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Cleaning validation of biologicals: Determination of a worst-case product for RTH 258

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ABSTRACT

Before pharmaceutical companies can produce a product several validation studies have to be done, such as cleaning validation. The main objective of cleaning validation is to minimize the risk of cross-contamination, a topic which has become more relevant due to the evolution of medicines. During the last decades, more potent and complex drugs, such as biologicals, have been developed in which pharmaceutical companies show increasing interest. In order to test new compounds or drugs in the context of cleaning validation, large amounts of these valuable and expensive products would be lost. Therefore, alternatives to these molecules are looked for and used as worst-case product. Moreover, if one worst-case product is found for several products, one cleaning validation of the worst-case product can cover the validation of the rest of the products, saving time and money.

The goal of this thesis is to find a good alternative to use as worst-case product for the cleaning validation of brolucizumab (formerly RTH 258), a potentially new drug for the treatment of wet age-related macular degeneration. The following products were investigated as possible worst-case product: four vegetable proteins, namely soy peptone, brown rice protein, pea protein isolate, whey protein isolate and one chemical substance riboflavin. Afterwards, the cleaning validation of reactor 88, which can be used for the production of the RTH 258 formulation, with the predetermined worst-case product was done.

In order to determine a worst-case product for RTH 258, cleanability and solubility tests were performed for the five candidate worst-case products. Such a worst-case product should be harder to clean than the reference product by the investigated conditions, so as to obtain cleaning settings that assure an acceptable cleaning of the reference product. Based on the results of the cleanability tests it can be concluded that all of the products are harder to clean than RTH 258, except for soy peptone no conclusive statements can be made. In addition, a worst-case product should be less soluble in the investigated cleaning agent than the reference product, indicating a more difficult cleaning by the investigated conditions. For all of the five products, the solubility in water was lower than the solubility of RTH 258 in water. Combining the results of the cleanability and solubility experiments, riboflavin, ‘whey protein isolate/riboflavin’, pea protein isolate and brown rice protein show to be the best candidates to use as worst-case product for RTH 258. However, the final ease of cleaning should also be taken into account in order to choose the ultimate, most suitable product. Therefore it might be better to select riboflavin or ‘whey protein isolate/riboflavin’ as worst-case product, since their cleanability results are less different from RTH 258 than the other two products. To work more efficiently only one substance should be used, therefore it probably is better to choose for riboflavin

alone. Moreover, riboflavin has the advantage of being easily detectable due to its fluorescent character.

The cleaning validation of reactor 88, which can be used for the production of the RTH 258 formulation, was performed using soy peptone. The choice for this protein resulted from the fact that an ELISA kit specific for soy proteins was already available in-house at the start of the project. First, the recovery of soy was determined on stainless steel (68.20%) and on glass (75.84%). The criterion determining the acceptable amount of residue was calculated by the 'Permitted Daily Exposure' principle and resulted in a brown solution, which is not useful: if a rinsing sample would have this color, one would never conclude that the reactor is clean. The criterion determining the acceptable amount of residue, as calculated by the 1/1000 principle, gave low results. Therefore, a more specific analysis method such as ELISA is necessary, instead of the non-specific analysis method, TOC. Since the first cleaning did not give satisfying results the cleaning procedure was modified. More favorable results were obtained after the second cleaning, however, some samples still exceeded their limits. The results from the third cleaning were doubtful and therefore neglected. Furthermore, the results of the fourth cleaning are unreliable due to high results of the blank samples. It could be concluded that a more specific analysis method is required to analyze the contribution of soy to the result of the sample. TOC is a non-specific method and will also include other substances. ELISA could be a good alternative for TOC, however some problems were experienced (Master thesis, Van de Voorde - Onderbeke Julie, 2017).

In conclusion, no conclusive statements can be made about the cleanability of soy peptone, extra tests should be performed to conclude if soy is suitable as worst-case product for RTH 258 or not. However the other four products could be used as worst-case product for RTH 258 based on the obtained information. Nevertheless, we contend that the analysis method should change to a more specific method to measure the exact amount of soy residue in order to obtain more reliable results. Moreover, cleaning validation has to be done by three acceptable cleaning cycles, which has not occurred due to a lack of time.

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LIST OF ABBREVIATIONS

AMD	Age-related Macular Degeneration
API	Active Pharmaceutical Product
BSE	Bovine Spongiform Encephalopathy
BSL	Bulk Syringe Line
CFU	Colony Forming Units
CIP	Clean-In-Place
CNV	Choroidal Neovascularization
FTIR	Fourier Transform Infrared
HPLC	High Performance Liquid Chromatography
INN	International Nonproprietary Names
MS	Mass Spectrometry
NaOH	Sodium hydroxide
P	Octanol-water partition coefficient
PDE	Permitted Daily Exposure
SOP	Standard Operating Procedure
TACT	Time, Action, Concentration/Chemistry, Temperature
TOC	Total Organic Carbon
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
WINS	Water, Individual, Nature of the soil, Surface
λ_{max}	Wavelength giving maximal absorbance

Brolucizumab = RTH 258

1. Introduction

1.1. Biologicals

Biologicals, commonly referred to as biopharmaceuticals or biologics, are medicinal products that are manufactured in or extracted from biological sources. One of the first examples is insulin, previously extracted from animals, but now more produced using modern molecular biology technology. The interest in biologicals strongly increased in the last decade, due to the discovery of many targets as a result of the expanding genetic knowledge and the growing understanding of cell processes and diseases. In this way, recently discovered biologicals provided new perspectives for the treatment of cancer, cystic fibrosis and diabetes [1].

Despite most drugs being manufactured by chemical synthesis, biologicals form a group of medicines that are produced in a living system, such as microorganisms or plant and animal derived cells. The production of many biologicals involves the use of recombinant DNA technology, a fast growing field based on combining DNA from different organisms in order to produce the product of interest [2]. To do so, a certain DNA fragment is isolated from human cells and inserted into a suitable expression vector, which is then be introduced into a bacterial or mammalian cell system. The cells containing the vector will thereupon express the gene of interest, after which the recombinant protein can be conducted to further downstream processing steps [1].

The enormous interest in biologicals is mainly due to their high selectivity and potent therapeutic efficacy, resulting in limited side effects. Furthermore, their behavior is easier to predict under *in vivo* conditions. Unfortunately, there are also disadvantages associated with biologicals. Short plasma half-lives compel the patients to take the drug several times a day, resulting in poor patient compliance. Biologicals are typically very large and complex molecules, hence, there is a greater chance for an immune response. The immune system will consider the biological as foreign and will eliminate the drug. The size of the drug will also limit the route of administration to injection or infusion [1]. Moreover, in contrast to chemical drugs which are generally more pure, better characterized and easy to produce, the production process of biologics is more complex, therefore giving rise to smaller quantities. Finally, physical conditions need to be fixed on behalf of the stability of the biological molecules, resulting in an increased price tag.

A few examples of biologicals are peptides, proteins, antibodies, small interfering RNAs and enzymes [3]. Three monoclonal antibodies will be discussed below.

1.1.1. Biologicals for the treatment of age-related macular degeneration

1.1.1.1. Disease mechanism

The most important cause of severe, irreversible vision loss among people with the age of fifty and older is age-related macular degeneration (AMD). This condition is characterized by degeneration of the oval-shaped pigmented region near the center of the retina, the macula, responsible for observing the central part of our field of vision, which is important for ordinary daily activities such as reading, driving and recognizing faces (Figure 1.1). The macula consists of millions of photoreceptors, most of which are cones providing detail and color. These photoreceptors continuously capture light signals, which are translated into electrical signals by the retina. These electrical signals are sent through the optic nerve to the brain, where they are translated into the image what is called our vision.

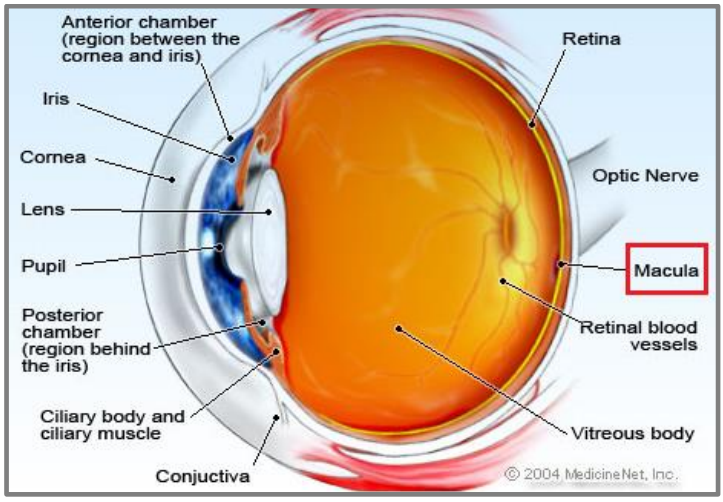


Fig. 1.1: The anatomy of the eye, with the macula framed in red [4]

When the macula is damaged, the central region of the field of view will be blurry, deformed and dark, as can be seen in Figure 1.2 [5]. The term AMD comprises two different pathologies, a dry and wet form. The dry form of AMD, affecting almost 90% of AMD patients, is characterized by yellow protein deposits in the macula. In more advanced stages of dry AMD, the layers of the macula get progressively thinner and less functional leading to geographic atrophy and blind spots in the center of the vision. The exact cause of dry AMD is unknown and neither does an approved treatment exist, although several clinical trials are underway [6]. The wet form of AMD is characterized by choroidal neovascularization (CNV) or the growth of new, pathologic blood vessels from under the retina toward the macula. Wet AMD develops more quickly and severely than the dry form, affecting almost 10-15% of AMD patients. However, almost 90% of cases who suffer from severe vision loss are due to wet AMD. One of the most critical and thoroughly studied activators of angiogenesis in wet AMD is the vascular endothelial growth factor (VEGF) [7].

Fig. 1.2: The effect of wet AMD on your vision [8]

The standard treatment for wet AMD is VEGF inhibitors. Nevertheless, the disease can also be treated using several other biologicals and drugs, such as tyrosine kinase inhibitors, pigment epithelium-derived factor, nicotinic acetylcholine receptor antagonists, small interfering RNA and sirulimus.

1.1.1.2. VEGF inhibitors: working mechanism and history

VEGF inhibitors or anti-VEGF drugs inhibit the effect of VEGF by binding to the protein itself. Due to the competitive inhibition of VEGF, the growth factor is no longer able to bind its receptors VEGFR-1 and VEGFR-2, thus, preventing VEGF of stimulating angiogenesis and leakage from blood vessels, the main causes of wet AMD. VEGF inhibitors are injected into the vitreous by a qualified ophthalmologist where it binds VEGF (Figure 1.3) [9].

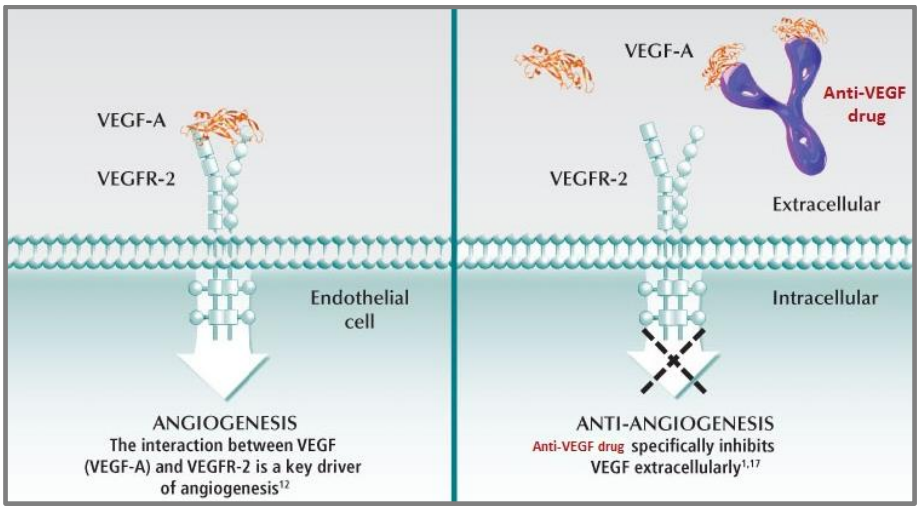


Fig. 1.3: The mode of action of VEGF inhibitors [10]

Examples of VEGF inhibitors used in the treatment of wet AMD are ranibizumab, bevacizumab and brolucizumab. The suffix -zumab, according to the guidelines for International Nonproprietary Names (INN) by the World Health Organization (WHO), indicates that these molecules are humanized monoclonal antibodies, with *humanized* signifying a 90-95% human nature of the molecules making them therefore less likely to elicit an immune response. Other INN suffixes are -momab, which

indicates that the monoclonal antibodies are 100% murine, -iximab, where 60-70% of the antibodies is human, and -mumab for antibodies that are 100% human [11].

Ranibizumab (Lucentis®, Genentech), on the Belgian market since 2007 by Novartis, was the first drug approved for the treatment of wet AMD [12]. The drug is a humanized monoclonal antibody fragment which is produced in *Escherichia coli* cells by recombinant DNA technology and has a high affinity towards the VEGF-A isoforms, such as $VEGF_{110}$, $VEGF_{121}$ and $VEGF_{165}$. Patients suffering from wet AMD need one injection per month until maximum visual acuity is obtained and/or until signs of disease activity have disappeared. For most patients, three or more consecutive, monthly injections are initially needed. Studies have shown that 30-40% of the patients experience visual improvement, the best of which were determined in patients with non-advanced wet AMD and younger patients [13].

Despite the promising results for the patients, the high cost of Lucentis® (€686 per syringe [14]) forced scientists to look for a low-cost alternative, resulting in the discovery of **bevacizumab** (Avastin®, Genentech). This drug is approved for the treatment of certain metastatic cancers, such as colon cancer, lung cancer and ovarian cancer. However, ophthalmologists use bevacizumab off-label, since the drug has not yet been approved by the Food and Drug Administration for the treatment of wet AMD [2]. Although bevacizumab differs from ranibizumab in structure, molecular size and half-life, research has shown comparable efficiency and safety. Moreover, Avastin® (€327 per syringe [14]) costs more than 50% less than Lucentis® [15, 16].

Currently, phase 3 clinical trials for **brolucizumab** (formerly RTH 258), a potentially new drug for the treatment of wet AMD, are ongoing. The anti-VEGF antibody fragment is developed by ESBAtech (Schlieren, Switzerland) and evaluated in a series of clinical trials by Novartis. Brolucizumab and ranibizumab have a similar mode of action, therapeutic dose, mode of administration, binding kinetics and other kinetic specifications. It is a fusion protein with a molecular weight of 26.3 kDa, making it the smallest VEGF inhibitor (Figure 1.4). Its small size results in an outstanding tissue penetration and the ability to administer a higher dose of the drug, potentially leading to a longer effect, which in turn is positive in the context of patient compliance due to the reduction of treatment frequency [9, 17].

Fig. 1.4: Comparison of structure and molecular size between ranibizumab and brolucizumab [9]

Some physicochemical properties of brolucizumab are already known. It has an isoelectric point of 5.25 and a solubility of 60 mg/mL in 15 mM citrate, 6.75% sucrose, 0.05% Tween 80 when the pH is adjusted to 6.75 with hydrochloric acid. Furthermore, it has a solubility of 300mg/mL in water. The composition of the formulation is represented in Table 1.1 [18].

Table 1.1: Composition of the formulation of brolucizumab. The quantities of each component are not determined at this moment. [18]

Chemical name	Weight percentage (%)	Function
RTH 258 = brolucizumab	12	Active ingredient
Citric acid (Monohydrate)	-	Buffering agent
Trisodium citrate (Dihydrate)	-	Buffering agent
Sucrose	-	Tonicity agent
Polysorbate 80	-	Solubilizing agent
Hydrochloric Acid	-	pH adjust
Sodium Hydroxide	-	pH adjust
Water for injection	q.s. 100	Vehicle

1.2. Cleaning validation

Although cleaning has always been a part of GMP regulations, it has received little attention in the past. In 1996 however, the FDA suggested revisions to the GMPs in order to redefine the manufacturing process, beginning with the cleaning process. One aspect of this process is cleaning validation, a topic that people commonly know the least about [19, 20]. The FDA's requirement regarding cleaning validation is the necessity to have documented evidence to guarantee removal of residues to predetermined levels of acceptability. Therefore, a minimum of usually three consecutive successful trials are needed.

Cleaning validation is a multidisciplinary activity, where one has to define the equipment, understand the properties of the drug and describe the analytical methods. Moreover, how to sample and collect residues from surfaces has to be known. This requires expertise of different disciplines and lots of interdisciplinary cooperation. An orderly approach to cleaning validation is needed in order to support all activities in a scientific way, a flow chart is shown in Figure 1.5.

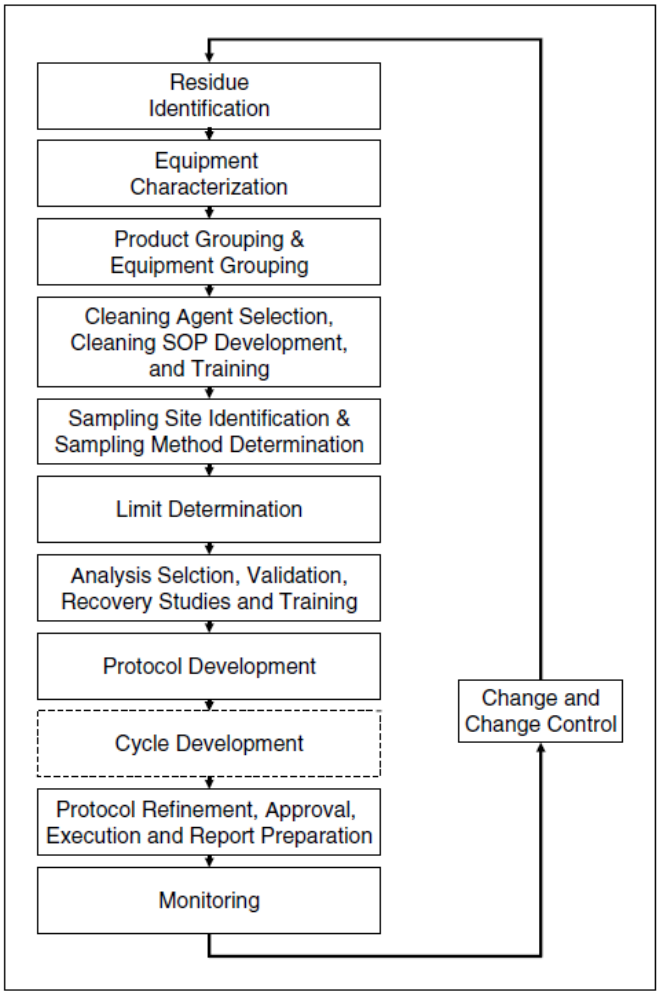


Fig. 1.5: Cleaning validation process flow chart [21]

The main objective of cleaning validation is to minimize the risk of cross-contamination, a topic which has become more relevant due to the evolution of medicines. During the last decades, more potent and complex drugs have been developed in which pharmaceutical companies show increasing interest. The complexity of these medicines, however, produce a greater risk of interactions between these products, resulting in damaging effects to patients [20].

1.2.1. Important steps during cleaning validation

1.2.1.1. Residue identification

The identification of residues is the first step in performing a cleaning validation, which will determine the consecutive steps of the validation process. One has to take into account the potential presence of a variety of residues, ranging from Active Pharmaceutical Products (API(s)), excipients of the formulation and metabolites to cleaning agents, precursors or starting material and buffers.

As a first screening, nonspecific methods, such as Total Organic Carbon (TOC), determination of the pH and conductivity, can be used to look for *all* types of residues. Next, specific methods, such as High Performance Liquid Chromatography (HPLC), Mass Spectrometry (MS) and Fourier Transform Infrared (FTIR) spectroscopy, are able to identify specific residue(s) of interest, although requiring more time and money.

The most important residues are APIs, precursors, cleaning agents, and preservatives, since they could potentially harm patients. API and precursors exhibit a certain activity while cleaning agents are not intended for consumption and preservatives could be toxic. During cleaning validation, it is preferred to consider all possible residues and choose the most important ones for further investigation.

To determine whether a residue is important or not five selection criteria are defined:

- Residues that define the dosage form or process.
- Residues that are the most active or toxic.
- Residues that would damage the quality, purity, efficacy and appearance of the next batch.
- Residues that would damage the next process.
- Residues that are the hardest to remove.

During the cleaning process, it is important to realize that cleaning could potentially change properties of the residues. Physicochemical changes of residues on the surface can take place due to alkaline or acid conditions, the presence of air or heat during cleaning. Therefore, the influence of these degradation products or denatured materials on the cleaning process has to be kept in mind [19, 20].

For example, in the case of biotechnological products degradation is a common issue during cleaning validation. Proteins will degrade into smaller fragments or larger molecules can be formed. This degradation is the result of hot, aqueous, alkaline or acidic cleaning conditions. Several specific methods exist for the identification of such degradants [21].

1.2.1.2. Equipment characterization

Besides the removal of residues, it is essential that all the pieces of the equipment associated with the process have been cleaned to acceptable levels in a reliable and reproducible way. Therefore the design of the equipment has to be well understood. The following two marks are examples of applications which are helpful for the characterization of the equipment. Firstly, locations that are hard to clean and locations with a high risk of contamination have to be identified in order to select sample sites [20] (Figure 1.6). For example, rough surfaces increase the area for soil contact which makes it more difficult for cleaning agents to penetrate through. Moreover, residues can attach to surfaces due to van der Waals forces, electrostatic effects and other forces. [21]

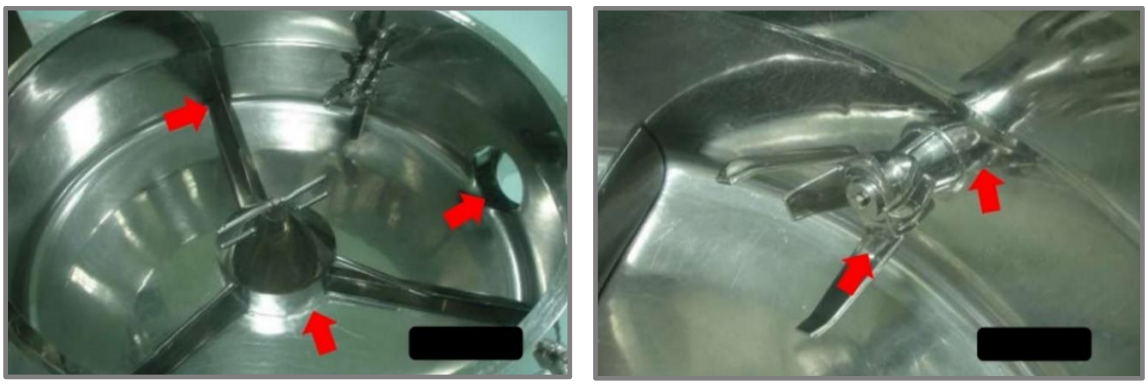


Fig. 1.6: Examples of difficult to clean locations [22]

Secondly, it is important to verify the compatibility of the cleaning procedures (cleaning agent, temperature) with the materials of construction, in order to minimize the influence on the materials [20].

