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RESEARCH ARTICLE

Challenges Related to Extractables/ Leachables During Bioprocessing

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ABSTRACT

As the production of biologics grows, so does the need to control impurity contamination. Some compounds have the possibility to leach into the products during their manufacturing, hampering their production. The presence of these compounds in the final product may also have dangers to the end user if not removed. These compounds are either simple organic molecules or heavy metals, and are often utilized during production, or are part of the disposable plastics in the processing. Some of the organics can be broken down into toxic materials, while the metals are shown to lead to undesirable effects at higher concentrations. Hence, monitoring their presence and concentrations in the final product is necessary for bioproduction efficiency and ultimately patient safety. This review discusses the most common organic and metallic extractables/leachables found in plastics that are utilized in the synthesis of biologics.

Keywords: Leachables; extractables; metals; organics; bioreactors

1.0 Introduction

Biologic drugs encompass a variety of therapeutic products, including vaccines, gene therapy, tissues, and recombinant proteins. The Food and Drug Administration (FDA) defines biologics as complex molecules derived from natural sources or manufactured using biotechnology. These products are highly susceptible to heat and easily contaminated by microbes.[1] Hence, their manufacturing process requires specific precautions in order to avoid any contamination. Over the past decades, many manufacturing companies have turned to single-use, polymeric, bioprocessing materials for biologic product development, including bioreactor bags, containers, transfusion kits, transfer tubing, syringes, and other production equipment.[2] Due to the advantages of disposable bioreactor systems in large-scale synthesis, their use has steadily increased in the biopharmaceutical manufacturing industry.

Traditional bioreactor designs were composed of stainless steel or glass vessels. These have evolved into disposable plastic bioprocessing container systems. These systems can decrease cost, ease handling, reduce cross-contamination, and expedite the pharmaceutical development processes.[3] Thermoplastics, commonly used in biologics production, include polypropylene, polyethylene, polystyrene, and polyvinyl chloride (PVC). Due to some clear advantages of these systems, there has been a doubling in their use over the last 20 years.[4] However, despite the increase in productivity that these systems provide, there are critical drawbacks and regulatory concerns surrounding their use. Among these concerns is the risk of extractables and leachables entering the bioreactor system and affecting the overall yield and purity of the biologic.[3]

Extractables are materials` extracted from a plastic under more aggressive conditions than simple contact between the plastic and the system.[3] Conversely, leachables are present in the final product via the relatively benign interaction between the plastic and the system.[3] Differentiating between extractables and leachables is highly challenging since they may be present as process contaminants, impurities, or reaction by-products.[4] Furthermore, the interaction between extractables, leachables, and the product's ingredients have been shown to potentially change the physicochemical properties of the biologic and thus negatively impact the final product's quality.[5] Examples of identified extractables include metals, Irganox 1076, Lupersol 221, DEHP, caprolactam, etc. Most notably, Irgafos 168 is an extractable, when broken down, generates bDtBPP, which is toxic to cell growth in media.[6] Thus, it is important to identify and quantify these extractables and leachables in bioprocessing materials to ensure that cell growth is optimal and patient safety is maintained.

Information regarding the plastic materials used is typically provided by the suppliers, however, the vendor of the biologic has the legal responsibility to demonstrate the safety and efficacy of the final drug product.[4] The FDA assesses the manufacturing of biologic products, including inherent variations, which is in part due to varying impurity concentrations. Herein, is an overview of many common extractables and leachables that have been found in plastic materials and their threshold concentrations.

2.0 Organics

2.1. Irgafos

Irgafos 168 (tris(2,4-di-tert-butylphenyl)phosphite) is a stabilizer used in plastic bioprocessing

materials to prevent discoloration, maintain viscosity, and protect the substance from thermo-oxidative degradation. Irgafos acts as a secondary antioxidant to inactivate hydroperoxides formed from plastic oxidation. This prevents induced degradation and extends the performance of primary antioxidants, such as Irganox 1076, which deactivates radicals.[6] (Djouani et al., 2011). Irgafos 168 primarily protects polymers from high temperatures during sterilization. However, it can breakdown to bis(2,4-di-tert-butylphenyl) phosphate (bDtBPP) (Figure 1).[7] The primary pathway to bDtBPP formation is hypothesized to be via ionizing radiation of oxidized Irgafos.[8] Leaching of bDtBPP has also been directly linked to gamma radiation sterilization and has been shown to be cytotoxic at concentrations of 0.2-0.73mg/L.[9] The FDA guidance per the International Conference on Harmonization (ICH) recommends organic impurities to be reported if greater than 0.05%, if found in the drug substance (daily dose less than 2g/day).[1]

Within growth media, CHO cell lines have variable levels of sensitivity and toxicity. CHO-K1 and CHO-DP12 are the two commonly used cell lines to measure growth inhibition. Concentrations of bDtBPP of 0.035mg/L has been shown to inhibit 50% growth after 96 hours on CHO-K1 cell lines.[10] With CHO-DP12, concentrations of 0.1mg/L are needed to inhibit 50% growth after 96 hours. At 0.25mg/L, both CHO-K1 and CHO-DP12 display apoptosis, cell cycle arrest, and cytostaticity.[10] In a study by Kelly and colleagues, large growth arrest was identified in the G1 phase of the cell cycle of CHO-DP12 at 0.25 mg/L of bDtBPP.[7] This was associated with reduced entry to the S1-phase and the inhibition of cell replication. Additionally, the cell cycle related proteins, CDK1 and CDC42, were downregulated due to the cytostatic effects of bDtBPP.[11] It was demonstrated that the bDtBPP's effects on protein production were due to oxidative damage to the CHO cells.[11]

