Dissertation zur Erlangung des Doktorgrades
der Fakultät für Chemie und Pharmazie
der Ludwig–Maximilians–Universität München

Leachable and Extractable Studies on Single-Use System Technologies in commercial scale Drug Filling Lines

Nicole Marion Doris Scherer

aus
Otjiwarongo, Namibia

2019

Erklärung:
Diese Dissertation wurde im Sinne von § 7 der Promotionsordnung vom 28. November 2011 von Herrn Prof. Dr. Gerhard Winter betreut.

Eidesstattliche Versicherung:
Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

München, den 19.08.2019

Nicole Scherer

Dissertation eingereicht am: 05.04.2019
Erstgutachter: Prof. Dr. Gerhard Winter
Zweitgutachter: Prof. Dr. Wolfgang Frieß
Tag der mündlichen Prüfung: 30.04.2019

Danksagung

In the following I want to acknowledge all supervisors, colleagues and friends who contributed to this work. I express my thanks in my native language German.

An dieser Stelle möchte ich mich bei allen Betreuern, Kollegen und Freunden bedanken die mich während dieser spannenden vier Jahre begleitet haben und an dem Entstehen dieser Arbeit beteiligt waren. Mein besonderer Dank gilt hierbei:

Herrn Prof. Dr. Gerhard Winter für sein großes Vertrauen und seine fortwährende, flexible Unterstützung, ohne die diese Projekte nicht möglich gewesen wären.

Besonderer Dank gilt auch meinem Betreuer Dr. Tobias Posset dessen unermüdlicher Forscherdrang meine Promotionsarbeit im industriellen Umfeld der Roche GmbH ermöglichte und mich in meiner Entwicklung maßgeblich gefördert hat.

Meiner Betreuerin Dr. Klaudia Marcseková danke ich ganz besonders dafür, dass sie stets für mich da war, mich bei jeglichen Fragen unterstützt hat und in allen Situationen und Lebenslagen mit Rat und Tat zur Seite Stand.

Meinen weiteren Kollegen in der Abteilung Inprozesskontrolle und Partikellabor der Roche GmbH für eine freundliche, heitere und stets produktive Arbeitsatmosphäre, aufgrund derer es für mich stets eine Freude war ins Büro zu kommen. Die sehr aufbauenden und stets lustigen Essenstermine mit Steffen Westkemper und Evgenija Stockinger.

Arno Wortmann, Margot Reth und Bertrand Gessier dafür, dass sie mich in Ihrer Abteilung herzlich aufgenommen haben, mir bei meinen ersten Gehversuchen mit LC Messungen unterstützt haben und für eine stets erfreuliche Kooperation und Atmosphäre.

Und dem Rest der Pharmazeutischen Technologie-Gruppe für eine tolle Zeit, inklusive tollem Skiurlaub.

Ich danke auch Herrn Prof. Dr. Wolfgang Frieß für die gute Kooperation.

Zu guter Letzt möchte ich meiner Familie, meiner Mutter Jeanette, meinen Brüdern Maik und Kai, meinem Freund Sebastian und meiner Oma Marion für die vorbehaltlose Unterstützung, den Rückhalt und die Bodenhaftung, die ich stets von euch erfahre, danken.

Contents

Nomenclature

ADI Acceptable Daily Intake

AET Analytical Evaluation Threshold

ASME-BPE American Society of Mechanical Engineers-Bioprocessing Equipment

ASTM American Society for Testing and Materials

BHT 3,5-Di-tert-butyl-4-hydroxytoluol

BPOG BioPhorum Operations Group

BPSA Bio-Process Systems Alliance

CIP/SIP Cleaning In Place / Sterilization In Place

CIS Cold Injection System

DP Drug Product

EG Ethylen-Glycol

EI Electron Ionization

EMA European Medical Affairs

ESI Electron Spray Ionization

FDA Food and Drug Administration

GC Gas Chromatography

He Helium

ICH International Conference of Harmonisation

ICP Inductively Coupled Plasma

IPA Isopropanol

ISPE International Society of Pharmaceutical Engineering

LC Liquid Chromatography

LOD Limit of Detection

LOQ Limit of Quantification

MCE Mixed Cellulose Esters

MS Mass Spectrometry

P Placebo

PDMS Polydimethylsiloxane

PP Polypropylene

ppb parts per billion

ppt parts per trillion

PRCA Pure Red-Cell Aplasia

PSU Polysulfone

PTV Programmed Temperature Vaporizing

PVC Polyvinyl Chloride

PVDF Polyvinylidene Fluoride

QTOF Quadrupole Time-of-Flight

SBSE Stir-Bar Sorptive Extraction

SI Silicone

SNR Signal to Noise Ratio

SUS Single-Use System

TBA 2,4,6-tribromoanisole

TBP Tribromophenol

TD Thermal Desorption

TD-GC-MS Thermal Desorption Gas Chromatography Mass Spectrometry

TDS Thermal Desorption System

THF Tetrahydrofurfuryl

TTC Threshold of Toxicological Concern

UPLC Ultra Performance Liquid Chromatography

USP United States Pharmacopeia

WfI Water for Injection

List of Figures

List of Tables

Chapter 1

General Introduction & Objectives

1.1 Single-Use-Technologies in Biopharmaceutical Manufacturing

Single-Use System (SUS) technology has increasingly emerged as the processing equipment of choice for modern biopharmaceutical lines [1, 2], as depicted in Figure 1.1. These polymer-based, ready-to-use systems were initially designed as lab-scale equipment for pilot projects. Recently SUSs including large-scale disposable bioreactors, storage bags, mixing systems, tubing, sensors, connectors, filter cartridges and chromatography systems, have become widely deployed throughout up- and downstream processing [3, 4]. The disposable concept is an innovative alternative to fixed stainless steel equipment, as it can outperform the latter in terms of flexibility, productivity and handling. Recently, the technology has become even more attractive being applicable to a more comprehensive product range and size scale [5]. The variety of available SUS components facilitates end-to-end single-use manufacturing facilities [6]. This modular approach of SUSs makes a customized configuration of unit operations possible and can be adapted in a product-specific way to save space and to provide the ease of maneuverability around the facility [7]. In addition to the saving of work space, the application of SUSs can reduce costs by up to 50%. Among other factors this is due to the elimination of cleaning validations especially in the case of multi-product lines. By implementing a single-use 3D bag for buffer preparation and, hence, avoiding the need of cleaning intervals, Weitbrecht et al. [8] were able to significantly increase the productivity from 2.5 to 4 batches per week. However, despite many distinct advantages of SUSs, the industry-wide application remains low (10%) with a market growth reported to be around 20% in the past three years [9]. This rather low number is mainly based on the concern of pharmaceutical-manufacturers that polymer-related impurities, so called “leachables”, might migrate into the production stream and persist throughout the manufacturing process (Fig. 1.1) [1]. As the main advantage of disposable systems stem from the lower risk of cross-contamination and thus increased patient safety [10], a contamination by leachables that can be of toxicological concern would be contrary and has to be monitored.

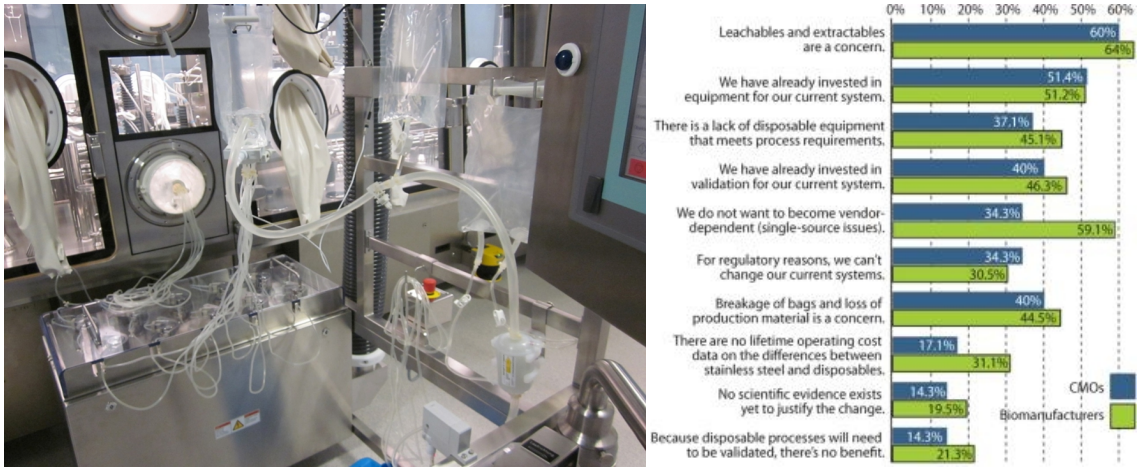


Figure 1.1: Comparison of assets and drawbacks of single-use system applications. Left: Biopharmaceutical manufacturing site fully equipped with disposables, enabling a configurable solution for the final formulation step (Production line, Roche Diagnostics GmbH, Mannheim Germany. Rights reserved). Right: Main factors restricting the use of disposable systems by biomanufacturers. The primary concern is the presence of leachables and extractables[1].

1.2 Polymer Additives and Possible Impurities

A number of additives are used in the processing of SUSs and pose risk as potential impurities. While these additives tend to be soluble in solvents, are volatile or interact with the product, they can potentially leach out of the polymer matrix. Due to direct contact of the SUSs to the reagents, reaction solutions, intermediates and drug products during the production process, these substances can potentially end up as leachables in the final drug product formulation, hence eventually contaminating the pharmaceutical product [11, 12].

Among these impurities are low molecular weight substances that originated from the polymer production process and remained in the polymer matrix, including initiators, solvents, crosslinking substances, block builders and unpolymerized monomers [13, 14, 15, 16]. These substances do not have covalent bonds to the matrix and can easily migrate through the polymer matrix. Further additive compounds are required to enable extrusion processing. These additives can include lubricants, flame-retardants, adhesion promoters and slip agents [17]. While necessary for the polymer processing, these additives can potentially impair the drug product.

Further compounding is done to achieve desired material properties by adapting the material to specific applications. These carefully selected additives are used to enhance

performance, but can potentially migrate out of the polymer matrix into the drug product under specific process conditions. The most commonly applied additives are plasticizers, antioxidants and UV stabilizers [18]. Different polymers require different additives depending on the application. Hence, certain additives can be expected for some polymers but not for others. The polymer polyvinyl chloride (PVC) for example, is in its pure form comparatively rigid and requires plasticizers to provide a desired increase in flexibility and formability [19, 20]. When a plasticizer is introduced to PVC, it affects the stress-strain relationship of the constrained polymer chain, resulting in a reduction in stiffness [21]. Plasticizers, such as phthalates, enable a degradation in melt viscosity of the material as well as an increase in its light stability and durability. They provide resistance to oxidizing acids, but at the same time lower the polymer resistance to fungal contaminants. In order to counteract, an approach of co-compounding of multiple additives in a single mixture is adopted, where different preservatives and additional anti-infective agents are introduced to the material. In the case, that certain properties cannot be achieved with one particular polymer, multi-layer structures, in which layers of different polymers are combined, enable an even wider range of properties [22]. However, each layer may feature different additives with typically low molecular weight and impair the product. Hence, the potential a wider spectrum of substances can pose risk as potential leachables.

In order to meet pharmaceutical needs single-use components have to be gamma-sterilized prior to use. Resistance against oxidation during the sterilization procedure and desirable durability are accomplished by antioxidant compounding [23]. This group of additives can stabilize the polymer against high temperature and prevents degradation over the operating lifetime induced by oxygen absorption [24]. There are two classes of antioxidants: Primary antioxidants are free-radical scavengers, while secondary antioxidants are classified as peroxide scavenger. These antioxidants, along with oxidized break-down fragments, are commonly found as impurities [25]. Typically stabilizers are compounded using UV-light in order to withstand oxidative deterioration. Besides organic stabilizers, inorganic elements, such as barium, cadmium, cobalt, tin and lead can be used. Inorganic reinforcement can be applied in form of fillers to reduce density and to improve stability, like glass or mineral fibers in silicone tubing. Furthermore, non-soluble complexes of metals are classically applied as active catalyst and initiators for the polymerization process [26, 27].

Development of novel and improved additives is an active area of research and new substances are quickly adopted in polymer processing [11]. This further complicates the determination of this broad set of diverse additives. To detect and quantify organic as well as inorganic non-target substances, analytical techniques with appropriate sensitivity are required. Depending on the organic substance's volatility and polarity, gas chromatography (GC) and complementary liquid chromatography (LC) in combination with mass spectrometry (MS) are commonly applied [28, 29]. Consequently, only one example, long-chained fatty acids used as lubricants are detected with LC analytic, compared to volatile solvents, which are verified by GC-MS. For inorganic substances, inductively coupled plasma

(ICP)-MS is applied.

1.3 Impact of Leachables and Extractables

The aim of this thesis is to monitor accumulation of leachables in drug products. This investigation is warranted, as low binding substances in polymers are unavoidable and can be harmful or potentially carcinogenic or genotoxic, posing a threat to the patient's health. In particular, as treatment duration can span several years up to a lifetime, even trace concentrations down to ppb ($\mu g/kg$) or ppt (ng/kg) are to be considered as critical thresholds to avoid any potential health hazards [30]. Parenteral pharmaceuticals are of special concern, as leachables can get directly transferred into the bloodstream of a patient and, thus, pose an increased safety risk [31].

Besides the immediate health risk due intake of leachables by the patient, another concern is potential alteration of the product's active ingredients leading to reduced efficacy or other undesired side-effects [32]. In 1998 a first case of leachables causing an unanticipated and adverse cross-reaction of pure red-cell aplasia (PRCA) was reported [33]. However, the leachables originated primarily in the packaging and not in SUSs. According to the US Food and Drug Administration (FDA) in this instance chemical components of the alkylphenol disulfide vulcanization agent within the rubber stopper of the Johnson & Johnson's Eprex pre-filled syringes were extracted by the drug component polysorbate 80 [34]. This reactive compound bound to the active protein, leading to a modification, which in turn induced an immunomodulatory effect in patients. Affected patients eventually started producing antibodies against the very protein that was supposed to replace their intrinsic EPO.

Other secondary effects can impact the appearance and other physical properties of the product. For example acidic or basic leachables can shift the product's pH-value. Leachables with spectral property can influence discoloration, turbidity and high UV absorbance in the product. Finally, leachables are also able to form particulate matters. In general these secondary effects can again influence the efficacy of the drug product. A consumer recall relating to an unusual moldy odor of the drug product Tylenol Arthritis Pain Caplet 100 was reported in 2010 [35], which was caused by the chemical 2,4,6-tribromoanisole (TBA), a breakdown-product of the leachable tribromophenol (TBP). This substance is used by wooden pallet manufacturers as a wood preservative and flame retardant. It migrated through the drug packaging from the wooden transport pallets, entered the drug product and caused gastrointestinal illness including nausea, vomiting and diarrhea.

To estimate the patient exposure and to ensure the integrity of the drug product it is imperative to identify and quantify accumulation of leachables in the final product. This includes testing for potential leachables in advance, before the actual SUS application [36, 37, 38]. However, formulation information provided through Material Safety

Data Sheet disclosures is typically incomplete or non-specific, as compounding is typically carefully protected as intellectual property. Therefore, so called extractable-studies are conducted [39, 40, 41]. These tests are performed to simulate worst case scenarios, in order to identify substances that can be extracted by exposing the SUS to appropriate solvents under exaggerated material stress including temperature and exposure time. This approach induces increased leaching and is regulated through government agencies such as the European Medical Affairs (EMA) [42] and the FDA [43]. Additionally, guidelines are available through the USP (<665> review stage, <1665> [44, 45]) and FDA (US 21 CFR 211.65 [46]) specifying the evaluation of SUS compatibility with the drug formulation. Beside authorities, industry collaboration groups such as the Bio-Process Systems Alliance (BPSA), BioPhorum Operations Group (BPOG), American Society for Testing and Materials (ASTM) International, International Society of Pharmaceutical Engineering (ISPE) and the American Society of Mechanical Engineers-Bioprocessing Equipment (ASME-BPE) provide strategies on determining whether a single-use component has been sufficiently and appropriately qualified. Although extractables can be considered a heuristic approximation of potential leachables, not all extractables leach into drug products. Furthermore, drug formulation components or buffers may interact with SUSs in various ways and form leachables that were not previously identified during extractables analysis with solvents [47]. Therefore, leachable studies of the whole manufacturing process are highly warranted in addition to the extractable-footprints of each SUS, in order to elucidate the actual leachable exposure and ensure the patient's safety and the product's integrity.

1.4 Objectives of this Thesis

The aim of this thesis was to obtain detailed information about the identities, the amount and the toxicological properties of leachables detected in real-world industrial drug-filling lines. This data can shed light on the potential actual patient exposure and can be used to verify the concern of pharmaceutical manufacturers regarding SUS applications [1]. For this purpose, samples of several intravenously administered protein drug formulations with long treatment duration, representative of a pharmaceutical production portfolio, were observed. Samples were drawn during the actual filling process after contact with various SUSs with different polymer parts under production conditions. The general outline of this thesis is depicted in Figure 1.2.

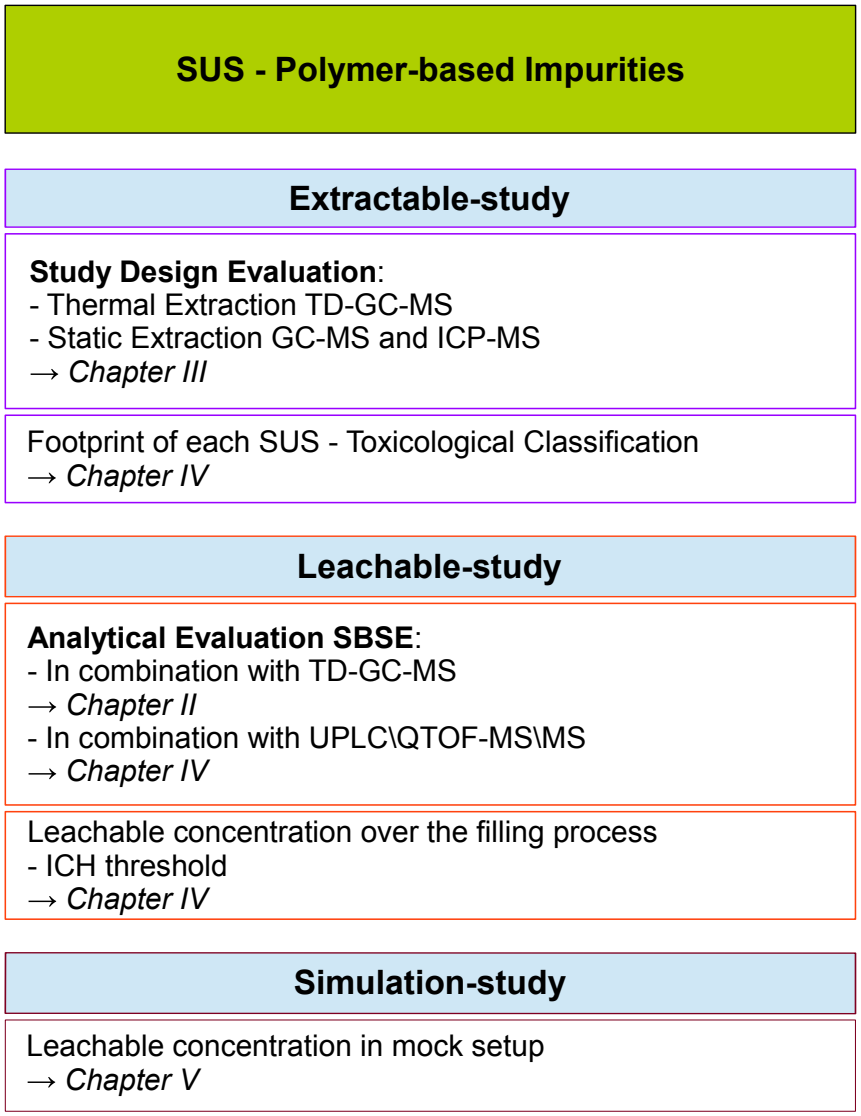


Figure 1.2: Thesis outline in four chapters, describing three different study-designs for the verification of polymer-based impurities, stem from SUSs in pharmaceutical processing. By extractable footprints of each SUS, leachable testing of the whole pharmaceutical filling-process was conducted as a classical approach. A potential risk-based approach was tested in form of a simulation-study, combining the other two studies in one test.

Gaschromatography-mass spectroscopy (GC-MS) served as the primary analytic for the experiments conducted in this thesis. An important aspect for the identification and quantification of leachables in the drug products with interfering protein and surfactants was the implementation of a pre-conditioning step. chapter 2 evaluates the applicability of the stir-bar sorptive extraction (SBSE) as sample preparation prior to thermal desorption (TD)-GC-MS for the detection of trace amounts of impurities in pharmaceutics. Tests were performed to verify sufficient sensitivity, reproducibility and linearity over a broad

spectrum of possible leachables with concentrations in the part per billion range and in presence of different drug product matrices. Further, the effect of the stir-bar coating material was examined and different optimization procedures were evaluated. Moreover, we investigated the conditioning of the stir-bar to mitigate the inhibitory effects of the drug matrix.

In chapter 3, the design of extractable-studies was adapted to identify impurities that may leach from process equipment into the contacting solution during production, to provide qualitative data for the selection of target leachable-analysis. Therefore, different harsh extraction methods were employed on the SUSs and assessed by their achieved extraction-profiles. In addition static and thermal extraction of different single-use filters were conducted. For the static procedure WfI was used as extraction solution with different pH-values and further an isopropanol/WfI mixture. Those extraction-procedures were compared to the direct material thermal extraction at 150°C. To ensure complete analysis the diverse extractions were studied in combination with GC-MS analytic and elemental impurity characterization with ICP-MS analytic.

In chapter 4 samples from real world filling lines were processed in order to detect leachable contamination. A differently coated stir-bar in combination with a solvent back extraction (SBE) step with subsequent Ultra Performance Liquid Chromatography equipped with a Quadrupole Time-of-Flight UPLC/QTOF-MS/MS analytic was used beside the GC-MS application. The development and adoption of extractable-studies greatly facilitated the ability to address the trace amounts of complex leachables to their origin SUSs in the process. These leachable-studies enabled to investigate if there was a link between the amount of leachables and the filled protein based drug products with their different formulations. Furthermore, SUSs with the highest leachable potential were identified for each drug product. Finally, the amount of leachables and their toxicity and/or carcinogenicity were evaluated by comparing to thresholds given by the ICH guideline M7, to expose potential health hazards [30].

In chapter 5, simulation-studies were assessed as an alternative to the extractable/ leachable concept to evaluate the patients exposure to polymer-based impurities. The new study-design was tested with the filling tube, which was reused up to six times in consecutive filling batches. After each application, the remaining additives in the polymer matrix of the tube were determined and compared to the extractable-footprint of an unused tube. The comparison allowed a determination of the leached substances during the batch-applications and even of substances, that were absorbed by the silicone. The new approach was tested in regards to its applicability and sensitivity.

In the last chapter 6 a summary and outlook on new approaches for the detection of polymer-based impurities in drug products is given.

Bibliography

[1] W. Ding and J. Martin. Implementing Single-Use Technology in Biopharmaceutical Manufacturing. *BioProcess International*, 8, 2010.

[2] Sade Mokuolu. New Standards Define Single-Use Materials Qualification. *PharmTech*, 42(2):52–53, 2018.

[3] Regine Eibl and Dieter Eibl, editors. *Single-use technology in biopharmaceutical manufacture*. Wiley, Hoboken, NJ, 2011.

[4] Susan Haigney. Integrating Single-Use Systems in Pharma Manufacturing. *Pharmaceutical Technology*, 40(6):42–44, 2016.

[5] Ben Madsen, Jeff Hurd, Chris Brau, and Nephi Jones. Simplify Upstream Process Development and Scale-Up: Single-Use 5:1 Turndown-Ratio Bioreactor Technology. *BioProcess International*, 15(11), 2017.

[6] Nick Hutchinson, Christian Manzke, and Miriam Monge. Could Commercial Manufacturing with Single-Use Technologies Increase Agility? *Pharmaceutical Processing*, 6(28):6–9, 2018.

[7] Andrew Sinclair and Miriam Monge. Concept Facility Based on Single-Use Systems, Part 2. *BioProcess International*, 3(9):51–55, 2005.

[8] Trinh Weitbrecht, Kallmeyer, Georg, Klotz, Udo, Posset, Tobias, and Mang, André. Einsatz von Disposables in der Pharma-Parenteralia-Produktion. *Pharmind*, 75(11):1810–1823, 2013.

[9] Feliza Mirasol. Industry Adoption of Single-Use Systems Remains Low. *BioPharm International*, 31(2):33–35, 2018.

[10] E. Isberg. The Overlooked Benefits of Single-Use Technology: Improved Quality and Increased Patient Safety. *BioPharm Int.*, pages 9–10, 2013.

[11] V. Ambrogi, C. Carfagna, P. Cerruti, and V. Marturano. Additives in Polymers. In *Modification of Polymer Properties*, pages 87–108. Elsevier, 2017.

[12] A. Michael Ruberto. Polymers and Additives Used in Fabrication of Disposable Bioprocess Equipment. *BioProcess International*, 2010.

[13] Hal F. Brinson and L. Catherine Brinson. *Polymer Engineering Science and Viscoelasticity*. Springer US, Boston, MA, 2015.

[14] Jiri E. Kresta. *Polymer Additives.* Springer Verlag, 2011. OCLC: 968635622.

[15] D. W. van Krevelen, Klaas te Nijenhuis, TotalBoox, and TBX. *Properties of Polymers.* Elsevier Science, 2009. OCLC: 969016936.

[16] J. Murphy. Additives for Plastics Handbook. *Elsevier Science*, 2001. OCLC: 969014030.

[17] Myer Kutz. Applied plastics engineering handbook: processing and materials, 2016. OCLC: 965367732.

[18] J Stepek and H Daoust. *Additives for Plastics.* Springer New York, New York, 2012. OCLC: 958524498.

[19] C. E. Wilkes, J. W. Summers, C. A. Daniels, and Mark T. Berard, editors. *PVC handbook*. Hanser, Munich ; Cincinnati, 2005.

[20] Michael Schiller. PVC additives: performance, chemistry, developments, and sustainability. *Hanser*, 2015. OCLC: ocn880755830.

[21] George Wypych, TotalBoox, and TBX. *Handbook of Plasticizers.* Elsevier Science, 2013. OCLC: 969091890.

[22] Handbook of plastic films. *Rapra*, 2003. OCLC: ocm51993517.

[23] Hans Zweifel, Ralph-Dieter Maier, and Michael Schiller, editors. *Plastics additives handbook*. Hanser, München, 6th ed edition, 2009. OCLC: 423775370.

[24] Hans Zweifel. *Stabilization of Polymeric Materials*. Springer Berlin Heidelberg, Berlin, Heidelberg, 1998. OCLC: 851371263.

[25] Kenneth C. Waterman, Roger C. Adami, and Jin Yang Hong. Impurities in drug products. In *Separation Science and Technology*, volume 5, pages 75–88. Elsevier, 2004.

[26] Peter Eyerer, editor. *Polymer Engineering: Technologien und Praxis ; mit 155 Tabellen*. VDI. Springer, Berlin, 2008. OCLC: 246623993.

[27] Jan Bart. *Additives in Polymers Industrial Analysis and Applications*. John Wiley & Sons, New York, NY, 2005. OCLC: 904828592.

[28] C. L. M. Stults, J. M. Ansell, A. J. Shaw, and L. M. Nagao. Evaluation of Extractables in Processed and Unprocessed Polymer Materials Used for Pharmaceutical Applications. *Pharm. Sci. Tech.*, 16(1):150–164, 2015.

[29] Dennis Jenke. Organic Polymer Additives: Identification and Quantification. *Encyclopedia of Chromatography*, 2009.

[30] International Conference of Harmonisation (ICH). M7 Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk. *Guidance for Industry*, 2015.

[31] Food and Drug Administration. Container Closure Systems for Packaging Human Drugs and Biologics, 2002.

[32] D. Liu, Y. Nashed-Samuel, P. V. Bondarenko, D. N. Brems, and D. Ren. Interactions between Therapeutic Proteins and Acrylic Acid Leachable. *PDA Journal of Pharmaceutical Science and Technology*, 66(1):12–19, 2012.

[33] T. Feinberg. Extractables and Leachables: Best Practices to Ensure Patient Safety. *BioPharm International*, 26(5), 2013.

[34] June M. McKoy, Robin E. Stonecash, Denis Cournoyer, Jerome Rossert, Allen R. Nissenson, Dennis W. Raisch, Nicole Casadevall, and Charles L. Bennett. Epoetin-associated pure red cell aplasia: past, present, and future considerations. *Transfusion*, 48(8):1754–1762, 2008.

[35] Denise Bohrer. *Sources of contamination in medicinal products and medical devices*. 2012. OCLC: 1042128104.

[36] C. Pan, F. Harmon, K. Toscano, F. Liu, and R. Vivilecchia. Strategy for identification of leachables in packaged pharmaceutical liquid formulations. *Journal of Pharmaceutical and Biomedical Analysis*, 46(3):520–527, 2008.

[37] I. Markovic. Evaluation of safety and quality impact of extractable and leachable substances in therapeutic biologic protein products: a risk-based perspective. *Expert Opin. Drug Saf.*, 6(5):487–491, 2007.

[38] Y. Gao and N. Allison. Extractables and leachables issues with the application of single use technology in the biopharmaceutical industry: Extractables and Leachables in Disposable Manufacture. *Journal of Chemical Technology & Biotechnology*, 91(2):289–295, 2016.

[39] D. Norwood, A. T. Granger, and D. M. Paskiet. Extractables and Leachables in Drugs and Packaging. *Encyclopedia Pharm. Sci. Technol.*, 2013.

[40] D. Bestwick and R. Colton. Extractables and Leachables from Single-Use Disposables. *Bioprocess Int.*, 2009.

[41] D. Jenke. Extractable/Leachable Substances from Plastic Materials Used as Pharmaceutical Product Containers/Devices. *J. Pharm. Sci. Technol.*, 56(6):332–371, 2002.

[42] European Medicines Agency. Guideline on the Limits of Genotoxic Impurities, 2006.

[43] Food and Drug Administration. Genotoxic and Carcinogenic Impurities in Drug Substances and Products. *Guidance for Industry*, 2008.

[44] U.S. Pharmacopeia. Draft: Characterization of Polymeric Components and Systems Used to Manufacture Pharmaceutical and Biopharmaceutical Drug Products. *USP 1665*, 2016.

[45] U.S. Pharmacopeia. Draft: Polymeric Components and Systems Used to Manufacture Pharmaceutical and Biopharmaceutical Drug Products. *USP 665*, 2016.

[46] Food and Drug Administration. Section 211.65 - Equipment construction. *FDA US 21*, 2010.

[47] D. J. Ball. Leachables and extractables handbook safety evaluation, qualification, and best practices applied to inhalation drug products. *John Wiley & Sons*, 2012.

Chapter 2

Evaluation of Stir-Bar Sorptive Extraction coupled with TD-GC-MS

Parts of the following chapter are published in the Journal of Pharmaceutical and Biomedical Analysis, titled "Evaluation of stir-bar sorptive extraction coupled with thermal desorption GC-MS for the detection of leachables from polymer single use systems to drugs" [1]

Stir-bar sorptive extraction (SBSE) in combination with thermal desorption and gas chromatography-mass spectrometry (TD-GC-MS) is widely accepted as the gold–standard analysis method for trace amounts of organic substances, including leachables in aqueous matrices. Meanwhile, as far as pharmaceutical quality control in protein-based parenteral drugs is concerned, the use of SBSE analysis remains unexplored. Previous studies reported a strong influence of the matrix on the method's recovery. The scope of the present work was to fill in the unexplored territory in a fourfold manner 1) by quantifying the effects that various matrices commonly found in pharmaceutical processing have on the recovery, 2) by comparing between different coating materials for stir bar (namely between polydimethylsiloxane (PDMS) material and ethylene-glycol (EG)-PDMS), 3) by determining the concentration behavior of SBSE in alcoholic solutions compared to the direct injection and 4) by proposing among possible optimizations a preparation step for stir-bar to mitigate inhibitory effects. The current study shows no inhibition of SBSE by protein matrices ($p > 0.15$). Further the influence of various drug matrices on the recovery of leachables with a log $K_{O/W} \geq$ 3.6 is negligible (-3.9 to 3.8%). In contrast, the inhibition effect caused by an alkaline media led to a recovery decrease of -42.9%. For leachables with a log $K_{O/W} < 3.6$, the relative recovery in the presence of various proteins ranged from -72.8% to 15.6%, depending on the excipients of the drug product and not on the protein itself. The highest loss in sensitivity was observed when the excipient benzyl alcohol was present in the drug. Nonetheless, the limit of detection for the tested leachables in the inhibitory matrices was still below 3 μg/L (ppb), due to the concentration behavior of SBSE. Additionally, SBSE was observed to be quantitatively reliable in all tested drug matrices for concentrations from 0.005 to 0.1 mg/mL ($r^2 > 0.992$). On average, the conventional

PDMS coating resulted in a 28-fold higher signal-to-noise ratio compared to EG-PDMS. Moreover, the PDMS coated stir-bar reached better reconcentrate in inhibitory alcoholic solutions. Furthermore, a broader range of leachables was detectable with the PDSM coating. Preceding stir-bar preparation consisting of a simple soaking step improved the enrichment by 14%, effectively lowering the limit of detection.

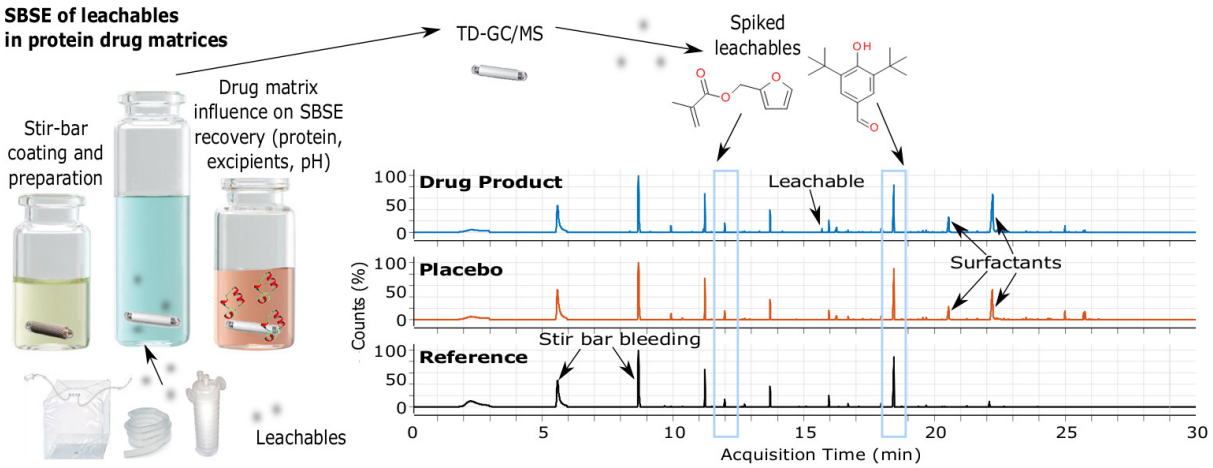


Figure 2.1: Graphical abstract - Evaluation of the performance of the stir-bar sorptive extraction (SBSE) technique in combination with TD-GC-MS analytic for the detection of leachables in drug products. Determination of the influence by the drug matrix, stir-bar coating and extraction procedure parameters on the SBSE recovery.

2.1 Introduction

Single-use systems (SUSs) are increasingly preferred in pharmaceutical production lines [2]. SUSs are polymers that contain certain low binding chemical substances that purposively have been added (e.g. antioxidants and plasticizers) in order to achieve desired material properties (preservation and flexibility), or unwontedly present such as production impurities. These substances are not incorporated in the polymer structure, are usually of low or medium molecular weight, and bear high potential to migrate into the drug product as leachables [3, 4]. Since there exist leachables, that have been classified as genotoxic and carcinogenic, and since international safety regulations have set an acceptable lifetime intake for such compounds in a daily dose as 1.5 μg [5], detection and monitoring is paramount for safe and responsible manufacturing of pharmaceuticals. Contamination monitoring requires measurement techniques that provide sufficient sensitivity for detection of trace amounts on the order of parts per billion (ppb).

Dispersive liquid-liquid extraction was routinely used for monitoring organic substances in aqueous samples [6, 7, 8, 9]. Among others, the main drawback of this technique is the

low substance recovery leading to poor sensitivity. Alternative extraction techniques are the solvent free variants, the well-established stir-bar sorptive extraction (SBSE). SBSE was introduced as sample enrichment technique for leachables in aqueous matrices [10], allowing direct extraction of solutes from polar aqueous matrices [8, 9, 11]. The targeted analyte is absorbed on the coating material, while the phenomenon is governed by the partition coefficient of the substance between the coating and the matrix. After the thermal desorption step, desorbed analytes are detected using gas chromatography mass spectrometry (GC-MS).

Due to its excellent sensitivity, efficiency and availability, SBSE has a wide range of applications. Previous studies have demonstrated the use of PDMS-coated devices for the extraction of organic contaminants from various complex matrices such as beverages [12], oily food components [13, 14], fatty animal tissue [15, 16], digestive fluids and plasma [17, 18]. The sensitivity of SBSE depends on the partition coefficient, i.e., the relation between the hydrophobicity of the PDMS coating and the hydrophilicity of the aqueous matrix, and it might therefore be dependent on the composition of the matrix. Nonetheless, previous literature has reported excellent recovery for a wide range of complex and challenging matrices [13]. Large proteins in biofluids have formerly been reported to inhibit substance enrichment by fouling the absorption phase during extraction [19]. However, it remains unclear if recovery by SBSE is impaired by proteins in parenteral drug products.

In this study, we evaluate the applicability of SBSE for the detection of trace amounts of leachables in pharmaceutical manufacturing. We examine the optimal use of this technique for protein-based drug matrices, including diverse excipients and pH-values, the effect of the stir-bar coating material and evaluated the extraction and concentration behavior. Moreover, we investigate the conditioning of the stir-bar to mitigate the inhibitory effects of the drug matrix.

2.2 Materials and Methods

2.2.1 Experimental Set-up

SBSE analysis was performed using a 10 mm long glass-encapsulated magnetic stir-bar coated with 22 μg of PDMS (TwisterTM, Gerstel GmbH, Mülheim an der Ruhr, Germany). The PDMS-layer is 0.5 mm thick, which corresponds to a volume of 24 μL. The analytes absorbed on stir bar coating material were released to the system by using a thermal desorption system (TDS 3, Gerstel GmbH) equipped with a multi-purpose autosampler (TDS A2, Gerstel GmbH). In accordance with the relevant manufacturer guideline, the stir-bars were used for a maximum of 50 applications. Furthermore, it was ensured that the number of times the stir-bar has been used before is comparable for each stir-bar in a set of experiments. The system was coupled with a gas chromatography mass spectrometer (7890B GC-System, 5977A MSD, Agilent Technologies, Santa Clara, US) equipped with

a 30 m column (DB-1 MS, Agilent Technologies) packed with 0.25 μm of PDMS coating. The absorption of analytes by the PDMS is a thermodynamically driven process to reach an equilibrium state of the analyte concentrations in water (c_W) and in the PDMS-phase (c_{PDMS}), corresponding to the analyte's polarity [20]:

$$c_{PDMS} = K_{PDMS/W} * c_W \tag{2.1}$$

The absorption is driven by the analyte's partition coefficient $K_{PDMS/W}$. The mass balance for the initial analyte mass in the sample (m_{sample}) comprises the mass in the PDMS (m_{PDMS}) and in water (m_W):

$$m_{sample} = m_{PDMS} + m_W. \tag{2.2}$$

Combining (2.1) and (2.1) yields

$$K_{PDMS/W} = \frac{c_{PDMS}}{c_W} = \frac{m_{PDMS}}{m_W} * \frac{V_W}{V_{PDMS}}. \tag{2.3}$$

Thus, the recovery of a substance by SBSE, defined as the extraction efficiency of the stir-bar's coating, depends on the analyte's partition coefficient $K_{PDMS/W}$ and the phase ratio between the aqueous sample and the PDMS coating (24 μL). Since the process of analyte partitioning from water into the PDMS coating is comparable to the partitioning from water into octanol the partition coefficient $K_{PDMS/W}$ is proportional to the octanol/water distribution coefficient $K_{O/W}$. Hence, the theoretical recovery can be described as

$$R[\%] = \frac{m_{PDMS}}{m_{sample}} = 100/(\frac{V_{sample}}{V_{PDMS} * K_{O/W}} + 1) \tag{2.4}$$

Therefore, the extraction of substances by the PDMS coating layer from aqueous samples of a volume of 5 mL is most effective (theoretical of recovery 95%) for substances with partitioning coefficients of $\log K_{O/W}$ greater than 3.6 [10]. In order to cover a broad range of theoretical recoveries (high and low), two analytes as spiking standards were chosen, namely: 3,5-di-tert-butyl-4-hydroxybenzaldehyde (BHT-aldehyde) with a $\log K_{O/W}$-value of 4.2 (theoretical recovery of 98.7%) and tetrahydrofurfuryl-methacrylate (THF-methacrylate) with a $\log K_{O/W}$-value of 1.8 (theoretical recovery of 23.24%). Both substances can be found in common drug manufacturing processes as leachables, which are a plastic adhesive and an antioxidant, respectively (Table 2.1) [21, 22].