1.2.1.3. Product and equipment grouping

The following step includes the grouping of products and equipment, which means that products or equipment with similar characteristics or purposes in the context of cleaning validation are put together. It is important that cleaning validation must always be carried out to meet the lowest limit

of the total product group. Furthermore, the surface area used in residue calculation must be the largest of all the equipment.

A few reasons to group **products** are:

- Products that are cleaned with the same cleaning agent
- Products that are manufactured on the same equipment group
- Products cleaned with the same cleaning procedure
- Products with similar formulations and manufacturing processes
- Products with similar patient risk levels

A few arguments to group **equipment** are:

- Equipment used to produce products from the same product group
- Equipment cleaned with the same cleaning agent
- Equipment cleaned with the same cleaning method
- Equipment that has an equivalent role in manufacturing processes
- Equipment that has a similar functionality and design [20]

1.2.1.4. Selection of the cleaning agent, SOP development and training

Cleaning processes are based on the principle of TACT-WINS: Time, Action, Concentration/Chemistry, Temperature - Water, Individual, Nature of the soil, Surface. This acronym summarizes the parameters that have to be controlled in any cleaning procedure (TACT) and those that affect the soil's removal from the surface (WINS).

Time represents the contact time on the surface being cleaned. *Action* is defined as the mechanism used to administer the cleaning agent, such as scrubbing, soaking or turbulent flow. Scrubbing and soaking are mostly used for manual cleaning, while automated cleaning will use a turbulent flow. *Concentration/Chemistry* refers to the concentration of a specifically selected type of cleaning agent, which will determine the effectiveness of the cleaning process. On the one hand, the cleaning process could fail as a result of an insufficient amount of detergent used. On the other hand, the use of an excessive amount of cleaning agent could result in the presence of detergent residues after cleaning, requiring extra rinsing steps. Moreover, there exist different cleaning chemistries, divided into four broad categories, which will be elaborated more in depth later in this paragraph. *Temperature* indicates that for each step of the cleaning process, the optimal temperature has to be chosen. During rinsing, for instance, ambient temperatures are set to minimize denaturing effects on proteins and maximize dilution effects. However, the effectiveness of cleaning agents increases with higher

temperature [21]. In order to apply TACT, proper knowledge and understanding of WINS is required, since each WINS parameter can affect the action of the TACT parameters. Furthermore, adjustment of one of the these parameters might influence one another, making it of great importance to find the optimal balance between the TACT parameters [20].

Cleaning agents are generally divided into four classes: water, organic solvents, commodity chemicals and formulated cleaning agents. Water is a universal solvent which is cheap and non-invasive. If the product can be adequately cleaned without undue time or physical labor, water alone should be used. In contrast, solvents are mostly used in processes where solvents are already used for manufacturing. However, it is recommended to reduce solvent use due to environmental reasons. Commodity chemicals, such as sodium hydroxide (NaOH), are used for cleaning due to their high alkalinity, making them capable of stimulating oxidation and reduction of soils. Still, they do not have a good cleaning capacity. Furthermore, they are sometimes difficult to rinse out and need a larger amount of water than formulated cleaning agents. These formulated cleaning agents are the biggest group of cleaning agents, including solvent-based formulations and aqueous formulations. Most of these products contain alkalinity or acidity sources, surfactants, chelants and either a solvent or water. They are often produced in such a way that they have a low-foaming capacity and therefore are more easily rinsable and suitable to be used in high turbulence cleaning [19, 20].

To select an appropriate cleaning agent, there are a few parameters that have to be well understood, such as the properties of the soil and surface, the availability of cleaning methods and utilities, safety considerations and effluent properties. It is important to look at the formulation as a whole and not only at the API to choose a cleaning agent, because excipients determine the biggest part of the formulation as well as the rate to release the API, thus affecting the API's solubility. Furthermore, the detergent has to be completely removed after cleaning, an additional aspect which has to be taking into account [20].

When the cleaning agent is chosen, a cleaning method must be determined. There are three different cleaning methods, namely manual, semi-automated and automated cleaning. For manual cleaning an operator will use a brush or hose to clean the equipment. Multiple operators will not clean on the exact same way, therefore it will be hard to obtain reproducible cleaning cycles. A tool to minimize these changes is to compose a detailed cleaning procedure and checklist based on the available standard operating procedures (SOPs). For (semi)- and automated cleaning processes there are part washers and Clean-In-Place (CIP) systems available. The reproducibility of these cleaning cycles will be better, however there is more concern about human interfaces with the system, for example loading of the parts washer, breaking connections, etc [20].

Another critical aspect of cleaning validation is the training of personnel regarding cleaning. It is necessary to discuss the importance of cleaning with operators, since they must understand each cleaning step that they perform and have to guarantee that cleaning procedures are properly sequenced, so to avoid contamination of locations that have already been cleaned. This familiarization of personnel with suitable techniques is a big advantage for cleaning validation, in particular for manual cleaning. For example, visual inspection is a valuable asset, because it gives a global image of the whole equipment and not only of the sampling sites. Operators have to know where they have to look and how they have to recognize residues on the surfaces [19, 20].

1.2.1.5. Identification of the sampling site and sampling method

After cleaning it is important to validate the effectiveness of the cleaning process. Therefore, samples are taken and examined for the presence of soil. Several specific areas of the equipment make up interesting sample sites, which are selected by the difficulty of cleaning and on the intended sampling methods. For example, inaccessible and difficult to clean geometries of the equipment, areas that are expected to become filthy during manufacturing, areas that are important to be cleaned in order to avoid contamination to the next batch and areas which might experience recirculation of soils during cleaning. Additionally, the variety of affinity of materials to soils also has to be considered.

Several tools exist to help select sampling sites: reviews of the equipment characterization, such as geometry and material of construction, and cleaning SOPs for potential areas of weakness both provide an important source of information for the characterization of interesting sampling sites. In addition, interviews with instrument operators might also be helpful to check on their experience.

Swab sampling and rinse sampling are the two sampling methods most commonly used for cleaning validation, with the preference for swab sampling. The first technique involves the use of an absorptive swab used to physically wipe the surface and recover the analyte. Sampling sites have to be accessible to reach with the human hand or arm. The swab can be used in combination with a diluent, such as water or a solvent, which has to be compatible with the analyte. It will improve the physical removal of the residue and help to dissolve it. The swab can also be used in its dry state. There are multiple swab techniques, one of which is shown in Figure 1.7. The second technique comprises the collection of fluid which has been in contact with the surface. The solvent used for rinse sampling has to be compatible with the residue. Most often, rinse water is used [19, 20].

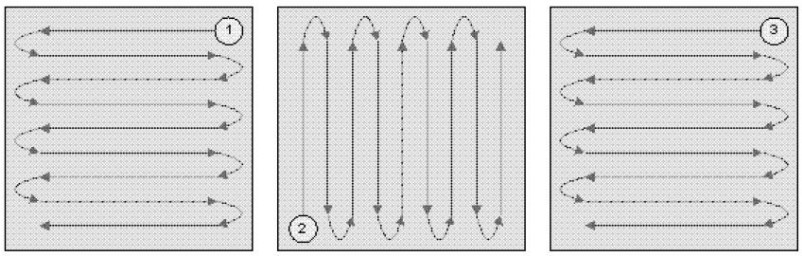


Fig 1.7: An example of a swabmethod[1]

Recovery studies are performed to evaluate the performance of sampling methods by the determination of the residue's recovery from the surface. A specific amount of residue of interest will be spiked onto the surface. After a drying period, mimicking the dirty hold time, the sampling method will be carried out. The sample is then analyzed and compared to a reference with the used amount of residue. Finally, the amount of recovery can be calculated. In general, a recovery greater than 50% is required, however most companies accept a recovery greater than 75%. [20]

1.2.1.6. Determination of limits[2]

Limits for cleaning validation have to be defined by the manufacturers and will depend on the specific situation. These limits represent the dosage that is safe for cleaning carry-over, therefore cleaning has to be continued until these acceptable limits are reached. Although most of the time only the API is of great importance to determine these limits, there is little guidance to determine limits for cleaning agents or other intermediate products that were not intended to be in the final product administered to patients. A formula for pharmaceutical manufacturers is worked out to determine limits in accordance with some well-documented mathematical standards. This formula consists of a combination of limits which guarantees that surfaces are visibly clean and safety limits are achieved by minimizing the total contamination towards the next batch. The following qualifications have to be met: first of all, the surfaces have to be visibly clean. Secondly, not more than 1/1000th of a therapeutic dose should arrive in the next batch. Lastly, not more than 10 ppm should enter the next batch. The lowest limit of the last two has to be followed. The limit of 1/1000th is chosen because at this level the product would no longer have a pharmacological effect on the next patient. The most basic formula to determine the limit is given as follows [20]:

[1] Figure obtained by Alcon
[2] Formulae obtained by Alcon

$$MAC = \frac{LTD * B * SF}{MTD}$$

- *MAC: Maximum Allowable Carry-over (mg)*
- *LTD: Lowest therapeutic dose considered for the product that has to be cleaned (mg/day)*
- *B: Minimal size batch of the next product (mL)*
- *SF: Safety Factor (typically 1/1000)*
- *MTD: The largest daily dose considered for the next product (mL/day)*

Nevertheless, these limits are very conservative and not based on a scientific background, since every product is treated in the same way, despite their toxicological differences. Therefore, the concept of Permitted Daily Exposure (PDE) was introduced, representing a substance-specific dose that is unlikely to cause an adverse effect if one is exposed to this or a lower dose every day for a lifetime [23]. PDE can be calculated by the following formula:

$$PDE = \frac{NOAEL * BW}{UFc * MF * PK}$$

- *PDE: Permitted daily exposure (mg/day)*
- *NOAEL: No Observed Adverse Effect Level (mg/kg/day)*
- *BW: The weight of an average adult (e.g. 70 kg)*
- *UFc: Composite Uncertainty Factor: Combination of factors which reflect the interindividual variability, interspecies differences, sub-chronic-to-chronic extrapolation, LOEL-to-NOEL extrapolation, database completeness*
- *MF: Modifying factor: a factor to address uncertainties not covered by the other factors*
- *PK: Pharmacokinetic adjustments [24]*

Based on the definition of PDE a limit of maximum carry-over after cleaning is given in the following formula:

$$Limit\ (mg) = \frac{PDE * B}{L}$$

- *PDE: Permitted daily exposure (mg/day)*
- *B: Minimal size batch of the next product (mg)*
- *L: Maximum daily dose of the next product (mg/day)*

More specific limits, based on both concepts, depend on the way of sampling. For example, if a homogeneous distribution on all surfaces of the equipment is assumed, which is usually not the case, a recommended value for the content in a swab after **swab sampling** can be set [24].

Formula based on the safety factor (1/1000):

$$MAC\ \left(\frac{mg}{mL}\right) = \frac{LTD * B * Aswab * SF * R\ item}{Atot * MTD * Vswab}$$

- *LTD: Lowest therapeutic dose (mg/day)*
- *B: Minimal size batch of the next product (mL)*
- *A_{swab}: Swabbed surface (cm²)*
- *SF: Safety factor (0.001)*
- *R_{item}: Recovery of the product to be investigated on a specific type of surface*
- *A_{tot}: The entire surface of the equipment train (cm²)*
- *MTD: Maximum therapeutic dose (mL/day)*
- *V_{swab}: Volume to dissolve the swab sample (mL)*

Formula based on the PDE concept:

$$Criterion\ \left(\frac{mg}{mL}\right) = \frac{PDE * B * A\ swab * R\ item}{L * Atot * V\ swab}$$

- *PDE: Permitted Daily Exposure (mg/day) of the API of the worst-case product*
- *B : Minimal size batch of the next product (mg)*
- *A_{swab}: Swabbed surface (cm²)*
- *R_{item}: Recovery of the product to be investigated on a specific type of surface*
- *L: Maximum daily dose of the next product (mg/day)*
- *A_{tot}: The entire surface of the equipment train (cm²)*
- *V_{swab}: Volume to dissolve the swab sample (mL)*

Furthermore, in the case of **rinse samples**, a limit for cleaning validation can be calculated by the following formula [24].

Formula based on the safety factor:

$$MAC\left(\frac{mg}{mL}\right) = \frac{LTD * B * SF * Arinse\ sample}{Atot * MTD * V}$$

- *LTD: Lowest therapeutic dose (mg/day)*
- *B: Minimal size batch of the next product (mL)*
- *SF: Safety factor (0.001)*
- $A_{rinse\ sample}$: *Rinsed surface (cm²)*
- A_{tot}: *The entire surface of the equipment train (cm²)*
- *MTD: Maximum therapeutic dose (mL/day)*
- *V: Volume of rinsate (mL)*

Formula based on the PDE concept:

$$Criterion\ \left(\frac{mg}{mL}\right) = \frac{PDE * B * Arinse\ sample}{V * L * Atot}$$

- *PDE: Permitted Daily Exposure (mg/day) of the API of the worst-case product*
- *B: Minimal size batch of the next product (mg)*
- $A_{rinse\ sample}$: *Rinsed surface (cm²)*
- *V: Volume of rinsate (mL)*
- *L: Maximum daily dose of the next product (mg/day)*
- A_{tot}: *The entire surface of the equipment train (cm²)*

1.2.1.7. Analysis selection and validation

As mentioned earlier, there are two kinds of analysis methods: specific or direct methods and nonspecific or indirect methods. Direct methods, such as HPLC, MS and FTIR, will identify the analyte by its specific composition. Indirect methods will measure a general property of the residue. For example TOC, the measurement of pH and conductivity will respectively measure the carbon content, the acid-base character or the ion strength. Of these indirect methods, TOC is most often used for cleaning validation, because it generates results rapidly [19, 20]. Figure 1.8 gives an overview of the properties of the most common analysis methods [20].

These methods must be validated, which is done in a similar way as or any other validation. The validation includes accuracy, precision, linearity, robustness, specificity and range. The quantification limit and detection limit are also important, because after cleaning only residues will remain. Other considerations included in the ICH standards must be observed, namely system suitability, standards & controls and robustness [20].

Attribute	pH	Conductivity	Total organic carbon	HPLC	Ion mobility spectrometry	Direct surface FTIR
Nonspecific	○	○	○	●	●	●
Does NOT detect in the presence of solvents	●	●	○	●	●	●
Requires a soluble/ semi-soluble residue	○	○	○	●	●	●
Requires an ionizable residue	●	○	●	●	○	●
Is NOT typically rapid/real time	●	●	●	○	●	●
Does NOT typically have any on/at-line capability	●	●	●	○	●	●
Uses reagents/mobile phase/specialty gases	●	●	○	○	●	●
Requires special sample preparation	●	●	●	○	●	●

Key: ●, No (advantage); ○, yes (potential disadvantage).

Fig. 1.8: Comparison of the most common analysis methods [20]

1.2.2. Microbiological contamination

Besides assuring the chemical cleanliness, microbiological contamination needs to be considered. All areas of equipment that have direct contact with the raw material need to be examined. Microbiological samples have to be taken before and after the cleaning procedure to determine the efficacy of the detergents and disinfectants. For non-sterile production microbiological cleanliness should be less than 100 CFU/mL, while for sterile production the level of microbiological contamination of the rinse water should be 10 CFU/100 mL. Sampling should take place in triplicate [19, 25].

1.2.3. Clean hold time - Dirty hold time

Clean hold time and dirty hold time are two concepts that are part of cleaning validation. Clean hold time is the time between cleaning and the start of the subsequent manufacturing operation. It is obvious that clean equipment has a higher chance of becoming soiled when the clean hold time increases. Dirty hold time is defined as the time between the end of manufacturing and the initiation of the cleaning process. The longer the dirty hold time, the harder it will be to clean the equipment [26].

1.2.4. Cleaning validation of biologicals

Cleaning validation of biologicals has more strict requirements due to their inherent characteristics, such as the stickiness of proteins, parenteral product purity obligation, the complexity of the used equipment and many materials that have to be cleaned. Specific analysis methods are recommended for cleaning validation in general, however for biologicals product-specific assays such as

immunoassays may not be adequate: a negative result can be the result of denaturation of the protein. Therefore product-specific assays should be coupled with the non-specific method TOC to detect protein residues. Large amounts of these valuable and expensive products are necessary to perform a cleaning validation. Therefore, alternatives to these molecules are looked for and used as worst-case product [27].

1.2.5. Determination of a worst-case product

The concept of grouping was already explained in section 1.2.1.3. Products that are similar regarding their cleaning can be assigned to one group, for which it is possible to determine a worst-case product. This is the substance that is the most difficult to remove during cleaning, representing all other materials in its group. This approach minimizes the number of required validation runs. The conditions to clean this worst-case product, will definitely be good enough to remove the other products [20].

To determine the worst-case product, it is important to analyze its solubility and degradation, since these affect the selection of a cleaning agent, procedure design and method. **Solubility** is important because at one point the residue has to be dissolved, emulsified or suspended to a certain level by the cleaning agent. A product's solubility will depend on the difficulty of solvating dislodged molecules, where the concept of 'like dissolves like' is valid. Moreover, the difficulty of dislodging solute molecules from the crystal structure must be taken into account. Important parameters, such as hydrophilicity/lipophilicity, ionizability, temperature effects and surface activity that will influence the solvation will be further explained.

Hydrophilic substances, also known as *water-loving* molecules, exhibit high polarity and intermix well with water molecules. This mixture is stabilized by dipole-dipole, dipole-charge, or hydrogen bonding interactions. Conversely, the introduction of hydrophobic substances, also known as *fat-loving* molecules, into water forces the water molecules to take a less random orientation, because the possibility of hydrogen bonds are reduced. Non-polar molecules dissolve more readily in non-polar solvents. The **hydrophilicity/lipophilicity** of a molecule can be characterized by its octanol-water partition coefficient (P), which is defined as the ratio of concentrations of a non-ionized molecule dissolved in octanol and water, two immiscible phases, at equilibrium [28]. This coefficient is often described in its logarithmic form (log P).

$$P = \frac{[X]octanol}{[X]water}$$

The **ionization** of molecules will greatly increase their solubility in water, because ions are far more polar than non-ionized substances. Many APIs contain regions in their molecular structure that can dissociate when dissolved in water, forming positively and negatively charged ions. Since the pH of the solution has a great impact on the dissolution and solubilization of many drug substances, the modification of the solution's pH is an easy way to enhance the solubilization of poorly soluble substances.

Higher **temperatures** generally increase the solubility of molecules. However, in some cases the solubility will decrease with an increase in temperature. An example of this last group are proteins. At higher temperatures protein denaturation occurs, because heat will cause the disruption of hydrogen bonds and non-polar hydrophobic interactions, resulting in a lower solubility.

Lastly, some APIs and formulation excipients are **surface-active**, i.e. their molecular structure contains a non-polar and a polar part. These surface-active substances, also called surfactants, promote wetting, emulsification and dispersion of solids to enable cleaning [28].

Besides solubility, the **degradation** of molecules is also an important aspect to determine a worst-case product. Unlike chemical degradation, referring to the conversion of the molecular structure resulting in degradation products that may not be detectable using an analytical method designed to quantify the API, physical degradation especially affects the ease or difficulty of removing the API from the surface. Physical degradation indicates the change of the physical form and the change in physical properties of a solid. This is relevant for cleaning since several properties such as solubility, hygroscopicity, chemical stability, and others might be influenced. Solid phase transition can for instance alter the cleanability if a water soluble solid is converted into a less water soluble form. It is essential to understand how APIs and excipients may degrade during cleaning in order to develop a reliable cleaning process.

Although solubility and degradation will determine the chemical cleaning mechanisms, residues can also be removed in a physical way. Physical removal depends on the size of the residue and its degree of adhesion to the equipment surface [21]. Normally, API candidates have a sufficient chemical and physical stability so that they can be produced and marketed. During cleaning operations, however, they are exposed to an environment capable of changing their properties, therefore potentially changing the ease of cleaning, which is a crucial aspect to understand in the context of cleaning validation [28, 29].

1.3. Alternatives to biologicals for cleaning validation

In order to test new compounds or drugs in the context of cleaning validation, large amounts of these valuable and expensive products would be lost. Therefore, alternatives to these molecules are looked for and used as worst-case product. The goal of this thesis is to find a good alternative vegetable protein to use as worst-case product for the cleaning validation of brolucizumab [30].

Below, a short theoretical discussion is given about vegetable proteins. Afterwards the vegetable proteins that will be used during this project are discussed in order to compare their properties. Besides these vegetable proteins, riboflavin has also been investigated as a potential candidate, due to its poor solubility and ease of detection.

1.3.1. Theoretical discussion

Proteins are the building blocks of all forms of life. They are in fact a sequence of amino acids which fold into a structurally complex and functionally active product. Proteins can be classified by their origin. For example, milk and egg proteins belong to the animal proteins, while fish and crustacean proteins are part of the marine proteins. The group of plant proteins, which will be focused on in this project, can be further subdivided in three different classes, an overview of which is given in Table 1.2.

