Juliana Münzer, Graz University of Technology, BSc.

Evaluation of Extractables and Leachables from Filter Materials and their Effect on Human Granulocyte-Colony Stimulating Factor (G-CSF)

MASTER'S THESIS

to achieve the university degree of

Master of Science

Master's degree programme: Biochemistry and Molecular Biomedical Sciences

submitted to

Graz University of Technology

Supervisor

Ao.Univ.-Prof. Mag. Dr., Hans Jörg Leis

Institute of Molecular Biosciences

Graz, January 2016

AFFIDAVIT

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly indicated all material which has been quoted either literally or by content from the sources used. The text document uploaded to TUGRAZonline is identical to the present master‘s thesis dissertation.

Date

Signature

Acknowledgement

First of all I would like to thank my supervisor Prof. Hans Jörg Leis for making this master thesis possible.

Moreover I want to thank Dr. Verena Wahl and Dr. Stefan Mohr for their great support and helpful input.

I would also like to express my sincere thanks to my beloved husband Markus, who supported me wherever possible.

Special thanks goes to my parents, who enabled my studies, and to my dear friend and colleague Reingard for her support.

Abstract

During the manufacturing of biopharmaceutical formulations, proteins are in contact with various polymeric components. Some of those components are filter membranes which have been commonly applied for sterile filtration. When sterile filtration is performed, the protein and the filter membrane are in close contact because of the sponge-like structure of the filter membrane.

Potential leachables from the filters may interact with the protein and increase the aggregation propensity. The resultant protein aggregation can either cause immunogenic responses in patients or loss of potency of the protein. In addition, leachables can interact directly with the protein and lead to protein damage.

The study examines the extractable and leachable profile of five different polymeric filter membranes by various chromatographic techniques including LC-MS, headspace GC-MS and GC-MS. In order to investigate their effect on protein stability hG-CSF (human granulocyte colony stimulating factor) formulations were spiked with filter leachable stock solutions at two different pH levels. The spiked formulations were analyzed with respect to their aggregation behavior. Significant differences were observed between formulations with and without leachable stock solutions.

Kurzfassung

Bei der Herstellung von biopharmazeutischen Formulierungen treten Proteine mit verschiedensten polymeren Komponenten in Kontakt. Diese Komponenten können unter anderem Filtermembranen sein, die üblicherweise für die Sterilfiltration eingesetzt werden. Auf Grund der „schwammartigen“ Struktur des Filters sind die Kontaktzeiten der Proteinformulierungen und der Filtermembrane während der Sterilfiltration sehr lange.
Potentielle Leachables dieser Filter können sich an das Protein anlagern und zur Bildung von Aggregaten führen. Die resultierende Proteinaggregation kann sowohl zur Immunantwort in Patienten führen als auch zum Wirksamkeitsverlust des Proteins. Außerdem können Leachables auch direkt mit dem Protein interagieren und so zu Proteinschädigungen führen.
Für eine ordnungsgemäße Analyse der auftretenden Extractables und Leachables von Filtern, wurden fünf verschiedene polymere Filtermembranen mit Hilfe unterschiedlicher chromatographischer Techniken, einschließlich LC-MS, Headspace GC-MS und GC-MS untersucht.
Um den Effekt von Extractables und Leachables auf die Proteinstabilität von hG-CSF Formulierungen zu untersuchen wurden diese bei zwei unterschiedlichen pH-Werten mit Leachable Stocklösungen versetzt und anschließend hinsichtlich ihres Aggregationsverhaltens analysiert. Signifikante Unterschiede wurden zwischen Formulierungen mit und ohne Leachable Stocklösungen detektiert.

Table of contents

List of figures

List of tables

Abbreviations

2-EH	2-Ethylhexanol
ACN	Acetonitrile
AUC	Area under the curve
CES	Controlled extraction study
CP-buffer	Citrate-phosphate buffer
CTA	Cellulose triacetate
DSC	Differential scanning calorimetry
EIC	Extracted ion chromatogram
E/L	Extractables and leachables
ESI	Electrospray ionization
E-stock	Extractable stock solution
GC	Gas chromatography
HPLC	High performance liquid chromatography
HS-GC	Gas chromatography of headspace volatiles
kDa	Kilo Dalton
LC	Liquid chromatography
m/z	Mass-to-charge ratio
MFI	Micro flow imaging
MS	Mass spectrometry
n.d.	not determinable
PA	Polyamide
PC	Polycarbonate
PES	Polyethersulfone
PSS	Protein stability study
PTFE	Polytetrafluoroethylene
RC	Regenerated cellulose
RP	Reversed phase
Rt	Retention time
SEC	Size exclusion chromatography
SPME	Solid phase micro extraction
TIC	Total ion current
UV	Ultraviolet

1. Introduction

Polymeric components, such as solvent containers, filter membranes and package material, are widely used in the pharmaceutical industry. Filter membranes are often used for sterile filtration, which is a major step during manufacturing of protein formulations (see Figure 1) [1][2].

Sterilization needs to be performed in pharmaceutical manufacturing processes to remove microorganisms. Due to the sensitivity of proteins regarding to heat, sterilization is performed via sterile filtration in biopharmaceutical formulations. Sterile filtration is also called a “cold” method of sterilization because the sterilization process is not based on destroying microorganisms by heat; it is based on the separation of microbial mass from the residual-formulation [3].

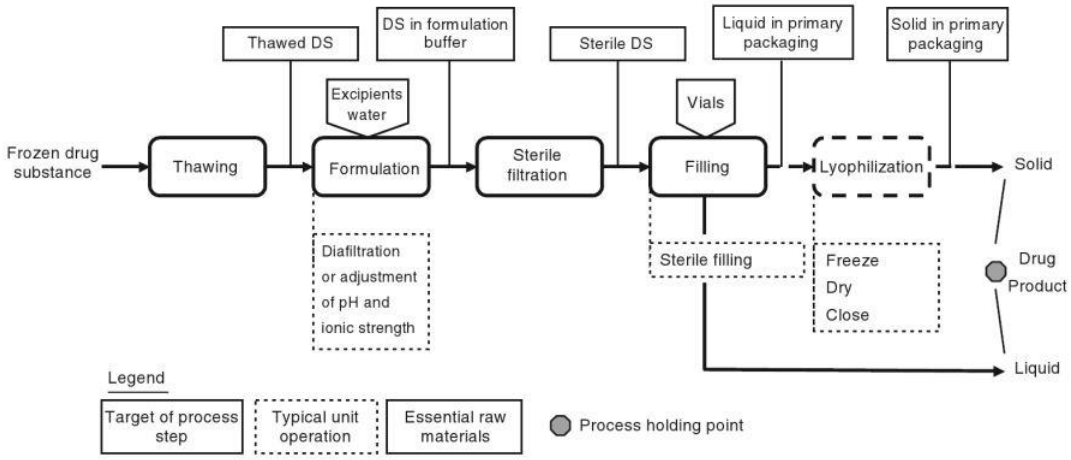


Figure 1: Schematic of formulation and filling of pharmaceutical proteins. DS = drug substance [2].

Ideally, these filter membranes should be inert to avoid the release of filter material into pharmaceutical products as well as potential interactions. As a matter of fact no filtration membrane is absolutely inert, thus extractables and leachables are a concern [1][4].

Extractables are compounds that can be released from a contact material under exaggerated extraction conditions. Therefore, multiple solvents of different polarity at a certain temperature are used. Leachables otherwise are compounds that migrate from the contact material into the product solution under normal using conditions [4][5]. Ideally, leachables are a subset of extractables, but that is not always the case [6][7]. Extractables and leachables (E/L) can be a risk for the patient either because of the toxicity of the E/L itself or the potential negative

influence on efficacy and stability of the pharmaceutical product [6]. Identifying these impurities presents an analytical, but necessary, challenge [5][6].

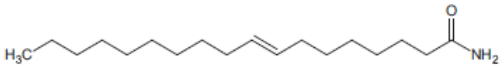
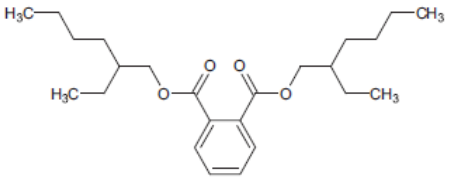
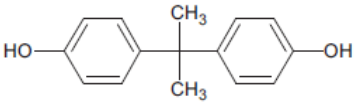
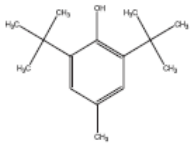
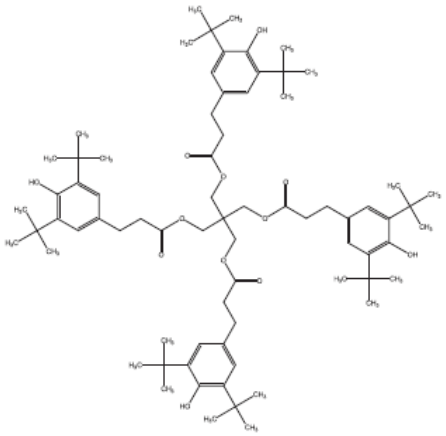
During sterile filtration the filtration area is often very small, but the contact surface that actually interacts with the solution being filtered is thousand times larger, because of the porous structure within the filter. Beside the sponge structure, other parts of the filter, like O-rings, filter housings or other polymeric components can be a source of leachables. However, drug manufacturers, not filter fabricators are responsible for filter compatibility with the manufacturing process. Therefore, the drug manufacturers need to ensure that levels of E/L that could negatively influence stability, quality or efficacy of the product are not leached into the product. For identifying these potential filter incompatibilities, extractable and leachable studies have to be performed [5].

1.1 Sources of extractables and leachables

Any contact material can be a source of extractables and leachables. Mostly plastics and elastomers are associated with the appearance of E/L. However, they can also come from metal or glass. The evaluation of E/L from pharmaceutical processing materials like filters started to be important, since the application of disposable use materials became more frequent [5].

For better processability and stability of the polymers, usually additives are used in the manufacturing of polymer-based plastic materials. Those additives are connected to the polymer via non-covalent bonds, which implies possible release into a contacting solution. The migrating additive amount varies depending on the properties of the solution itself and the contacting conditions. Pharmaceutical formulations are commonly water-based whereas most polymers and additives are naturally hydrophobic and organic. Therefore, the migration of organic polymers and their additives into the formulation is unlikely, but cannot be excluded. Whereas water for injection (WFI) does not leach excessive amounts out of hydrophobic polymers, water-based pharmaceutical formulations can act substantial different than WFI. Exemplarily the leaching propensity of a formulation may change significantly if solubilizing agents, like surfactants, are added to enhance the solubility. It is common to add more than one organic additive to a biopharmaceutical formulation. Typical examples for common leachables are listed in Table 1 [5][6].

Table 1: Examples of common leachables [6].

Class	Specific Example	Structure of Specific Examples
Lubricants	Oleamide	
Plasticizers	Bis(2-ethylhexyl) phthalate	
Monomers	Bisphenol A	
"Small" antioxidants	Butylhydroxytoluene	
"Large" antioxidants	Irganox 1010	
Organic impurities (alkanes, alcohols and aldehydes)	Butanol	

Lubricants

Typical examples for polymer additives are external and internal lubricants. External lubricants are used to lubricate the interface of the manufacturing equipment and the polymer material during processing. Internal lubricants are applied for lubrication of the polymer chains. Common lubricants are silicones, fatty acids or amides of fatty acids e.g. oleamides (see Table 1) [5][6].

Plasticizers

Plasticizers, e.g. Bis(2-ethylhexyl)phthalate (see Table 1), are common polymer additives used for enhancing the flexibility of polymers. However, the use of plasticizers in the filter production is rather unusual [1][5].

Monomers and oligomers

Monomers and oligomers present another class of potential impurities. They can either be a degradation product from oxidation of the readily manufactured polymer or a result of incomplete polymerization [5].

Antioxidants

Oxidation is a major problem in plastics made of polymers. Polymers which contain C-OH, C-H or C=O bonds are targets for oxidation and therefore, most polymers are rapidly degraded. Polymers without such bonds, like polytetrafluoroethylene (PTFE), are usually inert to degradation through oxidation. To prevent polymers from oxidation, antioxidants are used as additives during the manufacturing process. Mostly, phenolic antioxidants and hindered phenolic antioxidants as for example found in the product lines Irganox 1010 (see Table 1), Ethanox or Lowinox are added. These compounds provide an antioxidant effect according to their phenolic rings incorporated to their structure. The latter can capture free radicals, thus they are not able to oxidize the polymer anymore [5].

Wetting Agents

Wetting agents are frequently used in filters, most frequently when compared to other single-use polymeric materials in pharmaceutical manufacturing. Many filter materials like polyethersulfone (PES) and polyvinylidine fluoride are hydrophobic by nature. For producing the hydrophobic filter materials, hydrophilic wetting agents are added during manufacturing. Wetting agents can be either covalently bonded to the polymer or the polymer is impregnated or coated with the wetting agent to make the filter surface hydrophilic. Polyethylene glycol, polyvinyl pyrrolidone and polyacrylates are commonly used examples for wetting agents [5].

Moreover, sources for extractables and leachables can be molding agents, curing agents for elastomers, stabilizers, residual solvents, residual polymerization initiators and catalysts or reaction by products [8][9].

1.2 Extractable and leachable testing

Prior to the development of an analytical method for leachable testing, potential leachables need to be identified. This is achieved by performing extraction studies under exaggerated conditions with the aim to identify the detected extractables. The latter are compounds that can be extracted from an observed contact material that possibly become leachables. The correlation between extractables and leachables is illustrated in Figure 2.

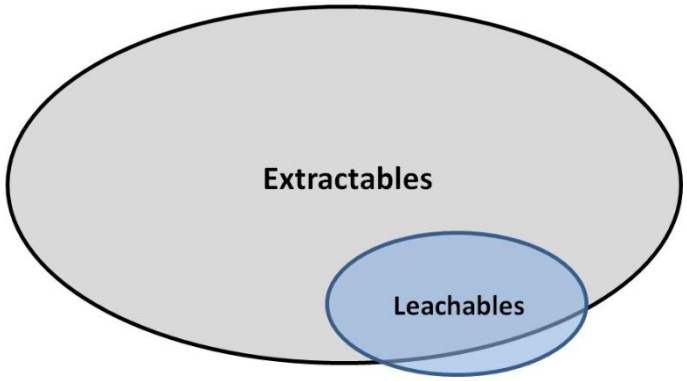


Figure 2: Relationship between extractables and leachables [8].

Conditions for extraction studies are chosen resting upon the drug product. Furthermore, extraction studies are designed to simulate a worst case scenario for the pharmaceutical product. In the selection process it must be borne in mind that conditions need to be aggressive enough to ensure that the extractables include all leachables; but not too aggressive, thus having a large number of extractables which are not leachables [6]. In addition, extraction conditions should enhance the migration of compounds out of contact materials but should not lead to a complete deformation of the material [5][10].

1.3 E/L study design

1.3.1 Fingerprint of the material

Prior to performing an extractable/leachable study, analyses of the contact material are accomplished to get an overview of the complete constitution of the material. The identification of the material composition makes it possible to select target compounds for the following steps of the E/L study. A few analytic techniques are available such as Chromatoprobe-GC coupled to MS, Thermodesorption GC/MS or Headspace GC/MS. At least one method should be used for the fingerprinting of the observed material [11].

1.3.2 Extraction study

In the second step toward evaluating leachables two different types of extraction studies can be performed: controlled extraction studies (CES) and simulated use extraction (SU) studies. They can be done in parallel or in series. In some cases doing just one of these two extractions may be sufficient.

In a controlled extraction study, also called material characterization study, the observed contact material is extracted with two or three solvents of different polarities. The different solvents are chosen based upon the physicochemical properties of the applied contact material where one of these solvents should represent a worst case scenario. For a CES accelerated extraction conditions are used, for example reflux or soxhlet extraction. With the combination of these aggressive extraction conditions and the solvent that mimic the worst case scenario it is possible to obtain a high number of extractables. As a result of this study type all potential leachables will be identified.

For simulated use (SU) extraction studies, also called simulation studies, the observed contact material is extracted with at least two solvents of different polarities. The solvents in SU studies are also selected based upon the properties of the contact material and should present an environment that is slightly more aggressive than normal using conditions. The extraction conditions in this study type are mostly static or agitated soaking of the contact material in the different solvents at a temperature slightly above the processing conditions of the pharmaceutical formulation. As a result of this extraction study, extractables which are likely to become leachables will be identified.

In contrast to a CE study, simulated use extraction studies are designed to be less aggressive. Therefore in SU studies a smaller amount of extractables is expected. SU studies are more likely to identify extractables that actually become leachables. CE studies on the other hand are likely to identify plenty of extractables, but they are not necessarily becoming leachables. This also means that a SU study is more likely to miss a potential leachable than a CE study.

Regardless which kind of extraction study was performed the resulting extracts are subsequently analyzed [6]. There is no single analytical technique which is sufficient to detect and identify all possible extractables from a contact material, thus multiple analytical techniques should be applied to ensure complete evaluation [11]. Since extractables from filters are expected to be below the concentration of 10 ppm and often below 1 ppm, sensitive analytical methods, including liquid chromatography coupled to mass spectrometry (LC–MS) and gas chromatography coupled to mass spectrometry (GC–MS), are required [5]. The aim of these analyses is to determine as many extractables as achievable [6].

1.3.3 Leachable study

The analytical methods, applied in the extraction studies, are further used in the leachable study. Leachable tests can either be performed as a part of product stability testing or in a separate migration study [6]. The aim of a leachable study is to evaluate the compounds migrating from the contact material into the pharmaceutical product under typical process conditions. The conditions for the study are selected based upon the highest acceptable range of the actual process conditions [5].

1.4 Filter materials

1.4.1 Polyamide

Polyamide (PA) is a collective term for polymers which contain amide bonds (the structural formula is shown in Figure 3) [12]. Since PA filters are naturally hydrophilic there are no wetting agents added during manufacturing of the filter [13]. Polyamide filter membranes are chemical resistant to organic solvents and alkaline solutions. They are appropriate for particle removing filtration of solvents, water and aqueous based solutions for analytical measurements such as HPLC. Also sterile filtration of these liquids can be achieved by PA filters. Their application is limited due to the fact that Polyamides have a high non-specific adsorption which can lead to loss of important substances of the sample [14].

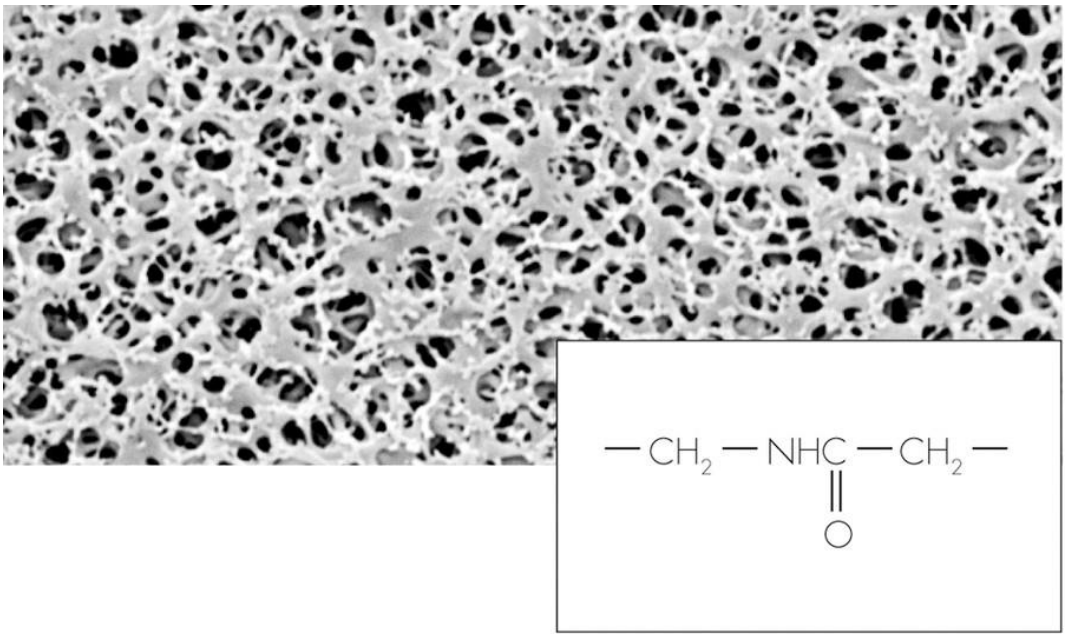


Figure 3: Microscopic image and structural formula of PA filter membranes [14].

1.4.2 Polycarbonate

Polycarbonates (PC) are polyesters of carbonic acid and aliphatic or aromatic dihydroxy-compounds (see Figure 4). Polycarbonate films are mostly manufactured by the reaction of bisphenol A and phosgene [15]. Under the use of track-etch technology a polycarbonate filter membrane is produced from a high grade PC film. PC filters are hydrophilic and have sharply defined pore sizes with a small range of pore size distribution [16]. Further on they have a good thermal stability and are highly chemical resistant, thus they can be used for a broad range of samples. Because of their flat and smooth surface they can achieve a high particulate visibility. Additionally, PC filter membranes have a low protein adsorption [17].