2.2.2 Chemicals and Materials

All spiking standards (see Tab. 2.1) (> 97%) were purchased from Sigma Aldrich (Steinheim am Albuch, Germany) and prepared in absolute ethanol (99.5%, Merck, Darmstadt, Germany). For dilution purposes Water for Injection (WfI) was prepared on a Milli-Q Advantage A10 system (Merck, Darmstadt, Germany). All tested drug products were obtained from Roche Diagnostic GmBH (Mannheim, Germany). The tested excipient benzyl alcohol (99%) and tritrisol buffer were purchased from Merck (Darmstadt, Germany). Further, the three different surfactants were obtained from BASF (Ludwigshafen, Germany) and isopropyl alcohol from VWR (Darmstadt, Germany) with a GC purity of 99.5%.

Table 2.1: Spiking standards used in the different experiments, along with the theoretical recovery based on the log $K_{O/W}$ and the phase ratio (see equation (2.4)). These substances can be found as potential leachables.

Experiment	**Spiking Standard [CAS No.]**	**log $K_{O/W}$**	**Theoretical recovery [%]**	**Average mass [Da]**	**Leachable characterization**
Applied in all experiments	Tetrahydrofurfuryl methacrylate [2455-24-5]	1.80	23.2	170.2	Adhesive
Applied in all experiments	3,5-Di-tert-butyl-4-hydroxybenzaldehyde [1620-98-0]	4.20	98.7	234.3	Antioxidant; BHT breakdown product
Effects of drug matrices; Stir-bar coating comparison	4-ethylphenol [123-07-9]	2.55	63.0	122.1	Antioxidant 2,6-di-tert-butyl-4-ethylphenol breakdown product
Stir-bar coating comparison	Tris(2-butoxyethyl) phosphate [78-51-3]	3.0	82.8	398.5	Flame retardant
Stir-bar coating comparison	Tetracosane [646-31-1]	13.12	100	338.6	Lubricant
Optimization of SBSE	Di-pentyl phthalate [131-18-0]	5.62	99.99	306.4	Plastizicer

2.2.3 Sample Preparation, Extraction and Analytical Methods

The spiking standards were dissolved in ethanol at a concentration of 0.1 g/L. This solution was used to prepare a stock solution by adding WfI, resulting in a final concentration of 0.05 mg/L. Two milliliters of this stock solution were mixed with 1 mL matrix sample and further diluted with 2 mL WfI in a 20 mL glass vial. Therefore, 4 mL of the 5 mL are WfI. The final solution contains 0.1 μg of each spiking substance, as well as 1 mL matrix sample, leading to a spiking to sample ratio of 0.1 mg/L (i.e. 0.1 ppm contamination). The high dilution with approximately 4 mL WfI was applied in order to reduce inhibitory matrix effects [23]. Furthermore, linearity solutions were prepared from the same stock solution of standards described above (concentration 0.1 g/L) diluting with WfI to 0.001, 0.005, 0.01, 0.05 and 0.1 mg/L for each spiking substance.

For the handling of the stir-bar, like depicted in Figure 2.2, the stir-bar was first exposed to the solution for 1 h and stirred at 800 rpm at a temperature of 19°C. Subsequently, to the extraction step the stir-bar was rinsed with WfI, dried and placed in the sample holder of the TDS for GC-MS analysis. The thermal desorption comprises the following four steps: 1) On the TDS the splitless mode was selected and the stir-bar was rapidly heated from 20°C up to 300°C at a rate of 60°C/min. The final temperature was maintained for 10 min. 2) The desorbed substances were transferred by a constant helium-flow at a transfer temperature of 320°C to the cold injection system (CIS). 3) The re-cryofocusing occurs in a programmed temperature vaporizing (PTV) injector with a Tenax containing liner at 0°C. 4) Finally, the CIS temperature was ramped up to 300°C at a rate of 8°C/s and was kept at the final temperature for 20 min. A solvent vent with a split vent purge flow of 40 mL/min was selected for the transfer to the column. The initial separation temperature in the column was 40°C. After 2 min the temperature was ramped up to 300°C at a rate of 10°C/min under a constant carrier gas flow rate of 1.0 mL/min and held for another 2 min at the final temperature. The mass spectrometric detection was performed in scan mode for m/z values between 29 and 650 with the electron ionization (EI) energy set to 70 eV. The MS transfer line and ion source temperatures were 300°C and 230°C, respectively. To evaluate the effectiveness of SBSE in pharmaceutical matrices, the present study comprises three sets of experiments. The first set investigates the impact of diverse parenteral drug products on the SBSE enrichment. The second set contains experiments performed with a recently developed copolymer device [24] that was compared to the established PDMS-stir-bar regarding its sorptive properties and sensitivity. The last set contains experiments performed for optimization and with a simple and cost-effective method to mitigate inhibitory effects for the detection of a broad range of leachable classes.

2.2.4 Effects of Drug Matrices on SBSE Recovery

Drug formulations are complex matrices and contain usually proteins as the active ingredient, along with a system of inactive ingredients, the excipients, which are preservatives, surfactants, tonicity modifiers, pH-buffers, stabilizers and bulking agents. In order to in-

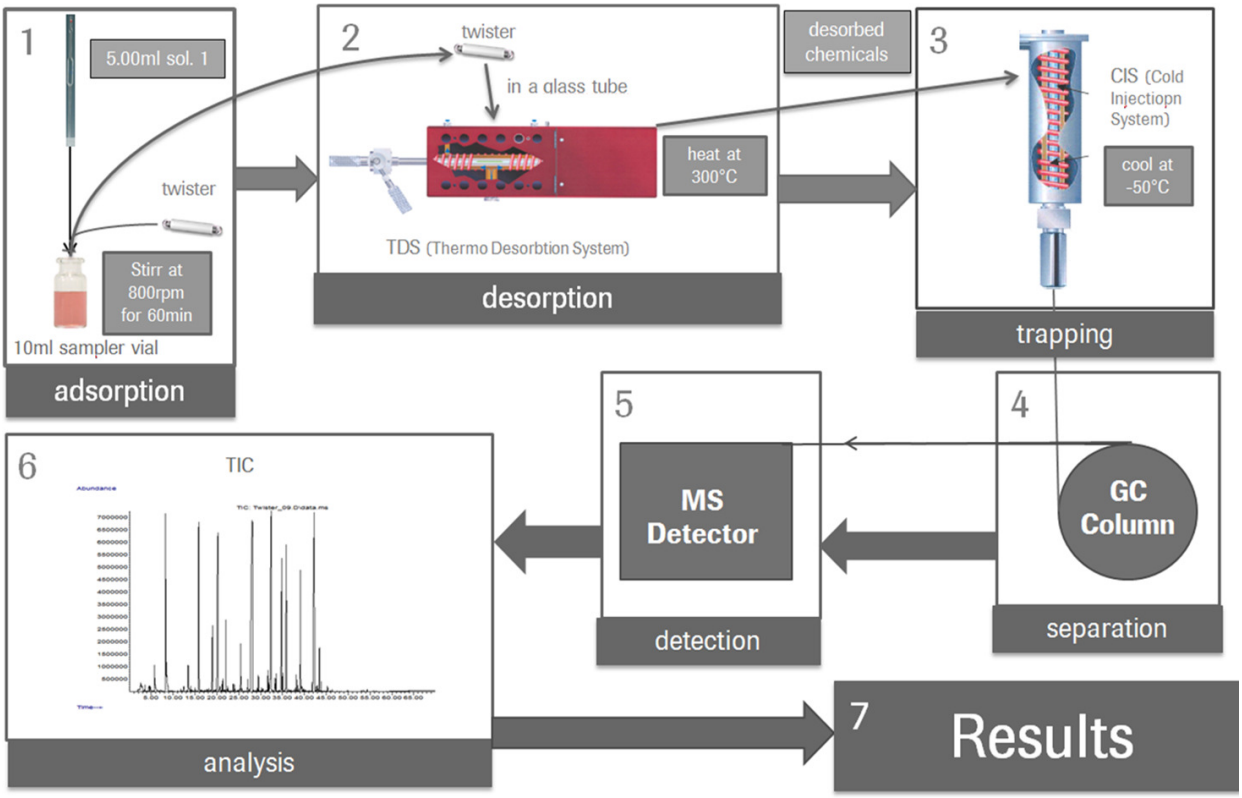


Figure 2.2: Scheme of the stir-bar handling in combination with TD-GC-MS analytic, including adsorption, thermal desorption, cold injection trapping and subsequent gas chromatography and mass spectrometry analysis.

vestigate the effect of components of the complex matrices on the PDMS-coated stir-bar sportive properties, four protein drugs (D: 1-4) along with four systems of their placebos (P: 1-4) (formulations that contain excipients without the active ingredients) and four corresponding systems of single excipients (E: 1-4) (benzyl alcohol for product 1; polysorbate 80 for product 2; poloxamer 188 for product 3 and polysorbate 20 for product 4) were examined. Table 2.2 lists the tested systems. For the four parenteral drugs the active ingredient varied considerably in protein molar weight (40, 140, 145 and 150 kDa, for the drug product 1, 2, 3, 4 respectively) and in concentration (0.1-30 mg). The four matrix formulations, which were chosen to cover a broad range of formulations, were compared to the corresponding placebo drugs in order to comparatively study the effect of the active ingredients. To provide further insights, single excipients were compared to the corresponding placebo formulations in order to show the impact of a single excipient on the sorptive properties of the PDMS stir-bar. Blank measurements were performed using a conditioned stir-bar subjected to WfI only. Additionally, reference measurements were performed where the stir-bars were subjected to spiked WfI, i.e., WfI containing controlled amounts of simulated impurities. Relative recovery was defined as the change in response (peak area) of the spiking substance in the drug product matrices compared to the response of the

same spiking substance in pure WfI using the same extraction and quantification method. The effect of proteins on SBSE recovery was statistically assessed using Student's T-test. Dependence between SBSE recovery, sensitivity and protein molar weight or concentration was analyzed using Pearson's correlation coefficient. Statistical significance was assessed using F-statistics, and p-values below 0.05 were considered to be significant. To determine the absolute recovery of the analytical instrument direct injection was conducted by injecting the spiking substances into a glass wool prepared thermal desorption tube.

To study the effects of sample pH on the SBSE recovery, measurements of two drugs (D: A-B) with extreme pH values were compared to a spiked water sample (pH = 7) (Table 2.2). The two drug products had pH values of 3.5 and 11.0, respectively. This covers the range for all marketable acidic and alkaline injectables. For further verification of the impact of an acidic and alkaline pH range without interacting excipient or drug molecules, a phosphate buffer, a Tris buffer and an acetic acid buffer were also tested.

2.2.5 Quantitative Measurement and Limit of Detection

To obtain quantitative measurements, the linear range of the studied SBSE based analysis was assessed by analyzing WfI, drug product 1, 2 and B (see Table 2.2) (n=4) containing spiking solutions of THF-methacrylate and BHT-aldehyde at concentrations of 0.001, 0.005, 0.01, 0.05 and 0.1 μg/mL. Linear regression analysis was performed, and the correlation coefficient (r^2) as well as the precision (root-mean square deviation, RSD) were estimated using Masshunter Quantitative Analysis (Gerstel GmbH, Mülheim an der Ruhr, Germany). The limits of detection (LOD) and quantification (LOQ) were calculated as the minimal amount of analytes that results in a signal-to-noise-ratio (SNR) of 3 or 10, respectively. Here, the SNR is defined as the peak height of the spiking substance and the baseline noise adjacent to the peak including a range of 0.05 min before and after the peak. The noise value for each noise range is computed as the standard deviation of the baseline over the selected noise region. The SNRs were determined at four concentrations, and the results were linearly interpolated. Confidence intervals were calculated using the fit residuals.

2.2.6 Different Stir-Bar Coatings

Recently, a new two-phase stir-bar coating with 5% ethylene glycol (EG) and 32 μL PDMS (EG-PDMS, Gerstel GmbH) was proposed. Due to the polar nature of EG and its siloxane base, the extraction capability for both, polar and non-polar substances, is claimed [20, 21, 22]. Additional substances spanning log $K_{O/W}$ from 1.80 to 13.12 and thus differences in polarity were used for our evaluation (Table 2.1). These substances, which can be found as leachables, were 4-ethylphenol (log $K_{O/W} = 2.55$), tris(2-butoxyethyl) phosphate (log $K_{O/W} = 3.0$), tetracosane (log $K_{O/W} = 13.12$), THF-methacrylate and BHT-aldehyde again. The main application of EG-PDMS stir-bars is the absorption of phenolic substances, like 4-ethylphenol [12, 25, 26]. The recovery and SNR were determined for each substance and stir-bar coating as described above. Desorption- and analysis parameters

Table 2.2: Drug products and placebo matrices used for the determination of the matrix influence on the SBSE-recovery.

Matrix	**Protein size** $[kg/mol]$	**Special characteristic**	**pH-value**	**Surfactant**
Drug product 1	~ 40	High content of benzyl alcohol ($\sim 1.0\%$)	6.0	Polysorbate 80
Placebo product 1	-	High content of benzyl alcohol	6.0	Polysorbate 80
Excipient 1	-	Benzyl alcohol only	-	-
Drug product 2	~ 140	High content of sodium chloride ($\sim 1.0\%$) and polysorbate 80 ($\sim 0.07\%$)	6.5	Polysorbate 80
Placebo product 2	-	High content of sodium chloride and polysorbate 80	6.5	Polysorbate 80
Excipient 2	-	Polysorbate 80 only	-	Polysorbate 80
Drug product 3	~ 145	L-Histidin content	6.0	Poloxamer 188
Placebo product 3	-	L-Histidin content	6.0	Poloxamer 188
Excipient 3	-	Poloxamer 188 only	-	Poloxamer 188
Drug product 4	~ 150	High concentration of protein ($\sim 3\%$) and trehalose	5.5	Polysorbate 20
Placebo product 4	-	High content of trehalose	5.5	Polysorbate 20
Excipient 4	-	Polysorbate 20 only	-	Polysorbate 20
Drug product A	-	High content of salt; no protein	3.5	-
Acetic acid buffer	-	-	4.75	-
Phosphate buffer	-	-	6.0	-
Tris buffer	-	-	8.5	-
Drug product B	~ 4.5	Polypeptide	10.8	-

had to be adjusted for the EG-PDMS stir-bar due to the heat-sensitivity of the EG coating (maximum temperature 220°C).

2.2.7 Sample Preparation for SBSE in Alcoholic Solutions

To identify possible leachables in advance, extractable-studies are conducted under harsh conditions to simulate worst-case scenarios. Regarding the USP recommendation for the design of extractable-studies by the Draft <1663> [27], binary mixtures of miscible solvents, such as alcohol/water mixtures have been utilized to simulate drug products with a high polysorbate content. As SBSE is perfectly suited for aqueous solutions [11], the extraction and concentration behaviour in solutions containing additional alcohol, like isopropanol (IPA), has to be evaluated. Therefore, SBSE was tested in an IPA/WfI (ratio 50/50) solution, spiked with THF-methacrylate and BHT-aldehyde, and compared to the recovery observed in pure WfI. Further, the concentration behavior was evaluated by comparing the technique to the direct injection of the spiked IPA/WfI sample into a TD-GC-MS system. For SBSE, 1 mL of the spiked sample was diluted with 4 mL WfI and stirred with a PDMS coated stir-bar for 1 h prior to analysis. The direct injection was conducted by injecting a sample volume of 5 μL into a glass wool prepared thermal desorption tube. The two measuring methods only differed in a lower TDS rate of 20 °C/min as well as a lower required CIS hold time of 10 min for the direct injection procedure.

2.2.8 SBSE Optimization

To mitigate inhibitory factors and to optimize SBSE sensitivity in drug products, we tested different set-ups for enhancing the extraction procedure plus purposed an additional preparation step of the stir-bar. Possible improvements were evaluated in form of the optimal level of stirring speed and stirring time for the stir-bar, a simultaneous adsorption by two stir-bars with different coatings and last a soaking procedure step of the stir-bar prior to usage:

Stirring Optimum

In order to determine the variables time and speed affecting the SBSE extraction process, the PDMS coated stir-bar was immersed in THF-methacrylate and di-pentyl phthalate spiked WfI. The absorption of the stir-bar was tested at 500, 800, 1000 and 1300 rpm within a stirring duration of 0.5, 1, 2 and 12 h.

Multi Stir-Bar

As each stir-bar coating is differently suited for the absorption of a specific polarity range of substances, an approach to combine the extraction power of both was conducted [22]. While the plain PDMS coated stir-bar is stirring in the solution, the EG/PDMS coated stir-bar is attached on the inner wall of the vial covered by the solution, like in Figure

2.3 depicted. This enables a circulation of the sample around both stir-bars without a steric hindrance of them. After the extraction procedure, both stir-bars were transferred in a single desorption tube (Fig. 2.3) and were at the same time thermally extracted at 220°C. Again, the study was carried out with a THF-methacrylate and di-pentyl phthalate spiked WfI solution. The determined recoveries were compared to the absorption by a single PDMS coated stir-bar.

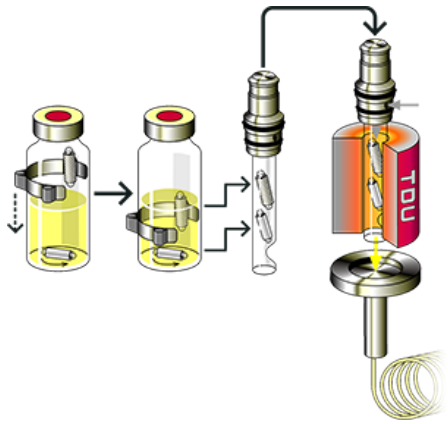


Figure 2.3: Representation of the multi stir-bar handling of a PDMS and an EG/PDMS coated stir-bar [22], including the simultaneously sample extraction and thermal desorption.

Soaking Procedure

Last, the improvement of the recovery by the PDMS stir-bar was tested in terms of a soaking step. However, the stir-bar was stirred in pure WfI for an hour at room temperature to soak the coating before application. The effect of a soaking preparation on SBSE sensitivity was evaluated with the two spiking-substances, THF-methacrylate and BHT-aldehyde, in water and in the matrices that showed the strongest inhibitory effects.

2.3 Results

2.3.1 Effects of Drug Matrices on SBSE

Figure 2.4 a) depicts representative chromatograms of a drug product, placebo and a WfI reference measurement. The reference shows peaks corresponding to the location of the spiking substances and additionally contamination caused by stir-bar bleeding. Additional peaks in the placebo and drug chromatograms correspond to surfactant and trace amounts of leachables. Absolute recovery of 55.7% and 88.6% was observed for THF-methacrylate and BHT-aldehyde, respectively. The recovery from spiked protein drug matrices compared to spiked protein-free placebo matrices and single excipients is displayed in Figure 2.4 b)

for THF-methacrylate and c) for BHT-aldehyde. Exposure to either, the excipients (E), the placebo (P) or drug product (D) caused a mild to decisive impact of SBSE-recovery, depending on the distribution coefficient of the spiking substance (Table 2.3). The influence of the active ingredient on the detection of the spiking substances was very low compared to the placebo effect (4.5% vs. placebo effect). For most of the products the influence of the active ingredient did not even exceed the standard deviation of the measurement variability. Furthermore, no protein influence on the recovery of the spiking substances was observed ($p > 0.15$) regardless of the protein's molar weight or concentration applying Pearson's Correlation Coefficient (protein molar weight: $r^2 < 0.49$; $p > 0.29$; protein concentration: $r^2 < 0.81$; $p > 0.09$).

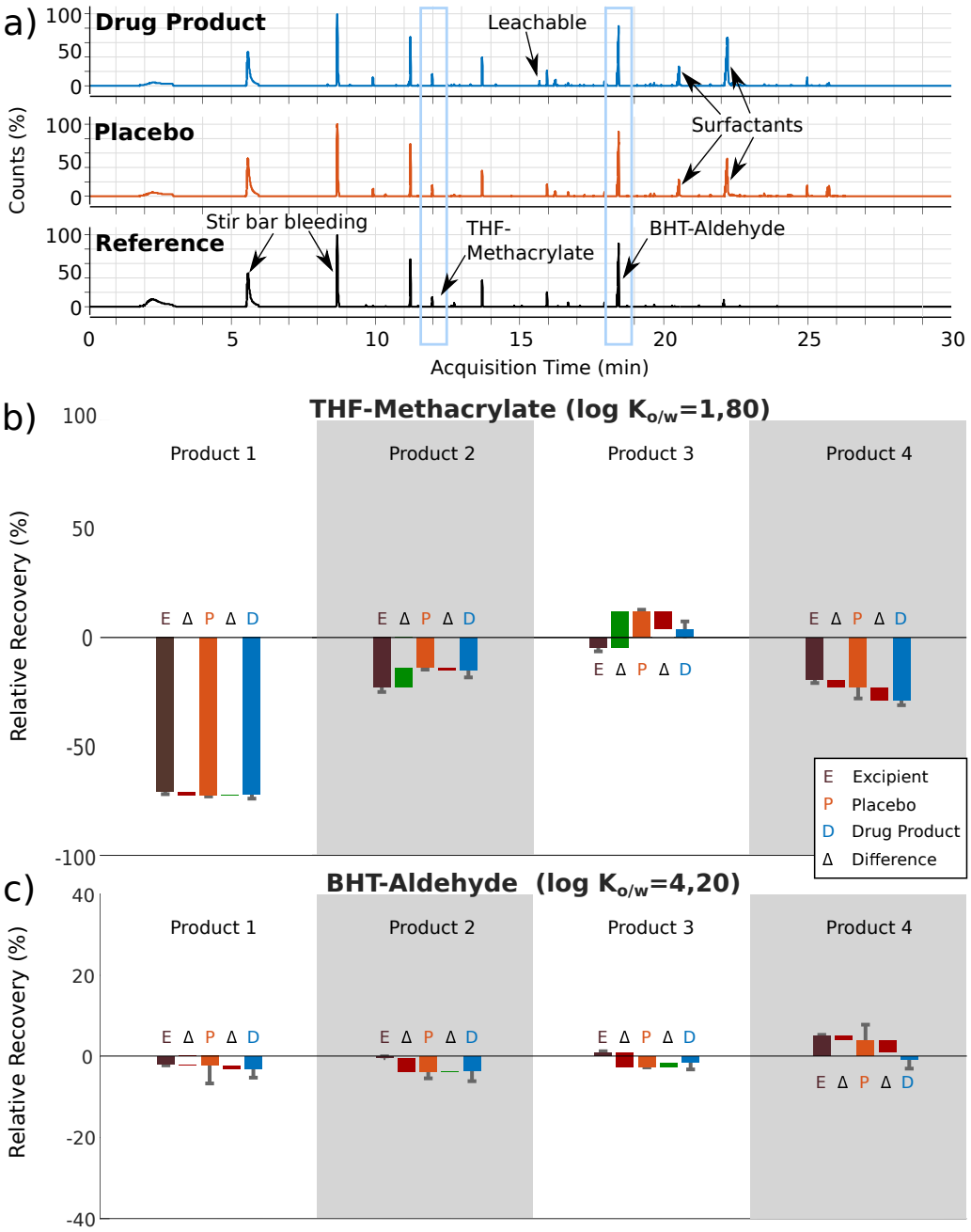


Figure 2.4: a) Example chromatograms of drug product 2, placebo and WfI reference measurements. Peaks corresponding to spiking (12 min: THF-methacrylate; 18.5 min: BHT-aldehyde), stir-bar bleeding, surfactants and leachables are indicated by black arrows. b) Influence of different drug products, their placebos and corresponding single excipients (product 1: benzyl alcohol; product 2: polysorbate 80; product 3: poloxamer 188; product 4: polysorbate 20) on the SBSE sorptive properties of THF-methacrylate and c) of BHT-aldehyde. Differences are shown as an increase/decrease of the mean detected concentration in the matrix using PDMS stir-bar (n=4) compared with the reference PDMS stir-bar (n=4) without matrix contact.

Table 2.3: Relative recovery and quantitative results of SBSE observed for THF-methacrylate and BHT-aldehyde in all tested matrices.

Matrix	Spiking	Relative recovery [%] spiked at 0.1 ppm	System precision [%] RSD (n = 4)	Relative recovery [%] spiked at 0.01 ppm	System precision [%] RSD (n = 4)	Corr. coeff. (r^2) (Origin: Force)	Precision method repeatability [%] RSD (n = 4)	LOD [ppb]	LOQ [ppb]
Wfl	THF-methacrylate	-	-	-	-	0.9970	8.0	0.2	0.7
	BHT-aldehyde	-	-	-	-	0.9978	3.4	0.2	0.6
Product 1	THF-methacrylate	-72.5	1.8	-77.2	1.5	0.9970	2.4	2.8	9.3
	BHT-aldehyde	-3.1	2.2	-7.6	1.4	0.9930	5.0	0.9	3.3
Placebo 1	THF-methacrylate	-72.8	0.5	-	-	-	-	-	-
	BHT-aldehyde	-2.4	4.4	-	-	-	-	-	-
Excipient 1	THF-methacrylate	-71.1	1.1	-	-	-	-	-	-
	BHT-aldehyde	-2.0	0.3	-	-	-	-	-	-
Product 2	THF-methacrylate	-14.9	3.5	-20.7	8.2	0.9989	7.7	2.2	7.3
	BHT-aldehyde	-3.7	2.5	-4.6	12.5	0.9954	7.3	0.6	2.1
Placebo 2	THF-methacrylate	-13.7	1.1	-	-	-	-	-	-
	BHT-aldehyde	-3.9	1.6	-	-	-	-	-	-
Excipient 2	THF-methacrylate	-23.0	2.1	-	-	-	-	-	-
	BHT-aldehyde	-0.3	0.2	-	-	-	-	-	-
Product 3	THF-methacrylate	3.5	3.9	-	-	-	-	-	-
	BHT-aldehyde	-1.5	1.8	-	-	-	-	-	-
Placebo 3	THF-methacrylate	12.1	0.7	-	-	-	-	-	-
	BHT-aldehyde	-2.6	0.2	-	-	-	-	-	-

Matrix	Spiking	Relative recovery [%] spiked at 0.1 ppm	System precision [%] RSD (n = 4)	Relative recovery [%] spiked at 0.01 ppm	System precision [%] RSD (n = 4)	Corr. coeff. (r^2) (Origin: Force)	Precision method repeatability [%] RSD (n = 4)	LOD [ppb]	LOQ [ppb]
Excipient 3	THF-methacrylate	-4.5	1.9	-	-	-	-	-	-
	BHT-aldehyde	0.9	0.3	-	-	-	-	-	-
Product 4	THF-methacrylate	-28.8	2.4	-	-	-	-	-	-
	BHT-aldehyde	-0.8	2.3	-	-	-	-	-	-
Placebo 4	THF-methacrylate	-22.8	5.3	-	-	-	-	-	-
	BHT-aldehyde	3.8	4.2	-	-	-	-	-	-
Excipient 4	THF-methacrylate	-19.3	1.7	-	-	-	-	-	-
	BHT-aldehyde	4.9	0.3	-	-	-	-	-	-
Product A	THF-methacrylate	15.6	8.0	-	-	-	-	-	-
	BHT-aldehyde	0.5	2.0	-	-	-	-	-	-
Acetic acid buffer	THF-methacrylate	-18.8	6.6	-	-	-	-	-	-
	BHT-aldehyde	-0.4	2.2	-	-	-	-	-	-
Phosphate buffer	THF-methacrylate	-0.2	6.4	-	-	-	-	-	-
	BHT-aldehyde	-7.6	6.2	-	-	-	-	-	-
Tris buffer	THF-methacrylate	-23.2	4.1	-	-	-	-	-	-
	BHT-aldehyde	-15.0	28.0	-	-	-	-	-	-
Product B	THF-methacrylate	-32.1	5.2	-23.3	13.0	0.9932	5.6	2.2	7.4
	BHT-aldehyde	-42.9	21.4	-60.9	18.3	0.9920	18.2	0.7	2.4

Placebos exhibited a more profound impact on the sorptive properties of the stir-bar. A major influence in sensitivity was observed for the THF-methacrylate spiking, while there were only minor effects caused by BHT-aldehyde. Recovery of THF-methacrylate was most profoundly impaired by product 1 and the corresponding placebo (-72.8%), which contained the preservative benzyl alcohol (not present in any of the other drug products). These results were further substantiated by the analysis of another substance with a low log $K_{O/W}$-value, the 4-ethylphenol. Here we could prove the negative influence of the benzyl alcohol. Further, the correlation to the distribution coefficient, as the recovery of 4-ethylphenol (-59.6%) was less influenced than the one of THF-methacrylate by benzyl alcohol. Benzyl alcohol could be shown to be the dominant source of extraction inhibition, whereas the other excipients, including the surfactants, exerted mild to moderate reduction of the THF-methacrylate recovery (polysorbate 80: -23.0%; polysorbate 20: -19.3%; poloxamer 188: -4.5%) (Table 2.3).

The impact of the matrix's pH on the SBSE of the three placebos and the two drug products exhibiting the highest and lowest pH values is shown in Figure 2.5. A trend of diminishing SBSE sensitivity was observed for matrices with a pH value substantially different from a neutral pH (Table 2.3). For BHT-aldehyde the alkaline pH value caused strong inhibition compared to an acidic pH value, as shown in Figure 2.5. Substantial loss in reproducibility is also observed in the highly alkaline regime. The only increase in recovery was observed for THF-methacrylate in product A, which is characterized by a high salt content.

2.3.2 Quantitative Measurement and Limit of Detection

To evaluate the applicability of SBSE based analytics in drug matrices, the repeatability, linearity, LOD and LOQ were investigated for three drug products compared to WfI. The calibration plots were linear over the range of 0.005–0.1 μg/mL with correlation coefficients (r^2) between 0.9920 and 0.9989 for both tested spiking substances (Table 2.3). The LODs ranged from 2.2 to 2.8 μg/L and from 0.6 to 0.9 μg/L for THF-methacrylate and BHT-aldehyde, respectively. LOQs reached values between 7.3 and 9.3 μg/L for THF-methacrylate and between 2.1 and 3.3 μg/L for BHT-aldehyde.

2.3.3 Stir-bar Coating

Higher recovery and substantially increased SNR were achieved with the conventional PDMS coating compared to EG/PDMS coating, as shown in Figure 2.6. For the polar substance 4-ethylphenol, moderately higher recovery was achieved for the two phase coated stir-bar, however at reduced values (18.0±3.4 vs 11.7±16.0). The PDMS stir-bar achieved consistently higher recovery and up to 20-fold improvement in SNR in comparison to the EG/PDMS stir-bar for all other tested substances/leachables, including the second polar substance tested with log $K_{O/W}$ = 4.2 (Figure 2.6). The non-polar THF-methacrylate

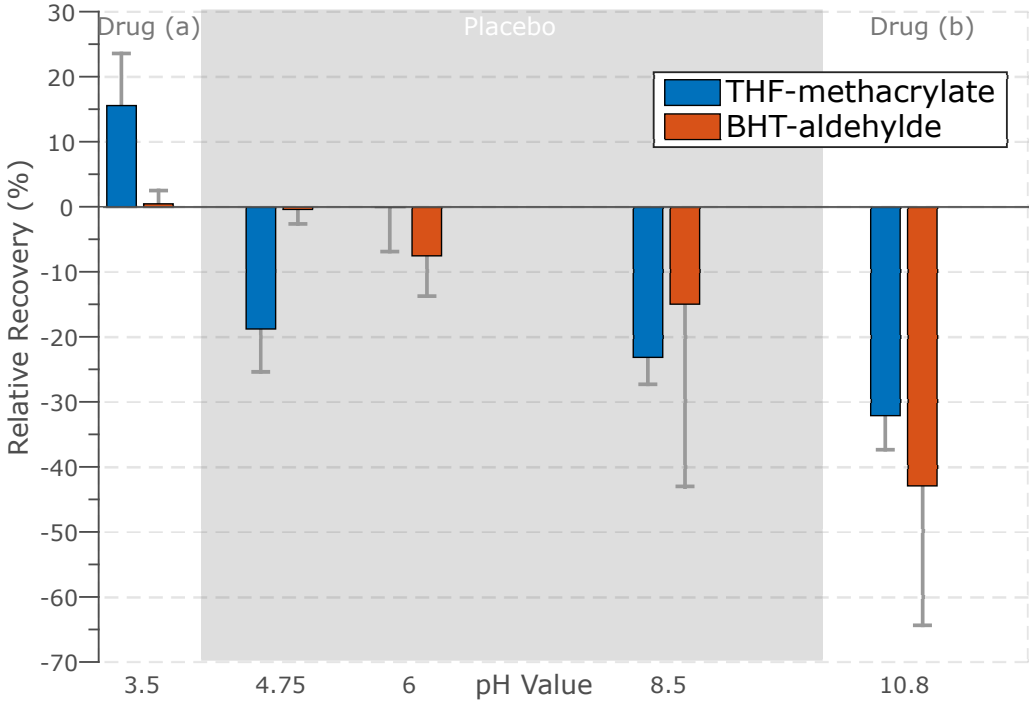


Figure 2.5: Influence of pH on the SBSE sorptive properties for THF-methacrylate and BHT-aldehyde. The effect of pH is shown as the percentage change of recovery in the pH samples (n=4 for acidic and neutral pH samples; n=7 for alkaline samples) versus a pH-neutral water sample (n=4). The horizontal position of the bar pairs represents pH values on the x-axis.

could not be detected with the polar EG/PDMS coated stir-bar, and detection of TBEP was close to the noise level (SNR: 3.7±7.8).

2.3.4 Sample Preparation for SBSE in Alcoholic Solutions

The extraction and concentration behavior of SBSE was tested for an IPA/WfI sample by comparing the recoveries to those observed within pure WfI. Further it was examined, if the method was better suited than the direct injection for IPA/WfI solutions. The negative influence of the alcohol on the uptake of the THF-methacrylate spiking, shown in Table 2.4, is in accordance with the observed data of the drug product 1 matrix with its benzyl alcohol content (see subsection 2.3.1). Nonetheless, SBSE still reached much better recoveries for both spiking substances (Table 2.4) compared to the direct injection without a sample preparation prior to GC-MS analysis.

2.3.5 SBSE Optimization

To achieve reduced LODs by optimizing the extraction procedure, different preparation and handling steps of the stir-bar were tested.

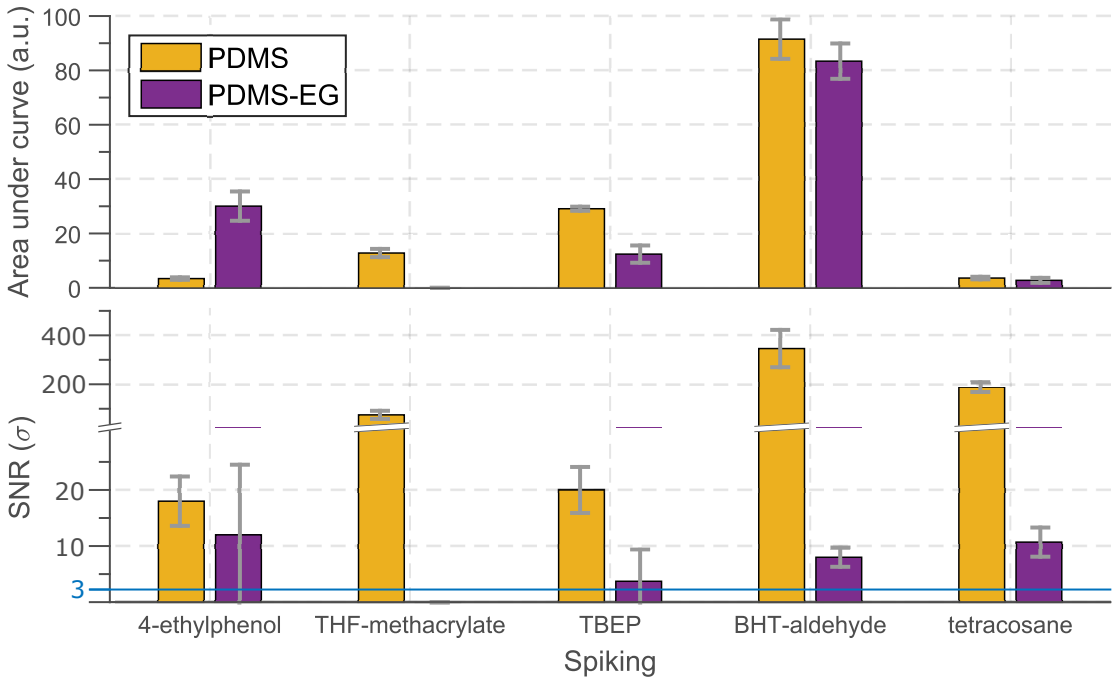


Figure 2.6: Sorptive properties of the PDMS phase compared to the EG-PDMS phase in 0.1 mg/L (ppm) spiked WfI. The detected peak area ratio and the corresponding SNR using noise samples adjacent to the peaks of the five leachables 4-ethylphenol, THF-methacrylate, Tris(2-butoxyethyl) phosphate, BHT-aldehyde and tetracosane (n=4).

Stirring Optimum

An important parameter affecting the extraction efficiency is the extraction time. For the poorly absorbed spiking substance THF-methacrylate an extended extraction time resulted in an increase in recovery (see Figure 2.7). In comparison, the extraction of di-pentyl phthalate spiking substance with an already high recovery of 95% did not impressively increase. Contrary, a sticking to the PDMS-phase of the substance was observed, as artefacts were detected in subsequent analysis of the same stir-bar. The recovery of both spikings during the 12 h test-time was not significantly influenced by the stirring speed of the stir-bar.

Multi Stir-Bar

As already shown in subsection 2.3.3, the PDMS coated stir-bar is better suited for the application of leachable-testing compared to the EG/PDMS coated stir-bar. The simultaneous multi-handling of both differently coated stir-bars and therefore the combination of both optimum range applications was not possible. For both spiking substances, recoveries below 5% were reached compared to the single application of a PDMS coated stir-bar.

Table 2.4: Observed recoveries of the two spiking substances THF-methacrylate and BHT-aldehyde affected by the IPA content. Comparison of the sample preparation step SBSE in combination with GC-MS analytic to the direct injection without any sample preparation for samples with IPA content.

	SBSE WfI solution (area under the curve)	SBSE IPA/WfI 50/50 solution (area under the curve)	Relative recovery - "alcohol" impact	Direct injection IPA/WfI 50/50 solution (area under the curve)	Relative recovery - "direct injection" impact
THF-methacrylate	$20.8 * 10^6$	$1.8 * 10^6$	-91.3%	$0.3 * 10^6$	-81.6%
BHT-aldehyde	$10.8 * 10^7$	$9.4 * 10^6$	-12.7%	$0.04 * 10^6$	-99.6%

Soaking Procedure

The tested one hour soaking in WfI successfully increased the recovery by 14% – 22% for spiking substances that showed inhibited recovery in matrices containing benzyl alcohol or exhibiting a pH value of 8.5. Detailed results are given Table 2.5.