CHO-DG44 has a higher concentration threshold of sensitivity and toxicity toward bDtBPP.[7] At concentrations of 0.3mg/L, approximately 50% growth inhibition after 96 hours was observed. The toxic dose required for the cell to experience apoptosis was found to be ≥0.84mg/L.[7]

Additionally, bDtBPP has been shown to decrease CHO cell density and viability.[7] When 0.1mg/L of bDtBPP was added to cell-based assays, 35% of cell growth was inhibited after 72 hours. However, at toxic doses of 0.25mg/L, cell growth was inhibited by an additional 40%.[7] Hammond and colleagues determined that bDtBPP concentrations between 0.12mg/L and 0.73mg/L led to a decrease in the viable cell density (VCD).[12] Increasing bDtBPP levels led to a progressive decrease in the mitochondrial membrane potential (MMP), apoptosis, and a decline in cell health. It has been hypothesized that bDtBPP induces cell death through the intrinsic mitochondrial pathway.[12]

Studies have also shown an exponential increase in bDtBPP generation, due to extraction temperature and increased incubation time. Since the primary pathway of bDtBPP generation is through irradiation of oxidized Irgafos, single-use biomanufacturing components decrease this extractable's introduction into the bioprocessing.[12]

Cells are often cultured in serum-free, chemically defined media. Shah *et. al.*, found

that CHO-K1 cells' effective concentration to cause 50% cell death (EC_{50}) from bDtBPP significantly lowered when the cells were adapted to serum-free culture, compared to CHO-K1 cells grown in fetal bovine serum (FBS).[13] FBS can mask the cytotoxic effects of bDtBPP as well as change the properties of CHO cells. It was found that suspended CHO cells which were cultured in serum-free media had a smaller surface area compared to adherent cells grown in FBS. The presence of FBS in the cell media appears to mask the toxicity of bDtBPP, hence, when cells are adapted to serum-free media, a change in cell properties is observed. Suspended CHO-K1 cells are found to be most susceptible to minute amounts of bDtBPP causing the EC_{50} for bDtBPP to drop significantly with increased exposure time.[13]

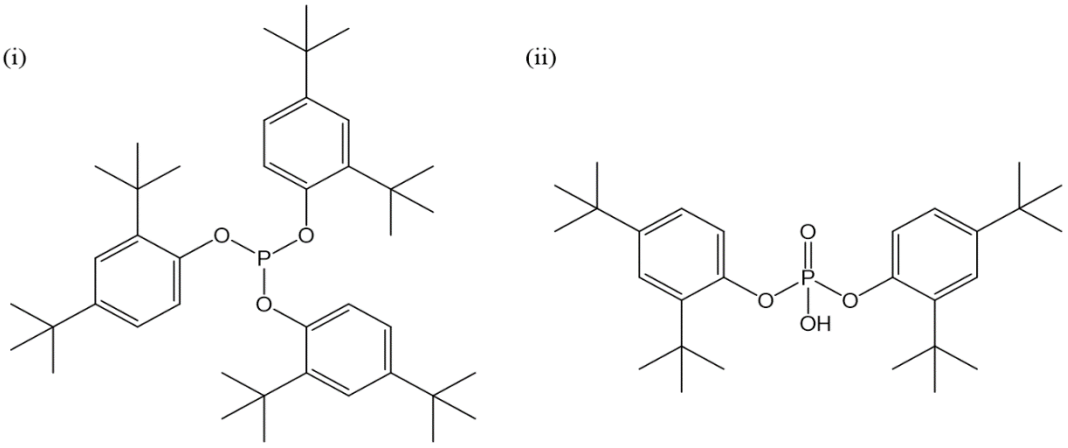

Figure 1: Chemical structure of (i) Irgafos 168 and (ii) bDtBPP, the toxic detergent

2.2 Irganox

Irganox is a common leachable encountered in biologic manufacturing. This organic leachable is usually present in its Irganox 1076 and 1010 forms (Figure 2), both of which may pose a risk to patients. Irganox 1010 (i.e. pentaerythritol tetrakis (3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate) is an antioxidant that is utilized as a protectant for various plastics in container closure systems. The National Institute for Occupational Safety and Health (NIOSH) has determined dose levels that could lead to lethal effects.[14] In rats, via inhalation, concentrations of Irganox 1010 greater than 1,950 mg/m^3 over the course of 4 hours is lethal. When administered orally, concentrations greater than 5 g/kg is lethal and can lead to acute toxicity in rats.[14] Regardless, it was determined that Irganox 1010 has a low risk of pulmonary, oral, or dermal toxicity in rabbits due to the lack of lethal concentrations present in biologic processing.[15] Irganox 1010 does not produce toxic effects on fertility or gestation and is not a skin or eye irritant based on *in vivo* experiments on rabbits. It is classified as both a non-carcinogenic and non-teratogenic leachable.[15] However, a study assessing developmental toxicity in mice reported that high doses led to a reduction in bone formation in the sternebrae.[16] With these results, a no-observed -adverse-effects-level (NOAEL) was set to 1000 mg/kg/day for maternal toxicity and 500 mg/kg/day for developmental toxicity.[16] Utilizing a repeat-dose toxicity study in dogs to determine a permitted daily exposure (PDE), 8 mg/person/ day was declared the limit for Irganox 1010.[15]

