addition of TRIS should be avoided. However, since the TRIS additive helps to stabilize the pH value, removing it can lead to a decrease in reproducibility, so the approach of using an alternative peptide was tested.

Since the mechanism behind peptide degradation could not be clarified unequivocally, a peptide was chosen that would be generated in the presence of chymotrypsin/trypsin and is specific for rituximab. For peptide specificity testing, rituximab, as well as 80 other mAbs, were digested in silico and the peptides were compared with each other with the goal of identifying peptides unique to rituximab. A listing of the mAbs can be found in the supplementary part. Thus, four theoretically unique peptides QTPGR, GAGTTVTVSAASTK, ATSNLASGVPVR, and TSNPPTF were identified. Peptide QTPGR was not recovered from digestion by IMER, while TSNPPTF (12 ppm error) was recovered only at a very low intensity. The other two peptides

had very high intensity and thus are potentially both suitable. However, peptide GAGTTVTVSAASTK (1.8 ppm error) was chosen because of its intensity being highest.

This peptide was used to determine the optimal contact time with the IMER. The results are shown in Figure 8-3. The intensity was expected to initially increase and once the protein was fully digested, a constant intensity should be obtained. However, this was not the case. A constant increase of the analyte signal was observed. Hence, no final statement can be made whether digestion proceeded until completion. Increasing the contact time was only possible to a limited extent, as it was accompanied by a reduction of the flow rate. As the standard deviation of the data at a contact time of 20 min shows, the flow rate was already so low that the reproducibility of the system began to decrease. An increase in standard deviation from 3.5-4.0% to 10.6% compared to the other contact times was observed.

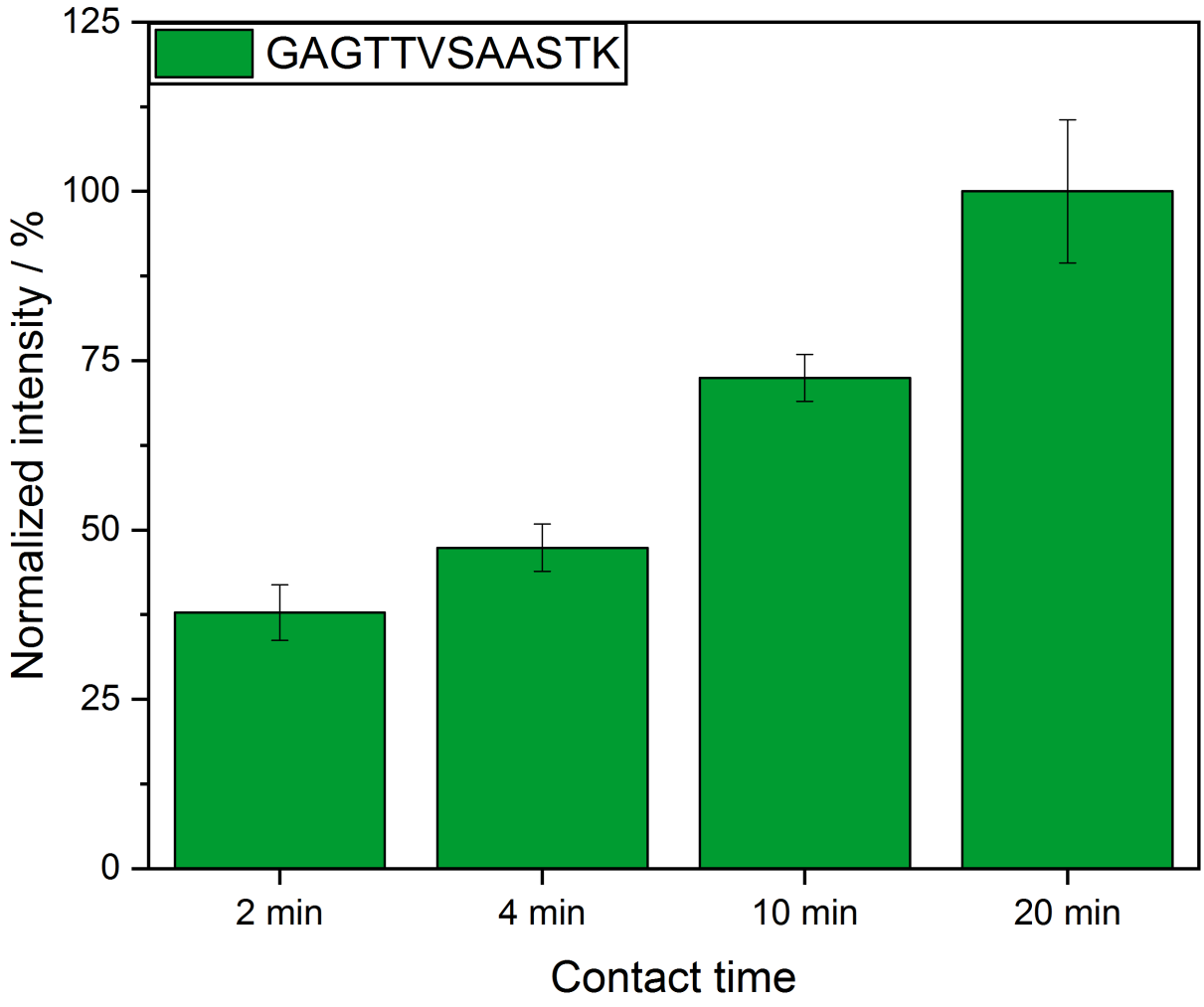


Figure 8-3: of the peptide GAGTTVSAASTK at different contact times in the IMER. Analyses were performed as triplicates.

Since complete digestion could not be assured, the selection of the optimal contact time was based on the intended application. The goal of the overall procedure was to ensure at-line analysis using tryptic digestion within one hour. However, the method time of standard tryptic digestion corresponds to ten times the contact time. The correlation between contact time and method time is explained in more detail in the Supplementary information. Additional time is needed for the analysis of the peptides. A typical analytical method for peptide separation requires 18 minutes. The selection of a contact time of 4 minutes and thus a total digestion time of 40 minutes allows all analyses to be performed in one hour and thus close to the time of sampling. The data shown in Figure 8-1B support the selection of the method parameters, as most of the protein was digested at this contact time. Nevertheless, it cannot be excluded that larger antibody fragments were accumulated at the column head.

In addition to selecting a different peptide, the optimal conditions for the original model peptide FSGSGSGTSYSLTISR without TRIS addition were determined, as it is stable under these conditions. Here, it was shown that the signal intensity is not increased for a contact time higher than 2 min, which means that the digestion can be considered complete. Nevertheless, if a buffer is not used, this can lead to reproducibility issues.

8.3.4 Characterization of the immobilized enzyme reactor

With the optimal parameters determined (2 min contact time without TRIS and 4 min contact time with TRIS), the overall system was characterized. An offline tryptic digest and the peptide FSGSGSGTSYSLTISR was chosen as a reference. The results are summarized in Table 8-5. A different peptide (GAGTTVTVSAASTK) was used for the comparison when using the TRIS buffer because the model peptide is unstable under these conditions, as mentioned earlier. Since these are different analytes, this can lead to different ionization properties and thus quantitation limits. Regardless of the digestion buffer used, a higher sensitivity is achieved for the offline tryptic digest (0.3 ng vs. 50 ng on column) when compared to the digestion by the IMER.

However, it should be noted that the dilution factors during sample preparation are different for offline and online tryptic digestion. Taking into account the dilution factors, the quantitation limits with respect to the untreated starting solution are 0.45 µg mL^{-1} for offline and 10 µg mL^{-1} for tryptic digestion using IMER. The quantitation limit using an IMER is thus worse by a factor of about 22.

Table 8-5: Results of method validation of tryptic digestion, which was performed either offline or by IMER. The quantitation limit is defined as the lowest calibration point. The calibration range was described by a ten-point calibration. The recovery was determined by a tenfold injection in the middle working range. n. d.: not detected. A: Without TRIS addition; B: With TRIS addition. C: The recovery at 100 ng on column is 99.6% ± 0.7% and at 450 ng on column 97.8% ± 2.0%, respectively.