Table 2.5: Effect of soaking preparation on recovery of two spiking substances (THF-methacrylate and BHT-aldehyde) in three matrices (WfI; Benzyl alcohol 5 mg/mL; Tris-buffer pH-value = 8.5). Consistent increase is observed for recovery of THF-methacrylate across all tested matrices. Recovery of BHT-aldehyde was successfully increased in the presence of a Tris buffer, and remained unchanged in the other matrices.

	WfI		Benzyl alcohol matrix		Tris buffer	
	THF-methacrylate	BHT-aldehyde	THF-methacrylate	BHT-aldehyde	THF-methacrylate	BHT-aldehyde
No preparation	-	-	-71.5±1.5	-3.0±2	-11.5±0.5	-22.0±9.0
Soaking	14.5±5.5	1.0±3.0	-66.5±2.5	-2.5±8.5	-7.5±5.5	0.5±2.5

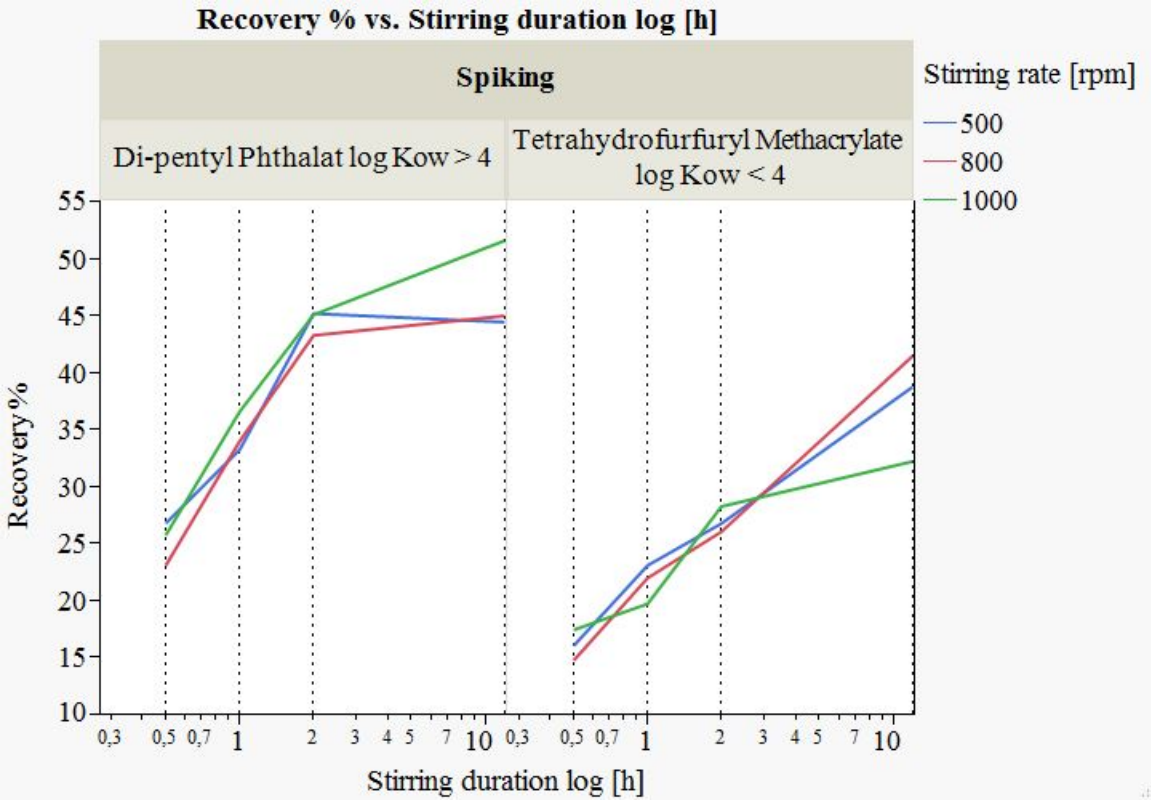


Figure 2.7: SBSE stirring time and duration optimum for dipentyl phthalate and tetrahydrofurfuryl methacrylate.

2.4 Discussion

The present study evaluated the performance of SBSE in pharmaceutical applications. The results suggest that SBSE can be highly valuable for the detection of leachables in drugs with diverse formulations. Optimal sensitivity was obtained using PDSMS stir-bars in the presence of matrices containing salts, stabilizer or proteins. The LOD of the PDMS stir-bar even in the presence of inhibiting benzyl alcohol and alkaline pH values was still in the ppb-range compared to the LOD reached by the direct injection. The influence of the pharmaceutical matrix on the SBSE recovery could be further mitigated by soaking the PDMS stir-bar in water prior to extraction.

Protein inhibition of PDMS extraction devices is a controversially discussed topic. Whereas A. Oomen et al. [28] found no protein inhibition, fouling of the PDMS phase by proteins was described by S. Ulrich et al. [19]. Both tested the influence of protein on PDMS coated fibers. Our results regarding stir-bar extraction indicate that the concentration and the size of the proteins have a negligible impact on the system's sensitivity. The absence of protein fouling in our experiments is most likely caused by the constant motion of the stir-bar, which reduces clogging by large proteins, and suggests good applicability

as a sample preparation for drug matrix analysis. No major effect of the drug matrix was observed on the recovery of BHT-aldehyde. Hence, sufficient uptake of substances with unfavorable log $K_{O/W}$ appears to be achieved by reaching the thermodynamic equilibrium between the drug matrices and the PDMS coating. Additionally, low inter-measurement variability indicates high reproducibility of the partition equilibrium [13]. In contrast, the recovery of THF-methacrylate (log $K_{O/W} < 3.6$) shows some dependence on the product matrix. Benzyl alcohol caused the most significant decrease in recovery. Benzyl alcohol is a strong hydrogen bond donor and can bind to the PDMS absorbent phase, potentially hampering the absorption of substances with a low partitioning coefficient. Additionally, it influences the equilibration time of THF-methacrylate, which was shown to have a prominent effect in a previous study with methanol [29, 30]. For compounds with low log $K_{O/W}$-values, the alcohol increases their solubility in the solution and therefore decreases the partitioning into the PDMS phase. Our further results with 4-ethylphenol, however, indicated comparable inhibition. Surfactants with diverse log $K_{O/W}$ values affect the recovery of analytes in the sample differently by changing the distribution coefficient. A mild to moderate decrease of recovery was observed for high log $K_{O/W}$ polysorbate 80 whereas recovery increased for low log $K_{O/W}$ poloxamer 188 as expected from Equation 1. However, product 2 with polysorbate 80 achieved better recovery for THF-methacrylate than product 4 with polysorbate 20. This effect could be caused by the high salt concentration of product 2, which leads to a salting-out-effect and fosters partitioning into the sampling phase [29, 31]. Further, it was observed that product 3 improved the recovery. This might be caused by deprotonation of THF-methacrylate by the bulking agent L-histidine.

The pH of the drug product is an important factor affecting the dissociation equilibrium in aqueous solutions and therefore the partitioning of leachables into the PDMS phase [12]. Our results confirm that the non-ionic form yields maximum extraction efficiency, as previously shown by Loconto et al. [17]. As the tested spiking substances both are weak organic acids that can be deprotonated, a pH > pKa results in a decrease of the log $K_{O/W}$-value [32]. Therefore, an alkaline pH of 8.5 - 10.8 led to reduced binding of both spiking substances to the absorbent phase of the stir-bar due to conversion to the dissociated form. An acidic pH led to a recovery decrease only for THF-methacrylate due to its chemical structure [33]. The increase of recovery of THF-methacrylate in the very acidic drug product could be caused by the high concentration of the excipient sodium chloride in the drug, which overcompensated for the negative effect of pH by increasing the log $K_{O/W}$.

The use of different polymers in the absorbent phase of the stir-bar has been previously proposed as a possible way to enhance the detection of polar and non-polar leachables [22]. The dual-phase stir-bar EG/PDMS has the polar EG part [34], which enables specific binding by polar hydrogen bond donors by H-bonding [11, 20, 22]. However, due to the heat sensitivity of the polar phase, this stir-bar cannot be exposed to high desorption temperatures. Therefore, a broader detection profile of leachables is reached by the PDMS stir-bar, as most leachables are desorbed from the absorbent phase at higher temperatures [29]. Furthermore, higher phase bleeding of the sensitive EG phase led to a substantial

decrease of the SNR. As a consequence, our results indicate that PDMS is to be preferred over EG-PDMS for optimal sensitivity in a pharmaceutical matrix.

The limits of detection in the tested drug matrices are well below regulatory guidelines. SBSE was shown to be not only suited for leachable-studies with interferring drug matrices but also for extractable-studies on the basis of solvent extracts, by concentrating analytes from a bigger sample volume, which can not be enabled by direct injection [35]. Despite the inhibiting alcohol, SBSE enabled by concentrating of the analytes better recoveries than without any sample preparation. Further, the SBSE technique was able to measure quantitatively despite drug matrix effects. Nonetheless several methods of improving SBSE recovery have been previously explored. As a first possibility to improve the uptake of analytes with a low distribution coefficient, the addition of salt has been proposed [25]. Furthermore, pH adjustment to a neutral value has been proposed for matrices with extreme pH values [12]. However, these steps may not be applicable for complex drug matrices in pharmaceutical manufacturing. As for product A with a salt content of 8 mg/mL, additional salt provides no improvement in recovery for THF-methacrylate. The pH adjustment for product B is precluded in pharmaceutical experiments as it may lead to protein degradation. Furthermore, both preparation steps only modify specific classes of leachables in the sample while hampering the detection of others. For optimization purposes, we tested the influence of the stirring time and the simultaneous application of two stir-bars with different coatings. However, for some substances an increase of stirring lead to a sticking to the coating of the stir-bar, which distorted following measurements by artefacts. Although an application of multi stir-bars probably improves the absorption of analytes, a decrease in recovery of the tested substances was observed, due to the allowed low thermal extraction temperature for the EG/PDMS stir-bar. However, we have successfully introduced a soaking step, which does not induce sensitivity bias across the range of analytes of interest. The method is simple and fast to apply and has to be further investigated in future studies.

2.5 Summary and Conclusion

SBSE is suitable for usage in pharmaceutical matrices due to its high sensitivity over a wide range of chemical classes. No matrix inhibition by large protein drugs and only mild reduction of recovery were observed in the relevant pH range. Limits of detection were found to be well below the applicable thresholds in a worst case scenario analysis.

Bibliography

[1] N. Scherer, K. Marcseková, T. Posset, and G. Winter. Evaluation of stir-bar sorptive extraction coupled with thermal desorption GC–MS for the detection of leachables from polymer single use systems to drugs. *Journal of Pharmaceutical and Biomedical Analysis*, 152:66–73, 2018.

[2] U. Gottschalk. Disposables in Downstream Processing. *Adv. Biochem. Eng. Biotechnol.*, 115:171–183, 2009.

[3] D. Jenke. A general assessment of the physiochemical factors that influence leachables accumulation in pharmaceutical drug products and related solutions. *J. Pharm. Sci. Technol.*, 65(2):166–176, 2011.

[4] D. Norwood, A. T. Granger, and D. M. Paskiet. Extractables and Leachables in Drugs and Packaging. *Encyclopedia Pharm. Sci. Technol.*, 2013.

[5] Food and Drug Administration. Genotoxic and Carcinogenic Impurities in Drug Substances and Products. *Guidance for Industry*, 2008.

[6] M. Rezaee, Y. Yamini, and M. Faraji. Evolution of dispersive liquid–liquid microextraction method. *J. Chromatog. A*, 1217(16):2342–2357, 2010.

[7] M. Rezaee, Y. Assadi, M. Milani Hosseini, E. Aghaee, F. Ahmadi, and S. Berijani. Determination of organic compounds in water using dispersive liquid–liquid microextraction. *J. Chromatogr. A*, 1116(1-2):1–9, 2006.

[8] O. Krueger, G. Christoph, U. Kalbe, and W. Berger. Comparison of stir bar sorptive extraction (SBSE) and liquid-liquid extraction (LLE) for the analysis of polycyclic aromatic hydrocarbons (PAH) in complex aqueous matrices. *Talanta*, 85(3):1428–1434, 2011.

[9] F. Nogueira. Novel sorption-based methodologies for static microextraction analysis: A review on SBSE and related techniques. *Analytica Chimica Acta*, 757:1–10, 2012.

[10] B. Armstrong, A. Senyurt, V. Narayan, X. Wang, L. Alquier, and G. Vas. Stir bar sorptive extraction combined with GC-MS/MS for determination of low level leachable components from implantable medical devices. *J. Pharma. Biomed. Analysis*, 74:162–170, 2013.

[11] E. Baltussen, P. Sandra, F. David, and C. Cramers. Stir bar sorptive extraction (SBSE), a novel extraction technique for aqueous samples: Theory and principles. *J. Microcolumn. Sep.*, 11(10):737–747, 1999.

[12] Q. Zhou, Y. Qian, and M. Qian. Analysis of volatile phenols in alcoholic beverage by ethylene glycol-polydimethylsiloxane based stir bar sorptive extraction and gas chromatography-mass spectrometry. *J. Chromatogr. A*, 1390:22–27, 2015.

[13] A. Jahnke and P. Mayer. Do complex matrices modify the sorptive properties of polydimethylsiloxane (PDMS) for non-polar organic chemicals? *J. Chromatogr. A*, 1217(29):4765–4770, 2010.

[14] M. Kawaguchi, A. Takatsu, Rie Ito, and Hiroyuki Nakazawa. Applications of stir-bar sorptive extraction to food analysis. *Trends Analyt. Chem.*, 45:280–293, 2013.

[15] A. Jahnke, P. Mayer, D. Broman, and M. McLachlan. Possibilities and limitations of equilibrium sampling using polydimethylsiloxane in fish tissue. *Chemosphere*, 77(6):764–770, 2009.

[16] E. A. Pfannkoch, J. R. Stuff, J. A. Whitecavage, J. M. Blevins, K. A. Seely, and J. H. Moran. A High Throughput Method for Measuring Polycyclic Aromatic Hydrocarbons in Seafood Using QuEChERS Extraction and SBSE. *Int. J. Anal. Chem.*, 2015:1–8, 2015.

[17] P. Loconto. Evaluation of Automated Stir Bar Sorptive Extraction-Thermal Desorption-Gas Chromatography Electron Capture Negative Ion Mass Spectrometry for the Analysis of PBDEs and PBBs in Sheep and Human Serum. *J. Chromatogr. Sci.*, 47(8):656–669, 2009.

[18] A. Stopforth, C. J. Grobbelaar, A. M. Crouch, and P. Sandra. Quantification of testosterone and epitestosterone in human urine samples by stir bar sorptive extraction – thermal desorption – gas chromatography/mass spectrometry: Application to HIV-positive urine samples. *J. Sep. Sci.*, 30(2):257–265, 2007.

[19] S. Ulrich, S. Kruggel, H. Weigmann, and C. Hiemke. Fishing for a drug: solid-phase microextraction for the assay of clozapine in human plasma. *J. Chromatogr. B, Biomed. Sci. App.*, 731(2):231–240, 1999.

[20] E. Baltussen, C. Cramers, and P. Sandra. Sorptive sample preparation - a review. *Analyt. Bioanalyt. Chem.*, 373(1-2):3–22, 2002.

[21] M. Nagao, W. P. Beierschmitt, D. J. Ball, A. J. Shaw, and S. Beck. The ELSIE Extractables and Leachables Database. *Pharma. Outsourcing*, 2011.

[22] N. Ochiai, K. Sasamoto, T. Ieda, F. David, and P. Sandra. Multi-stir bar sorptive extraction for analysis of odor compounds in aqueous samples. *J. Chromatogr. A*, 1315:70–79, 2013.

[23] N. Ochiai, K. Sasamoto, M. Takino, S. Yamashita, S. Daishima, A. Heiden, and A. Hoffmann. Simultaneous determination of preservatives in beverages, vinegar, aqueous sauces, and quasi-drug drinks by stir-bar sorptive extraction (SBSE) and thermal desorption GC-MS. *Analyt. Bioanalyt. Chem.*, 373(1-2):56–63, 2002.

[24] B. Sgorbini, C. Cagliero, C. Cordero, E. Liberto, P. Rubiolo, M. R. Ruosi, and C. Bicchi. New medium-to-high polarity twister coatings for liquid and vapour phase sorptive extraction of matrices of vegetable origin. *J. Chromatogr. A*, 1265:39–45, 2012.

[25] J. Cacho, N. Campillo, P. Viñas, and M. Hernández-Córdoba. Stir bar sorptive extraction with EG-Silicone coating for bisphenols determination in personal care products by GC-MS. *J. Pharma. Biomed. Analysis*, 78-79:255–260, 2013.

[26] K. Serrano de la Hoz, R. Salinas, and A Ferrandino. Different coatings for the HS-SBSE grape volatile analysis in model solution: Preliminary results. *Food Chemistry*, 212:814–820, 2016.

[27] U.S. Pharmacopeia. Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems and. 2015.

[28] A. Oomen, P. Mayer, and J. Tolls. Nonequilibrium Solid-Phase Microextraction for Determination of the Freely Dissolved Concentration of Hydrophobic Organic Compounds: Matrix Effects and Limitations. *Analyt. Chem.*, 72(13):2802–2808, 2000.

[29] A. Prieto, O. Basauri, R. Rodil, A. Usobiaga, L.A. Fernandez, N. Etxebarria, and O. Zuloaga. Stir-bar sorptive extraction: A view on method optimisation, novel applications, limitations and potential solutions. *J. Chromatogr. A*, 1217(16):2642–2666, 2010.

[30] B. Kolahgar, A. Hoffmann, and A. Heiden. Application of stir bar sorptive extraction to the determination of polycyclic aromatic hydrocarbons in aqueous samples. *J. Chromatogr. A*, 963(1-2):225–230, 2002.

[31] A. Noubigh, M. Abderrabba, and E. Provost. Salt addition effect on partition coefficient of some phenolic compounds constituents of olive mill wastewater in 1-octanol-water system at 298.15 K. *J. Iranian Chem. Soc.*, 6(1):168–176, 2009.

[32] S. Borrirukwisitsak, H. E. Keenan, and C. Gauchotte-Lindsay. Effects of Salinity, pH and Temperature on the Octanol-Water Partition Coefficient of Bisphenol A. *Int. J. Environ. Sci. Develop.*, pages 460–464, 2012.

[33] J. Y. Barletta, P. C. F. de Lima Gomes, Á. J. dos Santos-Neto, and F. M. Lancas. Development of a new stir bar sorptive extraction coating and its application for the determination of six pesticides in sugarcane juice. *J. Sep. Sci.*, 34(11):1317–1325, 2011.

[34] Y. Nie and E. Kleine-Benne. Using Three Types of Twister Phases for Stir Bar Sorptive Extraction of Whisky, Wine and Fruit Juice. *AppNote Gerstel*, 2011.

[35] E. Pfannkoch, J. Whitecavage, and A. Hoffmann. Stir Bar Sorptive Extraction: Capacity and Competition Effects. *AppNote Gerstel*, 4, 2002.

Chapter 3

Extractable-studies of Single-Use Systems

3.1 Introduction

The overall strategy of extractable and leachable testing follows a consecutive approach [1, 2, 3]. Thereby, the primary purpose of extractables studies is to identify chemical substances that may leach from process equipment into the contacting solution during production, i.e. to provide qualitative data for the selection of target leachables [4, 5, 6]. In general, the extraction parameters, including the contact-solvent, time and temperature are chosen to cover a wide range of conditions in order to maximize the recovery of potential leachables than to simulate any individual product (Fig. 3.1).

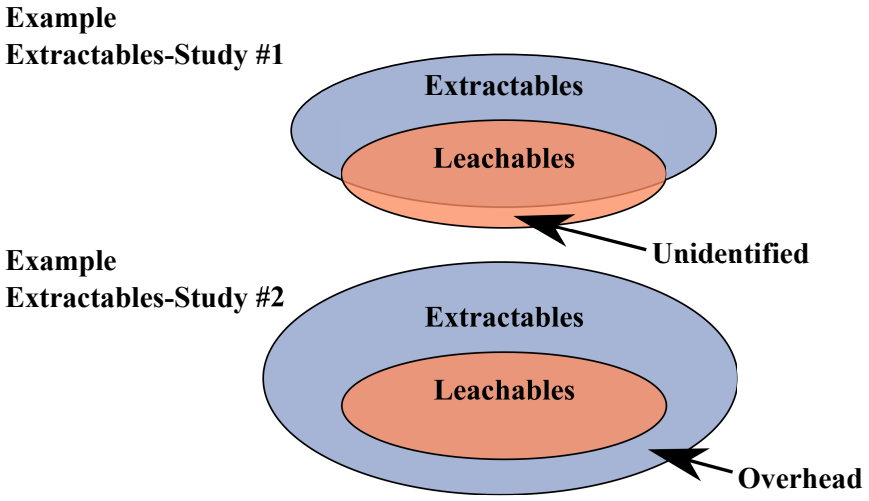


Figure 3.1: Correlation between extractable and leachable-data in dependence on the applied study design. (Top) Worst-case scenario: Possible occurring leachables are not totally covered by extractable-footprints. (Bottom) Harsher extractable-study design: Leachables are a subset of extractables.

The purpose of the study was to create a comprehensive extractable data-set of a sterile filter, which is applied in drug product processing, by using model solvents and conditions that mimic harsh scenarios. The extracted analytes were subsequently analyzed with stir-bar sorptive extraction (SBSE) in combination with gaschromatography mass spectrometry (GC-MS), used for the detection of organic substances and for the identification of elemental impurities inductively coupled plasma (ICP)-MS analytic, respectively. According to classical extractable testings USP <661.2> [7], the standard procedure involves incubation of the drug product contact materials in polar solutions such as water or ethanol. In a first class of experiments, the disposables were exposed to water solutions with different pH-values for long time periods under heat-influence. In a second step, increased material stress was induced by a more aggressive alcoholic contact solution. Direct injection of the alcoholic sample into a thermal desorption tube prepared with glass wool with subsequent TD-GC-MS analytic was performed for the trade-off against slightly compromised sensitivity, due to the small injection volume compared to the SBSE technique. Finally, an unprepared material-sample of the disposable was directly exposed to thermal desorption. Compared to the pyrolysis technique, the applied temperature maximum for the direct material thermal desorption was well below the melting point of the respective material. However, increased amounts of extractables are expected to migrate out of the filter with these kind of extractable study-design, which allows covering of all possible occurring leachables. In a second part of the study, extractable-profiles of further single-use filters, which differ in their material composition and manufacturer, were generated and compared among each other regarding their extractable-output.

3.2 Materials and Methods

The main investigated piece of equipment, a Millipore Durapore Cartridge sterile filter, designed for single-use, comprises the materials polyvinylidene fluoride (PVDF) as membrane, polypropylene (PP) as supporting and finally silicone (SI) as sealing material. Further tested filters included a filter with the exact same material composition and three others with deviant polymers. The chosen filters which demonstrate the effect of a single material change and the effect of the applied extraction-methods are listed in Table 3.1.

For the contact solutions, chemicals were purchased from the following providers: From SigmaAldrich (Steinheim am Albuch, Germany) high purity NaOH, H_3PO_4, butylhydroxytoluol (BHT) and n-eicosane were obtained. Absolute ethanol (99.7% optigrade, Promochem LGC, Wiesel, Germany), nitric acid, hydrocloric acid and isopropyl alcohol (IPA) from VWR (Darmstadt, Germany) with a GC purity of 99.5% were used. Water for Injection (WfI) was prepared on a Milli-Q Advantage A10 system (Merck GmbH, Darmstadt, Germany). Non-treated glass wool was purchased from SigmaAldrich and baked out at 300°C under helium flow for 30 min prior to use. For ICP measurements an ICP multi-element standard solution was purchased from AccuStandard (AccuStandard Europe, Switzerland).

Table 3.1: Description and material-composition (1: Filter membrane; 2: Housing material; 3: Seal material) of the investigated filters with the applied extracting agents and conditions. Abbreviations: Polyvinylidene fluoride (PVDF); Polypropylene (PP); Silicone (SI); Polysulfone (PSU); Mixed cellulose esters (MCE); Nylon (with an embedded base material of polyester fleece).

Nr.	Filter	Equipment composition	Extraction method				
			WfI - pH			Isopropyl /WfI	Thermal Desorption
			< 3	approx. 7	> 9.5		
1	Millipore Durapore Cartridge	1: PVDF; 2: PP; 3: SI;	x	x	x	x	x
2	Millipore Opticap XLT 10 Capsule 0.22 μm	1: PVDF; 2: PP; 3: SI;		x			
3	Millipore Opticap XLT 10 Capsule 1.2 μm	1: MCE; 2: PP; 3: SI;			x		
4	Millipore Millidisk 20 Cartridge	1: PVDF; 2: PSU; 3: SI;	x	x	x	x	
5	Pall Ultipor N66 Cartridge	1: Nylon; 2: PP; 3: SI;	x				

3.2.1 Sample Preparation

As the disposable filter is intended for the application of different drug product fillings, the study was performed with diverse extract solutions in harsh conditions. For this purpose the filter was analyzed in contact with water with different pH values for an incubation time of 3 days at a temperature of 95°C. Moreover, an IPA/water mixture was tested as a more aggressive solvent-mix at a temperature of 75°C. The incubation for all static extractions was carried out in the dark for three days. Lastly, to further increase the material stress, the filter materials were directly subjected to thermal desorption for 10 min at 150°C.

For the preparation of the stock solution of NaOH, approximately 800 mg of sodium hydroxide was given in a 20 mL volumetric flask and dissolved in WfI (eq. 1000 mmol/L). Subsequently, 1.0 mL of this solution was diluted to volume with WfI in a 10 mL volumetric flask (eq. 100 mmol/L). Again, 1.0 mL of the resulting solution was diluted to volume with WfI in a 100 mL volumetric flask (eq. 1 mmol/L). In turn for the stock solution of H_3PO_4, approximately 2.31 g of phosphoric acid were given in a 20 mL volumetric flask and dissolved in WfI (eq. 1000 mmol/L). The same dilution steps as used for the stock solution of NaOH were applied to reach a concentration of 1 mmol/L. Last, WfI and isopropyl alcohol with a ratio of 1/1 were prepared.

Before extraction, the filters were autoclaved for 30 min at 123°C. Afterwards the filters were incubated according to the extracting conditions described above (see Table 3.1). The filters, without housing were placed into closeable glass flasks and covered with the particular solvents as depicted in Figure 3.2. Additionally, for the preparation of the blank solution, 25 ml of WfI and of the IPA/WfI mixture were placed separately in Erlenmeyer glass flasks, which were closed with ground glass stoppers. According to the different solvents and their boiling points, the filters as well as the blank solutions were incubated at the particular temperature in the dark for three days. After incubation, the samples were cooled down to room temperature and decanted into glass vials for TD-GC-MS measurements. For the ICP-MS detection the samples were stored in polystyrene tubes to avoid absorbing glass contact.

3.2.2 Extractable Screening - SBSE TD-GC-MS

The PDMS coated stir-bar was exposed to 5 mL of the sample for 1 h and stirred with 800 rpm at 19°C. Following the extraction step, the stir-bar was rinsed with WfI, dried by dabbing and placed in the autosampler (TDS A2, Gerstel GmbH). Blank measurements were performed by immersing a stir-bar in pure WfI, to determine the system contamination by estimating the background noise. Reference measurements were done with stir-bars subjected to n-eicosane spiked WfI and measured at the beginning and at the end of the injection sequence. N-eicosane, a long chain saturated aliphatic hydrocarbon with a molecular mass of 282 g/mol, was chosen as a spiking substance, due to its late eluting timepoint at the end of the chromatographic separation and thus allowed semi-quantification.

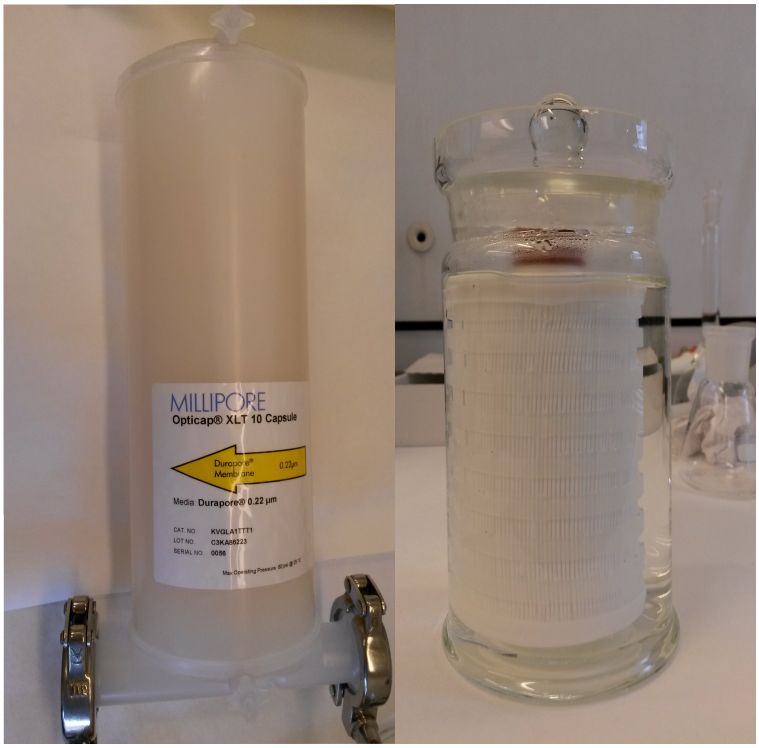


Figure 3.2: (Left) Filter preparation: Filter with housing filled with the extraction medium and sealed with steel clamps. (Right) Filters without housing placed in glass flasks and covered with the respective extraction medium.

Because of its low volatility the compound's response factor can be used as the lower limit of quantification. By semi-quantifying with aforementioned spiking substance, a worst-case quantity calculation of the extractables was carried out. It is used to demonstrate that the method/system is suitability for extractables with a higher boiling point (higher molecular mass and higher retention time). The spiking standard was dispersed in ethanol with the help of sonication at a concentration of 0.1 g/L and diluted with WfI to simulate a 0.1 ppm (0.05 µg/mL) concentration. The extraction of (semi-)volatile substances was performed using a thermal desorption (TD) system (TDS 3, Gerstel GmbH) equipped with a multi-purpose autosampler. During the extraction-process, volatilized analytes were purged under helium-flow into the precooled cold injection system (CIS, Gerstel GmbH), concentrating the extracted compounds in the inlet for subsequent GC-MS analysis. A detailed list of all operating parameters is provided in chapter chapter 2. Extractables with concentrations above 0.01 ppm were further characterized. Mass spectra of all compounds were matched with the NIST library 2.0 [8]. In case of no match the m/z spectrum was analyzed manually using the proprietary, internal databases of the Roche GmbH.

3.2.3 Extractable Screening - TD-GC-MS

Using a microliter syringe, 30 µL of the IPA/WfI sample and the pertaining blank solution were given into preconditioned, glass-wool prepared thermo desorption tubes (Gerstel GmbH). Reference measurements were performed by injecting n-eicosane on a 0.1 ppm level. Therefore, n-eicosane was dispersed in IPA and diluted with WfI to simulate a 1/1 ratio. The tubes were transferred into the autosampler and directly analyzed. Operating parameters were identical to the stir-bar analysis except the CIS setting in order to avoid icing of the WfI in the CIS system. Therefore, the applied initial CIS temperature was set to 20°C instead of 0°C. Again, only extractables with concentrations above 0.01 ppm were further characterized. Mass spectra of all compounds were matched with the NIST library 2.0 [8]. In case of no match the m/z spectrum was analyzed manually using the proprietary, internal databases of the Roche GmbH.

3.2.4 Extractable Screening - Direct TD-GC-MS

For the direct thermal desorption, ∼15 mg of both main polymer materials of the filters were taken and examined. The sample pieces were placed in preconditioned tubes and exposed to thermal extraction at a temperature of 150°C with a hold-time of 10 min (TDS rate: 60°C/min). Reference measurements were provided by simulating a 15 mg material-piece with an incorporated additive, by injecting BHT in a prepared glass wool tube with a concentration of 0.1 ppm [9]. For the analysis of the extractables, only substances with a semi-quantified concentration above 0.01 ppm were further analyzed. Mass spectra of all compounds were matched with the NIST library 2.0 [8]. In case of no match the m/z spectrum again was analyzed tentatively using the proprietary, internal databases of the Roche GmbH.

3.2.5 Extractable Screening - ICP-MS

The two WfI extract-samples obtained at low and high pH were used for the detection of elemental impurities. It is recommended to test for elements, which are known to impact product quality/stability. Therefore, a quantitative screening for inorganic extractables was performed by ICP-MS (Agilent 7700x) for the following elements: Ag, As, Au, Ba, Cd, Co, Cr, Cu, Hg, Ir, Li, Mo, Ni, Pb, Pd, Pt, Rh, Ru, Sb, Se, Sn, Tl, V. Further elements listed in the ICH Q3D Elemental Impurities guideline are Ti, W, Zn, Zr, Ca, Fe and Al [10] and were tested. As reference substances a set of typical elements was used in order to confirm the system suitability. The standard spiking elements were the following: Bi, Ge, In, Li6, Sc, Tb, V and Hg with a concentration of 10 µg/mL each. The standard solution was then diluted to reach a concentration of 1 µg/mL. Triple blank measurements were prepared in 50 mL plastic tubes with matrix solution, containing a 3% HNO3 and 1% HCl solution. For the autosampler, a rinsing solution of 5% HNO3 and 1% HCl was prepared, which was applied 60 sec after each sample with a rinse speed (nebulizer pump) of 0.1 rps. Each sample was measured twice, with and without spiking. For the spiked samples 25 µL

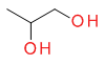
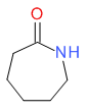
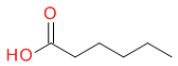
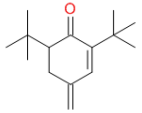
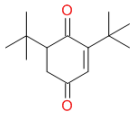
of the standard solution (1 µg/mL) were transferred into a 15 mL tube and completed to 5 mL with the sample. The ICP run parameters of the nebulizer pump (Micro mist) were configured at 0.3 rps with an uptake time of 60 sec and a stabilization time of 30 sec. For the MS a He gas-flow rate of 5.0 mL/min was adjusted.

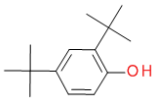
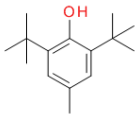
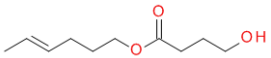
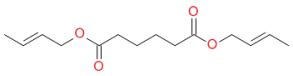
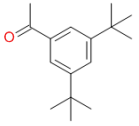
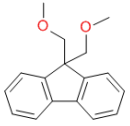
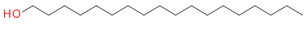
3.3 Results

3.3.1 Evaluation of the Extraction Techniques

Table 3.2 summarizes all detected extractables, extracted from the disposable filter Nr. 1, Millipore Durapore Cartridge, and their concentrations depending on the applied extraction technique. Static extraction based on water with different pH-values was shown to release only small amounts of extractables with a concentration above 0.01 ppm. Higher concentrations plus different substances were observed in the static extraction, using IPA/WfI extract. However, highest concentrations of extratables were extracted in the direct material thermal stress testing compared to the other tested extraction methods. It can be concluded, that substances with concentrations above 1 ppm, identified by direct material thermal desorption, were also found with other extraction methods with lower stress-levels on the polymer-material, including the substance 9,9-bis(methoxymethyl)fluorene. Furthermore, it can be observed that with different extraction techniques, different substances were extracted. With the IPA/WfI mixture the extracted substances display lower polarities, compared to the pure water extract solution. By the use of direct material thermal extraction, non-fragmented substances were determined, such as 2,6-bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione and adipic acid, di(but-2-en-1-yl) ester. The corresponding fragments 2,6-di-tert-butyl-4-methylene-2,5-cyclohexadienone and (E)hex-4-enyl,4-hydroxybutanoate were extracted with the other methods. Additionally, substances that were only determined by direct material thermal extraction were the very volatile R-(-)-1,2-propanediol and the non-polar caprolactam. The different pH-values of the water extracts did not have a major impact. With ICP-MS analysis no non-organic extractables were identified for the investigated single-use filter.

Table 3.2: Extractables of the single-use filter, Millipore Durapore Cartridge, detected and semi-quantified by TD-GC-MS. Direct material extraction, static extraction with WfI of three different pH-values and with an isopropane/WfI (1/1) mix were conducted.

Extractables	Structure	Molecular weight [g/mol]	Extractable concentration [ppm] - static extraction				Extractable concentration [ppm] - direct material extraction
			pH 3	neutral	pH 9.5	Iso/WfI 1/1	
R-(-)-1,2-propanediol		76	-	-	-	-	0.84
Caprolactam		113	-	-	-	-	0.34
Hexanoic acid		116	-	-	-	0.25	-
2,6-Di-tert-butyl-4-methylene-2,5-cyclohexadienone		218	-	-	0.02	0.59	-
2,6-Bis(1,1-dimethylethyl)-2,5-Cyclohexadiene-1,4-dione		220	-	-	-	-	1.17

2,4-Di-tert-butylphenol		206	0.03	0.02	0.02	0.47	7.15
Butylated hydroxytoluene		220	-	-	-	-	0.69
(E)hex-4-enyl 4-hydroxybutanoate		186	0.01	0.03	-	-	-
Adipic acid, di(but-2-en-1-yl) ester		254	-	-	-	-	1.95
1-(3,5-Di-tert-butylphenyl)ethanone		232	-	0.01	-	-	0.47
9,9-Bis(methoxymethyl)fluorene		254	0.02	0.02	0.02	-	1.05
Unknown: Alkene (cis-2-Octadecene)	-	252	-	-	-	0.50	-
Unknown: Alkene	-	-	-	-	-	2.15	-
1-Octadecanol		270	-	-	-	-	36.73

3.3.2 Different Single-Use Filters

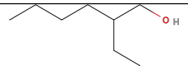
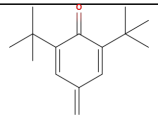
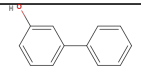
The effect of different filter materials on the extractable spectrum is displayed in Table 4.12. Comparing the extractable-profile of filter 1 (Millipore Durapore Cartridge - see table 3.2) to filter 2 (Millipore Opticap XLT 10 Capsule 0.22 μm - see table 4.12), which comprised both the same material compositions, showed similar extractable-profiles.

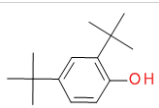
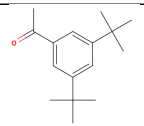
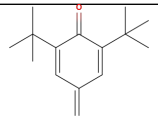
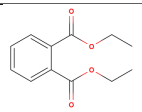
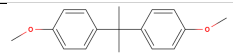
In contrast, filter 3 (Millipore Opticap XLT 10 Capsule 1.2 μm) uses a mixed cellulose ester (MCE) as filtration membrane rather than PVDF as for filter 1. The PP housing of filter 3 is much smaller and provides therefore much less material in contact to the extracting solution than filter 1. For filter 3 only one extractable, namely the 2,6-di-tert-butyl-4-methylene-2,5-cyclohexadienone was detected, which was also observed in case of filter 1.

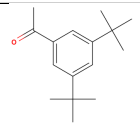
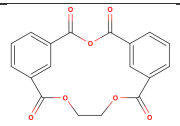
Although filter 1 and filter 4 (Millipore Millidisk 20 Cartridge) differed only in the housing material, no single matching extractable was observed for both filters. For the latter filter, the substance 1,1-(1-methylethylidene)bis[methoxy-benzene was detected, which is a classical building block of the polymer polysulfone (PSU) [11]. Further, the antioxidant diethyl phthalate was found, which functions as a protector for the vulnerable PSU-material. In case of the last filter (Pall Ultipor N66 Cartridge) from a different manufacturer and device material (nylon with an embedded base material of polyester fleece) no corresponding extractables with the extractable-profile of filter 1 were found. As expected, nylon building blocks were extracted, including 1-(3,5-di-tert-butylphenyl)ethanone and polyester polyol.

