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Evaluation of solvent systems for optimized extractables studies of single use bioprocessing solutions

Noemí Dorival-García[a], Jonathan Bones[a,b,*]

[a] Characterisation and Comparability Laboratory, NIBRT–The National Institute for Bioprocessing Research and Training, Foster Avenue, Mount Merrion, Blackrock, Co., Dublin, Ireland
[b] School of Chemical and Bioprocess Engineering, University College Dublin, Belfield, Dublin 4, Ireland

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ABSTRACT

Despite their advantages, there is concern that single-use systems used in biopharmaceutical manufacture might release potentially toxic substances during standard unit operations that negatively impact cell growth. Characterization of the extractables profile for single-use systems is necessary to know which compounds potentially become leachables under operational cell culture conditions. A key issue in the design of extractables studies is the composition of the model solvent, in particular its pH and polarity. In this study, a new approach, based on design of experiments (DoE), has been applied to determine the composition of the model solvent for extractable profiling of single-use bags (SUBs). Particular focus was placed on the determination of the degradation products of the antioxidant Irgafos 168®, due to evidence that some of these degradation products have cytotoxic effects on CHO cells. Results indicated that 2-propanol:water is the most appropriate solvent for the extraction of highly hydrophobic compounds with polar groups and/or acid-base properties from SUBs. The described DoE approach simplifies the number of experiments, evaluates all possible solvent water mixtures to select the best extraction solvent based on polarity, establishes the influence of each variable and provides information about variable interaction, which represents an important improvement over current best practice. The developed approach was applied to seven SUBs from different vendors and production dates facilitating the identification of potentially non-satisfactory films for cultivation of CHO cell lines under process conditions.

1. Introduction

Disposable or single-use systems (SUS) are being increasingly implemented by the biopharmaceutical industry as a key part of producing a safe, regulatory compliant products through robust and efficient cell culture processes [1]. SUS offer distinct advantages such as elimination of cleaning requirements, reduced risk of cross-contamination, faster turnaround between campaigns, increased convenience and flexibility, reduced time for a new facility to become operational [2], along with reduced capital, time and energy expenditure [3]. It has been estimated that designing a new production facility based on SUS can reduce capital costs by up to 40% compared to a conventional hard-piped facility [4] Economic benefits of disposable technologies are becoming more important as biopharmaceutical manufacturers face increasing pressure to reduce product costs while maintaining product quality [2].

SUS such as single-use bags (SUBs) are manufactured from various polymeric materials whose chemical and physical properties are influenced by the chemical structure, polymerization process, presence of additives, as well as the associated manufacturing process. Furthermore, this material may also be affected by external factors such as heat, light, oxygen and sterilization conditions [3], as well as by incubation conditions, such as time and temperature, as significant amounts of chemical entities might be continuously extracted after days or weeks of incubation, depending on the particular working conditions of the SUB [5]. A significant concern exists that these plastic materials might release potentially toxic substances that could affect cell growth and product titers [6], so called extractables and leachables (E&Ls). These substances may include a variety of chemicals or their derivatives used in the manufacture of SUS such as catalysts, polymeric initiators, additives (*e.g.* antioxidants, lubricants, anti-tack and anti-static agents), oligomers with a low degree of polymerization, adhesives, anchoring agents, adhesive resins, irradiation-induced degrada-

* Corresponding author at: Characterisation and Comparability Laboratory, NIBRT–The National Institute for Bioprocessing Research and Training, Foster Avenue, Mount Merrion, Blackrock, Co., Dublin, Ireland.
E-mail address: jonathan.bones@nibrt.ie (J. Bones).

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tion products [7], and breakdown compounds from oxidation and hydrolysis degradation processes, such as hindered-amine stabilizers (HLAS) or phenolic antioxidants [8,9], which might occur during film manufacture or through post-migration degradation processes [10]. Recent studies have shown poor cell growth of some Chinese Hamster Ovary (CHO) cell lines in various SUBs [11,12]. One compound, bis(2,4-di-*tert*-butylphenyl) phosphate (bDtBPP), a degradation product derived from Irgafos 168®, a commonly used secondary antioxidant, was experimentally shown to inhibit cell growth of CHO cell lines even at trace concentrations [13]. Other degradation products from Irgafos 168® have been demonstrated to exhibit toxicological potential; 2,4-di-*tert*-butylphenol (DtBP) has been shown to be toxic to fish [14] and also mammalian cells [15].

Although potentially problematic, antioxidants are necessary to keep SUBs stable as they protect the polymer film during gamma irradiation and from oxidative degradation during extrusion and storage. However, it is important to optimize and control the concentration of such antioxidants to prevent these detrimental effects on cells as the levels of additives are linked with the film manufacturing process. It is known that melting and extrusion steps are mainly responsible for the degradation of polymers and also for the generation of breakdown products from additives [3,8], with bDtBPP being a noted example [3]. It was shown that by varying the settings for extruder temperature (*i.e.*, temperature during melting and processing of the resin), and output, that reflects residence time and shear heating in the extruder, levels of degradation products from additives in the resulting film could be deliberately altered [3]. Consequently, these specific manufacturing steps, along with sterilization by γ-irradiation [3,5], specifically dose and irradiation time, should be carefully optimized to reduce these side effects, leading to a significant reduction in the degradation products with potential biological effects. The chemistry of the base polymer itself can be as equally as important for cell growth as the additive package. Base polymer properties, such as molecular weight, will determine polymer-melting temperature, which is directly correlated to the temperature needed for processing of a resin during extrusion, which in turn, controls the formation of degradation products from additives [3].