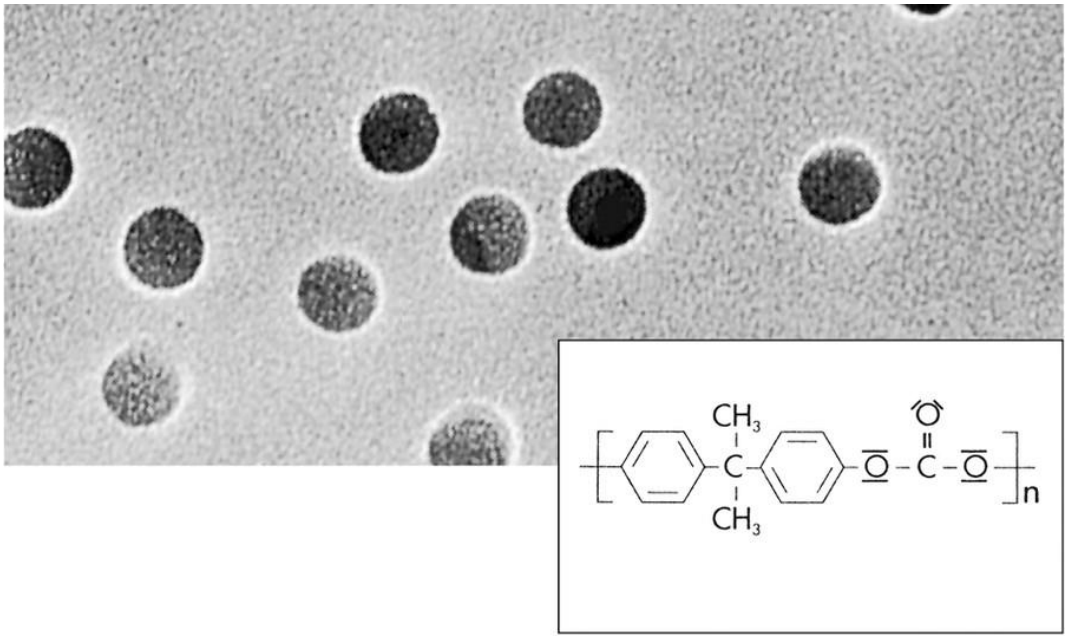


Figure 4: Microscopic image and structural formula of PC filter membranes [16].

1.4.3 Polyethersulfone

Polyethersulfones (PES) are polymers consisting of repeated units linked to each other through sulfone bonds (see Figure 5) [18]. PES filter membranes have a very high filterable volume since they allow the use of high flow speeds [19]. Their reaction to water is hydrophilic and they are acid and base resistant [20]. Because of the low protein adsorption of the filter, pharmaceutical solutions from pH 2 to pH 12 can be filtered. A microscopic image and the structural formula from a PES filter membrane is shown in Figure 5 [19].

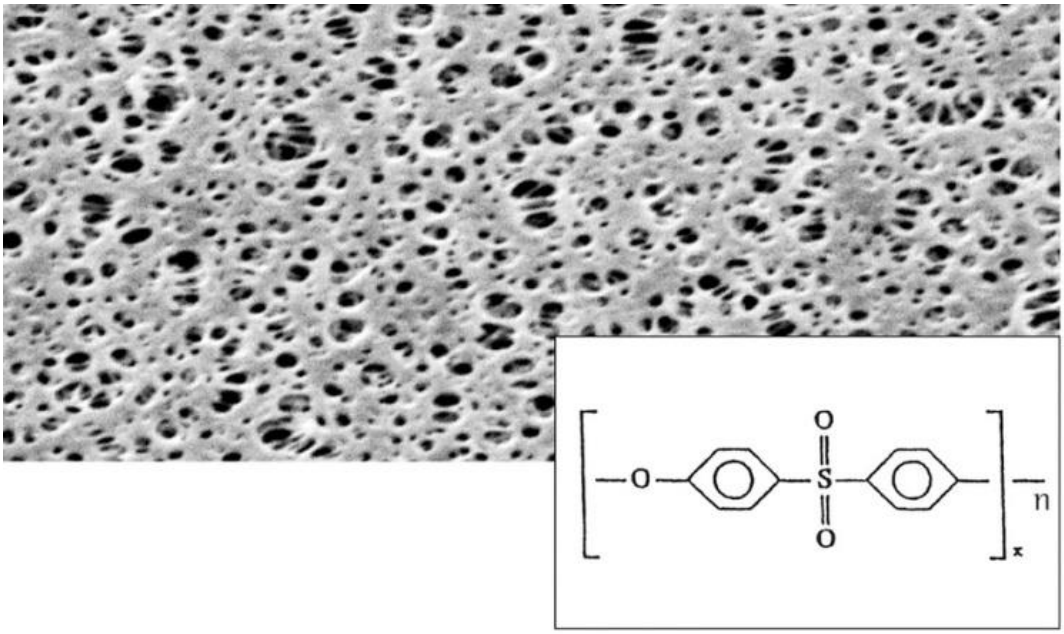


Figure 5: Microscopic image and structural formula of PES filter membranes [19].

1.4.4 Polytetrafluorethylene

Polytetrafluoroethylene (PTFE), better known as Teflon, is a fluoropolymer that consists of repeated units of tetrafluoroethen monomers (structural formula see Figure 6) [21]. PTFE filter membranes are mainly used for air/gas-filtration. Since they are made of pure PTFE they are permanently hydrophobic. Therefore, they cannot be wetted by air humidity in contrary to hydrophilic filters [22]. PTFE filter membranes have an excellent chemical resistance so that they are used for filtration of aggressive bases, acids and solvents that are incompatible with other filter membranes. Because of their hydrophobicity they need to be pre-wetted with alcohol when aqueous based formulations are filtrated [23].

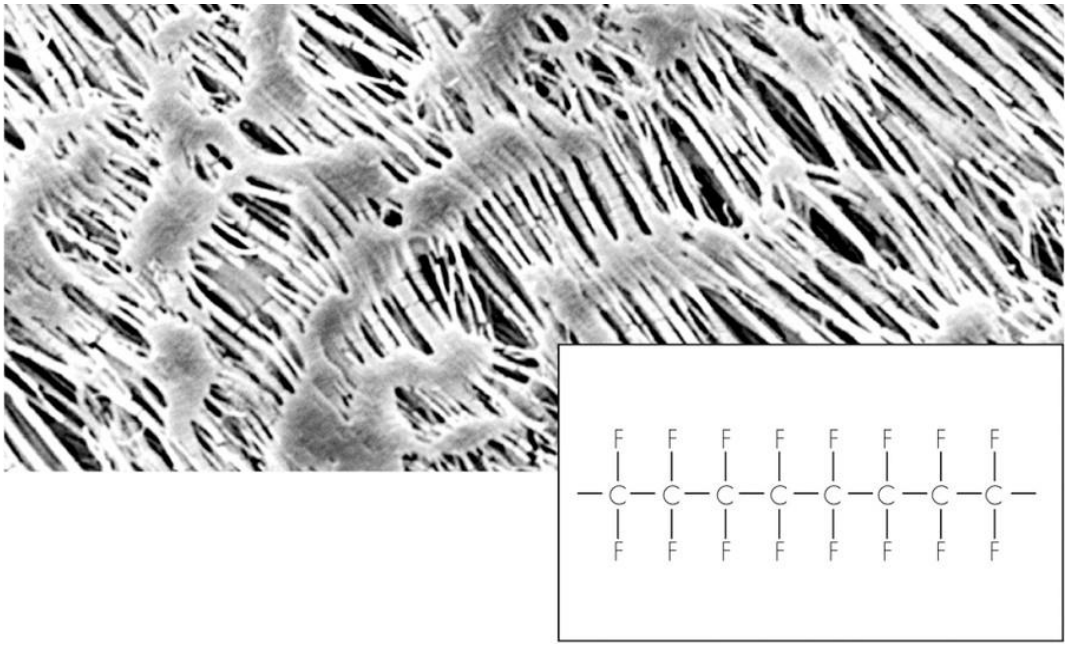


Figure 6: Microscopic image and structural formula of PTFE filter membranes [22].

1.4.5 Regenerated cellulose

Regenerated cellulose (RC) is a collective term for regained cellulose through precipitation of cellulose solutions or cellulose derivatives [24]. RC filter membranes are hydrophilic and highly resistant towards aqueous and organic solvents. They are primarily used for filtration of biological solutions because of their low non-specific protein adsorption. Furthermore they are applied to de-gas and ultraclean mobile phases and solvents for HPLC [25][26]. A microscopic image and the structural formula from a RC filter membrane is shown in Figure 7.

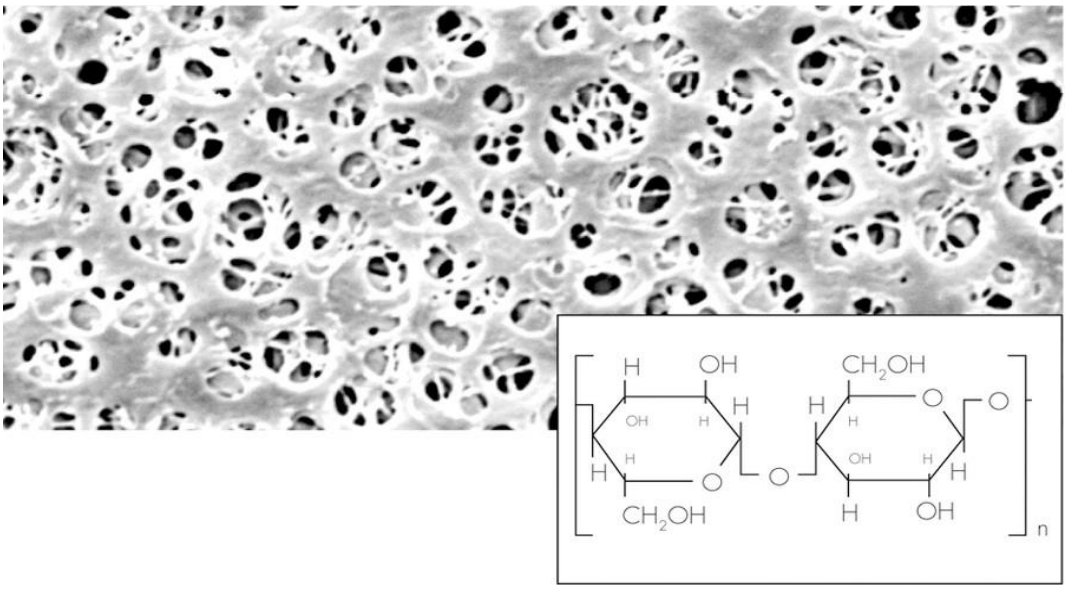


Figure 7: Microscopic image and structural formula of RC filter membranes [25].

1.5 Human granulocyte-colony stimulating factor

Human granulocyte-colony stimulating factor (hG-CSF) is a therapeutically relevant glycoprotein that belongs to the four-α-helix-bundle class of cytokines [27]. It is encoded by the G-CSF gene which is located on chromosome 17 q21-22. In its native conformation it has a molecular weight of 19.6 kDa and is o-glycosylated at Thr133. The glycosylation stabilizes the protein by protecting it from aggregation and change in conformation. The G-CSF protein is mainly produced by macrophages and therefore plays an important role in inflammatory response. Furthermore, it is involved in the proliferation of neutrophils, differentiation of precursor cells for neutrophil production and stimulates the activity of full-fledged neutrophil granulocytes. Because of its diverse functions it has various fields of application. On the one hand it is used for the treatment of neutropenia, which is a major side effect of chemotherapy. On the other hand, it is used for granulocyte transfusion therapy and to enable hematopoietic transplantations [28][29]. In addition G-CSF was tested in various studies as an alternative strategy for the treatment of Alzheimers's disease [30].

1.6 Motivation and aim of the work

The prevention of protein aggregation and protein denaturation respectively unfolding is a major goal in the successful formulation of biopharmaceuticals. Protein aggregates may cause immunogenic responses or lead to plugging during parenteral delivery and thus, can be harmful for the patient. Furthermore, protein unfolding/denaturation causes the loss of potency of the protein [31]. Via the use of size exclusion chromatography, electrophoretic light scattering, flow microscopy, differential scanning calorimetry and Raman spectroscopy potential protein aggregation and denaturation were extensively analyzed in this study. Protein unfolding and accumulation can be caused by various reasons for example heat, mechanical stress, radiation, strong acids or bases, detergents and organic solvents [32]. Some of those triggers may be descend from extractables and leachables from single use materials applied in the manufacturing process. In addition to the interaction with proteins, E/L itself may also be harmful for the patient. Therefore, an evaluation of extractables and leachables from applied materials is of great interest. In addition to the investigation of the effect on hG-CSF, used as model protein, extractables and leachables from filter materials were evaluated using various chromatographic methods.

2. Material and Methods

2.1 Materials

Polyamide, polycarbonate, polyethersulfone, polytetrafluoroethylene and regenerated cellulose filter membranes with a diameter of 47 mm and a pore size of 0.2 µm were kindly provided by Sartorius Stedim Biotech GmbH (Göttingen, Germany). Human granulocyte colony-stimulating factor (hG-CSF) from Sandoz (Kundl, Austria) was utilized.

2.2 Preparation of the extractable solutions for the controlled extraction study

For the controlled extraction study solvents of different polarities, namely ethanol (gradient grade for LC, Merck Millipore, Billerica, MA, USA), hexane (Rotisolv HPLC, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and purified water (TKA MicroPure system, TKA GmbH, Niederelbert, Germany) were used. Three filter membranes of each filter material were extracted in 10mL of extraction solvent at a temperature slightly under the solvent's boiling point (see Table 2). Each filter extractable stock (e-stock) was prepared in duplicate. Both, the ethanol and the hexane extracts were prepared in an orbital incubator shaker operated at a rotational speed of 100 rpm for 48 hours. The water extractable stocks were prepared in a compartment dryer at 90°C. Because of technical limitations the water extracts haven't been agitated (see Table 2).

Table 2: Extraction parameters for the controlled extraction study.

Sample ID	Filter type	Solvent	T [°C]	Agitation [rpm]	t [h]
PA_EtOH_I	Polyamide	Ethanol	70	100	48
PA_EtOH_II	Polyamide	Ethanol	70	100	48
PC_EtOH_I	Polycarbonate	Ethanol	70	100	48
PC_EtOH_II	Polycarbonate	Ethanol	70	100	48
PES_EtOH_I	Polyethersulfone	Ethanol	70	100	48
PES_EtOH_II	Polyethersulfone	Ethanol	70	100	48
PTFE_EtOH_I	Polytetrafluoroethylene	Ethanol	70	100	48
PTFE_EtOH_II	Polytetrafluoroethylene	Ethanol	70	100	48
RC_EtOH_I	Regenerated cellulose	Ethanol	70	100	48
RC_EtOH_II	Regenerated cellulose	Ethanol	70	100	48
PA_H_2O_I	Polyamide	Water	90	not agitated	48
PA_H_2O_II	Polyamide	Water	90	not agitated	48
PC_H_2O_I	Polycarbonate	Water	90	not agitated	48
PC_H_2O_II	Polycarbonate	Water	90	not agitated	48

PES_H_2O_I	Polyethersulfone	Water	90	not agitated	48
PES_H_2O_II	Polyethersulfone	Water	90	not agitated	48
PTFE_H_2O_I	Polytetrafluoroethylene	Water	90	not agitated	48
PTFE_H_2O_II	Polytetrafluoroethylene	Water	90	not agitated	48
RC_H_2O_I	Regenerated cellulose	Water	90	not agitated	48
RC_H_2O_II	Regenerated cellulose	Water	90	not agitated	48
PA_Hexane_I	Polyamide	Hexane	60	100	48
PA_Hexane_II	Polyamide	Hexane	60	100	48
PC_Hexane_I	Polycarbonate	Hexane	60	100	48
PC_Hexane_II	Polycarbonate	Hexane	60	100	48
PES_Hexane_I	Polyethersulfone	Hexane	60	100	48
PES_Hexane_II	Polyethersulfone	Hexane	60	100	48
PTFE_Hexane_I	Polytetrafluoroethylene	Hexane	60	100	48
PTFE_Hexane_II	Polytetrafluoroethylene	Hexane	60	100	48
RC_Hexane_I	Regenerated cellulose	Hexane	60	100	48
RC_Hexane_II	Regenerated cellulose	Hexane	60	100	48

2.3 Preparation of the leachable stock for the protein stability study

The leachable stocks for the protein stability study were prepared by extraction of PA, PC, PES, PTFE and RC filters in citrate-phosphate buffer (10mM) at pH 4 and pH 8. The different filter membranes were pre-wetted in purified water for 5 minutes and subsequently autoclaved by two cycles at 1 bar for 45 minutes. Further three membranes per filter type were extracted in 10mL CP- buffer at pH 4 and pH 8 in an orbital incubator operated at 100 rpm for 66 hours. The extraction conditions were selected based on previous studies (see Table 3) [4]. The leachable stocks for each type of filter were prepared either at 30°C or 50°C. Each filter leachable stock was prepared in duplicate for the following protein stability study.

Table 3: Extraction conditions of the leachable stocks for the protein stability study.

Sample ID	**Filter type**	**Solvent**	**T [°C]**	**Agitation [rpm]**	**t [h]**
PA_pH4_I	Polyamide	CP-buffer pH4	30/50	100	66
PA_pH4_II	Polyamide	CP-buffer pH4	30/50	100	66
PC_pH4_I	Polycarbonate	CP-buffer pH4	30/50	100	66
PC_pH4_II	Polycarbonate	CP-buffer pH4	30/50	100	66
PES_pH4_I	Polyethersulfone	CP-buffer pH4	30/50	100	66
PES_pH4_II	Polyethersulfone	CP-buffer pH4	30/50	100	66

PTFE_pH4_I	Polytetrafluoroethylene	CP-buffer pH4	30/50	100	66
PTFE_pH4_II	Polytetrafluoroethylene	CP-buffer pH4	30/50	100	66
RC_pH4_I	Regenerated cellulose	CP-buffer pH4	30/50	100	66
RC_pH4_II	Regenerated cellulose	CP-buffer pH4	30/50	100	66
PA_pH8_I	Polyamide	CP-buffer pH8	30/50	100	66
PA_pH8_II	Polyamide	CP-buffer pH8	30/50	100	66
PC_pH8_I	Polycarbonate	CP-buffer pH8	30/50	100	66
PC_pH8_II	Polycarbonate	CP-buffer pH8	30/50	100	66
PES_pH8_I	Polyethersulfone	CP-buffer pH8	30/50	100	66
PES_pH8_II	Polyethersulfone	CP-buffer pH8	30/50	100	66
PTFE_pH8_I	Polytetrafluoroethylene	CP-buffer pH8	30/50	100	66
PTFE_pH8_II	Polytetrafluoroethylene	CP-buffer pH8	30/50	100	66
RC_pH8_I	Regenerated cellulose	CP-buffer pH8	30/50	100	66
RC_pH8_II	Regenerated cellulose	CP-buffer pH8	30/50	100	66

2.4 Preparation of the protein formulations for the protein stability study

Human granulocyte colony-stimulating factor was obtained in acetate buffer with a concentration of 1 mg/mL. The protein stock was stored in a freezer at -20°C. In order to exchange the acetate buffer with the 10mM CP-buffer, ultra-filtration was applied. Therefore, spin columns (Vivaspin 20mL, Sartorius Stedim, Göttingen, Germany) with a molecular weight cut-off membrane of 5000 Dalton were used. The frozen protein was centrifuged in a spin column with 5000g at 4°C and the buffer was exchanged two times. The concentrated protein was then re-suspended in the desired CP-buffer. The achieved protein concentration was determined via UV absorbance measurements at 280 nm. For the preparation of the protein formulations the hG-CSF solutions were diluted with the different leachable stocks to achieve a final protein concentration of 0.6 mg/mL in the formulations. The leachable stock concentration in the final protein formulations was 0.65-fold in relation to the total sample volume. As a reference a hG-CSF-formulation without leachable stock was prepared in an analogous manner as the formulations with leachable stocks.

2.5 Analytical Methods

2.5.1 Solid phase micro extraction of headspace volatiles and add-on GC/MS

To obtain an overview of volatile compounds in the filters a solid phase micro extraction (SPME) of headspace volatiles from each filter membrane was performed. For this purpose the membranes were sliced in small pieces and 0.5 g weighed into a 20mL headspace-GC

(HS-GC) vial, containing a glass coated magnetic stir bar. Prior to this the HS-GC vials as well as the magnetic stir bars were heated at 200°C for at least 30 minutes. The vials were sealed with a PTFE lined silicone septum. The samples were preheated for 5 minutes at 50°C using a Combi Pal autosampler system. The resulting headspace volatiles were extracted at 50°C over 20 minutes onto a 2cm Stableflex 50/30µm DVB/Carboxen/PDMS SPME fiber (Supelco, Bellefonte, USA). For the following analysis of the headspace volatiles a 7890A gas chromatograph (Agilent Technologies, Santa Clara, USA) coupled to a 5975C mass spectrometer (Agilent Technologies, Santa Clara, USA) and a CTC Combi Pal autosampler (CTC Analytics AG, Zwingen, Switzerland) with SPME option was used. The separation was accomplished by the use of a ZB-5MSi column with a film thickness of 0.25 µm, an inner diameter of 0.25 mm and a length of 30 meters. The oven program was as follows: initial hold: 35°C for 1 minute, heating up to 230°C with a heating rate of 5.5°C per minute, final hold: 230°C for 1 minute. Helium was used as carrier gas with a flow rate of 0.86 mL/min. The MS interface temperature was set to 280°C and mass detection was operated in scan mode from 35 to 300 m/z with a delay time of 5 minutes after injection. Volatile compounds were identified via comparison of their mass spectra with the NIST mass spectral library. Compounds with a match quality higher that 90% were considered identified.