Digestion type	Offline	IMER[a]	IMER[b]
Peptide	FSGSGSGTSYSLTISR		GAGTTVTVSAASTK
Contact time (min)	n/a	2	4
Digestion time (min)	1260	20	40
Limit of Quantitation (ng)	0.3	50	50
Calibration range (ng)	0.3-3.0	50-500	50-500
Calibration model	First order		Second order
Recovery (%)	99 ± 3	96 ± 7[C]	100 ± 9
Carry-Over (%)	n. d.	15	20

In terms of recovery of a quality control sample, manual digestion and digestion by IMER are comparable (99% vs. 96% and 100%). Interestingly, omitting TRIS leads to comparable reproducibility in IMER (7% vs. 9%). Furthermore, when IMER was used without TRIS addition, it was tested whether the recovery depends on concentration. This is not the case. The recovery varies independently of the concentration in the range of 98%-100% (See Table 8-5 for more details). With respect to the calibration model, the IMER shows a second-order correlation with TRIS use. This could be an indication that the reaction kinetics are inhibited, resulting in all immobilized enzyme already occupied by analyte. This behavior does not occur without TRIS addition.

Initially, a high carry-over of 15% or 20% was observed. Increasing the proportion of organic mobile phase (acetonitrile) during the rinsing step from 20% to 50% resulted in the carry-over being reduced to 3%. Increasing the organic content also resulted in a continuous loss of signal intensity (15% signal reduction after 5 samples), indicating that the IMER is damaged. This behavior did not occur during a rinse step with 20% of acetonitrile, so the organic content should not be increased further. For further carry-over reduction, a blank sample should therefore be taken after the sample instead of increasing the organic content.

With respect to digestion time, the use of an IMER results in a significant time advantage. When using a TRIS free solution the required time could be reduced by more than 98% (1260 min vs. 20 min), making it possible to obtain the analysis result on the same day instead of the next.

This speed advantage offers the possibility to react early to the result, as is important for at-line process control.

In summary, the manual tryptic digestion is more sensitive, whereas the enzyme reactor leads to a speed advantage. A TRIS additive can be omitted, as this has no negative influence on the reproducibility.

8.4 Conclusion

Sample preparation by immobilized enzyme reactor, as well as by classical overnight digestion, both have specific advantages and disadvantages. Digestion of the model protein rituximab took 20 min with the IMER and 1260 min with the overnight protocol used. For the model protein, the IMER approach leads to a significant time reduction. Nevertheless, the quantification limit of the IMER is worse with 10 µg mL^{-1} compared to 0.45 µg mL^{-1}. Therefore, it has to be considered on a case-by-case basis whether a low quantification limit or a fast analysis result is needed.

8.5 Supplementary Information

8.5.1 Setup of the IMER system used

For the use of an immobilized enzyme reactor (IMER) with online analysis of the peptides formed, two binary pumps, an autosampler, two column ovens, a detector and a six-way valve is required. Furthermore, a diverter valve is recommended when using a mass spectrometer (MS) to avoid that the non-volatile salt load will contaminate the ion source. The schematic system design is shown in Figure 8-4A-C.

At the beginning of the analysis, as shown in Figure 8-4A, the IMER is in the direct flow path with a C18 column. This results in the protein (in the study: rituximab) being digested by the IMER (in the study: trypsin column) and converted to peptides. The peptides are focused on the C18 column. Care should be taken to avoid the addition of organic solvent during tryptic digestion, otherwise focusing of the peptides will be impeded.

After digestion of the protein is complete, the six-way valve is switched as shown in Figure 8-4B, leaving the IMER in the flow path of pump 1 (green) and switching the C18 column to the flow path of pump 2 (red). Without the C18 column in the flow path, the trypsin column can now be rinsed with organic solvent (in the study: acetonitrile) to reduce carry-over for subsequent analyses. This is followed by re-equilibration with the digestion buffer. In the flow path of the second dimension (red), rinsing to the starting conditions required for the analysis

begins at the same time. If an MS is used for the detection of the peptides, the diverter valve is still switched to waste so that the remaining salt load can be separated.

Once the salt load has been separated, the diverter valve switches, as shown in Figure 8-4C, placing the C18 column in the flow path of the detector. The newly generated peptides can now be analyzed.

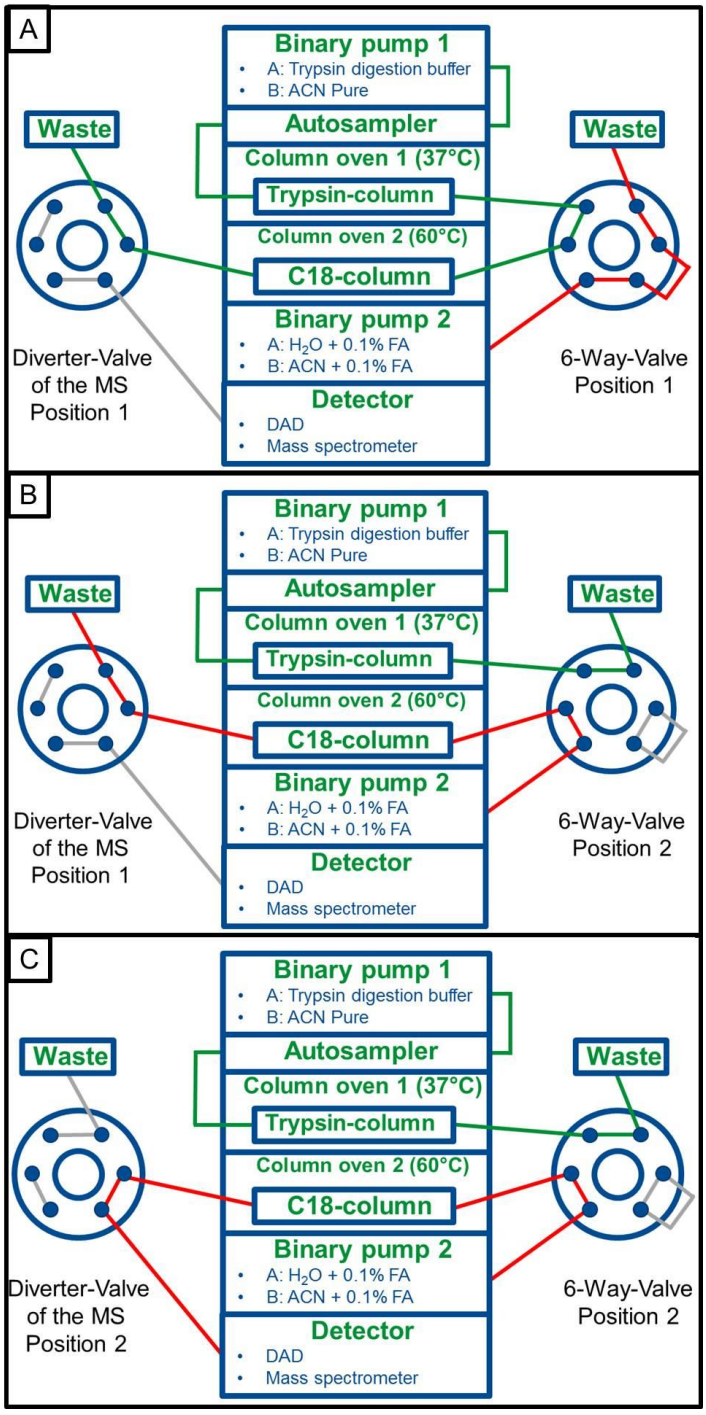


Figure 8-4: System design of the 2D HPLC system used for the immobilized enzyme reactor.

8.5.2 Derivation of the used digestion buffer

Although immobilized and dissolved enzyme are the same, the immobilisation step can lead to a change in the optimal digestion conditions [28]. This means that a different digestion buffer should be used for IMER than for digestion in solution. In terms of pH, this implies that pH values around 9 are preferred after immobilisation [29,30]. However, since online coupling to a chromatographic separation column is to take place and many reversed phase chromatography (RP) phases have a pH limit of 8, the pH of the digestion buffer was set to 8. Šlechtová et al. were able to show for the IMER used that a difference in digestion efficiency between pH 8 and 9 is present, but acceptable [29]. In order to obtain an unbiased result, a buffer must be chosen that does not react with the peptides formed and induces modifications such as deamidation, which is why a TRIS buffer was used [31]. Furthermore, $CaCl_2$ was added to the buffer to improve the stability of trypsin [32].

8.5.3 Optimisation of tryptic digestion through acetonitrile addition

Direct injection of rituximab onto the IMER has not resulted in complete conversion to peptides. The addition of 20% acetonitrile also did not lead to the desired result, as shown in Figure 8-5.