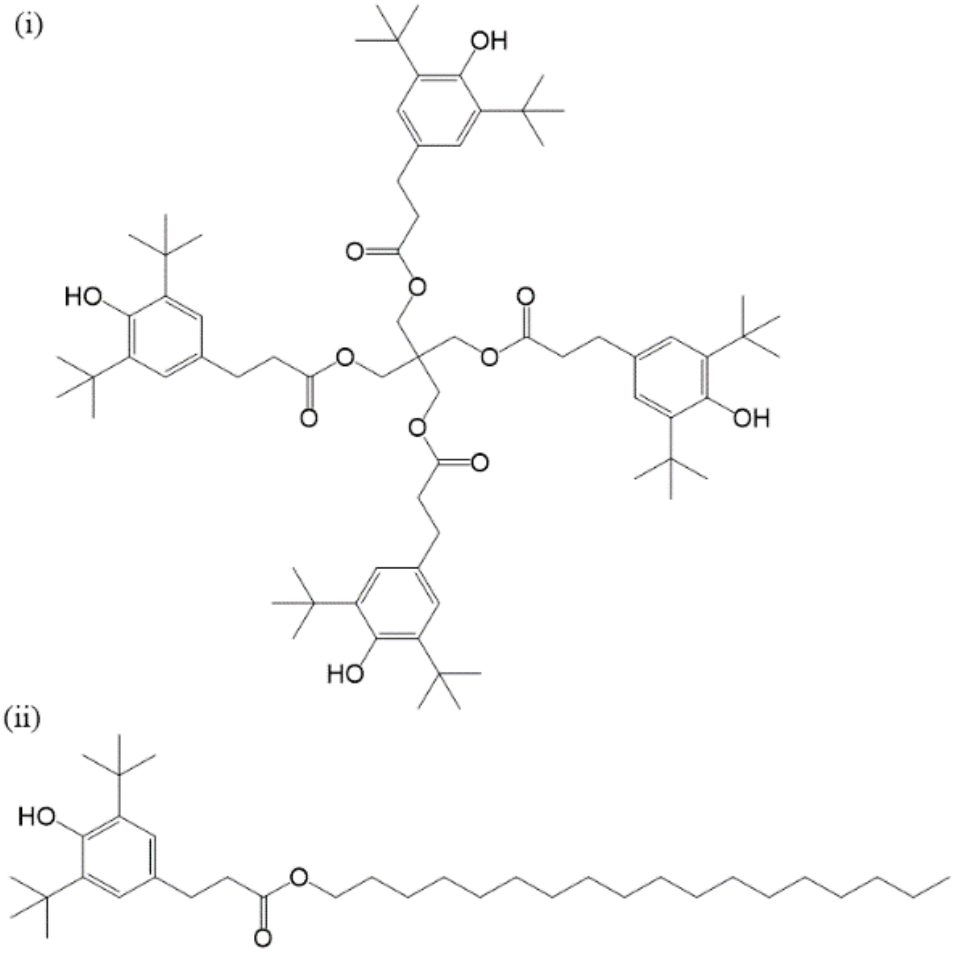

Figure 2: Chemical structure of (i) Irganox 1010 and (ii) Irganox 1076

Irganox 1076 (i.e., octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate) is an antioxidant that is used in the synthesis of polypropylene, polyethylene, polycarbonate, PVC, and polyurethane. In an extractable safety assessment, Irganox 1076 had low toxicity risk in animals following acute administration at doses exceeding the limits set by the United States Environmental Protection Agency (EPA).[17] However, at repeated doses, an increase in liver weight in rats was observed, due to morphological changes in their hepatocytes.[17] Irganox 1076 also increases hepatic microsomal xenobiotic metabolism in rats when administered orally.[18] The induction of uridine 5'-diphospho-glucuronosyltransferase, oxidases, and cytochrome P-450 enzymes can lead to potential altered drug concentrations. Furthermore, hypertrophy of hepatic centrilobular cells revealed considerable liver enlargement in both male and female rats. Additionally, the smooth endoplasmic reticulum showed apparent proliferation when examined by ultrastructural microscopic studies.[18] It was thus determined that the permitted daily exposure (PDE) of Irganox 1076 should not exceed 1mg/day as a leachable in parenteral biologics.[17] This calculation accounts for a 50-kg individual and an absorption rate of approximately 30%.[17]

It is important to note that Irganox 1076 also has four associated leachables, all with

structural similarities.[4] 3,5-Bis(1,1-dimethylethyl)-4-hydroxy-benzenepropanoic acid and 1-octadecanol are formed via hydrolysis (Figure 3). The former can subsequently undergo oxidation to 3,5-bis(1,1-dimethylethyl)-4-oxo-2,5-cyclohexadiene-1-propanoic acid, which can further be lactonized to 7,9-di-t-butyl-1-oxaspiro [4,5] deca-6,9-diene-2,8-dione.

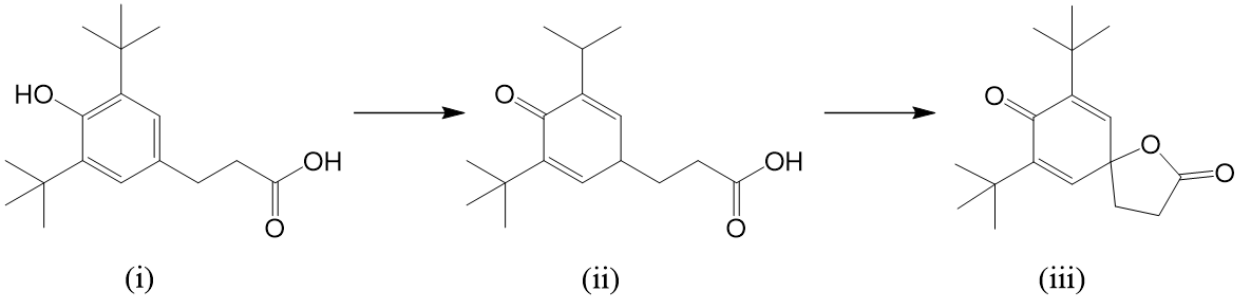

Figure 3: Chemical structure of (i) 3,5-bis(1,1-dimethylethyl)-4-hydroxy-benzenepropanoic acid, (ii) 3,5-bis(1,1-dimethylethyl)-4-oxo-2,5-cyclohexadiene-1-propanoic acid, and (iii) 7,9-di-t-butyl-1-oxaspiro [4,5] deca-6,9-diene-2,8-dione