2.5.2 GC/MS

Gas chromatography was applied for the controlled extraction study to obtain an overview of the semi-volatile compounds in the extracts. Therefore the hexane and ethanol extractable stocks were analyzed by a Shimadzu GC-2010 Plus gas chromatograph coupled to a GCMS-QP2010SE mass spectrometer (Shimadzu, Kyōto, Japan). A HP5MS column with a film thickness of 0.25 µm, a diameter of 0.25 mm and a length of 30 m was used as stationary phase. The following temperature program was applied: initial hold: 50°C for 1 minute, heating up to 310°C with a heating rate of 10°C per minute, final hold: 310°C for 3 minutes. Helium was used as carrier gas at a flow rate of 0.8 mL/min. Injection of 1 µl of sample solution was performed automatically by an AOC-20I auto injector (Shimadzu, Kyōto, Japan). The interface temperature of the mass spectrometer was set to 250°C and a mass range from 30 to 600 m/z was scanned starting 3.5 minutes after the injection. Detected compounds were identified by the comparison of their mass spectra with the NIST mass spectral library. Compounds with a match quality higher that 90% were considered identified.

2.5.3 RP-HPLC/UV/MS

HPLC/UV and HPLC/MS measurements were performed for the extracts of the controlled extraction study and for the extracts of the protein stability study. Beside the hexane extracts all other extracts were injected directly after extraction. Hexane extracts from the CES were treated as follows: 1mL of each extract, as well as a blank, were filled in a HPLC vial and

evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 1mL mobile phase (10% acetonitrile, 90% purified water). 40µL of each vial were injected and analyzed by an Alliance 2695 Separations Module (Waters Corp., Milford, MA, USA) coupled with a 2996 Photodiode Array Detector (Waters Corp., Milford, MA, USA) and a Micromass® Quattro micro™ API mass spectrometer (Waters Corp., Milford, MA, USA). A C-18 reversed phase column (Purospher® STAR, Merck Millipore, Billerica, MA, USA) was used as stationary phase at a temperature of 20°C. Acetonitrile (A) (Sigma Aldrich, St.Louis, Missouri, USA) and water (B) were used as mobile phase using the following gradient program: 0-40 minutes linear gradient from 10% Acetonitrile (ACN) to 100% ACN, 40-45 minutes isocratic hold 100% ACN, 45-45.1 minutes from 100% ACN to 10% ACN and 45.1-55 minutes isocratic 10% ACN. The flow rate was set to 1mL/min and UV detection was performed at 220nm. In order to enable appropriate electrospray ionization the flow towards the MS detector was split in a 1:1 ration resulting in 0.5mL/min flowrate. To improve ionization a 30mM ammonium acetate solution in water was infused post column via a "T" connector into the mobile phase at a flow rate of 0.1mL/min using an external pump (LC10AD, Shimadzu, Kyōto, Japan). The operation parameters for the mass spectrometer measurements are shown in Table 4. A mass range from 110 to 1200 m/z was scanned. Data analysis was performed with the corresponding MassLynx 4.1 software (Waters Corp., Milford, MA, USA).

Table 4: Operating parameters for HPLC/MS measurements.

Operating parameters	Operating value
Voltages	
Capillary [kV]	3.00
Cone [V]	35.00
Temperatures	
Source [°C]	130
Desolvation [°C]	400
Gas flows	
Desolvation [L/hr]	750
Cone [L/hr]	50.0

2.5.4 SEC-HPLC

For the size exclusion chromatography measurements a Merck Hitachi LaChrom liquid chromatography system with an L-7400 UV detector was used (Hitachi, Darmstadt, Germany). The separation was accomplished by a TSK-GEL G3000SWXL column that was equipped with a guard column from Tosoh Bioscience (Tosoh Biosience, Tokyo, Japan). Citrate-phosphate

buffer at pH 4 and pH 8 were used as mobile phases with a flow rate of 0.5 mL/min, depending on the pH-value of the protein formulation. The column temperature was set to 30°C [33]. Before the measurements, the column was saturated with rhG-CSF at a concentration of 1.71mg/mL.

2.5.5 Density

Prior to surface tension measurements, density needs to be determined. The solvents from the controlled extraction study and the protein stability study were analyzed using a DSA 5000 M density and sound velocity meter (Anton Paar GmbH, Graz, Austria). Triplets were performed for each solvent and the results were averaged.

2.5.6 Surface tension analysis

The surface tension of the ethanol and water extracts from the controlled extraction study as well as the leachable stocks of the protein stability study was determined using the EasyDrop System (Krüss, Hamburg, Germany). The measurements were performed with the pendant drop method using a 500 µL Hamilton syringe with a 0.8 mm needle diameter (Hamilton, Reno, Nevada, USA). A ten-fold determination was executed for each sample and subsequently averaged.

2.5.7 Zeta potential

Electrophoretic light scattering was applied to analyze the zeta potential of the protein formulations using a Malvern ZetaSizer Nano-ZS (Malvern Instruments, UK) equipped with a 532 nm laser. A sample of approximately 1 mL was filled in a folded capillary cell (Malvern Instruments, UK), equilibrated at 25°C and measured three times. The resulting values were averaged. Data analysis was conducted by the Malvern Zetasizer software.

2.5.8 Microflow Imaging

The protein aggregate formation of the different protein formulations was analyzed using micro-flow imaging (MFI^{TM}). Therefore a MFI 5100 flow microscope, for the acquisition of protein particles between 2-300 µm, from ProteinSimple (Santa Clara, CA, USA) was used. Samples of about 330 µL from the different protein formulations were analyzed. A three-fold determination was performed for each formulation and the particle concentrations were averaged.

2.5.9 Differential scanning calorimetry (DSC)

The different protein formulations were analyzed for thermal events using a differential scanning calorimeter (DSC 204F1 Phoenix, Netzsch GmbH, Germany). Samples of each protein formulation of about 10-20 mg were placed into an aluminum pan. The aluminium pan was subsequently closed with an closed aluminium lid. As a reference an empty aluminum pan was used. For the characterization of the samples a modulated DSC method with a period of

40 seconds and an amplitude of 0.212 was performed. The formulation samples were heated from ambient temperature to 120°C with a heating rate of 2K/min. Pure nitrogen (Linde AG, Munich, Germany) with a flow rate of 20mL/min was used as purging gas. The data analysis was accomplished via Proteus Thermal Analysis Software (Netzsch GmbH, Germany).

2.5.10 Raman spectroscopy

To analyze the secondary structure of the protein formulations Raman spectroscopy was applied. Therefore, a RamanRXN2™ Hybrid Spectrometer (Kaiser Optical systems, Ann Arbor, MI, USA) with a 785nm laser was used. Samples of 3.5 mL were taken from the different protein formulations and stirred with 100 revolutions per minute. During the agitation the samples were scanned between a Raman shift of 100 cm^{-1} and 1900cm^{-1} six times for 45 seconds using a MR probe, which was immersed into the formulation. The corresponding iC Raman 4.1™ software was used to record the spectra. For the following data analysis SIMCA 13.0 software (Umetrics, Umeå, Sweden) was utilized and a SNV correction was performed.

3. Results and Discussion

This chapter is divided into two sections (see Figure 8). The first section deals with the evaluation of extractables and leachables from filter materials via the use of SPME-GC/MS, GC/MS and LC/UV/MS. The second part is dealing with the analysis of the effect of leachable stock solutions on human granulocyte-colony stimulating factor (rh-GCSF), which is termed protein stability study (PSS) in the following.

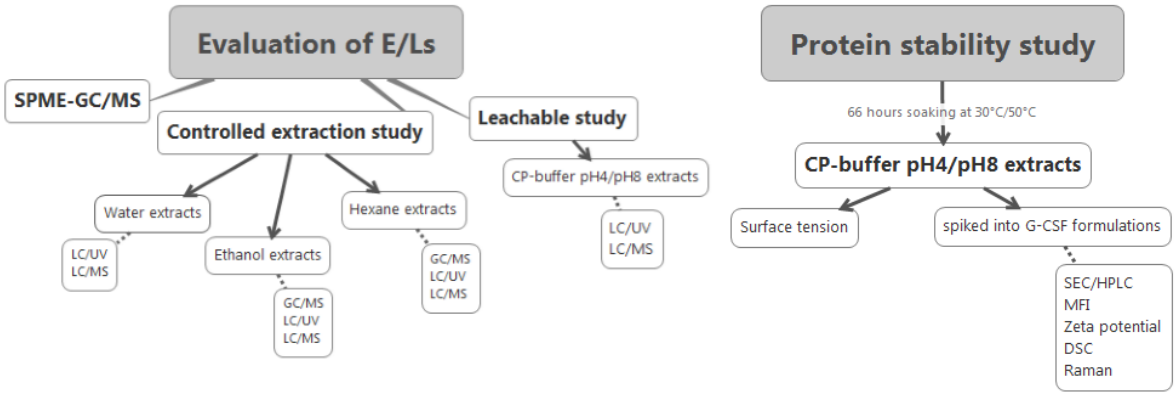


Figure 8: Flowchart for the evaluation of E/L and the following protein stability study.

3.1 Evaluation of extractables and leachables

3.1.1 Solid phase micro extraction (SPME) of headspace volatiles and add-on GC/MS

By using solid phase micro extraction, followed by GC/MS, it was possible to identify diverse volatiles in all filters. The total ion chromatogram of polyamide is shown in Figure 9; the detected compounds are given in Table 5. Five main peaks at Rt 5.97 min, 8.39 min, 12.32 min, 22.00 min and 27.00 min respectively were observed. Those peaks were identified as hexanal, styrene, 2-ethylhexanol, tetradecane and isopropyl laurate. In addition various peaks of lower intensity were detected and identified as hexamethylcyclotrisiloxane, nonanal, N,N-dimethylformamide and several branched and unbranched aliphatic hydrocarbons of short chain length. In addition a large number of small peaks was detected which could not be identified.

Some of the determined substances could be associated with compounds used in polymer fabrication such as styrene, 2-ethylhexanol and N,N-dimethylformamide. Styrene is a toxic liquid and is known to be used in the manufacturing of rubbers and polymers [34]. 2-ethylhexanol (2-EH) is a high-boiling solvent and has various fields of application. Most of the

produced 2-EH is used as an esterification component for the production of plasticizers [35]. N,N-dimethylformamide is known to be a common solvent during the manufacturing of diverse polymers like polyamides, polyurethanes and others [36].

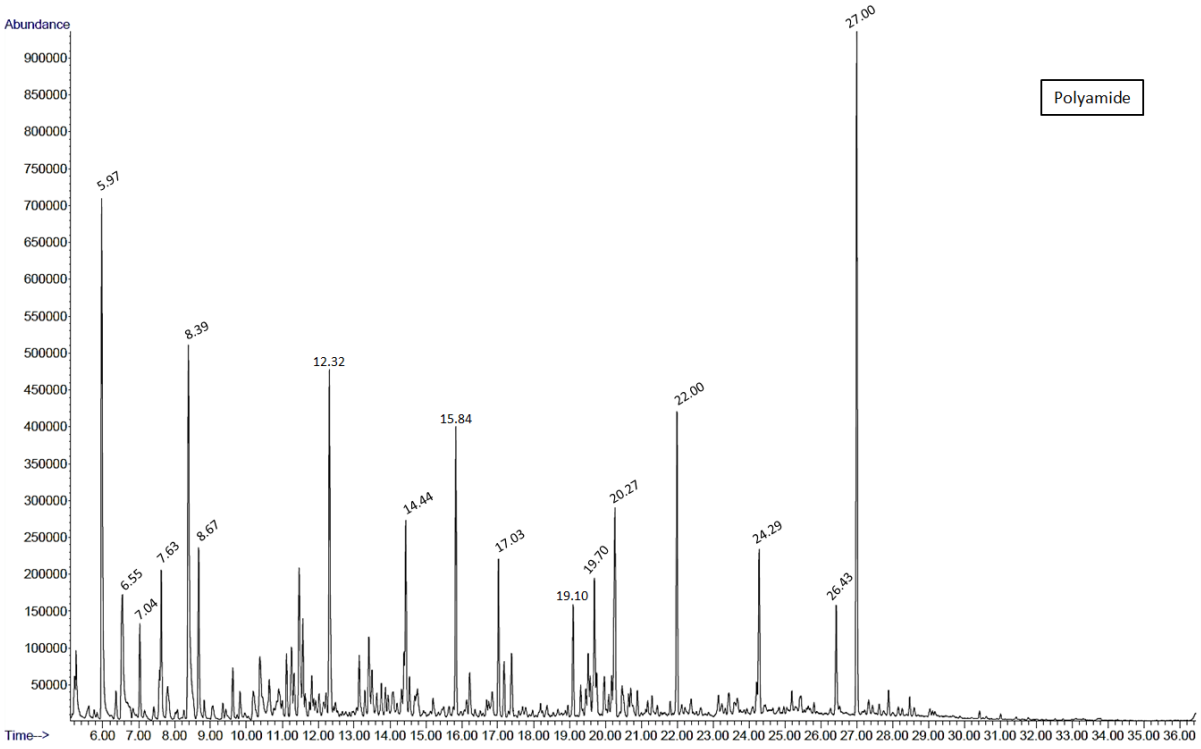


Figure 9: SMPE-HS-GC/MS total ion chromatogram of polyamide filters.

Table 5: Results of SPME-HS-GC/MS from polyamide filters.

RT [min]	Compound	Molecular weight	Cas #
5.97	Hexanal	100.16	66-25-1
6.55	Hexamethylcyclotrisiloxane	222.46	541-05-9
7.04	2,4-Dimethyl-1-hepten	126.24	19549-87-2
7.63	4-Methyloctan	128.26	2216-34-4
8.39	Styrene	104.15	100-42-5
8.67	n.d.	-	-
12.32	2-Ethylhexanol	130.23	104-76-7
14.44	Nonanal	142.24	124-19-6
15.84	n.d.	-	-
17.03	Dodecane	170.33	112-40-3
19.10	n.d.	-	-
19.70	N,N-Dimethylformamide	157.25	761-65-9

20.27	n.d.	-	-
22.00	Tetradecane	198.39	629-59-4
24.29	n.d.	-	-
26.43	Hexadecane	226.44	544-76-3
27.00	Isopropyl laurate	242.40	10233-13-3

n.d. = not determinable

A lower number of peaks was detected in polycarbonate samples (Figure 10). The most prominent peak was detected at a retention time of 7.19 minutes and was identified as chlorobenzene. Peaks of lower intensity were detected at a retention time of 5.97 min, 8.39 min and refer to hexanal and styrene, as already seen in polyamide samples. In addition, traces of toluene, butylacetate, xylene, siloxanes and carene were detected (see Table 6). The chromatogram also shows various small peaks, which were not identifiable.

Some of the identified compounds could be associated with polymer manufacturing such as chlorobenzene, toluene and xylene. Chlorobenzene is an important solvent in the manufacturing of polycarbonate and can be used as a solvent for dissolving chain terminator- or branching agents. It can also be applied as the organic phase for interphase polycondensation. Chlorobenzene can be used either alone or mixed with other suitable solvents. Toluene is another solvent that could be used during the manufacturing of polycarbonates [37][38]. Xylene is available as a mixture of three isomers: ortho-xylene, para-xylene and meta-xylene. They are usually not separated from each other. Pure o-xylene is used as a source material for the production of phthalic acid or phthalic anhydride, which is an intermediate for the manufacturing of plasticizers or polyesters. As the use of plasticizers is very common in polycarbonate, the appearance of xylene as an extractable from PC filters was an expected result [37][39]. Otherwise p-xylene is primarily used for the production of terephthalic acid which is further converted to polyesters. Meta-xylene is often isomerized to its ortho- or para isomer [39].

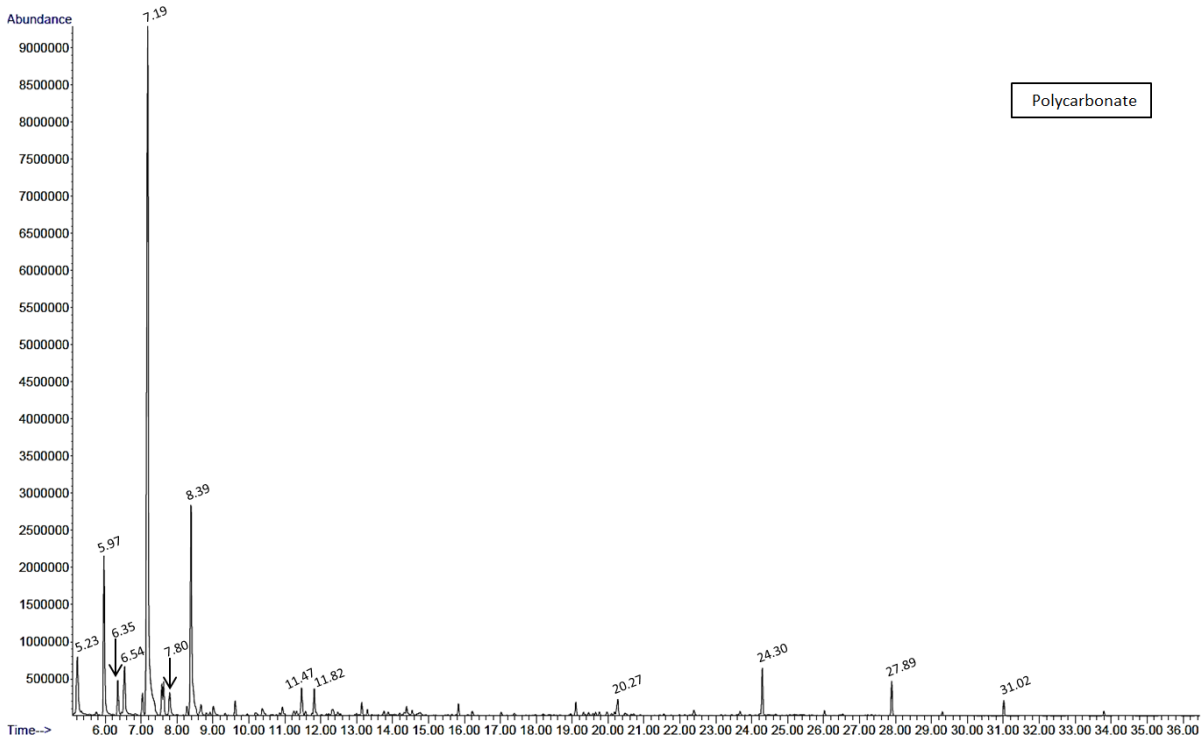


Figure 10: SPME-HS-GC/MS total ion chromatogram of polycarbonate filters.

Table 6: Results of SPME-HS-GC/MS from polycarbonate filters.

RT [min]	Compound	Molecular weight	Cas #
5.23	Toluene	92.14	108-88-3
5.97	Hexanal	100.16	66-25-1
6.35	Butyl acetate	116.16	123-86-4
6.54	Hexamethylcyclotrisiloxane	222.46	541-05-9
7.19	Chlorobenzene	112.56	108-90-7
7.80	m-Xylene	106.17	108-38-3
	p-Xylene	106.17	106-42-3
8.39	Styrene	104.15	100-42-5
11.47	Octamethylcyclotetrasiloxane	296.62	556-67-2
11.82	Carene	136.23	498-15-7
20.27	n.d.	-	-
24.30	n.d.	-	-
27.89	n.d.	-	-
31.02	n.d.	-	-

n.d. = not determinable

The chromatogram of polyethersulfone showed a high number of peaks. In general the intensity of the peaks was times lower compared to polyamide, polycarbonate and regenerated cellulose samples (see Figure 11), implying lower amounts of volatile components. There were several peaks at a higher intensity and many smaller peaks. It was possible to identify five branched saturated and unsaturated aliphatic hydrocarbons, one siloxane, hexanal, styrene, α-pinene and longifolene (see Table 7).

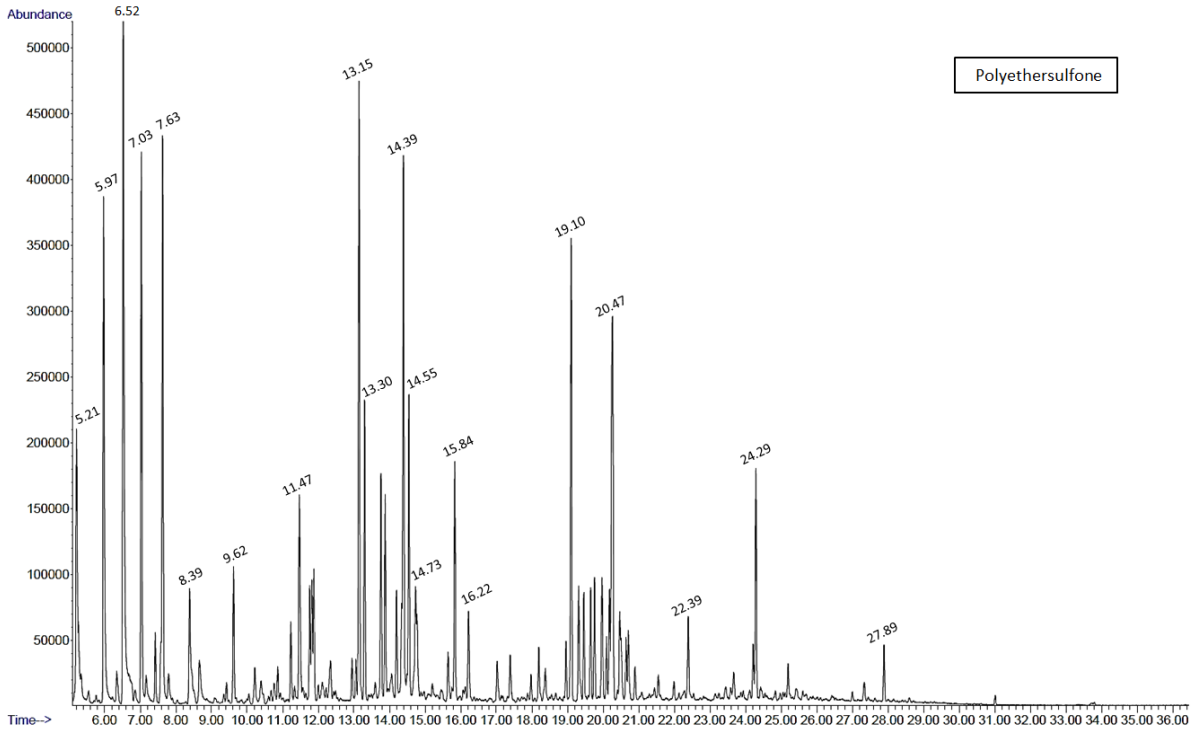


Figure 11: SPME-HS-GC/MS total ion chromatogram of polyethersulfone filters.