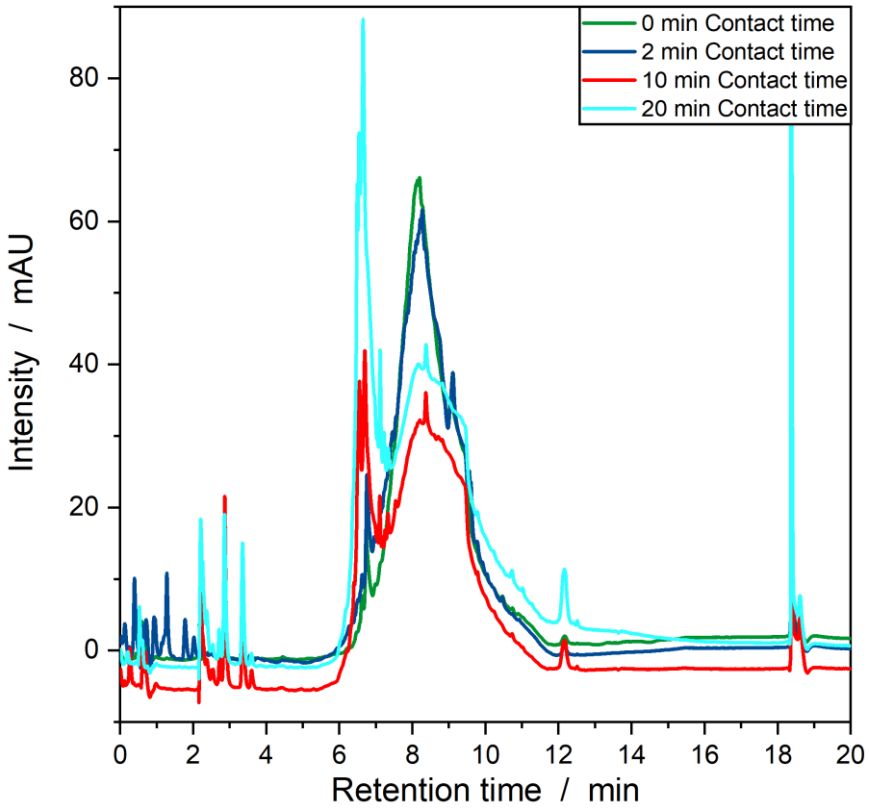


Figure 8-5: Digestion of rituximab with the addition of 20% acetonitrile using an immobilized enzyme reactor at different contact times.

Manual denaturation of rituximab prior to delivery to the IMER resulted in the majority of the protein being converted after only 2 min of contact time. The further addition of 20% acetonitrile to the digestion buffer did not lead to any additional improved conversion (Figure 8-6). However, additional signals were observed in the chromatogram, which is why it is assumed that the addition of acetonitrile accelerates the degradation of the IMER.

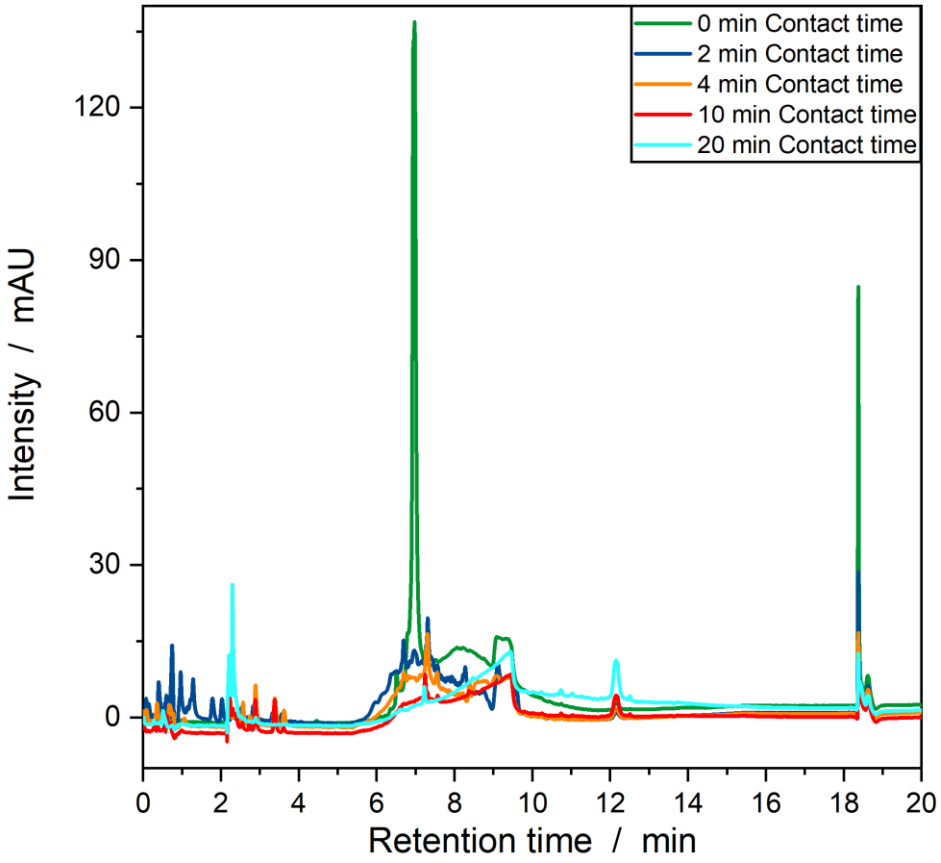


Figure 8-6: Digestion of denatured rituximab with the addition of 20% acetonitrile using an immobilized enzyme reactor at different contact times.

8.5.4 Correlation of the contact time and the digestion time

The contact time of the analyte with the IMER depends on the flow rate and is specified by the manufacturer. For example, this is 2 min for a flow rate of 50 µL min^{-1}. As Figure 8-7 demonstrates, it is not the same as the required digestion time. The highest signal intensity in Figure 8-7 is obtained at a retention time of 2.64 min, although the signal does not have an intensity of 0 mAU until after approximately 17.5 min. Therefore, the systematic approach was chosen to use ten times the contact time as the required digestion time. This ensures that the carry-over is kept as low as possible.

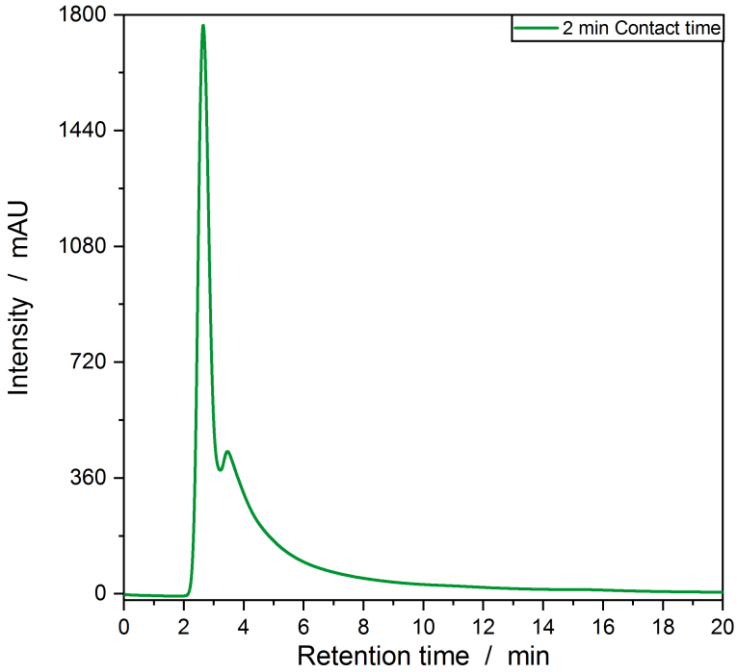


Figure 8-7: Detector signal at 214 nm at the column exit of the immobilized enzyme reactor during digestion of rituximab at a concentration of 0.5 mg mL^{-1}. The contact time is 2 min, resulting in a digestion time of 20 min.