Extractables are defined as "compounds that migrate from any product-contact material when exposed to an appropriate solvent under exaggerated conditions of time and temperature' [16], while leachables are "compounds, typically a subset of extractables, that migrate into the drug formulation from any product-contact material as a result of direct contact with the drug formulation under normal process conditions or accelerated storage conditions and are found in the final drug product' [16]. Extractables screening is crucial to provide a comprehensive view of potential substances that may become leachables [17]. A challenge facing the industry is the generation of meaningful data on extractables with model solvents that provide an accurate assessment of potential leachables into buffers or culture media during standard unit operations. The partitioning and migration of these substances between dissimilar phases depends on the properties of the plastic, the substance itself and the properties of the solution housed in a SUS. Factors like pH, media components (*e.g.* salts, sugars, amino acids, other ionic and non-ionic ingredients) and the presence of solubilising agents (*e.g.* organic solvents, surfactants) play important roles in partitioning behaviour. Other factors, like incubation/contact time, temperature and ratio of a sample's surface area to the volume of solvent (cm^2/mL) are also critical and should be included as part of the E&L testing protocols [18]. In this study it was intended to simulate the working incubation and temperature times for SUBs, which usually vary in a defined range during upstream processes, and consequently, these parameters were set in the simulation studies. It has been shown that the composition of the leachables profile is influenced mainly by pH and solubilising agents, whereas the presence of 'inactive' formulation components will not appreciably affect the partitioning phenomenon or the accumulation of leachables in such solutions [19,20]. Therefore, the most important characteristics of a model solvent for extractables studies are pH and polarity [10]. Whilst binary mixtures of organic solvents and water are commonly used as model solvents *e.g.* ethanol [21,22], acetonitrile [23], 2-propanol [24] and glycols [24,25] with in some cases, surfactants and buffers [23–26], the broad applicability of these systems is questionable. As the implementation rate of SUS within the biopharmaceutical industry increases, a pressing need exists for systematically designed studies for E&L analysis to provide comfort to companies using these platforms regarding their applicability and safety to both the manufacturing process and ultimately the patients who will receive the expressed product.

Here, an approach for establishing model solvent systems for extractables studies of SUS using design of experiments (DoE) is reported. DoE enables simplified optimisation of the extraction system across the design space through the performance of a reduced number of controlled experiments using response surface methodology. The presented approach enables the identification of the most appropriate solvent extraction system for a particular SUS to ensure an accurate representation of a range of extractables present that are also potential leachables.

2. Experimental

2.1. Chemicals and reagents

Water used throughout was from a Sartorius Stedim Biotech Arium® 61316 system (Göttigen, Germany). Analytical grade standards of Irgafos 168® (TBPP), 2,4-di-tert-butylphenol (DtBP) and tri-o-tolyl phosphate (TOP) were purchased from Sigma-Aldrich (Wicklow, Ireland). bis(2,4-di-tert-butylphenyl)phosphate (bDtBPP) (98% purity) and 2,4-di-*tert*-butylphenyl dihydrogen phosphate (DtBPP) were custom synthesized by Albany Molecular Research Inc. (Albany, NY). Tris(2,4-di-*tert*-butylphenyl)phosphate, that is Irgafos oxidized form (TBPP-ox) was synthesized by dissolution of Irgafos 168® in chloroform/isopropanol, 2:1 mixture and addition of a 5% molar excess of hydrogen peroxide (3% aqueous solution) [13]. Structures and chemical properties of the compounds under study are shown in Table 1 (A). All extraction solvents, acids, bases and general reagents used throughout the study were ACS reagent grade or better and were from Sigma-Aldrich (Wicklow, Ireland). Hydrogen peroxide (30% wt, non-stabilised) was supplied by Acros organics (Dublin, Ireland). LC-MS Optima grade water and acetonitrile were obtained from Fisher Scientific (Dublin, Ireland). Individual stock solutions of compounds (2000 μg mL^{-1}) were prepared in acetonitrile with the exception of Irgafos 168 which was prepared in acetone. All standards were stored at 4 °C for a maximum of one month. Working standard mixtures were prepared by diluting the individual stock solution in the initial mobile phase immediately before use.

Plastic material used in this study were multilayer films from single-used bags. For the extractables study, the material contacting the bioprocess fluid was always the inner layer from the bag that was made of ethylene-vinyl acetate (EVA) and different density grades of polyethylene (PE): linear low-density PE (LLDPE), low-density PE (LDPE), and ultralow-density PE (ULDPE). A detailed description of the film layers are given in Table S1, (supplementary material).

2.2. Sample preparation

Extraction was performed at small-scale, on the basis of a previous study [13] which consisted in cutting the polymer film with