Table 7: Results of SPME-HS-GC/MS from polyethersulfone filters.

RT [min]	Compound	Molecular weight	Cas #
5.21	4-Methylheptane	114.23	589-53-7
5.97	Hexanal	100.16	66-25-1
6.52	2,4-Dimethylheptan	128.26	2213-23-2
7.03	2,4-Dimethyl-1-heptene	126.24	19549-87-2
7.63	4-Methyloctan	128.26	2216-34-4
8.39	Styrene	104.15	100-42-5
9.62	α-Pinene	136.23	80-56-8
11.47	Octamethylcyclotetrasiloxane	296.62	556-67-2
13.15	n.d	-	-
13.30	n.d	-	-

14.39	n.d	-	-
14.55	n.d	-	-
14.73	4-Methylundecane	170.33	2980-69-0
15.84	n.d	-	-
16.22	n.d.	-	-
19.10	n.d.	-	-
20.47	n.d.	-	-
22.39	Longifolene	204.36	475-20-7
24.29	n.d	-	-
27.89	n.d	-	-

n.d. = not determinable

In PTFE samples many peaks were detected, but most of it could not be identified (see Figure 12 and Table 8). Like polyethersulfone, the chromatogram of the PTFE sample showed a lower abundance compared to polyamide, polycarbonate and regenerated cellulose samples. Two siloxanes, two aliphatic hydrocarbons, verebenone, 4-butylnonane and isopropyl laurate were successfully determined.

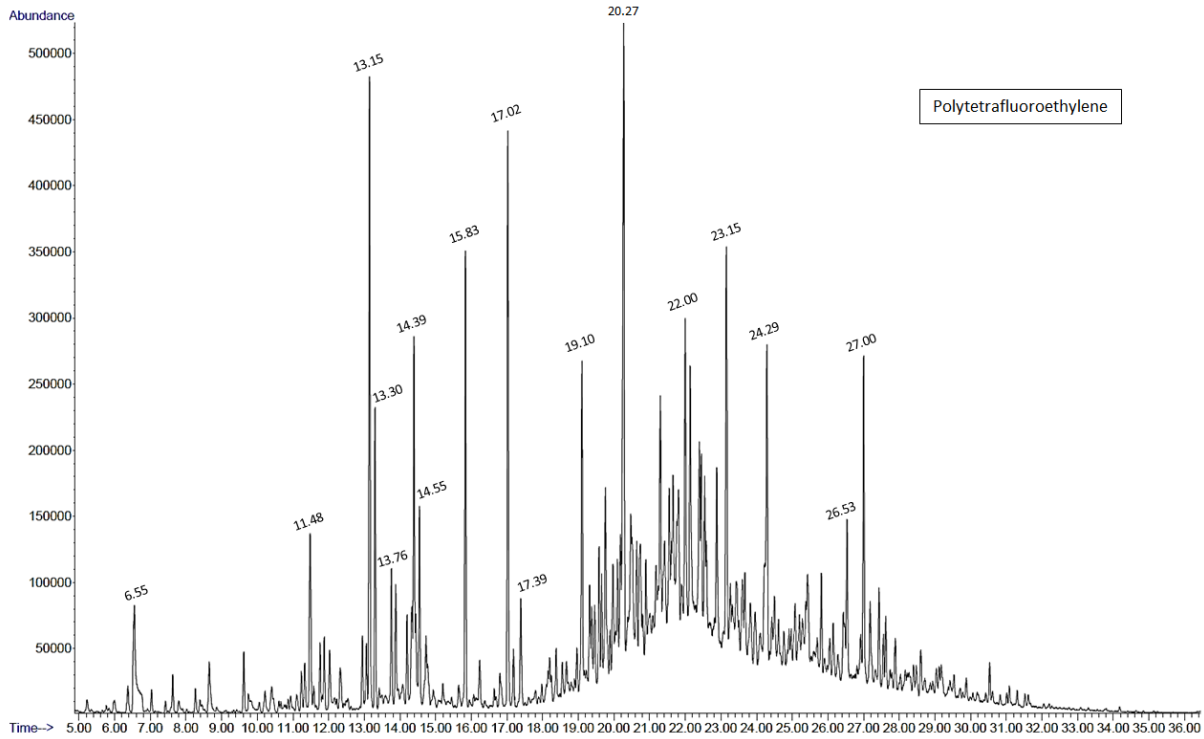


Figure 12: SPME-HS-GC/MS total ion chromatogram of polytetrafluoroethylene filters.

Table 8: Results of SPME-HS-GC/MS from polytetrafluoroethylene filters.

RT [min]	Compound	Molecular weight	Cas #
6.55	Hexamethylcyclotrisiloxane	222.46	541-05-9
11.48	Octamethylcyclotetrasiloxane	296.62	556-67-2
13.15	n.d	-	-
13.30	n.d	-	-
13.76	n.d	-	-
14.39	n.d	-	-
14.55	n.d	-	-
15.83	n.d	-	-
17.02	Dodecane	170.33	112-40-3
17.39	Verebenone	150.22	1196-01-6
19.10	n.d.	-	-
20.27	n.d	-	-
22.00	Tetradecane	198.39	629-59-4
23.15	n.d	-	-
26.53	4-Butylnonane	184.36	17312-63-9
27.00	Isopropyl laurate	242.40	10233-13-3

n.d. = not determinable

In regenerated cellulose samples, fewer peaks were detected. The most prominent peak was detected at a retention time of 9.83 minutes and was identified as 6-methyl-3-heptanone. Identifiable peaks with lower peak intensity were detected at a retention time of 5.96 min, 8.38 min, 8.43 min, 12.32 min, 14.43 min, 17.02 min and 21.99 min and were identified as hexanal, styrene, cyclohexanone, 2-ethylhexanol, nonanal, dodecane and tetradecane. Due to literature, cyclohexanone plays an important role in RC manufacturing. RC can be produced via the hydrolysis of cellulose triacetate (CTA) films. For the production of CTA films a solution of CTA in a solvent mixture of cyclohexanone and methylene chloride is prepared. In the second step, the solution is casted and the membrane is precipitated through the immersion of the cast film into a methanol bath. The recovered CTA membrane can then be hydrolyzed to obtain regenerated cellulose membranes [40].

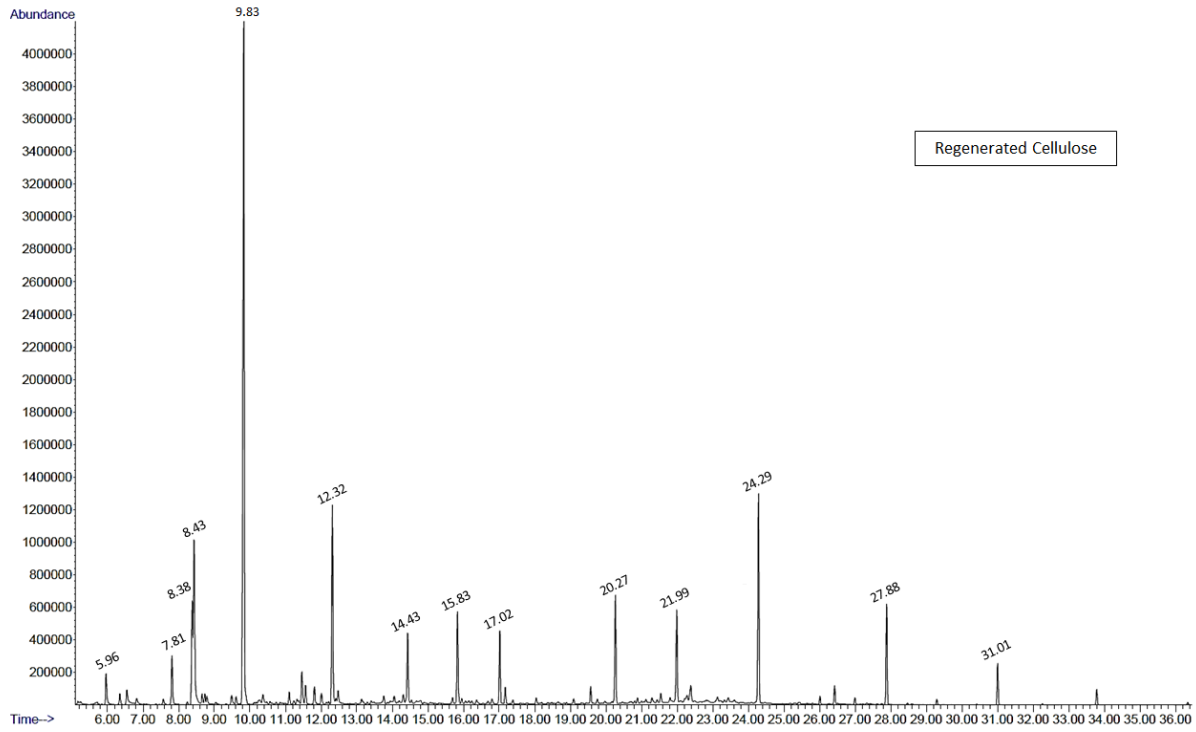


Figure 13: SPME-HS-GC/MS total ion chromatogram of regenerated cellulose filters.

Table 9: Results of SPME-HS-GC/MS from regenerated cellulose filters.

RT [min]	Compound	Molecular weight	Cas #
5.96	Hexanal	100.16	66-25-1
7.81	n.d.	-	-
8.38	Styrene	104.15	100-42-5
8.43	Cyclohexanone	98.15	108-94-1
9.83	6-Methyl-3-heptanone	128.21	624-42-0
12.32	2-Ethylhexanol	130.23	104-76-7
14.43	Nonanal	142.24	124-19-6
15.83	n.d.	-	-
17.02	Dodecane	170.33	112-40-3
20.27	n.d.	-	-
21.99	Tetradecane	198.39	629-59-4
24.29	n.d	-	-
27.88	n.d	-	-
31.01	n.d	-	-

n.d. = not determinable

3.1.2 GC/MS

Gas chromatography coupled to mass spectrometry was applied to detect and identify semi-volatiles in the hexane and ethanol extracts from the CES. Chromatograms of the hexane extracts showed no significant peaks compared to the co-extracted hexane blank. Regarding the ethanol extracts only polyamide and polycarbonate showed significant peaks compared to the corresponding ethanol blank (see Figure 14 and Figure 15). In the polyamide-ethanol extract two intensive peaks, at a retention time of 11.35 min and 22.50 min, and one peak of lower intensity at a retention time of 23.48 min were detected. The first peak, which appeared at 11.35 minutes was identified as caprolactam. The second peak was determined to be bisphenol A. The third peak could not be identified (see Table 10).

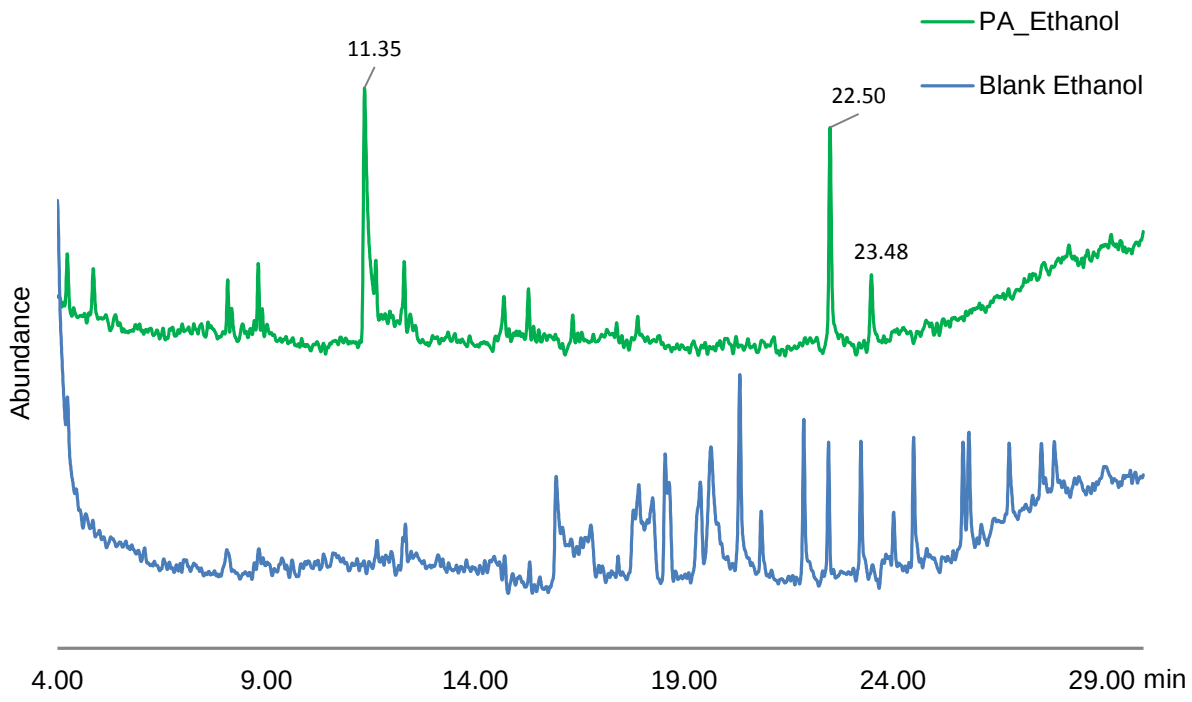


Figure 14: GC/MS total ion chromatogram of the polyamide-ethanol extract.

Table 10: Results of GC/MS analysis of the polyamide-ethanol extract.

RT [min]	Compound	Molecular weight	Cas#
11.35	Caprolactam	113.16	105-60-2
22.50	Bisphenol A	228.28	80-05-7
23.48	n.d.	-	-

Because caprolactam is the base material for the production of polyamides via polycondensation, it was expected to be an extractable from polyamide filters and could be successfully verified [41]. The chromatogram of the polycarbonate-ethanol extract showed two

intensive peaks at a retention time of 6.72 min and 22.50 min, which were identified as phenol and bisphenol A. In addition various smaller peaks, which were not determinable, had been detected. Bisphenol A was present in both filters which confirms the information found in literature. In references bisphenol A is described to be either the base material for polycarbonate production or used as an antioxidants for plasticizers [42].

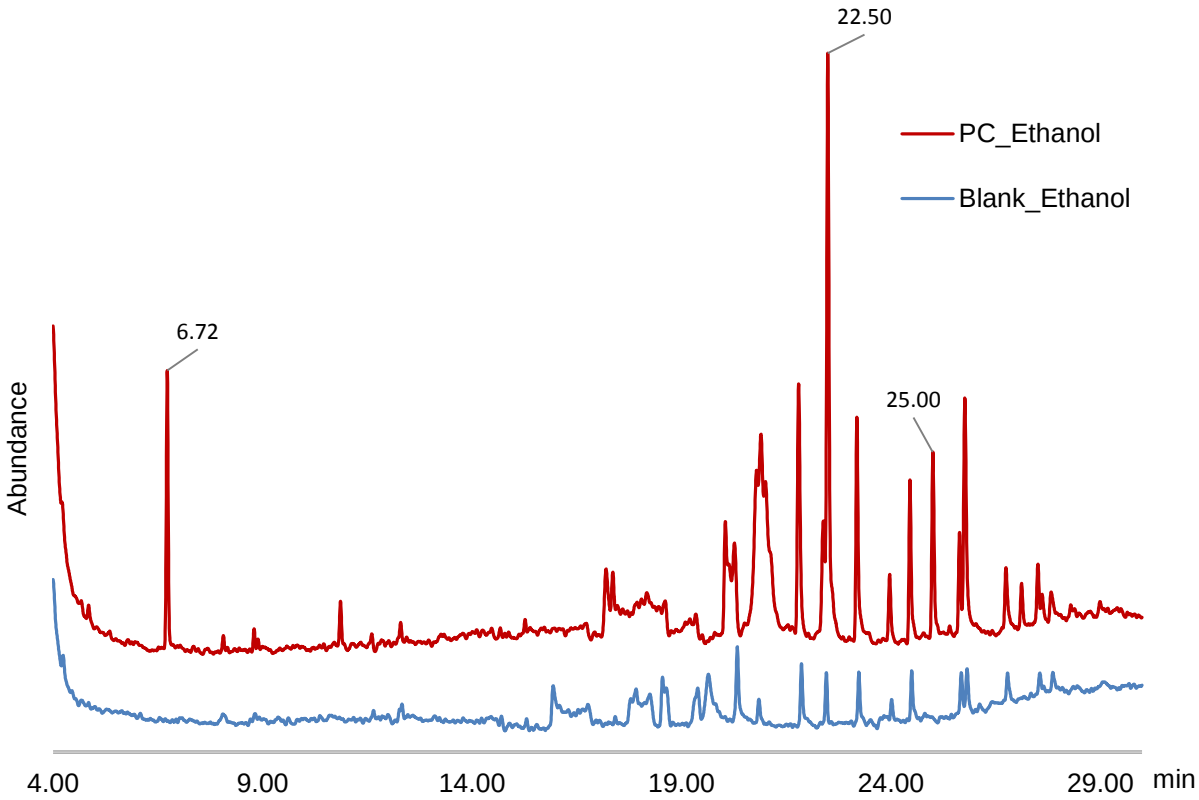


Figure 15: GC/MS total ion chromatogram of the polycarbonate-ethanol extract.

Table 11: Results of GC/MS analysis of the polycarbonate-ethanol extract.

RT [min]	Compound	Molecular weight	Cas#
6.72	Phenol	94.11	108-95-2
22.50	Bisphenol A	228.28	80-05-7
25.00	n.d.	-	-

3.1.3 HPLC/UV/MS analysis for the controlled extraction study

By using liquid chromatography coupled with an UV detector and a mass spectrometer, non-volatiles were detected in every filter. HPLC/UV chromatograms of each filter extractable stock are shown in Figure 16 – Figure 20. The UV-chromatograms of the water and the ethanol blank showed a signal without any significant peaks, which means that there are no contaminants present in the solvents. The chromatogram of the hexane blank showed a few peaks, which indicates that the introduced hexane was contaminated. Peaks of contaminants were detected at a retention time of 9.08, 33.41 and 42.41 minutes and were considered when analyzing the hexane samples. No molecular weights of the contaminants could be determined by mass spectrometry.

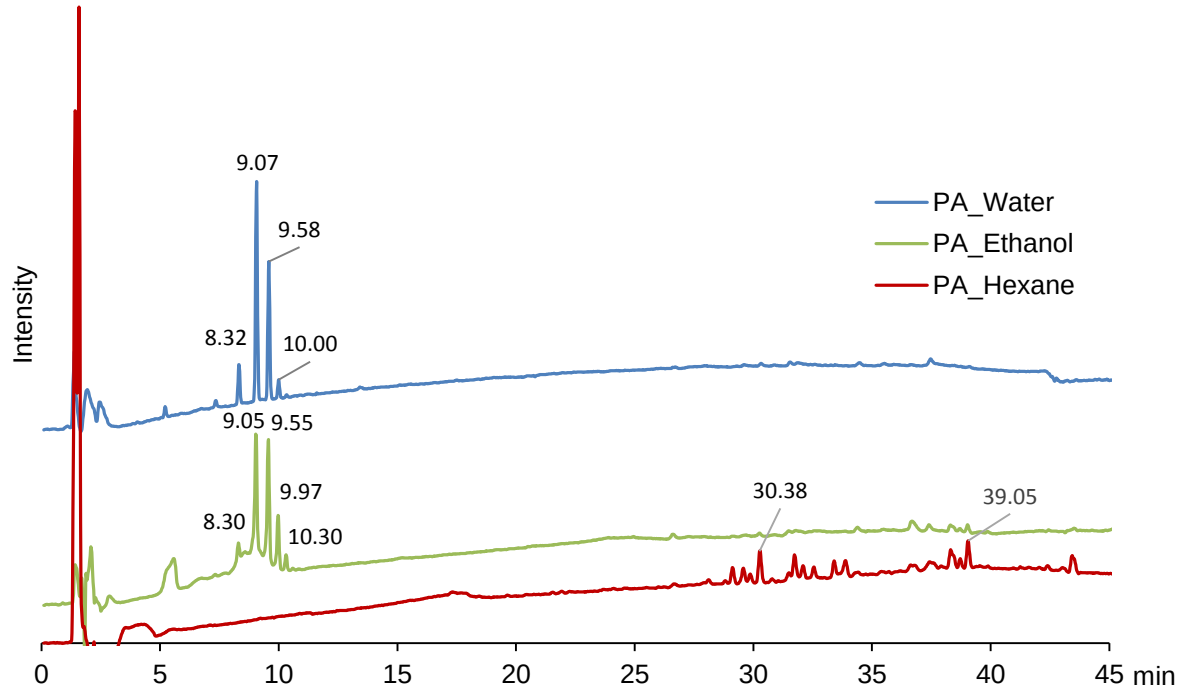


Figure 16: HPLC/UV chromatograms of the polyamide extracts from the controlled extraction study.