8.5.5 Sequences of monoclonal antibodies used

Listed below are all monoclonal antibodies whose primary sequences have been screened to identify a peptide specific for rituximab:

Abciximab, Adalimumab, Alemtuzumab, Alirocumab, Atezolizumab, Avelumab, Basiliximab, Benralizumab, Bezlotoxumab, Blinatumomab, Brentuximab vedotin, Brodalumab, Brolucizumab, Burosumab , Canakinumab, Caplacizumab, Cemiplimab, Certolizumab pegol, Crizanlizumab, Daclizumab, Denosumab, Dinutuximab, Dupilumab, Durvalumab, Eculizumab, Edrecolomab, Efalizumab, Elotuzumab, Emapalumab , Emicizumab, Eptinezumab, Erenumab, Evolocumab, Fremanzezumab, Galcanezumab, Gemtuzumab ozogamicin, Golimumab, Guselkumab, Ibalizumab, Ibritumomab tiuxetan, Idarucizumab, Infliximab, Ipilimumab, Isatuximab, Ixekizumab, Lanadelumab, Leronlimab, Mepolizumab, Mogamulizumab, Moxetumomab pasudotox, Muromonab- CD3, Narsoplimab, Natalizumab, Necitumumab, Nivolumab, Obiltoxaximab, Obinutuzumab, Ocrelizumab, Ofaturmumab, Olaratumab, Palivizumab, Panitumumab, Pembrolizumab, Pertuzumab, Ramucirumab, Ranibizumab, Ravulizumab-ALXN1210, Riskanizumab, Romosozumab, Sacituzumab govitecan, Sarilumab, Satralizumab, Secukinumab, Siltuximab, Tafasitamab, Teprotumumab, Tildrakizumab, Tocilizumab, Tositumomab, Ustekinumab, Vedolizumab

8.5.6 Confirmation of the completeness of the tryptic offline digestion

The completeness of the digestion protocol used was verified with the antibodies trastuzumab and daratumumab. For this purpose, the signal intensity of the single miscleavage of a specific peptide was observed. The tryptic digest was considered complete as soon as the signal could no longer be detected. The data are shown in Figure 8-8.

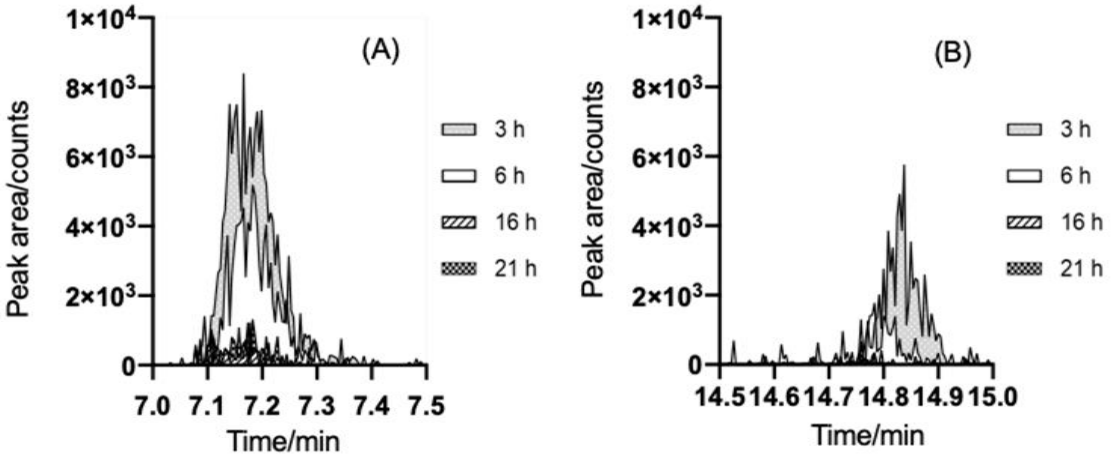


Figure 8-8: Intensity of LLIYDASNRATGIPAR (A) and LLIYSASFLYSGVPSRFSGSR (B) peptides as a function of the required digestion time, which occurs during the digestion of daratumumab and trastuzumab, respectively.

The data show that a digestion time of 21 h is required for the monoclonal antibody daratumumab (Figure 8-8A), whereas 16 h is already sufficient for trastuzumab (Figure 8-8B). In order to extend the digestion protocol to other monoclonal antibodies, the higher time (21 h) was selected.

8.6 References

1. Beck A, Wagner-Rousset E, Ayoub D, Van Dorsselaer A, Sanglier-Cianferani S (2013) Characterization of Therapeutic Antibodies and Related Products. Anal Chem 85 (2):715-736. doi:10.1021/ac3032355

2. Shukla AA, Thommes J (2010) Recent advances in large-scale production of monoclonal antibodies and related proteins. Trends Biotechnol 28 (5):253-261. doi:10.1016/j.tibtech.2010.02.001

3. Beck A, Sanglier-Cianferani S, Van Dorsselaer A (2012) Biosimilar, Biobetter, and Next Generation Antibody Characterization by Mass Spectrometry. Anal Chem 84 (11):4637-4646. doi:10.1021/ac3002885

4. Ma J, Zhang L, Liang Z, Zhang W, Zhang Y (2007) Monolith-based immobilized enzyme reactors: Recent developments and applications for proteome analysis. J Sep Sci 30 (17):3050-3059. doi:10.1002/jssc.200700362

5. Slechtova T, Gilar M, Kalikova K, Moore SM, Jorgenson JW, Tesarova E (2017) Performance comparison of three trypsin columns used in liquid chromatography. J Chromatogr A 1490:126-132. doi:10.1016/j.chroma.2017.02.024

6. Hsieh YLF, Wang HQ, Elicone C, Mark J, Martin SA, Regnier F (1996) Automated analytical system for the examination of protein primary structure. Anal Chem 68 (3):455-462. doi:DOI 10.1021/ac950421c

7. Krenkova J, Lacher NA, Svec F (2009) Highly Efficient Enzyme Reactors Containing Trypsin and Endoproteinase LysC Immobilized on Porous Polymer Monolith Coupled to MS Suitable for Analysis of Antibodies. Anal Chem 81 (5):2004-2012. doi:10.1021/ac8026564

8. Moore S, Hess S, Jorgenson J (2016) Characterization of an immobilized enzyme reactor for on-line protein digestion. J Chromatogr A 1476:1-8. doi:10.1016/j.chroma.2016.11.021

9. Safdar M, Spross J, Janis J (2014) Microscale immobilized enzyme reactors in proteomics: Latest developments. J Chromatogr A 1324:1-10. doi:10.1016/j.chroma.2013.11.045

10. Freije JR, Mulder PPMFA, Werkman W, Rieux L, Niederlander HAG, Verpoorte E, Bischoff R (2005) Chemically modified, immobilized trypsin reactor with improved digestion efficiency. J Proteome Res 4 (5):1805-1813. doi:10.1021/pr050142y

11. Fang P, Liu MQ, Xue Y, Yao J, Zhang Y, Shen HL, Yang PY (2015) Controlling nonspecific trypsin cleavages in LC-MS/MS-based shotgun proteomics using optimized experimental conditions. Analyst 140 (22):7613-7621. doi:10.1039/c5an01505g

12. Millan-Martin S, Jakes C, Carillo S, Buchanan T, Guender M, Kristensen DB, Sloth TM, Orgaard M, Cook K, Bones J (2020) Inter-laboratory study of an optimised peptide mapping workflow using automated trypsin digestion for monitoring monoclonal antibody product quality attributes. Anal Bioanal Chem 412 (25):6833-6848. doi:10.1007/s00216-020-02809-z

13. Gstottner C, Klemm D, Haberger M, Bathke A, Wegele H, Bell C, Kopf R (2018) Fast and Automated Characterization of Antibody Variants with 4D HPLC/MS. Anal Chem 90 (3):2119-2125. doi:10.1021/acs.analchem.7b04372

14. Bobaly B, D'Atri V, Goyon A, Colas O, Beck M, Fekete S, Guillarme D (2017) Protocols for the analytical characterization of therapeutic monoclonal antibodies. II - Enzymatic and

chemical sample preparation. J Chromatogr B 1060:325-335. doi:10.1016/j.jchromb.2017.06.036

15. Switzar L, Giera M, Niessen WMA (2013) Protein Digestion: An Overview of the Available Techniques and Recent Developments. J Proteome Res 12 (3):1067-1077. doi:10.1021/pr301201x

16. Abe K, Shibata K, Naito T, Karayama M, Hamada E, Maekawa M, Yamada Y, Suda T, Kawakami J (2020) Quantitative LC-MS/MS method for nivolumab in human serum using IgG purification and immobilized tryptic digestion. Anal Methods-Uk 12 (1):54-62. doi:10.1039/c9ay02087j

17. Shida H, Naito T, Shibata K, Yamada Y, Kawakami J (2018) LC-MS/MS method for denosumab quantitation in human serum with rapid protein digestion using immobilized trypsin. Bioanalysis 10 (18):1501-1510. doi:10.4155/bio-2018-0161

18. Mao YH, Krischke M, Kulozik U (2019) beta-Lactoglobulin hydrolysis by a flow-through monolithic immobilized trypsin reactor in ethanol/aqueous solvents. Process Biochem 82:84-93. doi:10.1016/j.procbio.2019.04.017

19. Bartolini M, Greig NH, Yu QS, Andrisano V (2009) Immobilized butyrylcholinesterase in the characterization of new inhibitors that could ease Alzheimer's disease. J Chromatogr A 1216 (13):2730-2738. doi:10.1016/j.chroma.2008.09.100