Beside the organic extractable examination, the filters were tested for inorganic additives with ICP-MS analytic. A single elemental impurity, namely antimony (Sb), which is classically used as fire retardant for diverse polymers [12], was determined for the Millipore Opticap XLT 10 Capsule 1,2 μm and Pall Ultipor N66 Cartridge filter with a concentration of 2.1 mg/L and 2.2 mg/L, respectively.

Table 3.3: Detected and semi-quantified extractables of each filter by TD-GC-MS analytic. Static extraction with WfI of three different pH-values and with an IPA/WfI (1/1) mix were conducted.

Nr. Filter	Extractables	Structure	Extractable concentration [ppm] - static extraction			
			pH 3	neutral	pH 9.5	Iso/WfI 1/1
2 - Millipore Opticap XLT 10 Capsule 0.22 μm	2-ethyl-1-hexanol		n.a.	0.02	n.a.	n.a.
2 - Millipore Opticap XLT 10 Capsule 0.22 μm	Acetophenone		n.a.	0.02	n.a.	n.a.
2 - Millipore Opticap XLT 10 Capsule 0.22 μm	2,6-Di-tert-butyl-4-methylene-2,5-cyclohexadienone		n.a.	0.02	n.a.	n.a.
2 - Millipore Opticap XLT 10 Capsule 0.22 μm	3-hydroxy-biphenyl		n.a.	0.01	n.a.	n.a.

Nr. Filter	Extractables	Structure	Extractable concentration [ppm] - static extraction			
			pH 3	neutral	pH 9.5	Iso/WfI 1/1
2 - Millipore Opticap XLT 10 Capsule 0.22 μm	2,4-Di-tert-butylphenol		n.a.	0.01	n.a.	n.a.
2 - Millipore Opticap XLT 10 Capsule 0.22 μm	1-(3,5-Di-tert-butylphenyl)ethanone		n.a.	0.02	n.a.	n.a.
3 - Millipore Opticap XLT 10 Capsule 1.2 μm	2,6-Di-tert-butyl-4-methylene-2,5-cyclohexadienone		n.a.	n.a.	0.02	n.a.
4 - Millipore Millidisk 20 Cartridge	Diethyl phthalate		-	0.02	-	-
4 - Millipore Millidisk 20 Cartridge	1,1-(1-Methylethylidene)bis[methoxybenzene		0.01	0.01	0.01	0.18
5 - Pall Ultipor N66 Cartridge	Tetrahydrofuran		0.18	n.a.	n.a.	n.a.

Nr. Filter	Extractables	Structure	Extractable concentration [ppm] - static extraction			
			pH 3	neutral	pH 9.5	Iso/WfI 1/1
5 - Pall Ultipor N66 Cartridge	1-(3,5-Di-tert-butylphenyl)ethanone		0.03	n.a.	n.a.	n.a.
5 - Pall Ultipor N66 Cartridge	Polyester polyol		0.02	n.a.	n.a.	n.a.

3.4 Discussion

In the previous chapter, SBSE was shown to be the gold standard method for the detection of leachables in drug product matrices. A similar setup can be used for an extractable study. Hereby, we found direct injection of single-use material probes into a TDS to be more useful as an initial, quick-shot analysis. While the range of compounds and compound groups which are detected in direct material thermal desorption experiments are quite similar to those extracted with an organic solvent-mix from polymeric materials, the concentrations and fragments are much higher and larger, respectively. No impact of water extract pH-value was observed, as the level of extraction was mostly driven by the incubation temperature of 100°C. Subsequent studies in accordance with USP <661> can then be employed to fully characterize the material for the application of different drug products [7]. In summary the three analysis methods offer very different levels of material stress, with water solution being the most benign and direct material desorption the most aggressive one.

Importantly we found that a single material change e.g. in the housing in the composition of a polymer-mix of a SUS, can heavily influence the extractable-outcome. Although a material-change from the inert material PVDF to a non-classical polymer material such as MCU does not have an impact on the organic extractable-outcome, it can alter the inorganic impurity level. Therefore, extractable-studies on the SUS are indispensable prior to the leachable-study itself. Nonetheless, it was also shown that SUSs with the same material composition yield comparable extractable-footprints. However, the overall number of extractables from the filters was low.

3.5 Conclusion

In summary it was shown that direct material TD stress testing can be a time saving first step, as it does not require any sample preparation nor solvents, that may contaminate the sample. However, for a comprehensive evaluation multiple and complementary stress tests should be performed to fully characterize a disposable and to allow for subsequent leachable analysis. Nonetheless, before any extraction of a new SUS is conducted, already performed studies of different SUSs, but with the same polymer-mixes, should be consulted in order to point out possible extractables in advance and thus allow target analysis.

Bibliography

[1] C. Pan, F. Harmon, K. Toscano, F. Liu, and R. Vivilecchia. Strategy for identification of leachables in packaged pharmaceutical liquid formulations. *Journal of Pharmaceutical and Biomedical Analysis*, 46(3):520–527, 2008.

[2] I. Markovic. Evaluation of safety and quality impact of extractable and leachable substances in therapeutic biologic protein products: a risk-based perspective. *Expert Opin. Drug Saf.*, 6(5):487–491, 2007.

[3] Y. Gao and N. Allison. Extractables and leachables issues with the application of single use technology in the biopharmaceutical industry: Extractables and Leachables in Disposable Manufacture. *Journal of Chemical Technology & Biotechnology*, 91(2):289–295, 2016.

[4] D. Norwood, A. T. Granger, and D. M. Paskiet. Extractables and Leachables in Drugs and Packaging. *Encyclopedia Pharm. Sci. Technol.*, 2013.

[5] D. Bestwick and R. Colton. Extractables and Leachables from Single-Use Disposables. *Bioprocess Int.*, 2009.

[6] D. Jenke. Extractable/Leachable Substances from Plastic Materials Used as Pharmaceutical Product Containers/Devices. *J. Pharm. Sci. Technol.*, 56(6):332–371, 2002.

[7] U.S. Pharmacopeia. Plastic Packaging Systems for Pharmaceutical Use <661.2>. 2016.

[8] V. Shen. *Standard Reference Simulation Website, NIST Standard Reference Database 173*. National Institute of Standards and Technology, 2006.

[9] R. L. Gray, R. E. Lee, and B. M. Sanders. Low volatility antioxidants for scorch protection of polyurethane foams. *Journal of Vinyl and Additive Technology*, 2(3):265–269, 1996.

[10] International Conference of Harmonisation (ICH). Guideline for Elemental Impurities. 2014.

[11] S. Al-Gharabli, J. Kujawa, M. Mavukkandy, and H. Arafat. Functional groups docking on PVDF membranes: Novel Piranha approach. *European Polymer Journal*, 96:414–428, 2017.

[12] V. Babushok, P. Deglmann, R. Krämer, and G. Linteris. Influence of Antimony-Halogen Additives on Flame Propagation. *Combustion Science and Technology*, 189(2):290–311, 2017.

Chapter 4

Studies on Leachables in commercial scale Protein Drug Filling Lines

Parts of the following chapter are published in the International Journal of Pharmaceutics, titeled "New Studies on Leachables in commercial scale Protein Drug Filling Lines using Stir Bar Sorptive Extraction coupled with TD-GC-MS and UPLC/QTOF-MS/MS analytics" [1]

The increasing application of Single-Use Systems (SUSs) in pharmaceutical manufacturing lines poses a potential risk of polymer-related impurities leaching into the process stream and persisting through the manufacturing process. To minimize any potential toxicity and impairment to the product's quality, safety thresholds are strictly regulated and enforced in particular for parenteral solutions. At present, impurities are estimated from extractable profiles, which are generated for each SUS with thermal or static extraction. In this study we employed target leachable-testing by taking samples directly from an industrial filling line probed during real-life processing of three parenteral drugs (n=2) under actual process-conditions, to estimate the concentration of leachables throughout drug-manufacturing. At five different points, samples were drawn to study the individual impact of SUSs on the leachable accumulation within the drug-filling process. The drug products were examined for leachables using stir-bar-sorptive-extraction (SBSE) with polydimethylsiloxane (PDMS) and ethylene glycol (EG)-PDMS coated stir-bars. Subsequent extraction from the stir-bars and analysis of the substances was performed with TD-GC-MS and solvent-back-extraction (SBE)-UPLC/QTOF-MS/MS analytics. Our study revealed the following main results: 1) Leachables were found in extremely low concentrations, all below toxicological thresholds (highest leachable concentration in the final drug product 1 (DP1): 0.274 ppm $<$ drug specific threshold: 6.0 ppm; DP2: 0.010 ppm $<$ 0.2 ppm; DP3: 0.011 ppm $<$ 0.5 ppm). All compounds identified in the leachables study were found to be non-genotoxic. 2) Most of the leachables (68%) that were found were already observed at the beginning of the filling process, delivered by the API Neither a common source of leaching could be identified within the filling-line nor a specific product influence on quality or quantity of leachables. 3) No leachable increase could be observed over the

filling process. On the contrary leachable concentrations declined with 83%, which was partly due to dilution by buffer-feed and to a proven absorption of leachables by filters and silicon tubing. 4) No active ingredient influence on the leachable-outcome was observed by placebo to drug product comparison. In contrast, the pH-setting of the drug product influenced the leaching-behavior to the greatest possible extent.

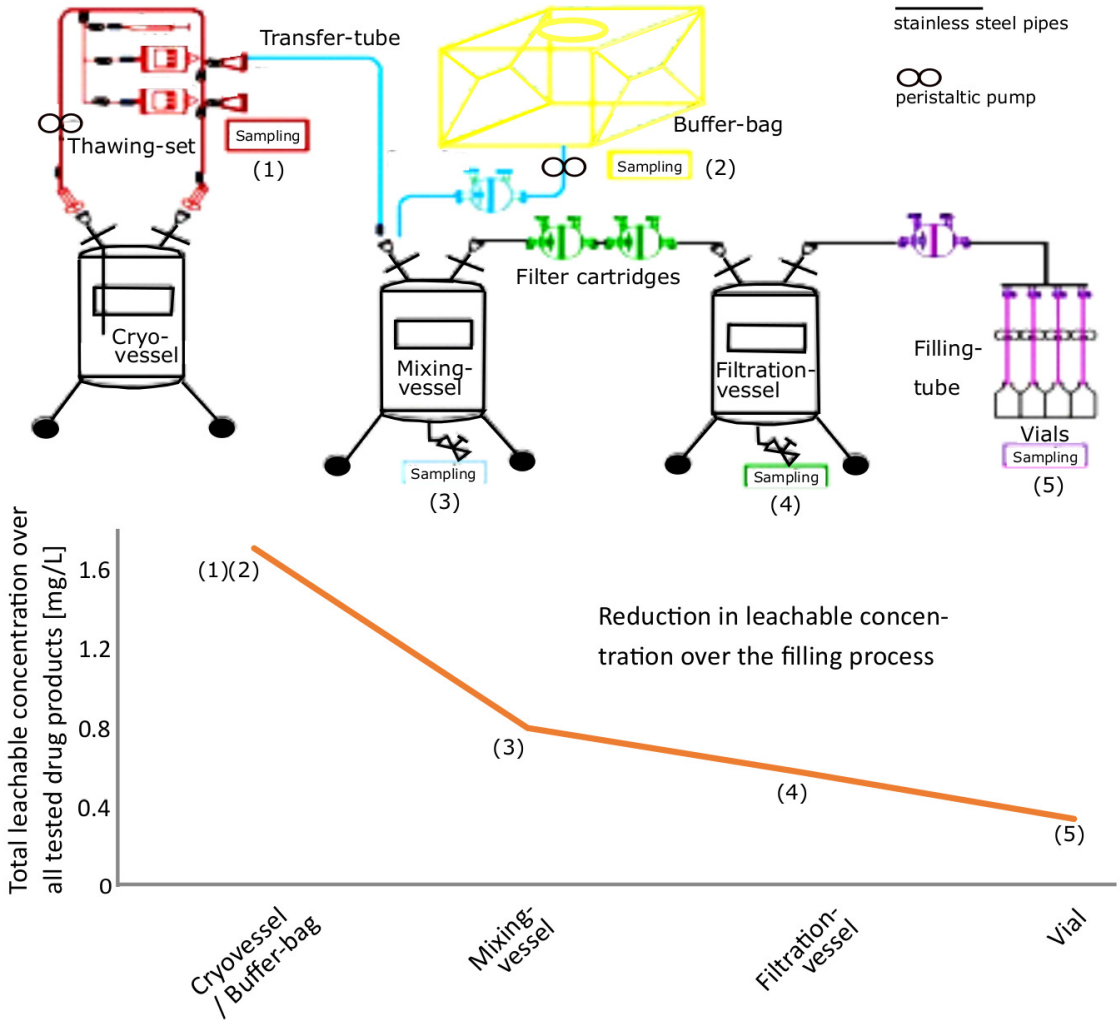


Figure 4.1: Graphical abstract of a filling line equipped with diverse Single-Use Systems: Major reduction in concentration of leachables during the filling process.

4.1 Introduction

Single-Use Systems (SUSs), including silicone tubing, filter cartridges and formulation bags with diverse polymeric materials, recently emerged as the material of choice for various

pharmaceutical production lines [2, 3, 4]. However, an abundance of polymeric surfaces with direct or indirect contact to the product in filling lines poses a risk of contaminating the final product and to impair its quality. Hence, SUSs are subject to multiple regulations including USP (<665> review stage, <1665>) and FDA (US 21 CFR 211.65) specifying the evaluation of their compatibility with the drug formulation [5, 6, 7]. This includes testing for potential leaching of functional additives (e.g. antioxidants and plasticizers). These tests are performed before the actual usage and simulate worst case scenarios to identify extractable substances by exposing the material to appropriate solvents under exaggerated conditions of temperature and time [8, 9, 10, 11]. However, this kind of testing does not identify or quantify the exact amount of individual leachables, as observed in real-life processing. Although extractables can be considered as potential leachables, not all extractables leach into drug products. In addition, drug formulation components or buffers may interact with SUSs in various ways and form leachables that were not previously identified during extractable analysis with solvents [12, 13, 14, 15]. The risk of residual leachables, beyond detection in extractable studies, is particularly pronounced in injectable solutions compared to other dosage forms, as with this route of administration toxic substances can be most harmful [16]. As leachables may be of genotoxic concern, international safety regulations (ICH M7) have set an acceptable daily intake dose (ADI) concept derived from the maximum lifetime drug treatment days [17, 18]. Therefore, leachable studies of the whole filling process are highly warranted in addition to the extractable-profiles of each SUS, to elucidate the actual leachable exposure and to ensure the patient's safety and the product's integrity.

The quantification of leachables poses several requirements on the measurement technique and the sample preparation [19, 20]: 1) Sufficient sensitivity over a broad spectrum of substances. 2) Avoidance of plastic parts in the analysis, including e.g. Eppendorf-tubes. 3) Minimizing interference of drug components with the analysis, such as protein clogging in columns. To meet these requirements stir-bar sorptive extraction (SBSE) was recently introduced in combination with high performance liquid chromatography (UPLC) and gas chromatography (GC) for detection of non-volatile and semi-volatile leachables in drug matrices [21, 22, 23, 24]. This solvent-less sample enrichment technique allows direct extraction of solutes from aqueous matrices and works well in the presence of protein drugs. In SBSE, a glass-lined magnetic stir-bar, coated with either a polydimethylsiloxane (PDMS) or a polar ethylene glycol (EG)-PDMS copolymer layer, is immersed into the sample [25]. After sufficient stirring with the bar, analyte partition equilibrium is reached on the coating. The recovery of substances is based on each analyte's octanol/water distribution coefficient ($K_{O/W}$) and the phase ratio between the aqueous drug sample and the coating of the stir-bar. GC- and UPLC-mass spectrometry (MS) analysis of the stir bar can then be performed by transferring the analytes into the system via thermal desorption (TD) and solvent back extraction (SBE), respectively.

The purpose of this study was to identify and quantify accumulation of leachables in real-world commercial scale industrial drug-filling lines for protein drug products in order

to shed light on the actual patient exposure. To understand the accumulation of leachables in the final drug formulation it was indispensable to outline the leachable-concentrations within the drug manufacturing over different processing steps, like the buffer fed-in or the purification-steps, beyond extractables-studies [26]. For the first time actual leachable results were published beside the designing and implementing requirements [20, 27, 28]. Therefore, samples of several injectable drug formulations, representative of a typical pharmaceutical production portfolio, were drawn during the actual filling process after contact with a SUS at five points under actual commercial scale production conditions. Leachables were traced back to their origin (SUS) by identifying the first point of appearance in the filling line and by comparing them to previously obtained extractable-footprints of each SUS. This was studied for multiple product formulations. Our study also includes the filling tube, which is subjected to mechanic stress and poses an elevated risk, as no further purification or filtration step follows the surface contact to this SUS [29]. Furthermore, filters, with their large contact surfaces, and the buffer bag, used for the preparation of the buffer, with its material mix and long contact time to the buffer-solution were expected to be particularly relevant. Further the extraction-power of pH-setting of the drug product and its active ingredient, the protein, during the filling process in contact to SUSs was investigated. Therefore, we tested different drug products with diverse pH-settings and further compared a placebo-run with its pertaining drug product-run. Finally, the impact on patient's safety was classified by evaluating the amount, toxicity and carcinogenicity of the detected substances.

4.2 Materials and Methods

4.2.1 Chemicals

To assess the exposure to leachables from modern drug filling lines, five commonly used SUSs (Table 4.1) with large contact surfaces were studied for polymer related impurities. The potential leachable-footprint can vary substantially between the tested SUSs, due to differences in material compositions, compounded additives and fabrication. All tested SUSs were certified as USP class VI biocompatible. Samples were drawn from a filling line comprising the listed SUSs during processing of three parenteral drugs (Roche Diagnostic GmbH, Mannheim, Germany), which were selected to be representative of a pharmaceutical manufacturer's portfolio (Table 4.2).

Sample analysis was performed with GC and UPLC. Nine spiking substances were added to allow semi-quantitative evaluation of the GC and UPLC analysis. The substances were selected because they are commonly observed as typical leachables. Substances that are commonly observed as leachables in pharmaceutical processing [29] were purchased from Sigma Aldrich (Steinheim am Albuch, Germany) with purity $> 97\%$. For GC analytic: Tetrahydrofurfuryl (THF) methacrylate log $K_{O/W}$ 1.8; Di-ethyl-phthalate log $K_{O/W}$ 2.45; 3,5-Di-tert-butyl-4-hydroxybenz(BHT)-aldehyde log $K_{O/W}$ 4.2 and Butylated hydroxyl-

toluene (BHT) log $K_{O/W}$ 5.3. UPLC analytic, positive ionization-mode: 2-(2-hydroxy-5-methyl-phenyl)benzotriazole (UVA P) log $K_{O/W}$ 6.1; 2-(2H-Benzotriazol-2-yl)-4,6-bis(1,1-dimethylpropyl)phenol (Tinuvin 328) log $K_{O/W}$ 7.25; 2,5-Bis(5-tert-butyl-benzoxazol-2-yl)thiophene (Uvitex OB) log $K_{O/W}$ 8.0; Tris(2,4-Di-tert-butylphenyl)phosphite (Irgafos 168) log $K_{O/W}$ 15.5; UPLC analytic, negative ionization-mode: Pentaerythritol tetrakis(3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate) (Irganox 1010) log $K_{O/W}$ 19.4. Water for Injection (WfI) was prepared on a Milli-Q Advantage A10 system (Merck GmbH, Darmstadt, Germany). Absolute ethanol (99.7% optigrade, Promochem LGC, Wiesel, Germany), methanol (UPLC-grade, Biosolve, Dieuze, France) and ethyl acetate (99.8% Sigma Aldrich) were applied as solvents and/or eluents. Formic acid (Sigma Aldrich) was used for LC ion suppression and non-treated glass wool (Sigma Aldrich) for direct TD spiking application.

Table 4.1: The tested SUSs are listed along with their corresponding drug product (DP 1-3) volume passing by, exposure time and material-composition, as well as a possible leachable footprint obtained from literature.

SUSs / Manufacturer	Material-Characterization	Typical extractable footprint [30]	Drug product (DP) volume contact [L]			Max. allowed contact time [h] to the DP
			DP 1	DP 2	DP 3	
Thawing tubing set / Millipore Techno Plast	Platinum-catalyzed silicone (SI) tube	Oligomers; solvent residues like isopropyl alcohol; preservatives like hydroxybiphenyl	115	90	110	17
	Polysulfone (PS) connector	Bisphenol A; polycarbonate oligomers; solvent residues like chlorbenzene				
Transfer tubing / Techno Plast	Fabric hose: Platinum-catalyzed SI tube with glass silk braid	Oligomers; solvent residues like isopropyl alcohol; preservatives like hydroxybiphenyl	115	90	110	24
Buffer-Bag / Sartorius	Bag: Polyethylene (PE) ethylene vinyl acetate (EVOH)	Aliphatic hydrocarbones C4 - C30; antioxidants like BHT and Irganox 1010	200	90	n.a.	24
	Bag-port and tubing: Thermoplastic elastomer (TPE) and SI tube	Oligomers; solvent residues like isopropyl alcohol; preservatives like hydroxybiphenyl				
Filter / Merck	Filter membrane: Polyvinylidene fluoride (PVDF)	n.a.	1000	275	650	8

SUSs / Manufacturer	Material-Characterization	Typical extractable footprint [30]	Drug product (DP) volume contact [L]			Max. allowed contact time [h] to the DP
			DP 1	DP 2	DP 3	
	Filter cartridge: Polypropylene (PP)	Aliphatic hydrocarbones C3 - C34; fatty acids; lubricants; antioxidants like Irganox 1010, 1076 and Irgafos 168				
Filling tubing / Techno Plast	Platinum-catalyzed SI tube	Oligomers; solvent residues like isopropyl alcohol; preservatives like hydroxybiphenyl	125	35	85	72

4.2.2 Extractables

Extractable footprints were obtained in order to restrict the possible leachables for each SUS, and to enable association of different substances with source SUSs in the leachable study. Best practice recommendations and compendial standards for extractables and leachables note that a leachables-extractables correlation, either qualitative or quantitative, should be established by linking actual drug product leachables with extractables from corresponding controlled extraction studies performed on each SUSs. The flowchart (Figure 4.3) depicts the overall strategy of the semi-quantified leachable-testing approach based on toxicological extractable profiling and other analytical techniques. Extractable-testing was performed by exposing the polymer component to appropriate solvents at high temperatures to detect potentially toxic additives that might leach into the drug product. The considered SUSs were in the same condition (e.g. gamma sterilized) as those integrated in the actual filling line for the leachable-testing. No water flush step prior to testing was performed, in order to prevent a possible loss of extractables. For the detection of (semi)-volatile extractables all SUS materials were analyzed with direct TD with subsequent GC-MS (7890B GC-System, equipped with a DB-1ms column, 5977A MSD, Agilent Technologies, Waldbronn, Germany), to minimize sample preparation and eliminate sample contamination from the solvents. This extraction procedure was previously shown to provide a comprehensive list of leachable candidates [31, 32]. Conventional static extraction with a solvent-mix under heat-influence was performed to yield substances with higher polarity and molecular weight, as well as non-volatile substances. These were subsequently analyzed by UPLC (Acquity, Waters GmbH, Eschborn, Germany) combined with electron spray ionization (ESI) and MS/MS analysis (Xevo G2-S Tof, Waters GmbH).

GC-MS Measurement of (Semi)-Volatile Extractables

For the direct TD, ∼15 mg of each SUS material was taken and examined. The sample pieces were placed in preconditioned tubes (Gerstel GmbH, Mülheim an der Ruhr, Germany) and exposed to thermal extraction at a temperature of 150°C with a hold-time of 10 min (rate: 60°C/min). The extraction of (semi-)volatile substances was performed using a thermal desorption system equipped with a multi-purpose autosampler (TD 3 and TD A2, Gerstel GmbH). During the extraction-process, volatilized analytes were purged under helium-flow into the precooled (-50°C) cold-injection-system (CIS, Gerstel GmbH), concentrating the extracted compounds in the inlet for subsequent GC-MS analysis (heat program: 40°C for 2 min – rate 10°C/min – 320°C for 10 min). A detailed list of all operating parameters is provided in Table 4.3. The compounds were identified in the extraction study by comparing the chromatograms to a blank analysis of an empty analytical tube. Reference measurements were provided using a BHT prepared glass wool tube with a concentration simulating a 0.1 ppm contamination of a 15 mg material-piece. Mass spectra (scan range 33-650 m/z) of all compounds were matched with the NIST library 2.0 [33]. In case of no match the m/z spectrum was analyzed manual using proprietary, internal databases of the Roche GmbH.

Table 4.2: Tested drug product formulations and their individual analytical evaluation threshold (AET) regulated by the ICH M7 guideline for single and multiple impurities, as well as the contact to the individual SUSs are listed.

Drug Product	Formulation-Characterization	AET [ppm] for single impurities [17]	AET [ppm] for multiple impurities [17]	Contact to SUSs
Drug Product 1	Protein size: ∼140 kg/mol; High content of sodium chloride (∼1.0%) and polysorbate 80 (∼0.07%)	6.0	6.0	Thawing tubing; Transfer tubing; Bag in combination with filter; 2 x Filters; Filling tubing
Drug Product 2	Protein size: ∼1 kg/mol (∼0.7%); Phosphate based buffered with a high content of trehalose (∼6%)	0.2	0.6	Thawing tubing; Transfer tubing; Bag; 2 x Filters; Filling tubing
Drug Product 3	Protein size: ∼150 kg/mol; High concentration of protein (∼3%) and trehalose; Polysorbate 20	0.5	1.5	Thawing tubing; Transfer tubing; 3 x Filters; Filling tubing
Drug Product 4	Protein size: ∼150 kg/mol; High content of L-Histidine; Poloxamer 188	0.5	1.5	Thawing tubing; Transfer tubing; 2 x Filters; Filling tubing
Placebo Product 4	High content of L-Histidine; Poloxamer 188	0.5	1.5	Bag; 2 x Filters; Filling tubing
Drug Product 5	pH: ∼3.5; High content of sodium chlorid	20	20	Thawing tubing; Transfer tubing; Filter; Filling tubing
Drug Product 6	Protein size: ∼150 kg/mol; pH: ∼6.0; Polysorbate 20	12	12	Thawing tubing; Transfer tubing; Filter; Filling tubing
Drug Product 7	Protein size: ∼30 kg/mol; pH: ∼7.5; Polysorbate 20	2.5	7.5	Thawing tubing; Transfer tubing; Filter; Filling tubing
Drug Product 8	pH: Sodium carbonate and mannitol ∼10.0;	0.75	2.25	Thawing tubing; Transfer tubing; Filter; Filling tubing

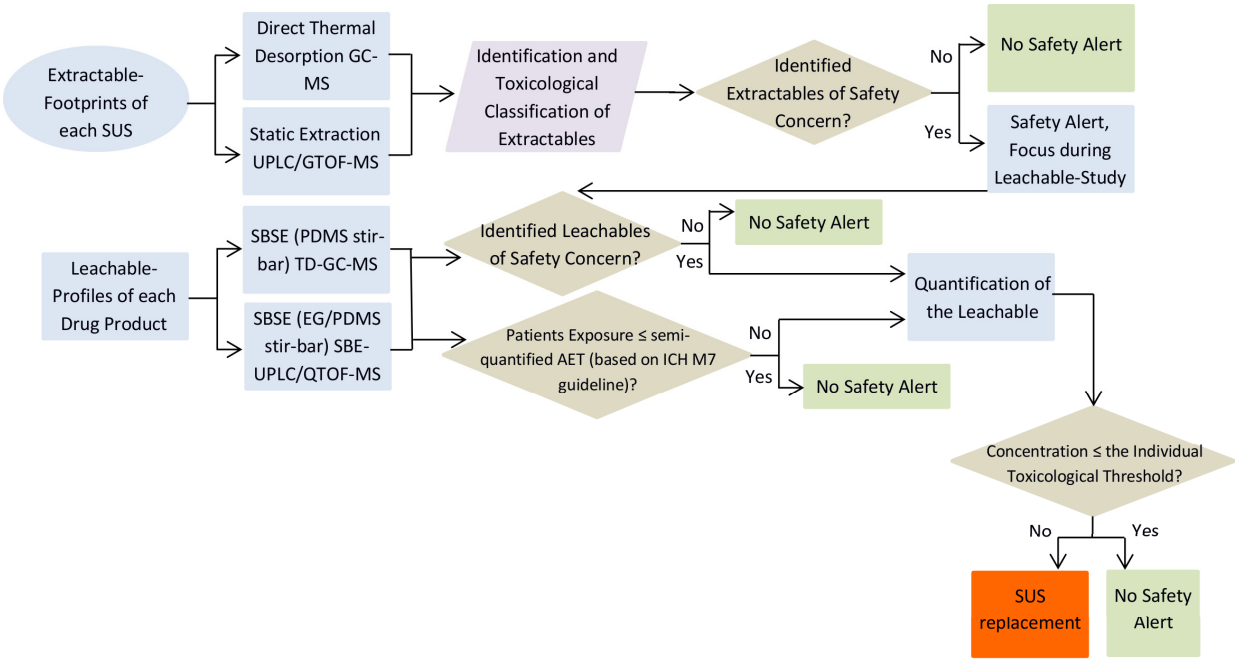


Figure 4.2: Strategy for toxicological risk-based leachable-testing based on extractable-footprints.

Table 4.3: Operating parameters: A) TDS, CIS, GC and MS parameters for the thermal extraction study. B) TDS, CIS, GC and MS parameters for the leachable-study. C) UPLC and QTOF-MS/MS parameters for the extractable- and leachable-study.

A) Method: TD-GC-MS Extractable-Study GERSTEL TDS		B) Method: TD-GC-MS Leachable-Study GERSTEL TDS		C) Method: UPLC/GTOF-MS Extractable/Leachable-Study Waters UPLC
TDS	**Setting**	**TDS**	**Setting**	
Temperature	Program	Temperature	Program	
Initial Temperature	20°C	Initial Temperature	20°C	
Delay Time	3.00 min	Delay Time	3.00 min	
Rate	60.0 °C/min	Rate	60.0 °C/min	
End Temp.	150°C	End Temp.	300°C	
Hold Time	10.0min	Hold Time	10.0min	

Transfer Temp:	320°C	Transfer Temp:	320°C	
Desorption Mode:	Splitless	Desorption Mode:	Splitless	

CIS	**Setting**	**CIS**	**Setting**	
Liner type with glass	packed wool	Liner type with glass	packed wool	
Mode:	Solvent Vent	Mode:	Solvent Vent	
Total Flow:	40.5 mL/min	Total Flow:	40.5 mL/min	
Vent Pressure:	49 kPa until 0.01 min	Vent Pressure:	49 kPa until 0.01 min	
Purge Flow to Split Vent:	36.5 mL/min at 1.51 min	Purge Flow to Split Vent:	36.5 mL/min at 1.51 min	
Temperature	Program	Temperature	Program	
Initial Temp.:	-50°C	Initial Temp.:	-20°C	
Equilibration Time:	0.50 min	Equilibration Time:	0.50 min	
Initial Time:	0.10 min	Initial Time:	0.10 min	
Rate:	8.00°C/s	Rate:	8.00°C/s	
End Temp:	300°C	End Temp:	300°C	
Hold Time:	20.00 min	Hold Time:	20.00 min	

GC	**Setting**	**GC**	**Setting**	**UPLC**	**Setting**
Column Agilent	122-0132: A002	Column Agilent	122-0132: A002	Column Agilent	InfinityLab Poroshell 120
DB-1ms:	30 m x 250 μm x 0.25 μm	DB-1ms:	30 m x 250 μm x 0.25 μm	HPH C8:	2.1 x 50 mm 2.7 μm
He Column Flow:	1 mL/min	He Column Flow:	1 mL/min	Column Flow:	350 μL/min
Temperature	Program	Temperature	Program	Temperature	Program
Initial Temp.:	40°C	Initial Temp.:	40°C	Column Temperature:	40°C
Hold Time	2.0min	Hold Time	2.0min	Injection	Volume

Rate	10.0 °C/min	Rate	6.5 °C/min	Leachable-Study:	10 μL
End Temp.	320°C	End Temp.	300°C	Extractable-Study:	0.5 μL
Hold Time:	10.00 min	Hold Time:	3.00 min	Mobile Phase	A: Water 0.1% formic acid; B: Methanol
MSD Transfer Temp.:	300°C	MSD Transfer Temp.:	300°C	Gradient Time / Eluent A / Eluent B	0.0 min 80% 20% 0.5 min 80% 20% 8.0 min 3% 97% 16.0 min 3% 97% 16.1 min 80% 20% 20.0 min 80% 20%

MS	**Setting**	**MS**	**Setting**	**MS**	**Setting**
MS Source:	230°C	MS Source:	230°C	MS Source:	130°C
MS Quad:	150°C	MS Quad:	150°C	Desolvation Temp.:	550°C
				Desolvation Gas Flow:	790 L/h
Scan range:	33 - 650	Scan range:	33 - 650	Scan range:	100 - 1200
Threshold:	150	Threshold:	150	Capillary:	1.85 kV
Frequency:	2.4 scans/sec	Frequency:	2.4 scans/sec	Scan Time	1.0 sec

UPLC/QTOF-MS/MS Measurement of Non-Volatile Extractables

The extraction of non-volatile substances was achieved by using exaggerated conditions. A solvent mix of water and ethanol with a ratio of 50:50 was applied in order to mimic a water based protein drug product with surfactant content [5]. The tested SUSs were filled with the solvent mix and sealed with glass stoppers. In order to prevent evaporation, the extractable-studies for the tubes were performed in glass bottles containing ∼10 mL of the solvent mixture. The filter with the non-polymer filter-housing was stored in a glass container and fully submerged in the mixture. For the 200 L buffer-bag an identical scale-down version of 3 L was chosen. The extractable-study was performed at approximately

70°C with exposure time that matched the maximally allowed contact-time of each SUS in the filling-process (see Table 4.1). Note that the temperature was chosen just below the boiling point of ethanol. Blank measurements were performed with aliquots of the extracting solvent mixture in glass bottles and carried out under the same conditions. Non-volatile compounds in the generated extract-solutions (0.5 μL sample) were identified by reversed phase UPLC/QTOF-MS/MS analytic (equipped with C8 column) [34] at a column temperature of 40°C and flow rate of 350 μL/min. The ESI source was operated with a desolvation temperature of 40°C and flow rate of 350 μL/min. Further operating parameters are listed in Table 4.3. Reference measurement was performed by injecting predefined spiking substances on water ethanol basis into the UPLC-system, simulating a 0.1 ppm contamination. The compounds were identified at a scan range of 100-1200 m/z by matching accurate monoisotopic masses to a proprietary, internal database (Roche GmbH) and the handbook of Bolgar et al. [30].

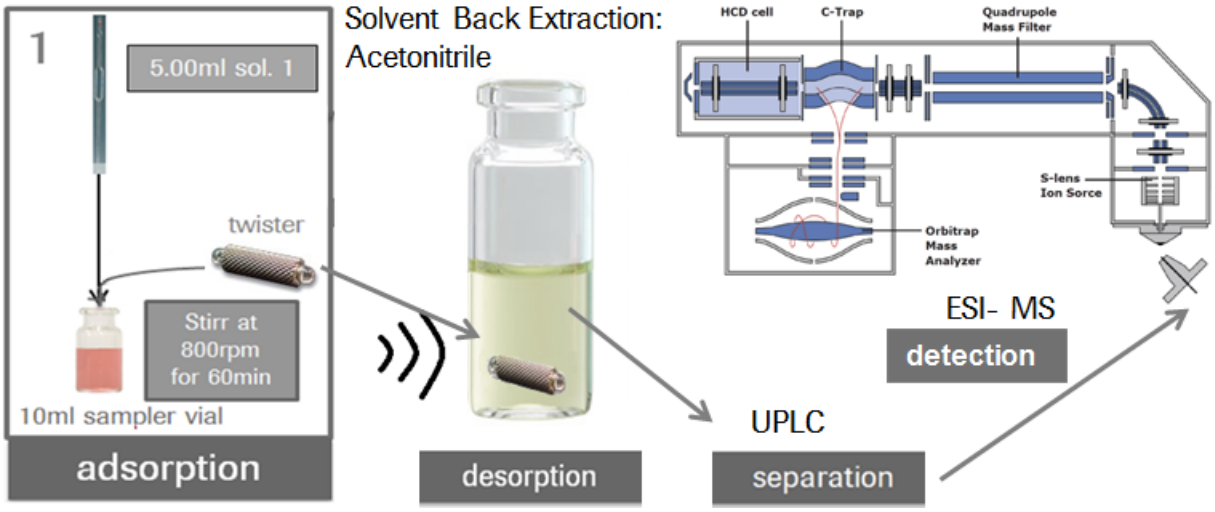


Figure 4.3: Schematic representation of the EG/PDMS coated stir bar handling, including the adsorption, the solvent-back-extraction (SBE) and the subsequent UPLC/QTOF-MS/MS analysis of the sample.

Risk assessments of extractables

All identified extractables were evaluated regarding potential health and safety concerns [15, 35]. The detected substances were classified in three categories with a generic threshold of toxicological concern (TTC) according to the Cramer Class concept, which provides guidelines for structural safety alerts [36, 37]. Categorization was performed based on the data collected by Jenke et al. [38], and using the ELSIE-database [39] and Toxtree Software (IDEAconsult Ltd) [40]. Beside aflatoxin-, N-nitroso- or azoxy-compounds that were considered as high-potent mutagen, in vitro mutagenicity data was collected of ames

salmonella typimurium and mouse lymphoma tests by literature [18].

4.2.3 Leachables

In order to evaluate the leaching behavior of SUSs in pharmaceutical drug manufacturing, the leachable-study was carried out by taking multiple samples directly from the filling line (sampling points marked in Figure 4.4). Two separate filling runs were examined (n=2) for each drug product (drug product 1-3, Table 4.2). To demonstrate a worst-case scenario, the maximum detected concentration of the two runs was listed and not the average.

To report leachables above a relevant toxic concentration an analytical evaluation thresh-

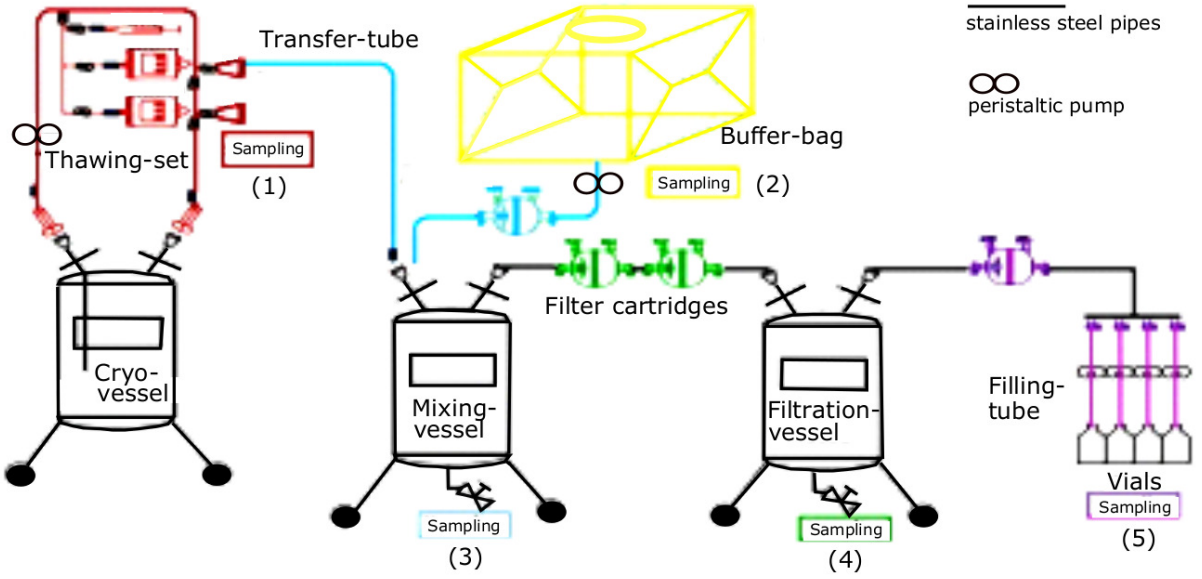


Figure 4.4: Simplified schematic representation of the filling line that was considered in this study. The different SUSs (pictured in color) and the representative sampling points are highlighted. The filling process for all tested products started with thawing of the deep-frozen drug substance in stainless steel vessels using a SUS, comprised of a thawing tube and a connector (circulating pump: 1 L/min). Subsequently, the respective drug substance was pooled by transferring the product from various cryo-vessels via silicon tubing to a steel-based mixing vessel. For the drug products 1 and 2 buffer, prepared in a disposable bag, was fed with varying volumes in using a peristaltic pump (300 rpm). Furthermore, the product was filtered using a product specific number of single-use filters in polymer cartridges. Finally, a silicon filling hose in combination with a peristaltic pump and shut-off devices was used for injection of the product into vials.

old (AET) concept was applied. Therefore, the ICH M7 guideline defines an acceptable

daily intake (ADI) based on the worst-case assumption that all impurities may be of genotoxic concern. For the calculation the maximum lifetime dosing days, which are the number of days are considered, which describes the number of days in a patient's life for which the drug is administered [17].