2.3 Silicone

Silicone oils are often utilized as a lubricating agent in various devices in the manufacturing of syringes, needles, and plastic containers and has been identified as a leachable.[19] Silicone impurities can directly affect intermolecular interactions on biologic protein surfaces, leading to their aggregation and drug instability. Precipitation can also occur if there is an incompatibility present between silicone and the biologic of interest.[20]

In an investigation concerning the aggregation of four model proteins of differing molecular weights and isoelectric points, incubation at elevated temperatures with silicone oil at a concentration of 0.5% (w/v), resulted in significant levels of protein aggregation.[20] Hydrophobic proteins (i.e. Bovine Serum Albumin and Concanavalin A) were more likely to aggregate in comparison to hydrophilic ones (i.e. Lysozyme and Ribonuclease A) in the presence of silicone.[20] Another study assessed the effects of agitation, temperature, pH, and ionic strength on silicone oil-induced protein loss on a monomeric anti-streptavidin IgG1 monoclonal antibody.[19] Evaluation of oil droplet size in a silicone oil emulsion revealed that size distribution increased with time, suggesting antibody instability. In conclusion, presence of silicone oil can directly lead to the aggregation of biologic products.[19]

2.4 Acetyl tributyl citrate (ATbC)

Acetyl tributyl citrate (ATbC) is a plasticizer of PVC, cellulose resin, and synthetic rubber. It is formed by the esterification of citric acid with butanol and is biodegradable (Figure 4).[21] It is insoluble in water, but soluble in alcohol and other organic solvents.

Hassouna *et. al.*, reported that after aging 6 months at ambient temperature, polylactide (PLA) with 20% ATbC causes plasticizer leaching.[21] This resulted in a re-concentration of the plasticizer into the amorphous phase,

leading to a 10°C lowering of the glass transition temperature (T_g) from 26°C to 16°C. ATbC expelled from the blend during the crystallization contributed to an even greater amorphous phase and thus further reduction in T_g. Fortunately, after 6 months of aging no phase separation was observed. However, leaching was not observed in malleated polylactide (MAG-PLA) with 20% ATbC. This indicated that mobility restrictions derived from the mechanism of reactive extrusion grafting of ATbC onto anhydride, was at play. It was suggested that hydrogen bonding, in addition to the direct reactions between the hydroxyl of de-esterified ATbC and the anhydride from MAG-PLA, enabled the reduction of leaching and provided safer outcomes.[21]

ATbC, within bioprocessing, does have the potential to leach into the final drug product. Ingested ATbC has been reported to increase CYP3A4 messenger RNA levels (mRNA). This increase in enzyme activity was observed in intestinal cells, however not in the liver.[22] Regardless, this has the potential to cause drug-drug interactions or increase metabolism within the body.[22] Some findings also suggest that low levels of ATbC ingestion may be detrimental to ovarian function.[23] ATbC is rapidly absorbed and eliminated, however it does have a bioavailability of 27.4%. Metabolic clearance may play an important role in determining the systemic exposure of ATbC.[23]

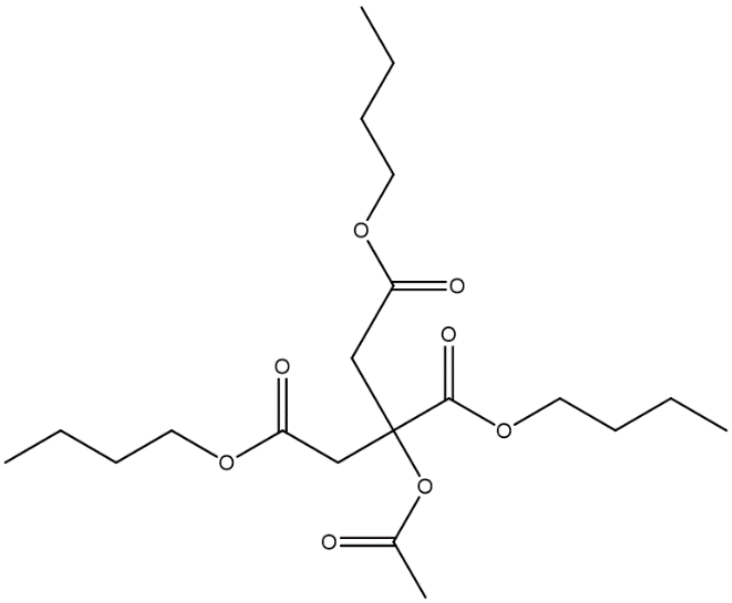

Figure 4: Chemical structure of acetyl tributyl citrate (ATbC)

2.5 Acrylic Acid

Acrylic acid is the simplest unsaturated carboxylic acid, with both functionalities allowing for polymerization, thus giving rise to three-dimensional networks (Figure 5). Acrylates are important in the polymer manufacturing process of plastics, adhesives, coatings, and elastomers.[24]