20. Reinders LMH, Klassen MD, Jaeger M, Teutenberg T, Tuerk J (2018) Development of an analytical method to assess the occupational health risk of therapeutic monoclonal antibodies using LC-HRMS. Anal Bioanal Chem 410 (11):2829-2836. doi:10.1007/s00216-018-0966-1

21. Sun Y, Frenkel-Pinter M, Liotta CL, Grover MA (2020) The pH dependent mechanisms of non-enzymatic peptide bond cleavage reactions. Phys Chem Chem Phys 22 (1):107-113. doi:10.1039/c9cp05240b

22. Li AQ, Sowder RC, Henderson LE, Moore SP, Garfinkel DJ, Fisher RJ (2001) Chemical cleavage at aspartyl residues for protein identification. Anal Chem 73 (22):5395-5402. doi:10.1021/ac010619z

23. Song Y, Schowen RL, Borchardt RT, Topp EM (2001) Formaldehyde production by Tris buffer in peptide formulations at elevated temperature. J Pharm Sci 90 (8):1198-1203. doi:DOI 10.1002/jps.1073

24. Metz B, Kersten GFA, Hoogerhout P, Brugghe HF, Timmermans HAM, de Jong A, Meiring H, ten Hove J, Hennink WE, Crommelin DJA, Jiskoot W (2004) Identification of formaldehyde-induced modifications in proteins - Reactions with model peptides. J Biol Chem 279 (8):6235-6243. doi:10.1074/jbc.M310752200

25. Tayri-Wilk T, Slavin M, Zamel J, Blass A, Cohen S, Motzik A, Sun X, Shalev DE, Ram O, Kalisman N (2020) Mass spectrometry reveals the chemistry of formaldehyde cross-linking in structured proteins. Nat Commun 11 (1). doi:10.1038/s41467-020-16935-w

26. Secundo F (2013) Conformational changes of enzymes upon immobilisation. Chem Soc Rev 42 (15):6250-6261. doi:10.1039/c3cs35495d

27. Xie HW, Chakraborty A, Ahn J, Yu YQ, Dakshinamoorthy DP, Gilar M, Chen WB, Skilton S, Mazzeo JR (2010) Rapid comparison of a candidate biosimilar to an innovator monoclonal antibody with advanced liquid chromatography and mass spectrometry technologies. Mabs-Austin 2 (4):379-394. doi:DOI 10.4161/mabs.11986

28. Mao YH, Fan R, Li RK, Ye XY, Kulozik U (2021) Flow-through enzymatic reactors using polymer monoliths: From motivation to application. Electrophoresis. doi:10.1002/elps.202000266

29. Slechtova T, Gilar M, Kalikova K, Moore SM, Jorgenson JW, Tesarova E (2017) Performance comparison of three trypsin columns used in liquid chromatography. J Chromatogr A 1490:126-132. doi:10.1016/j.chroma.2017.02.024

30. Mao YH, Krischke M, Kulozik U (2019) beta-Lactoglobulin hydrolysis by a flow-through monolithic immobilized trypsin reactor in ethanol/aqueous solvents. Process Biochem 82:84-93. doi:10.1016/j.procbio.2019.04.017

31. Hao P, Ren Y, Datta A, Tam JP, Sze SK (2015) Evaluation of the Effect of Trypsin Digestion Buffers on Artificial Deamidation. J Proteome Res 14 (2):1308-1314. doi:10.1021/pr500903b

32. Sipos T, Merkel JR (1970) Effect of calcium ions on the activity, heat stability, and structure of trypsin. Biochemistry 9 (14):2766-2775. doi:10.1021/bi00816a003

Chapter 9 General Conclusion and Outlook

The cytostatics and monoclonal antibodies used for cancer therapy are prepared individually for each patient. However, they have carcinogenic, mutagenic, or reproductive toxicity (CMR) properties. Therefore, patient and personnel safety are linked in all analytical considerations.

A good example of this is the quality control of patient-specific cytostatic preparations. The European Pharmacopoeia stipulates volume control in open vessels for the quality control of these preparations [1], which is questionable for reasons of occupational safety. Within the framework of this work, it could be shown that volume determination in a closed vessel is possible (see chapter 3). The measurement uncertainty of this procedure is 0.4%, whereas the measurement uncertainty of the pharmacopoeia method is 0.5-1.0%. Both methods are thus equivalent, whereby working in a closed vessel significantly reduces the occupational risk. A deviation from the pharmacopoeia is therefore justifiable for safety reasons.

Furthermore, preparations with cytostatics and monoclonal antibodies were tested for their content and active substance identity. For monoclonal antibodies in particular, a suitable workflow first had to be developed, as the European Pharmacopoeia does not explicitly provide one for this [2]. It could be shown that 96% (n=136) of the cytostatic preparations (see chapter 3) and 100% (n=11) of the monoclonal antibody preparations (see chapter 4) comply with the pharmaceutical rules. Studies in which cytostatic drugs were analysed using the sample preparation method described in the pharmacopoeia yield comparable analysis data with conformities of 99.2% (n=123) and 100% (n=33), respectively [3,4]. This comparison also highlights that the modified sample preparation method has no influence on the analytical result, but significantly improves work safety.

Additionally, the study design was chosen in such a way that pharmacies did not know which preparations were being analysed before manufacture, so that the results reflect the preparation quality very well. Although the preparation quality is very high, there are few random samples that are objectionable. This shows that analytical quality control can make a decisive contribution to identifying and eliminating deficits. In perspective, a mandatory analytical quality control should be implemented at intervals in order to further increase the preparation quality. Studies in which the surface contamination with cytostatic drugs was regularly quantified instead of the preparation quality could prove a process optimisation [5]. A comparable effect can therefore also be expected for the quality control of preparations.

In the deeper investigation of monoclonal antibodies, it has been demonstrated that it is possible to correlate structural changes with their properties in the mode of action (see chapter 5).

Nevertheless, a variety of complementary analytical techniques are required to enable a complete characterisation. When evaluating the data, it must be taken into account that the active substances are biotechnologically produced, which means that there is microheterogeneity. This renders the identification of changes and the differentiation from biosimilars (see chapter 6) more difficult. In the future, however, the demands on analytics and evaluation will continue to grow, as the complexity of the active substances continues to increase. Currently, conjugated monoclonal antibodies are approved in addition to the non-conjugated monoclonal antibodies investigated. The conjugated species consist of a monoclonal antibody, a linker and a cargo. The cargo can be, for example, a cytotoxic molecule [6-9].

As the production of the patient-specific preparations is carried out by pharmaceutical personnel, occupational health and safety aspects must also be taken into account. Prior to this work, there was no analytical information on workplace exposure to monoclonal antibodies. Theoretical considerations indicate that the greatest health risk comes from airborne monoclonal antibodies. Therefore, a method of analysis was established to quantify them (see chapter 7). It was shown that when fresh drug vials were punctured with a cannula, a release of 15 ng of active substance were detected. Other working groups had estimated the release potential, based on theoretical considerations, at 7-70 ng [10,11]. The analytical data collected for the first time thus confirm the expected value. However, as soon as pressure equalisation systems such as spikes are used, no release can be detected. From this, it can be deduced as a recommendation for action that working with pressure equalisation systems such as spikes is essential for occupational safety. Furthermore, toxicologists can use these data to optimise the risk assessment for the preparation.

Since a release could be detected, the next step should be to address the question of how to remove the contamination. The multi-stage cleaning procedure of surfaces developed for cytostatics consists of an alkaline and alcoholic cleaner [5]. However, the conditions of both cleaning agents lead to aggregation of monoclonal antibodies, which can cause them to precipitate [12]. In subsequent work, it should therefore be examined whether the currently used cleaning method for surfaces is also suitable for monoclonal antibodies. For this, however, a sampling procedure for contaminated surfaces needs to be developed first. In the future, this could also be used for environmental monitoring in order to identify contaminated surfaces. As long as the procedure to be developed is based on a peptide-based workflow using LC-MS/MS, like the air tests, the analysis method already developed can be adapted.

The same applies for the automated sample preparation procedure (see chapter 8). This is based on the use of immobilised trypsin and enables the tryptic digestion of denatured rituximab within 20 minutes, as well as the subsequent mass spectrometric detection of the specific peptide. The developed method can already be used, but offers further potential for optimisation. For example, the current two-dimensional system can be extended to three dimensions in order to implement the denaturation step as well.