The UV chromatograms of polyamide samples showed significant peaks in each extract (see Figure 16). In the polyamide water extract, peaks were detected at a retention time of 8.33, 9.07, 9.58 and 10.00 minutes. The same peaks were detected in the polyamide ethanol extract with an additional peak at 10.30 minutes. In the polyamide hexane extract, peaks of low intensity were observed at a retention time of 30.38 and 39.05 minutes. For a rough estimation of the extracted amounts of the different compounds the area under the peak, or area under the curve (AUC), was calculated at a wavelength of 220nm (see Table 12). By evaluating the AUC data it can be seen that the concentration of the first three peaks (at Rt 8.33 min, 9.07 min and 9.58 min) is approximately the same in water and in ethanol extracts. Only the fourth

peak (at Rt 10.00 min) showed a higher concentration in the ethanol extract than in the PA water sample. The total ion chromatograms of the water and the ethanol extract in ESI positive mode showed peaks at the same retention times like those in the corresponding UV chromatograms. The mass to charge (m/z) ratios for all peaks were successfully determined and were as follows: 566 (Rt 8.33 min), 679 (Rt 9.07 min), 792 (Rt 9.58 min), 906 (Rt 10.00 min) and 1019 (Rt 10.30 min) (see Table 12). Representative total ion chromatograms and extracted ion chromatograms are shown in Figure 21. By having a closer look at the m/z ratios, it can be seen that the next higher m/z value is always 113 higher than the previous m/z ratio. Because a caprolactam monomer was already detected via GC/MS, its molecular weight is approximately 113 g/mol, and caprolactam monomers and their related oligomers were already documented by Jenke et al., it is obvious that the occurring peaks belong to caprolactam oligomers. [43]. Whereby m/z 566 represents the caprolactam pentamer (5 x 113 + H^+), m/z 679 the hexamer, m/z 792 the pentamer, m/z 906 the octamer and m/z 1019 the caprolactam nonamer. As already mentioned above the concentration of the caprolactam octamer was higher in ethanol extracts than in water extracts. This is because the polarity of the caprolactam oligomer decreases with the length of the hydrocarbon chain which means that the caprolactam octamer is better soluble in ethanol compared to the more polar water.

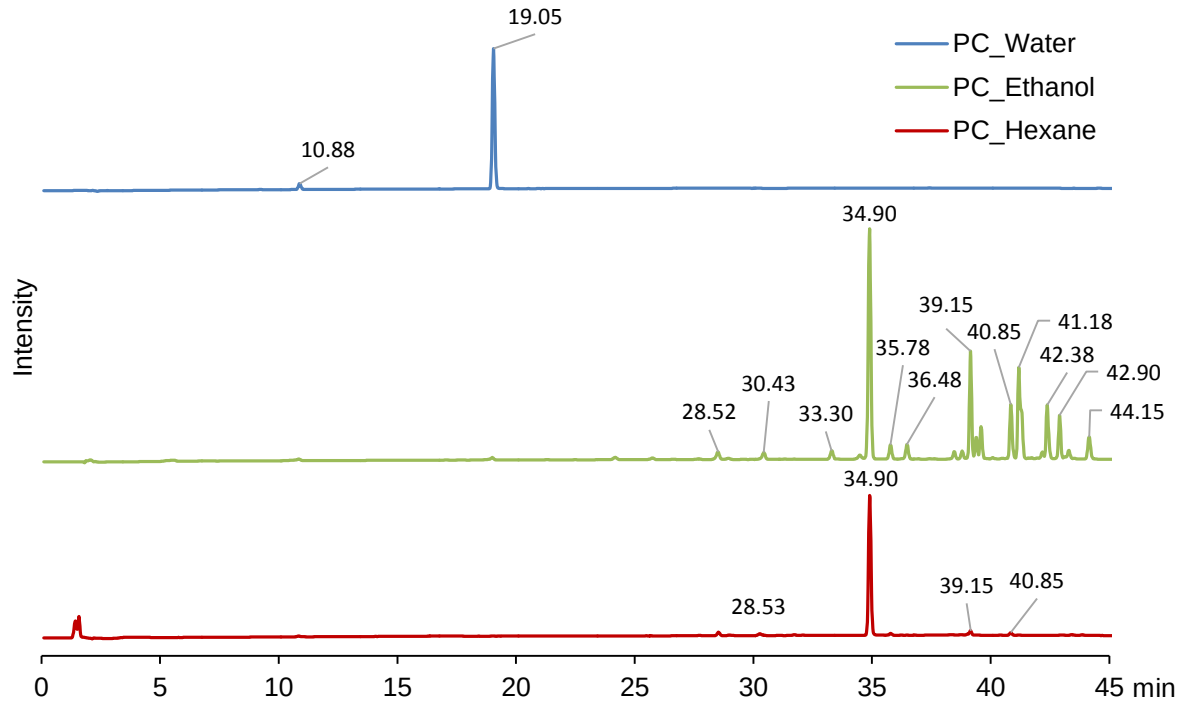


Figure 17: HPLC/UV chromatograms of the polycarbonate extracts from the controlled extraction study.

In all polycarbonate samples peaks could be identified. In the polycarbonate water extract two peaks were observed, one peak with a lower and another peak with a high intensity. The peak of lower intensity was detected at a retention time of 10.88 min. The second peak occurred at 19.05 min. Both peaks, detected in the polycarbonate water extract, are exclusively present in the water sample, which indicates that those peaks belong to hydrophilic compounds. The UV chromatogram of the PC ethanol extract showed many significant peaks. The retention times of the peaks are listed in Table 12. Most of the peaks were only present in the ethanol sample except for the peaks at 34.90 and 39.15minutes, which were also present in the PC hexane extract. Regarding the AUC data it can be seen that the concentration of both peaks is much higher in ethanol than in hexane samples (see Table 12). Mass-to-charge ratios in ESI positive mode were successfully determined for some peaks with the following mass spectrometry and were as follows: 438 (Rt 33.30 min), 486 (Rt 34.90 min), 740 (Rt 39.15 min), 780 (Rt 40.85 min) and 995 (Rt 41.18 min). Unfortunately no compounds were successfully associated with the present m/z values. The total ion chromatograms and representative extracted ion chromatograms at the indicated m/z values are shown in Figure 22.

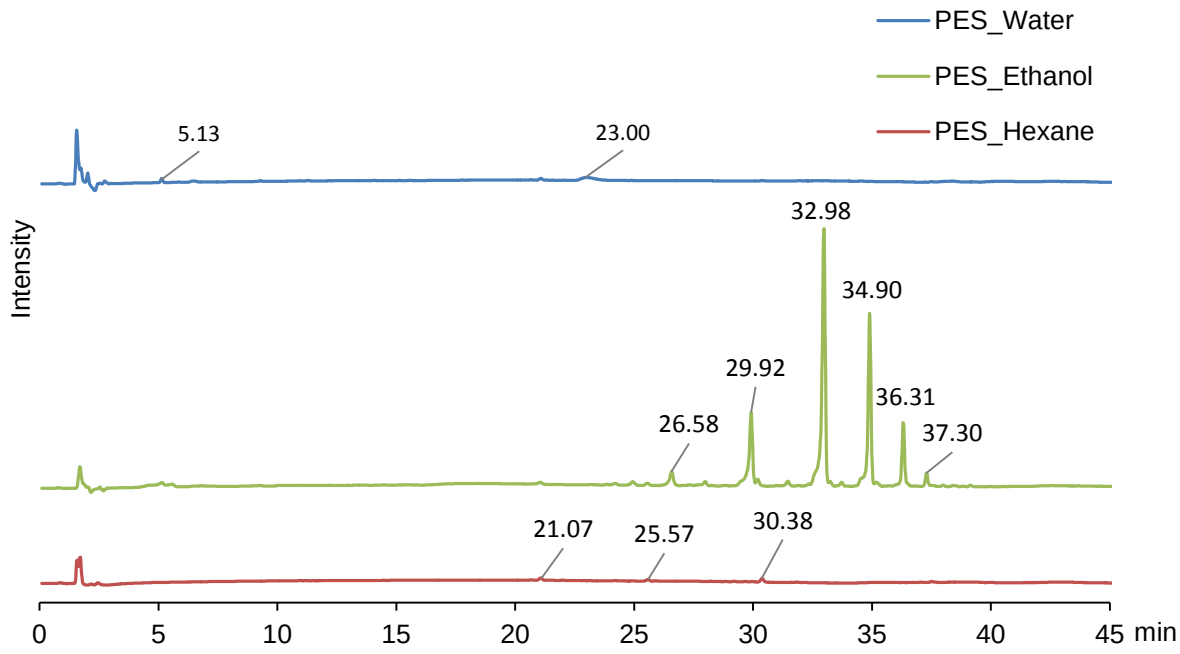


Figure 18: HPLC/UV chromatograms of the polyethersulfone extracts from the CES.

The chromatograms of the polyethersulfone samples showed peaks in the water and ethanol extract as well as in the hexane extract. Only two peaks of relatively low intensity were detected in the water extract. The most peaks were found in the ethanol extract and detected at the retention times of 26.58, 29.92, 32.98, 34.90, 36.31 and 37.30 minutes. The UV chromatogram

of the PES hexane extract showed peaks of low intensity at 21.07, 25.57 and 30.38 minutes. No peak occurring in one extract was present in another extract. The total ion chromatograms of all PES extracts showed no significant peaks which indicates that the occurring compounds were poorly ionizable under the applied conditions.

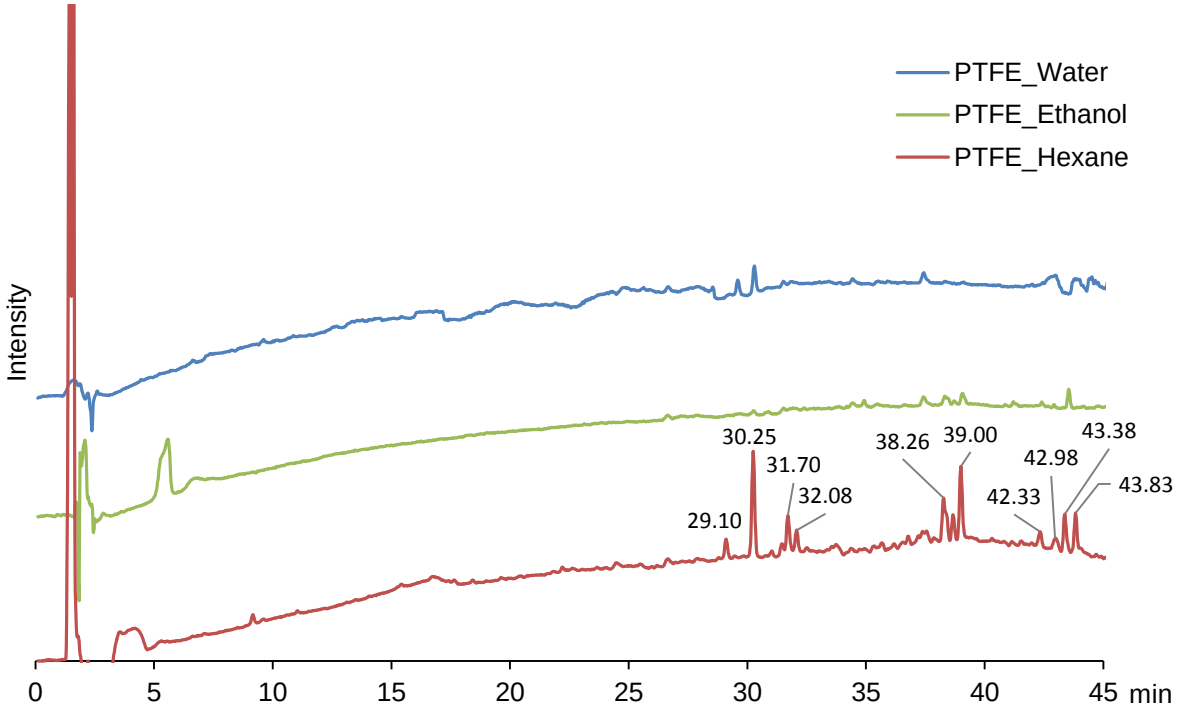


Figure 19: HPLC/UV chromatograms of the PTFE extracts from the CES.

The UV Chromatograms of PTFE extracts showed significant but small peaks only in the hexane extract. The peaks were detected with a low intensity at a retention time of 30.25, 38.26, 42.98, 43.38 and 43.83 minutes. No peaks were detected in the total ion chromatograms of all extracts.

The regenerated cellulose extracts showed significant peaks in the water and the hexane extract. In the water extract one peak with a higher intensity and three smaller peaks were detected. The intensive peak was observed at a retention of 4.82 minutes, the other peaks were detected at 2.38, 5.07 and 5.62 minutes. Compared to the other filter extracts the occurring substances have a relatively low retention time. Due to the fact that a non-polar stationary phase was used for this measurement it is obvious that the present peaks belong to hydrophilic compounds. In the chromatogram of the RC hexane sample a significant peak at a retention time of 30.23 minutes was observed. For the RC samples no significant peaks were observed via MS-detection.

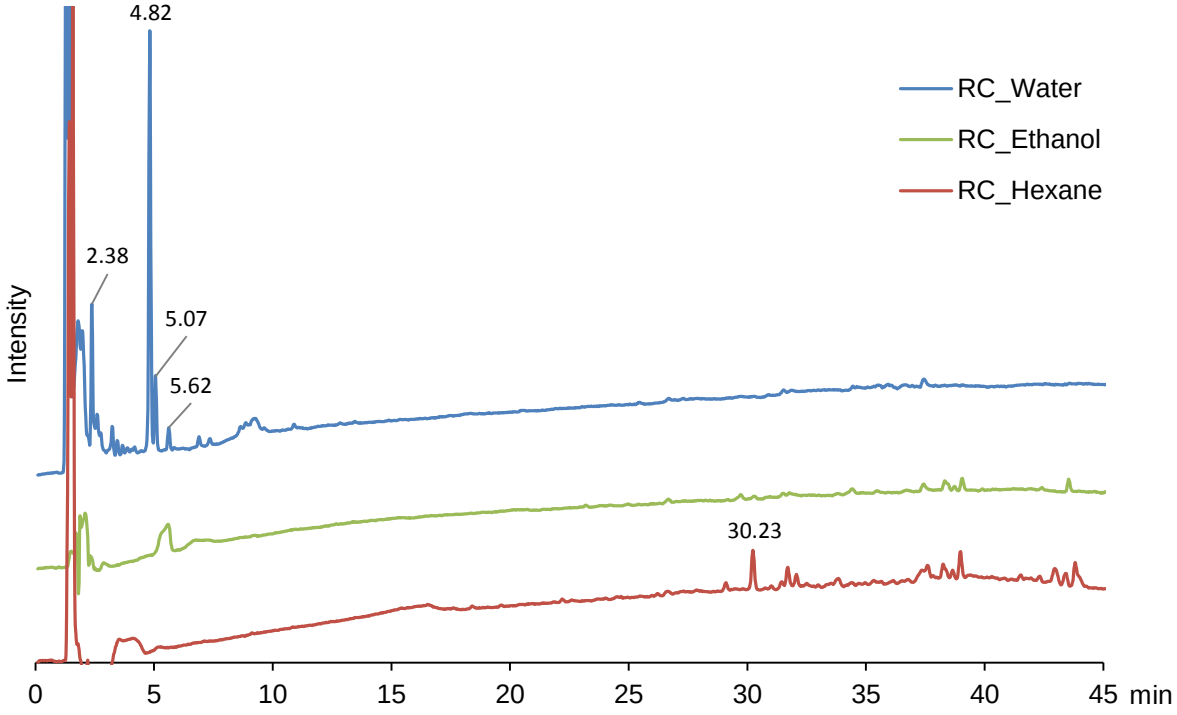


Figure 20: HPLC/UV chromatograms of the regenerated cellulose extracts from the CES.

In general UV detection showed many significant peaks in the different filter extracts compared to the sample blanks. Thus, the use of ethanol as an extraction solvent seemed to mimic the worst-case scenario in this study. The detection via mass spectrometry showed just a few significant peaks which means that most extractables, detected in the controlled extraction study, were not ionizable under applied conditions. Interestingly mass detection was exclusively possible in positive ion mode. The total ion chromatograms in negative ion mode showed no significant peaks despite the use of ammonium acetate as ionization source should normally enable positive and negative ionization. Regarding the compounds detected via mass spectrometry only the peaks occurring in polyamide samples were successfully identified. The other detected m/z ratios were not further analyzed in this study.

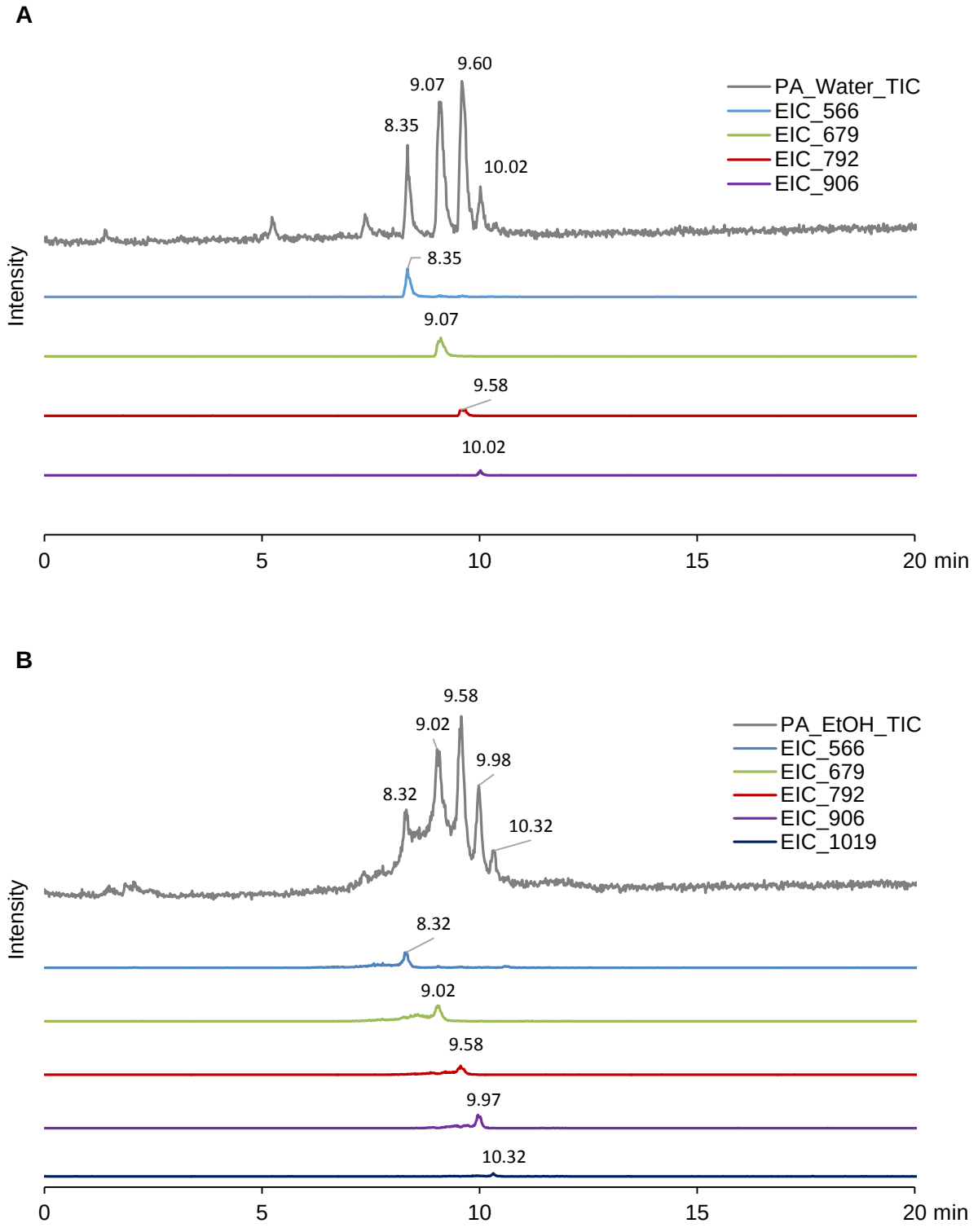


Figure 21: HPLC/MS chromatograms of the polyamide-water (A) and the polyamide-ethanol (B) extract. The upper chromatogram is the total ion current (TIC) chromatogram of the extract; the remaining chromatograms are extracted ion chromatograms at the indicated mass value.