Leachable acrylic acid has the ability to modify the physico-chemical properties of proteins, such as charge and hydrophobicity.[25] Liu *et.*

al. found that acrylic acid has the potential to affect proteins at three distinct sites. Firstly, the amino group of a lysine side chain can react in a Michael's addition with acrylic acid, thus increasing its lipophilicity. A similar process can occur with the amino group of the N-terminus. The third modification is on histidine side chains, where the imidazole ring can react with acrylic acid, again via Michael's addition. These three modifications were found to occur with concentrations as low as 5µg/mL of acrylic acid.[25]

Acrylic acid can also undergo polymerization if exposed to light, heat, or oxygen. This has been reported to interfere with thymidines incorporation into DNA and for uracil into RNA, which can result in the inhibition of protein synthesis.[24]

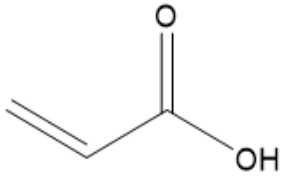

Figure 5: Chemical structure of acrylic acid

2.6 Additional Organic Extractables/Leachables

Caprolactam has also been identified as a potential leachable originating from PVC bags (Figure 6).[26] PVC bags are commonly used in the biopharmaceutical industry due to their flexibility. Caprolactam contamination can occur in the heat sterilization phase of bioprocessing. It was also found that caprolactam can migrate from the adhesion substance in the plastic overwrap of PVC bags. Plasticizer migration from PVC bags into an intravenous solution resulted in findings of trace amounts of caprolactam at levels of 1.2 to 15.0 mg.[26] Like Irganox 1076, caprolactam has associated leachables, including 1,8-diazacyclotetradecane-2,9-dione, with the corresponding hydrolyzed dimer, 1,8,15-triazacycloheneicosane-2,9,16-trione, and 1,8,15,22-tetraazacyclooctacosane-2,9,16,23-tetrone.[4] However, it should be noted that caprolactam has not been identified at substantial levels.[27] Generally, caprolactam is highly water soluble, which thus increases its potential to concentrate in various drug products.

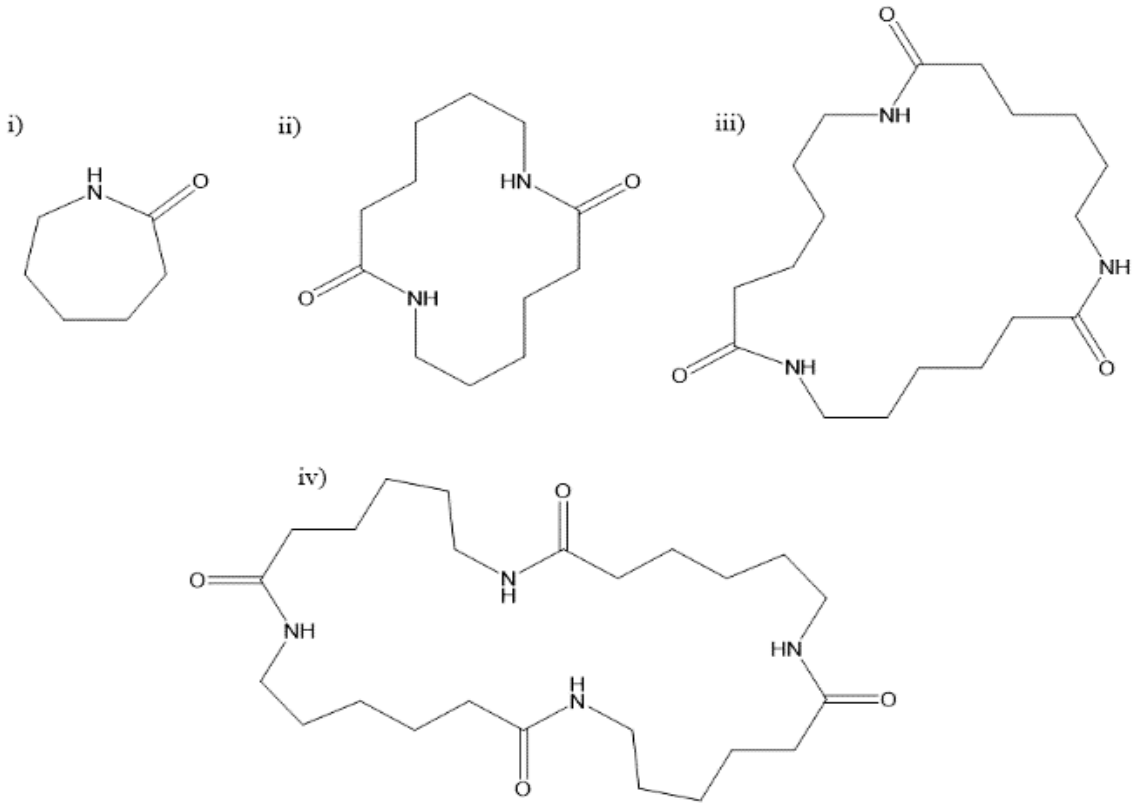

Figure 6: Chemical structure of (i) caprolactam, (ii) 1,8-diazacyclotetradecane-2,9-dione, (iii) 1,8,15-triazacycloheneicosane-2,9,16-trione, and (iv) 1,8,15,22-tetraazacyclooctacosane-2,9,16,23-tetrone

Lupersol 221 is used as an initiator in the synthesis of ethylene-vinyl acetate (EVA) resins, and as an additive in plastic bioprocessing bags. Trace amounts of Lupersol 221 have been detected in manufacturing, along with its associated leachables, as a diester with either 1,2-ethanediol, 1,4-butanediol, 1-acetoxy-1,2-ethanediol, and 1-acetoxy-1,4-butanediol.[4] Conclusive toxic effects have not been determined for either caprolactam or Lupersol 221. Due to this, there is insufficient evidence regarding safety concern thresholds for these two leachables.