9.1 References

1. Europäisches Arzneibuch 2.9.17 Bestimmung des entnehmbaren Volumens von Parenteralia.

2. Europäisches Arzneibuch V10

3. Ministerium für Arbeit, Gesundheit und Soziales des Landes Nordrhein-Westfalen (2018) Ergebnisse von Apothekenkontrollen liegen vor. https://www.mags.nrw/pressemitteilung/ergebnisse-von-apothekenkontrollen-liegen-vor. Accessed 20.01.2023

4. Gieskes V (2021) Probenuntersuchungen im Land Brandenburg. NZW-Sommer

5. Kiffmeyer T (2008) Monitoring-Effekt-Studie für Wischproben in Apotheken (MEWIP). bgw Forschung

6. Khongorzul P, Ling CJ, Khan FU, Ihsan AU, Zhang J (2020) Antibody-Drug Conjugates: A Comprehensive Review. Mol Cancer Res 18 (1):3-19. doi:10.1158/1541-7786.MCR-19-0582

7. Beck A, Goetsch L, Dumontet C, Corvaia N (2017) Strategies and challenges for the next generation of antibody drug conjugates. Nature Reviews Drug Discovery 16 (5):315-337. doi:10.1038/nrd.2016.268

8. Polakis P (2016) Antibody Drug Conjugates for Cancer Therapy. Pharmacol Rev 68 (1):3-19. doi:10.1124/pr.114.009373

9. Perez HL, Cardarelli PM, Deshpande S, Gangwar S, Schroeder GM, Vite GD, Borzilleri RM (2014) Antibody-drug conjugates: current status and future directions. Drug Discov Today 19 (7):869-881. doi:10.1016/j.drudis.2013.11.004

10. Halsen G, Kramer I (2011) Assessing the risk to health care staff from long-term exposure to anticancer drugs--the case of monoclonal antibodies. J Oncol Pharm Pract 17 (1):68-80. doi:10.1177/1078155210376847

11. Reinders LMH, Noelle D, Klassen MD, Jaeger M, Schmidt TC, Tuerk J, Teutenberg T (2022) Development and validation of a method for airborne monoclonal antibodies to quantify workplace exposure. J Pharm Biomed Anal 221:115046. doi:10.1016/j.jpba.2022.115046

12. Nowak C, Cheung JK, Dellatore SM, Katiyar A, Bhat R, Sun J, Ponniah G, Neill A, Mason B, Beck A, Liu HC (2017) Forced degradation of recombinant monoclonal antibodies: A practical guide. Mabs 9 (8):1217-1230. doi:10.1080/19420862.2017.1368602

Appendix

List of Figures

List of Tables

List of Abbreviations

2D-HPLC-HRMS	Two-dimensional liquid chromatography-mass spectrometry
5-FU	5-Fluorouracil
ACN	Acetonitrile
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADLM	Adalimumab
API	Active pharmaceutical ingredient
ASM	Active solvent modulation
Biosimilar	Follow-on product
BVCZ	Bevacizumab
CA	Cellulose acetate
$CaCl_2$	Calcium chloride
ccs	Cell-free culture supernatant
CDC	Complement-dependent cytotoxicity
CE	Collision energy
CHO	Chinese hamster ovary cells
CMR	Carcinogenic, mutagenic or toxic to reproduction
CP	Cyclophosphamide
CTX	Cetuximab
CV	Column volume
CXP	Collision cell exit potential
D	Detected sugar modification within a peak
D_c	Daratumumab content
D_{max}	Expected recovered daratumumab content
D_{min}	Minimum expected recovered daratumumab content
DP	Declustering potential
DRTM	Daratumumab

DTT	Dithiothreitol
EMA	European Medicines Agency
FA	Formic acid
Fab	Fragment antigen binding
FALD	Formaldehyde
Fc	Fragment crystallizable
FcR	Fragment crystallisable receptor
FTIR	Fourier transform infrared spectroscopy
Gem	Gemcitabine
GF	Glass fibre
GlcNAc	N-Acetylglucosamine
GuHCl	Guanidine hydrochloride
H_2O	Water
HCl	Hydrochloric acid
HIC	Hydrophobic interaction chromatography
HILIC	Hydrophilic interaction liquid chromatography
HPLC-DAD	High-performance liquid chromatography and diode array detector
HPLC-MS/MS	High-performance liqiod chromatography with tandem mass spectrometry
HRMS	High-resolution mass spectrometry
IF	Ifosfamide
IgG1	Immunoglobulin G subclass 1
IMER	Immobilized enzyme reactors
IPA	Isopropanol
Irino	Irinotecan
k	Coverage factor

LC	Liquid Chromatography
LCxLC	Comprehensive two-dimensional liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
M	Most intensive glycan modification within a peak
mAbs	Monoclonal antibodies
MAGS	Ministry of Labour, Health and Social Affairs of the state of North Rhine-Westphalia
MaxEnt	Maximum Entropy
MS	Mass spectrometer
MS/MS	Tandem mass spectrometry
MW	Molecular weight
n. d.	Not detected
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH_4HCO_3	Ammonium bicarbonate
OctopoleRPPeak	Octopole rod repel voltage
OMLZ	Omalizumab
Originator	Original product
P	Peak in which the glycan modification is most intense
PA	Polyamide
Pac	Paclitaxel
PBS	Phosphate buffered saline
PC	Polycarbonate
PET	Polyethylene terephthalate
Ph. Eur	European Pharmacopoeia
Ph. Helv.	Pharmacopoea Helvetica

PTFE	Polytetrafluoroethylene
PTZ	Pertuzumab
pur	Purified
PVDF	Polyvinylidene fluoride
Q	Volume flow rate
QC	Quality control
$q_{Density}$	error of the density determination
QF	quartz fibre
$q_{Random\_HPLC\text{-}DAD}$	Random error of HPLC-DAD
$q_{Random\_Raman/UV}$	Random error of Raman/UV
q_{Random_sp}	Random error of sample preparation
$q_{Systematic\_HPLC\text{-}DAD}$	Systematic error of HPLC-DAD
$q_{Systematic\_Raman/UV}$	Systematic error of Raman/UV
$q_{Systematic\_sp}$	Systematic error of sample preparation
R	Recovery
Raman/UV	Raman spectroscopy and UV absorption
RC	Regenerated cellulose
RP	Reversed Phase
RSD	Relative standard deviation of the recovery
RT	Retention time
RTX	Rituximab
S/N	Signal-to-noise
SCX	Strong cation exchange
SD	Standard deviation
SEC	Size exclusion chromatography
SMEPAC	Standardised measurement of equipment particulate airborne concentration

STOP	Substitution, technical measures, organisational measures and personal protective equipment
t	Sampling time
TCLZ	Tocilizumab
TRISHCl	Tris(hydroxymethyl)aminomethane hydrochloride
TTZ	Trastuzumab
u_{csp}	Combined uncertainty of the sample preparation
$u_{HPLC-DAD}$	Combined uncertainty of HPLC-DAD
$U_{HPLC-DAD}$	Extended combined uncertainty of HPLC-DAD
$u_{Raman/UV}$	Combined uncertainty of Raman/UV
$U_{Raman/UV}$	Extended combined uncertainty of Raman/UV
USP	United States Pharmacopeia
UTKM	Ustekinumab
V	Colume Unit
VCap	Capillary voltage
w	Active pharmaceutical ingredient content

List of Publications

Articles in peer-reviewed journals

Reinders, L.M.H., Klassen, M.D., Jaeger, M., Schmidt, T.C., Teutenberg, T., Tuerk, J.
Quality control of personalized drug products – Identity and quantity of monoclonal antibodies as active pharmaceutical ingredient
J. Pharm. Sci. (2023), Status: Submitted

Reinders, L.M.H., Klassen, M.D., Endres, P., Krumm, A., Jaeger, M., Schmidt, T.C., Teutenberg, T.
Development of a two-dimensional liquid chromatography high resolution mass spectrometry method for the characterization of monoclonal antibodies in cell-free culture supernatant via FcR affinity chromatography
Chromatographia 86, 79-85 (2023) https://doi.org/10.1007/s10337-022-04228-x

Reinders, L.M.H., Noelle, D., Klassen, M.D., Jaeger, M., Schmidt, T.C., Tuerk, J., Teutenberg, T.
Development and validation of a method for airborne monoclonal antibodies to quantify workplace exposure
J. Pharm. Biomed. Anal. 221, 115046 (2022) https://doi.org/10.1016/j.jpba.2022.115046

Reinders, L.M.H., Klassen, M.D., Teutenberg, T., Jaeger, M., Schmidt, T.C.
Comparison of originator and biosimilar monoclonal antibodies using HRMS, Fc affinity chromatography, and 2D-HPLC
Anal. Bioanal. Chem. 414, 6761-6769 (2022) https://doi.org/10.1007/s00216-022-04236-8