Table 1.2: Overview of plant proteins

Class	Source
Vegetable proteins	Pea, potato
Cereal proteins	Wheat, barley, rye, oat, maize, rice
Seed proteins	Soy, peanut, bean, sunflower seeds, cotton seed

Brolucizumab is a monoclonal antibody, i.e. an immunoglobulin consisting of four amino acid chains. Therefore it was decided to work with proteins instead of small chemicals because the structure of proteins and their associated properties determining the ease of cleaning, are more related to those of brolucizumab. Vegetable proteins are preferable because of their low price and low risk. In contrast, animal proteins are less suitable, since they contain the risk of being contaminated with viral and bovine spongiform encephalopathy (BSE). The same holds for human proteins, which, moreover, are too expensive to use as worst-case product.

Food proteins are mostly difficult to remove in a cleaning process, because they can be very sticky after denaturation. Therefore these vegetable proteins can be chosen as possible worst-case product to perform a cleaning validation for a biological product [31, 32].

1.3.2. Substances to be investigated

1.3.2.1. Soy peptone

Soy is derived from the soybean plant and consists of 11S or glycinin and 7S or β-conglycinin. Glycinin is the most important component, responsible for adhesion strength and water resistance [33]. Soy proteins have an isoelectric point around 4.5. Their charged groups give rise to a good solubility in water, while basic conditions improve their solubility even more. β-conglycinin is more sensitive to heat than glycinin and has a denaturation temperature of 70°C and 80°C [33].

1.3.2.2. Brown rice protein

Rice mainly consists of albumin, globulin, prolamin and glutelin [34]. Albumin is the most soluble protein followed by globulin. Prolamin and glutelin show a lower solubility as a result of their lower aspartic acid and glycine content. Rice proteins have an isoelectric point between 4 and 6, which has an influence on their solubility when the pH changes. Rice protein isolate has a denaturation temperature around 83.4°C [35].

1.3.2.3. Pea protein isolate

Pea protein isolate mainly consist of 11S legumin and 7S vicilin. It has a poor solubility in water due to its hydrophobic surface structure and low surface charge [36]. No stability data are available in literature.

1.3.2.4. Whey protein isolate

Whey proteins belong to a group of milk proteins classified as globular proteins consisting of mainly lactoglobulin and α-lactalbumin. They have a good water solubility over a wide range of pH values. However, whey proteins are sensitive to heat denaturation at temperatures above 70°C, resulting in protein aggregation [37].

1.3.2.5. Riboflavin

Riboflavin, also known as vitamin B12, is a yellow-orange solid substance with poor water solubility, when compared to other B vitamins. The protein has a high stability at high temperatures and in acidic media. Riboflavin is often used as tracer due to its fluorescence under UV light. The vitamin however is very sensitive to visible light [38].

2. Objectives

The interest in biologicals has strongly increased in the last decade due to the discovery of many targets, as a result of the expanding genetic knowledge and the growing understanding of cell processes and diseases. Pharmaceutical companies are also focusing more on biologicals in order to replace the pipeline of chemical molecules, which is getting increasingly smaller. In general, the enormous interest in biologicals is due to their high selectivity and potent therapeutic efficacy, resulting in limited side effects. Furthermore, their behavior is easier to predict under *in vivo* conditions.

Before pharmaceutical companies can produce a product several validation studies have to be done, such as cleaning validation. The main objective of cleaning validation is to minimize the risk of cross-contamination, a topic which has become more relevant due to the evolution of medicines. During the last decades, more potent and complex drugs have been developed in which pharmaceutical companies show increasing interest. In order to test new compounds or drugs in the context of cleaning validation, large amounts of these valuable and expensive products would be lost. Therefore, alternatives to these molecules are looked for and used as worst-case product. Moreover, if one worst-case product is found for several products, one cleaning validation of the worst-case product can cover the validation of the rest of the products, saving time and money.

The goal of this thesis is to find a good alternative to use as worst-case product for the cleaning validation of brolucizumab (formerly RTH 258), a potentially new drug for the treatment of wet age-related macular degeneration. Currently, phase 3 clinical trials for brolucizumab are ongoing. This anti-VEGF antibody fragment is developed by ESBAtech (Schlieren, Switzerland) and evaluated in a series of clinical trials by Novartis. The molecule is a monoclonal antibody, i.e. an immunoglobulin consisting of four amino acid chains. Therefore it was decided to work with proteins instead of small chemicals because the structure of proteins and their associated properties determining the ease of cleaning, are more related to those of brolucizumab. Vegetable proteins are preferable because of their low price and low risk. In contrast, animal proteins are less suitable, since they contain the risk of being contaminated with viral and bovine spongiform encephalopathy (BSE). The same holds for human proteins, which, moreover, are too expensive to use as worst-case product.

In the present thesis, the following products were investigated as possible worst-case product: four vegetable proteins, namely soy peptone, brown rice protein, pea protein isolate and whey protein isolate and one chemical substance, riboflavin. Afterwards, the cleaning validation of reactor 88 with the predetermined worst-case product was done. In this reactor, the production of the formulation of brolucizumab can occur.

3. Materials and Methods

3.1. Materials

3.1.1. Reagents

- ◊ Oxoid VGO300 Veggietones GMO-free soya peptone (Oxoid, Basingstoke, United Kingdom)
- ◊ Bulk Powders super pea pea protein isolate (Bulk Powders, Colchester, United Kingdom)
- ◊ Bulk Powders pure whey isolate™ 97 unflavored (Bulk Powders, Colchester, United Kingdom)
- ◊ Bulk Powders brown rice protein 80% unflavored (Bulk Powders, Colchester, United Kingdom)
- ◊ Riboflavin (Sigma-aldrich, St. Louis, United States of America)
- ◊ Sucrose (Obtained by Alcon)
- ◊ Demi water (Obtained by Alcon)
- ◊ Destil water (Obtained by Alcon)

3.1.2. Supplies

- ◊ Small & large low TOC swabs (small & large) (Texwipe, Kernersville, United States of America)
- ◊ BBL™ RODAC™ plates (BD, Franklin Lakes, USA)
- ◊ OPTIMAX™ 365 UV-A LED Inspection Flashlight (Spectronics corporation, Westburry, United States of America)
- ◊ Molybdenum test kit (Ironhaven BV, Hoofddorp, the Netherlands)

3.1.3. Equipment

- ◊ Brinox REA 88 (Brinox Process Systems, Medvode, Slovenia) (More information: see appendix 7.1 and 7.2)
- ◊ Total Organic Carbon Analyzer Sievers 900 Series (GE instruments, Boulder, USA)
- ◊ UV-1650PC spectrophotometer (Shimadzu, Kyoto, Japan)

3.2. Methods

3.2.1. Molybdenum test

The molybdenum test kit allows to quickly and easily determine whether stainless steel contains molybdenum or not. To compare the type of stainless steel between the reactor and the tubes used for the determination of the cleanability, the test was conducted on both materials as follows: after shaking the reagent, one or two drops were applied on the surface. A color change was observed if the investigated stainless steel contained molybdenum. This type of stainless steel was categorized as stainless steel 316. If no color change was observed, the stainless steel contained no molybdenum and was categorized as stainless steel 304. Afterwards, the surfaces were rinsed with water [39].

3.2.2. Determination of the cleanability

In order to determine a worst-case product for RTH 258, the cleanability of the five products (soy peptone, brown rice protein, pea protein isolate, whey protein isolate and riboflavin) was compared with RTH 258. Therefore, testing mixtures of the five products were prepared. Specifically, 12 g of the product was dissolved in 100 mL cooled distilled water. To allow a similar detection, 5 g riboflavin was added to the solutions of the proteins. Riboflavin has a yellow color and is fluorescent, therefore it is more accurate to determine if the stainless steel tube is visually clean. In addition, 3 mL of the commercial stock solution of RTH 258 (12% w/v) was mixed with 150 mg riboflavin to obtain the same riboflavin concentration as the other samples. In this way, solutions of the five products and RTH 258 were obtained with a concentration of 12% w/v.

For each of the five products, three spots of 300 µL were applied on the stainless steel tube at a uniform distance. After overnight drying of the spots, the test was performed: first, a flow of 150 ± 5 mL/min was set by collecting cold demineralized water in a graduated cylinder and determining the amount of water in the cylinder after 3 minutes. Next, a small tab was attached to the tube, to connect the tube and the water tap. As soon as the water hit the first spot, the chronometer was started. A visual control of the cleanliness was done at the following times:

- 0 to 30 minutes: every minute
- 30 to 60 minutes: every five minutes
- From 60 minutes: every fifteen minutes

An additional check of cleanliness was done using a UV light. Finally, the time was noted when visual cleanliness was reached. The longer the time, the harder to clean the product, indicating a better choice as worst-case product.

3.2.3. Determination of the solubility

The equilibrium solubility of the five products was determined by the shake-flask method. Three independent shake flask experiments were carried out for each product. A solution with a solid excess of the sample was made by adding the desired product to 100 mL of water in a flask. These flasks were placed on a stirring plate for 24 hours at room temperature. After centrifugation, three aliquots of supernatant were taken from the saturated solution and measured by UV/VIS spectrophotometry [40]. The proteins were measured at a wavelength of 280 nm, based on the absorbance of UV light by the aromatic amino acids, tryptophan and tyrosine, and by cysteine in protein solutions [41]. While for riboflavin, the wavelength giving maximal absorbance (λ_{max}) was first determined by measuring the whole absorption spectrum, after which, the riboflavin samples were measured at the found λ_{max}.

In order to determine the concentration of the saturated solutions, a calibration curve was first made by measuring samples with a known concentration of a certain product with UV/VIS spectrophotometry at the given wavelength (Table 3.1) [40]. A lower solubility indicates a better choice as worst-case product.

Table 3.1: Weighed amounts of product in 100 mL to obtain a calibration curve

	Soy peptone	Brown rice	Pea protein	Whey protein	Riboflavin
Calibration point 1 (mg)	251.4	1.0	1.2	500	1.0
Calibration point 2 (mg)	500.7	2.0	2.1	1,000	2.2
Calibration point 3 (mg)	749.2	3.2	3.3	1,504	3.1
Calibration point 4 (mg)	1,000	4.0	4.1	2,001	4.2
Calibration point 5 (mg)	1,256	5.0	5.0	2,498	5.1

3.2.4. Cleaning validation

The cleaning validation of reactor 88, which can eventually be used for the production of the RTH 258 formulation, was performed using soy peptone. The choice for this protein resulted from the fact that an ELISA kit specific for soy proteins was already available in-house at the start of the project. This ELISA kit can replace the unspecific TOC method to analyze the samples of remnants of soy. To prove a reproducible and effective cleaning, the manual cleaning was done in triplicate.

3.2.4.1. Risk assessment

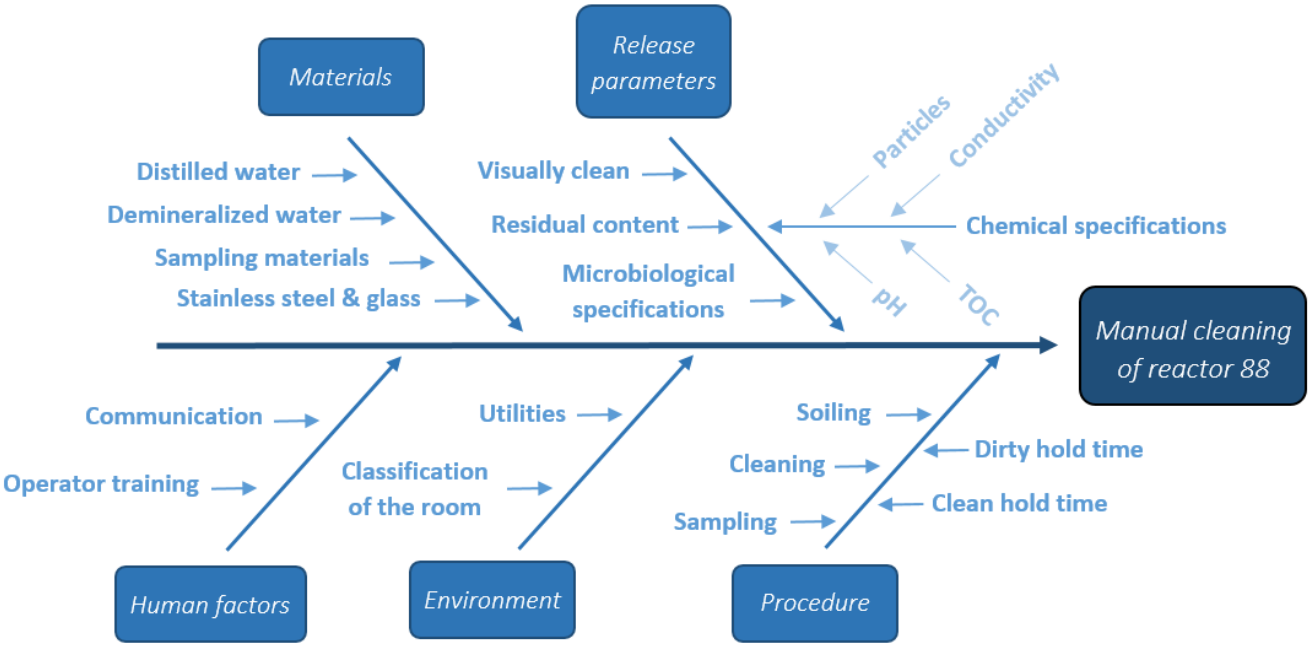


Fig. 3.1: Risk assessment of the manual cleaning of reactor 88

3.2.4.2. Determination of the recovery of swabbing

- **Calculation of the criterion**

In order to calculate the criterion for biologicals, PDE was used using the following formula from section 1.2.1.6. The equipment train consists of two Biological-reactors (2 x 10,000 cm²) and the Bulk Syringe Line (BSL) (5,000 cm²), making a surface of 25,000 cm². To obtain a more worst-case situation a surface of 30,000 cm² is used to calculate the criterion. This initial criterion, used for the recovery studies, was based on early research on RTH 258. However, after obtaining more information and change of reactor a recalculation of the criterion was done in section 3.2.4.4.

$$Criterion\ \left(\frac{mg}{mL}\right) = \frac{PDE * B * A\ swab}{L * Atot * V\ swab} * R\ item$$

$$Criterion\ \left(\frac{mg}{mL}\right) = \frac{0.003 * 2{,}000 * 100}{0.2 * 30{,}000 * 1} * 0.50 = 0.05$$

Table 3.2: Explanation of the formula to calculate the criteria for to perform the recovery test

Factor	Explanation of the factor	Amount
PDE	Permitted daily exposure (mg/day) of RTH 258	0.003
B	Minimal size batch of the next product (mL)	2,000
A_{swab}	Swabbed surface (cm²)	100
R_{item}	Fixed recovery during recovery studies to calculate the criterion	0.05
L	Maximum daily dose of the next product = RTH 258 (mg/day)	0.2
A_{tot}	The entire surface of the equipment train (cm²)	30,000
V_{swab}	Volume to dissolve the swab sample (mL)	1

Based on this criterion, a stock solution of 0.05 mg/mL of soy peptone was prepared for the recovery study.

- **Performing of the test**

To determine the recovery of soy peptone on stainless steel, five different samples were analyzed, all tested in triplicate, thus using three different plates of stainless steel type 316. A schematic representation of the four reference samples is given in Figure 3.2. A **positive control** was made by adding 1 mL of the stock solution to a TOC vial with 39 mL cooled distilled water. The **diluent control** consisted of the water used to prepare the samples. Therefore a TOC vial was filled with cooled distilled water. The **swab control** was prepared by adding the top of a swab to 40 mL of cooled distilled water in a TOC vial. The **coupon control** was made by swabbing a clean surface of the stainless steel plate, that will be used for the test sample. The swabbing technique was shown earlier in Figure 1.7. The top of the swab was cut and placed in a TOC vial filled with 40 mL cooled distilled water. To obtain the **test sample**, the plates of stainless steel were first cleaned and dried. Then, 1 mL of the stock solution (0.05 mg/mL) was spotted on a plate and a TOC vial was filled with 40 mL cooled distilled water. The swab was moistened by dipping the swab in the TOC tube. Finally, the plate was swabbed in a manner described by Figure 1.7 (section 1.2.1.5.), followed by cutting the top of the swab into the TOC vial.

After sonication of the samples for five minutes, TOC measurements were performed. Based on the obtained results, the recovery was calculated by the following formula:

$$Recovery = \frac{swab\ result - coupon\ control}{positive\ control - diluent\ control} * 100\%$$

To determine the recovery on glass, the same test was also performed on glass instead of on stainless steel.

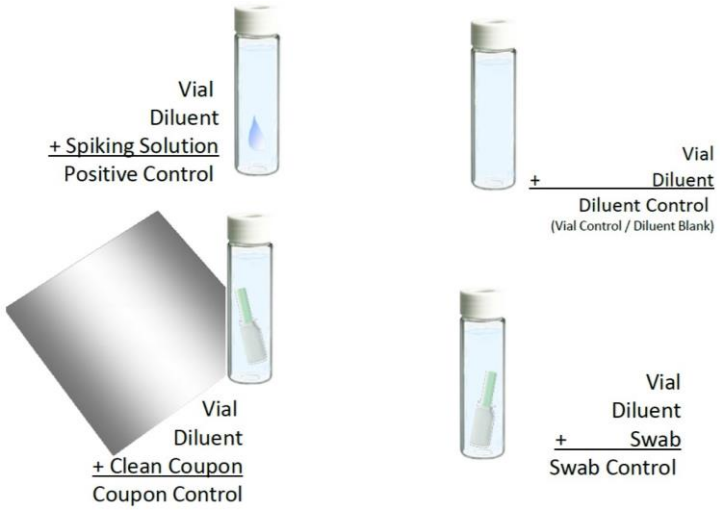


Fig. 3.2: Reference samples for the determination of the recovery of swabbing for TOC analysis[3]

3.2.4.3. Recalculation of the criteria for sampling

After change of reactor, used to perform the cleaning validation of RHT 258, and results from the recovery study, a new criterion for the cleaning validation of reactor 88 was calculated in two ways: 1) criterion based on 1/1000 limit and 2) criterion based on the PDE concept by the two formulae given in section 1.2.1.6. The entire surface of the equipment train consists of the reactor surface (36,925 cm^2) (calculation of the reactor surface is presented in Appendix 7.2.) and the BSL (5,000 cm^2). The most strict criterion will be applied during cleaning validation. Solutions with the corresponding concentrations were made for analysis by TOC in order to obtain the corresponding TOC values. No swab was added to the solutions representing the criteria for swab samples, in order to obtain a worst-case value. With these data it is possible to compare the TOC results of the test samples, obtained after cleaning, and the criterion to conclude if the cleaning was acceptable or not.

[3] Figure obtained by Alcon

- **Criteria for swab samples on stainless steel**

PDE concept

$$Criterion\ \left(\frac{mg}{mL}\right) = \frac{PDE\ * B * A\ swab *\ R\ item}{L * Atot * V\ swab}$$

$$Criterion\ \left(\frac{mg}{mL}\right) = \frac{0.003\,\frac{mg}{day} * 52{,}935{,}000\ mg * 100\ cm^2 * 0.682}{0.2\,\frac{mg}{day} * 41{,}495\ cm^2 * 40\ mL} = 32.63\ mg/mL$$

Table 3.3: Explanation of the formula to calculate the criteria for swab samples on stainless steel via the PDE concept

Factor	Explanation of the factor	Amount
PDE	Permitted daily exposure (mg/day) of RTH 258	0.003
B	Minimal size batch of the next product (mg)	52,935,000*
A_{swab}	Swabbed surface (cm²)	100
R_{item}	Recovery of soy peptone on stainless steel	0.682
L	Maximum daily dose of the next product (RTH 258) (mg/day)	0.2
A_{tot}	The entire surface of the equipment train (cm²)	41,495
V_{swab}	Volume to dissolve the swab sample (mL)	40

*corresponds with 50 L of the current formulation of RTH 258 with a density of 1.0587 g/mL:

$batch\ size\ (mg) = 50{,}000\ ml * 1.0587\,\frac{g}{ml} * 1000 = 52{,}935{,}000\ mg$

Table 3.4: Corresponding criteria based on the PDE concept for the sampling locations

Sampling location	Swabbed Surface (cm²)	Corresponding criterion (mg/mL)
Wall of the reactor	100	32.63
Bottom of the reactor	100	32.63
Bottom of the stirrer	122.7	40.03
Endings of the bottom piping	107.4	35.04
Connection 1 on the top of the vessel	75.39	24.60
Connection 2 on the top of the vessel	94.24	30.75
Connection 3 on the top of the vessel	94.24	30.75
Inside of the lid	100	32.75

1/1000 limit

$$Limit\ \left(\frac{mg}{mL}\right) = \frac{LTD * B * Aswab *\ SF * R\ item}{Atot * MTD *\ Vswab}$$

$$Limit\ \left(\frac{mg}{mL}\right) = \frac{0.1\,\frac{mg}{day} * 50{,}000\ mL * 100\ cm^2 *\ 0.001 * 0.682}{41{,}495\ cm^2 * 1.67\,\frac{mL}{day} * 40\ mL} = 1.230 * 10^{-4}\ mg/mL$$

Table 3.5: Explanation of the formula to calculate the criteria for swab samples on stainless steel via the 1/1000 limit

Factor	Explanation of the factor	Amount
LTD	Lowest therapeutic dose of RTH 258 (mg/day)	0.1
B	Minimal size batch of the next product (mL)	50,000
A_{swab}	Swabbed surface (cm²)	100
SF	Safety factor	0.001
R_{item}	Recovery of soy peptone on stainless steel	0.682
A_{tot}	The entire surface of the equipment train (cm²)	41,495
MTD	Maximum therapeutic dose of RTH 258 (mL/day)	1.67
V_{swab}	Volume to dissolve the swab sample (mL)	40

Table 3.6: Corresponding criteria based on the 1/1000 limit for sampling locations

Sampling location	Swabbed surface (cm²)	Corresponding criterion (mg/mL)
Wall of the reactor	100	$1.230 * 10^{-4}$
Bottom of the reactor	100	$1.230 * 10^{-4}$
Bottom of the stirrer	122.7	$1.509 * 10^{-4}$
Endings of the bottom piping	107.4	$1.292 * 10^{-4}$
Connection 1 on the top of the vessel	75.39	$0.9273 * 10^{-4}$
Connection 2 on the top of the vessel	94.24	$1.159 * 10^{-4}$
Connection 3 on the top of the vessel	94.24	$1.159 * 10^{-4}$
Inside of the lid	100	$1.230 * 10^{-4}$