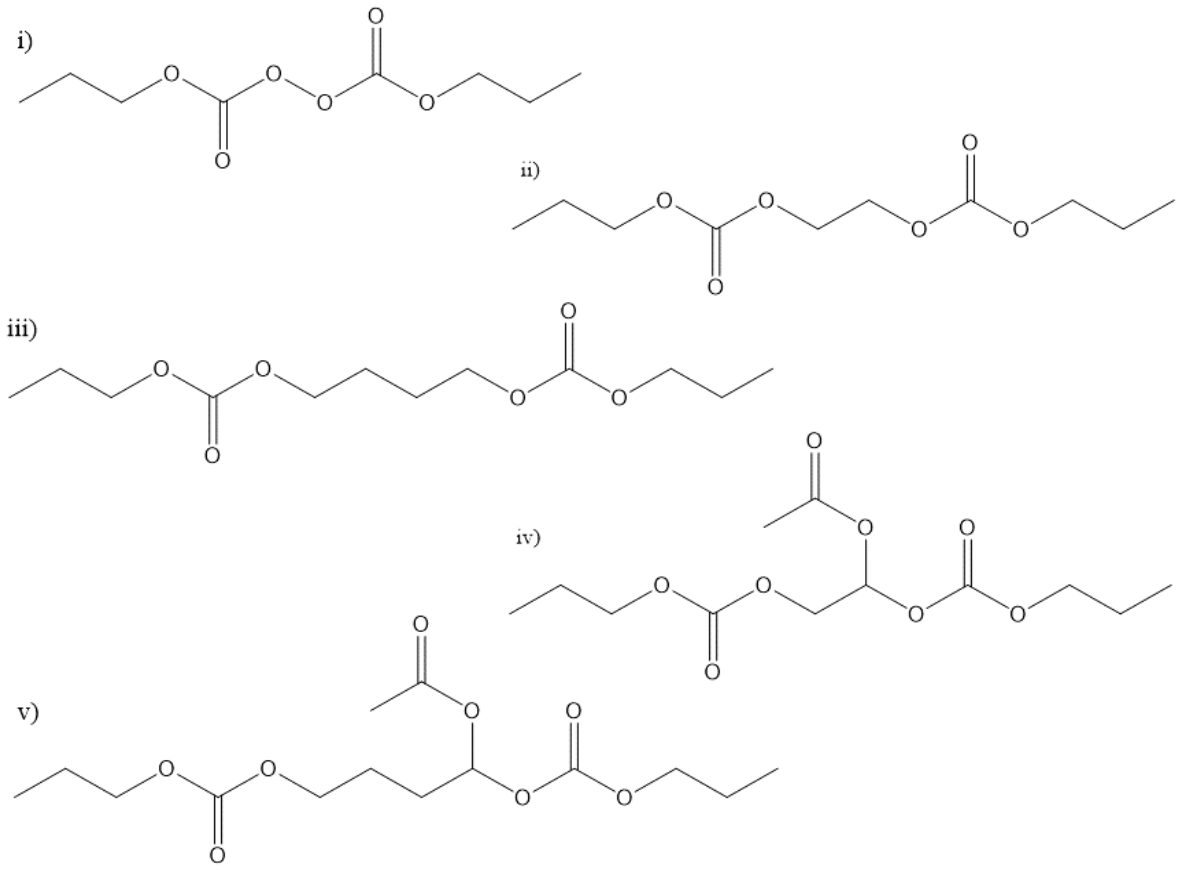

Figure 7: Chemical structure of (i) Lupersol 221 and (ii-v) associated leachables.

The extractable, di-2-ethylhexyl phthalate (DEHP), is a plasticizer used in the manufacturing of soft and flexible plastics (Figure 8). Cremophor and ethanol are known to promote leaching of DEHP from PVC bags.[28] Large amounts of DEHP can also leach into solutions containing either polysorbate 20 or 80.[28] This plasticizer is known to be hepatotoxic, and thus it is essential to minimize human exposure.[29] Blood stored in PVC bags for patients receiving blood transfusions are at higher risk of DEHP exposure. It has been reported that it can accumulate in lipophilic drugs, such as cyclosporine, paclitaxel, and miconazole.[29] To limit DEHP leaching into drug products, it has been suggested that lipophilic medication should be administered via PVC free bags and tubing, or administered directly after preparation.[30]

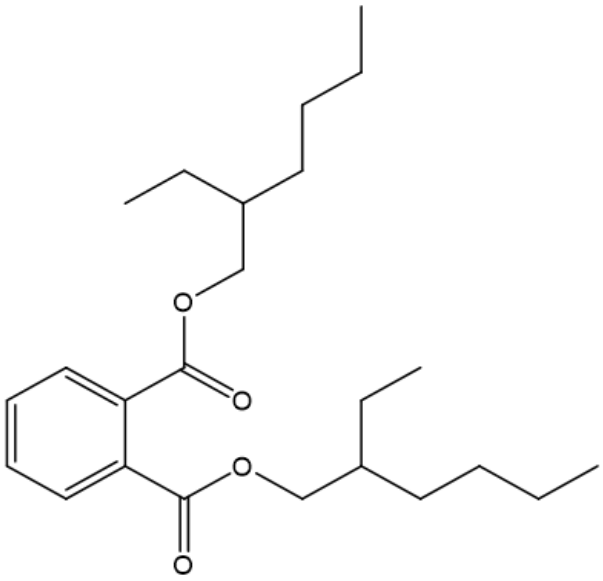

Figure 8: Chemical structure of DEHP

3.0 Metals

Heavy metals are one of the foremost extractables and leachables that can lead to physicochemical changes in the desirable biological product. Toxic metal levels can influence cell growth, viability, and the production of IgG. Careful monitoring and controlling levels are essential in bioprocessing.