Sample Preparation

To analyze and quantify leachables with relevant concentrations in protein drug products an enrichment and preparation step for GC and UPLC analytic was required. To ensure sufficient sensitivity, SBSE was performed using a stir-bar (Twister, Gerstel GmbH) consisting of a 10mm long glass-encapsulated magnetic stir-bar. Stir-bars used with GC were coated 24 μL of pure PDMS and those used with UPLC were coated with 32 μL of the more polar EG(5%)-PDMS mix. As the SBSE technique is highly dependent on the substances' octanol/water coefficient (log $K_{O/W}$) [22], spiking-substances were chosen to represent both, the lower limit of recovery and the optimal field of application.

(PDMS)-SBSE Coupled with TDS-GC-MS

For the GC-analysis, 1 mL drug sample was mixed with 4 mL WfI to ensure a total cover of the stir-bar. The PDMS coated stir-bar was exposed to the solution for 1 h and stirred at 800 rpm and 19°C. Following the extraction step the stir-bar was rinsed with WfI, dried by dabbing and placed in the autosampler. The operating parameters differ from the extractable-method regarding the TDS end temperature of 300°C and the CIS initial temperature of -20°C (listed in Table 4.3). Blank measurements were performed by immersing a stir-bar in pure WfI, to verify the system contamination by estimating the background noise. Reference measurements were done with stir-bars subjected to THF-methacrylate and BHT-aldehyde spiked drug matrices. To make sure that the spiking substances are not confounded by the same leachables in the drug product, drug product blanks were performed as well. For this purpose, the spiking standards were dispersed in ethanol by sonication at a concentration of 0.1 g/L and diluted with WfI to simulate the drug product specific AET. To obtain semi-quantitative measurements, linearity solutions were prepared from the same stock solution by diluting with WfI to 0.001, 0.005, 0.01, 0.05 and 0.1 mg/L. Linear regression analysis was performed, and the correlation coefficient (r^2) was estimated using Masshunter Quantitative Analysis (Gerstel GmbH). The limits of detection (LOD) and quantification (LOQ) were calculated as the minimal amount of analytes that result in a signal-to-noise-ratio (SNR) of 3 or 10, respectively. Here, the SNR is defined as the peak height of the spiking substance and the baseline noise adjacent to the peak including a range of 0.05 min before and after the peak. The noise value for each noise range is computed as the standard deviation of the baseline over the mean of the selected noise region. The SNRs were determined at four concentrations, and the results were linearly interpolated. Confidence intervals were calculated using the fit residuals.

(EG/PDMS)-SBSE Coupled with SBE-UPLC/QTOF-MS/MS

The EG/PDMS coated stir-bar was immersed in 5 mL drug sample and stir-bar handling was performed as described above. For the SBE-step, the stir-bar was transferred in 1 mL pure acetonitrile and stirred for another 30 min, followed by a sonication-step of 20 min. Of the generated solution 10 µL were directly injected into the UPLC system (same operating parameters listed as the extractable-method, in Table 4.3). For the blank measurement the SBSE procedure was conducted in pure WfI. The reference measurement was performed by dissolving the polar spiking substances in ethyl acetate under sonication at a concentration of 1 g/L. Again, the specific AET was simulated and linearity solutions were prepared by diluting the stock solution with WfI corresponding to concentrations of 0.01, 0.025, 0.05, 0.075, 0.1 and 0.2 mg/L, respectively. For each of the spiking substances linearity, LOD and LOQ were analyzed as described above.

Safety Concern Thresholds for Mutagenic Leachables

Leachables with concentrations above the estimated AET were characterized using the NIST library and an internal library (Roche GmbH), their exact concentration determined and reported for potential toxicological assessments. To demonstrate leachable-profiles over the filling process and to assign leachables to each SUS by matching to the generated extractable-footprints, even leachables below the AET were identified and semi-quantified for this study.

4.2.4 Absorbance and Filtering of Leachables

In order to retrace leached substances inside the filling line, a scale-down version of the filling line was designed. The endless circle filling line comprised an identical scale-down model of the applied filter, the transfer and the filling tubing. A peristaltic pump was used to circulate the test-solution in the system for 3 h at a speed of 3.5 L/h. The system was run with 0.1 ppm diethyl phthalate spiked WfI to observe a potential absorption of the plasticizer by SUSs in the system. The concentration of the spiking substance in the solution was analyzed after the test-run with the use of the SBSE method in combination with TD-GC-MS, as described above.

4.2.5 Impact of the Active Ingredient on the Leachable Spectrum

To uncover potential interaction between the active ingredient and the leachable release, samples were taken during the drug product filling, as depicted in Figure 4.4, for fillings of drug product product 4 and placebo (Table 4.2). Leachable profiles were compared between the runs regarding leachable types and concentrations. This enabled to reveal possible extraction from the SUS driven by the protein during the filling process of the drug product. Furthermore, the identification of some leachables may be hampered for samples from the actual filling process as interaction with the active protein prevents detection with GC-and LC-MS. The impact of the protein on leachable extraction was analyzed with

SBSE and subsequent TDS-GC-MS analysis, as described above. The compared runs were performed with identical filling lines, except for two modifications. For the placebo-run, the buffer was prepared in a disposable bag instead of being dispensed from a cryovessel. Hence, no thawing set and no transfer tubing was applied for processing of the placebo.

4.2.6 Impact of the Drug Product pH

The impact of the drug product's pH-value on the leaching behavior during the filling process was evaluated. Four drug products with different pH-values were tested (pH ∼3.5 to ∼10.0, Table 4.2). The samples were drawn from two spots, the cryovessel and the filled vial at the end of the filling process. All other SUSs, except the buffer-bag were applied for the filling of the the four drug products (see Table 4.2). Filters with different membrane materials had to be used for different products, due to the major variation in pH-values. The extractable-footprints of all filters can be found in chapter 3. The samples were analyzed by SBSE and subsequent TDS-GC-MS analysis, as described above.

4.3 Results

4.3.1 Extractable-Profiles

Extractable profiles for all SUS as generated with thermal and static extraction are listed in the Tables 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 4.10, 4.11, 4.12 and 4.13,. After identification, the detected extractables were compared to the expected foot print of each material as taken from literature [41] (Table 4.1).

Table 4.4: Extractable-footprints conducted for the disposable bag by direct material thermal extraction in combination with TD-GC-MS. Detected extractables are categorized by their application, SCT and cancerogenic and mutagenic alerts. *SCT: Cramer Class: (1) =1800 μg/day; (2) = 540 μg/day; (3) = 90 μg/day; Special cases = 70 μg/day; Sulfates = 53 μg/day; Organophosphates and Carbamates = 18 μg/day; **Structural alert: Indicated based on in-silico studies by Benigni/Biossa rulebase (Toxtree V.2.6.13); ** Pos. Ames or Mouse Lymphoma test 0.15 μg/day

Chemical group	Extractables identified by TDS-GC-MS	Application	SCT	Cancero-genicity alert
Acids	Acetic acid	Monomer and solvent	(1)	Neg.
	Fatty acids: $C_{10}H_{20}O_2 - C_{18}H_{36}O_2$ (Decanoic acid - Octadecanoic acid)	Plasticizers, intermediats, slip agents and lubricants	(1)	Neg.
Alcohols	Benzyl alcohol	Solvents, preservatives and epoxy coating	(1)	Neg.
	2-Hexyl-1-decanol; 2-Hexyl-1-octanol	Metabolites of diethyl-hexylphthalate	(1)	Neg.
Aldehydes	Aldehydes with and without double bonds (trimethyl): $C_8H_{16}O - C_{17}H_{28}O$ (Octanal - 5,9,13-trimethyl-4,8,12-Tetradecatrienal)	Plasticizers and lubricants	(1)	Structural alerts
Alkanes	Straight chain and non-straight alkanes: $C_{10}H_{22} - C_{28}H_{58}$ (Decane / 9-methylheptadecane - Octacosane / 9-Octyl-eicosane)	Intermediates and lubricants	(1)	Neg.
Alkene	1-Nonadecene	Intermediate	(1)	Neg.
Amides	Pentanamide; 2-Methyl-1-ethylpyrrolidine	Polyamid and block builder	(3)	Neg.

Chemical group	Extractables identified by TDS-GC-MS	Application	SCT	Cancero-genicity alert
Aromatic compounds	o-Hydroxyacetophenone	Polymer product and solvent (fragment of antioxidants)	70 μg/day	Neg.
	1,3-Di-tert-butylbenzene; 3,4',5,6'-Tetrakis(2-methyl-2-propanyl)-2,3'-biphenyldiol	Polymer product and solvent (fragment of antioxidants)	(1)	Neg.
	2,4-Di-tert-butylphenol; 3-(4-(tert-Butyl)phenyl)-2-methylpropanal	Antioxidants (Irgafos 168 and BHT degradation product)	(1)	Neg.
	2,6-Di-tert-butyl-hydroquinone; 2,6-Bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione; 7,9-Di-tert-butyl-1-oxaspiro(4,5)-deca-6,9-diene-2,8-dione	Antioxidants	(2)	Structural alerts
	Octyl salicylate	Light stabilizer / UV absorber	(3)	Neg.
Ester	Triacetin; Isopropyl myristate	Anti-Infective Agent and polymer builder	(1)	Neg.
	Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester; Dibutyl adipate; Di(2-ethylhexyl) adipate	Plasticizers and solvents	(2)	Structural alerts
Lactams	Caprolactam	Monomer, Cross-linking agent for polyurethanes, coating and plasticizer	(3)	Neg.
Phosphates	Triethyl phosphate	Catalyst, plasticizer and strength agent	18 μg/day	Structural alerts

Chemical group	Extractables identified by TDS-GC-MS	Application	SCT	Cancero-genicity alert
Phthalate	Phthalic acid, butyl tetradecyl ester; Diisobutyl phthalate	Plasticizer (phthalate ester that increases flexibility, transparency, durability and longevity of plastics	(1)	Structural alerts
Siloxanes	Unknown	Polymer	(3)	Neg.
Sulfides	Disulfide, di-tert-dodecyl; 6-Tetradecanesulfonic acid, butyl ester; Sulfurous acid, octadecyl pentyl ester	Lubricants and lubricant additives	53 μg/day	Structural alerts
Unknown	m/z: 363; 378			

Table 4.5: Extractable-footprints conducted for the disposable bag by static extraction in combination with UPLC/QTOF-MS/MS. Detected extractables are categorized by their application, SCT and cancerogenic and mutagenic alerts. *SCT: Cramer Class: (1) =1800 μg/day; (2) = 540 μg/day; (3) = 90 μg/day; Special cases = 70 μg/day; Sulfates = 53 μg/day; Organophosphates and Carbamates = 18 μg/day; **Structural alert: Indicated based on in-silico studies by Benigni/Biossa rulebase (Toxtree V.2.6.13); ** Pos. Ames or Mouse Lymphoma test 0.15 μg/day

m/z	Chemical structure plus Probability [%]	Application	SCT*	Cancero-genicity Alerts**
Pos.: 237,1104	$C_8H_{21}N_2OP$ 97.8%			
Pos.: 329,1477	Unknown			
Pos.: 251,1263	$C_8H_{19}N_4O_3P$ 97,48%			
Pos.: 407,6746	$C_{19}H_{21}O_4P_3$ 89,71%			
Pos.: 307,1887	$C_{14}H_{22}N_6O_2$ 94,87%			
Pos.: 491,1694	Unknown			
Pos.: 337,0757	$C_6H_{20}N_6O_3Si_4$ 91,57%			

m/z	Chemical structure plus Probability [%]	Application	SCT*	Cancero-genicity Alerts**
Pos.: 463,1378	$C_{21}H_{19}N_8O_3P$ 91.31%			
Pos.: 485,2178	$C_{22}H_{29}O_4P_3$ 98,86%			
Pos.: 499,2334	Tetraethyl 1,4-diphenyl-1,1,4,4-butanetetracarboxylate $C_{28}H_{34}O_8$ 93.52%	Oligomer	(3)	Neg.
Pos.: 505,1842	Unknown			
Pos.: 519,2009	Unknown			
Pos.: 527,2623	$C_{27}H_{45}O_4P_3$ 90.43%			
Pos.: 521,3118	$C_{31}H_{36}N_8$ 67.22%			
Pos.: 535,3256	Unknown			
Pos.: 541,2781	$C_{26}H_{40}N_2O_{10}$ 70,62%			
Pos.: 555,2922	$C_{28}H_{38}N_6O_6$ 76,58%			
Pos.: 733,3194	Unknown			
Pos.: 563,3574	Disodium 4,4'-bis(2-sulfostyryl)biphenyl $C_{28}H_{20}Na_2O_6S_2$ tentative	Bleaching	53 μg/day	Structural alerts
Pos.: 577,3714	Unknown			
Pos.: 761,3491	Unknown			
Pos.: 591,3891	Unknown			
Pos.: 775,3652	Unknown			
Pos.: 789,3814	Unknown			
Pos.: 423,2520	Unknown			
Pos.: 501,3134	$C_{29}H_{43}N_4P$ 92,08%			
Pos.: 523,2955	$C_{29}H_{50}P_4$ 71,67%			
Pos.: 560,3869	$C_{32}H_{52}N_5P$ 86,14%			
Pos.: 699,4151 Neg.: 471,2693	$C_{39}H_{59}N_2O_7P$ 96,94%			
Neg.: 675,4207	Unknown siloxane	Polymer	(3)	Neg.

Table 4.6: Extractable-footprints conducted for the filter by direct material thermal extraction in combination with TD-GC-MS. Detected extractables are categorized by their application, SCT and cancerogenic and mutagenic alerts. *SCT: Cramer Class: (1) =1800 μg/day; (2) = 540 μg/day; (3) = 90 μg/day; Special cases = 70 μg/day; Sulfates = 53 μg/day; Organophosphates and Carbamates = 18 μg/day; **Structural alert: Indicated based on in-silico studies by Benigni/Biossa rulebase (Toxtree V.2.6.13); ** Pos. Ames or Mouse Lymphoma test 0.15 μg/day

Chemical group	Extractables	Application	SCT	Cancero-genicity alert
Alcohols	Ethanol, 1-(2-butoxyethoxy)-	Solvent	(1)	Structural alerts
	2-Hexyl-1-octanol; 2-hexyl-1-Decanol; 1-Hexadecanol	Metabolites of bis(2-ethylhexyl) phthalate (DEHP)	(1)	Neg.
	1-Dodecanol, 3,7,11-trimethyl-; 2-Isopropyl-5-methyl-1-heptanol; Tridecanol, 2-ethyl-2-methyl-; Octadecanol	Intermediate	(1)	Neg.
Aldehydes	Decanal	Plasticizers and lubricants	(1)	Structural alerts
Alkanes	Straight chain and non-straight alkanes: C_8H_{18} – $C_{28}H_{58}$ (4-methyl-Heptane - Octacosane)	Intermediates, antifoaming agents and lubricants	(1)	Neg.
Alkene	2,4-Dimethyl-1-heptene	Intermediate	(1)	Neg.
Aromatic com-pounds	2,4-Di-tert-butylphenol; Butylated Hydroxy-toluene (BHT), p-Octylacetophenone; 3,5-di-tert-Butyl-4-hydroxybenzaldehyde; 1H-Indene-4-acetic acid, 6-(1,1-dimethylethyl)-2,3-dihydro-1,1-dimethyl-	Antioxidants (Irgafos 168 and BHT degradation prod-uct)	(2)	Neg.

Chemical group	Extractables	Application	SCT	Cancero-genicity alert
	2,6-Bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione; 2.6-di-tert-butyl-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	Antioxidants	(2)	Structural alerts
Diol	2,4,7,9-Tetramethyl-5-decyn-4,7-diol	PE-polymer, Antifoaming agent, Wetting agent	(3)	Neg.
Ester	Di-(2E)-2-buten-1-yl adipate; Di(2-ethylhexyl) adipate (DEHA)	Plasticizers and solvents	(1)	Structural alerts
Lactams	Caprolactam	Monomer, Cross-linking agent for polyurethanes, coating and plasticizer	(3)	Neg.
Phthalate	Diethyl phthalate	Plasticizer	(1)	Structural alerts
Polycyclic aromatic hydrocar-bon	9,9-bis(methoxymethyl)fluorene	Catalyst	(3)	Structural alerts
Siloxanes	Cyclosilioxanes ($C_6H_{18}O_3Si_3 - C_{20}H_{60}O_{10}Si_{10}$); Siloxane chains ($C_7H_{21}O_2Si_3 - C_{16}H_{48}O_6Si_7$)	Monomers	(3)	Neg.
Unknown	m/z: 79; m/z: 219	unknown aromatic sub-stance		

Table 4.7: Extractable-footprints conducted for the filter by static extraction in combination with UPLC/QTOF-MS/MS. Detected extractables are categorized by their application, SCT and cancerogenic and mutagenic alerts. *SCT: Cramer Class: (1) =1800 μg/day; (2) = 540 μg/day; (3) = 90 μg/day; Special cases = 70 μg/day; Sulfates = 53 μg/day; Organophosphates and Carbamates = 18 μg/day; **Structural alert: Indicated based on in-silico studies by Benigni/Biossa rulebase (Toxtree V.2.6.13); ** Pos. Ames or Mouse Lymphoma test 0.15 μg/day

m/z	Chemical structure plus Probability [%]	Application	SCT*	Cancero-genicity Alerts**
Pos.: 519,2415	$C_{16}H_{41}N_4O_{11}P$ 91,53%			
Pos.: 213,1469	Propyl gallate $C_{10}H_{12}O_5$ tentative	Antioxidant	(2)	Neg.
Pos.: 573,2533	Unknown			
Pos.: 822,5035	Unknown			
Pos.: 806,5090 Neg.: 564,354	Unknown			
Neg.: 403,0623	Unknown			

Table 4.8: Extractable-footprints conducted for the thawing tubing and connector by direct material thermal extraction in combination with TD-GC-MS. Detected extractables are categorized by their application, SCT and cancerogenic and mutagenic alerts. *SCT: Cramer Class: (1) =1800 μg/day; (2) = 540 μg/day; (3) = 90 μg/day; Special cases = 70 μg/day; Sulfates = 53 μg/day; Organophosphates and Carbamates = 18 μg/day; **Structural alert: Indicated based on in-silico studies by Benigni/Biossa rulebase (Toxtree V.2.6.13); ** Pos. Ames or Mouse Lymphoma test 0.15 μg/day

Chemical group	Extractables	Application	SCT	Cancero-genicity alert
Alcohols	Ethanol, 1-(2-butoxyethoxy)- ; Ethanol, 2-(2-ethoxyethoxy)-	Solvents, preservatives and epoxy coating; used for the siloxane production	(1)	Structural alerts
	Benzyl alcohol	Solvents, preservatives and epoxy coating	(1)	Neg.
	2-Hexyl-1-decanol; 2-Ethyl-1-hexanol	Metabolites of diethyl-hexylphthalate	(1)	Neg.
	1-Tetradecanol	Intermediate	(1)	Neg.
Aldehydes	Aldehydes with and without double bonds (trimethyl): $C_9H_{18}O - C_{17}H_{28}O$ (Nonanal - 5,9,13-trimethyl-4,8,12-Tetradecatrienal)	Plasticizers and lubricants	(1)	Structural alerts
Alkanes	Straight chain and non-straight alkanes: $C_{12}H_{26} - C_{19}H_{40}$ (Dodeacane - 5,5-Diethylpentadecane)	Intermediates and lubri-cants	(1)	Neg.
Alkene	6-methyl-5-Hepten-2-one; 6,10-dimethyl-5,9-Undecadien-2-one; 1-Tetradecene	Intermediate	(1)	Neg.
Amides	Pentanamide; 2-Methyl-1-ethylpyrrolidine	Polyamid and block builder	(3)	Neg.

Chemical group	Extractables	Application	SCT	Cancero-genicity alert
Aromatic compounds	Acetophenone	Degradation product	70 μg/day	Neg.
	Benzaldehyde	Polymer products and solvents (fragments of antioxidants)	0.15 μg/day	Pos. Ames or Mouse Lymphoma test
	1-Phenoxypropan-2-ol; p-Octylacetophenone; 2,4-Di-tert-butylphenol; 1,3-Di-tert-butylbenzene;	Antioxidants (Irgafos 168 and BHT degradation product)	(1)	Neg.
	Bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione	Antioxidants	(2)	Structural alerts
	Tetramethylbutyl phenol	Antioxidants	35 μg/day	Neg.
	Phenol, 2,4-di-t-butyl-6-nitro- ; o-Hydroxybiphenyl	Preservative	0.15 μg/day	Pos. Ames or Mouse Lymphoma test
Benzene	Benzene, 1,1'-(1-methylethylidene)bis[4-methoxy]- ; 1-methoxy-4-[(E)-2-phenylethenyl]benzene	Based on Bisphenol A; epoxy resins used for polycarbonates; processing for polyvinyl chloride; monomer	(3)	Neg.

Chemical group	Extractables	Application	SCT	Cancero-genicity alert
Ester	Trimethylolpropane trimethacrylate	Intermediate and adhesive; Photosensitive	(1)	Neg.
	Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester; Benzoic acid, 4-(4-butylcyclohexyl)-, 4-butoxy-2,3-dicyanophenyl ester	Plasticizers and solvents	(2)	Neg.
Ketone	Cyclohexanone	Solvent and intermediate for caprolactam	(2)	Structural alerts
	Benzophenone	Photoinitiator; building block; UV-absorber	(3)	Neg.
Lactams	Caprolactam	Monomer, Cross-linking agent for polyurethanes, coating and plasticizer	(3)	Neg.
Phthalates	Diethyl phthalate; Phthalic acid, hept-4-yl isobutyl ester	Plasticizer	(1)	Structural alerts
Polycyclic aromatic hydrocar-bon	1,3-di-iso-propylnaphthalene, 2,6-Diisopropylnaphthalene	Production of phthalates; Monomer; Anti-Infective Agents	(3)	Structural alerts
Siloxanes	Silanediol, dimethyl-; 2,5-Bis[(trimethylsilyl)oxy]benzaldehyde; Cyclosiliox-anes ($C_6H_{18}O_3Si_3$ – $C_{20}H_{60}O_{10}Si_{10}$); Siloxane chains ($C_7H_{21}O_2Si_3 - C_{16}H_{48}O_6Si_7$)	Monomer	(3)	Neg.
Triazine	1,3,5-Triazine-2,4,6(1H,3H,5H)-trione, 1,3,5-tri-2-propenyl-	Crosslinking and curing agent	(3)	Neg.
Unknown	m/z: 401			

Table 4.9: Extractable-footprints conducted for the thawing tube by static extraction in combination with UPLC/QTOF-MS/MS. Detected extractables are categorized by their application, SCT and cancerogenic and mutagenic alerts. *SCT: Cramer Class: (1) =1800 μg/day; (2) = 540 μg/day; (3) = 90 μg/day; Special cases = 70 μg/day; Sulfates = 53 μg/day; Organophosphates and Carbamates = 18 μg/day; **Structural alert: Indicated based on in-silico studies by Benigni/Biossa rulebase (Toxtree V.2.6.13); ** Pos. Ames or Mouse Lymphoma test 0.15 μg/day

m/z	Chemical structure plus Probability [%]	Application	SCT*	Cancero-genicity Alerts**
Pos.: 114,0920	Caprolactam $C_6H_{11}NO$ 99,99%	Monomer	(3)	Neg.
Pos.: 195,0897	Butyl 4-hydroxybenzoate $C_{11}H_{14}O_3$ 89.98%	Antioxidant	(3)	Neg.
Pos.: 371,0658	$C_{22}H_{14}N_2S_2$ 80,56%			
Pos.: 499,1291 Neg.: 223,0284	$C_{26}H_{24}N_2O_5S$ 86,87%			

m/z	Chemical structure plus Probability [%]	Application	SCT*	Cancero-genicity Alerts**
Unknown Siloxanes	Pos.: 237,0794 (Silquest® A-187 gamma-glycidoxypropyltrimethoxysilane); Pos.: 263,0575 Neg.: 239,0592 ($C_6H_{20}O_4Si_3$ 81,83% Hexamethyltrisiloxane-1,5-diol); Pos.: 337,0771 Neg.: 313,0778; Pos.: 411,0954 Neg.: 165,0405 ($C_{10}H_{32}O_6Si_5$ 96,66% Decamethyldihydroxypentasiloxane); Pos.: 485,1141 Neg.: 239,0589; Pos.: 397,0429 Neg.: 373,0449 ($C_8H_{26}O_7Si_5$ 99,99% 2,6-dihydroxy-2,4,4,6,8,8,10,10-octamethylcyclopentasiloxane); Pos.: 559,1352; Pos.: 633,1509 Neg.: 313,0780; Pos.: 573,1484; Pos.: 702,2165 Neg.: 729,1756; Pos.: 642,2109; Pos.: 656,2278 Neg.: 723,0951; Pos.: 776,2320 Neg.: 857,1155; Pos.: 716,2287 Neg.: 797,1146; Pos.: 850,2512; Pos.: 730,2446; Pos.: 790,2484; Pos.: 924,2723; Pos.: 610,1854	Polymer	(3)	Neg.
Neg.: 299,0258	Unknown substance			
Neg.: 345,0319	Bisphenol P $C_{24}H_{26}O_2$ tentative	Epoxy resins used for polycarbonates; processing for polyvinyl chloride; oligomer	(3)	Neg.

Table 4.10: Extractable-footprints conducted for the transfer tubing by direct material thermal extraction in combination with TD-GC-MS. Detected extractables are categorized by their application, SCT and cancerogenic and mutagenic alerts. *SCT: Cramer Class: (1) =1800 μg/day; (2) = 540 μg/day; (3) = 90 μg/day; Special cases = 70 μg/day; Sulfates = 53 μg/day; Organophosphates and Carbamates = 18 μg/day; **Structural alert: Indicated based on in-silico studies by Benigni/Biossa rulebase (Toxtree V.2.6.13); ** Pos. Ames or Mouse Lymphoma test 0.15 μg/day

Chemical group	Extractables	Application	SCT	Cancero-genicity alert
Acids	Hexanoic acid, 2-ethyl-	Plasticizers, intermediats, slip agents and lubricants	(1)	Neg.
Alcohols	Ethanol, 1-(2-butoxyethoxy)-; Ethanol, 2-(2-ethoxyethoxy)-;	Solvents, preservatives and epoxy coating; used for the siloxane production	(1)	Structural alerts
	Benzyl alcohol	Solvents, preservatives and epoxy coating	(1)	Neg.
	1-Hexanol, 2-ethyl-,	Metabolites of diethyl-hexylphthalate	(1)	Neg.
	Cyclohexanol, 4-(1,1-dimethylethyl)-	Catalyst and precursor DEHP	(2)	Neg.
	2-Propanol, 1-(2-methoxy-1-methylethoxy)-; E-10-Pentadecenol, 1-Undecanol, 1-Dodecanol, 3,7,11-trimethyl-	Intermediate	(1)	Neg.
Aldehydes	$C_9H_{18}O - C_{12}H_{24}O$ (Nonanal – Dodecanal)	Plasticizers and lubricants	(1)	Structural alerts
Alkanes	Straight chain and non-straight alkanes: $C_{12}H_{26} - C_{28}H_{58}$ (Dodecane / 9-methylheptadecane - Octacosane)	Intermediates and lubricants	(1)	Neg.

Chemical group	Extractables	Application	SCT	Cancero-genicity alert
Alkene	5-Hepten-2-one, 6-methyl. 11,13-Dimethyl-12-tetradecen-1-ol acetate, 5,9-Undecadien-2-one, 6,10-dimethyl-	Intermediate	(1)	Neg.
Amides	1-Ethyl-2-pyrrolidinone	Polyamid and block builder	(3)	Neg.
Aromatic compounds	Acetophenone	Degradation product	70 μg/day	Neg.
	1,3-Di-tert-butylbenzene	Polymer products and solvents (fragments of antioxidants)	(1)	Neg.
	Phenol, m-tert-butyl-; 2,4-Di-tert-butylphenol; unknown aromatic compound: 3,5-di-tert-Butyl-4-hydroxybenzaldehyde, m-Toluic acid, 4-pentadecyl ester	Antioxidants (Irgafos 168 and BHT degradation product)	(1)	Neg.
	2,6-Di-tert-butyl-hydroquinone	Antioxidants	(2)	Structural alerts
	Phenol, 2,4-di-t-butyl-6-nitro-,	Preservative	0.15 μg/day	Pos. Ames or Mouse Lymphoma test
	1,1'-Biphenyl, 2,2',5,5'-tetramethyl	Oligomer	(3)	Neg.
Ester	Glycerol 1,2-diacetate	Anti-Infective Agents	(3)	Neg.
	Benzothiazole, 1(3H)-Isobenzofuranone	Rubber accelerator, adjuvant and dye additive	(3)	Neg.
	Acetic acid, phenylmethyl ester, Pentadecanoic acid, 14-methyl-, methyl ester, Benzoic acid, 4-(4-butylcyclohexyl)-, 2,3-dicyano-4-(pentyloxy)phenyl ester	Plasticizers and solvents	(2)	Structural alerts

Chemical group	Extractables	Application	SCT	Cancero-genicity alert
Ketone	Benzophenone	Photoinitiator; building block; UV-absorber	(3)	Neg.
Polycyclic aromatic hydrocar-bon	2,6-Diisopropylnaphthalene; 9,9-Dimethyl-9-silafluorene	Production of phthalates; Monomer; Anti-Infective Agents	(3)	Structural alerts
Lactams	Caprolactam	Monomer, Cross-linking agent for polyurethanes, coating and plasticizer	(3)	Neg.
Phosphates	Triethyl phosphate	Catalyst, plasticizer and strength agent	18 μg/day	Structural alerts
Phthalate	Diethyl Phthalate; Phthalic acid, hept-4-yl isobutyl ester; Phthalic acid, isobutyl octadecyl ester	Plasticizer	(1)	Structural alerts
Siloxanes	Silanediol, dimethyl-; 2,5-Bis[(trimethylsilyl)oxy]benzaldehyde; Cyclosiliox-anes ($C_6H_{18}O_3Si_3$ – $C_{20}H_{60}O_{10}Si_{10}$); Siloxane chains ($C_7H_{21}O_2Si_3 - C_{16}H_{48}O_6Si_7$)	Monomer	(3)	Neg.
Triazine	1,3,5-Triazine-2,4,6(1H,3H,5H)-trione, 1,3,5-tri-2-propenyl-	Crosslinking and curing agent	(3)	Neg.
Unknown	m/z: -415; 327; 73			
	- 401; 489; 163; 327; 415;			

Table 4.11: Extractable-footprints conducted for the transfer tube by static extraction in combination with UPLC/QTOF-MS/MS. Detected extractables are categorized by their application, SCT and cancerogenic and mutagenic alerts. *SCT: Cramer Class: (1) =1800 μg/day; (2) = 540 μg/day; (3) = 90 μg/day; Special cases = 70 μg/day; Sulfates = 53 μg/day; Organophosphates and Carbamates = 18 μg/day; **Structural alert: Indicated based on in-silico studies by Benigni/Biossa rulebase (Toxtree V.2.6.13); ** Pos. Ames or Mouse Lymphoma test 0.15 μg/day

m/z	Chemical structure plus Probability [%]	Application	SCT*	Cancero-genicity Alerts**
Pos.: 191,0233	$C_3H_{12}O_5P_2$ 99,77%	Initiator for [1,3-phenylenebis(methy1ene)] bisphosphonic acid anti-inflammatory agent	18 μg/day	Structural alerts
Pos.: 217,1056 Neg.: 327,9723	$C_4H_{17}N_4O_4P$ 94.86%			
Pos.: 114,0921	Caprolactam $C_6H_{11}NO$ 99.99%	Monomer	(3)	Neg.
Pos.: 171,1002	Phthalic anhydride 87.42%	Plasticizer	(1)	Structural alerts
Pos.: 233,0702	Polyethylene glycol, ubiquitous polyether $[C_2H_4O]$nH_2O 99.99%	Polymer; Lubricating coating	(1)	Neg.
Pos.: 245,0652	$C_4H_{13}N_4O_6P$ 87.42%			
Pos.: 239,0907	Dimethyl 2-hydroxy-1,3-cyclohexanedicarboxylate $C_{10}H_{16}O_5$ 84,72%		(1)	Neg.
Pos.: 241,0690	Triacetin $C_9H_{13}O_6$ 99.99%	Anti-Infective Agent and polymer builder	(1)	Neg.
Pos.: 227,0695	Unknown			
Pos.: 185,1155	Diethylene glycol butyl ether (2-(2-Butoxyethoxy)ethanol) $C_8H_{18}O_3$ 99.99%	Solvent	(1)	Structural alerts

m/z	Chemical structure plus Probability [%]	Application	SCT*	Cancero-genicity Alerts**
Pos.: 223,0952 Pos.: 245,0794	Diethyl Phthlalate C12H14O4 99.99%	Plasticizer	(1)	Structural alerts
Pos.: 205,0610	Triethyl phosphate $C_6H_{15}O_4P$ 99,99%	Catalyst, plasticizer and strength agent	18 μg/day	Structural alerts
Pos.: 227,1262	$C_{10}H_{20}O_4$ 96,09%			
Pos.: 213,1469	2-[(4,6-diamino-1,3,5-triazin-2-yl)amino]-2-ethylpropane-1,3-diol C8H16N6O 96.54%	Crosslinking agent	(3)	Neg.
Pos.: 407,0753	$C_{18}H_{10}N_6O_6$ 98.89%			
Pos.: 495,1280	Unknown			
Pos.: 451,1020	$C_{12}H_{20}O_9$ 58.12%			
Pos.: 293,2096	1,9-dioxacyclooctadecan-2-one $C_{16}H_{30}O_3$ 58.03%	Polymer	(1)	Neg.
Pos.: 337,2358	n-butyl-phthalybutyl glycolate $C_{18}H_{24}O_6$ 99.90%	Plasticizer	(1)	Structural alerts
Pos.: 599,1168	$C_{28}H_{26}O_{12}$ 47.27%			
Pos.: 643,1439	Unknown			
Pos.: 577,1358	Ethylene Terephthalate Cyclic Trimer $C_{30}H_{24}O_{12}$ 87.33%	Oligomer	(1)	Neg.
Pos.: 401,2893	$C_{17}H_{37}N_8OP$ 89.84%			
Pos.: 411,0947	Decamethyldihydroxypentasiloxane $C_{10}H_{32}O_6Si_5$ 96,66%	Polymer	(3)	Neg.
Pos.: 791,1589	Unknown			
Pos.: 485,1136	Unknown Siloxane Polymer (3) Neg.			
Pos.: 481,3511	Bis(2,2,6,6-tetramethyl-4-piperidyl) sebacate (TInuvin 770) $C_{28}H_{52}N_2O_4$ tentative	Light stabilizer	(3)	Neg.
Neg.: 209,0457	Unknown			
Neg.: 154,9902	Unknown			
Neg.: 188,9501	Unknown			

m/z	Chemical structure plus Probability [%]	Application	SCT*	Cancero-genicity Alerts**
Neg.: 401,0865	Acetoxy-1,2,3-propanetricarboxylic acid, tributyl ester $C_{20}H_{34}O_8$ tentative	Intermediate and adhesive; Photosensitive	(1)	Neg.
Neg.: 357,0597	Irganox® 1081 6,6'-di-tert-butyl-2,2'-thiodi-p-cresol $C_{22}H_{30}O_2S$ tentative	Antioxidant	53 μg/day	Structural alerts

Table 4.12: Extractable-footprints conducted for the filling tubing by direct material thermal extraction in combination with TD-GC-MS. Detected extractables are categorized by their application, SCT and cancerogenic and mutagenic alerts. *SCT: Cramer Class: (1) =1800 μg/day; (2) = 540 μg/day; (3) = 90 μg/day; Special cases = 70 μg/day; Sulfates = 53 μg/day; Organophosphates and Carbamates = 18 μg/day; **Structural alert: Indicated based on in-silico studies by Benigni/Biossa rulebase (Toxtree V.2.6.13); ** Pos. Ames or Mouse Lymphoma test 0.15 μg/day

Chemical group	Extractables	Application	SCT	Cancero-genicity alert
Acids	n-Decanoic acid, 9-Hexadecenoic acid	Plasticizers, intermediats, slip agents and lubricants	(1)	Neg.

Chemical group	Extractables	Application	SCT	Cancero-genicity alert
Alcohols	Ethanol, 1-(2-butoxyethoxy)-; Ethanol, 2-(2-ethoxyethoxy)-; Ethanol, 2-(2-butoxyethoxy)-, acetate,	Solvents, preservatives and epoxy coating; used for the siloxane production	(1)	Structural alerts
	Benzyl alcohol	Solvents, preservatives and epoxy coating	(1)	Neg.
	2-Hexyl-1-octanol	Metabolites of diethylhexylphthalate	(1)	Neg.
	Cyclohexanol, 4-(1,1-dimethylethyl)-	Catalyst and precursor DEHP	(2)	Neg.
	2-Propanol, 1-(2-methoxy-1-methylethoxy)-; 1-Dodecanol, 1-Decanol, 2-hexyl-, 1-Tetradecanol, 2-Methyl-1-undecanol, 1-Hexadecanol, 2-methyl-	Intermediate	(1)	Neg.
Aldehydes	$C_9H_{18}O - C_{12}H_{24}O$ (Nonanal – Dodecanal)	Plasticizers and lubricants	(1)	Structural alerts
Alkanes	Straight chain and non-straight alkanes: $C_{12}H_{26}$ – $C_{28}H_{58}$ (Dodecane / 9-methylheptadecane - Octacosane)	Intermediates and lubricants	(1)	Neg.
Alkene	17-Pentatriacontene	Intermediate	(1)	Neg.
Amides	2-Pyrrolidinone, 1-methyl-, 4(1H)-Pteridinone, 2-amino-	Polyamid and block builder	(3)	Neg.