- **Criteria for swab samples on glass**

PDE concept

$$Criterion\ \left(\frac{mg}{mL}\right) = \frac{PDE\ *B*A\ swab*\ R\ item}{L*Atot*V\ swab}$$

$$Criterion\ \left(\frac{mg}{mL}\right) = \frac{0.003\frac{mg}{day}*52{,}935{,}000\ mg*38.46\ cm^2*0.7584}{0.2\frac{mg}{day}*41{,}495\ cm^2*40\ mL} = 13.95\ mg/mL$$

Table 3.7: Explanation of the formula to calculate the criteria for swab samples on glass via the PDE concept

Factor	Explanation of the factor	Amount
PDE	Permitted daily exposure (mg/day) of RTH 258	0.003
B	Minimal size batch of the next product (mg)	52,935,000*
A_{swab}	Swabbed surface = surface of the window of reactor 88 (cm²)	38.46
R_{item}	Recovery of soy peptone on glass	0.7584
L	Maximum daily dose of the next product (RTH 258) (mg/day)	0.2
A_{tot}	The entire surface of the equipment train (cm²)	41,495
V_{swab}	Volume to dissolve the swab sample (mL)	40

*corresponds with 50 L of the current formulation of RTH 258 with a density of 1.0587 g/mL:

$batch\ size\ (mg) = 50{,}000\ ml * 1.0587\frac{g}{ml} * 1000 = 52{,}935{,}000\ mg$

1/1000 limit

$$Limit\ \left(\frac{mg}{mL}\right) = \frac{LTD * B * Aswab * SF * R\ item}{Atot * MTD * Vswab}$$

$$Limit\ \left(\frac{mg}{mL}\right) = \frac{0.1\frac{mg}{day} * 50{,}000\ mL * 38.46\ cm^2 * 0.001 * 0.7584}{41{,}495\ cm^2 * 1.67\frac{mL}{day} * 40\ mL} = 5.261 * 10^{-5}\ mg/mL$$

Table 3.8: Explanation of the formula to calculate the criteria for swab samples on glass via the 1/1000 limit

Factor	Explanation of the factor	Amount
LTD	Lowest therapeutic dose of RTH 258 (mg/day)	0.1
B	Minimal size batch of the next product (mL)	50,000
A_{swab}	Swabbed surface = surface of the window of reactor 88 (cm²)	38.46
SF	Safety factor	0.001
R_{item}	Recovery of soy peptone on glass	0.7584
A_{tot}	The entire surface of the equipment train (cm²)	41,495
MTD	Maximum therapeutic dose of RTH 258 (mL/day)	1.67
V_{swab}	Volume to dissolve the swab sample (mL)	40

- **Criteria for rinse samples[4]**

PDE concept

$$Criterion\ \left(\frac{mg}{mL}\right) = \frac{PDE * B * Arinse\ sample}{V * L * Atot}$$

$$Criterion\ \left(\frac{mg}{mL}\right) = \frac{0.003\frac{mg}{day} * 52{,}935{,}000\ mg * 36{,}925\ cm^2}{5{,}000\ mL * \ 0.2\frac{mg}{day} * 41{,}495\ cm^2} = 141.3\ mg/mL$$

Table 3.9: Explanation of the formula to calculate the criteria for rinse samples via the PDE concept

Factor	Explanation of the factor	Amount
PDE	Permitted daily exposure (mg/day) of RTH 258	0.003
B	Minimal size batch of the next product (mg)	52,935,000*
$A_{rinse\ sample}$	Rinsed surface = total surface of reactor 88 (cm²)	36,925
V	Volume of rinsate (mL)	5,000
L	Maximum daily dose of the next product (RTH 258) (mg/day)	0.2
A_{tot}	The entire surface of the equipment train (cm²)	41,495

*corresponds with 50 L of the current formulation of RTH 258 with a density of 1.0587 g/mL:

$batch\ size\ (mg) = 50,000\ ml * 1.0587\frac{g}{ml} * 1000 = 52{,}935{,}000\ mg$

[4] Recovery studies are not required for rinse samples

1/1000 limit

$$Limit\left(\frac{mg}{mL}\right) = \frac{LTD * B * SF * Arinse\ sample}{Atot * MTD * V}$$

$$Limit\left(\frac{mg}{mL}\right) = \frac{0.1\frac{mg}{day} * 50{,}000\ mL * 0.001 * 36{,}925\ cm^2}{41{,}495\ cm^2 * 1.67\frac{mL}{day} * 5{,}000\ mL} = 5.329 * 10^{-4}\ mg/mL$$

Table 3.10: Explanation of the formula to calculate the criteria for rinse samples via the 1/1000 limit

Factor	Explanation of the factor	Amount
LTD	Lowest therapeutic dose of RTH 258 (mg/day)	0.1
B	Minimal size batch of the next product (mL)	50,000
SF	Safety factor	0.001
$A_{rinse\ sample}$	Rinsed surface = total surface of reactor 88 (cm^2)	36,925
A_{tot}	The entire surface of the equipment train (cm^2)	41,495
MTD	Maximum therapeutic dose of RTH 258 (mL/day)	1.67
V_{swab}	Volume to dissolve the swab sample (mL)	40

3.2.4.4. Determination of the worst-case places of the reactor

The sampling plan is presented in Appendix 7.3. A list of the sampling places is given in Table 3.11.

Table 3.11: Worst-case places of the reactor where samples will be taken

Number	Sampling location	Statement
1	Wall of the reactor	Representative area for the reactor
2	Bottom of the reactor	Representative area for the reactor
3	Bottom of the stirrer	Difficult to clean
4	Endings of the bottom piping	Difficult to clean
5	Window	Other kind of material (glass)
6	Connection 1 on the top of the vessel	Difficult to reach
7	Connection 2 on the top of the vessel	Difficult to reach
8	Connection 3 on the top of the vessel	Difficult to reach
9	Inside of the lid	Risk of contamination

3.2.4.5. Schematic overview of a cleaning cycle

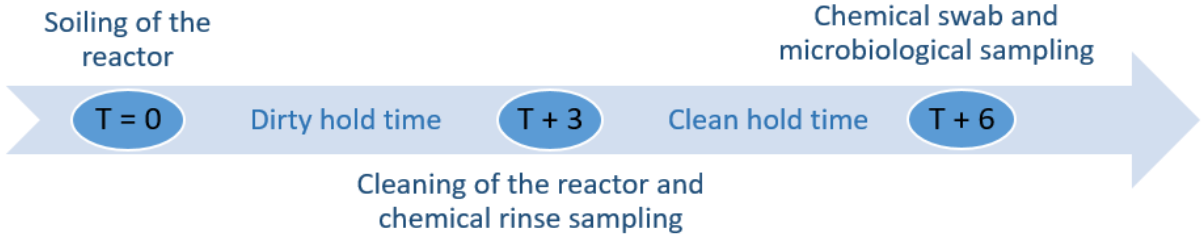


Fig. 3.3: Schematic overview of a cleaning cycle expressed in number of days

3.2.4.6. Soiling of the reactor

The content of soy peptone in the soiling batch was chosen such that the concentration of soy should be higher than the concentration of RTH 258 (12%) making it a worst-case batch. Moreover sucrose was added to the batch as viscosity enhancer to make it more worst-case. A concentration of 10% is chosen, because the currently investigated formulation of RTH 258 contains 10% of sucrose. Thus, a soiling batch of 15% soy peptone and 10% sucrose was made by adding 300 g soy peptone and 200 g sucrose to a total volume of 2 L cooled distilled water. Finally, the solution was transferred to a non-transparent bottle to protect the protein-solution from light and denaturation. Moreover, the solution was stored in the refrigerator. No riboflavin was added as visual tracer because it would interfere the ELISA analysis. The reactor was soiled by *painting* it with a brush after dipping the brush into the solution. Every area, where a sample would be taken after cleaning, was covered with the solution. Finally, a dirty hold time of minimum 3 days was taken into account.

3.2.4.7. Cleaning of the reactor

- **Procedure**

In advance of the start of the first cleaning a cleaning-procedure was drawn up. When the results were out of specifications and corrective actions were needed, a revision of the cleaning procedure was written. A plan of the reactor is shown in Appendix 7.3.

- Preparation:

After moving reactor 88 to the cleanroom, two tubes were connected to a water tap: the first one was connected to demineralized water, the second one to distilled water. A third tube was connected to the drain point of tap 38 and the sewerage. Finally, bottom tap 14 was opened.

Before cleaning, the lid of the reactor was opened to perform a visual control. Irregularities were reported to the supervisor.

- Cleaning of the connection to tap 38:

To clean the connection to tap 38, tap 37 was closed and tap 38 was opened. The tube connected to demineralized water was connected to drain point of tap 37. It was rinsed with demineralized water for 55 seconds. Next, the same cycle was performed with distilled water by connecting the tube attached to distilled water to drain point of tap 37. In this way the bottom line was cleaned underneath.

- Cleaning of the inside of the reactor:

Firstly, a flow of 0,390 L/s was set via a flow meter. This flow was chosen, because it is worst-case to the flow used in routine cleaning. On this way cleaning is executed under worst-case circumstances. Next, the lid of the reactor was opened and the seal of the lid was removed and cleaned for 55 seconds with demineralized water and afterwards for 55 seconds with distilled water.

The lid, the mixing baffle and the inside of the reactor were then cleaned four times with demineralized water for 55 seconds. Between each rinse, one had to wait until the reactor was empty. Afterwards, a visual control of cleanliness was done. If the reactor was not clean, an extra cleaning cycle was performed. Additionally, the lid, the mixing baffle and the inside of the reactor were cleaned two times with distilled water for 55 seconds. Between each rinse, one had to wait until the reactor was empty.

Lastly, the cleaned seal was attached to the lid of the reactor before the lid and bottom tap 14 were closed.

- Cleaning of bottom tap 14:

The drain point connected to bottom tap 14 and bottom tap 14 itself were opened. The tap was cleaned for 55 seconds with demineralized water and 55 seconds with distilled water. The reactor was not dried to obtain a worst-case scenario for microbiological growth during the clean hold time of 3 days.

- **Revision 1**

After receiving the results of the first cleaning run, the following changes to the procedure were implemented in order to obtain better cleaning results. Firstly, the connection to tap 38 was cleaned at the end of the cleaning run. Secondly, an extra cleaning cycle was introduced: the attachments on the top of the reactor for automatic cleaning were cleaned with demineralized water for 15 seconds followed by cleaning with distilled water for 15 seconds.

3.2.4.8. Chemical and microbiological sampling

- **Chemical sampling**

Before sampling, visual inspection of the cleanliness of the reactor was performed by two certified persons. Extra attention was given to the critical sites of the reactor. After cleaning a rinse sample was taken, whereas swab samples and microbiological samples were taken after three days of clean hold time.

1. Rinse sample

A rinse sample was taken by rinsing the lid and the inside of the reactor with approximately 5 L of distilled water. The sample was collected in a clean, sterile glass bottle at tap 38. Then 40 mL was taken and analyzed by TOC to evaluate the water quality. If the result was lower than 500 ppb, a European standard for the quality of water [25], indicating an effective cleaning, the remaining sample was divided in three clean, sterile glass bottles. These bottles were given at the appropriate laboratories for the analysis of pH and conductivity and compared to their specifications recorded by the "European Pharmacopeia 9.0 – Water for injections" [42]. Furthermore, the amount of particles was analyzed and compared to their specifications recorded by the "USP – Particulate matter in ophthalmic solutions" [43]. The specifications for the acceptable amount of particles for the cleaning validation of RTH 258 are more strict, namely 50% of the amount of particles recorded by the USP for ophthalmic solutions, to assure an acceptable amount of particles in the final product. A summary of the specifications is given in Table 3.12.

Afterwards, the content of the rinse sample was determined by TOC. In order to decide whether the content of the rinse sample was acceptable, the result of the rinse sample obtained with TOC was compared with the TOC result of a sample containing a concentration equal to the calculated criterion in section 3.2.4.3.

Table 3.12: Summary of the specifications of the chemical analysis of the rinse sample

Chemical analysis	TOC water	Particle analysis			pH	Conductivity
Specification	500 ppb	> 10 µm	> 25 µm	> 50 µm	5 -7	< 1.3 µS/cm
		25/mL*	2/mL*	1/mL*		

*50% of the amount of particles recorded by the USP for ophthalmic solutions

2. Swab sample

After a minimum of three days of clean hold time, swab samples were taken at the predetermined places, in the same way as explained in section 3.2.3.1. These samples were analyzed by TOC.

In order to determine whether the content of the swab sample was acceptable, the result of each swab sample obtained with TOC was compared with the TOC result of a sample containing a concentration equal to the calculated criterion presented in section 1.2.1.6.

- **Microbiological sampling**

After three days of clean hold time, but preceding the swab sampling, a microbiological rinse sample was taken. In this way, the rinse sample was not affected by the swabs. Rinse samples were taken by rinsing the lid and the inside of the reactor with approximately 800 mL of distilled water and collecting the fluid in a clean, sterile glass bottle with a 0.9% w/v saline solution. After analysis the results were compared with its specification (100 CFU/100mL), which is 100 times smaller than the specification for water described in the European Pharmacopeia (100 CFU/mL) to control any microbiological contamination.

After taking microbiological rinse samples and swab samples, additional contact surface samples were taken. Therefore the bottom of the stirrer, the wall, the bottom and the lid of the reactor were sampled using RODAC plates, which were then incubated to determine the total colony forming units (CFU). After analysis, the results were compared with the specifications managed by Alcon for class C units. A summary of the specifications is given in Table 3.13.

Table 3.13: Summary of the specifications of the microbiological analysis

Microbiological analysis	Specification
Rinse sample	< 100 CFU/100mL
RODAC plate	< 1 CFU/cm²

4. Results and discussion

4.1. Molybdenum test

The molybdenum test was carried out on the reactor and the tubes used for the determination of the cleanability. Both tests resulted in a red color, meaning that the reactor as well as the tubes contain molybdenum and thus consist of stainless steel 316. Therefore, it can be concluded that the results of the cleanability are representative for the reactor.

4.2. Determination of the cleanability

4.2.1. RTH 258

After five minutes, every spot was rinsed away. When a UV lamp was used, however, some fluorescent residues were seen for 25 minutes. As with the other samples, the third part of the tube remained dirty for a longer period than the other parts, because water was already soiled when it had reached the last part. It seems that RTH 258 prevents adhesion of riboflavin to the tube, because it was harder to clean riboflavin alone. It can be concluded that RTH 258 was the most easy product to clean, when the results were compared to those from the other products. A time lapse image of 'RTH 258/riboflavin' is given in Appendix 7.4.1.

Table 4.1: Flow before and after the experiment and time for complete removal of RTH 258

	Flow before (mL/min)	Flow after (mL/min)	Time for complete removal (min)
1	154.7	152.3	25
2	152.3	152.6	25
3	152.6	152.3	25

4.2.2. Soy peptone

Before the start of the test, a separation between soy and riboflavin was noticed at the spots on the stainless steel tube, shown in Appendix 7.4.2. The same observation was made with the stock solution of soy, shown in Figure 4.1. Therefore it can be concluded that soy and riboflavin possibly are immiscible. Within the first two minutes after the test was started, all the spots were already disappeared, however, when a UV lamp was used, some residues were still visible. These residues probably derive from riboflavin, because the mixture 'soy peptone/riboflavin' has a similar time of complete removal as riboflavin alone. It is a little bit shorter than riboflavin, probably due to a smaller content of riboflavin (5%) in the mixture 'soy peptone/riboflavin' than riboflavin alone (12%). It is

assumed that soy peptone was already cleaned after approximately five minutes in the absence of riboflavin, which could be tested in the future. Due to the immiscibility of soy and riboflavin no conclusive statements can be made of the cleanability results of soy, but probably soy peptone on its own has a less or similar cleanability than RTH 258, indicating that soy probably will not be applicable as worst-case product. However, extra tests are necessary to conclude if soy could be used as worst-case product for RTH 258 or not.

Fig. 4.1.: Stock solution of soy before, directly after and one hour after adding riboflavin

Table 4.2: Flow before and after the experiment and time for complete removal for soy peptone

	Flow before (mL/min)	Flow after (mL/min)	Time for complete removal (min)
1	145.7	146.3	90
2	148.0	145.8	60
3	150.3	149.2	60

4.2.3. Brown rice protein

Before the start chapped, hard spots were observed, as shown in Appendix 7.4.3. When the spots were observed in detail, one can see that brown rice protein was partially separated from riboflavin. However, the precipitated stock solution of the mixture 'brown rice/riboflavin' did not show any separation of the two substances, as was observed with soy. One hour of rinsing resulted in the disappearance of the yellow spots, however fluorescent residues were seen for two hours and 25 minutes. Brown rice probably inhibits the cleaning of riboflavin since fluorescent residues were seen for a longer time than when riboflavin alone was applied. The third part of the tube remained dirty for a longer time than the other parts, because water was already soiled when it had reached the last part of the tube. Eventually, white spots remained on the tube until the end, as shown in Figure 4.2. Brown rice protein probably has a strong adhesion strength on stainless steel, therefore one can conclude that it is hard to clean brown rice by these conditions. Perhaps by other conditions, such as the addition of a detergent or a faster flow, cleaning would be easier. When comparing the results with those of

RTH 258, it can be concluded that brown rice protein is a potential worst-case product candidate, because it is harder to clean than RTH 258 at these conditions.

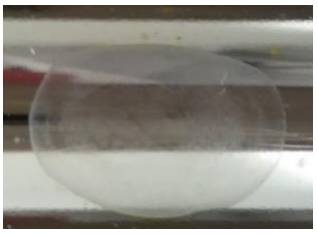


Fig. 4.2.: White spot after cleaning

Table 4.3: Flow before and after the experiment and time for complete removal of brown rice

	Flow before (mL/min)	Flow after (mL/min)	Time for complete removal (h)
1	145.5	145.7	> 6
2	146.6	150.8	> 6
3	155.9	151.2	> 6

4.2.4. Pea protein isolate

The stock solution of pea protein isolate was more viscous than the other solutions, therefore the small spots were not spread on the tube. Overnight drying resulted in porous and crumbling spots. After five minutes of rinsing, the contour of the spots was still visible. Even after thirty minutes, some residues of the spots were still noticeable. The third part of the tube remained dirty for a longer time than the other parts, because water was already soiled when it reached the last part of the tube. After one hour, residues were still detectable with a UV light and white spots remained on the tube until the end, as was seen earlier with brown rice protein. Thus, similar results were obtained for pea protein isolate and brown rice protein, concluding that pea protein isolate is also a potential worst-case product candidate. A time lapse image of 'pea protein isolate/riboflavin' is given in Appendix 7.4.4.

Table 4.4: Flow before and after the experiment and time for complete removal for pea protein

	Flow before (mL/min)	Flow after (mL/min)	Time for complete removal (h)
1	153.3	148.1	> 6
2	151.7	149.4	> 6
3	151.5	153.2	> 6

4.2.5. Whey protein isolate

The stock solution of whey protein isolate was, just like soy, not precipitated. After five minutes all spots were disappeared, however, when a UV lamp was used, some residues were still visible for one hour, as shown in the time lapse image of 'whey protein isolate/riboflavin' given in Appendix 7.4.5. These residues probably derive from riboflavin, because it has similar results as riboflavin alone. However this statement cannot be certified. Some interactions between the two substances could have occurred. The time for complete removal is a little bit shorter than riboflavin alone, probably due to a smaller content of riboflavin (5%) in the mixture 'whey protein isolate/riboflavin' than riboflavin alone (12%). Thus, one cannot conclude with certainty that this result was only determined by riboflavin. Therefore only conclusions can be taken about the mixture: 'whey protein isolate/riboflavin' is harder to clean than 'RTH 258/riboflavin' and could be used as worst-case product by these conditions. As with the other samples, the third part of the tube remained dirty for a longer time than the other parts, because water was already soiled when it reached the last part of the tube.

Table 4.5: Flow before and after the experiment and time for complete removal of whey protein

	Flow before (mL/min)	Flow after (mL/min)	Time for complete removal (min)
1	154.2	153.4	60
2	145.0	151.7	75
3	147.2	153.1	60

4.2.6. Riboflavin

After five minutes all the spots were disappeared, however, when a UV lamp was used, some residues were still visible for one hour. As with the other samples, the third part of the tube remained dirty for a longer time than the other parts, because water was already soiled when it had reached the last part of the tube. When the results are compared with those of RTH 258, it can be concluded that it is harder to clean riboflavin indicating that riboflavin is a potential worst-case product candidate. A time lapse image of riboflavin is given in Appendix 7.4.6.

Table 4.6: Flow before and after the experiment and time for complete removal of riboflavin

	Flow before (mL/min)	Flow after (mL/min)	Time for complete removal (min)
1	153.0	154.0	90
2	151.2	153.9	90
3	151.2	153.4	90

4.2.7. Overall conclusion cleanability

An overall scheme of the cleanability of the six mixtures under the investigated conditions is given in Figure 4.3. Up to now, potential candidates for the worst-case product of RTH 258 are riboflavin, 'whey protein isolate/riboflavin', 'pea protein isolate/riboflavin' and 'brown rice protein/riboflavin'.