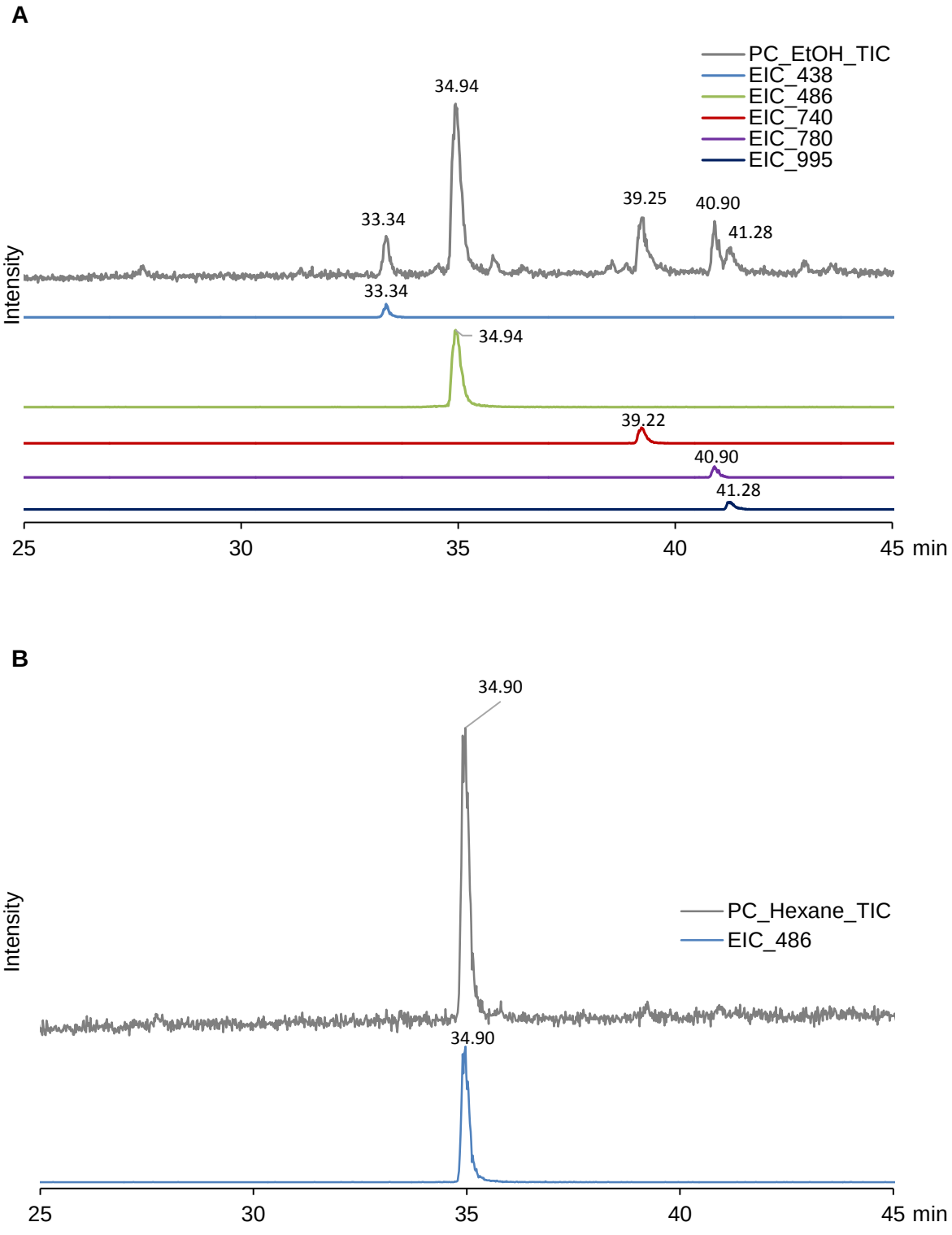


Figure 22: HPLC/MS chromatograms of the polycarbonate-ethanol (A) and the polycarbonate-hexane (B) extract. The upper chromatogram is the total ion current (TIC) chromatogram of the extract; the remaining chromatograms are extracted ion chromatograms at the indicated mass value

Table 12: Results of HPLC/UV/MS analysis of the extracts from the controlled extraction study.

Sample ID	RT	AUC	m/z
PA_Water	8.33	240	567
	9.07	1293	680
	9.58	806	793
	10.00	110	906
PA_Ethanol	8.32	274	567
	9.03	1273	680
	9.55	820	793
	9.97	330	906
	10.30	81	1019
PA_Hexane	30.28	280	-
PC_Water	10.88	1372	-
	19.05	29125	-
PC_Ethanol	28.52	2073	-
	30.43	1866	-
	33.30	2128	438
	34.90	50842	486
	35.78	3117	-
	36.48	3425	-
	38.46	1650	-
	38.80	1687	-
	39.15	20288	740
	39.40	3591	-
	39.60	6402	-
	40.85	10733	780
	41.18	25008	995
	42.38	12540	-
	42.90	8436	-
	43.30	2233	-
	44.15	5122	-
	45.48	2646	-
PC_Hexane	28.53	824	-
	34.90	29314	486
	39.15	1209	-
	40.85	644	-

PES_Water	5.13	226	-
	23.00	1028	-
PES_Ethanol	26.58	1505	-
	29.92	7821	-
	32.98	22470	-
	34.90	13894	-
	36.31	4332	-
	37.30	792	-
PES_Hexane	21.07	180	-
	25.57	138	-
	30.38	288	-
PTFE_Hexane	30.25	597	-
	32.08	93	-
	38.26	281	-
	42.98	84	-
	43.38	187	-
	43.83	256	-
RC_Water	2.38	579	-
	4.82	2339	-
	5.07	380	-
	5.62	137	-
RC_Hexane	30.23	327	-

3.1.4 HPLC/UV/MS analysis for the leachable study

For the leachable study filter extracts at pH 4 and pH 8 were examined. The UV chromatograms of each filter extract are shown inFigure 23 and Figure 24. In the sample blanks no significant peaks were detected, which implies that neither UV absorbing nor ionizable contaminants were present in the buffer solutions.

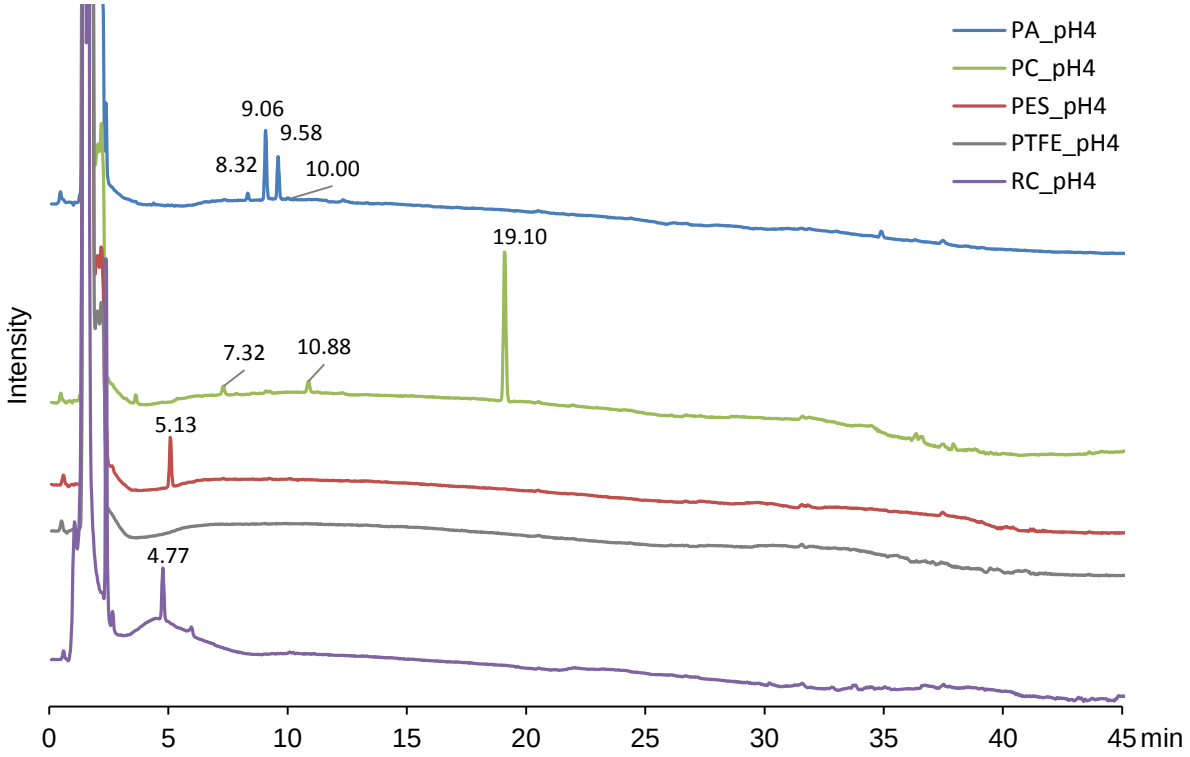


Figure 23: HPLC/UV chromatograms of the different filter extracts at pH 4 for the leachable study.

The UV chromatogram of the polyamide pH 4 extract showed two peaks at a retention time of 9.06 and 9.60 minutes and two peaks of low intensity at 8.32 and 10.00 minutes. In the chromatogram of the PA buffer pH 8 extract two peaks at 8.33 and 9.08 minutes were detected and one peak with a low intensity at 9.62 minutes (see Table 13). With respect to the data from the controlled extraction study it is obvious that the determined peaks were already detected in the PA water samples. Via mass spectrometry, it could be proven that the detected peaks were the same than those detected in the CES of polyamide, which were identified to be caprolactam oligomers (see Figure 25). This means that the detected extractables in the CES actually became leachables. The only exception was the caprolactam nonamer which was detected in the controlled extraction- but not in the leachable study. Moreover it is apparent that a more acidic pH (pH 4) seems to leach more compounds out of polyamide filters.

Polycarbonate pH 4 samples showed only one peak at a retention time of 19.10 minutes. In the pH 8 samples two peaks occurred at a retention time of 10.83 and 19.08 minutes. Referring to the AUC data listed in Table 13 it is shown that the peak at a retention time of 19.10 minutes was substantial higher in buffer at pH 8 than in buffer at pH 4. Interestingly the peak occurring at 10.83 minutes was exclusively present in pH 8 samples. With regard to the peaks found in the polycarbonate samples of the CES it, can be seen that both peaks were also present in the water extract. Thus, the determined extractables in the water extracts actually became leachables. The detection of mass-to-charge ratios through mass spectrometry was unsuccessful.

The UV chromatograms of the polyethersulfone buffer extracts showed only one peak which was detected in the pH 4 buffer at a retention time of 5.13 minutes. The same peak was also observed in the water extracts of the CES, which indicates that the corresponding substance is an extractable that became a leachable. No m/z ratio could be determined for the observed peak.

The polytetrafluoroethylene samples showed no significant peaks compared to the sample blanks. This was somehow an expected result, because the water extract of the controlled extraction study showed no peaks either.

In the UV chromatograms of the regenerated cellulose buffer extracts two peaks were determined at both pH values. The first peak was detected at 2.38 minutes and was more intensive than the second peak, which was detected at 4.77 minutes. Both peaks were also detected in the controlled extraction study. With the following mass spectrometry no peaks were detected.

In general no leachables were detected, which were not already identified as extractables. This observation confirms the general thesis according to literature that leachables are typically a subset of extractables. In addition it was observed that the pH of the solvents has an impact on the amount of extractables and leachables leached from the contact materials.

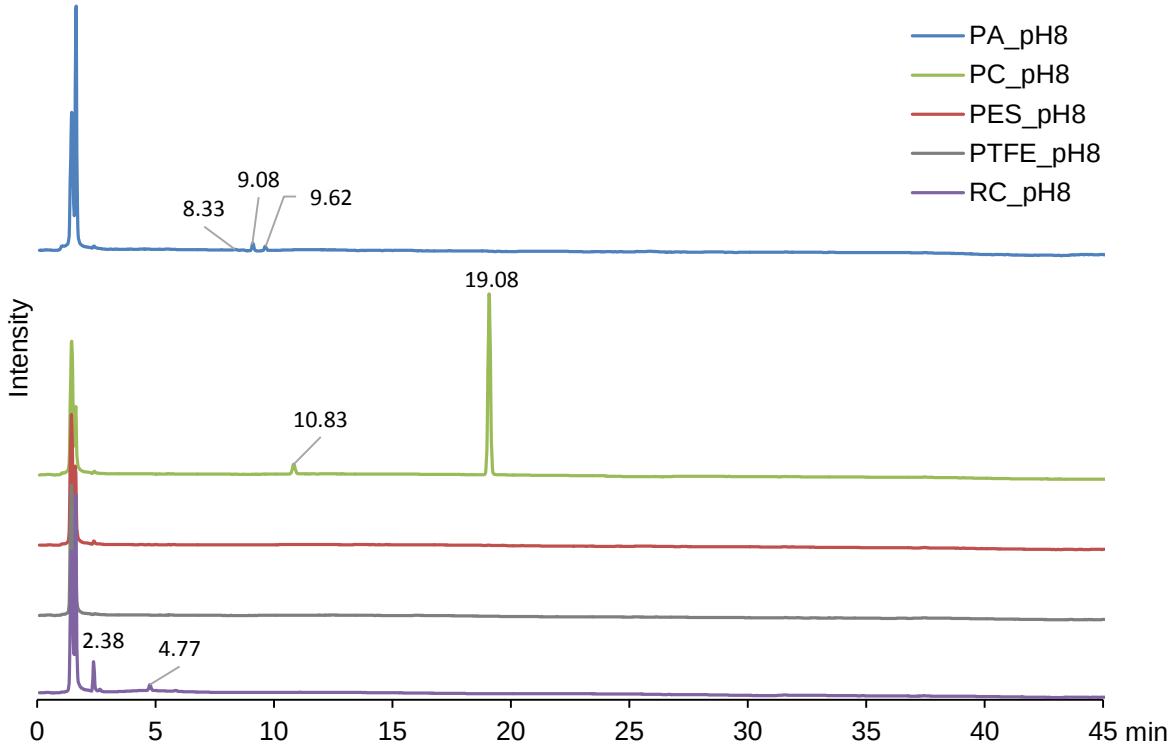


Figure 24: HPLC/UV chromatograms of the different filter extracts at pH 8 for the leachable study.

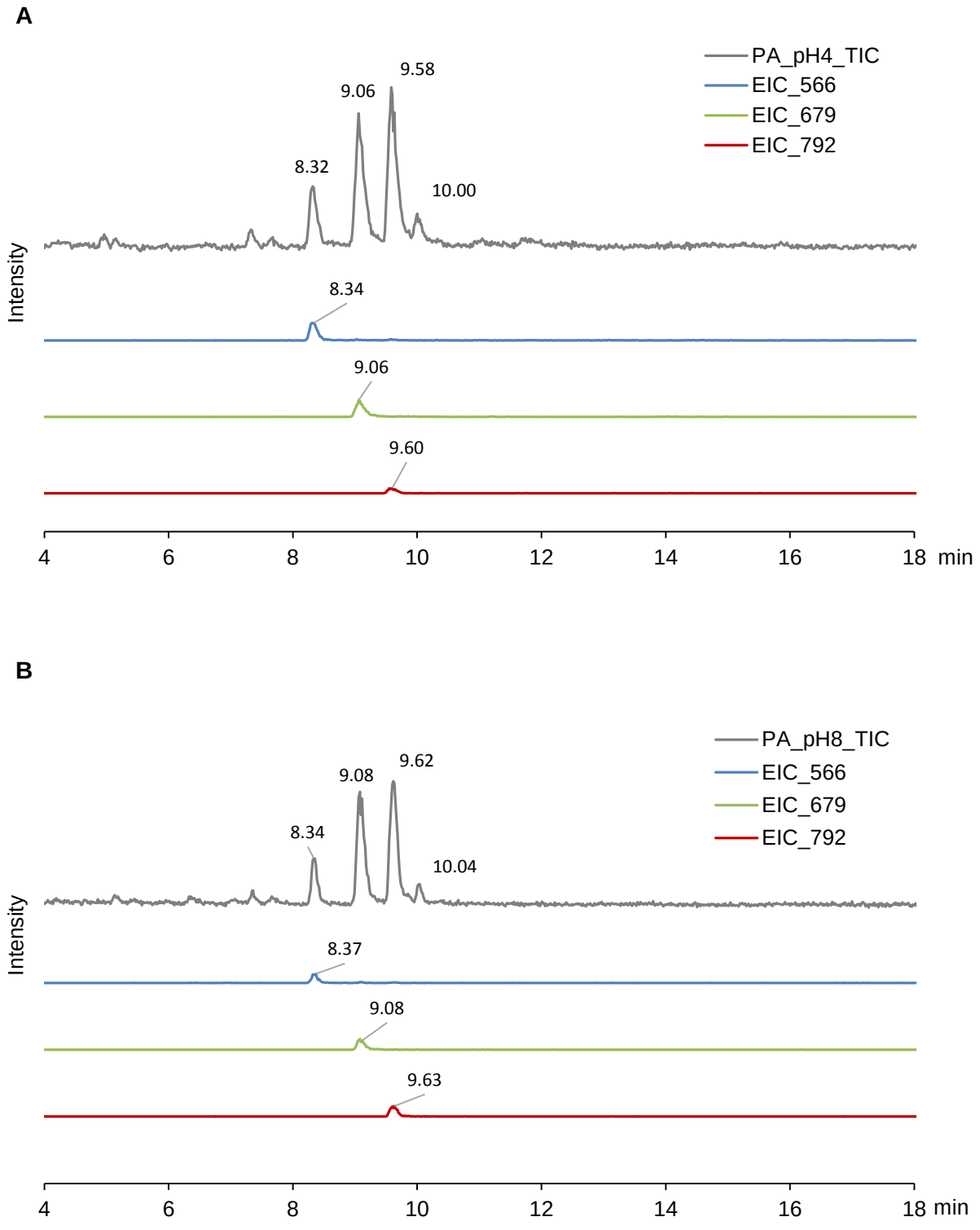


Figure 25: HPLC/MS chromatograms of the polyamide-CP-buffer pH 4 (A) and the polyamide-CP-buffer pH 8 extract. The upper chromatogram is the total ion current (TIC) chromatogram of the extract; the remaining chromatograms are extracted ion chromatograms at the indicated mass value.

Table 13: Results of HPLC/UV/MS analysis of the filter extracts for the leachable study.

Sample ID	RT	AUC	ESI+
PA_Buffer_pH4	8.32	84	567
	9.06	584	680
	9.58	352	793
	10.00	13	-
PA_Buffer_pH8	8.33	60	567
	9.08	550	680
	9.62	317	793
PC_Buffer_pH4	19.10	1578	-
PC_Buffer_pH8	10.83	1008	-
	19.08	16816	-
PES_Buffer_pH4	5.13	397	-
RC_Buffer_pH4	2.38	2124	-
	4.77	479	-
RC_Buffer_pH8	2.38	1552	-
	4.77	484	-

3.2 Protein stability study (PSS)

3.2.1 Surface tension analysis

Surface tension is an important material attribute in the formulation of biopharmaceuticals. Surface active agents, also called surfactants, are frequently added to protein formulations. Their purpose is to prevent protein molecules from accumulation at solid-liquid or air-liquid interfaces. Such interfaces often occur during common unit operations of manufacturing, like fill-finishing, or transportation etc. Surfactants are stabilizing the protein formulation because they have a higher affinity to occurring interfaces than proteins. Thus, they are preventing the protein from adsorption and in the following protein denaturation. Despite the addition of surfactants may be important for some protein formulations; the accidental introduction of surface active leachables is considered an adulteration and could alter quality, purity or safety of the drug product [4].

The surface tension was measured for each filter material in CP-buffer at pH 4 and pH 8. The extraction conditions were set to 30°C and to 50°C. In the first trail the temperature was set to 30°C because sterile filtration is rarely performed above this temperature [44]. In the second trail it was set to 50°C to mimic a slightly more aggressive scenario. The results are illustrated in Figure 26. As a control, a buffer solution without E/Ls from filters was used. At the first glance it is noticeable that the surface tension decreased significantly with leachables from regenerated cellulose membranes at both extraction conditions, whereas the effect is markedly increased at pH 4 than pH 8. This fact indicates that a lower, and therefore more acidic, pH value might dissolve more leachables out of the membrane filter. Compared to the AUC data achieved in the leachable study (see Table 13) it is obvious that the compound occurring at a retention time of 2.38 minutes is higher concentrated in CP-buffer at pH 4. This might imply that this peak belongs to a compound which is surface active. A closer look, however, shows that other filter materials led to a decrease of surface tension either. Polyethersulfone extracts showed a decreased surface tension value at both extraction conditions at pH 4 and pH 8. Polyamide extracts at pH 8 showed a slightly decrease in surface tension at both extraction temperatures. At pH 4 a decrease in PA samples was only proven at 50°C extraction temperature. This might be due to the fact that a higher temperature leaches more compounds out of the filter, which may be surface active. In polycarbonate and polytetrafluoroethylene extracts no significant decrease of surface tension was observed.

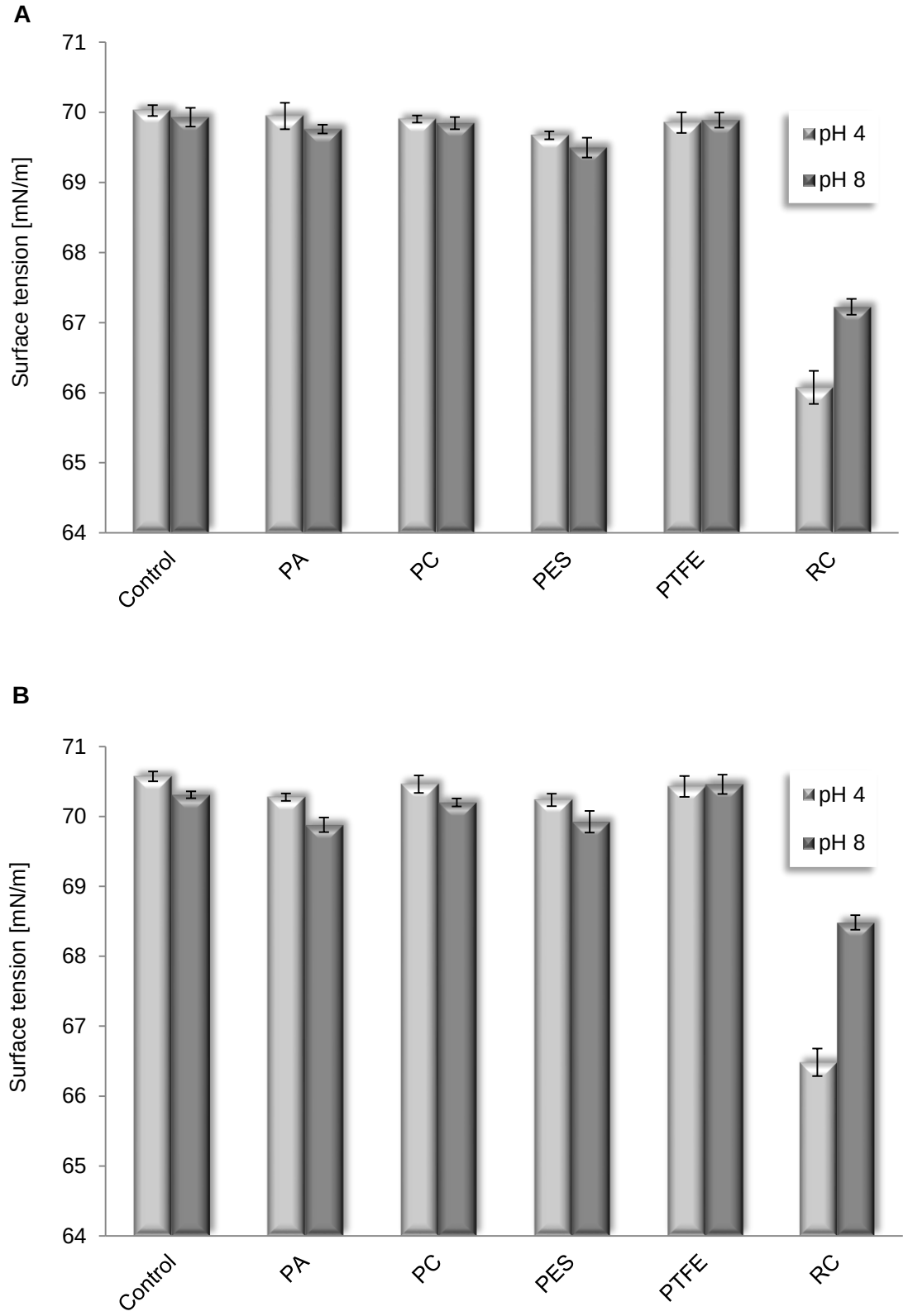


Figure 26: Results of surface tension measurements from filter leachable stocks which were extracted at 30°C (A) and 50°C (B).