3.1 Cobalt

Cobalt is an extractable/leachable metal found in single-use bags used in biological bioprocessing and is considered a high-risk factor. A recent study assessed multiple risk factors of cobalt during bioprocessing, specifically its impact on the galactosyltransferase enzyme activity in CHO cells and its effect on IgG expression.[31] It was determined that concentration changes differently influence cell growth, hence leading to altered effects on biological products.

An experiment was conducted by Prabhu et al. to measure the effects of manganese, an essential cofactor for cell growth, along with cobalt at varying concentrations between 0 and 1 mM.[31] Cobalt's effect on galactosyltransferase was dose-dependent. Upon increasing cobalt concentrations, enzyme activity decreased. It was suggested that this was due to the enzyme having two binding sites that are occupied by both manganese and cobalt. Furthermore, cobalt binding led to a lower activation compared to manganese.[31]

Moreover, the impact of cobalt on the IgG expression in recombinant CHO cells was evaluated to understand its effect on glycosylation.[31] Different cobalt concentrations were employed and after 3 days, it was shown that 300 uM led to cell death. Additionally, there was a 50% reduction in IgG titer, starting at concentrations of 200 uM. No observed effect on cell growth was seen at concentrations of 50 uM or less.[31]

Although concentrations between 1-100uM of cobalt increased the galactosylation levels in CHO cells, it was concluded that the addition of manganese supplement could slightly reverse and improve this galactosylation effect.[31] Cobalt also led to the increase in ROS generation, which itself stimulates a hypoxia-mediated reaction by lowering the dissolved oxygen levels. In addition, 50 uM of cobalt led to an increase in G1F glycan (mono galactosylated) by 3%, while at 200 uM the non-galactosylated (G0F) was increased by 5%. It was also noted that there was an increase in lactate production in the presence of 200 uM cobalt.[31]

3.2 Nickel

Nickel is also considered a trace metal leading to toxicity in CHO cells during biological bioprocessing. Glycosylation is an important post translational modification that impacts glycoproteins in CHO cells. It contributes to the quality of the biologic and is monitored and analyzed throughout bioprocessing.[31] Nickel has been reported to leach from different equipment used in bioprocessing, with the degree of leaching affected by the solution's pH, concentration, salt level, and temperature.[31]

A study done by Prabhu and Gadgil investigated the effect of nickel on CHO cells.[31] Nickel inhibited both fructosyltransferase and galactosyltransferase. Furthermore, nickel led to the formation of ROS, resulting in oxidative

stress and reduced galactosylation. Consequently, it was demonstrated that nickel caused a significant reduction in glycosylation of recombinant IgG.[31]

The effect of nickel on cell growth and viability was also tested.[31] Up to 50 uM of nickel showed no alteration on either growth or viability. Above 200 uM the cell growth was significantly reduced, while the viability remained >60% until nickel concentration reached 1 mM. Lactate production increased when the nickel concentration reached 200 uM. Reduction from 32% to 25% in glycosylation of the IgG was also observed at nickel concentration >1 mM. However, no effect on fructosylation was observed at any nickel concentration.[31]

Nickel's effect on galactosyltransferase was also evaluated.[31] As mentioned prior, manganese is an essential cofactor for the enzyme galactosyltransferase; thus, different concentrations of manganese were added with nickel to determine whether this would reverse the glycosylation reduction. Although the addition of manganese increased glycosylation, it was not sufficient enough to fully inhibit the reduction.[31]

Finally, Wang, *et. al*, evaluated nickel as one of the major leachables during bioprocessing to determine its effect on protein aggregation and precipitation.[32] Very small amounts of nickel from different equipment, such as nickel-plated connectors, led to protein precipitation. This precipitation was explained by the possible chelation between nickel and protein monomers. It was also seen that trace amounts of nickel were found in almost all excipients evaluated.[32]

3.3 Copper

Although copper is considered a fundamental element in multiple metabolic pathways, high concentrations can be toxic due to the generation of ROS, leading to cell damage.[33] Consequently, copper leaching from bioprocessing equipment can corrupt the optimal balance, induce toxicity, and prevent cell growth.[33]

Yuk investigated the maximum level of copper within a growth media, without causing toxicity. CHO cells were treated with different copper levels over 14 days.[33] The minimum threshold of copper to obtain regular cell growth without any toxicity, ranged between 30 to 60 nM, per packed cell volume. CHO cells treated with high levels of copper negatively impacted the quality of the biologic through the increase in the modification of IgG products. It was also demonstrated that CHO cells require a minimum copper level to survive and provide optimum performance. C-terminal proline amidation is known to be catalyzed by peptidyl glycine-hydroxylating monooxygenase which requires binding to copper in order to be activated. Higher copper concentration increased this reaction, leading to more IgG production.[33]

A study performed by Qian, *et. al*. showed that copper levels greater than 50uM led to higher protein aggregation, which further led to a reduction in protein quality.[34] Chaderjian, *et al*, demonstrated that cell viability was higher in a control group compared to those treated with lower copper levels.[35] Hence, it was assumed that this was due to high copper levels having a slower accumulation inside the cells. They also concluded that copper levels ranging between 0-100 uM copper do not impact cell growth.[35]

Dorival-García, *et. al* demonstrated that copper caused a bioaccumulation behavior in CHO cells, hence interrupted cell growth.[36] This accumulation was further explained by copper's effect on the glutathione-redox balance, which is known to be an essential protector against toxicity. This imbalance can lead to an interruption in cell growth. It was proposed that the copper concentration in packed cell volume should be greater than 30nM to obtain the desired benefits, while remaining below 60 nM to avoid copper toxicity.[36]