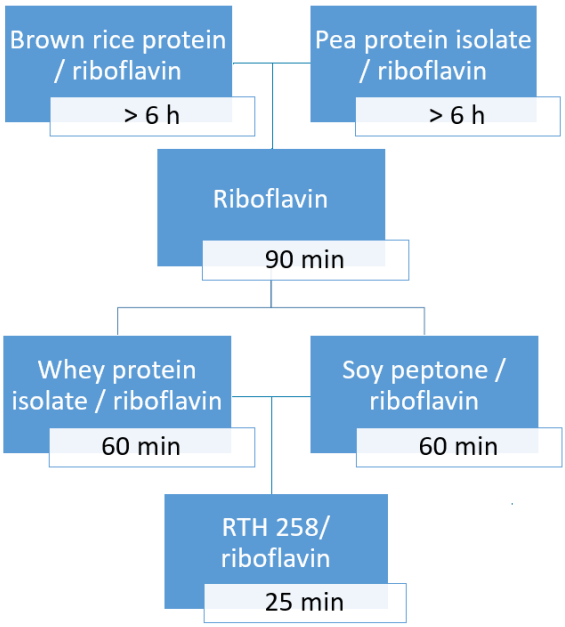


Fig. 4.3: Scheme of the cleanability results of the six products at the investigated conditions

4.3. Determination of the solubility

4.3.1. Soy peptone

To obtain a calibration curve, samples with a predetermined concentration were measured by UV/VIS spectrophotometry. The results and the calibration curve are represented in Appendix 7.5.1. The unknown concentration of the samples with an excess of soy can be calculated by the Lambert-Beer law:

$$E = \varepsilon * c * l$$

- E: Extinction
- ε : Molar extinction coefficient (L/(mol*cm))
- c : Concentration (mol/L)
- l : Path length through the sample (cm)

It can be concluded that soy has a solubility of approximately **6.580 g/100 mL** in water at room temperature, presented in Table 4.7. Thus, soy has a lower solubility in water than RTH 258 (30 g/100mL) which indicates that soy peptone can be used as worst-case product for RTH 258 in terms of solubility in water.

Table 4.7: Calculation of the concentration of the samples with an excess of soy

Sample	Absorbance	1/100 diluted concentration (mg/100mL)	Concentration (g/100mL)	Mean concentration (g/100mL) ± stdev	Total mean concentration (g/100mL) ± stdev
Excess 1	0.541	64.9	6.493	6.509 ± 0.03125	**6.580 ± 0.1**
	0.540	64.8	6.481		
	0.546	65.5	6.552		
Excess 2	0.537	64.5	6.445	6.521 ± 0.05353	
	0.546	65.5	6.552		
	0.547	65.6	6.564		
Excess 3	0.555	66.6	6.660	6.711 ± 0.03923	
	0.560	67.2	6.719		
	0.563	67.5	6.755		

4.3.2. Brown rice protein

A calibration curve was made by measuring samples with a predetermined concentration by UV/VIS spectrophotometry. The results and the calibration curve are represented in Appendix 7.5.2. The unknown concentration of the samples with an excess of brown rice protein are calculated by the Lambert-Beer law. The results are presented in Table 4.8, concluding that brown rice protein has a

solubility of **6.774 mg/100 mL** in water at room temperature. Brown rice has thus a lower solubility in water than RTH 258 (30 g/100mL) indicating that brown rice protein could be a good worst-case product for RTH 258 while cleaning is performed using water.

Table 4.8: Calculation of the concentration of the samples with an excess of brown rice

Sample	Absorbance	Concentration (mg/100mL)	Mean concentration (mg/100mL) ± stdev	Total mean concentration (mg/100mL) ± stdev
Excess 1	0.043	7.945	7.764 ± 0.2571	**6.774 ± 0.7576**
	0.043	7.945		
	0.040	7.400		
Excess 2	0.034	6.309	6.248 ± 0.3736	
	0.036	6.673		
	0.031	5.764		
Excess 3	0.035	6.491	6.309 ± 0.1485	
	0.033	6.127		
	0.034	6.309		

4.3.3. Pea protein isolate

A calibration curve, made in the same way as for the other products, together with the results is represented in Appendix 7.5.3. The unknown concentration of the samples with an excess of pea protein was calculated. The results are presented in Table 4.9, indicating that pea protein isolate has a solubility of **183.3 mg/100 mL** in water at room temperature. Thus, pea protein has a lower solubility in water than RTH 258 (30 g/100mL) indicating that pea protein isolate could be a good worst-case product for RTH 258 in terms of solubility in water.

Table 4.9: Calculation of the concentration of the samples with an excess of pea protein

Samples	Absorbance	Calculated 1/10 diluted concentration (mg/100mL)	Calculated concentration (mg/100mL)	Mean concentration (mg/100mL) ± stdev	Total mean concentration (mg/100mL) ± stdev
Excess 1	0.221	11.39	113.9	116.5 ± 1.910	**183.3 ± 48.39**
	0.228	11.73	117.3		
	0.230	11.83	118.3		
Excess 2	0.456	23.02	230.2	229.5 ± 0.6174	
	0.455	22.97	229.7		
	0.453	22.87	228.7		
Excess 3	0.401	20.30	203.0	204.0 ± 0.8084	
	0.403	20.40	204.0		
	0.405	20.50	205.0		

4.3.4. Whey protein isolate

A calibration curve was made in the same way as the other products. The calibration curve and results are represented in Appendix 7.5.4. The unknown concentration of the samples with an excess of whey protein was calculated. The results are presented in Table 4.10, concluding that whey protein isolate has a solubility of **5.559 g/100 mL** in water at room temperature. Thus, whey protein has a lower solubility in water than RTH 258 (30 g/100mL) indicating that whey protein isolate can be used as worst-case product for RTH 258 in terms of solubility in water.

Table 4.10: Calculation of the concentration of the samples with an excess of whey protein

Samples	Absorbance	Calculated 1/100 diluted concentration (g/100mL)	Calculated concentration (g/100mL)	Mean concentration (g/100mL) ± stdev	Total mean concentration (g/100mL) ± stdev
Excess 1	0.586	0.057	5.688	5.622 ± 0.06169	**5.559 ± 0.2960**
	0.581	0.056	5.639		
	0.571	0.055	5.540		
Excess 2	0.559	0.054	5.421	5.503 ± 1.057	
	0.568	0.055	5.510		
	0.575	0.056	5.579		
Excess 3	0.570	0.055	5.530	5.553 ± 0.02032	
	0.572	0.055	5.550		
	0.575	0.056	5.579		

4.3.5. Riboflavin

First the wavelength giving maximal absorbance (λ_{max}) was determined by measuring the whole absorption spectrum of riboflavin, given in Appendix 7.5.5. The highest absorbance was found at λ = 223 nm. Thus, first the samples for obtaining the calibration curve were measured at this wavelength, presented in Appendix 7.5.5. Next, the samples with an excess of riboflavin were measured at the same wavelength, giving the results shown in Table 4.11. It can be concluded that riboflavin has a solubility **14.25 mg/100 mL** at room temperature. Thus, riboflavin has a lower solubility in water than RTH 258 (30 g/100mL) indicating that riboflavin could be a good worst-case product for RTH 258 in terms of solubility in water.

Table 4.11: Calculation of the concentration of the samples with an excess of riboflavin

Samples	Absorbance	Calculated 1/10 diluted concentration (mg/100mL)	Calculated concentration (mg/100mL)	Mean concentration (mg/100mL) ± stdev	Total mean concentration (mg/100mL) ± stdev
Excess 1	0.896	1.380	13.80	13.86 ± 0.1131	**14.25 ± 14.25**
	0.910	1.402	14.02		
	0.894	1.377	13.77		
Excess 2	0.950	1.466	14.66	14.48 ± 0.1300	
	0.935	1.442	14.42		
	0.931	1.435	14.35		
Excess 3	0.934	1.440	14.40	14.40 ± 0.02596	
	0.936	1.443	14.43		
	0.932	1.437	14.37		

4.3.6. Overall conclusion solubility

Using UV/VIS spectrophotometry, the investigated products were ranked from low to high solubility in water. The less soluble, the more worst-case. Thus brown rice protein is the most worst-case product in terms of solubility in water.

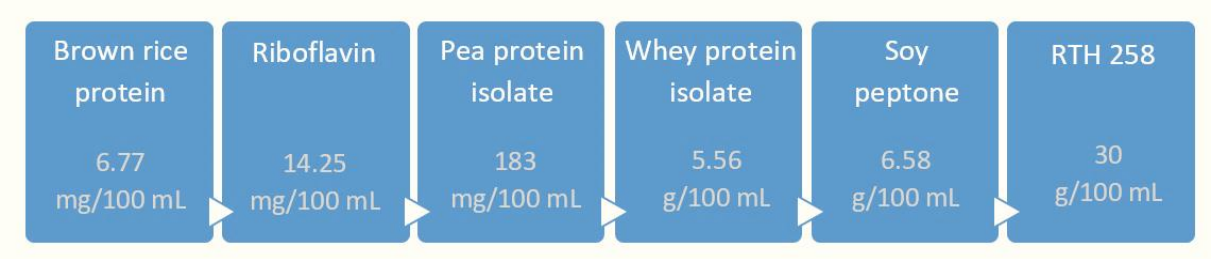


Fig. 4.4: Scheme of the results of the solubility in water at room temperature of the six products ranked from low to high solubility in water

4.4. Cleaning validation

4.4.1. Determination of the recovery of swabbing on stainless steel and glass

- **Stainless steel**

A stock solution of soy peptone (0.05 mg/mL) was spotted on three different stainless steel plates with a surface area of 100 cm². Next, the recovery experiment was performed as was told in section 3.2.4.2. The results of the experiment are presented in Table 4.12. The recovery was calculated by formula given in section 3.2.4.2. All values are situated above 50% and do not differ more than 5% of the average. Therefore, it can be concluded that the average recovery of soy peptone on stainless steel equals 68.20%.

The obtained recovery is lower than the recovery most of the companies ask (75%), however it was acceptable for Alcon, because research was still ongoing to determine if soy peptone could be used as worst-case product for RTH 258. Therefore this swab method and TOC analysis are accepted to use for the cleaning validation of soy peptone. However, in the future, when soy peptone would be elected as worst-case product, extra recovery studies can be performed to obtain a higher recovery, meaning that the cleaning validation must be performed again.

Table 4.12: Calculation of the recovery of soy peptone on stainless steel

Sample	Result (ppb)			Mean result (ppb)	Recovery (%)
Positive control	574	584	585	581	-
Diluent control	63.2	66.3	63.6	64.37	-
Swab control	89.8	96.2	96.9	94.3	-
Coupon control 1	160	167	168	165	-
Coupon control 2	162	168	165	165	-
Coupon control 3	158	165	172	165	-
Test 1	508	506	511	508.3	66.46
Test 2	528	528	528	528.0	70.26
Test 3	507	521	519	515.7	67.88
Mean recovery (%)					**68.20**

- **Glass**

A stock solution of soy (0.05 mg/mL) was spotted on three different glass plates with a surface area of 100 cm². The results of the recovery experiment (section 3.2.4.2) are presented in Table 4.13. The recovery was calculated by the formula given in section 3.2.4.2. All values are situated above 50% and do not differ more than 5% of the average. Therefore, it can be concluded that the average recovery of soy peptone on glass equals 75.84%. This value is acceptable, meaning that the swab method and analysis by TOC are suitable for the cleaning validation of soy peptone.

Table 4.13: Calculation of the recovery of soy peptone on glass

Sample	Result (ppb)			Mean result (ppb)	Recovery (%)
Positive control	495	495	495	495.00	-
Diluent control	67.5	67.6	67.5	67.53	-
Swab control	120	121	121	120.67	-
Coupon control 1	232	232	232	232.00	-
Coupon control 2	261	268	261	263.62	-
Coupon control 3	261	258	258	259.00	-
Test 1	566	567	567	566.67	78.29
Test 2	587	590	576	584.33	75.02
Test 3	572	581	576	576.33	74.21
Mean recovery (%)					**75.84**

4.4.2. Recalculation of the criteria for sampling

- **Criterion for swab samples on stainless steel**

PDE concept

Due to the brown color of the solution, as shown in Figure 4.5, and to avoid damage to the TOC apparatus, the samples were not measured by TOC. Visual inspection can be done in order to compare the color of the samples taken during cleaning validation with the corresponding criterion. Nevertheless, the color of the samples is doubtful: if a rinsing sample would have this color, one would in no case conclude that the reactor is clean. Furthermore since the 1/1000 criterion is more strict, the 1/1000 criterion will be used during cleaning validation.

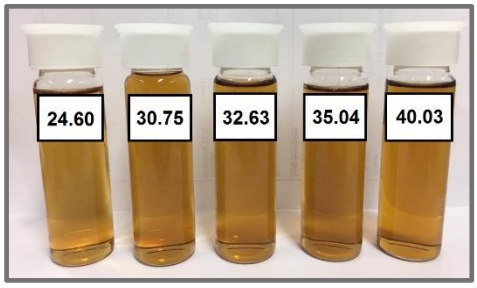


Fig. 4.5: Solutions with a concentration (mg/mL) of the criterion

1/1000 limit

A low corresponding TOC result was measured for every sample, due to the low criterion based on the 1/1000 limit. These values are more strict than the specification of water after cleaning (500 ppb). Therefore a specific analysis method, such as ELISA, is recommended, because it will be hard to obtain lower results, since water and the swab itself will also contribute to the obtained value.

Table 4.14: TOC results based on the 1/1000 limit for every sampling location

Sampling location	Surface (cm^2)	Corresponding criterion (mg/mL)	Corresponding TOC result (ppb)
Wall of the reactor	100	$1.230 * 10^{-4}$	205
Bottom of the reactor	100	$1.230 * 10^{-4}$	205
Bottom of the stirrer	122.7	$1.509 * 10^{-4}$	173
Endings of the bottom piping	107.4	$1.292 * 10^{-4}$	187
Connection 1 on the top of the vessel	75.39	$0.9273 * 10^{-4}$	224
Connection 2 on the top of the vessel	94.24	$1.159 * 10^{-4}$	184
Connection 3 on the top of the vessel	94.24	$1.159 * 10^{-4}$	184
Inside of the lid	100	$1.230 * 10^{-4}$	205

- **Criterion for swab samples on glass**

Table 4.15: TOC result of the criterion for the swab sample on glass for the window of the reactor

	Concentration (mg/mL)	Corresponding TOC result (ppb)
PDE	13.95	Brown color - not measured
1/1000 limit	$5.261 * 10^{-5}$	180

- **Criterion for rinse samples**

Table 4.16: TOC results of the criterion for rinse samples

	Concentration (mg/mL)	Corresponding TOC result (ppb)
PDE	141.3	Brown color - not measured
1/1000 limit	$5.329 * 10^{-4}$	385

4.4.3. Chemical and microbiological sampling

4.4.3.1. Cleaning 1

After cleaning it can be concluded that the reactor was visible clean. The results of the water analysis by TOC, particle analysis, analysis of the pH and conductivity, and the result of the rinse sample are acceptable, as presented in Table 4.17 and 4.19. However, higher values were obtained for the swab samples at the endings of the bottom piping and the three connections on the top of the vessel, shown in Table 4.18 marked in red. Therefore the procedure was changed. Firstly, the connection to tab 38 was cleaned at the end of the cleaning procedure instead of at the beginning, because during the whole cleaning procedure soiled water passes these endings of the bottom piping making them dirty again. Secondly, an extra cleaning cycle was introduced to clean the attachments alone. The result of the inside of the lid does not differ a lot from the TOC value of the corresponding criterion, therefore no

change was made for this location. However, extra attention was given to the inside of the lid in the next cleaning cycles.

Table 4.17: Results of the chemical analysis of the first cleaning (part I)

Chemical analysis	TOC water	Particle analysis			pH	Conductivity
Specification	500 ppb	> 10 µm	> 25 µm	> 50 µm	5 -7	< 1.3 µS/cm
		25/mL	2/mL	1/mL		
Result	277 ppb	3/mL	0/mL	0/mL	5.790	0.92 µS/cm

Table 4.18: Results of the chemical analysis of the first cleaning (part II)

Chemical analysis: Content swab samples		
Sampling places	**Specification (ppb)**	**Result (ppb)**
Wall of the reactor	205	88.9
Bottom of the reactor	205	115
Bottom of the stirrer	173	149
Endings of the bottom piping	187	1060
Window	180	106
Connection 1	224	214
Connection 2	184	4140
Connection 3	184	2870
Inside of the lid	205	241
Blank water	-	49.9
Blank + swab	-	66.6

Table 4.19: Results of the chemical analysis of the first cleaning (part III)

Chemical analysis: Content rinse sample	
Specification (ppb)	**Result (ppb)**
385	227

The microbiological samples do not exceed their corresponding criteria as is shown in Table 4.20 and 4.21.

Table 4.20: Results of the microbiological analysis of the first cleaning (part I)

Microbiological analysis		Rinse sample
Specification		< 100 CFU/100mL
Result	**Test**	10 CFU/100mL
	Blank	1 CFU/100mL

Table 4.21: Results of the microbiological analysis of the first cleaning (part II)

Microbiological analysis		RODAC plate
Specification		< 1 CFU/cm²
Result	**Lid**	0 CFU/cm²
	Bottom of the stirrer	0.04 CFU/cm²
	Wall	0 CFU/cm²
	Bottom	0.16 CFU/cm²

4.4.3.2. Cleaning 2

After cleaning it can be concluded that the reactor was visible clean. Furthermore, better results were obtained after revision of the cleaning procedure, with the exception of the rinse sample, although these values do not exceed the limit of water (500 ppb) as shown in Table 4.22 - 4.24. A possible explanation could be that the same soiling batch as in the first cleaning cycle was used and was not always stored in the refrigerator. For the next cleaning procedures a new soiling batch will be made immediately before soiling of the reactor. Moreover a more specific analysis method is required to analyze the contribution of soy to the result of the sample. ELISA could be a good alternative for TOC, however a few problems were experienced (Master thesis, Van de Voorde - Onderbeke Julie, 2017). Although the results of the three connections do not exceed their specifications anymore, the result of the endings of the bottom piping is lower than the previous cleaning, but still exceeds its specification. Similar results were obtained for the inside of the lid. Extra attention will be given at these locations during the following cleaning cycle. Furthermore, the result of the bottom of the reactor exceeded its specification. During sampling, a person was standing on the bottom of the reactor, which could explain this high value. During the following cleaning, the swab sample of the bottom of the reactor will be taken first.

Table 4.22: Results of the chemical analysis of the second cleaning (part I)

Chemical analysis	TOC water	Particle analysis			pH	Conductivity
Specification	500 ppb	> 10 µm	> 25 µm	> 50 µm	5 -7	< 1.3 µS/cm
		25/mL	2/mL	1/mL		
Result	454 ppb	13/mL	0/mL	0/mL	5.761	1.28 µS/cm

Table 4.23: Results of the chemical analysis of the second cleaning (part II)

Chemical analysis: Content swab samples		
Sampling places	**Specification (ppb)**	**Result (ppb)**
Wall of the reactor	205	128
Bottom of the reactor	205	264
Bottom of the stirrer	173	117
Endings of the bottom piping	187	460
Window	180	114
Connection 1	224	112
Connection 2	184	134
Connection 3	184	147
Inside of the lid	205	247
Blank water	-	61.2
Blank + swab	-	78.9

Table 4.24: Results of the chemical analysis of the second cleaning (part III)

Chemical analysis: Content rinse sample	
Specification (ppb)	**Result (ppb)**
385	454

Although the same explanation as for the swab sample taken at the bottom of the reactor could be given for the higher result of the RODAC plate taken at the bottom of the reactor, it does not exceed its specification. RODAC samples were taken after the swab samples to prevent contamination of the sampling place, thus it is not possible to take this sample in the beginning. But the first microbiological sample will be taken at the bottom of the reactor in the following cleaning cycle. The results are given in Table 4.25 and 4.26.

Table 4.25: Results of the microbiological analysis of the second cleaning (part I)

Microbiological analysis		Rinse sample
Specifications		< 100 CFU/100mL
Result	**Test**	36.5 CFU/100mL
	Blank	3 CFU/100mL

Table 4.26: Results of the microbiological analysis of the second cleaning (part II)

Microbiological analysis		RODAC plate
Specification		< 1 CFU/cm²
Result	**Lid**	0 CFU/cm²
	Bottom of the stirrer	0 CFU/cm²
	Wall	0 CFU/cm²
	Bottom	0.24 CFU/cm²

4.4.3.3. Cleaning 3

After cleaning it can be concluded that the reactor was visible clean. The results of the water analysis by TOC, particle analysis, analysis of the pH and conductivity, and the rinse sample are acceptable, as presented in Table 4.27 and 4.29. Despite the fact that higher results of the swab samples were obtained after the third cleaning cycle, every result is lower than the specification of water (500 ppb), as shown in Table 4.28. According to the available analysis methods this cleaning cycle is not conform to the specifications, however there is a need for a more specific analysis method to analyze the contribution of soy to the result of the sample. TOC is a non-specific method and will also include other substances. ELISA could be a good alternative for TOC, although a few problems were experienced (Master thesis, Van de Voorde - Onderbeke Julie, 2017).

Table 4.27: Results of the chemical analysis of the third cleaning (part I)

Chemical analysis	TOC water	Particle analysis			pH	Conductivity
Specification	500 ppb	> 10 µm	> 25 µm	> 50 µm	5 -7	< 1.3 µS/cm
		25/mL	2/mL	1/mL		
Result	252 ppb	7/mL	0/mL	0/mL	6.003	0.93 µS/cm

Table 4.28: Results of the chemical analysis of the third cleaning (part II)

Chemical analysis: Content swab samples		
Sampling places	**Specification (ppb)**	**Result (ppb)**
Wall of the reactor	205	240
Bottom of the reactor	205	208
Bottom of the stirrer	173	487
Endings of the bottom piping	187	462
Window	180	278
Connection 1	224	349
Connection 2	184	386
Connection 3	184	160
Inside of the lid	205	251
Blank water	-	28.0
Blank + swab	-	34.0

Table 4.29: Results of the chemical analysis of the third cleaning (part III)

Chemical analysis: Content rinse sample	
Specification (ppb)	**Result (ppb)**
385	252

The microbiological rinse sample exceeded its specifications (Table 4.30), in contrast to the RODAC samples which were acceptable (Table 4.31). An explanation for the difference between the results of the samples could be due to the small piping at the bottom of the reactor with *'dead points'* were the rinse sample passes, but the RODAC samples do not have any contact with this part of the reactor. These pipings probably will be more moist and more sensitive to microbial growth. Another explanation could be to question the sampling material and method: Was the bottle sterilized? Were there gloves used? Etc. To explain the exceedance of the specification of the microbiological rinse sample, an early event should be reported. After the second cleaning, the soiling batch of already two weeks old, was used to soil the reactor. The reactor had to be cleaned after three days of dirty hold time, but an excess of fungus was observed when the reactor was opened. The reactor was cleaned thoroughly with demineralized and distilled water. In addition the reactor was sanitized with steam to assure a fungus free reactor. However, no validation was done of the microbiological cleanliness due to a lack of time. Afterwards a new soiling batch was made to soil the reactor to start the third cleaning. Therefore it could be that there were still some remnants of fungus, although no visual growth was

seen after three days of dirty hold time. In order to prevent microbial growth, an extra drying step could have been included, however, no time remained to validate this.