3.2.2 Micro flow Imaging

Protein aggregation is a major problem in biopharmaceutical formulations as already mentioned in section 3.2.1. It can lead to a higher particle concentration within the formulation which can be determined by micro flow imaging [45].

The results of the micro flow imaging analysis are shown in Figure 27. As a control G-CSF formulations without addition of leachable stocks were analyzed. The overall trend showed a higher particle concentration in pH 4 compared to pH 8 samples. However, it is noticeable that all G-CSF protein formulations at pH 4 showed an extremely high particle concentration when spiked with regenerated cellulose extracts. Whereas the formulations spiked with RC extracts at pH 8 showed lower concentrations of approximately the same level than that in the control.

By having a look at samples containing other filter extracts (extracted at 30°C) it is conspicuous that all protein formulations at pH 4 have a higher particle concentration compared to the control. Thereby the G-CSF samples with the polyamide pH 4 extracts showed a distinct higher concentration whereas the remaining pH 4 formulations showed only a slightly increased particle concentration. Concerning the pH 8 formulations the only observed trend was a higher particle formation in the samples containing leachables from PA filters.

With respect to pH 4 formulations containing PA, PC, PES and PTFE filter solutions (extracted at 50°C) it was observed that only samples containing polyamide extracts showed a higher particle concentration. In contrary PTFE-leachable containing samples showed a slightly lower particle concentration, whereas the other pH 4 formulations showed no significant trend. In the formulations containing extracts at pH 8, PA samples showed also a higher particle concentration. Interestingly PC and PTFE extract containing formulations showed a clearly lower particle level. Formulations with PES extracts showed no significant change in particle concentration compared to the control.

However, all G-SCF formulations at pH 4 showed a higher particle concentration than formulations at pH 8 even though G-CSF is usually more stable at pH 4 and should therefore have a lower particle concentration compared to pH 8 G-CSF. This indicates that at pH 4, more compounds with adverse effects on protein stability are leached from the filter membranes. Another interesting effect observed in this analysis is the extremely high particle concentration of formulations at pH 4 containing leachables from regenerated cellulose filters. Interestingly this effect was not seen in regenerated cellulose samples at pH 8. In respect of the data obtained in section 3.2.1 it is seen that regenerated cellulose stocks at pH 4 showed a significantly decrease in surface tension whereas pH 8 cellulose stocks showed a lower

decrease in surface tension. This implies that regenerated cellulose stocks at pH 4 contain more surface active compounds with the ability to promote protein aggregation. Other filter E/L stocks which seemed to alter stability of G-CSF in both extraction conditions at both pHs were polyamide extracts. All formulations containing leachables from polyamide filters showed a higher particle concentration compared to the corresponding control samples. In contrary to this formulations with leachables from PTFE filters, which were extracted at 50°C, seemed to have a stability enhancing impact on G-CSF formulations that leads to a decrease of particle concentration.

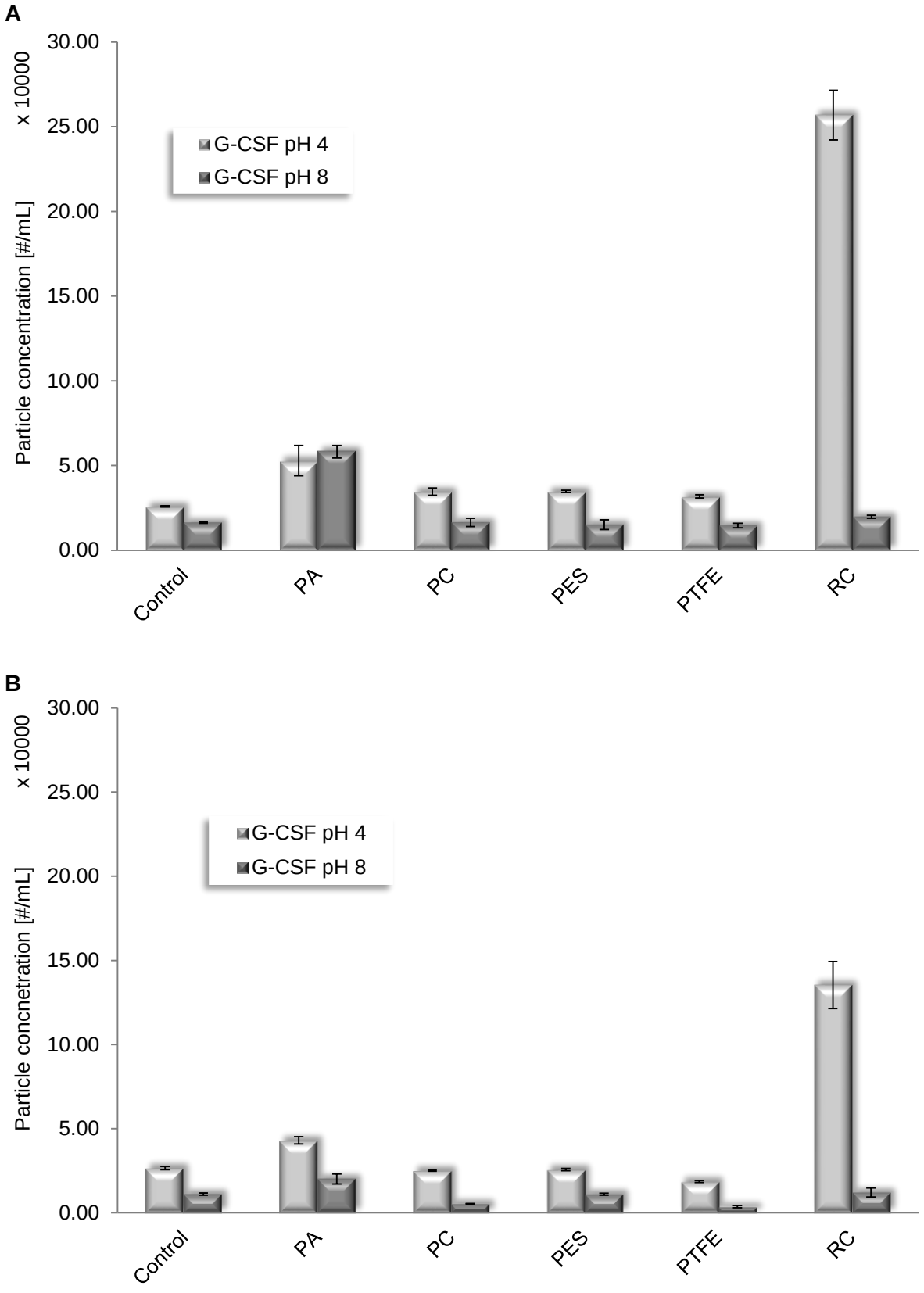


Figure 27: Results of MFI measurements from filter leachable stocks which were extracted at 30°C (A) and 50°C (B).

3.2.3 Size exclusion chromatography

Size exclusion chromatography is a technique which generally separates molecules by their size and in some cases by their molecular weight. The separation method is based on the fact that bigger molecules move faster through the stationary phase compared to smaller molecules, which can enter intermediate spaces of the packaging material. Therefore smaller molecules are eluting later than bigger molecules. Applied on protein aggregates this means that proteins in their native conformation have a higher retention time than dimers or oligomers of the protein. As a control a G-CSF formulation without filter extracts was analyzed. For the G-CSF protein in its native form a retention time of approximately 24 minutes in pH 4 and approximately 18.5 minutes in pH 8 was detected. Other peaks in the chromatograms with lower retention times were considered protein aggregates. Peaks with higher retention times were considered denatured protein molecules [46]. To obtain an overview of protein aggregation in the samples all AUC values for peaks with lower retention times than the monomer peak were summarized and given in percentage relation to the entire protein concentration (see Figure 28).

In pH 4 formulations no protein aggregates were detected. Shoulders at monomer peaks of PA containing samples are conspicuous. Possibly those shoulders indicate denaturation of protein monomers to a certain extent. Anyway, protein behavior seems to be different in PA samples compared to the control. Besides also MFI measurements showed an increase of particles in all PA containing samples. Formulations at pH 8 showed aggregates in all samples including the control. This is because G-CSF is more stable in pH 4 than pH 8 [33]. It is shown that formulations containing RC extracts (extracted at 30°C and 50°C) have a significantly lower aggregation rate than the control. PES and PTFE extracts (at 30°C) showed a slightly lower aggregate level. Formulations containing leachables from polyamide filters showed a prominent peak after the monomer peak, which implies that protein denaturation is occurring in those samples (see Figure 29).

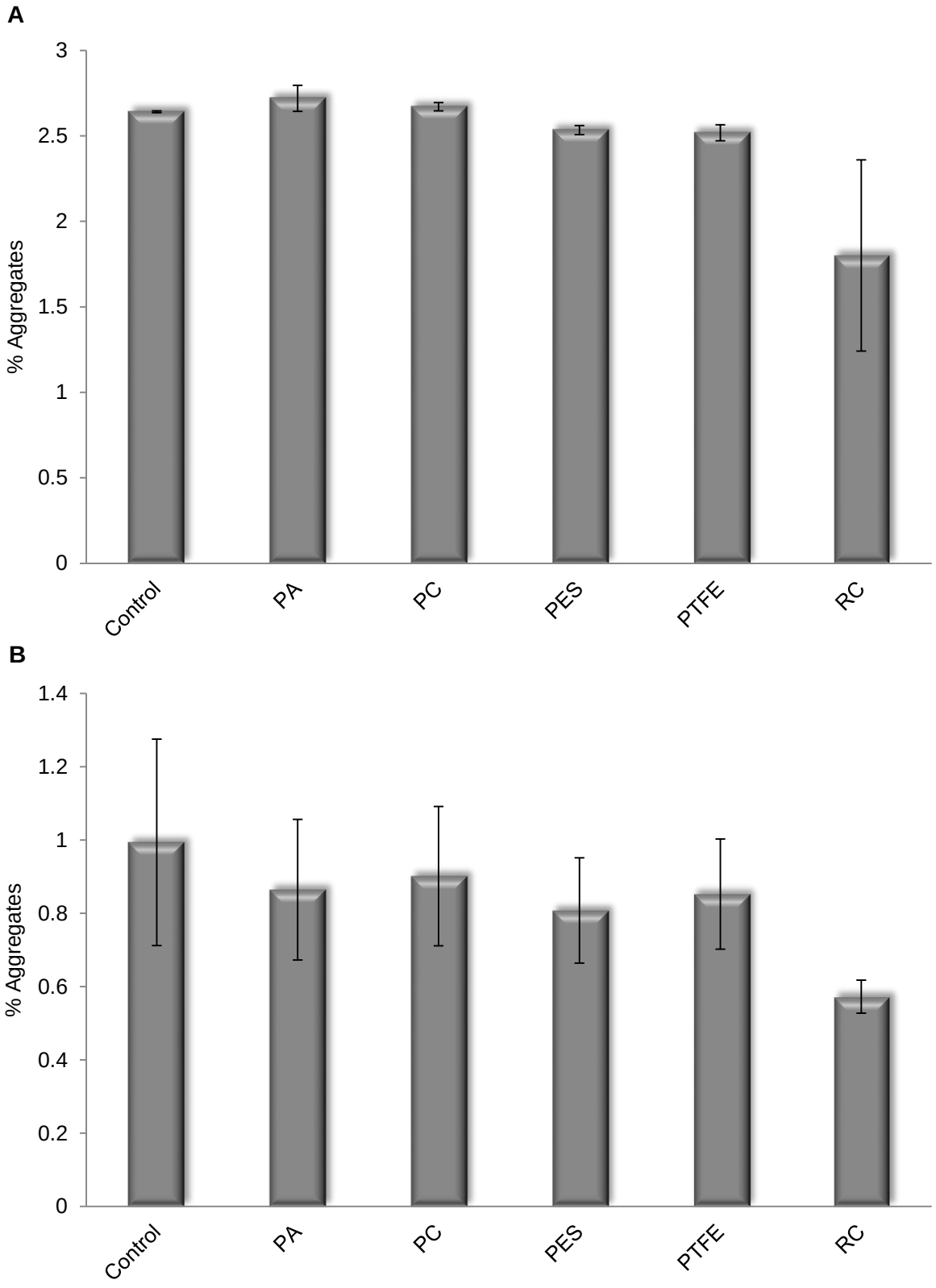


Figure 28: Aggregate levels in G-CSF formulations at pH 8 analyzed by SEC-HPLC after spiking with filter leachable stocks which were extracted at 30°C (A) and 50°C (B).

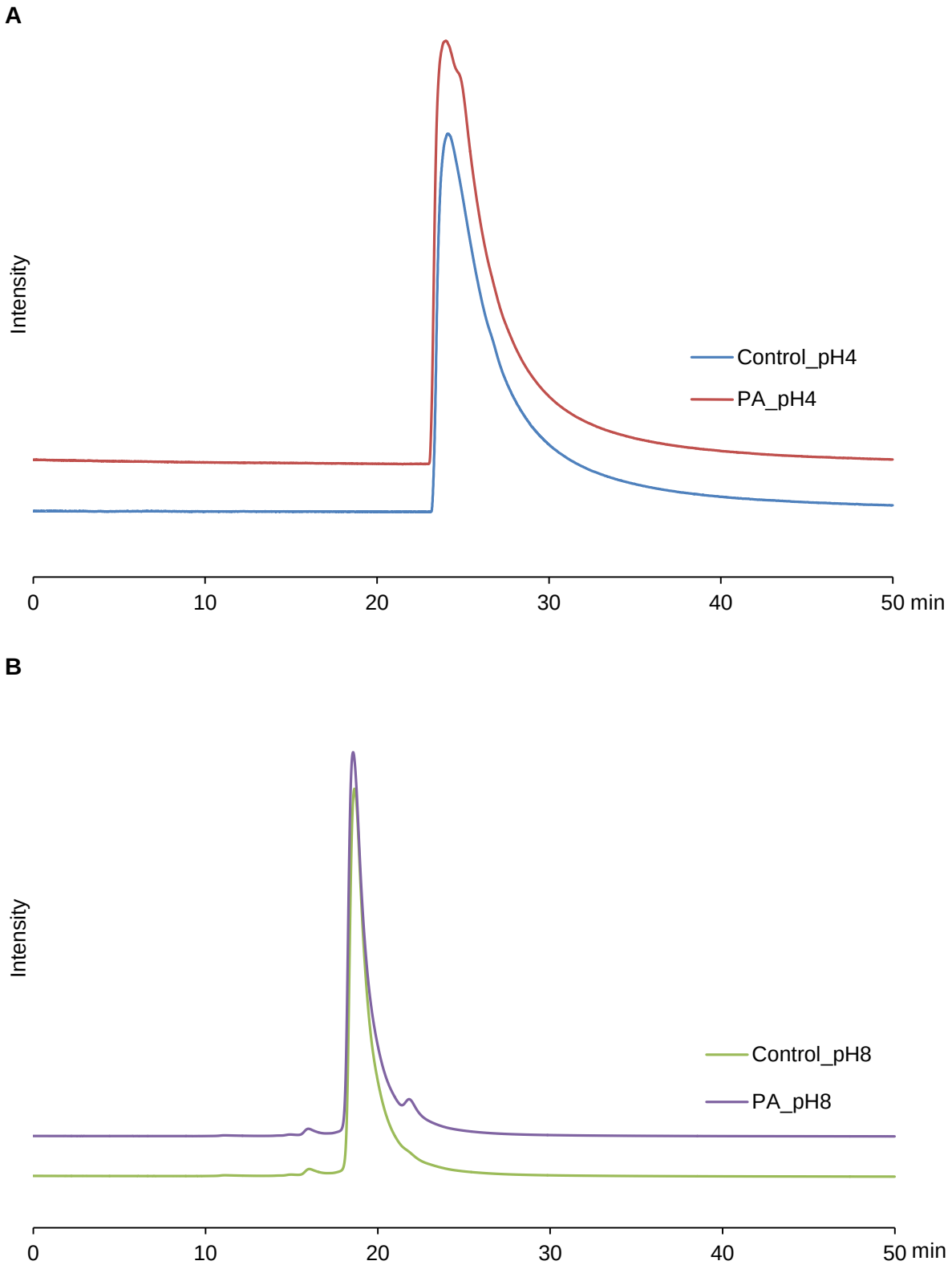


Figure 29: SEC chromatograms of the control and the polyamide samples at pH 4 (A) and pH 8 (B).

3.2.4 Zeta potential

By the use of electrophoretic light scattering the zeta potential of charged molecules like protein complexes can be determined. When charged particles are associated with an electric field its motion vector is the sum of its charge dependent motion in the direction of the oppositely charged electrode and its random motion. With the use of laser Doppler velocimetry, to determine particle velocity, the zeta potential can be calculated. The lower the zeta potential (absolute value) the more likely protein aggregation occurs. Accordingly a higher zeta potential value indicates that a formulation is more stable. As far as proteins are concerned the zeta potential decrease with increasing pH [31].

The results of zeta potential measurements are illustrated in Figure 30. G-CSF-formulations at pH 4 spiked with filter extracts (extracted at 30°C) showed no significant decrease in zeta potential except for samples spiked with regenerated cellulose filter extract. This implies that RC containing samples are less stable than the control and therefore, shows a higher propensity to form aggregates. This effect was already seen in MFI measurements (Figure 27) were RC containing samples at pH 4 showed an extremely higher particle concentration which also indicates that those samples were less stable. The pH 8 samples showed higher variability in zeta potential than pH 4 samples. Compared to the control PA, PC and RC samples showed a significant drop in zeta potential and are therefore more likely to form aggregates.

Regarding pH 4 samples spiked with e-stocks extracted at 50°C only RC samples showed a drop in zeta potential, as already seen in samples with extraction conditions set to 30°C. In pH8 samples all samples showed a lower zeta potential compared to the control.

Interestingly most of the zeta potential values of samples spiked with leachables extracted at 30°C are higher than those of the samples containing extracts which were extracted at 50°C. This may indicates that increasing temperature leaches more extractables and leachabels out of the filter, which may have destabilizing effect on protein formulations.

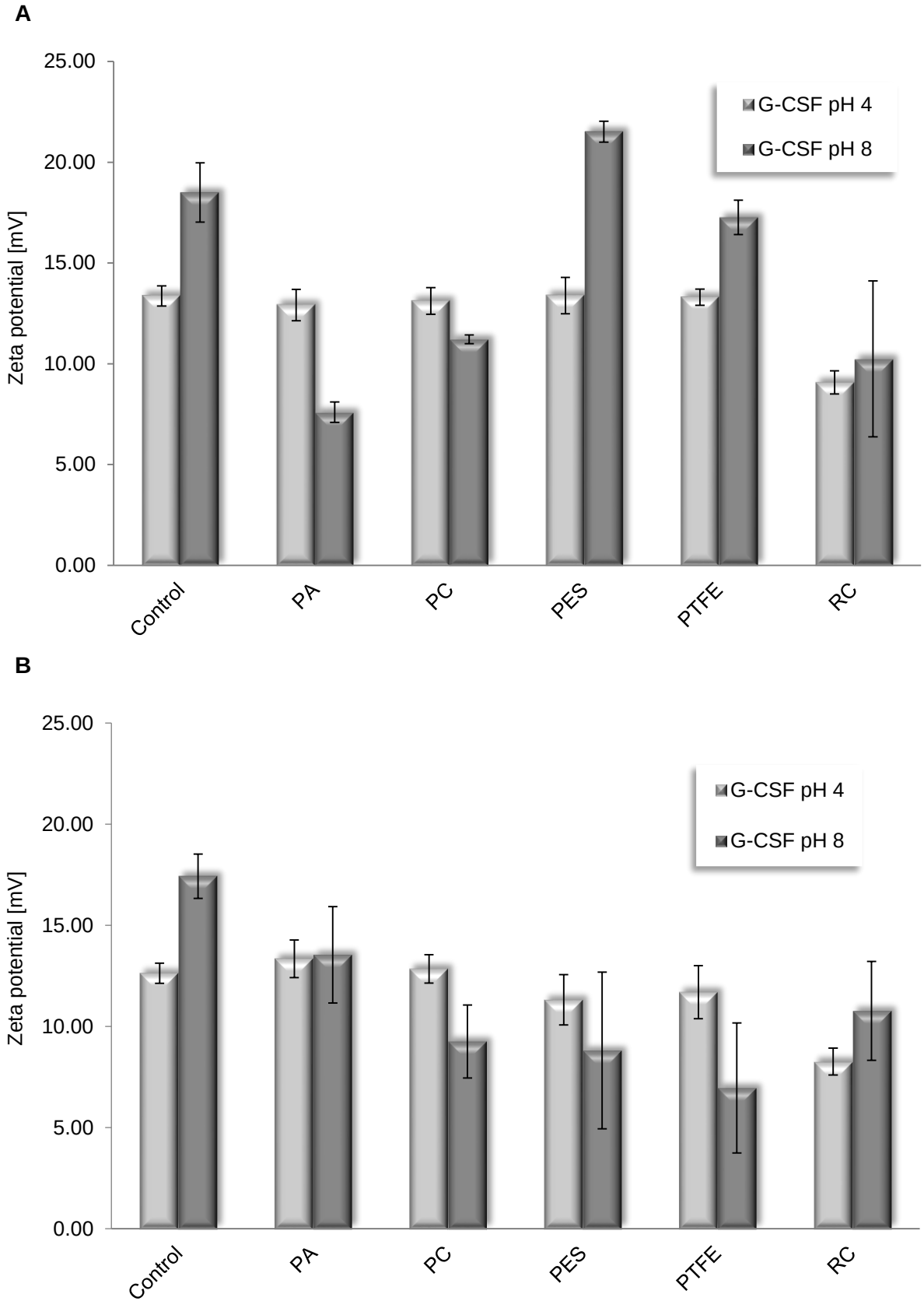


Figure 30: Results of zeta potential analysis from filter leachable stocks which were extracted at 30°C (A) and 50°C (B).