Chemical group	Extractables	Application	SCT	Cancero-genicity alert
Aromatic compounds	Acetophenone	Degradation product	70 μg/day	Neg.
	Benzaldehyde	Polymer products and solvents (fragments of antioxidants)	0.15 μg/day	Pos. Ames or Mouse Lymphoma test
	1,3-Di-tert-butylbenzene; Benzene, 1,2,4-trimethyl-5-(1-methylethyl)-	Polymer products and solvents (fragments of antioxidants)	(1)	Neg.
	2,6-Di-tert-butyl-hydroquinone; unknown aromatic compound; 2-Octyl benzoate	Antioxidants (Irgafos 168 and BHT degradation product)	(2)	Neg.
	2,6-Di-tert-butyl-hydroquinone	Antioxidants	(2)	Structural alerts
	Phenol, 2,4-di-t-butyl-6-nitro-,	Preservative	0.15 μg/day	Pos. Ames or Mouse Lymphoma test

Chemical group	Extractables	Application	SCT	Cancero-genicity alert
Ester	Glycerol 1,2-diacetate; 1-Methoxy-2-propyl acetate (Glycerol)	Anti-Infective Agents	(3)	Neg.
	Nonanoic acid, 9-oxo-, methyl ester, Dodecanedioic acid, bis(tert-butyldimethylsilyl) ester, Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester, m-Toluic acid, 4-hexadecyl ester, Pentadecanoic acid, 14-methyl-, methyl ester Acetic acid, phenylmethyl ester, Methacrylic acid, nonadecyl ester, Hexadecanoic acid, 2-methylpropyl ester	Plasticizers and solvents	(2)	Neg.
Ketone	Benzophenone	Photoinitiator; building block; UV-absorber	(3)	Neg.
Lactams	Caprolactam	Monomer, Cross-linking agent for polyurethanes, coating and plasticizer	(3)	Neg.
Polycyclic aromatic hydrocarbon	9,9-Dimethyl-9-silafluorene	Production of phthalates; Monomer; Anti-Infective Agents	(3)	Structural alerts
Lactams	Caprolactam	Monomer, Cross-linking agent for polyurethanes, coating and plasticizer	(3)	Neg.
Phosphates	Triethyl phosphate;Tributyl phosphate	Catalyst, plasticizer and strength agent	18 μg/day	Structural alerts
Phthalate	Diethyl Phthalate; Diisobutyl phthalate; Butyl Isodecyl Phthalate; Benzoic acid, 4-(4-butylcyclohexyl)-, 2,3-dicyano-4-ethoxyphenyl ester	Plasticizer	(1)	Structural alerts

Chemical group	Extractables	Application	SCT	Cancero-genicity alert
Siloxanes	Silanediol, dimethyl-; 2,5-Bis[(trimethylsilyl)oxy]benzaldehyde; Cyclosiliox-anes ($C_6H_{18}O_3Si_3$ – $C_{20}H_{60}O_{10}Si_{10}$); Siloxane chains ($C_7H_{21}O_2Si_3 - C_{16}H_{48}O_6Si_7$)	Monomer	(3)	Neg.
Triazine	1,3,5-Triazine-2,4,6(1H,3H,5H)-trione, 1,3,5-tri-2-propenyl-	Crosslinking and curing agent	(3)	Neg.

Table 4.13: Extractable-footprints conducted for the filling tubing by static extraction in combination with UPLC/QTOF-MS/MS. Detected extractables are categorized by their application, SCT and cancerogenic and mutagenic alerts. *SCT: Cramer Class: (1) =1800 μg/day; (2) = 540 μg/day; (3) = 90 μg/day; Special cases = 70 μg/day; Sulfates = 53 μg/day; Organophosphates and Carbamates = 18 μg/day; **Structural alert: Indicated based on in-silico studies by Benigni/Biossa rulebase (Toxtree V.2.6.13); ** Pos. Ames or Mouse Lymphoma test 0.15 μg/day

m/z	Chemical structure plus Probability [%]	Application	SCT*	Cancero-genicity Alerts**
Pos.: 202,1807	$C_{11}H_{23}NO_2$ 99.99%			
Pos.: 191,0239	$C_3H_{12}O_5P_2$ 99.77%			
Pos.: 173,0796	Triethylene glycol $C_6H_{14}O_4$ 96.05%	Solvent	(1)	Neg.
Pos.: 215,1260	Uvinul 3000 2,4-dihydroxybenzophenone $C_{13}H_{10}O_3$ tentative	Photoinitiator	(3)	Neg.
Pos.: 217,1056 Neg.: 327,9723	$C_4H_{17}N_4O_4P$ 94.86%			
Pos.: 163,0611 Neg.: 327,9812	$C_5H_{14}O_2Si_2$ 93.46%	Polymer	(3)	Neg.
Pos.: 261,1310	Pentaethyleneglycol $C_{10}H_{22}O_6$ 98,85%	Solvent	(1)	Neg.

m/z	Chemical structure plus Probability [%]	Application	SCT*	Cancero-genicity Alerts**
Pos.: 199,0587	Glycerine acetate $C_7H_{12}O_5$ 99.99%	Breakdown product of Triacetin	(1)	Neg.
Pos.: 305,1585	$C_8H_{25}N_4O_6P$ 96.15%			
Pos.: 114,0921	Caprolactam $C_6H_{11}NO$ 99.99%	Monomer	(3)	Neg.
Pos.: 177,1010	$C_8H_{14}N_2O$ 95.78%			
Pos.: 349,1844	Polyethylene glycol, ubiquitous polyether $[C_2H_4O]$nH2O 98.46%	Polymer, lubricating coating	(1)	Neg.
Pos.: 255,0850	$C_7H_{19}N_2O_3P$ 99.52%			
Pos.: 239,0900	Dimethyl 2-hydroxy-1,3-cyclohexanedicarboxylate $C_{10}H_{16}O_5$ 84.72%	Intermediate	(1)	Neg.
Pos.: 241,0700	Triacetin $C_9H_{13}O_6$ 99.99%	Anti-Infective Agent and polymer builder	(1)	Neg.
Pos.: 481,2631	Polyethylene glycol, ubiquitous polyether $[C_2H_4O]$nH2O 90.92%	Polymer, lubricating coating	(1)	Neg.
Pos.: 525,2886	Polyethylene glycol, ubiquitous polyether $[C_2H_4O]$nH2O 91.91%	Polymer, lubricating coating	(1)	Neg.
Pos.: 273,1678	$C_7H_{23}N_8P$ 91.01%			
Pos.: 205,0613	Triethyl phosphate $C_6H_{15}O_4P$ 99.99%	Catalyst, plasticizer and strength agent	18 μg/day	Structural alerts
Pos.: 244,1428	$C_8H_{18}N_7P$ 78.84%			
Pos.: 223,0946	Diethyl phthalate $C_{12}H_{14}O_4$ 99.99%	Plasticizer	(1)	Structural alerts
Pos.: 249,1111	$C_9H_{21}N_2OP$ 88.30%			
Pos.: 227,1262	Unknown			
Pos.: 279,0070	Dibutyl phthalate $C_{16}H_{22}O_4$ 99,99% Plasticizer (1) Structural alerts			
Pos.: 227,1266	$C_{10}H_{20}O_4$ 91.17%			
Pos.: 371,2419	$C_{17}H_{41}O_2P_3$ 71.94%			

m/z	Chemical structure plus Probability [%]	Application	SCT*	Cancero-genicity Alerts**
Neg.: 170,9881	Unknown			
Neg.: 471,2769	Unknown			
Neg.: 1012,4156	Unknown			
Neg.: 1142,0212	Unknown			

Silicone Tubes

GC-MS analysis reveals comparable chromatographic fingerprints with respect to the peak number and magnitude for all three tested silicone-tubes (see Figure 4.5, C-D-E). The chromatograms are dominated by large peaks attributable to a homologous series of polydimethyl-siloxanes (Si), as commonly observed in thermal extraction from silicone materials [42]. The largest peaks are associated with cyclic oligomers with the general structural formula of $[(CH_3)_2SiO]_n$. A number of minor peaks were identified as various phthalate based plasticizers, which are used to promote flexibility of the polymer structure of the tube. Residual substances detected in the chromatogram include, different kinds of naphthalenes, that are in turn used for the production of phthalates, 2-hexyl-1-decanol and 2-ethyl-1-hexanol, metabolites of the production in particular for diester bis(2-ethylhexyl) phthalate (DEHP) and 4-(1,1-dimethylethyl)-cyclohexanol, a catalyst and precursor of DEHP. Although many phthalates and intermediates of the production of DEHP were detected as extractables, DEHP itself was not observed. Furthermore, beside many intermediates and lubricants 1-(2-butoxyethoxy)-ethanol and 2-(2-ethoxyethoxy)-ethanol, which are commonly used as solvents and preservatives in siloxane production were found in moderate amounts in the extractable profile. Two substances were observed that are known to be carcinogen, the antioxidant 2,4-di-tert-butyl-6-nitro-phenol and traces of the solvent benzaldehyde [43]. The Si thawing tubing demonstrated a large number of siloxanes by UPLC with static extraction, which can likely be linked to its softer material properties (peaks from 7.5 to 9.5 min; Figure 4.6). The transfer and filling tube contained triethyl phosphate, tributyl phosphate and a high concentration of the anti-infective agent 1,2-diacetate-glycerol and triacetin (retention time of 2.32 min, Figure 4.6).

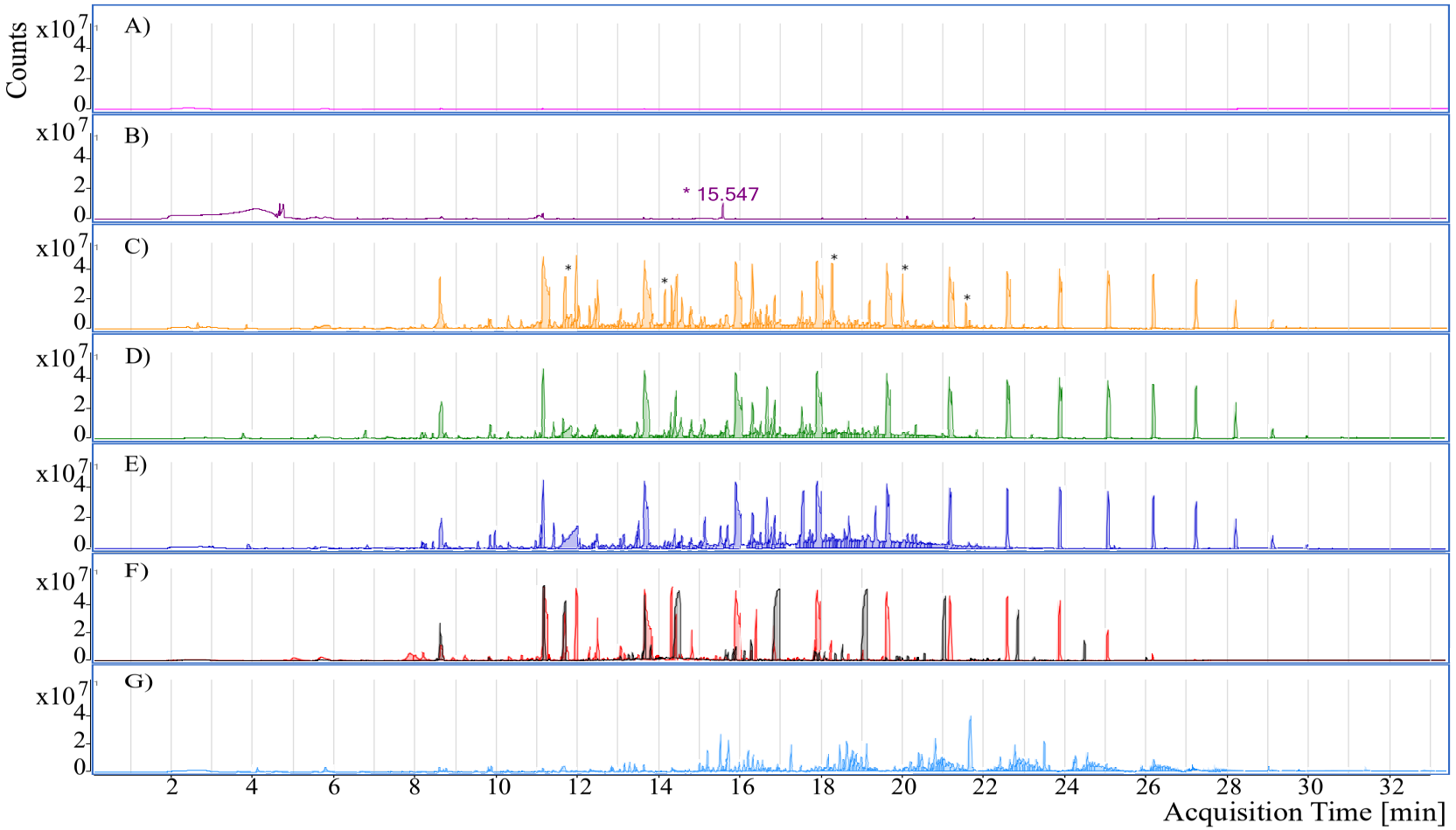


Figure 4.5: Direct thermal extraction at 150°C from the SUSs. Depicted are the GC-MS chromatograms of: A) Blank: Empty tube; B) Reference measurement: Glass wool spiked with 0.1 ppm BHT (RT: 15.547 min); C) Thawing system: Platinum-catalyzed SI tube and PS connector. D) Transfer system: Platinum-catalyzed SI tube. E) Filling system: Platinum-catalyzed SI tube. F) Bag system: Black peaks: Bag (PE; EVOH); Red peaks: Bag-port (TPE) and Tubing (SI). G) Filter system: PVDF and PP filter cartridge. (*marked peaks can be traced back as extractables of the PS material)

In addition to the above listed substances tetramethylbutyl phenol and trimethylolpropane trimethacrylate were extracted from the polysulfone (PS) connector of the thawing system. Two benzenes, 1,1'-(1-methylethylidene)bis[4-methoxy]-benzene and 1-methoxy-4-[(E)-2-phenylethenyl]benzene were found and are commonly observed as building blocks of the PS material [41]. UPLC analysis identified an additional benzene the large molecule bisphenol p. Further, sulfurous molecules were revealed as potential leachables of the PS.

Bag and Filter

Direct material TD analysis of the buffer-bag and filter indicated the presence of neither carcinogenic nor mutagenic substances. Figure 4.5 (F) depicts the bag profile marked with a homologous series of polydimethylsiloxanes, which can be attributed to the bag tubing. Moreover, broad peaks of alkanes, extracted from the polypropylene (PP) material are observed. In contrast, the filter cartridge chromatogram demonstrated recurring peak-blocks that are associated with an unknown substance (m/z: 79) and straight and non-straight chain alkanes. The major peak at 21.66 min can be identified as the alkane heptacosane. A further major peak is observed at 15.498 min, which is the antioxidant break-down 2,4-bis(1,1-dimethylethyl)-phenol. The filter resulted in the lowest amount of extractables of all tested SUSs. Only diethyl phthalate and two adipates, di-(2E)-2-buten-1-yl adipate and di(2-ethylhexyl) adipate (DEHA) could be identified as plasticizers. Additional peaks were found to correspond to antioxidants, including butylated hydroxytoluene (BHT), a molecule that is controversially discussed in literature regarding its toxicity [44, 45].

The polyethylene (PE) material of the buffer-bag revealed the oligomers 3,4',5,6'-tetrakis(2-methyl-2-propanyl)-2,3'-biphenyldiol and tetraethyl 1,4-diphenyl-1,1,4,4-butanetetracarboxylate, as detected with UPLC/QTOF-MS/MS. Furthermore, fragments including o-hydroxyacetophenone and 2,4-di-tert-butylphenol could be thermally extracted at 150°C and were detected with GC-MS. Di-tert-dodecyl disulfide, butyl ester 6-tetradecanesulfonic acid and octadecyl pentyl ester sulfurous acid were found as extractables of the thermoplastic elastomer bag port. These sulfurous chemicals are commonly used as lubricants and lubricant additives [39]. In comparison to the other SUSs, higher concentrations of fatty acids were found in the bag. These additives were likely employed to promote flexibility and to function as slip agents and lubricants for the diverse bag-materials. These are also stated reported to be commonly encountered extractables of polyethylene in literature [41]. The large peak with a retention of 9.28 min (in both ionization modes, Figure 4.6) could be attributed to a phosphor based additive with the chemical formula $C_{39}H_{59}N_2O_7P$.

4.3.2 Leachables

It was previously demonstrated (chapter 2) that SBSE provides sufficient sensitivity for semi-quantitative assessment [21]. Applicability of the proposed SBSE analytical set-up for leachable detection and quantification in drug products was verified by studying the range of measurement linearity (Table 4.14). Calibration plots depict high linearity ($r^2 > 0.990$)

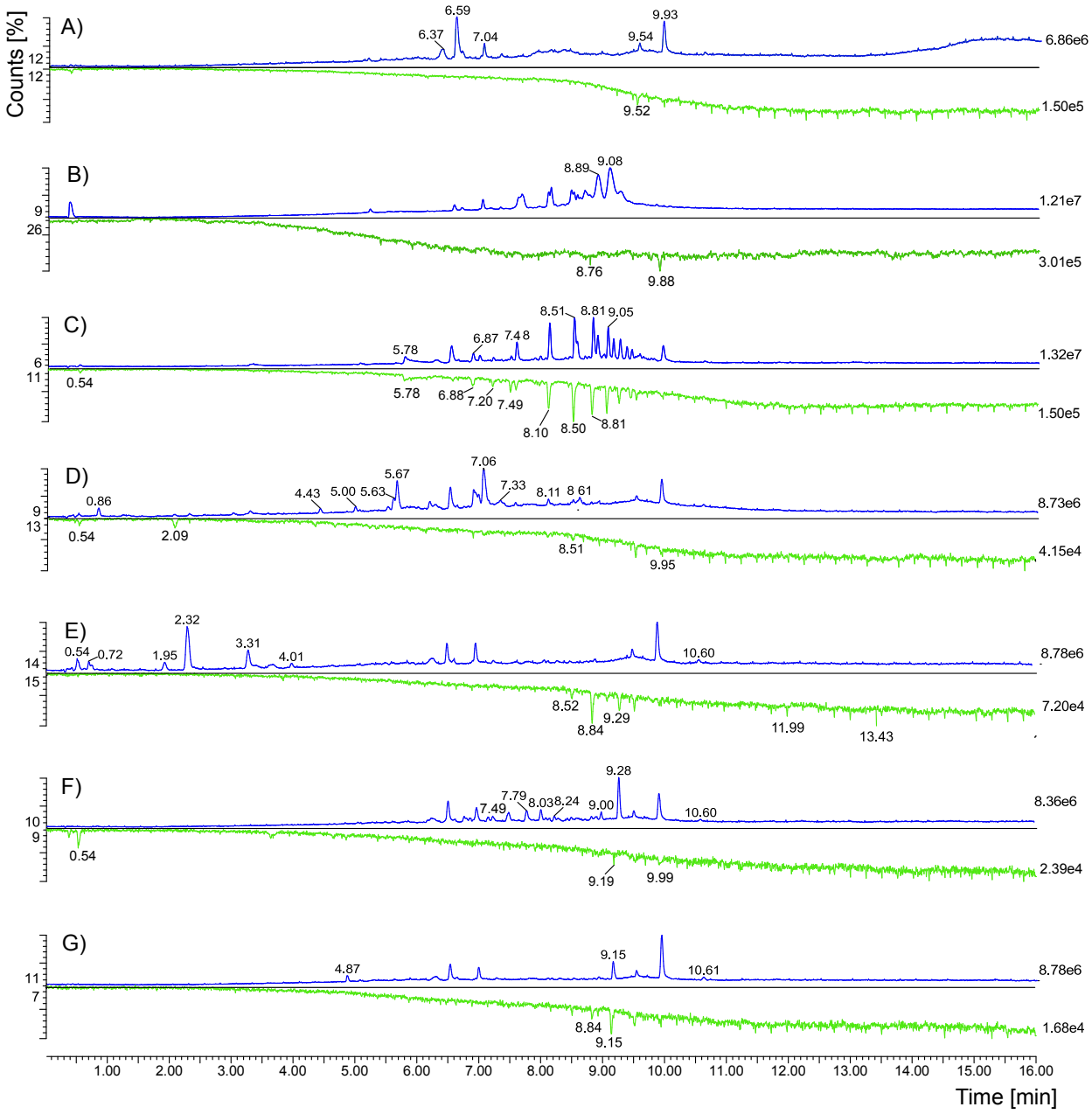


Figure 4.6: Static extraction with an ethanol water mix from the SUSs. Depicted are the UPLC/QTOF-MS/MS chromatograms in positive (color blue) and negative (color green) ionization mode: A) Blank: Aliquot of the ethanol/water mix; B) Reference measurement: Solvent mix spiked with 0.1 ppm of UVA P (pos. RT: 6.69 min), Tinuvin 328 (pos. RT: 9.08 min), Uvitex OB (pos. RT: 8.89 min) and Irganox 1010 (neg. RT: 9.88 min); C) Thawing system: Extraction time (ET) 17 h. D) Transfer system: ET 24 h. E) Filling system: ET 72 h. F) Bag system: ET 24 h. G) Filter system: ET 8 h.

up to the LOQ of 0.1 and 2.0 mg/L for TD-GC-MS and SBE-UPLC/QTOF-MS/MS, respectively see Table 4.14). The LOD for the SBSE method in combination with the TD-GC-MS was assessed as 2.2 - 0.6 μg/L and the LOQ as 2.1 and 7.3 μg/L for the two spiking substances, respectively. In combination with the SBE-UPLC/QTOF-MS/MS analytic, LODs between 0.003-0.025 mg/L and LOQs between 0.011-0.083 mg/L were achieved see Table 4.14). The ADI for all drug products examined in this study was between 20 and 120 μg/day per impurity. Dividing the regulated threshold by the drug specific dose results in the AET, which ranged from 0.2 to 6.0 ppm for single mutagenic leachables (multiple impurities 0.6 – 6.0 ppm) in the examined drug products (see Table 4.2).

Table 4.14: Correlation coefficient for the tested linear range of each spiking substance and the quantitative limits of SBSE observed for PDMS and EG/PDMS coated stir-bars in combination with TD- GC-MS and SBE-UPLC/QTOF-MS/MS analytic.

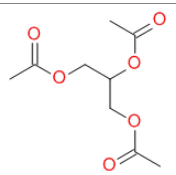
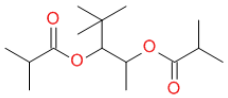
Analyte	Spiking substances	Linear range [ppm]	Correlation coefficient (r^2) (Origin: Force)	Precision method repeatability [%] RSD (n=4)	LOD [ppb]	LOQ [ppb]
PDMS stir-bar; TDS-GC-MS	THF-methacrylate	0.007-0.1	0.9989	7.7	2.2	7.3
	BHT-aldehyde	0.002-0.1	0.9954	7.3	0.6	2.1
EG/PDMS stir-bar; SBE-UPLC/ QTOF-MS/MS	UVA P	0.011-2.0	0.9934	6.5	3.3	10.9
	Tinuvin 328	0.028-2.0	0.9952	7.0	8.5	28.1
	Uvitex OB	0.025-2.0	0.9919	8.0	7.7	25.2
	Irgafos 168	0.074-2.0	0.9920	8.9	22.4	74.0
	Irganox 1330	0.08-2.0	0.9915	11.9	24.1	79.8
	Irganox 1010	0.083-2.0	0.9902	12.6	25.2	83.3

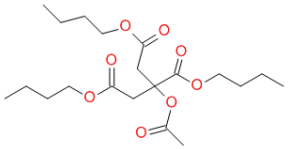
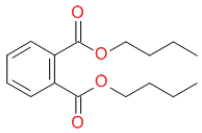
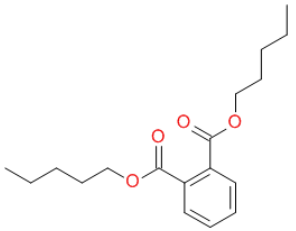
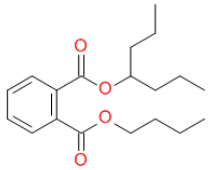
Samples were directly taken from the filling line during processing at five different spots. A full account of all leachables, as detected with SBSE, is provided in Table 4.15 and 4.16 ordered by the respective sampling spots. The results are in agreement with the extractable study, as leachables form a subset of previously identified extractables. The first run of drug product 1, which is characterized by a high protein concentration (Table 4.2), yields only four leachables with semi-quantified concentrations above the LOQ. The concentrations fall well below the AET of 6 ppm. One of the detected leachables was triacetin, a non-genotoxic, commonly used plasticizer, which was observed only at the first sampling point of the filling line with a sub-critical concentration of 33 ppb (RT: 17.22 min; Figure 4.7). Furthermore, dibutyl phthalate (see zoom Figure 4.7), a well-known plasticizer, originated from the buffer bag and remained detectable throughout the filling process, finally ending up in the vial with a negligible concentration below the LOQ. In

both sampling-runs of drug product 1 acetophenone was observed, a substance that results from antioxidant-breakdown. Further, two amides were detected as leachables with low concentration. Lastly, an unknown siloxane was detected with UPLC [m/z: 792.6278; 813.557] (RT: 9.87 min; Figure 4.7).

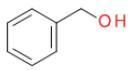
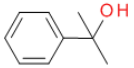
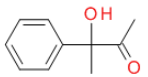
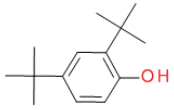
In the second run of drug product 1, a higher number of unknown substances was observed with UPLC analytic. One of the unknown substances [m/z: 362.099; 533.1563], with a concentration of 417 ppb, was present in the sample taken from the buffer-bag. However, this is still well below the drug product's individual AET. This substance could not be identified with respect to the extractable-footprint of the associated SUSs. Thus, it is considered to be a buffer-impurity. The substance consisted throughout the filling process and was detected with a concentration of 274 ppb in the vial.

Table 4.15: Leachables detected with TD-GC-MS and semi-quantified at the different sample spots during the filling process. For each leachable concentration the maximum concentration over the two runs was listed. Coding: Normal: Leachable found as well as extractable of the same SUS; Dark grey: Leachable not found as extractable of this SUS; Light grey: Leachable found as a possible breakdown molecule of an extractable of the same SUS. Leachables detected with TD-GC-MS and SBE-UPLC/QTOF-MS/MS and semi-quantified at the different sample spots during the filling process. For each leachable concentration the maximum concentration over the two runs was listed. Coding: Normal: Leachable found as well as extractable of the same SUS; Dark grey: Leachable not found as extractable of this SUS; Light grey: Leachable found as a possible breakdown molecule of an extractable of the same SUS.

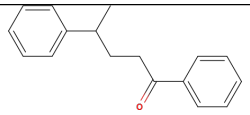
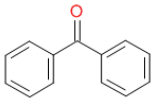
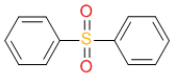
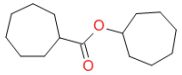
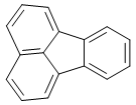
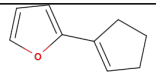
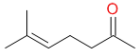
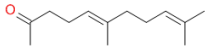
Leachables	Structure	Drug product	Cryo-vessel – Thawing set [ppm] Sampling (1)	Buffer Bag [ppm] Sampling (2)	Mixing-vessel – Transfer and bag tube [ppm] Sampling (3)	Filtration-vessel – Filter cartridge [ppm] Sampling (4)	Vial – Filling tube [ppm] Sampling (5)
Triacetin		1	0.033	-	-	-	-
Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester		2	0.016	-	-	-	-

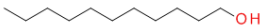
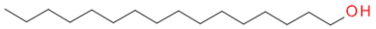
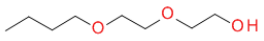
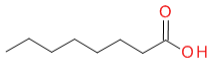
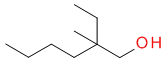
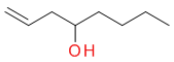
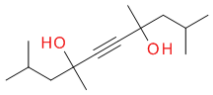
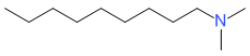
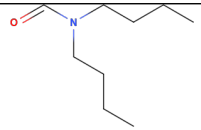
Leachables	Structure	Drug product	Cryo-vessel – Thawing set [ppm] Sampling (1)	Buffer Bag [ppm] Sampling (2)	Mixing-vessel – Transfer and bag tube [ppm] Sampling (3)	Filtration-vessel – Filter cartridge [ppm] Sampling (4)	Vial – Filling tube [ppm] Sampling (5)
Tributyl acetylcitrate		2	LOQ	-	-	-	-
Dibutyl phthalate		1	LOQ	0.005	LOQ	0.003	LOQ
		2	0.079	LOQ	LOQ	LOQ	LOQ
		3	-	n.a.	0.003	LOQ	-
Diamyl phthalate / Dipentyl phthalate		1	-	LOQ	-	-	-
		2	LOQ	-	-	-	-
		3	-	n.a.	LOQ	LOQ	-
Phthalic acid, butyl hept-4-yl ester		1	-	LOQ	-	0.003	LOQ
		2	0.049	-	-	-	-
		3	-	n.a.	LOQ	LOQ	-

Leachables	Structure	Drug product	Cryo-vessel – Thawing set [ppm] Sampling (1)	Buffer Bag [ppm] Sampling (2)	Mixing-vessel – Transfer and bag tube [ppm] Sampling (3)	Filtration-vessel – Filter cartridge [ppm] Sampling (4)	Vial – Filling tube [ppm] Sampling (5)
Diisobutyl phthalate		1	-	LOQ	LOQ	LOQ	LOQ
		2	LOQ	LOQ	-	-	-
Phthalic acid, butyl nonyl ester/ butyl octyl phthalate		1	-	LOQ	-	LOQ	LOQ
		2	-	-	-	-	LOQ
Phthalic acid		3	0.010	n.a.	0.008	LOQ	LOQ
Acetophenone		1	0.021	-	0.013	LOQ	LOQ
		2	0.041	-	-	LOQ	LOQ
		3	LOQ	n.a.	-	-	0.011

Leachables	Structure	Drug product	Cryo-vessel – Thawing set [ppm] Sampling (1)	Buffer Bag [ppm] Sampling (2)	Mixing-vessel – Transfer and bag tube [ppm] Sampling (3)	Filtration-vessel – Filter cartridge [ppm] Sampling (4)	Vial – Filling tube [ppm] Sampling (5)
Benzyl alcohol		1	LOQ	-	0.010	0.008	0.008
2-Phenyl-2-propanol		1	LOQ	-	LOQ	LOQ	LOQ
		2	0.012	-	-	-	-
		3	LOQ	n.a.	-	-	-
3-Hydroxy-3-phenylbutan-2-one		3	-	n.a.	LOQ	LOQ	-
2,4-Di-tert-butylphenol		1	LOQ	-	LOQ	LOQ	-
		2	0.002	-	-	0.005	-
		3	LOQ	n.a.	LOQ	LOQ	-

Leachables	Structure	Drug product	Cryo-vessel – Thawing set [ppm] Sampling (1)	Buffer Bag [ppm] Sampling (2)	Mixing-vessel – Transfer and bag tube [ppm] Sampling (3)	Filtration-vessel – Filter cartridge [ppm] Sampling (4)	Vial – Filling tube [ppm] Sampling (5)
Phenol, 4-(1,1-dimethylpropyl)-		1	-	LOQ	LOQ	-	LOQ
		2	LOQ	-	-	-	-
		3	LOQ	n.a.	LOQ	LOQ	LOQ
Butylated Hydroxytoluene		2	LOQ	-	-	-	-
3,5-di-tert-Butyl-4-hydroxybenzaldehyde		1	-	-	-	LOQ	LOQ
7,9-Di-tert-butyl-1-oxaspiro(4,5)-deca-6,9-diene-2,8-dione		2	LOQ	0.009	0.006	0.005	0.005
		3	LOQ	n.a.	-	-	-
p-Octylacetophenone		2	0.002	-	-	-	-

Leachables	Structure	Drug product	Cryo-vessel – Thawing set [ppm] Sampling (1)	Buffer Bag [ppm] Sampling (2)	Mixing-vessel – Transfer and bag tube [ppm] Sampling (3)	Filtration-vessel – Filter cartridge [ppm] Sampling (4)	Vial – Filling tube [ppm] Sampling (5)
1,4-Diphenyl-1-pentanone		1	-	-	-	-	LOQ
Benzophenone		1	-	-	LOQ	-	-
		3	0.007	n.a.	0.011	LOQ	LOQ
Diphenyl sulfone		2	0.002	-	-	-	-
Cyclohexanecarboxylic acid, cyclohexyl ester		2	LOQ	-	-	-	-
Fluoranthene		2	LOQ	-	-	-	-
3-(1-Cyclopentenyl)furan		1	-	-	-	LOQ	-
5-Hepten-2-one, 6-methyl-		1	-	-	LOQ	-	-
5,9-Undecadien-2-one, 6,10-dimethyl-		1	-	-	LOQ	-	-

Leachables	Structure	Drug product	Cryo-vessel – Thawing set [ppm] Sampling (1)	Buffer Bag [ppm] Sampling (2)	Mixing-vessel – Transfer and bag tube [ppm] Sampling (3)	Filtration-vessel – Filter cartridge [ppm] Sampling (4)	Vial – Filling tube [ppm] Sampling (5)
1-Undecanol		1	-	-	LOQ	LOQ	LOQ
1-Hexadecanol		1	-	-	-	-	0.009
		2	LOQ	-	-	-	-
		3	0.008	n.a.	-	-	-
Ethanol, 2-(2-butoxyethoxy)-, acetate		1	-	-	-	-	0.025
		2	0.044	-	-	-	0.010
		3	LOQ	n.a.	-	-	-
Octanoic acid		1	0.010	-	0.010	0.008	-
1-Hexanol, 2-ethyl-		1	LOQ	-	LOQ	LOQ	LOQ
1-Octen-4-ol		1	-	-	-	LOQ	-
2,4,7,9-Tetramethyl-5-decyn-4,7-diol		1	-	-	-	LOQ	-
		2	LOQ	-	-	-	-
1-Nonanamine, N,N-dimethyl-		1	-	-	-	-	LOQ
		3	-	n.a.	-	-	LOQ
Formamide, N,N-dibutyl-		1	-	-	-	LOQ	-
		2	LOQ	-	-	-	-

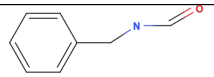
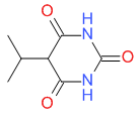
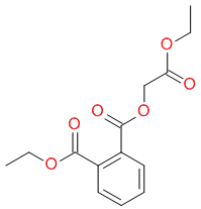
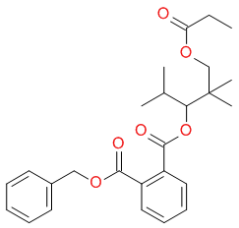
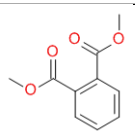
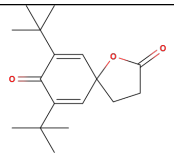
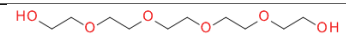
Leachables	Structure	Drug product	Cryo-vessel – Thawing set [ppm] Sampling (1)	Buffer Bag [ppm] Sampling (2)	Mixing-vessel – Transfer and bag tube [ppm] Sampling (3)	Filtration-vessel – Filter cartridge [ppm] Sampling (4)	Vial – Filling tube [ppm] Sampling (5)
		3	0.004	n.a.	LOQ	-	-
Tert-octyldiphenylamine	n.a.	2	0.003	-	-	-	-
n-Benzylformamide		2	LOQ	-	-	-	-
Isopropylbarbituric acid		3	LOQ	n.a.	LOQ	LOQ	LOQ
Unknown amid (tentative n-Octyl-acetamide)	n.a.	1	-	-	-	-	LOQ
Unknown acid	n.a.	1	-	LOQ	LOQ	-	LOQ
Unknown substance	n.a.	1	LOQ	-	LOQ	-	-
		3	LOQ	n.a.	-	-	-

Table 4.16: Leachables detected with SBE-UPLC/QTOF-MS/MS and semi-quantified at the different sample spots during the filling process. For each leachable concentration the maximum concentration over the two runs was listed. Coding: Normal: Leachable found as well as extractable of the same SUS; Dark grey: Leachable not found as extractable of this SUS; Light grey: Leachable found as a possible breakdown molecule of an extractable of the same SUS.

Leachables	Structure	Drug product	Cryo-vessel – Thawing set [ppm] Sampling (1)	Buffer Bag [ppm] Sampling (2)	Mixing-vessel – Transfer and bag tube [ppm] Sampling (3)	Filtration-vessel – Filter cartridge [ppm] Sampling (4)	Vial – Filling tube [ppm] Sampling (5)
2-Ethoxy-2-oxoethyl ethyl phthalate $C_{14}H_{16}O_6$		1	-	0.011	-	-	-

Leachables	Structure	Drug product	Cryo-vessel – Thawing set [ppm] Sampling (1)	Buffer Bag [ppm] Sampling (2)	Mixing-vessel – Transfer and bag tube [ppm] Sampling (3)	Filtration-vessel – Filter cartridge [ppm] Sampling (4)	Vial – Filling tube [ppm] Sampling (5)
Tentative Benzyl 3-isobutyryloxy-1-isopropyl-2,2-dimethylpropyl phthalate $C_{26}H_{40}O_5Na$		3	0.092	n.a.	-	-	-
Dimethyl phthalate $C_{10}H_{10}O_4$		2	-	-	0.038	-	-
7,9-Di-tert-butyl-1-oxaspiro(4,5)-deca-6,9-diene-2,8-dione $C_{17}H_{24}O_3$		2	-	-	-	0.035	-
Pentaethylene glycol $C_{10}H_{21}O_6$		2	LOQ	-	-	-	-

Leachables	Structure	Drug product	Cryo-vessel – Thawing set [ppm] Sampling (1)	Buffer Bag [ppm] Sampling (2)	Mixing-vessel – Transfer and bag tube [ppm] Sampling (3)	Filtration-vessel – Filter cartridge [ppm] Sampling (4)	Vial – Filling tube [ppm] Sampling (5)
Ethanol, 2-(2-Butoxyethoxy)- $C_8H_{18}O_3$		2	-	-	0.036	LOQ	-
Methanediylbis (Dioctylphosphane) Dioxide $C_{33}H_{72}O_2P_2$ (tentative)		2	0.021	-	-	-	-
4-Undecylbenzenesulfonic acid $C_{17}H_{28}O_3S$		3	0.352	n.a.	0.103	LOQ	LOQ
Methyl Tetrapropylenebenzenesulphonate $C_{19}H_{32}O_3S$		3	0.454	n.a.	0.236	0.167	LOQ
$C_{15}H_{33}N_2PS$	n.a.	3	0.495	n.a.	LOQ	-	-

Leachables	Structure	Drug product		Cryo-vessel – Thawing set [ppm] Sampling (1)	Buffer Bag [ppm] Sampling (2)	Mixing-vessel – Transfer and bag tube [ppm] Sampling (3)	Filtration-vessel – Filter cartridge [ppm] Sampling (4)	Vial – Filling tube [ppm] Sampling (5)
$C_{12}H_{24}O_3$	n.a.	3		0.013	n.a.	-	-	-
Unknown substance [m/z pos. 362,099; 533,1563]	n.a.	1		-	0.417	0.168	0.230	0.274
Aromatic substance $C_{11}H_{14}O_5$	n.a.	1		-	LOQ	-	-	-
Unknown Siloxane	n.a.	1		-	~	~	~	-
		2		-	-	-	-	~
		3		~	n.a.	-	-	-

Both runs of the second drug product, which has a high content of polysorbate 80 and a significantly lower AET, show that the majority of leachables was already found at the first sampling point in the filling line, i.e. in the cryo-vessel. Most of the detected leachables were substances that were also observed as extractables of the thawing tube with the PS-connector. Only two substances remained detectable throughout the filling process and ended up in the vial: 2-(2-butoxyethoxy)-ethanol acetate and the antioxidant 7,9-di-tert-butyl-1-oxaspiro(4,5)-deca-6,9-diene-2,8-dione. These two substances were consistently detected with both analytic techniques. The ethanol 2-(2-butoxyethoxy)-acetate was picked up by TD-GC-MS in the cryo-vessel and in the vial, and as 2-(2-butoxyethoxy)-ethanol it was detected with SBE-UPLC/QTOF-MS/MS in the mixing and filtration vessel. The concentration of this nontoxic leachable, which can be traced back as extractable of the thawing- and transfer tubing, decreased over the filling process from 44 to 10 ppb. At no sampling spot, the concentration of the leachable exceeded the drug based AET of 200 ppb. The other persistent substance, 7,9-di-tert-butyl-1-oxaspiro(4,5)-deca-6,9-diene-2,8-dione, was also confirmed with both analytics but with even lower concentrations. The leachables with the highest identified concentrations at any point of the filling line were phthalates in the cryo-vessel of the second run (cumulative of 128 ppb). However, the substances did not persist at subsequent sampling points.

For the last tested drug product, no SUS bag was applied and hence, no dilution by buffer inflow was necessary. Again, a consistent decrease of leachables was observed over the course of the filling process. Over both runs a total of twelve leachables whose concentrations exceeded the corresponding LOQ were detected. For this drug product three sulfurous leachables were observed in both runs, which were not present for the other drug products. These leachables included 4-undecylbenzenesulfonic acid, methyl tetrapropylenebenzenesulphonate and an unknown substance with the chemical formula $C_{15}H_{33}N_2PS$. Those sulfurous leachables occurred with comparably high concentrations (up to 495 ppb at the beginning of the filling process) but still fell below the AET of 500 ppb. Furthermore, a sharp decrease of the concentration over the course of the filling process was observed. Besides the sulfurous substances, all other detected leachables in the cryo-vessel could not be matched to extractables from the thawing-system. Remaining leachables that occurred during the process were diverse phthalates, with concentrations mostly below the LOD.