Table 4.30: Results of the microbiological analysis of the third cleaning (part I)

Microbiological analysis		Rinse sample
Specification		< 100 CFU/100mL
Result	Test	Overgrowth
	Blank	3 CFU/100mL

Table 4.31: Results of the microbiological analysis of the third cleaning (part II)

Microbiological analysis		RODAC plate
Specification		< 1 CFU/cm²
Result	Lid	0 CFU/cm²
	Bottom of the stirrer	0 CFU/cm²
	Wall	0 CFU/cm²
	Bottom	0.12 CFU/cm²

4.4.3.4. Cleaning 4

After cleaning it can be concluded that the reactor was visible clean. Similar results as the third cleaning cycle were obtained. The results of the blank solution and the blank + swab solution however were higher than 100 ppb, making these results unreliable. The results are presented in Table 4.32 - 4.34.

Table 4.32: Results of the chemical analysis of the fourth cleaning (part I)

Chemical analysis	TOC water	Particle analysis			pH	Conductivity
Specification	500 ppb	> 10 µm	> 25 µm	> 50 µm	5 -7	< 1.3 µS/cm
		25/mL	2/mL	1/mL		
Result	212 ppb	1/mL	0/mL	0/mL	6.046	1.0 µS/cm

Table 4.33: Results of the chemical analysis of the fourth cleaning (part II)

Chemical analysis: Content swab samples		
Sampling places	Specification (ppb)	Result (ppb)
Wall of the reactor	205	121
Bottom of the reactor	205	215
Bottom of the stirrer	173	280
Endings of the bottom piping	187	104
Window	180	213
Connection 1	224	211
Connection 2	184	157
Connection 3	184	420
Inside of the lid	205	114
Blank water	-	115
Blank + swab	-	156

Table 4.34: Results of the chemical analysis of the fourth cleaning (part III)

Chemical analysis: Content rinse sample	
Specification (ppb)	**Result (ppb)**
385	212

The microbiological samples did not exceed its specifications, as shown in Table 4.35 and 4.36. A slightly higher value was observed for the RODAC sample taken at the bottom of the reactor, an explanation for which was already given in the earlier sections.

Table 4.35: Results of the microbiological analysis of the fourth cleaning (part I)

Microbiological analysis		Rinse sample
Specification		< 100 CFU/100mL
Result	Test	11 CFU/100mL
	Blank	3 CFU/100mL

Table 4.36: Results of the microbiological analysis of the fourth cleaning (part I)

Microbiological analysis		RODAC plate
Specification		< 1 CFU/cm²
Result	Lid	0 CFU/cm²
	Bottom of the stirrer	0 CFU/cm²
	Wall	0 CFU/cm²
	Bottom	0.2 CFU/cm²

4.4.3.5. Overall conclusion cleaning validation

A summary of the results of the four cleaning cycles is given in Figure 4.6. According to the available analysis methods these cleaning cycles are not conform to the specifications, however there is a need for a more specific analysis method to analyze the exact amount of soy residue in order to obtain more reliable results. However, product-specific assays for biologicals, such as immunoassays, may not be adequate when they are used alone: a negative result can be the result of denaturation of the protein. Therefore product-specific assays should be coupled with the non-specific method TOC to detect protein residues. Moreover, cleaning validation has to be done by three acceptable cleaning cycles.

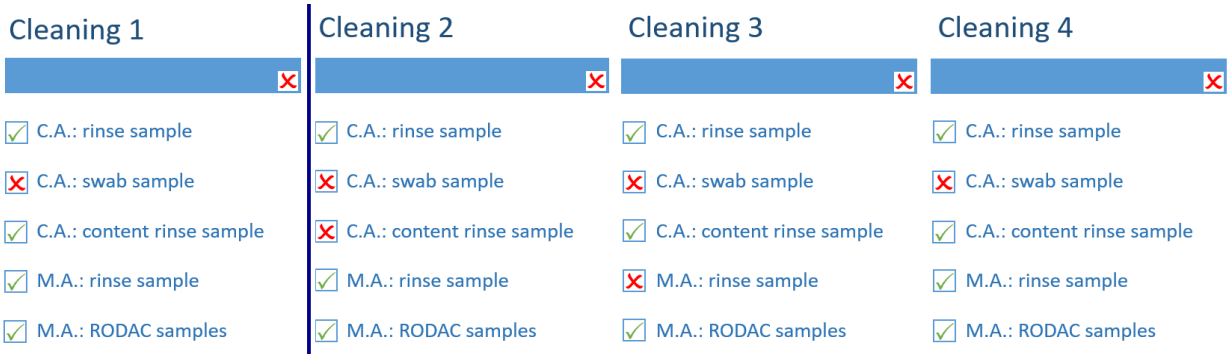


Fig. 4.6: Summary of the results of the four cleaning cycles, with C.A.: Chemical Analysis and M.A.: Microbiological analysis and a blue line indicating the revision after cleaning cycle 1

5. Conclusion

In order to determine a worst-case product for RTH 258, cleanability and solubility tests were performed for five candidate worst-case products: soy peptone, brown rice protein, pea protein isolate, whey protein isolate and riboflavin. Such a worst-case product should be harder to clean than the reference product by the investigated conditions, so as to obtain cleaning settings that assure an acceptable cleaning of the reference product.

Based on the results of the cleanability of **riboflavin** it can be concluded that riboflavin is harder to clean than RTH 258 with demineralized water at a flow of approximately 150 ± 5 mL/min. **Soy peptone** and riboflavin demonstrated to be immiscible, therefore no conclusive statements could be made of the cleanability results of soy itself, but soy peptone on its own probably has a less or similar cleanability than RTH 258, however extra tests are necessary to conclude if soy is a potential worst-case product for RTH 258 or not. For **Whey protein isolate** conclusions can only be made about the mixture 'whey protein isolate/riboflavin': the cleanability results of the mixture demonstrate a longer time for complete removal than RTH 258. Thus, the mixture could be used as worst-case product. The results of the cleanability experiments of **brown rice protein** and **pea protein** isolate indicate that these two products are the most difficult products to clean by the used conditions. Judging from the cleanability experiments, riboflavin, the mixtures 'whey protein isolate/riboflavin', 'brown rice protein/riboflavin' and 'pea protein isolate/riboflavin' are the best candidates to use as worst-case product for RTH 258.

In addition, a worst-case product should be less soluble in the investigated cleaning agent than the reference product, indicating a more difficult cleaning by the investigated conditions. For all of the five products, the solubility was lower than RTH 258 (30 g/100 mL): riboflavin (14.25 mg/100 mL), soy peptone (6.58 g/100 mL), whey protein isolate (5.56 g/100 mL), pea protein isolate (183.3 mg/100 mL) and brown rice protein (6.77 mg/100 mL). Based on the criterion of solubility, all of the five tested products demonstrate to be potential candidates to use as worst-case product for RTH 258.

Combining the results of the cleanability and solubility experiments, riboflavin, 'whey protein isolate/riboflavin', pea protein isolate and brown rice protein show to be the best candidates to use as worst-case product for RTH 258. However, the final ease of cleaning should also be taken into account in order to choose the ultimate, most suitable product. In this context, brown rice protein and pea protein isolate have the most extreme results, indicating good worst-case product characteristics. However, it must still be possible to clean the reactor with acceptable settings at all. Therefore it might be better to select riboflavin or 'whey protein isolate/riboflavin' as worst-case product, since their cleanability results are less different from RTH 258 than the other two products. To work more

efficiently only one substance should be used, therefore it probably is better to choose for riboflavin alone. Moreover, riboflavin has the advantage of being easily detectable due to its fluorescent character.

The cleaning validation of reactor 88, which can be used for the production of the RTH 258 formulation, was performed using soy peptone. The choice for this protein resulted from the fact that an ELISA kit specific for soy proteins was already available in-house at the start of the project. First, the recovery of soy was determined on stainless steel (68.20%) and on glass (75.84%). The criterion determining the acceptable amount of residue was calculated by the PDE principle and resulted in a brown solution. Visual inspection can be done by comparing the color of the samples taken during cleaning validation with the corresponding criterion. However, the color of the samples was doubtful: if a rinsing sample would have this color, one would never conclude that the reactor is clean. The criterion determining the acceptable amount of residue, as calculated by the 1/1000 principle, gave low results. Therefore, a more specific analysis method such as ELISA is necessary. However, product-specific assays for biologicals, such as immunoassays, may not be adequate when they are used alone: a negative result can be the result of denaturation of the protein. Therefore product-specific assays should be coupled with the non-specific method TOC to detect protein residues.

Since the first cleaning did not give satisfying results for the swab samples taken at the endings of the bottom piping, connection 2 & 3 and the inside of the lid, the cleaning procedure was modified. Firstly, the connection to tab 38 was cleaned at the end of the cleaning procedure instead of at the beginning, because during the whole cleaning procedure soiled water passes these endings of the bottom piping making them again dirty. Secondly, an extra cleaning cycle was introduced to clean the connections alone. The result of the inside of the lid did not differ a lot from the TOC value of the corresponding criterion, therefore no change was made for this location. More favorable results were obtained after the second cleaning, however, some samples still exceeded their limits. The results from the third cleaning were doubtful and therefore neglected. Furthermore, the results of the fourth cleaning are unreliable due to high results of the blank samples. It could be concluded that a more specific analysis method is required to analyze the contribution of soy to the result of the sample. TOC is a non-specific method and will also include other substances. ELISA could be a good alternative for TOC, however some problems were experienced (Master thesis, Van de Voorde - Onderbeke Julie, 2017).

In conclusion, extra cleanability tests for soy peptone are necessary to conclude if soy is suitable as worst-case product for RTH 258 or not, however riboflavin, ‘whey protein isolate/riboflavin’, pea protein isolate and brown rice protein are, with riboflavin being the best worst-case product candidate. Nevertheless, we contend that the analysis method should change to a more specific method to

measure the exact amount of soy residue in order to obtain more reliable results. Moreover, cleaning validation has to be done by three acceptable cleaning cycles, which has not occurred due to a lack of time.

6. References

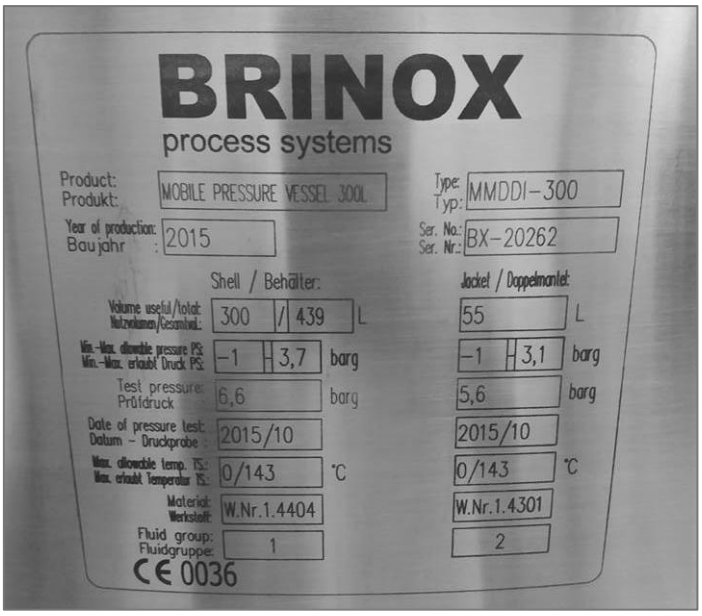
1. Morrow, T.H.F.L., *Defining the difference what makes biologics unique.* Biotechnology Health Care, 2004.
2. FDA. [cited 2016 26 oktober]; http://www.fda.gov/AboutFDA/Transparency/Basics/ucm194516.htm].
3. Skalko-Basnet, N., *Biologics: the role of delivery systems in improved therapy.* Biologics, 2014. **8**: p. 107-14.
4. Medicinet, I. *Picture of Eye Anatomy Detail*. 2004; Available from: http://www.medicinenet.com/image-collection/eye_anatomy_detail_picture/picture.htm.
5. *https://nei.nih.gov/*. [cited 2016 29 oktober]; https://nei.nih.gov/health/maculardegen/armd_facts].
6. Michalska-Małecka, K., et al., *Age related macular degeneration – challenge for future: Pathogenesis and new perspectives for the treatment.* European Geriatric Medicine, 2015. **6**(1): p. 69-75.
7. Veritti, D., V. Sarao, and P. Lanzetta, *Neovascular age-related macular degeneration.* Ophthalmologica, 2012. **227 Suppl 1**: p. 11-20.
8. *Wet Age Related Macular Degeneration Effect*. Available from: http://glaznoy-doctor.ru/prochie-zabolevaniya/lechenie-suxoj-i-vlazhnoj-formy-vozrastnoj-makulyarnoj-degeneracii-setchatki.html.
9. Chexal, S.G., I. Berger, B. Jhaveri, C., *A novel compound for the treatment of wet AMD.* Retina Today, 2016: p. 72 - 78.
10. Genentech. *The mechanism of action of Avastin*. 2017 [cited 2017 2th of February]; Available from: https://www.avastin-hcp.com/about-avastin/proposed-moa.html.
11. www.who.int. [cited 2016 29 oktober 2016]; http://www.who.int/medicines/services/inn/en/].
12. FDA. *FDA Approves New Biologic Treatment for Wet Age-Related Macular Degeneration*. 2006 [cited 2017 12th of march]; Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2006/ucm108685.htm.
13. Agency, E.M., *EPAR_Ranibizumab.* EMA, 2007: p. 146.
14. Informatie, B.C.v.F. 2016 [cited 2016 2nd of november]; Available from: http://www.bcfi.be/nl/start.
15. Alyamac Sukgen, E., et al., *The Process of Retinal Vascularization after Anti-VEGF Treatment in Retinopathy of Prematurity: A Comparison Study between Ranibizumab and Bevacizumab.* Ophthalmologica, 2016.
16. Comparison of Age-related Macular Degeneration Treatments Trials Research, G., et al., *Five-Year Outcomes with Anti-Vascular Endothelial Growth Factor Treatment of Neovascular Age-Related Macular Degeneration: The Comparison of Age-Related Macular Degeneration Treatments Trials.* Ophthalmology, 2016. **123**(8): p. 1751-61.
17. AG, N.P., *Health Hazard Assessment Monograph - RTH258.* 2016: p. 18.
18. Mayo, A., *Process Selection Summary: ESBA1008 IVT (A00740)*. 2014: Alcon. p. 10.
19. Nassani, M., *Cleaning Validation in the Pharmaceutical Industry.* Journal of Validation Technology, 2005: p. 58.
20. Agalloco, J.C., F., *Validation of Pharmaceutical Processes third edition*. 2007: Informa. 760.
21. Bicksler, R.C., J.; Ektefaie, E.; Guyader, M.; Hentschel, N.; LeBlanc, D.; Levy, P.; Rathore, A.; Sillivan, D.; Stevens, B., *Points to consider for biotechnology cleaning validation - technical reports no. 49*. 2010: Parenteral Drug Association.
22. Das, S. *Cleaning Validation*. 2014 [cited 2016 2nd of november]; Available from: https://www.slideshare.net/SambhujyotiDas/cleaning-validation-a-complete-know-how.
23. Agency, E.M., *Guidance on setting health based exposure limits for use in risk identification in the manufacture of different medicinal products in shared facilities.* 2014: p. 11.

24. (APIC), A.P.I.C., *Guidance on aspects of cleaning validation in active pharmaceutical ingredient plants.* 2014: p. 55.
25. *European Pharmacopeia (Ph.Eur. 9th edition)*.
26. Forsyth, R.J., *Equipment hold time for cleaning validation.* Pharmaceutical Technology, 2008. **32**(4).
27. Asgharian, R.H., F.; Heydari, A., *Step by Step How to Do Cleaning Validation.* International journal of pharmacy & life sciences, 2014. **5**(3): p. 3345 - 3366.
28. Porter, R., *Impact of API physicochemical properties on cleaning method design and cleaning validation.* Journal of Validation Technology, 2011: p. 96.
29. Pluta, L.S., R., *Avoiding Pitfalls of Cleaning Validation.* Journal of GSX Compliance, 2010. **14**(3): p. 62.
30. Solutions, R.P. *A cost-effective approach to cleaning validation and verification*

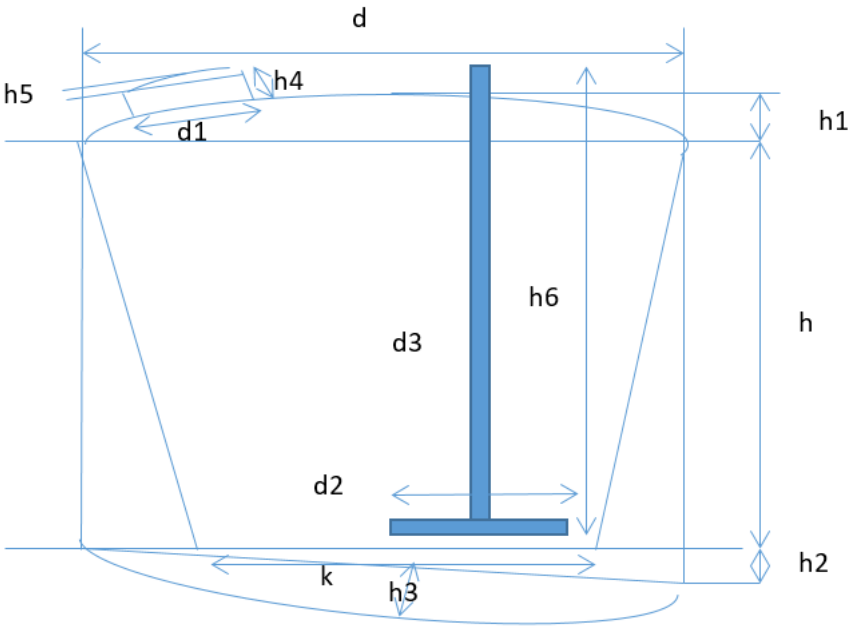
    [cited 2017 19th of march]; Available from: http://www.ropack.com/2015/10/20/a-cost-effective-approach-to-cleaning-validation-and-verification/.
31. Nehete, J.Y., et al., *Natural proteins: Sources, isolation, characterization and applications.* Pharmacogn Rev, 2013. **7**(14): p. 107-16.
32. Jackson, L.A.-t., F., Moorman, M.; Devries, J.; Tippett, R.; Swanson K.; Fu, T.; Salter, R.; Dunaif, G.; Estes, S.; Albills, S.; Gendel, S., *Cleaning and other control and validation strategies to prevent allergen cross-contact in food-processing operations.* Journal of food protection, 2007. **71**(2): p. 458.
33. Hettiarachchy, N.S. and U. Kalapathy, *Functional Properties of Soy Proteins.* 1998. **708**: p. 80-95.
34. Prakash, J., *Rice bran proteins: properties and food uses.* Crit Rev Food Sci Nutr, 1996. **36**(6): p. 537-52.
35. Wang, C., et al., *Physico-chemical and structural properties of four rice bran protein fractions based on the multiple solvent extraction method.* Czech Journal of Food Sciences, 2016. **33**(No. 3): p. 283-291.
36. Tamnak, S., et al., *Physicochemical properties, rheological behavior and morphology of pectin-pea protein isolate mixtures and conjugates in aqueous system and oil in water emulsion.* Food Hydrocolloids, 2016. **56**: p. 405-416.
37. Teo, A., et al., *Physicochemical properties of whey protein, lactoferrin and Tween 20 stabilised nanoemulsions: Effect of temperature, pH and salt.* Food Chem, 2016. **197**(Pt A): p. 297-306.
38. Bou, R., S. Cofrades, and F. Jiménez-Colmenero, *Physicochemical properties and riboflavin encapsulation in double emulsions with different lipid sources.* LWT - Food Science and Technology, 2014. **59**(2): p. 621-628.
39. b.v., S.J., *Molybdeen test 960 voor Roestvaststaal*. The Netherlands. p. 2.
40. Baka, E., J.E. Comer, and K. Takacs-Novak, *Study of equilibrium solubility measurement by saturation shake-flask method using hydrochlorothiazide as model compound.* J Pharm Biomed Anal, 2008. **46**(2): p. 335-41.
41. Simonian, M.H., *Spectrophotometric determination of protein concentration* Current protocols in cell biology (A.3B.1 - 1.3B.7), 2002.
42. *European Pharmacopeia (Ph. Eur.) 9th Edition: water for injections (01/2009:0169)*. Vol. 1. 2017.
43. *USP 36: Physical tests / Particulate matter in Ophtalmic Solutions*. 2013.