3.2.5 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is commonly used to investigate folding and unfolding of protein samples. Thus, it can be used as a screening method for thermal stability because the thermal melting temperature (T_m) of a protein, which can be determined by DSC, usually correlates with the proteins' relative thermal stability. Impurities can lead to an alteration of protein structure which leads to a change in the thermal melting temperature and enthalpy [4]. In this study DSC measurements were performed to evaluate the effect of filter leachables on the thermal stability of G-CSF formulations. The DSC data for G-CSF spiked with different filter extractable stocks is shown in Table 14 (extracted at 30°C) and Table 15 (extracted at 50°C). The overall trend of the obtained data showed slight differences in formulations containing E/Ls from polyamide filters. The other formulations showed no significant differences.

Table 14: Results of DSC analysis of G-CSF formulations spiked with filter extracts, extracted at 30°C.

Sample ID	Peak [°C]	Onset [°C]	Area
Control_GCSF_pH4	92.5±5.4	85.2±7.4	2.57±1.10
PA_GCSF_pH4	88.5±2.6	84.1±1.4	1.32±0.21
PC_GCSF_pH4	89.9	82.5	4.59
PES_GCSF_pH4	87.3	75.2	4.57
PTFE_GCSF_pH4	94.7±6.2	90.8±4.6	4.16±0.45
RC_GCSF_pH4	92.3±3.5	84.9±6.2	5.32±1.25
Control_GCSF_pH8	85.6±1.0	78.2±1.7	2.81±0.61
PA_GCSF_pH8	92.4±1.8	85.2±1.1	3.11±0.89
PC_GCSF_pH8	89.3±1.3	84.5±1.7	2.45±0.89
PES_GCSF_pH8	84.0±1.0	78.3±1.3	5.80±1.47
PTFF_GCSF_pH8	86.5±1.7	80.4±1.7	5.75±0.83
RC_GCSF_pH8	89.8±1.8	84.7±2.0	4.58±0.52

Table 15: Results of DSC analysis of G-CSF formulations spiked with filter extracts, extracted at 50°C.

Sample ID	Peak [°C]	Onset [°C]	Area
Control_GCSF_pH4	97.9	91.9	5.53
PA_GCSF_pH4	92.8±3.9	88.4±5.6	3.86±1.06
PC_GCSF_pH4	85.9	78.8	5.39
PES_GCSF_pH4	85.9±2.5	87.8±2.3	4.10±1.46
PTFE_GCSF_pH4	92.3±2.4	86.3±2.3	2.76±0.32
RC_GCSF_pH4	97.8	90.3	1.59
Control_GCSF_pH8	82.5	76.6	5.67

PA_GCSF_pH8	80.9±1.2	73.9±2.6	3.66±0.96
PC_GCSF_pH8	85.3±4.8	79.6±3.6	1.91±0.03
PES_GCSF_pH8	84.6±1.5	78.1±0.8	4.73±0.18
PTFE_GCSF_pH8	86.4±5.7	79.3±5.3	5.45±0.33
RC_GCSF_pH8	83.2±1.2	77.9±2.1	3.97±1.30

3.2.6 Raman spectroscopy

Raman spectroscopy is an essential type of molecular spectroscopy. It is used to gain information about properties and structure of a molecule based on their vibrational transitions. The observed Raman scattering is emitted by an induced dipole moment which is created by the interaction of the polarizability of the molecule with the incoming radiation [47].

In this study Raman spectroscopy was applied to detect potential changes in confirmation when G-CSF formulations were spiked with filter leachables. The results are illustrated in Figure 31 and Figure 32. In the illustration the amide III region for the G-CSF protein is depicted. The secondary structure of the formulations after spiking with filter extracts (extracted at 30°C and 50°C) did not vary from the conformation of the protein without filter extracts. Therefore, no significant changes in the proteins secondary structure was determined. Which was an unexpected result because SEC and zeta potential analysis showed significant changes regarding protein denaturation and aggregation. This may originates from the fact that the protein concentration in the formulations was too low to determine differences via Raman spectroscopy.

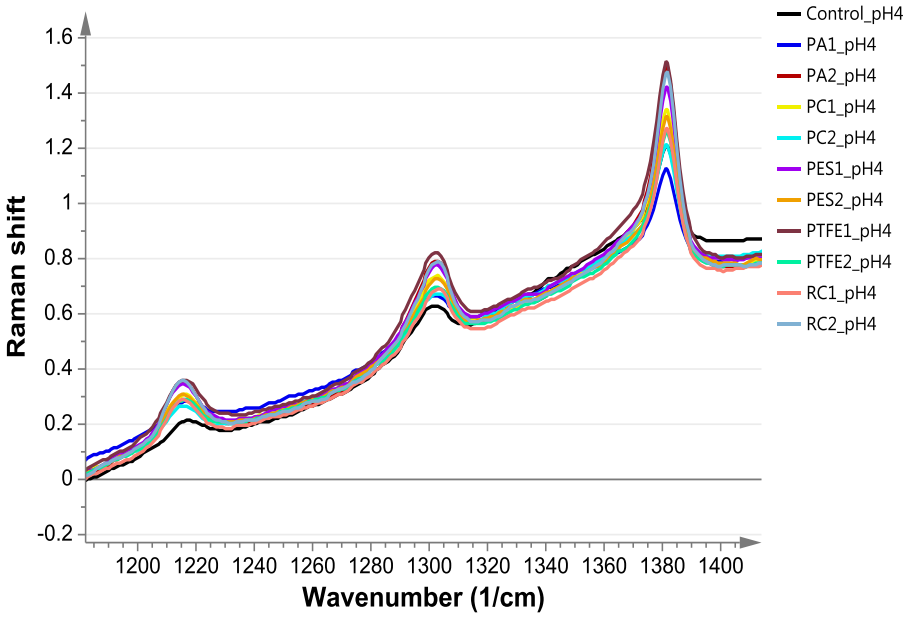


Figure 31: Results of the Raman spectroscopy analysis of G-CSF formulations spiked with pH 4 filter extracts extracted at 30°C.

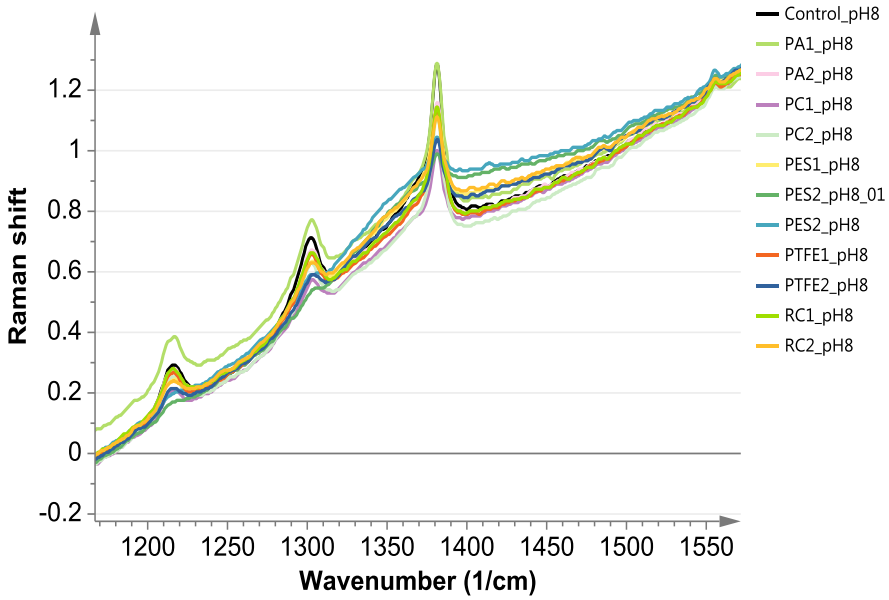


Figure 32: Results of the Raman spectroscopy analysis of G-CSF formulations spiked with pH 8 filter extracts extracted at 30°C.

4. Conclusion and Outlook

The goal of the first part of this thesis was to determine the extent to which organic substances were extracted from filter materials under specific conditions. Furthermore, by applying HPLC-UV/MS and different extraction conditions many E/Ls were detected. Via the application of exaggerated extraction conditions extractables of the filters were identified. Some of the identified extractables were known extractables from the specific material such as Bisphenol A from polycarbonate membranes, whereby many of those are known to be harmful for patients. Other detected extractables were not found in literature prior to the analysis and were successfully associated with polymer production processes. Remarkably, polyamide, polycarbonate and polyethersulfone filters showed the highest extractable content whereas polytetrafluoroethylene and regenerated cellulose membranes were low in extractables. By applying less aggressive extraction conditions, in order to mimic actual process or storage conditions, leachables were identified. As a result all the detected leachables were already determined as extractables which confirms the information found in literature that leachables are typically a subset of extractables. Because many E/Ls were detected but could not be identified, testing of fragmentation behavior using LC/MS would be of great interest. In addition to the qualification of the extractables and leachables, semi-quantitative determination could be performed to obtain an overview if the present concentration can be harmful for patients.

The second part geared towards the evaluation of the effect of E/Ls on protein formulations. It turns out that regenerated cellulose membranes are surface active, depending on the pH of the solvent used for the analysis. Further on polyethersulfone membranes led to a slightly decrease of surface tension. Regarding the anaylsis of the protein stability, significant differences between protein formulation with and without filter leachables were detected via microflow imaging, electrophoretic light scattering and size exclusion chromatography. Slight differences could be identified via differential scanning calorimetry. Most of all samples containing leachables from polyamide filters showed distinct changes. Unfortunately no differences were detected via Raman spectroscopy. For further analysis of the effect of polyamide leachables on G-CSF formulations it would be of interest to test the effect of some specific identified compounds, for example caprolactam monomers, on G-CSF.

References

[1] D. R. Jenke, J. M. Jene, M. Poss, J. Story, T. Tsilipetros, A. Odufu, and W. Terbush, "Accumulation of extractables in buffer solutions from a polyolefin plastic container.," *Int. J. Pharm.*, vol. 297, no. 1–2, pp. 120–33, Jun. 2005.

[2] S. Behme, "Formulation and Filling," in *Manufacturing of Pharamceutical Proteins: From Technology to Economy*, 2nd ed., Berlin, 2015, pp. 96–98.

[3] Michael J. Akers, "Sterile Filtration," in *Sterile Drug Products: Formulation, Packaging, Manufacturing and Quality*, 1st ed., London, 2010, pp. 267–269.

[4] M. Huang, T. S. Horwitz, C. Zweiben, and S. K. Singh, "Impact of Extractables / Leachables from Filters on Stability," *J. Pharm. Sci.*, vol. 100, no. 11, pp. 4617–4630, 2011.

[5] R. H. Colton and D. G. Bestwick, "Extractables and Leachables Evaluation for Filters," in *Filtration and Purification in the Biopharmaceutical Industry*, 2nd ed., M. Jornitz and T. Meltzer, Eds. Oregon,USA, 2008, pp. 389–411.

[6] K. Moyer and J. Scull, "Extractables and leachables," in *Specification of Drug Substances and Products*, C. M. Riley, T. W. Rosanske, and S. R. Rabel Riley, Eds. 2014, pp. 265–289.

[7] P. Peri, "Regulatory Perspectives on Extractables and Leachables Structures of potential leachables," Bethesda (MD), 2011.

[8] Y. Hua and D. Jenke, "Increasing the Sensitivity of an LC – MS Method for Screening Material Extracts for Organic Extractables via Mobile Phase Optimization," *J. Chromatogr. Sci.*, vol. 50, pp. 213–227, 2012.

[9] "Material Requirements for Plastics used in Medical Devices," in *Plastics in Medical Devices*, 2nd ed., Burlington, MA, USA: Elsevier, 2010, pp. 33–54.

[10] R. Colton, "Recommendations for Extractables and Leachables Testing," *Bioprocess Int.*, vol. 5, no. 11, pp. 36–49, 2007.

[11] S. Genot, "The Problematic and Concrete Cases concerning large Volume Parenteral Drugs," in *Extractables&Leachables 2012, 12-13 December*, 2012.

[12] "RÖMPP - Polyamide - Georg Thieme Verlag KG." [Online]. Available: https://roempp.thieme.de/roempp4.0/do/data/RD-16-03046. [Accessed: 15-Jan-2015].

[13] "Nylon Membrane Filters | Sterlitech Corporation." [Online]. Available: http://www.sterlitech.com/filters/membrane-disc-filters/nylon-membranes.html. [Accessed: 15-Jan-2015].

[14] "Nylon (NY) Membrane Filter 25007-293------N - Sartorius AG." [Online]. Available: http://www.sartorius.ie/en/product/product-detail/25007-293-n/. [Accessed: 15-Jan-2015].

[15] "RÖMPP - Polycarbonate - Georg Thieme Verlag KG." [Online]. Available: https://roempp.thieme.de/roempp4.0/do/data/RD-16-03130. [Accessed: 15-Jan-2015].

[16] “Polycarbonate (PC) Membrane Filter 23007--50------N - Sartorius AG.” [Online]. Available: http://www.sartorius.com/en/product/product-detail/23007-50-n/. [Accessed: 15-Jan-2015].

[17] “Whatman® Nuclepore Track-Etched Membranes - Analysis and Detection of Microorganisms | Sigma-Aldrich.” [Online]. Available: http://www.sigmaaldrich.com/labware/labware-products.html?TablePage=109469950. [Accessed: 16-Jan-2015].

[18] “RÖMPP - Polysulfone - Georg Thieme Verlag KG.” [Online]. Available: https://roempp.thieme.de/roempp4.0/do/data/RD-16-03598. [Accessed: 15-Jan-2015].

[19] “Polyethersulfon (PES) Membranfilter 15407--25----MIN - Sartorius AG.” [Online]. Available: http://www.sartorius-austria.at/de/product/product-detail/15407-25-min/. [Accessed: 03-Jan-2015].

[20] “Polyethersulfone (PES) Membrane Filters | Sterlitech Corporation.” [Online]. Available: http://www.sterlitech.com/filters/membrane-disc-filters/polyethersulfone-pes-membrane-filters.html. [Accessed: 15-Jan-2015].

[21] “RÖMPP - Polytetrafluorethylene - Georg Thieme Verlag KG.” [Online]. Available: https://roempp.thieme.de/roempp4.0/do/data/RD-16-03604. [Accessed: 14-Jan-2015].

[22] “Polytetrafluorethylene (PTFE) Membrane Filter 66042--47------N - Sartorius AG.” [Online]. Available: http://www.sartorius.com/en/product/product-detail/66042-47-n/. [Accessed: 15-Jan-2015].

[23] “PTFE Membrane Filters | Sterlitech Corporation.” [Online]. Available: http://www.sterlitech.com/filters/membrane-disc-filters/ptfe.html. [Accessed: 15-Jan-2015].

[24] “RÖMPP - Regeneratcellulose - Georg Thieme Verlag KG.” [Online]. Available: https://roempp.thieme.de/roempp4.0/do/data/RD-18-00636. [Accessed: 29-Jun-2015].

[25] “Regenerated Cellulose (RC) Membrane Filter 18406--25------N - Sartorius AG.” [Online]. Available: http://www.sartorius.com/en/product/product-detail/18406-25-n/. [Accessed: 26-Mar-2015].

[26] “DIAFIL RC Spritzenvorsatzfilter Membranmaterial: Regenerierte Cellulose.” [Online]. Available: http://filtershop.cc/contents/de/d2.html. [Accessed: 26-May-2015].

[27] S. W. Raso, J. Abel, J. M. Barnes, K. M. Maloney, G. Pipes, M. J. Treuheit, J. King, and D. N. Brems, “Aggregation of granulocyte-colony stimulating factor in vitro involves a conformationally altered monomeric state.,” *Protein Sci.*, vol. 14, no. 9, pp. 2246–2257, 2005.

[28] K. Hübel and a Engert, “Clinical applications of granulocyte colony-stimulating factor: an update and summary.,” *Ann. Hematol.*, vol. 82, no. 4, pp. 207–213, 2003.

[29] C. P. Hill, T. D. Osslund, and D. Eisenberg, “The structure of granulocyte-colony-stimulating factor and its relationship to other growth factors.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 90, no. 11, pp. 5167–5171, 1993.

[30] A. Prakash, B. Medhi, and K. Chopra, “Granulocyte colony stimulating factor (GCSF) improves memory and neurobehavior in an amyloid-β induced experimental model of Alzheimer’s disease,” *Pharmacol. Biochem. Behav.*, vol. 110, pp. 46–57, 2013.

[31] W. Jiskoot and D. Crommelin, “Light Scattering,” in *Methods for Structural Analysis of Protein Pharmaceuticals*, Utrecht, Netherlands, 2005, pp. 260–263.

[32] H. S. Stoker, “Biological Chemistry,” in *General, Organic and Biological Chemistry*, 6th ed., Belmont, CA, USA, 2013, p. 733.

[33] U. Roessl, J. Wiesbauer, S. Leitgeb, R. Birner-Gruenberger, and B. Nidetzky, “Non-native aggregation of recombinant human granulocyte-colony stimulating factor under simulated process stress conditions,” *Biotechnol. J.*, vol. 7, no. 8, pp. 1014–1024, 2012.

[34] “STYRENE | C8H8 - PubChem.” [Online]. Available: http://pubchem.ncbi.nlm.nih.gov/compound/styrene#section=3D-Conformer. [Accessed: 17-Jun-2015].

[35] “RÖMPP - 2-Ethylhexan-1-ol - Georg Thieme Verlag KG.” [Online]. Available: https://roempp.thieme.de/roempp4.0/do/data/RD-05-02039. [Accessed: 17-Jun-2015].

[36] “RÖMPP - Dimethylformamid - Georg Thieme Verlag KG.” [Online]. Available: https://roempp.thieme.de/roempp4.0/do/data/RD-04-01717. [Accessed: 17-Jun-2015].

[37] R. Dujardin and R.-V. Meyer, “Polycarbonate mit Aralkylphenylendgruppen, ihr Herstellung und Verwendung,” 0327951 A21989.

[38] W. Herrig, “Verfahren zur Herstellung von Polycarbonaten,” DE4241619 A11994.

[39] “RÖMPP - Xylole - Georg Thieme Verlag KG.” [Online]. Available: https://roempp.thieme.de/roempp4.0/do/data/RD-24-00154. [Accessed: 17-Jun-2015].

[40] B. M. Johnson, “Production of permeable cellulose triacetate membranes,” US 4631157 A1986.

[41] “RÖMPP - Perlon® - Georg Thieme Verlag KG.” [Online]. Available: https://roempp.thieme.de/roempp4.0/do/data/RD-16-01098. [Accessed: 25-Jun-2015].

[42] “RÖMPP - Bisphenole - Georg Thieme Verlag KG.” [Online]. Available: https://roempp.thieme.de/roempp4.0/do/data/RD-02-01737. [Accessed: 25-Jun-2015].

[43] D. Jenke, “Evaluation of the Chemical Compatibility of Plastic Contact Materials and Pharmaceutical Products ; Safety Considerations Related to Extractables and Leachables,” *J. Pharm. Sci.*, vol. 96, no. 10, pp. 2566–2581, 2007.

[44] S. Nema and J. D. Ludwig, “Validation of fill and finish processes for biotherapeutics,” in *Pharmaceutical dosage forms: Parenteral Medications, Volume 3: Regulations, Validation and the Future*, 3rd ed., New York, 2010, p. 30.

[45] A. Zhang, S. Singh, and M. Shirts, “Distinct aggregation mechanisms of monoclonal antibody under thermal and Freeze/Thaw-stresses revealed by hydrogen exchange.,” *Pharm. Res.*, vol. 29, no. 1, pp. 236–50, 2012.

[46] V. Uversky, “Use of Fast Protein Size-Exclusion Liquid Chromatography to Study the Unfolding of Proteins Which Denature through the Molton Globule,” *Biochemistry*, vol. 32, pp. 13288–13298, 1993.

[47] R. Lewis and H. G. M. Edwards, “Theory of Raman Scattering,” in *Handbook of Raman Spectroscopy: from the Research Laboratory to the Process Line*, New York, USA, 2001, pp. 1–3.