Jochums, M., Reinders, L.M.H., Tuerk, J., Teutenberg, T.
Flexible Digitization of Highly Individualized Workflows Demonstrated Through the Quality Control of Patient-Specific Cytostatic Application Bags: Digitization from the Perspective of Small and Medium-Sized Laboratories
Adv. Biochem. Eng. Biotechnol. 182, 115-129 (2022), https://doi.org/10.1007/10_2021_190

Reinders, L.M.H., Klassen, M.D., Teutenberg, T., Jaeger, M., Schmidt, T.C.
Development of a multidimensional online method for the characterization and quantification of monoclonal antibodies using immobilized flow-through enzyme reactors
Anal. Bioanal. Chem. 413, 7119-7128 (2021), https://doi.org/10.1007/s00216-021-03683-z

Reinders, L.M.H., Klassen, M.D., vom Eyser, C., Teutenberg, T., Jaeger, M., Schmidt, T.C., Tuerk, T.
Quality control of cytostatic drug preparations—comparison of workflow and performance of Raman/UV and high-performance liquid chromatography coupled with diode array detection (HPLC-DAD)
Anal. Bioanal. Chem. 413, 2587-2596 (2021), https://doi.org/10.1007/s00216-021-03223-9

Reinders, L.M.H., Klassen, M.D., Jaeger, M., Teutenberg, T., Tuerk, J.
Development of an analytical method to assess the occupational health risk of therapeutic monoclonal antibodies using LC-HRMS
Anal. Bioanal. Chem. 410, 2829-2836 (2018), https://doi.org/10.1007/s00216-018-0966-1

Technical Papers

Reinders, L.M.H., Teutenberg, T., Klassen, M.D., Esser, D., Tuerk, J.
Ionensuppression in der Massenspektrometrie von Biomolekülen – Notwendigkeit und Einfluss von Additiven in der mobilen Phase
GIT Labor-Fachzeitschrift 6/2020

Oral Presentations (Scientific)

Teutenberg, T., Tuerk, J., Gehrmann, L., Klassen, M.D., Reinders, L.M.H., Werres, T., Henning, I., Kochale, K., Jochums, M.
FutureLab.NRW – Von der Idee zur Realität: Impulsvorträge zu spezifischen Projekten im Kontext des FutureLab.NRW
6. IUTA-AnalytikTag, Duisburg, Deutschland (10.11.2022)

Tuerk, J., Reinders, L.M.H., Jochums, M., vom Eyser, C., Teutenberg, T.
FutureLab.NRW - Digitalisierung der Qualitätskontrolle von Zytostatika-Zubereitungen
3. ZytostatikaTag, Duisburg, Deutschland (09.11.2022)

Reinders, L.M.H., Klaßen M.D., Teutenberg, T.
Massenspektrometrische Analyse von monoklonalen Antikörpern - Einfluss von ein- und zweidimensionaler Chromatographie
Moderne Trenn- und Detektionsmethoden für Antikörper und Proteine, Online (27.09.2022)

Reinders, L.M.H
Bioanalytical methods to determine exposure to monoclonal antibodies
Virtual Analytical Summit 2022, Online (15.02.2022)

Reinders, L.M.H., Klaßen, M.D., Jäger, M., Schmidt, T.C., Teutenberg, T.
Entwicklung eines multidimensionalen Verfahrens mit tryptischen on-line Verdau zur Peptid-Analytik
32. Doktorandenseminar des AK Separation Science, Online (10.01.2022)

Reinders, L.M.H., Kochale, K., Jochums, M., Tuerk, J., Teutenberg, T.
Digitization of Workflows and Method Developments in a Pharmaceutical Testing Laboratory
PharmaLab 2021 – Optimization and Automation, Online (22.11.2021)

Reinders, L.M.H.
Massenspektrometrische Analyse von monoklonalen Antikörpern
Moderne Trenn- und Detektionsmethoden für Antikörper und Proteine: Herausforderungen und Lösungsansätze in der BioLC, Online (05.10.2021)

Reinders, L.M.H., Klassen, M.D., Constantinidis, P., Thissen, J., Teutenberg, T.
Characterisation and quantification of biomolecules
Wiley Analytical Science Conference, Online (22.04.2021)

Reinders, L.M.H., Klassen, M.D., Teutenberg, T.
Mehrdimensionale Flüssigkeitschromatographie in der Bioanalytik - Grundlagen, Routine, Forschung
HPLC-Praxistag, Online (18.03.2021)

Reinders, L.M.H., Klassen, M.D., Noelle, D., vom Eyser, C., Teutenberg, T., Tuerk, T.
Expositionsmessungen von monoklonalen Antikörpern
12. NZW-Dresden, Dresden, Deutschland (23.10.2020)

Reinders, L.M.H., Kerstein, J., vom Eyser, C., Korczowska, E., Tuerk, J.
Bewertungskonzepte und aktuelle Daten aus dem Umgebungsmonitoring mittels Wischproben
12. NZW-Dresden, Dresden, Deutschland (23.10.2020)

Reinders, L.M.H., Hermuth-Kleinschmidt, K.
Die Mikro-LC: Eine nachhaltige Methode in der Routineanalytik
PharmaLab, Neuss, Deutschland (13.11.2019)

Tuerk, J., Reinders, L.M.H.
Analytik to go: Arbeitssicherheit und Qualitätssicherung – Forum für kritische Diskussion
11. NZW-Dresden, Dresden, Deutschland (21.06.2019)

Oral Presentations (Educational)

Reinders, L.M.H, Tuerk, J.
Monoklonale Antikörper im Arbeitsschutz
Sicherheitstraining Zytostatika, Online (02.12.2022)

Reinders, L.M.H, Tuerk, J.
Monoklonale Antikörper im Arbeitsschutz
Sicherheitstraining Zytostatika, Online (11.11.2022)

Reinders, L.M.H., Klassen, M.D., Tuerk, J.
Effektive Reinigung nach unbeabsichtigter Substanzfreisetzung
Sicherer Umgang mit Zytostatika, Duisburg, Deutschland (08.11.2022)

Reinders, L.M.H., Tuerk, J., vom Eyser, C.
Effektive Reinigung belasteter Oberflächen und Materialien
14. NZW-Sommer, Berlin, Deutschland (30.09-01.10.2022)

Reinders, L.M.H., vom Eyser, C., Klassen, M.D., Teutenberg, T., Tuerk, J.
Qualitätssicherung in der Sterilherstellung und Gehaltsbestimmungen von Zytostatika-Applikationslösungen
Sicherer Umgang mit Zytostatika, Online (24.09.2021)

Reinders, L.M.H., Tuerk, J., vom Eyser, C.
Effektive Reinigung und Desinfektion in der Zytostatika-Herstellung
Sicherheitstraining für Zytostatika, Online (25-26.06.2021)

Reinders, L.M.H., Dieckhoff, M., Tuerk, J.
Verhalten in Notfallsituationen: Praktische Anwendung von SpillKits
Sicherheitstraining für Zytostatika, Online (25-26.06.2021)

Reinders, L.M.H., Tuerk, J.
Effektive Reinigung von Oberflächen, Desinfektion und Validierung
Sicherheitstraining für Zytostatika, Online (25-26.06.2021)

Reinders, L.M.H., Tuerk, J.
Monoklonale Antikörper im Arbeitsschutz
Sicherheitstraining für Zytostatika, Online (25-26.06.2021)

Reinders, L.M.H., Klassen, M.D., vom Eyser, C., Bruckmann, J., Teutenberg, T., Jaeger, M., Schmidt, T.C., Tuerk, J.
Monoklonale Antikörper und Arbeitsschutz
Sicherheitstraining für Zytostatika, Hamburg, Deutschland (06.11.2020)

Reinders, L.M.H.
Effektive Reinigung von Oberflächen, Desinfektion und Validierung
Sicherheitstraining für Zytostatika, Hamburg, Deutschland (16.11.2019)

Reinders, L.M.H.
Monoklonale Antikörper im Arbeitsschutz
Sicherheitstraining für Zytostatika, Hamburg, Deutschland (16.11.2019)

Reinders, L.M.H., Tuerk, J.
Effektive Reinigung und Desinfektion in der Zytostatika-Herstellung
Sicherheitstraining für Zytostatika, Hamburg, Deutschland (15.11.2019)

Reinders, L.M.H., Tuerk, J.
Effektive Reinigung und Desinfektion in der Zytostatika-Herstellung
Sicherheitstraining für Zytostatika, Hamburg, Deutschland (24.08.2019)