7. Appendix

7.1. Brinox Reactor 88



7.2. Calculation of the surface of reactor 88



Diameter cylinder	d	988 cm²
Height cylinder	h	585 cm²
Height lid	h1	185 cm²
Truncated cylinder	h2	105 cm²
Height bottom	h3	97 cm²
Diameter manhole	d1	350 cm²
Height manhole	h4	97 cm²
Length mixing baffle	h6	910 cm²
Diameter mixing baffle	d2	125 cm²
Diameter agitator shaft	d3	35 cm²
Length bottom line	l	858 cm²
Diameter cone	k	584 cm²

Part of the reactor	**Calculation**	**Area (cm²)**
Top of the reactor	= π*((d/2)²+h1²)	8742
Bottom of the reactor	= π*((d/2)²+h3²)	7962
Manhole elevation	= π*d1*h4	1067
Mixing baffle	= π*d3*h6	1001
Agitator shaft	=2π*(d2/2)²	245
Bottom line	= π*dia*l	701
Manhole lid	= π*((d1/2)²+h5²)	962
Truncated cylinder	= ((d+k)/2)* π*sqr(h²+((d-k)/2)²)	15282
Side of the truncated cylinder	= (π*k*h2)/2	963
Total		**36925**

7.3. Sampling plan of reactor 88

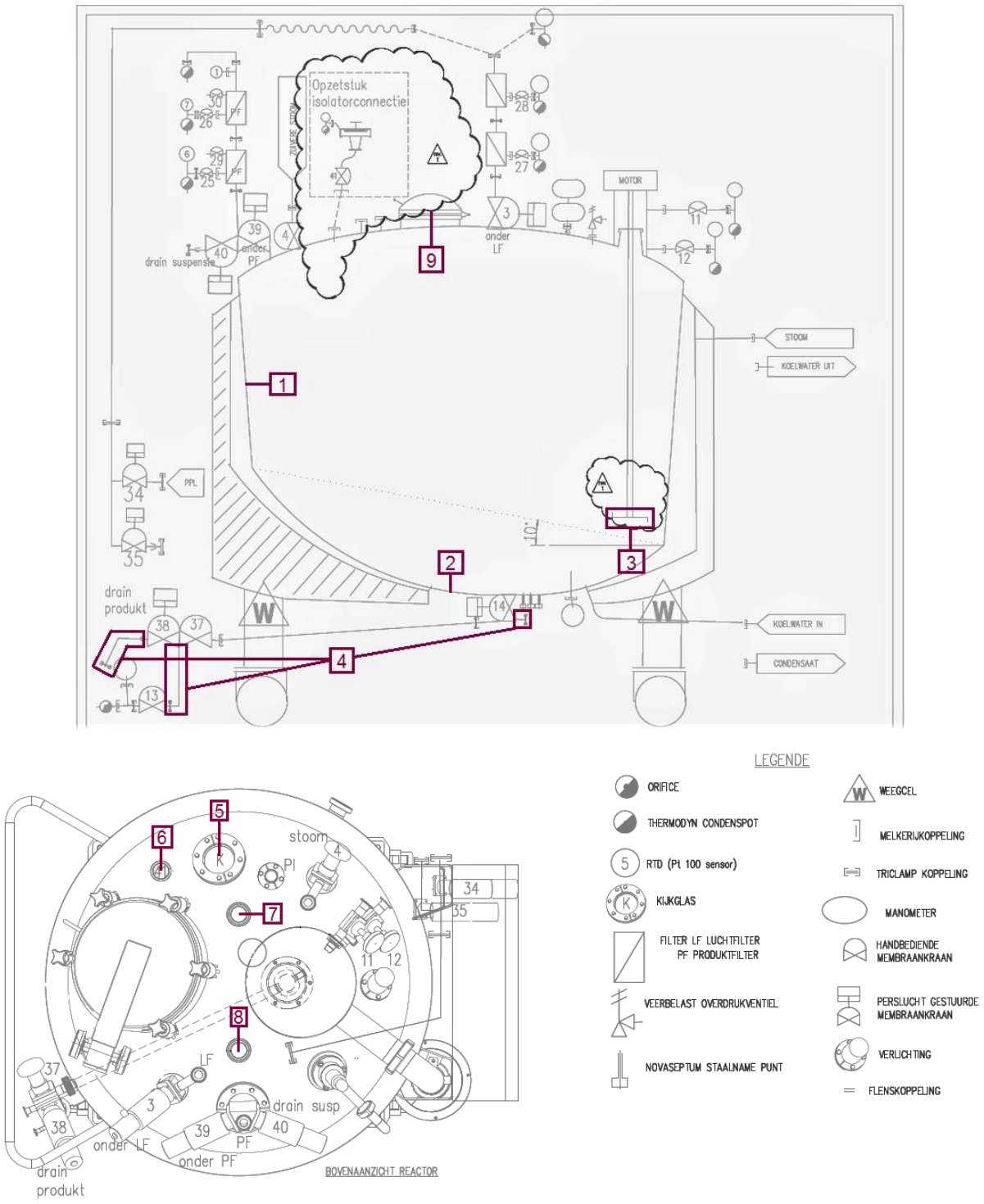
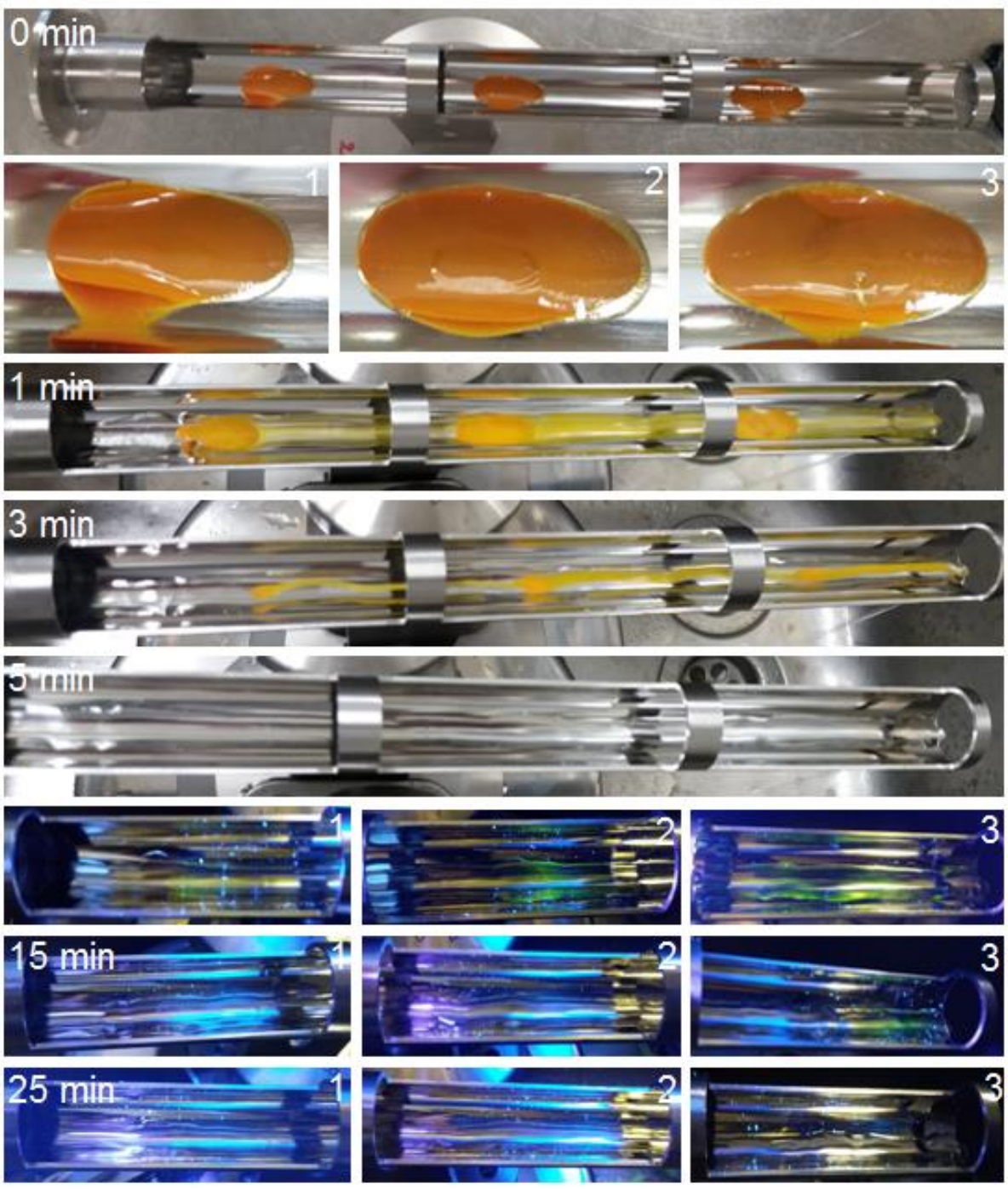


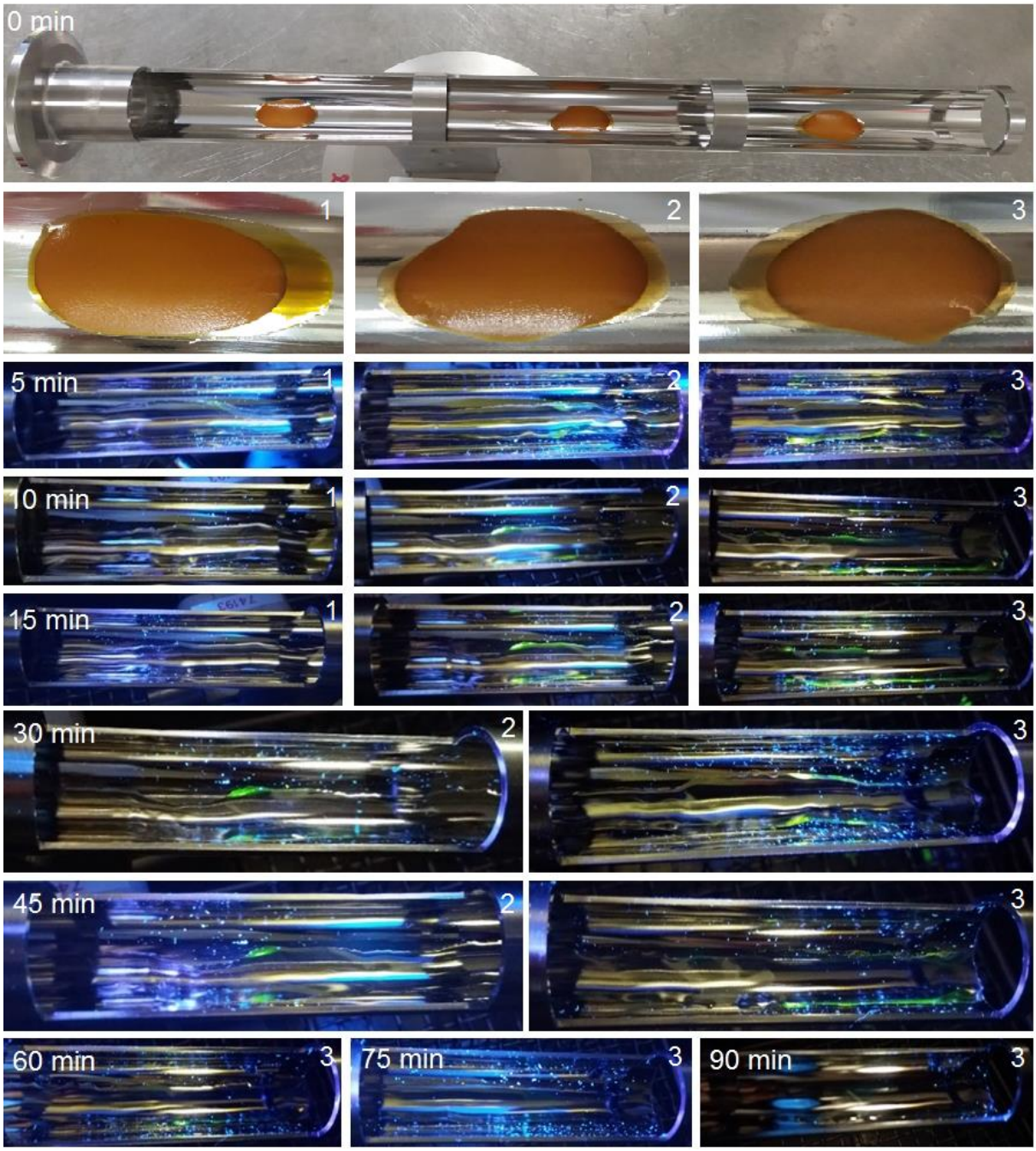
Figure 7.7: Sampling places indicated at reactor 88

7.4. Cleanability results

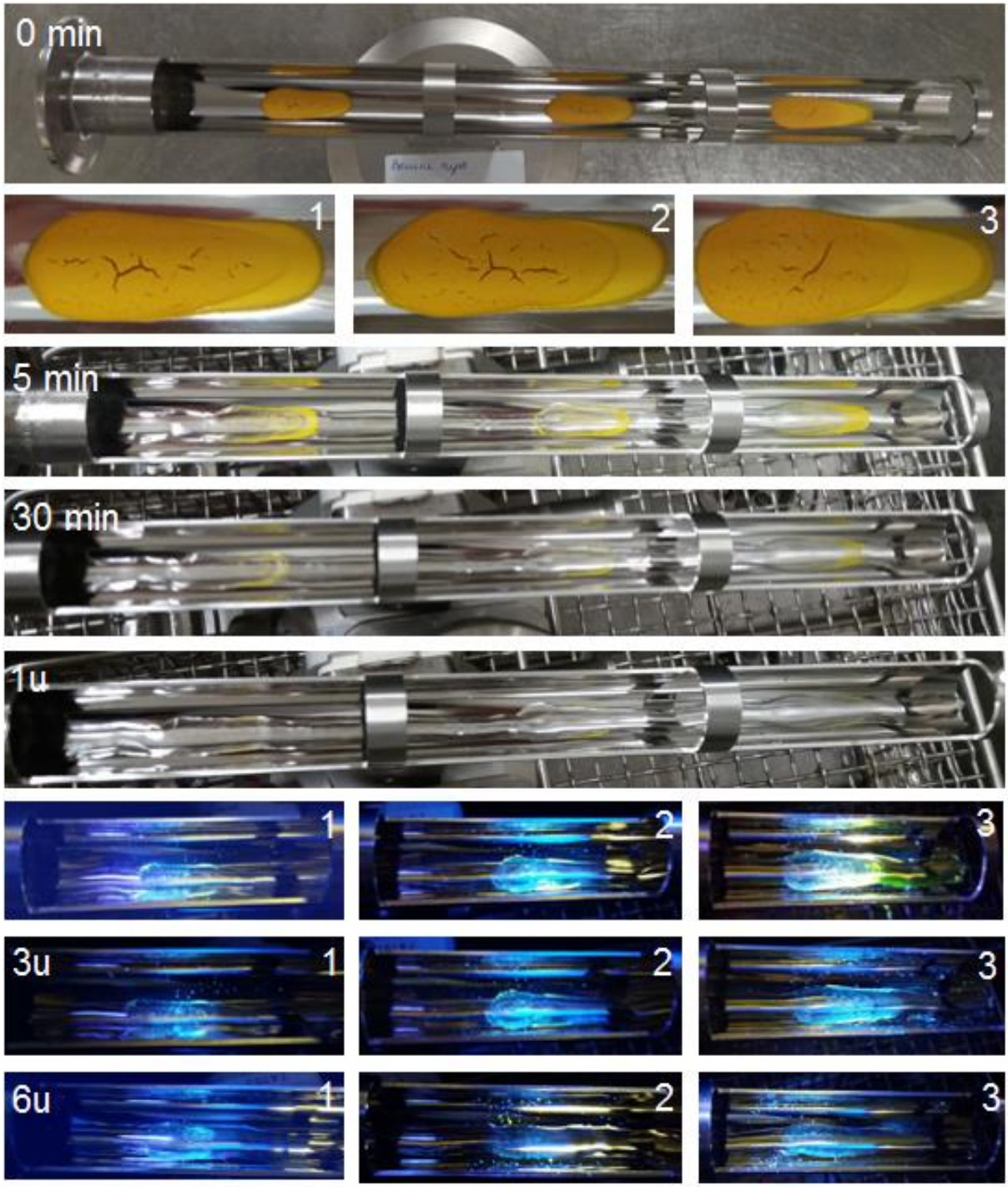
7.4.1. Time lapse images of ‘RTH 258/riboflavin’ on the stainless steel tube



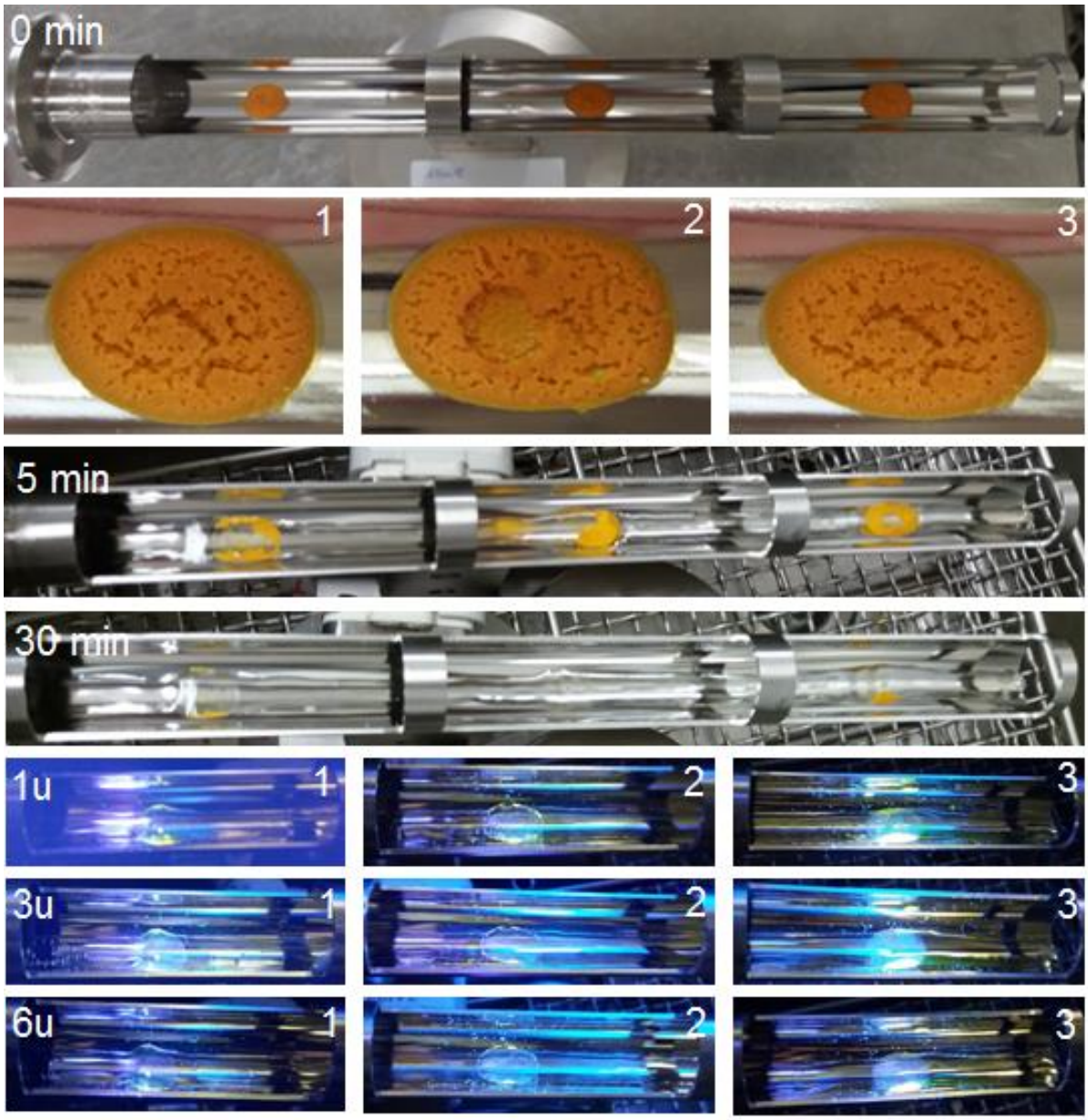
7.4.2. Time lapse images of ‘soy peptone/riboflavin’ on stainless steel tube



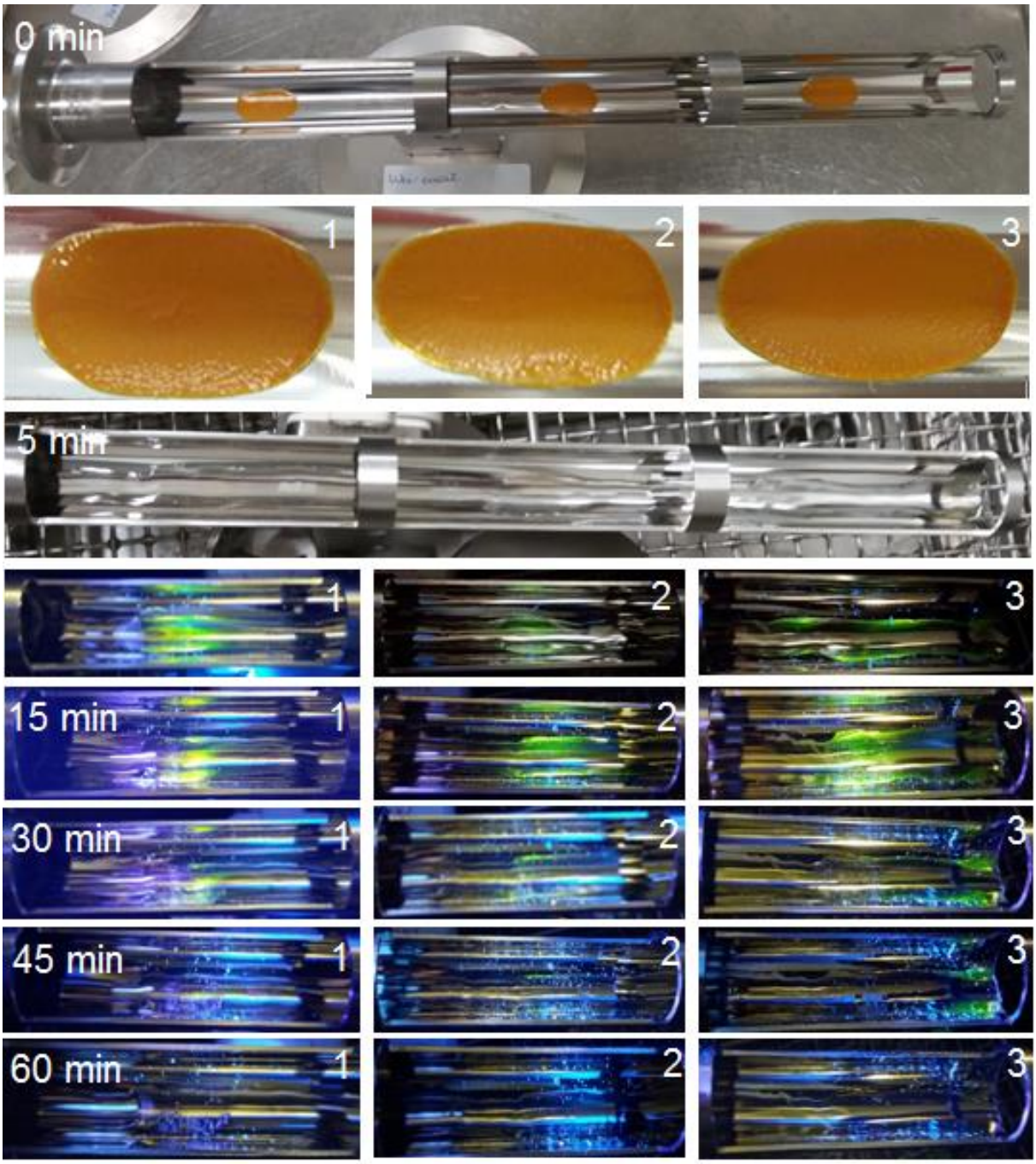
7.4.3. Time lapse images of ‘brown rice protein/riboflavin’ on the stainless steel tube