4.0 Conclusion

The use of disposable bioprocessing systems has steadily increased in the biopharmaceutical manufacturing industry however, this shift towards disposable plastic systems requires regulation and monitoring of extractables and leachables to ensure patient safety. Although the concentration of extractables and leachables are low in the final drug product, they have the ability to affect the quality and subsequently increase the risk of entering the patient. A number of safety thresholds have been evaluated for the most common extractables and leachables to ensure cell viability during biologic synthesis (Table 1). For example, cobalt, nickel, and copper were found to decrease cell growth at concentrations > 200 uM, >200 uM, and >60 nM, respectively.[31,33] Both cobalt & nickel lead to hypoxia-mediated reactions that lower the dissolved oxygen levels causing oxidative stress.[31] Maintaining safe concentrations for these toxic metals is essential in order to avoid any negative influence on cell growth, viability, and IgG production. Likewise, Irgafos 168 inhibits absolute CHO cell growth and is responsible for cell cycle arrest at concentrations greater than 0.035 mg/L. Other organic materials, such as silicone and acrylic acid, can also affect proteins via aggregation or modification at specific sites, compromising final product stability. Effectively monitoring the concentrations of these harmful extractables and leachables remains imperative to ensure the production of high-quality products and patient safety.

Table 1a: Extractables/Leachable Safety Concern Threshold

Extractable/Leachable	Cell Type	Safety Concern Threshold	Result	Ref.
bDtBPP (bis(2,4-di-tert -butylphenyl) phosphate)	CHO cells	0.2-0.73mg/L	Harmful to CHO cell growth and decreases cell density viability by 10-20x in 72h	7
	CHO-K1	0.035mg/L	50% growth inhibition in 96 hours	7
		0.25mg/L	Apoptosis, cell cycle arrest, cytostatic to cell growth and cytotoxic	7
	CHO-DP12	0.1mg/L	50% growth inhibition in 96 hours	7
		0.25mg/L	Apoptosis, G1 phase cell cycle arrest, cytostatic to cell growth, cytotoxic	7
	CHO-DG44	0.3mg/L	50% growth inhibition in 96 hours	7
		0.84mg/L	Apoptosis, cytostatic to cell growth, cytotoxic	7
Irganox 1076 Associated leachables: 3,5-bis(1,1-dimethylethyl) -4-hydroxy-benzenepropanoic acid 3,5-bis(1,1-dimethylethyl) -4-oxo-2,5-cyclohexadiene-1-propanoic acid 7,9-di-t-butyl-1-oxaspiro [4,5] deca-6,9-diene-2,8-dione 1-Octadecanol		1 mg/day	Hepatotoxicity UDP-glucuronyltransferase, oxidase, and CYP450 inducer Can cause proliferation of smooth endoplasmic reticulum	17,18
Irganox 1010		Inhalation (rat) -1,950mg/m^3 Oral (rats) - 5g/kg NOAEL (mice) Maternal toxicity – 100 mg/kg/day Developmental toxicity – 500 mg/kg/day PDE (based on study in dogs) - 8 mg/person/day	Cytotoxicity Reduction in bone formation in sternebrae	14-16

Table 1b: Extractables/Leachable Safety Concern Threshold

Silicone	Cyclic Olefin Copolymer (COC) Syringes	Extreme pH solutions (less than or equal to pH 2 and greater than or equal to pH 12) >1 ppm pH 2-12 solutions <0.2 ppm	Silicone oil can directly affect intermolecular interactions on biologic protein surfaces or indirectly affect solvents utilized leading to protein aggregation Proteins that are more hydrophobic (i.e. BSA, ConA) are more likely to aggregate than those that are more hydrophilic (i.e. lysozyme, RNase A)	20
Tribu- tylO-acetylcitrate (ATbC)		Polylactide (PLA) + 20% ATbC, aged for 6 months	Shift of T_g toward lower temperatures (26 to 16 degrees Celsius). ATbC expelled from the blend during the crystallization process of PLA contributed to plasticize the amorphous phase even more and therefore reduced further the Tg.	21
Acrylic Acid		5µg/mL	Can modify proteins at three different sites: 1) the lysine side chain, 2) the N-terminus, and 3) the histidine side chain, by the Michael reaction.	25
Caprolactam	Polyvinyl chloride (PVC) bags	N/A	Can migrate and leach into drug products due to high water solubility The resulting effect on products has not been concluded	26,27
Lupersol 221	Ethylene-vinyl acetate (EVA) resins	N/A	The resulting effect on products has not been concluded	4
di-2-ethylhexyl phthalate (DEHP)		Reaction with Polyvinyl chloride (PVC) container/closure systems	Hepatotoxic	29
2-ethylhexanoic acid		Reaction with styrene-butadiene-styrene block co-polymer	Accumulation can affect solution pH	37
Cobalt	CHO cells	> 200 uM	Reduction in IgG titer Reduction in cell growth	31
Nickel	CHO cells	> 200 uM	Nickel decreases Galactosylation of IgG Reduction in cell growth	31,32
Copper	CHO cells	> 50 uM	Increased IgG production Protein aggregation	33-36

Author contributions:

Natalie Ourfalian, Mahek Ramani, Grace Mosallam, David Priefer, and Aadya Jaipuria were responsible for the composition of the bulk of the manuscript including finding the referenced articles. Additionally, Natalie, Mahek, and Grace were responsible for creating the summative table within the manuscript. Ronny Priefer developed the idea of the manuscript and mentored the co-authors throughout the writing and proofread process.

Declaration of competing interest:

The authors have no competing interests to declare.

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