The total concentration of all leachables over all tested drug products listed in Table 4.17 depicts a remarkable decrease over the filling process to about 1/6 of the concentration at the end compared to the start of the filling process (Figure 4.1).

4.3.3 Absorbance and Filtering of Leachables

To confirm an uptake of leachables by SUSs, a scale-down version of the filling line was run with a commonly occurring leachable. Comparing the concentration of the plasticizer diethyl phthalate in the solution before and after processing in the test version of the filling line reveals a decrease in quantity. After a contact time of 3 h to the SUSs the

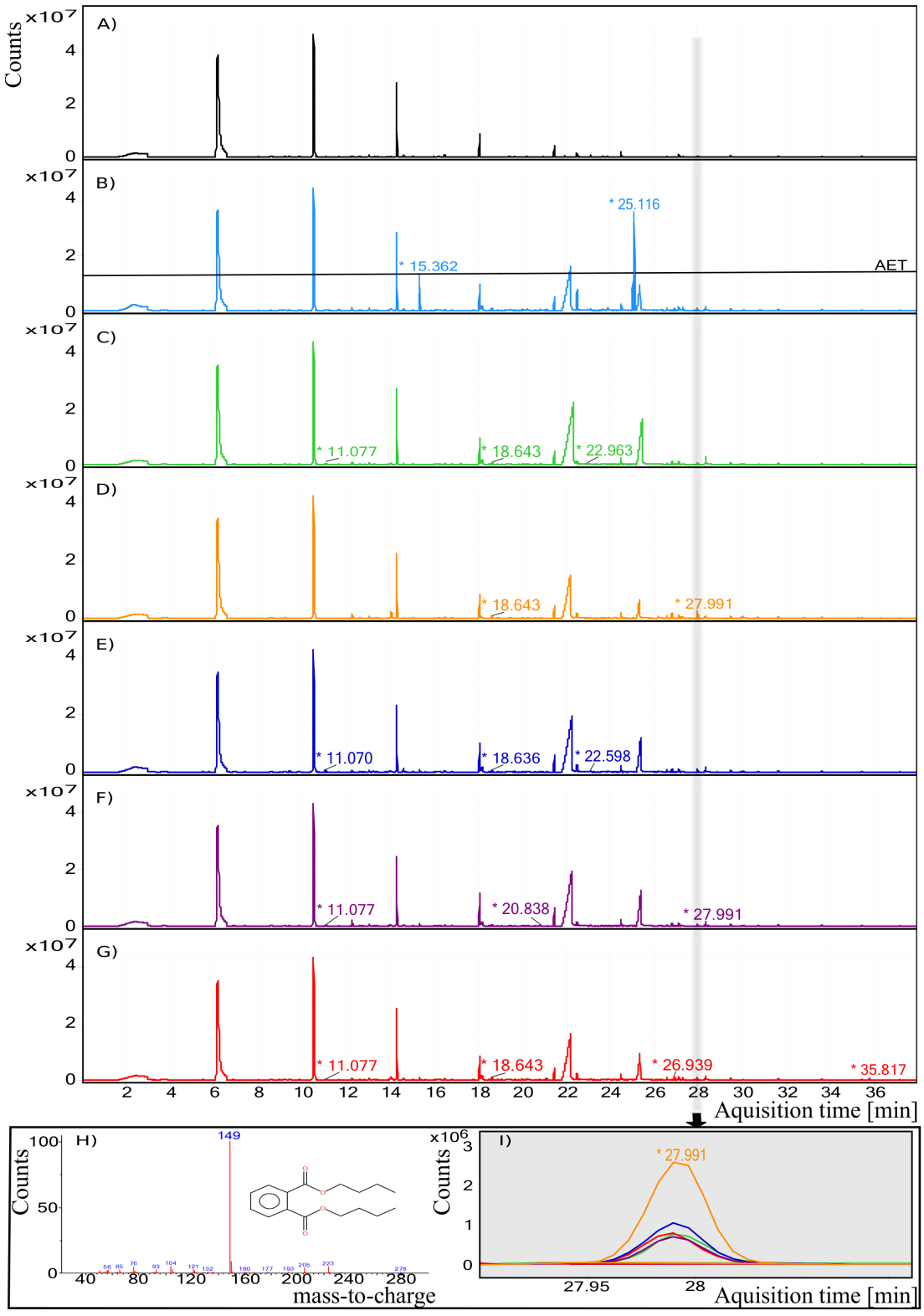


Figure 4.7: (Figure caption continued on page 116.)

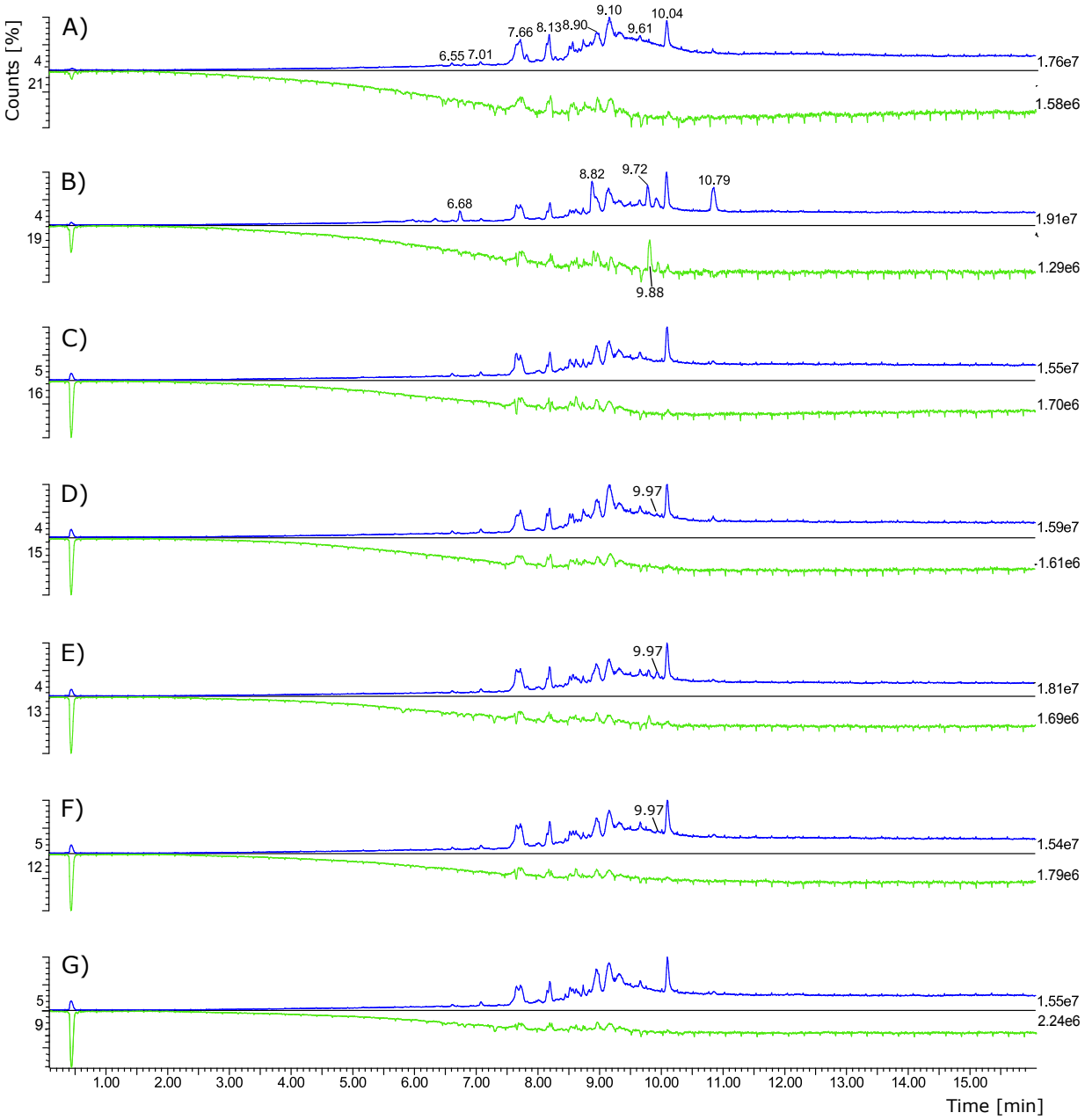


Figure 4.7: Leachable-study of drug product 1. Chromatograms using PDMS stir-bar in combination with TD-GC-MS are depicted: A) Blank: Stir-bar immersed in pure WfI. Peaks correspond to stir-bar bleeding; B) Reference measurement: Drug product spiked with 0.2 ppm (lowest AET-value 0.2 ppm/drug product 1 AET 6 ppm) of THF-methacrylate (RT: 6.69 min) and BHT-aldehyde (RT: 9.08 min); Additional peaks correspond to surfactant of the drug product. C) Drug sample: Cryo-vessel sampling point. Detected leachables above LOQ are indicated by RT-marks. D) Drug sample: Buffer-bag. E) Drug sample: Mixing-vessel. F) Drug sample: Filtration-vessel. G) Drug sample: Vial. H) NIST m/z spectrum of the leachable dibutyl phthalate at 27.991 min. J) Peak-overlay of dibutyl phthalate in the different drug samples.

Table 4.17: The in total concentration of all detected leachables for all three drug products over both sampling runs at each sampling point.

PDMS stir-bar TD-GC-MS; EG/PDMS stir-bar SBE-UPLC/QTOF-MS/MS Drug product 1-3, run 1 -2	Cryo-vessel – Sampling (1)	Buffer Bag - Sampling (2)	Mixing-vessel – Sampling (3)	Filtration-vessel – Sampling (4)	Vial – Sampling (5)
Summation of the concentration of all detected leachables over the three drug products [ppm]	2.020	0.442 (only for drug product 1 & 2)	0.798	0.576	0.342

concentration of the plasticizer was halved from 0.1 ppm to 0.053 ppm.

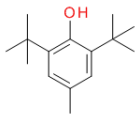
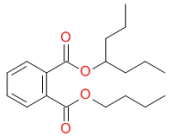
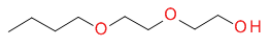
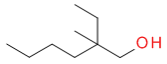
4.3.4 Impact of the Active Ingredient on the Leachable Spectrum

A full account of all leachables, as detected with SBSE combined with TD-GC-MS analysis, is provided along with the sample points in Table 4.18. Samples were taken directly from the filling line during processing of the drug product 4 and the corresponding placebo. The leachable profiles detected in the two runs were compared to assess the impact of protein in leachable-studies. However, some of the SUSs varied for the filling-process of the drug product compared to the placebo. The following three leachables were found in both runs: An unknown substance at the last sampling point, butylated hydroxytoluene (BHT), a well known antioxidant, and phthalic acid, butyl hept-4-yl ester, a plastizicer breakdown product. The last two substances were found with different concentrations in the filling process. For these two substances, a decrease over the filling line was detected. One substance was found in the drug product run only. This substance 2-(2-butoxyethoxy)-ethanol acetate leached out of the transfer tube during the contact to the drug product. All remaining leachables were present only at very low concentrations well below the LOQ.

4.3.5 Impact of the Drug Product pH

Four drug products with pH-values ranging from acidic to basic were examined. All leachables were detected with SBSE, combined with TD-GC-MS analysis, and provided in Table 4.19. A clear trend of increased leachable contamination with increasing pH-value of the drug product was observed. For the drug product with the highest pH-value (pH 10 - product 8), the highest cumulative leachable concentration (0.307 ppm) was detected. However, this was still well below the individual AET for multiple impurities, which was at 2.25 ppm. In addition, substances with non-polar surface area were found in higher concentrations in the basic drug product compared to the ic and neutral drug products.

Table 4.18: Leachables detected with TD-GC-MS and semi-quantitatively assessed at different sample spots during the filling process of drug product 4 (DP 4) and a placebo (P). For the DP-filling no disposable bag, and for the P-filling no single-use thawing set and transfer tube was applied.

Leachable	Structure	Sample	Cryo-vessel – Thawing set [ppm] Sampling (1)	Buffer Bag [ppm] Sampling (2)	Mixing-vessel – Transfer tube / bag tube [ppm] Sampling (3)	Filtration-vessel – Filter cartridge [ppm] Sampling (4)	Vial – Filling tube [ppm] Sampling (5)
Butylated Hydroxytoluene		DP 4	0.004	n.a.	0.005	0.003	LOQ
		P	n.a.	0.003	LOQ	LOQ	-
Phthalic acid, butyl hept-4-yl ester		DP 4	0.003	n.a.	0.005	0.004	0.003
		P	n.a.	LOQ	LOQ	-	-
Unknown substance (m/z 129)	n.a.	DP 4	-	n.a.	-	-	0.005
		P	n.a.	-	-	-	0.012
Ethanol, 2-(2-butoxyethoxy)-, acetate		DP 4	LOQ	n.a.	0.016	0.008	0.012
1-Hexanol, 2-ethyl-		P	n.a.	LOQ	LOQ	LOQ	LOQ

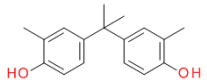
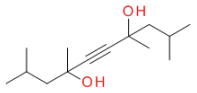
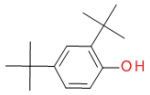
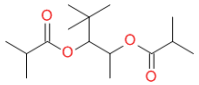
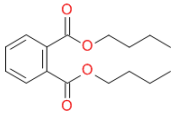
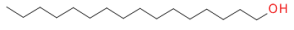
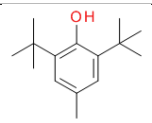
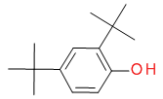
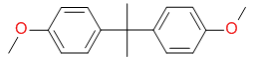
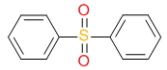
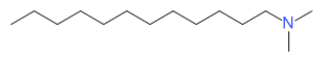
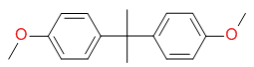
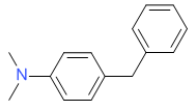
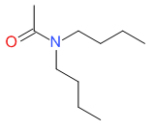
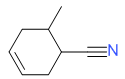
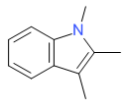
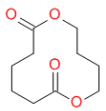
Leachable	Structure	Sample	Cryo-vessel – Thawing set [ppm] Sampling (1)	Buffer Bag [ppm] Sampling (2)	Mixing-vessel – Transfer tube / bag tube [ppm] Sampling (3)	Filtration-vessel – Filter cartridge [ppm] Sampling (4)	Vial – Filling tube [ppm] Sampling (5)
Bisphenol C		DP 4	-	n.a.	-	-	LOQ
2,4,7,9-Tetramethyl-5-decyn-4,7-diol		DP 4	-	n.a.	-	-	LOQ
2,4-Di-tert-butylphenol		P	n.a	LOQ	-	LOQ	-
Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester		P	n.a	LOQ	LOQ	-	LOQ
Dibutyl phthalate		P	n.a	LOQ	LOQ	-	-
1-Hexadecanol		P	n.a	-	LOQ	-	-

Table 4.19: Leachables detected with TD-GC-MS and semi-quantively assessed for different drug products with pH-values ranging from 3.5 to 10.0.

Drug product	pH-value	Leachable	Structure	Concentration [ppm]
DP 5	∼3.5	Butylated Hydroxytoluene		LOQ
		Unknown phenol	n.a.	0.009
DP 6	∼6.0	2,4-Di-tert-butylphenol		0.002
		Benzene, 1,1'-(1-methylethylidene)bis[4-methoxy-		LOQ
		Diphenyl sulfone		LOQ
		1-Dodecanamine, N,N-dimethyl-		LOQ
		Unknown substance	n.a.	0.003
		Unknown substance	n.a.	0.009

Drug product	pH-value	Leachable	Structure	Concentration [ppm]
DP 7	∼7.5	Benzene, 1,1'-(1-methylethylidene)bis[4-methoxy-		LOQ
		Benzene, 1-dimethylamino-4-phenylmethyl		LOQ
		Formamide n,n-diutyl		LOQ
		3-cyclohexen-1-nitrile,6-methyl		0.003
		1H-Indole,1,2,3 trimethyl		0.006
		1,6-dioxacyclododecane-7,12-dione		0.013

Drug product	pH-value	Leachable	Structure	Concentration [ppm]
DP 8	~10.0	2,4-Di-tert-butylphenol		0.029
		Benzene, 1,1'-[oxybis(methylene)]bis-		0.036
		Benzenemethanol, α,α-diphenyl-		0.021
		Benzophenone		LOQ
		2H-Cyclodeca[b]pyran,3,4,5,6,7,8,9,10,11,12-decahydro-4-methyl-		0.089
		Phenanthrene		0.006
		Triphenylmethane		0.126

4.4 Discussion

The present study evaluated the risk of leachables migrating from SUSs, applied throughout the pharmaceutical filling process, into the final drug product in order to assess potential patient hazards. Samples were drawn at multiple points from a real-life production line and were analyzed with TD-GC-MS and UPLC/QTOF-MS/MS to detect relevant impurities. Additionally, extractable-studies were conducted with thermal extraction of the polymers at high temperatures and static extraction, enabling the characterization of extractable foot prints of each SUS. Those footprints provide an overview of the extractables, which can be expected as typical substances of the different polymer-materials (listed in Table 1) or which are observed as additives subject to manufacturing variability. Our results indicate that all relevant impurities were well below the safety threshold, and often decreased in quantity throughout the manufacturing line. Thus, this study concludes, that leachables do not pose a major health hazard in pharmaceutical processing.

Thermal extraction was chosen for the extractable study due to its ease-of-use and the fast turnaround of this method. Compounds with comparably high mobility within the polymer matrix are most readily extracted using thermal extraction [32]. Hence, this method is well suited to provide an overview of the most relevant leachable-candidates. However, to obtain a more comprehensive picture of the extractable footprint, a static extraction procedure in combination with UPLC/QTOF-MS/MS analytic was performed in order to assess non-volatile high molecular weight substances. None of the substances that were only extracted under harsh conditions using a solvent-mix in the static extraction has been observed in the drug product as leachables. Thus, substances with lower mobility rates are unlikely to leach out of the SUS material during the filling process under normal process-conditions with a comparably short contact time. These results indicate that thermal extraction seems to be well suited for detection of leachable candidates in real world processing. Static extraction seems to be only necessary if a complete extractable-footprint is required. In addition, the levels of the leachables during the filling process detected with the GC-MS analytics were higher than for the UPLC/QTOF-MS/MS analytics.

For leachable-studies the applicability of the SBSE method in combination with TD-GC-MS and SBE-UPLC/QTOF-MS/MS was evaluated. The limits of detection in the tested drug matrices were well below the guideline regulated AET of the tested drug products [17]. SBSE analysis allowed fast and easy quantitative measurements even in presence of the drug matrix. However, our results indicate that no quantification of siloxane fragments in the drug product can be performed, as bleeding of the PDMS stir-bar coating is interfering. Despite this limitation the presented sample-preparation technique seems to be readily applicable and effective in detection of residual leachables with GC and UPLC analytics in drug products, with the potential for quantitative or semi-quantitative assessment.

SBSE has previously been used for detection of leachables and extractables in pharmaceutical processing [46]. B. Athenstädt developed GC-MS methods in combination with

SBSE for the identification and quantification of leachables from plastic packaging in dialysis solutions. In this study build upon this literature, by exploiting the high sensitivity of SBSE to analyze samples from a real-world filling line equipped with a variety of polymers for different drug products. Impurity studies in pharmaceutical drug products were largely restricted to extractable-data for polymer materials in previous literature [11, 47, 48]. In this study we present the analysis of three drug products in a real-world filling line. Besides the presented data no other pharmaceutical products were studied, and none were excluded from analysis. The results coherently demonstrate low exposure to leachables, far less than the relevant safety threshold.The majority of leachables was detected at the first sampling point, but only half of them were also observed as extractables of the thawing tubing with the connector. It is plausible that those substances have already been present in the delivered bulk drug substance solution and only partially originated in the thawing set. Other leachables that have not been identified as extractables were observed in the drug-product samples from the buffer-bag. This provides further evidence that most leachable exposure happened before entering the filling process, due to increased stress including longer contact times, and that impurities are contained in the buffer components that are fed into the drug.

Drug product 3 compared to the other tested drug products had the highest leachable concentration. However, among all leachables only sulfides were depending on the actually filled product. These leachables ($C_{24}H_{50}S_2$; $C_{18}H_{38}O_3S$; $C_{23}H_{48}O_3S$) were identified in the cryo-vessel of both runs of drug product 3, but for no other product. Those can be traced to the PS material of the connector. Although these substances were not identified by the extractable-studies, larger sulfurous substances like $C_{22}H_{14}N_2S_2$ and $C_{26}H_{24}N_2O_5S$ were extracted by static extraction. Therefore, these leachables are likely to be breakdown-fragments of the determined extractables of the connector, resulting from different interactions of the drug product formulation with the material compared which do not occur with the solvent mix during the extraction-study. However, again it cannot be fully excluded that these leachables were already in the delivered bulk and originated from a SUS of the preceding manufacturing. Besides this group, no other direct influence of the product composition on the nature of the detected leachables was observed.

The impact of proteins on the leachable spectrum was tested by comparing a drug product filling with a placebo filling. Identical leachables were observed in both runs, independent of the filled solution, although with different concentrations. These differences in concentration were mostly due to minor, random variations between fillings (compare subsection 4.3.2), and were not a result of protein interaction. Only one leachable that was not detected in the placebo-run, was observed in the drug product filling with a concentration higher than the LOQ. Based on the ectractable-footprints, this leachable can be traced to a single-use transfer tube, which was not applied for the placebo filling. Hence, it is questionable whether the leachable results from the protein impact. Furthermore, the impact of the drug product's pH was investigated. Although, not exactly the same SUSs were used for the different drug products, a pH effect on the leachables spectrum was

demonstrated.

The use of platinum-cured silicone tubing is expected to result in low extractable/leachable levels [49]. However, in pharmaceutical processes SI tubes are most commonly used. Our study revealed that larger amounts of extractables, including some with mutagenic concern were obtained from these tubing compared with other SUSs. The SUS with the highest exposure was the thawing tube with connector. Only few leachable substances exceeding the LOQ were found following the filters despite their high drug volume contact. Although, the bag had the highest contact surface, this SUS was irrelevant for the overall leachable outcome in the product. All in all, no significant leachable enrichment was observed throughout the filling process by any particular SUS.

Our results show that in the commercial manufacturing pipeline leachables were only found in extremely low concentrations, mostly below the LOQ. Despite the use of numerous SUSs in the process, the maximum detected leachable concentration did not once exceed the reporting threshold given by the ICH. The concentrations were particularly low in the final sampling point, i.e. in the filled vials. Leachables, which were observed to migrate from the SUSs into the drug products, were mostly plasticizers in form of phthalates and antioxidant breakdowns. All substances were categorized as non-mutagenic and non-carcinogenic. Neither benzaldehyde nor 2,4-di-t-butyl-6-nitro-phenol or o-hydroxybiphenyl, the only identified genotoxic substances in the extractable-study, were detected as leachables in the tested drug products. This highlights the importance of leachable studies, besides extractable analysis, to allow for accurate classification and to avoid misleading worst-case conclusions.

Many substances, which were present in the beginning of the filling line, could not be detected in the vial anymore, indicating a major reduction in concentration of leachables during the process in our study. This is most likely caused by API-pooling and buffer-dilution. However, even for drug product 3, which did not employ dilution by buffer, an extreme reduction over the filling process was observed. The absorbance of leachables by filters and tubes is a likely explanation for the reduction of the concentration, as they provide high surface areas for interaction with leachables. We were able to confirm this uptake of leachables by SUSs for a plasticizer in a scale-down filling line experiment. This result is in good agreement with a previous work by Hauk et al. [50], which describes filters used for sterile filtration, acting as scavengers of leachables in an experimental set-up.

4.5 Conclusion

With this study we were able to identify and quantify accumulation of leachables in real-world commercial scale industrial drug-filling lines for protein drug products in order to shed light on the actual patient exposure, beyond the classical extractable-studies. We have demonstrated that leachables have only a marginal contribution to parenteral solu-

tion contamination and that SUSs in the filling line even exert scavenging behavior for leachables, resulting in a decrease in concentration within the drug processing, instead of contaminating the product. But still, each drug product, especially its pH-value, and each SUS can influence the leachable-outcome of a filling-processing.

Bibliography

[1] N. Scherer, K. Marcseková, T. Posset, and G. Winter. New Studies on Leachables in commercial scale Protein Drug Filling Lines using Stir Bar Sorptive Extraction coupled with TD-GC-MS and UPLC/QTOF-MS/MS analytics. *International Journal of Pharmaceutics*, 30(555):404–419, 2018.

[2] A. A. Shukla and U. Gottschalk. Single-use disposable technologies for biopharmaceutical manufacturing. *Trends in Biotechnol.*, 31(3):147–154, 2013.

[3] U. Gottschalk. Disposables in Downstream Processing. *Adv. Biochem. Eng. Biotechnol.*, 115:171–183, 2009.

[4] E. S. Langer and R. A. Rader. Single-use technologies in biopharmaceutical manufacturing: A 10-year review of trends and the future. *Eng. Life Sci.*, 14(3):238–243, 2014.

[5] U.S. Pharmacopeia. Draft: Polymeric Components and Systems Used to Manufacture Pharmaceutical and Biopharmaceutical Drug Products. *USP 665*, 2016.

[6] U.S. Pharmacopeia. Draft: Characterization of Polymeric Components and Systems Used to Manufacture Pharmaceutical and Biopharmaceutical Drug Products. *USP 1665*, 2016.

[7] Food and Drug Administration. Section 211.65 - Equipment construction. *FDA US 21*, 2010.

[8] Steven A. Zdravkovic, Cindy T. Duong, Ashley A. Hellenbrand, Steven R. Duff, and Alyssa L. Dreger. Establishment of a reference standard database for use in the qualitative and semi-quantitative analysis of pharmaceutical contact materials within an extractables survey by GC–MS. *Journal of Pharmaceutical and Biomedical Analysis*, 151:49–60, 2018.

[9] A. Wakankar, Y. Wang, E. Canova-Davis, S. Ma, D. Schmalzing, J. Grieco, T. Milby, T. Reynolds, K. Mazzarella, Ed Hoff, S. Gomez, and S. Martin-Moe. On developing a process for conducting extractable-leachable assessment of components used for storage of biopharmaceuticals. *J. Pharm. Sci.*, 99(5):2209–2218, 2010.

[10] D. Jenke. Compatibility of pharmaceutical products and contact materials: safety considerations associated with extractables and leachables. *John Wiley & Sons*, 2009.

[11] C. Pan, F. Harmon, K. Toscano, F. Liu, and R. Vivilecchia. Strategy for identification of leachables in packaged pharmaceutical liquid formulations. *Journal of Pharmaceutical and Biomedical Analysis*, 46(3):520–527, 2008.

[12] D. Jenke. Linking Extractables and Leachables in Container/Closure Applications. *J. Pharm. Sci. Technol.*, 59(4):265–281, 2005.

[13] D. L. Norwood, D. Paskiet, M. Ruberto, T. Feinberg, A. Schroeder, Guirag Poochikian, Qingxi Wang, Tian Jing Deng, Fran DeGrazio, Melinda K. Munos, and Lee M. Nagao. Best Practices for Extractables and Leachables in Orally Inhaled and Nasal Drug Products: An Overview of the PQRI Recommendations. *Pharma. Research*, 25(4):727–739, 2008.

[14] K. Li, G. Rogers, Y. Nashed-Samuel, H. Lee, A. Mire-Sluis, B. Cherney, R. Forster, P. Yeh, and I. Markovic. Creating a Holistic Extractables and Leachables (E&L) Program for Biotechnology Products. *PDA Journal of Pharmaceutical Science and Technology*, 69(5):590–619, 2015.

[15] T. H. Broschard, S. Glowienke, U. S. Bruen, L. M. Nagao, A. Teasdale, Cheryl L.M. Stults, Kim L. Li, Laurie A. Iciek, Greg Erexson, Elizabeth A. Martin, and Douglas J. Ball. Assessing safety of extractables from materials and leachables in pharmaceuticals and biologics – Current challenges and approaches. *Regulatory Toxicology and Pharmacology*, 81:201–211, 2016.

[16] D. Jenke. Evaluation of the chemical compatibility of plastic contact materials and pharmaceutical products; safety considerations related to extractables and leachables. *J. Pharm. Sci.*, 96(10):2566–2581, 2007.

[17] International Conference of Harmonisation (ICH). M7 Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk. *Guidance for Industry*, 2015.

[18] A. Amberg, L. Beilke, J. Bercu, D. Bower, A. Brigo, K. Cross, L. Custer, K. Dobo, E. Dowdy, K. Ford, S. Glowienke, J. Van Gompel, J. Harvey, C. Hasselgren, M. Honma, R. Jolly, R. Kemper, M. Kenyon, N. Kruhlak, P. Leavitt, S. Miller, W. Muster, J. Nicolette, A. Plaper, M. Powley, D. Quigley, V. Reddy, H. Spirkl, L. Stavitskaya, A. Teasdale, S. Weiner, D. Welch, A. White, J. Wichard, and G. Myatt. Principles and procedures for implementation of ICH M7 recommended (Q)SAR analyses. *Regulat. Toxicol. Pharmacol.*, 77:13–24, 2016.

[19] B. Xiao, S. K. Gozo, and L. Herz. Development and validation of HPLC methods for the determination of potential extractables from elastomeric stoppers in the presence of a complex surfactant vehicle used in the preparation of parenteral drug products. *Journal of Pharmaceutical and Biomedical Analysis*, 43(2):558–565, 2007.

[20] D. L. Norwood, D. Jenke, C. Manolescu, S. Pennino, and N. Grinberg. HPLC and LC/MS Analysis of Pharmaceutical Container Closure System Leachables and Extractables. *J. Liq. Chromatogr. Related Technol.*, 32(11-12):1768–1827, 2009.

[21] N. Scherer, K. Marcseková, T. Posset, and G. Winter. Evaluation of stir-bar sorptive extraction coupled with thermal desorption GC–MS for the detection of leachables from polymer single use systems to drugs. *Journal of Pharmaceutical and Biomedical Analysis*, 152:66–73, 2018.

[22] B. Armstrong, A. Senyurt, V. Narayan, X. Wang, L. Alquier, and G. Vas. Stir bar sorptive extraction combined with GC-MS/MS for determination of low level leachable components from implantable medical devices. *J. Pharma. Biomed. Analysis*, 74:162–170, 2013.

[23] F. Nogueira. Novel sorption-based methodologies for static microextraction analysis: A review on SBSE and related techniques. *Analytica Chimica Acta*, 757:1–10, 2012.

[24] A. Prieto, O. Basauri, R. Rodil, A. Usobiaga, L.A. Fernandez, N. Etxebarria, and O. Zuloaga. Stir-bar sorptive extraction: A view on method optimisation, novel applications, limitations and potential solutions. *J. Chromatogr. A*, 1217(16):2642–2666, 2010.

[25] E. Baltussen, C. Cramers, and P. Sandra. Sorptive sample preparation - a review. *Analyt. Bioanalyt. Chem.*, 373(1-2):3–22, 2002.

[26] D. Jenke, T. Egert, A. Hendricker, J. Castner, T. Feinberg, C. Houston, D. G. Hunt, M. Lynch, K. Nicholas, D. L. Norwood, D. Paskiet, M. Ruberto, E. J. Smith, F. Holcomb, and I. Markovic. Simulated Leaching (Migration) Study for a Model Container-Closure System Applicable to Parenteral and Ophthalmic Drug Products. *PDA Journal of Pharmaceutical Science and Technology*, 71(2):68–87, 2017.

[27] S Görög. *Identification and determination of impurities in drugs*. Elsevier, Amsterdam; New York, 2000. OCLC: 162130921.

[28] D. Jenke. Guidelines for the Design, Implementation, and Interpretation of Validations for Chromatographic Methods used to Quantitate Leachables/Extractables in Pharmaceutical Solutions. *J. Liq. Chromatogr. Related Technol.*, 27(20):3141–3176, 2004.

[29] N. Magarian, K. Lee, K. Nagpal, K. Skidmore, and E. Mahajan. Clearance of extractables and leachables from single-use technologies via ultrafiltration/diafiltration operations. *Biotechnol. Prog.*, 32(3):718–724, 2016.

[30] M. Bolgar, J. Hubball, J. Groeger, and S. Meronek. *Handbook for the chemical analysis of plastic and polymer additives*. CRC Press, Taylor & Francis Group, Boca Raton, second edition edition, 2016.

[31] C. Zweiben and A. J. Shaw. Use of thermal desorption GC-MS to characterize packaging materials for potential extractables. *J. Pharm. Sci. Technol.*, 63(4):353–359, 2009.

[32] A. Hoffmann and T. Albinus. Extractables and Leachables Analysis of IV Bag Systems. *Agilent Technologies*, 2015.

[33] V. Shen. *Standard Reference Simulation Website, NIST Standard Reference Database 173*. National Institute of Standards and Technology, 2006.

[34] Y. Zhang, S. Sun, X. Xing, Z. Du, Q. Guo, and W. Yu. Detection and Identification of Leachables in Vaccine from Plastic Packaging Materials Using UPLC-QTOF MS with Self-Built Polymer Additives Library. *Analytical Chemistry*, 88(13):6749–6757, 2016.

[35] L. Müller, R. J. Mauthe, C. M. Riley, M. M. Andino, D. De Antonis, C. Beels, J. DeGeorge, A. G.M. De Knaep, D. Ellison, J. A. Fagerland, R. Frank, B. Fritschel, S. Galloway, E. Harpur, C. D.N. Humfrey, A. S. Jacks, N. Jagota, J. Mackinnon, G. Mohan, D. K. Ness, M. R. O'Donovan, M. D. Smith, G. Vudathala, and L. Yotti. A rationale for determining, testing, and controlling specific impurities in pharmaceuticals that possess potential for genotoxicity. *Regulatory Toxicology and Pharmacology*, 44(3):198–211, 2006.

[36] J. Zhuang, X. Xing, D. Wang, Z. Du, J. Wang, Y. Dong, W. Yu, and S. H. Siyal. Toxicity assessment of the extractables from multi-layer coextrusion poly ethylene bags exposed to pH=5 solution containing 4% benzyl alcohol and 0.1 M sodium acetate. *Regulatory Toxicology and Pharmacology*, 94:47–56, 2018.

[37] I.C. Munro, A.G. Renwick, and B. Danielewska-Nikiel. The Threshold of Toxicological Concern (TTC) in risk assessment. *Toxicology Letters*, 180(2):151–156, 2008.

[38] D. Jenke and T. Carlson. A Compilation of Safety Impact Information for Extractables Associated with Materials Used in Pharmaceutical Packaging, Delivery, Administration, and Manufacturing Systems. *J. Pharm. Sci. Technol.*, 68(5):407–455, 2014.

[39] M. Nagao, W. P. Beierschmitt, D. J. Ball, A. J. Shaw, and S. Beck. The ELSIE Extractables and Leachables Database. *Pharma. Outsourcing*, 2011.

[40] G. Patlewicz, N. Jeliazkova, R.J. Safford, A.P. Worth, and B. Aleksiev. An evaluation of the implementation of the Cramer classification scheme in the Toxtree software. *SAR and QSAR in Environmental Research*, 19(5-6):495–524, July 2008.

[41] X. Ding, X. Yu, and D. Wood. Extractables and Leachables Study Approach for Disposable Materials Used in Bioprocessing. *BioPharm. International*, 21(2), 2008.

[42] R. D. Jenke, J. Story, and R. Lalani. Extractables/leachables from plastic tubing used in product manufacturing. *International Journal of Pharmaceutics*, (315):75–92, 2006.

[43] E. Demir, S. Kocaoğlu, and B. Kaya. Assessment of genotoxic effects of benzyl derivatives by the comet assay. *Food and Chemical Toxicology*, 48(5):1239–1242, May 2010.

[44] A. L. Branen. Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. *Journal of the American Oil Chemists' Society*, 52(2):59, February 1975.

[45] G.M Williams, M.J Iatropoulos, and J Whysner. Safety Assessment of Butylated Hydroxyanisole and Butylated Hydroxytoluene as Antioxidant Food Additives. *Food and Chemical Toxicology*, 37(9-10):1027–1038, 1999.

[46] B. Athenstaedt. *Development of GC-MS methods for the identification and quantification of leachables from plastic packaging in dialysis solutions*. PhD thesis, Duisburg, 2013.

[47] I. Markovic. Evaluation of safety and quality impact of extractable and leachable substances in therapeutic biologic protein products: a risk-based perspective. *Expert Opin. Drug Saf.*, 6(5):487–491, 2007.

[48] Y. Gao and N. Allison. Extractables and leachables issues with the application of single use technology in the biopharmaceutical industry: Extractables and Leachables in Disposable Manufacture. *Journal of Chemical Technology & Biotechnology*, 91(2):289–295, 2016.

[49] A. Colas, R. Malczewski, and K. Ulman. Silicone Tubing For Pharmaceutical Processing. *Pharma. Chem.*, pages 30–36, 2004.

[50] A. Hauk, E. Jurkiewicz, I. Pahl, T. Loewe, and R. Menzel. Filtration membranes - Scavengers for leachables? *European Journal of Pharmaceutical Sciences*, April 2018.

Chapter 5

Simulation-studies

5.1 Introduction

Alternative study designs for improved assessment of potential impurities in pharmaceutical processing are an active area of research, marked as a hot topic in the most recent extractable and leachable conferences [1]. Simulation based studies are an emerging and promising alternative to real world testing. This new method of testing is an intermediate step between classic extractable- and leachable-studies. Classical extractable studies are well known for the comprehensive and almost exhaustive assessment of tentative leachables (see chapter 3). Simulation-studies provide more restrictive profiles comprising only substances which are most likely to leach out, thus providing a more realistic assessment. Furthermore, simulation-studies can estimate the potential amount of leachables without the need of real-sample analysis by leachable-studies (described in chapter 4). This new concept offers a real alternative to the best practice protocol of the BioPhorum Operation Group (BPOG) [2, 3]. The best practice guidelines recommend to assess the risks for biopharmaceutical manufacturing posed by SUSs using multiple analytical methodologies and a broad range of extraction solvents. The guidelines also highlight important parameters to consider when designing a study to ensure optimal information from the generated extractions. For example extracting solvents stronger than the drug product are recommended to obtain qualitative extraction profiles to be used for establishing quality control criteria. However, this design is time intensive with respect to preparation and evaluation. Simulation-studies on the other hand are intended to use more realistic and adaptable parameters, with respect to contact time and contact solution. Additionally, as each SUS application and its contact to the individual drug product varies, customized extraction design is achieved by simulation-studies. Finally, the surface to volume ratio is adjusted in order to increase both the concentrations of probable leachables and the rates of migration of these into the solvent [4, 5].