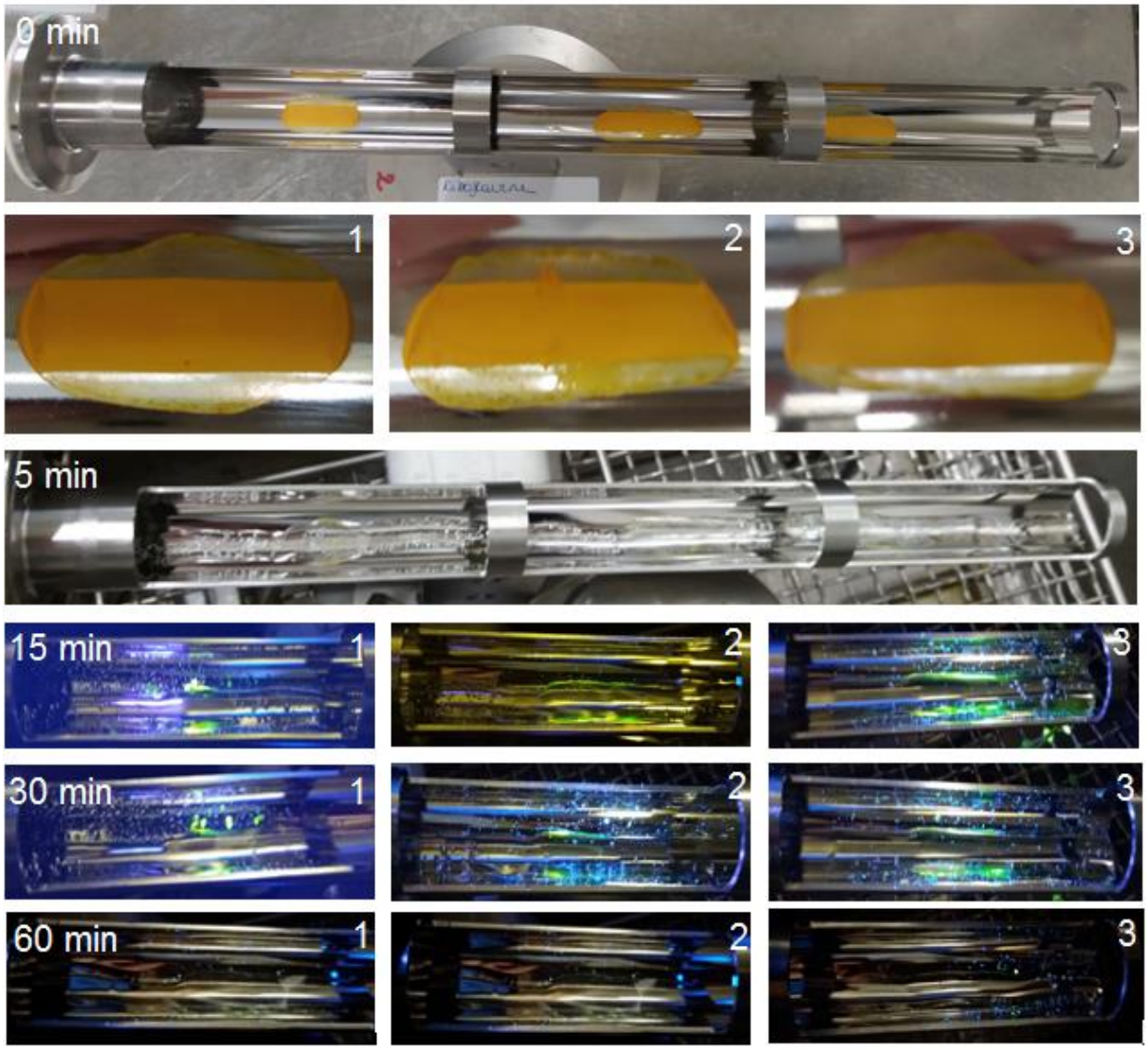
7.4.4. Time lapse images of ‘pea protein isolate/riboflavin’ on the stainless steel tube



7.4.5. Time lapse images of ‘whey protein isolate/riboflavin’ on the stainless steel tube



7.4.6. Time lapse images of riboflavin on the stainless steel tube



7.5. Solubility – calibration curve

7.5.1. Soy peptone

Table 7.1.: Measurement of the absorbance of the samples of soy peptone to create a calibration curve

Concentration (mg/100mL)	1/100 diluted concentration (mg/100mL)	Absorbance
0	0	0
251.4	2.514	0.018
		0.016
		0.019
500.7	5.007	0.038
		0.038
		0.037
749.2	7.492	0.056
		0.054
		0.056
1000.0	10.00	0.073
		0.075
		0.074
1256.0	12.56	0.108
		0.105
		0.107

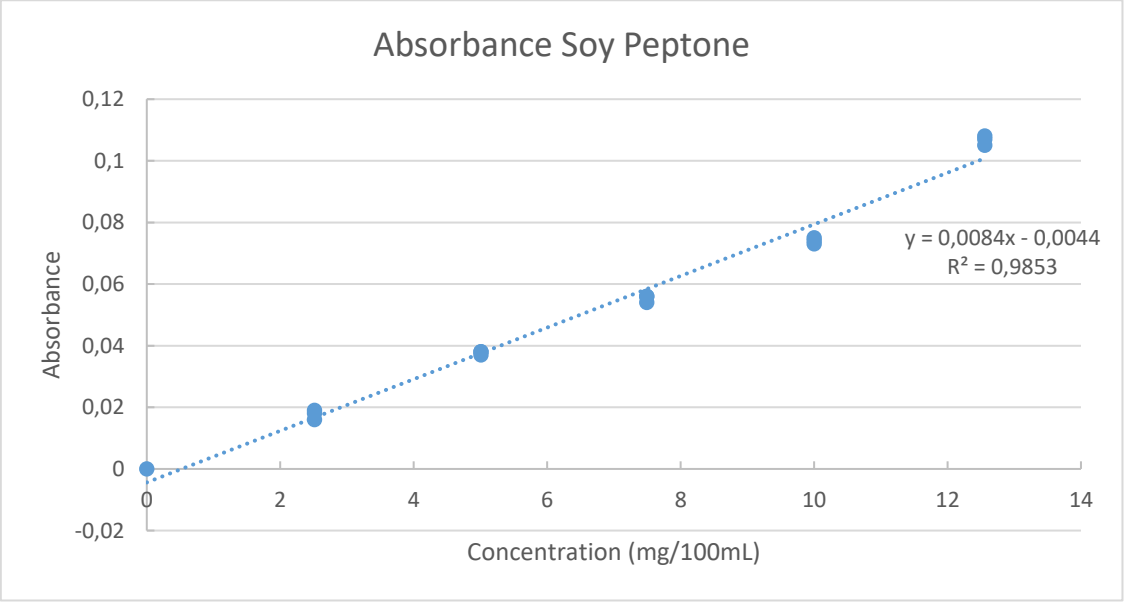


Fig. 7.1: Calibration curve of soy peptone obtained by UV/VIS spectrophotometry

7.5.2. Brown rice protein

Table 7.2.: Measurement of the absorbance of the samples of brown rice protein to create a calibration curve

Concentration (mg/100mL)	Absorbance
0	0
1.00	0.006
	0.005
	0.004
2.00	0.010
	0.009
	0.010
3.2	0.018
	0.016
	0.015
4.0	0.020
	0.022
	0.023
5.0	0.025
	0.027
	0.028

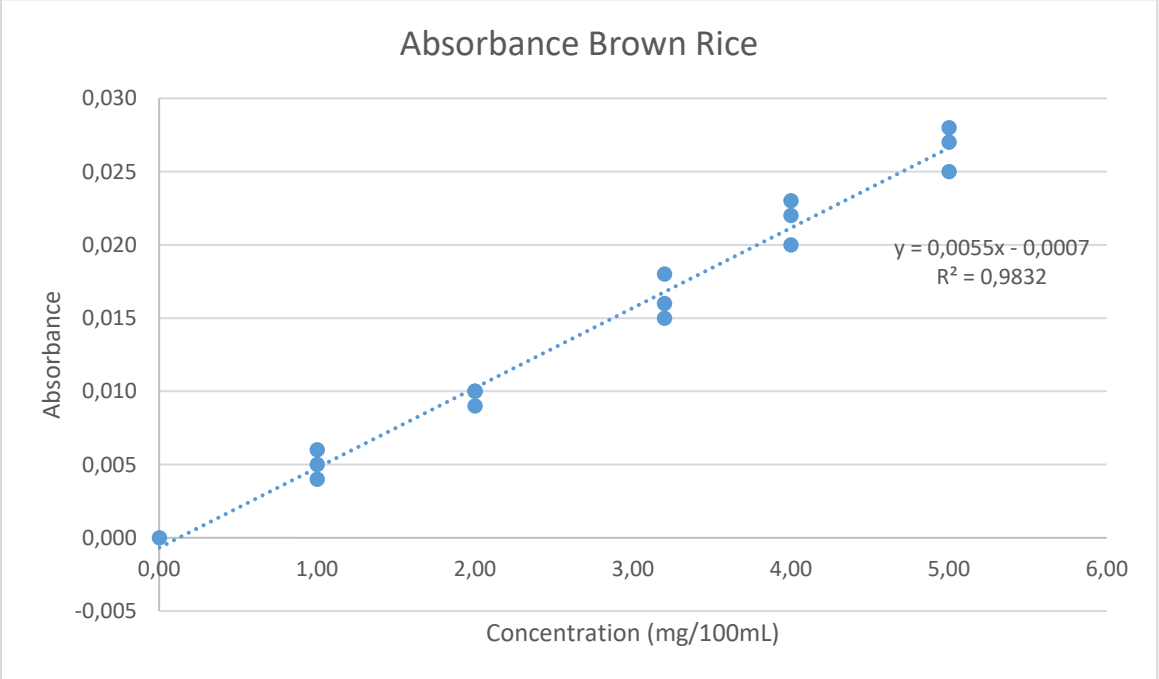


Fig. 7.2: Calibration curve of brown rice protein obtained by UV/VIS spectrophotometry

7.5.3. Pea protein isolate

Table 7.3.: Measurement of the absorbance of the samples of pea protein isolate to create a calibration curve

Concentration (mg/100mL)	Absorbance
0	0
1.2	0.016
	0.013
	0.016
2.1	0.029
	0.028
	0.032
3.3	0.05
	0.055
	0.06
4.1	0.07
	0.077
	0.079
5.00	0.09
	0.096
	0.097

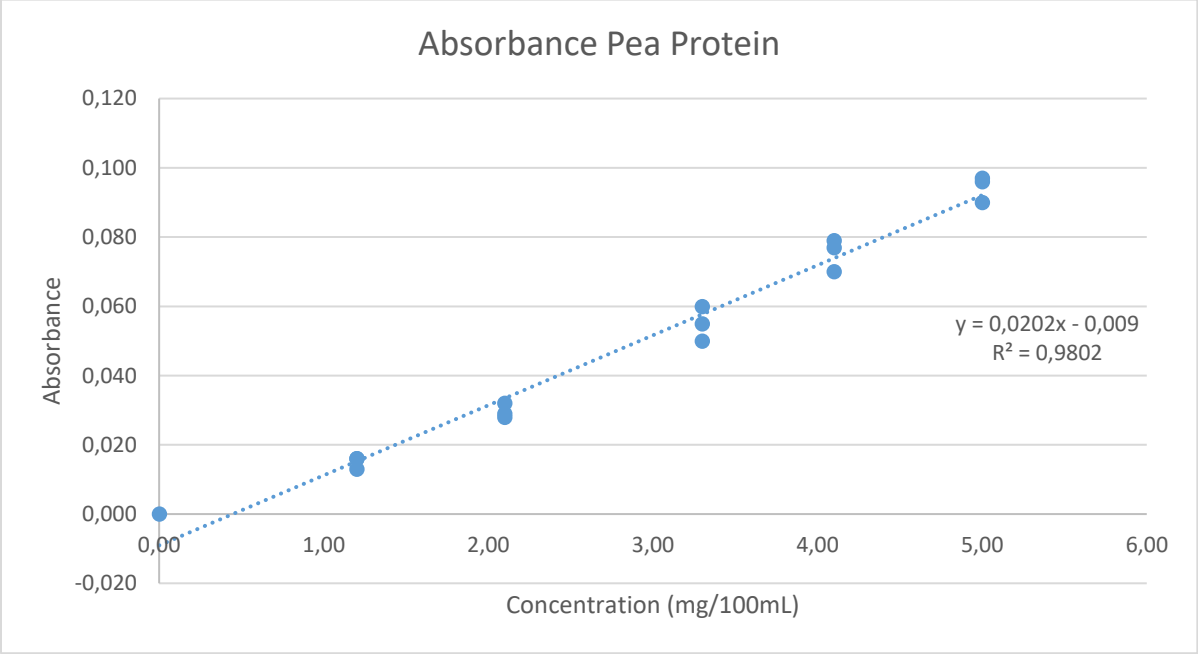


Fig. 7.3: Calibration curve of pea protein isolate obtained by UV/VIS spectrophotometry

7.5.4. Whey protein isolate

Table 7.4.: Measurement of the absorbance of the samples of whey protein isolate to create a calibration curve

Concentration (g/100mL)	1/50 diluted concentration (g/100mL)	Absorbance
0	0	0
0.500	0.01	0.082
		0.087
		0.090
1.000	0.0200	0.259
		0.258
		0.263
1.504	0.0301	0.299
		0.305
		0.310
2.001	0.0400	0.417
		0.423
		0.412
2.498	0.0500	0.507
		0.510
		0.505

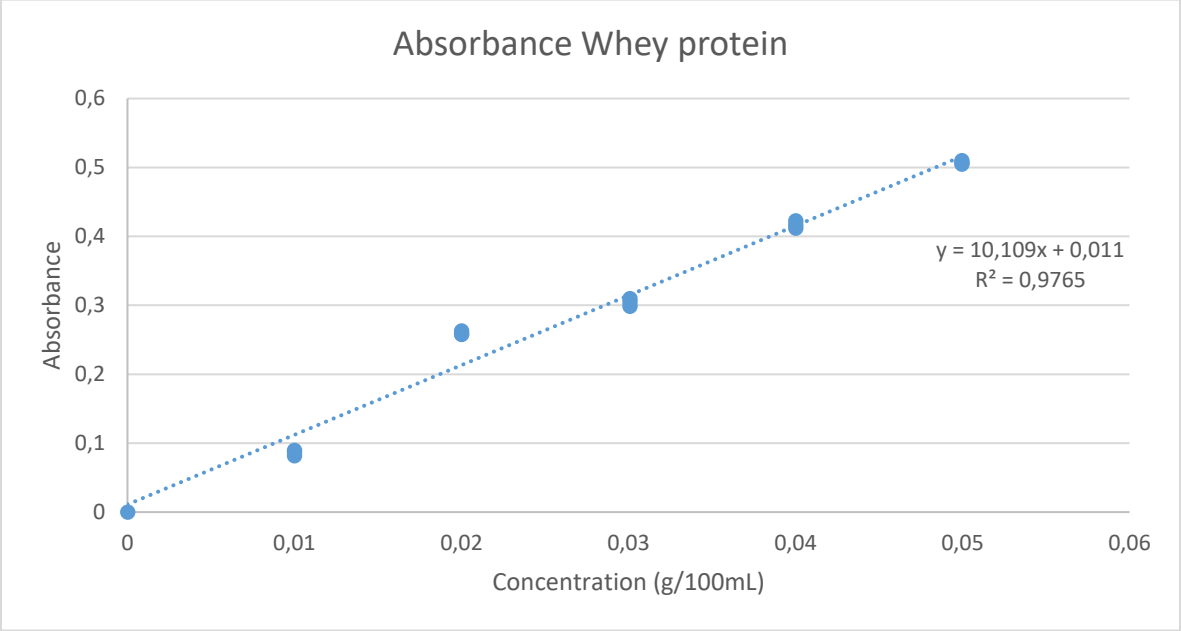


Fig. 7.4: Calibration curve of whey protein isolate obtained by UV/VIS spectrophotometry

7.5.5. Riboflavin

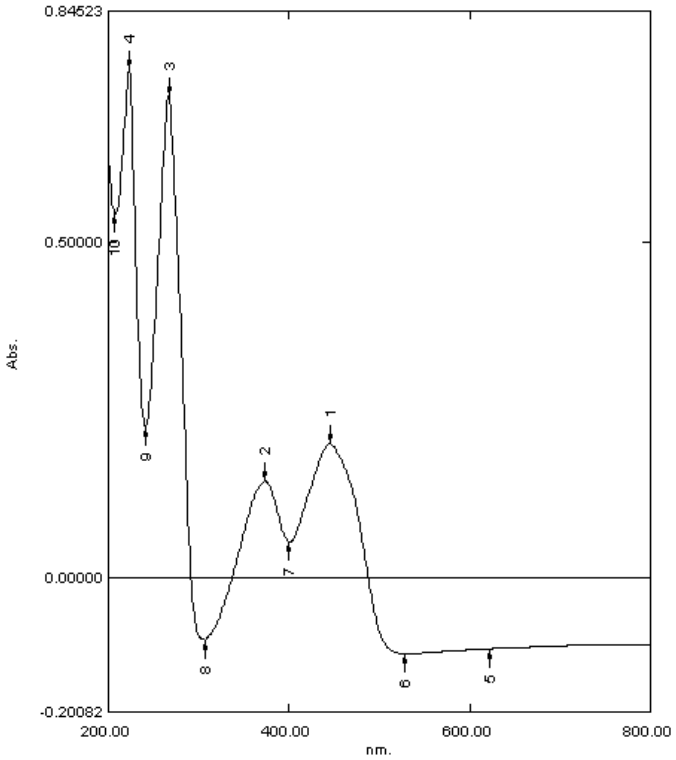


Fig. 7.5: Absorbance spectrum of riboflavin

Table 7.5.: Measurement of the absorbance of the samples of riboflavin to create a calibration curve

Concentration (g/100mL)	1/5 diluted concentration (g/100mL)	Absorbance
0	0	0
1.00	0.20	0.142
		0.149
		0.149
2.20	0.44	0.308
		0.324
		0.317
3.1	0.62	0.432
		0.435
		0.440
4.2	0.84	0.553
		0.566
		0.577
5.1	1.02	0.645
		0.649
		0.650

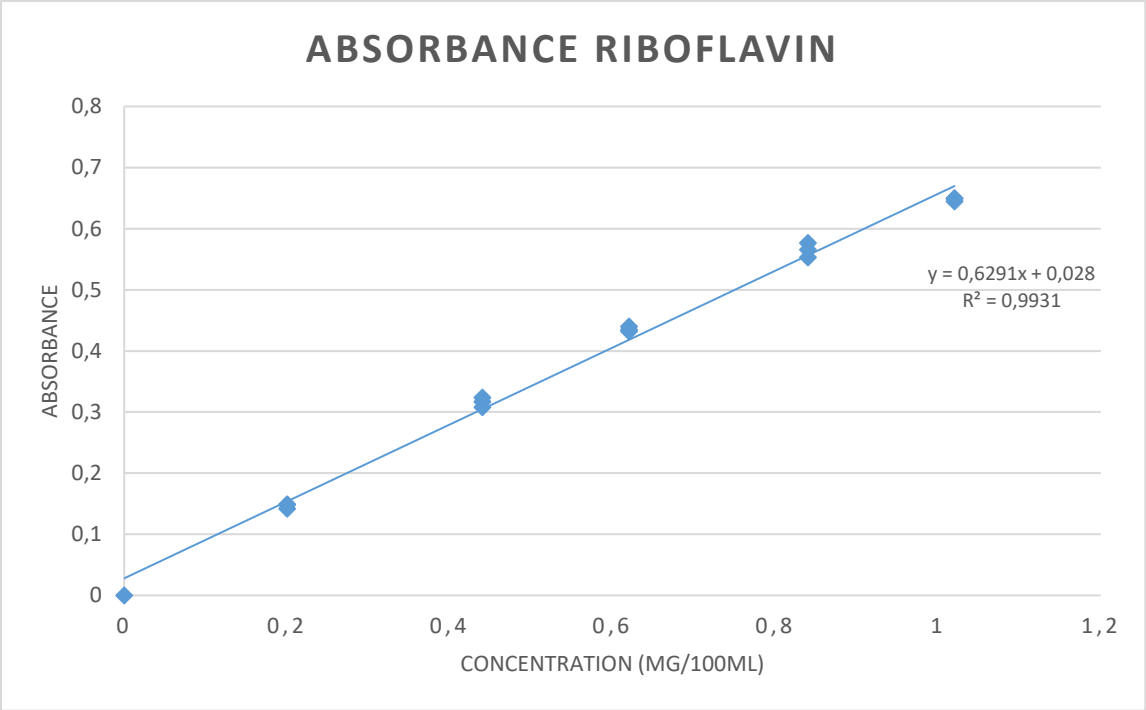


Fig. 7.6: Calibration curve of riboflavin obtained by UV/VIS spectrophotometry