Reinders, L.M.H.
Monoklonale Antikörper im Arbeitsschutz
Sicherheitstraining für Zytostatika, Hamburg, Deutschland (23.08.2019)

Reinders, L.M.H., Tuerk, J.
Effektive Reinigung und Desinfektion in der Zytostatika-Herstellung
Sicherheitstraining für Zytostatika, Hamburg, Deutschland (18.05.2019)

Reinders, L.M.H.
Monoklonale Antikörper im Arbeitsschutz
Sicherheitstraining für Zytostatika, Hamburg, Deutschland (17.05.2019)

Reinders, L.M.H.
Monoklonale Antikörper im Arbeitsschutz
Sicherer Umgang mit Zytostatika, Duisburg, Deutschland (05.04.2019)

Reinders, L.M.H.
Gehaltsbestimmung von Zytostatika-Applikationslösungen
Sicherer Umgang mit Zytostatika, Duisburg, Deutschland (04.04.2019)

Poster Presentations

Knoll, L., Thiesen, J., Klassen, M.D., Reinders, L.M.H., Tuerk, J., Kraemer, I.
Physicochemical stability of subcutaneous daratumumab (DARZALEX® 1800 mg injection solution) in plastic syringes over a period of 28 days stored refrigerated or at room temperature
25th European GERPAC Conference, Hyère, France (05-07.10.2022)

Reinders, L.M.H., Bruckmann, J., Klassen, M.D., vom Eyser, C., Jaeger, M., Schmidt, T.C., Teutenberg, T., Tuerk, J.
Quality control of infusions in patient specific preparations for oncological treatment
24th Congress of the European association of hospital pharmacists, Barcelona, Spain (27-29.03.2019)

Reinders, L.M.H., Bruckmann, J., Klassen, M.D., vom Eyser, C., Jaeger, M., Schmidt, T.C., Teutenberg, T., Tuerk, J.
Quality control of infusions in patient specific preparations for oncological treatment
ANAKON, Münster, Deutschland (25-28.03.2019)

Klassen, M.D., Reinders, L.M.H., Jaeger, M., Teutenberg, T., Tuerk, J.
Development of an analytical method to assess the occupational health risk of therapeutic monoclonal antibodies using liquid chromatography and high resolution mass spectrometry (LC-HRMS)
4th European Conference of Oncology Pharmacy, Nantes, France (24-26.10.2018)

Reinders, L.M.H., Klassen, M.D., Jaeger, M., Teutenberg, T., Tuerk, J.
Development of an analytical method to assess the occupational health risk of therapeutic monoclonal antibodies using liquid chromatography and high resolution mass spectrometry (LC-HRMS)
23rd Congress of the EAHP, Gothenburg, Sweden (21-23.03.2018)

vom Eyser, C., Reinders, L.M.H., Klassen, M.D., Jaeger, M., Teutenberg, T., Tuerk, T. Development of an analytical method to assess the occupational health risk of therapeutic monoclonal antibodies using liquid chromatography and high resolution mass spectrometry (LC-HRMS)
11th World Meeting on Pharmaceuticals, Biopharmaceuticals and Pharmaceutical Technology, Granada, Spain (19-22.03.2018)

Declaration of Scientific Contribution

This thesis includes work that was published in cooperation with co-authors. My own contributions are declared in the following:

Chapter 3

Reinders, L.M.H., Klassen, M.D., vom Eyser, C., Teutenberg, T., Jaeger, M., Schmidt, T.C., Tuerk, T., Quality control of cytostatic drug preparations—comparison of workflow and performance of Raman/UV and high-performance liquid chromatography coupled with diode array detection (HPLC-DAD). Anal Bioanal Chem 413, 2587–2596 (2021). https://doi.org/10.1007/s00216-021-03223-9

CRediT authorship contribution statement

Lars M. H. Reinders: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration, Funding acquisition. **Martin D. Klassen:** Conceptualization, Methodology, Writing - Review & Editing, Supervision, Funding acquisition. **Claudia vom Eyser:** Writing - Review & Editing, Project administration. **Thorsten Teutenberg:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition. **Martin Jaeger:** Writing - Review & Editing, Supervision. **Torsten C. Schmidt:** Writing - Review & Editing, Supervision. **Jochen Tuerk:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition.

Chapter 4

Reinders, L.M.H., Klassen, M.D., Jaeger, M., Schmidt, T.C., Teutenberg, T., Tuerk, J., Quality control of personalized drug products – Identity and quantity of monoclonal antibodies as active pharmaceutical ingredient. J Pharm Sci (2023) Submitted

CRediT authorship contribution statement

Lars M. H. Reinders: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration, Funding acquisition. **Martin D. Klassen:** Conceptualization, Methodology, Writing - Review & Editing, Supervision, Funding acquisition. **Thorsten Teutenberg:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition. **Martin Jaeger:** Writing - Review & Editing, Supervision. **Torsten C. Schmidt:** Writing - Review & Editing, Supervision. **Jochen Tuerk:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition.

Chapter 5

Reinders, L.M.H., Klassen, M.D., Endres, P., Krumm, A., Jaeger, M., Schmidt, T.C. Teutenberg, T., Development of a two-dimensional liquid chromatography high resolution mass spectrometry method for the characterization of monoclonal antibodies in cell-free culture supernatant via FcR affinity chromatography. Chromatographia 86, 79-85 (2023) https://doi.org/10.1007/s10337-022-04228-x

CRediT authorship contribution statement

Lars M. H. Reinders: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration, Funding acquisition. **Martin D. Klassen:** Conceptualization, Methodology, Writing - Review & Editing, Supervision, Funding acquisition. **Patrick Endres:** Conceptualization, Investigation, Resources, Writing – Original Draft, Writing – Review & Editing. **Andrea Krumm:** Writing – Review & Editing. **Thorsten Teutenberg:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition. **Martin Jaeger:** Writing - Review & Editing, Supervision. **Torsten C. Schmidt:** Writing - Review & Editing, Supervision.

Chapter 6

Reinders, L.M.H., Klassen, M.D., Teutenberg, T., Jaeger, M., Schmidt, T.C., Comparison of originator and biosimilar monoclonal antibodies using HRMS, Fc affinity chromatography, and 2D-HPLC. Anal Bioanal Chem 414, 6761–6769 (2022). https://doi.org/10.1007/s00216-022-04236-8

CRediT authorship contribution statement

Lars M. H. Reinders: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration, Funding acquisition. **Martin D. Klassen:** Conceptualization, Methodology, Writing - Review & Editing, Supervision, Funding acquisition. **Thorsten Teutenberg:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition. **Martin Jaeger:** Writing - Review & Editing, Supervision. **Torsten C. Schmidt:** Writing - Review & Editing, Supervision.

Chapter 7

Reinders, L.M.H., Noelle, D., Klassen, T., Jaeger, M., Schmidt, T.C., Tuerk, J., Teutenberg, T. Development and validation of a method for airborne monoclonal antibodies to quantify workplace exposure. J. Pharm. Biomed. Anal. 221, 115046 (2022) https://doi.org/10.1016/j.jpba.2022.115046

CRediT authorship contribution statement

Lars M. H. Reinders: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration, Funding acquisition. **Dennis Noelle:** Formal Analysis, Investigation. **Martin D. Klassen:** Conceptualization, Methodology, Writing - Review & Editing, Supervision, Funding acquisition. **Thorsten Teutenberg:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition. **Jochen Tuerk:** Conceptualization, Writing - Review & Editing, Supervision. **Martin Jaeger:** Writing - Review & Editing, Supervision. **Torsten C. Schmidt:** Writing - Review & Editing, Supervision.

Chapter 8

Reinders, L.M.H., Klassen, M.D., Teutenberg, T., Jaeger, M., Schmidt, T.C., Development of a multidimensional online method for the characterization and quantification of monoclonal antibodies using immobilized flow-through enzyme reactors. Anal Bioanal Chem 413, 7119–7128 (2021). https://doi.org/10.1007/s00216-021-03683-z

CRediT authorship contribution statement

Lars M. H. Reinders: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration, Funding acquisition. **Martin D. Klassen:** Conceptualization, Methodology, Writing - Review & Editing, Supervision, Funding acquisition. **Thorsten Teutenberg:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition. **Martin Jaeger:** Writing - Review & Editing, Supervision. **Torsten C. Schmidt:** Writing - Review & Editing, Supervision.

Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„Analytical monitoring of personalized drugs containing monoclonal antibodies: An alliance of patient and personnel safety"

selbst verfasst, keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet sind und die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Duisburg, Februar 2023

_____

Lars Reinders