According to USP <665> [6] the difference between a drug product leachable-study and a simulation-study with respect to the study design is that the drug product formulation is replaced with a simulating solvent. Depending on the extraction purposes the drug product

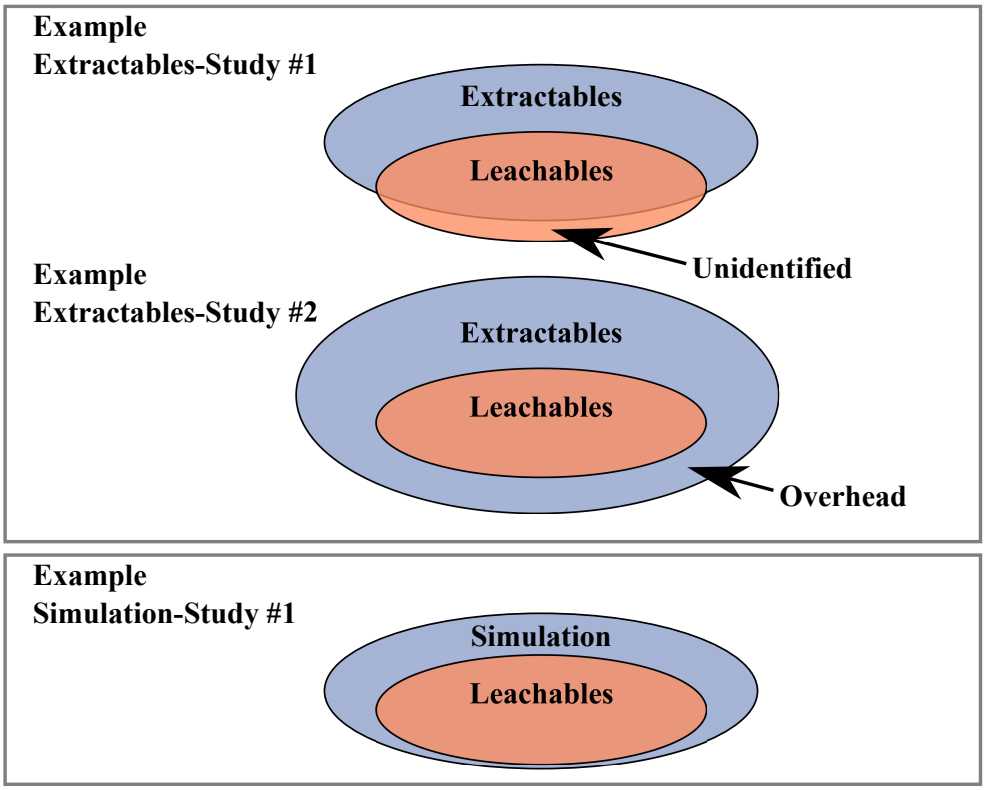


Figure 5.1: The relationship between extractables and leachables can vary from a full and complete correlation to a poor and largely incomplete correlation depending on the study-design, with extractables representing worst-case leachables. However, simulation-studies typically correlate well with leachables without a unnecessary large overhead.

can be mimicked by a precise extractation medium with similar propensity to extract chemicals from the components. A possible outlay of a simulating solvent with this advantage was shown by R. Haep et al. [7]. The authors used a screening assay with glutathione as a surrogate compound, which can be used in the SUS selection process to identify polymers with a risk of protein-reactive compounds leaching. This kind of testing detects leachables that otherwise might not be identified during a classical leachable-study, as they can be masked by reactions with the active protein of the drug product. These reaction products were also present under more realistic conditions during the simulation study. Therefore, simulation studies are not only an approach for more realistic extractable-studies, but also provide adjusted leachable-study with a clear proof of migrating substances by simulating the product and/or the process parameters.

In this chapter we demonstrate the potential of a new kind of simulation study as an alternative design to the classical extractable & leachable approach. Instead of analyzing the drug product with a classical leachable-study as described in chapter 4 or applying a simulating solvent, all used SUS were tested for residual additives after application in the drug

filling process. The post-application extractable footprint was compared to extractable footprints of identical but unused SUSs. This enabled the identification and quantification of substances that leached into the drug product during the application of the SUS. This approach indirectly estimates the leachable contamination without analyzing the drug product. Hence, this method is particularly well suited in cases where minimal disruption of the manufacturing process is desirable. Furthermore, the controlled test matrix in simulation studies is easy to analyze, due to the lack of any interfering drug product. At the same time the drug manufacturing process can remain largely unchanged as compared with real world processing. The simulation study, described in this work, was conducted with a filling tubing, which was reused over several drug product batches. The silicone tubing was used for up to six drug product runs, as it can be cleaned and sterilized in place (CIP/SIP). The repeated use of the silicon tubing enables the assessment of the leaching and the potential uptake of additives by the silicone matrix over the number of applications. To this end, some tubes were analyzed after 1, 3 or 6 runs in the manufacturing process. The majority of leachables can be expected in the first run, as most (semi-)volatile additives are likely to leach at first contact to the product. However, during each application the tube is exposed to mechanical stress by squeezing, which can potentially lead to variable leachable outcome in repeated use. Therefore, identified leachables were compared among tubes with different number of applications. All detected substances were also compared to the extractable-footprint obtained in chapter 3 (Table 3.2), to exclude substances that were absorbed by the porous silicone material, as described in subsection 4.3.2. Additionally, the outcome was compared to the results of the leachable-study (chapter 4) of the last processing step, the filling.

5.2 Materials and Methods

5.2.1 Chemicals

Sample analysis was performed with thermal desorption gas chromatography mass spectrometry (TD-GC-MS) (TDS 3 TDSA 2 Gerstel GmbH, Muelheim an der Ruhr, 7890B GC-System, 5977A MSD, Agilent Technologies, Waldbronn, Germany) and ultra performance liquid chromatography (UPLC/QTOF-MS/MS) (Xevo G2-S Tof, Acquity, Waters GmbH, Eschborn, Germany) combined with electron spray ionization (ESI). Nine spiking substances were added to allow for semi-quantitative analysis with GC-MS and UPLC/QTOF-MS/MS. These substances were selected as they are commonly observed leachables. GC analytic: Tetrahydrofurfuryl (THF) methacrylate $\log K_{O/W}$: 1.8; Diethyl-phthalate $\log K_{O/W}$: 2.45; 3,5-Di-tert-butyl-4-hydroxybenz-aldehyde $\log K_{O/W}$: 4.2 and Butylated hydroxyltoluene (BHT) $\log K_{O/W}$: 5.3. UPLC analytic, positive ionization-mode: 2-(2-hydroxy-5-methylphenyl) benzotriazole (UVA P) $\log K_{O/W}$: 6.1; 2-(2H-Benzotriazol-2-yl)-4,6-bis(1,1-di-methylpropyl) phenol (Tinuvin 328) $\log K_{O/W}$: 7.25; 2,5-Bis(5-tert-butylbenzoxazol-2-yl) thiophene (Uvitex OB) $\log K_{O/W}$: 8.0; Tris(2,4-DI-tert-butylphenyl) phosphite (Irgafos 168) $\log K_{O/W}$: 15.5; UPLC analytic, negative ionization-mode:

1,3,5-Trimethyl-2,4,6-tris(3,5-di-tert-butyl-4-hydroxybenzyl) benzene (Irganox 1330) log $K_{O/W}$: 17.7; Pentaerythritol tetrakis(3-(3,5-di-tert-butyl-4-hydroxy-phenyl)-propionate) (Irganox 1010) log $K_{O/W}$: 19.4. All substances were purchased from Sigma Aldrich (Steinheim am Albuch, Germany) with purity > 97%. Water for Injection (WfI) was prepared on a Milli-Q Advantage A10 system (Merck GmbH, Darmstadt, Germany). Absolute ethanol (99.7% optigrade, Promochem LGC, Wiesel, Germany), methanol (UPLC-grade, Biosolve, Dieuze, France) and ethyl acetate (99.8% Sigma Aldrich) were applied as solvents and/or eluents. Formic acid (Sigma Aldrich) was used for LC ion suppression and non-treated glass wool (Sigma Aldrich) for direct TD spiking application.

5.2.2 Sample Preparation, Extraction and Analytic Methods

The filling tubing (see Table 4.1 in chapter 4) was reused for up to six batches of drug product 1 (see Table 4.2 in chapter 4). To assess the impact of repeated use, filling tubes were analyzed after 1, 3 or 6 applications, respectively. The disposable tubing was gently flushed with water (at room temperature) prior to examination, in order to wash residual adherent protein off the inner wall of the tube. The tubes were filled with 6 mL of either pure WfI or a WfI/ethanol mix, depending on the subsequent analysis, and sealed with glass stoppers. Heated extraction was performed for three days in glass bottles containing $\sim$ 10 mL of extraction solvent, in order to prevent evaporation. Blank measurements were performed with aliquots of the extracting solvents in glass bottles and carried out under identical conditions. Stir-bar sorptive extraction (SBSE) was performed with the generated WfI samples using a 10 mm glass-encapsulated, magnetic stir-bar coated with 24 μL of pure polydimethylsiloxane (PDMS) (Twister, Gerstel GmbH). Subsequent analysis was performed with direct thermal desorption (TD) with subsequent GC-MS. The WfI/ethanol samples were directly analyzed with UPLC/QTOF-MS/MS analytic without any sample preparation.

TD-GC-MS Measurement of (Semi-)Volatile Substances

Extraction with WfI was performed at 100°C. For the GC analysis, 1 mL sample was mixed with 4 mL WfI to ensure complete submersion of the stir-bar. The PDMS coated stir-bar was then exposed to the solution for 1 h at a stirring speed of 800 rpm and a temperature of 19°C. Following the extraction step, the stir-bar was rinsed with WfI, dried by dabbing and placed in the autosampler (operating parameters listed in chapter 2). Blank measurements were performed by immersing the stir-bar in the blank solution, to verify the system contamination by estimating the background noise. Reference measurements were done with stir-bars subjected to THF-methacrylate and BHT-aldehyde spiked WfI. For this purpose, the spiking standards were dispersed in ethanol by sonication at a concentration of 0.1 g/L and diluted with WfI to simulate a contamination of 0.1 ppm.

UPLC/QTOF-MS/MS Measurement of Non-Volatile Substances

For the extraction of residual, non-volatile substances in the filling tubing, the tubes were filled with the solvent mix and extracted at 70°C. Note that the temperature was chosen just below the boiling point of ethanol. Non-volatile compounds in the generated extract-solutions were identified by UPLC/QTOF-MS/MS analytic [8] with operating parameters listed in chapter 4. Reference measurement was performed by injecting pre-defined spiking substances on water ethanol basis into the UPLC-system, simulating a 0.1 ppm contamination. The compounds were identified by matching accurate monoisotopic masses to a proprietary, internal database (Roche GmbH) and the handbook of Bolgar et al. [9].

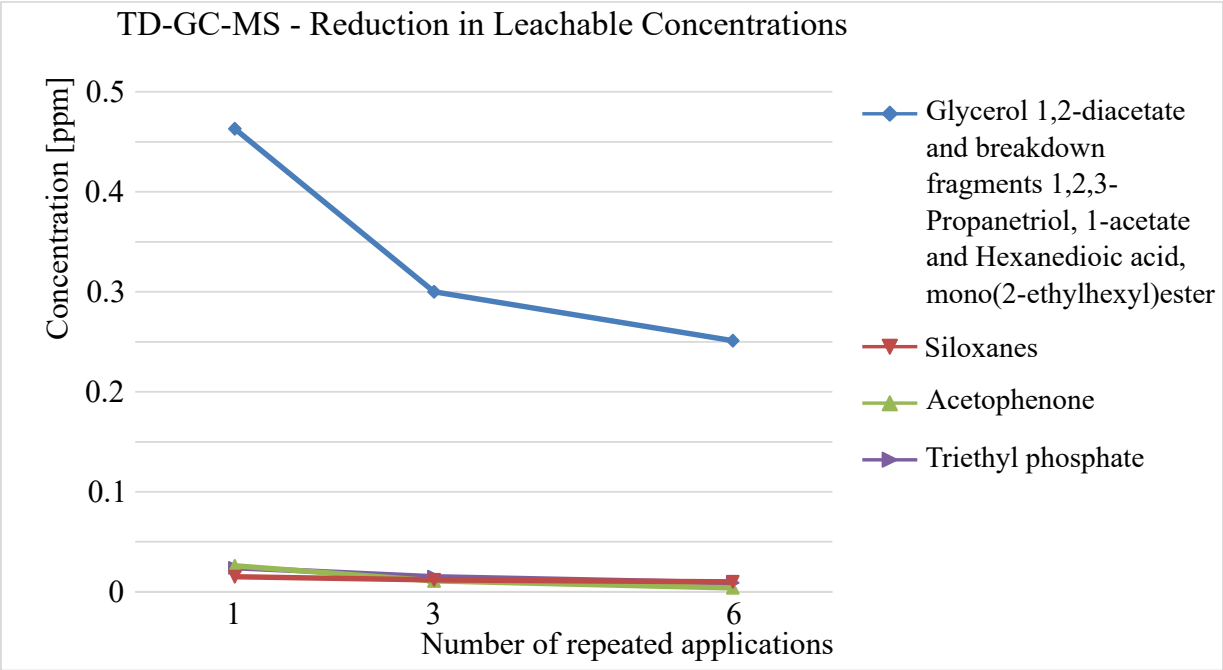
5.3 Results

5.3.1 Impurity-Output during Single Batch Applications

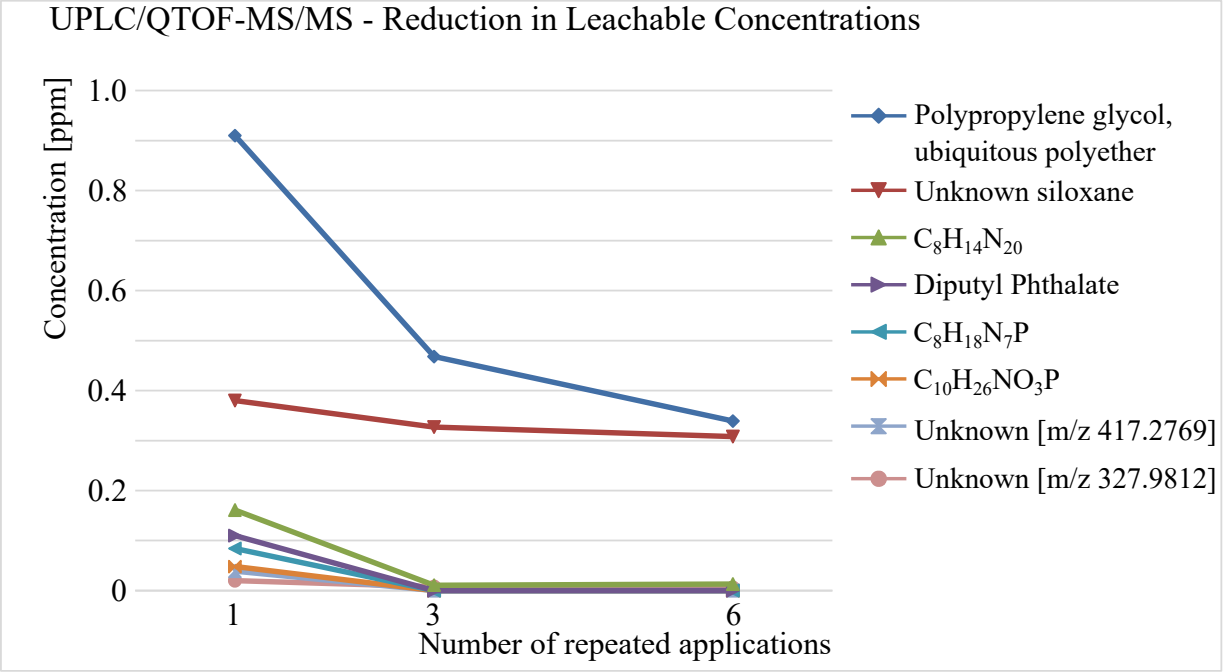
All residual substances found in the different filling tubes after varying number of reusage are depicted in Figures 5.2a-5.2d. All semi-volatile substances detected with TD-GC-MS analysis exhibited decreased concentration for increased number of aplications (Figure 5.2a), except benzaldehyde (Figure 5.2d), which displayed a largely stable concentration fluctuating around 0.04 ppm over the six batches. Even glycerol 1,2-diacetate and its breakdown fragments 1,2,3-propanetriol, 1-acetate and hexanedioic acid, mono(2-ethylhexyl)ester, which were found in high concentrations after one usage, decreased drastically in concentration. Also a decrease in concentration for higher molecular weight substances was observed by UPLC/QTOF-MS/MS analysis (Figure 5.2b). The most significant decrease was determined for oligomers, including silixones and ubiquitous polyether polypropylene glycols. On the other hand, some substances with lower mobility exhibited increased concentration after repeated application (Figure 5.2c). The largest increase in concentration was observed for the monomer caprolactam increased (0.55 ppm over the five repetitions). An unknown substance with a mass-to-charge ratio of 250.1280 m/z was the only substance which exhibited an increase in concentration that could not be identified as an impurity of the filling tubing (by comparison to the extractable-footprint, chapter 4 Table 3.2). Neither a decrease nor an increase was observed for the plasticizer triacetin and its fragment glycerine acetate (Figure 5.2d).

5.3.2 Determination of the Impurity-Outcome

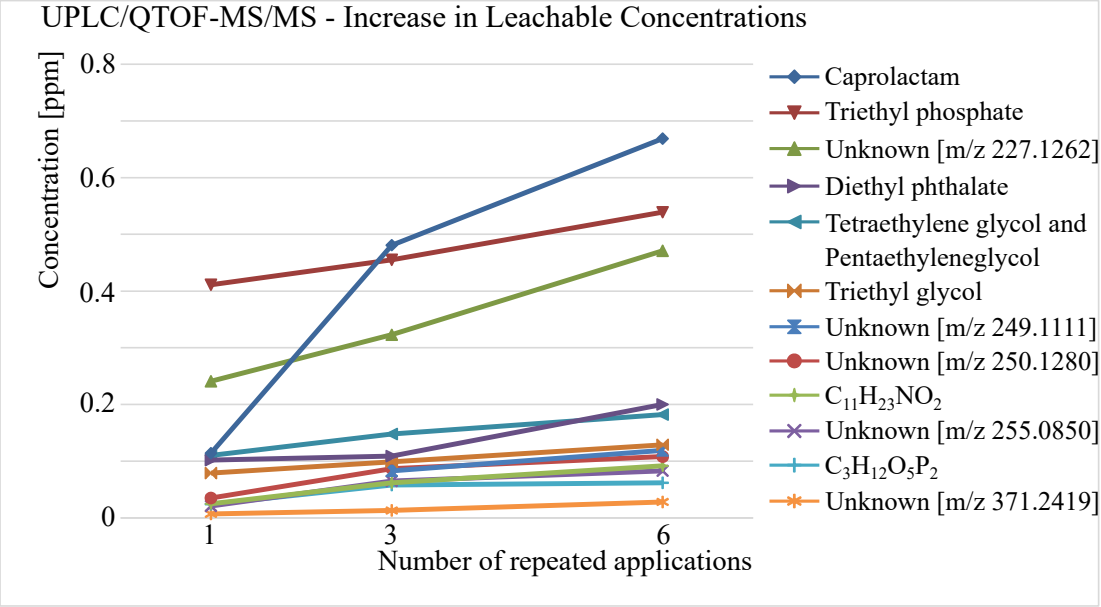
Based on the assumption of a constant leaching out of substances by the filling tubing during its application within the drug product processing, the simulation-study provides quantitative estimates of the amount of leachables in the final drug product. For example, polypropylene glycol, the substance observed with the highest concentration, exhibits a decrease of 0.442 ppm for two repeated applications (number of applications 1-3). Hence, the following quantity of leachable can be assumed to have migrated into the drug product:



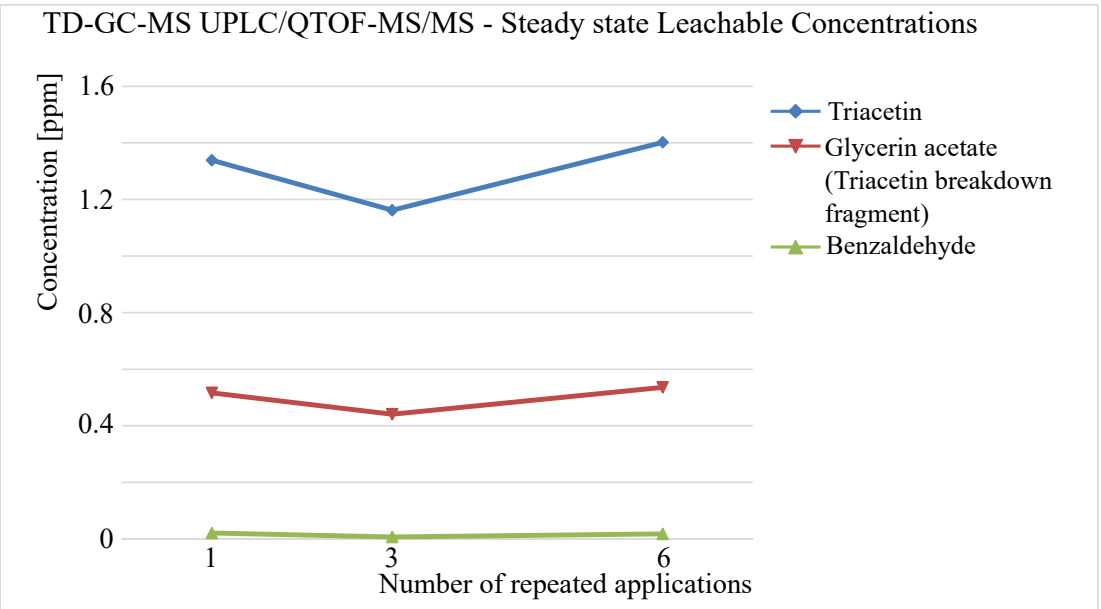
(a) Consistent decrease in leachable concentrations for increasing number of applications detected with TD-GC-MS.



(b) Consistent decrease in leachable concentrations for increasing number of applications detected with UPLC/QTOF-MS/MS.



(c) Increase in leachable concentration for increasing number of applications detected with UPLC/QTOF-MS/MS.



(d) Largely constant leachable concentrations for varying number of applications detected with TD-GC-MS and UPLC/QTOF-MS/MS analytic.

Figure 5.2: Residual impurities in the silicone matrix of the filling tube, extracted by static extraction, detected with TD-GC-MS and UPLC/QTOF-MS/MS analytic and semi-quantitatively assessed over a varying number of repeated applications. Substances are grouped based on the exhibited concentration behavior for a varying number of applications.

$$m_{Leachable} = c_{Leachable} * V_{Extract} = 0.442ppm * 6mL = 2.65\mu g \quad (5.1)$$

Accordingly a single filling tube is expected to excrete 2.65 μg of polypropylene glycol between the first and third repeated application. In the corresponding two batches a drug volume of 122.5 L was filled and was exposed to the tube, resulting in a leachable contamination of 1.1 * 10^{-5} mg per drug product volume L (= 11 ppt). This can be considered a worst case estimation as potential leakage during the intermediary CIP/SIP procedures of high temperature and pressure are not taken into account. Furthermore, higher leachable contamination is expected compared with the actual drug product filling, as higher temperatures are applied.

Furthermore, the impurity estimation based on the simulation study was compared to the observed leachables during the drug-product filling in chapter 4. The following three leachables were found in the last sample spot, the vial, but not in the filtration-vessel before (also listed in table 4.15): 1-Hexadecanol with a concentration of 0.009 ppm, ethanol, 2-(2-butoxyethoxy)-, acetate with a concentration of 0.025 ppm, both detected in drug product 1, and acetophenone with a concentration of 0.011 ppm in drug product 3. All three substances were also detected in the simulation-study, although partially in an unoxidized form and in much lower concentrations.

5.4 Discussion

A simulation-study was applied as a minimally disruptive alternative to conventional extractable and leachable studies. As a hybrid method between extractable and leachable-studies, simulation-studies potentially offer time efficient screening of impurities while also providing quantitative estimation.

The present simulation study showed only limited agreement with the reference result of the extractable and leachable studies, respectively. Only few of the substances detected in the extractables study of chapter 4 were observed in the simulation study (5.1). The leachable profile obtained in the simulation study showed also not exact agreement with the production line leachable study in chapter 4, as shown in 5.2. Our simulation-study indicates a total amount of leachables in the range of parts per trillion (ppt). These calculations are based on the worst case assumption that no leaching had taken place during the CIP/SIP procedure and steady leaching is observed. However, the simulation studies could not explain larger concentrations up to 0.025 ppm, as observed in the real-world leachable-study. This discrepancy warrants further investigation.

Table 5.1: Extractable-footprint conducted for the unused filling tubing (see chapter 4). Comparison to the Leachable-footprint (see chapter 4 - Leachables that occur between position 4 and 5) and to the Simulation-footprint of the used filling tubes.

Chemical group	Extractable-footprint	Overlap of the Leachable-footprint	Overlap of the Simulation-footprint
Acids	n-Decanoic acid		
	9-Hexadecenoic acid		
Alcohols	Ethanol, 1-(2-butoxyethoxy)-		
	Ethanol, 2-(2-ethoxyethoxy)-		
	Ethanol, 2-(2-butoxyethoxy)-, acetate,	x	
	Benzyl alcohol		
	2-Hexyl-1-octanol		
	Cyclohexanol, 4-(1,1-dimethylethyl)-		
	2-Propanol, 1-(2-methoxy-1-methylethoxy)-		
	1-Dodecanol		
	1-Decanol,2-hexyl-,		
	1-Tetradecanol		
	2-Methyl-1-undecanol		
	1-Hexadecanol, 2-methyl-	x	
Aldehydes	$C_9H_{18}O - C_{12}H_{24}O$ (Nonanal – Dodecanal)		
Alkanes	Straight chain and non-straight alkanes: $C_{12}H_{26}$ – $C_{28}H_{58}$ (Dodecane / 9-methylheptadecane - Octacosane)		
Alkene	17-Pentatriacontene		
Amides	2-Pyrrolidinone, 1-methyl-,		
	4(1H)-Pteridinone, 2-amino-		
Aromatic compounds	Acetophenone	x	x
	Benzaldehyde		x
	1,3-Di-tert-butylbenzene		
	Benzene, 1,2,4-trimethyl-5-(1-methylethyl)-		
	2,6-Di-tert-butyl-hydroquinone		
	unknown aromatic compound		
	2-Octyl benzoate		
	2,6-Di-tert-butyl-hydroquinone		
	Phenol, 2,4-di-t-butyl-6-nitro-,		
Ester	Glycerol 1,2-diacetate		
	1-Methoxy-2-propyl acetate (Glycerol)		
	Nonanoic acid, 9-oxo-, methyl ester		

	Dodecanedioic acid, bis(tert-butyldimethylsilyl) ester		
	Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester		
	m-Toluic acid, 4-hexadecyl ester		
	Pentadecanoic acid, 14-methyl-, methyl ester		
	Acetic acid, phenylmethyl ester		
	Methacrylic acid, nonadecyl ester		
	Hexadecanoic acid, 2-methylpropyl ester		
Ketone	Benzophenone		
Lactams	Caprolactam		
Polycyclic aromatic	9,9-Dimethyl-9-silafluorene		
Lactams	Caprolactam		
Phosphates	Triethyl phosphate		x
	Tributyl phosphate		
Phthalate	Diethyl Phthalate		
	Diisobutyl phthalate		
	Butyl Isodecyl Phthalate		
	Benzoic acid, 4-(4-butylcyclohexyl)-, 2,3-dicyano-4-ethoxyphenyl ester		
Siloxanes	Silanediol, dimethyl-; 2,5-Bis[(trimethylsilyl)oxy]benzaldehyde; Cyclosilioxanes ($C_6H_{18}O_3Si_3 - C_{20}H_{60}O_{10}Si_{10}$); Siloxane chains ($C_7H_{21}O_2Si_3 - C_{16}H_{48}O_6Si_7$)		x
Triazine	1,3,5-Triazine-2,4,6(1H,3H,5H)-trione, 1,3,5-tri-2-propenyl-		

Table 5.2: Extractable-footprint conducted for the unused filling tubing (see chapter 4). Comparison to the Leachable-footprint (see chapter 4 - Leachables that occur between position 4 and 5) and to the Simulation-footprint of the used filling tubes.

m/z	Extractable-footprint	Overlap of the Leachable-footprint	Overlap of the Simulation-footprint
Pos.: 202,1807	$C_{11}H_{23}NO_2$ 99.99%		
Pos.: 191,0239	$C_3H_{12}O_5P_2$ 99.77%		
Pos.: 173,0796	Triethylene glycol $C_6H_{14}O_4$ 96.05%		x

Pos.: 215,1260	Uvinul 3000 2,4-dihydroxybenzophenone C13H10O3 tentative		
Pos.: 217,1056 Neg.: 327,9723	$C_4H_{17}N_4O_4P$ 94.86%		
Pos.: 163,0611 Neg.: 327,9812	$C_5H_{14}O_2Si_2$ 93.46%		x
Pos.: 261,1310	Pentaethyleneglycol $C_{10}H_{22}O_6$ 98,85%		x
Pos.: 199,0587	Glycerine acetate $C_7H_{12}O_5$ 99.99%		x
Pos.: 305,1585	$C_8H_{25}N_4O_6P$ 96.15%		
Pos.: 114,0921	Caprolactam $C_6H_{11}NO$ 99.99%		x
Pos.: 177,1010	$C_8H_{14}N_2O$ 95.78%		
Pos.: 349,1844 Pos.: 481,2631 Pos.: 525,2886	Polyethylene glycol, ubiquitous polyether $[C_2H_4O]$nH2O 98.46%		x
Pos.: 255,0850	$C_7H_{19}N_2O_3P$ 99.52%		x
Pos.: 239,0900	Dimethyl 2-hydroxy-1,3-cyclohexanedicarboxylate $C_{10}H_{16}O_5$ 84.72%		
Pos.: 241,0700	Triacetin $C_9H_{13}O_6$ 99.99%		x
Pos.: 273,1678	$C_7H_{23}N_8P$ 91.01%		
Pos.: 205,0613	Triethyl phosphate $C_6H_{15}O_4P$ 99.99%		x
Pos.: 244,1428	$C_8H_{18}N_7P$ 78.84%		x
Pos.: 223,0946	Diethyl phthalate $C_{12}H_{14}O_4$ 99.99%		x
Pos.: 249,1111	$C_9H_{21}N_2OP$ 88.30%		x
Pos.: 227,1262	Unknown		x
Pos.: 279,0070	Dibutyl phthalate $C_{16}H_{22}O_4$ 99,99%		x
Pos.: 227,1266	$C_{10}H_{20}O_4$ 91.17%		
Pos.: 371,2419	$C_{17}H_{41}O_2P_3$ 71.94%		x
Neg.: 170,9881	Unknown		
Neg.: 471,2769	Unknown		
Neg.: 1012,4156	Unknown		
Neg.: 1142,0212	Unknown		

As expected, a decrease in concentration of volatile substances was observed for repeated use of silicon tubings. However, half of the detected substances (those with higher molecular weight) exhibited an increase in concentration. These results corroborate that

substances with low molecular weight are starting to leach right from the first application, while higher molecular weight substances are extracted comparatively late after increased mechanical stress.

The present results demonstrate poor agreement in the assessed leachable profiles with the reference leachable study and thus no improved accuracy compared to the extractable study. Potential leaching in the SIP/CIP step introduces some additional uncertainty in the quantification. Accordingly, improved accuracy in the quantitative assessment can be expected for SUSs where no intermediate preparation steps are required. Furthermore, the present study-design was based on the assumption that all tubes had the exact same amount of additives polymerized in their matrix.

5.5 Conclusion

Simulation-studies have been previously presented as an emerging technique for simple and minimally disruptive detection of impurities in pharmaceutical filling lines. However, no accurate leachable identification could be observed within this study compared to the well-known extractable & leachable concept especially for critical manufacturing processes. However, extensive testing is warranted to establish the level of accuracy of simulation studies in various conditions and qualify this method for different SUSs.

Bibliography

[1] C. T. Houston. *Crafting Controlled Extraction and Simulation Studies for Parenteral and Ophthalmic Drug Products: Impact of Formulation on Study Design.* Rockville, MD, 2013.

[2] W. Ding, G. Madsen, E. Mahajan, S. OConner, and K. Wong. Standardized Extractables Testing Protocol for Single-Use Systems in Biomanufacturing. *Pharma. Eng.*, 34(6), 2014.

[3] A. W. Sexton, A. D. Rusu, C. Smalley, D. Tapiawala, D. Lok, G. L. Madsen, K. McGohan, K. M. Wong, K. Dhamasiri, L. Litauszki, M. Jahn, P. Wang, S. O'Connor, S. Yi, and W. Ding. Guide for Evaluating Leachables Risk from Polymeric Single-Use Systems used in Biopharmaceutical Manufacturing. *BioPhorum Opterations Group*, 2017.

[4] Ca. Worsoe. *The simulation study – the replacement of extraction studies for PODP E&L documentation?* Europe, 2012.

[5] D. Jenke, T. Egert, A. Hendricker, J. Castner, T. Feinberg, C. Houston, D. G. Hunt, M. Lynch, K. Nicholas, D. L. Norwood, D. Paskiet, M. Ruberto, E. J. Smith, F. Holcomb, and I. Markovic. Simulated Leaching (Migration) Study for a Model Container-Closure System Applicable to Parenteral and Ophthalmic Drug Products. *PDA Journal of Pharmaceutical Science and Technology*, 71(2):68–87, 2017.

[6] U.S. Pharmacopeia. Draft: Polymeric Components and Systems Used to Manufacture Pharmaceutical and Biopharmaceutical Drug Products. *USP 665*, 2016.

[7] R. Haep, A. Stratmann, S.A. Watt, and L. Martens. Protein-reactive extractables a screening assay which adds additional value to the traditional E/L workflow. *Pharmazeutische Industrie*, 80(1):104–114, 2018.

[8] Y. Zhang, S. Sun, X. Xing, Z. Du, Q. Guo, and W. Yu. Detection and Identification of Leachables in Vaccine from Plastic Packaging Materials Using UPLC-QTOF MS with Self-Built Polymer Additives Library. *Analytical Chemistry*, 88(13):6749–6757, 2016.

[9] M. Bolgar, J. Hubball, J. Groeger, and S. Meronek. *Handbook for the chemical analysis of plastic and polymer additives.* CRC Press, Taylor & Francis Group, Boca Raton, second edition edition, 2016.

Chapter 6

Final Summary & Outlook

The aim of this thesis was to extent the current knowledge around the analytical characterization and the migration behaviour of leachables in bio-pharmaceutical drug product manufacturing lines, equipped with diverse single-use systems (SUSs) such as disposable bio-process bags, sterile filters and silicone tubing. chapter 1 provides the background for the various analytical methods and extraction studies used to analyze real-life leaching of polymer-based impurities.

In chapter 2 the applicability of stir-bar sorptive extraction (SBSE) was evaluated for the detection of trace amounts of leachables in pharmaceutical manufacturing. The impact of various drug product formulations on the extraction and concentration efficiency was assessed via spiking/simulation studies. Furthermore, the suitability of the absorption method was studied by demonstrating the uptake of a wide range of potential leachables, despite a large variety in the volatility and the log $K_{O/W}$-setting of the substances. No matrix inhibition by large protein drugs and only mild reduction of recovery were observed in the relevant pH range. In contrast, the chemical category of the extracted substance and the alcoholic content of the matrix-solution had a higher impact. Nonetheless, all limits of detection were found to be well below the applicable thresholds in a worst case scenario analysis. This was further corroborated by a high linearity of the measurement technique. The optimal use of this technique, including the effect of the stir-bar coating material, the combination of different stir-bars and the stirring time and speed, was examined for protein-based drug matrices. Additionally, a conditioning step of the stir-bar, used to mitigate the inhibitory effects of the drug matrix, was investigated . Our results indicate that in combination with gas-chromatography mass-spectrometer (GC-MS), the conventional poly-di-methyl-siloxane (PDMS) coating achieves better recovery on average compared with the ethylene-glycol-(EG)-PDMS. Furthermore, despite inhibitory effects of alcoholic solutions the SBSE method combined with a PDMS coated stir-bar reached satisfactory sensitivity, and large improvements compared with direct injection of the sample into the GC-MS system. Preceding stir-bar preparation consisting of a simple soaking step improved the enrichment, effectively lowering the limit of detection.

chapter 3 elaborates on the concept of extractable studies to delineate the leachable candidates for each SUS in prior to manufacturing. Our results validate that by modelling extraction parameters the detection of potential leachables is possible. It can be distinguished between static and thermal extraction-procedures, where the polymer is tested with different solvents and contact-times, or by direct exposure of the material to different heat-stages. Depending on the intensity of the extraction conditions, the amount and type of extracted substances can vary widely. For the detection of more volatile substances with GC-MS analytic, direct material thermal desorption of polymers was shown to be a fast and easily applicable study-design to obtain comprehensive additive-fingerprints of each SUS. Finally to fully characterize the samples, elemental analysis of inorganic impurities was conducted with inductively coupled plasma (ICP)-MS analytic, in addition to the GC-MS analytic.

chapter 4 describes the major contribution of this work, direct assessment of leachables in real-world filling lines. Multiple samples were drawn from various positions in a conventional SUS equipped manufacturing line during commercial processing of representative drug products. Exhaustive and quantitative leachable-profiles were generated for each sampling point using previously introduced stir-bar sorptive extraction (SBSE) with polydimethylsiloxane (PDMS) and ethylene glycol(EG)-PDMS coated stir-bars, with subsequent TD-GC-MS and ultra performance liquid chromatography (UPLC)-MS. The majority of contaminating substances exhibited a consistent decrease for all drug products and independent of the investigated SUSs. This can be explained by dilution with buffer-feed and by absorption of leachables in filters and silicon tubing. To corroborate the latter, absorption was independently proven in a mock set-up. Additionally, all leachables in the manufacturing line were found in extremely low concentrations, all below toxicological thresholds. Furthermore, all substance were found to be non-genotoxic. The previously obtained extractable-footprint also enabled to trace back each leachables to the SUSs, where they originated. This data shows that most of the detected leachables, were observed right from the start of the filling line and were likely already found in the delivered API. This indicates that harsher processing parameters necessary in drug manufacturing on substance side lead to increased leaching-rates. Benign conditions in the filling line, on the other hand, pose no threat in terms of leachable contamination and most often lead to a decrease in leachable concentration. Neither a common source of leaching could be identified within the filling-line nor a specific product influence on quality or quantity of leachables. Changes in the pH-value of the matrix were the product property that was found to markedly affect the amount of leaching during manufacturing. Specifically, no significant impact of the active ingredients on the leachable-outcome was observed.

chapter 5 explores a novel strategy for migration-studies, suited for real-life filling lines. So called simulation studies are an intermediate step between classic extractable- and leachable-studies. Simulation studies generate quantitative leachable estimates by simulating the stress during the manufacturing process in a controlled environment. Simulation studies provide more restrictive leachable estimates compared with the comprehensive

worst-case profiles obtained in extractable-studies. Furthermore, simulation studies provide approximate quantification of the expected impurities. However, the leachable-profiles obtained in simulation studies do not necessarily form a superset of the final real-life leachable outcome. In our experiments simulation studies were used to evaluate the leaching-behaviour of a reusable filling-tubing, which was applied over six batch-fillings. The results shows a decrease in concentration of volatile substances with increasing number of repeated applications of the reusable system. Furthermore, substances with low molecular weight leached out in the first applications of the reuseable system. Conversely, substances with higher molecular weight were extracted with increased concentration after the polymer matrix was affected by mechanic stress.

SUS are rapidly gaining importance in pharmaceutical manufacturing and become increasingly established as industry standard. To date extractable studies are the method of choice to ensure patient safety in light of leachable contamination of the drug product. Our results demonstrate that extractable studies are suitable for delineating a superset of potential leachables. However, novel results based on the comparison to real-world leachable measurements also show that extractable-footprints lead to overly conservative use of SUS and can lead to restriction of efficient drug manufacturing based on inaccurate worst-case estimations. In this work we explored the use of leachable detection during the commercial filling process, a process which sheds new light on the actual patient exposure. Initial extractable footprints can be used to design tailored measurement techniques sensitive to the expected contaminants. Tailored measurements enable in-line leachable assessment with high accuracy and sensitivity and open up new possibilities for commercial leachable studies, without the repercussions of overly conservative worst-case estimations based on extractable studies. Our results also demonstrate that multiple SUSs act as leachable scavenger in the manufacturing process. To minimize leachable contamination in manufacturing further this concept warrants exploration as dedicated “leachable filter” for pharmaceutical manufacturing. Besides filter SUSs, PDMS and EG-PDMS coating showed excellent absorption, facilitating sensitive measurements with the SBSE technique. This material property could conceivably be exploited for improved scavenging technology within the filling line, essentially eliminating any residual risks associated with polymer leaching. While accurate evaluation and monitoring are paramount to ensure safe use of SUS the results presented in this thesis corroborate the usefulness of this novel material in pharmaceutical manufacturing. Hence, techniques, such as proposed in this thesis, especially when established for improved in-line use of SUS system, will eventually facilitate large scale use of novel, efficient and safe manufacturing techniques and ensure effective and widespread access to crucial drugs for those who are in need.