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Toward a robust lipid supplementation for SP2/0 fed-batch culture: Improving process performance by leveraging lipoprotein variability

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ZUSAMMENFASSUNG

Im Zusammenhang mit der Produktion rekombinanter monoklonaler Antikörper stellt die Verwendung von Hybridomzellen aufgrund ihrer Lipid-Auxotrophie eine Herausforderung dar. Der Ersatz von Serum durch Lipoproteine zur Lipidsupplementierung in SP2/0-Zellen ist möglich, allerdings bleiben derzeit noch Probleme, die im Rahmen dieser Dissertation angegangen werden sollen. Die inhärente Variabilität von Lipoprotein-Chargen, die aus tierischen Quellen stammen, hat einen erheblichen Einfluss auf das Zellwachstum und die Gesamteffizienz des vorgeschalteten Zellkulturprozesses. Ziel dieser Forschungsarbeit ist es, die Beziehung zwischen der Variabilität von Lipoprotein-Chargen und der Leistung der vorgeschalteten Zellkulturprozesse zu klären. Es wurde eine Untersuchung unter Verwendung mehrerer Lipoprotein-Chargen desselben Lieferanten durchgeführt. Bestimmte Chargen wiesen während der Zellkulturprozesse schlechte Leistungsmerkmale auf, die sich in einer Apoptoseinduktion und einem vorzeitigen Rückgang der Lebensfähigkeit während der Fed-Batch-Produktion äußerten. Eine physikalisch-chemische Charakterisierung des Lipoproteinzusatzes bestätigte, dass Lipoproteine überwiegend Sterole, Fettsäuren und Apolipoproteine enthalten, wobei ein beträchtlicher Anteil dieser Bestandteile oxidiert ist. Die Induktion einer kontrollierten Oxidation in Lipoprotein-Formulierungen führte zu beobachtbaren Veränderungen, einschließlich einer Braunfärbung der Lipoproteinzusätze und einer erhöhten UV-Absorption, die wiederum mit einer verminderten Leistung in Zellkulturprozessen korrelierte. Diese Erkenntnis ermöglichte die Festlegung eines Schwellenwerts für die Absorption bei 276 nm, der die Identifizierung von oxidierten und schlecht funktionierenden Chargen von Lipoproteinzusätzen erleichtert. Diese Methode dient dazu, Lipoprotein-Chargen mit schlechter Leistung präventiv zu erkennen und aus dem Herstellungsprozess auszuschließen, wodurch die Produktionsstabilität erhöht wird. Insbesondere wurde festgestellt, dass die chemischen Verbindungen, die für das Absinken der Lebensfähigkeit verantwortlich sind, bereits im Rinderserum-Rohmaterial vorhanden sind und nicht während des Umwandlungsprozesses in Lipoproteinzusätze eingeführt wurden. Um die durch Lipoproteine verursachten Einschränkungen zu überwinden, wurden chemisch definierte Lipidzusätze formuliert. Unter Verwendung von Methyl-Beta-Cyclodextrin-Einschlusskomplexen wurden Fettsäuren und Cholesterin effektiv in Zellkulturmedien solubilisiert. Das Lipid-Cyclodextrin-Gleichgewicht sowie die Fettsäure- und Cholesterinverhältnisse wurden optimiert, um den Lipidbedarf der SP2/0-Zellen zu decken und gleichzeitig die zytotoxischen Auswirkungen der Cyclodextrinverwendung abzuschwächen. Obwohl die Cyclodextrin-Einschlusskomplexe mit Lipiden das Potenzial zeigten, Lipoproteine im Prozess zu ersetzen, erbrachten sie eine minderwertige Leistung, die durch einen frühen Rückgang der Lebensfähigkeit der Zellen und ein verringertes Gesamtwachstum gekennzeichnet war. Es wurde eine Supplementierung mit verschiedenen Verbindungen untersucht, wobei sich Trolox als vielversprechender Kandidat erwies, der die mit der Oxidation verbundenen Probleme wirksam abschwächt. Diese Ergebnisse sollen dazu beitragen, die Formulierung von Zellkulturmedien weiter zu optimieren und Parameter zu identifizieren, die das Wachstum und die Produktionsleistung von Zellen für die rekombinante Antikörperproduktion beeinflussen.

ABSTRACT

In the context of recombinant monoclonal antibody production, the utilization of hybridoma cells is challenging due to their lipid auxotrophy. Although substituting serum with lipoproteins for lipid supplementation in SP2/0 cells has mitigated certain limitations associated with serum utilization, it has not addressed all the obstacles. The inherent variability in lipoprotein batches, derived from animal sources, exerts a significant impact on cell growth and the overall efficiency of the upstream cell culture process. This research aims to elucidate the relationship between lipoprotein batch-to-batch variability and the performance of upstream cell culture processes. An investigation was conducted utilizing multiple lipoprotein supplement batches from the same supplier. Notably, certain batches exhibited low performance characteristics during cell culture processes, manifesting as apoptosis induction and premature viability decline during fed-batch production. A physicochemical characterization of the lipoprotein supplement confirmed that lipoproteins predominantly contain sterols, fatty acids, and apolipoproteins, with a notable proportion of these constituents being oxidized. The induction of controlled oxidation in lipoprotein supplements resulted in observable changes, including browning of the supplement and increased UV absorbance, which, in turn, correlated with diminished performance in cell culture processes. This insight enabled the establishment of a threshold absorbance level at 276 nm, facilitating the identification of oxidized and low-performing lipoprotein supplement batches. This method serves to preemptively detect and exclude low-performing lipoprotein batches from the manufacturing process, thereby enhancing production robustness. Notably, it was determined that the chemical compounds responsible for viability drops were already present in the bovine serum raw material and were not introduced during the transformation process into lipoprotein supplements. To overcome limitations caused by lipoproteins, chemically defined lipid supplements were formulated. Utilizing methyl-beta-cyclodextrin inclusion complexes, fatty acids, and cholesterol were effectively solubilized in cell culture media. Optimization of the lipid-cyclodextrin balance, as well as fatty acid and cholesterol ratios, was undertaken to match the lipid requirements of SP2/0 cells while mitigating any cytotoxic effects stemming from cyclodextrin utilization. Although the cyclodextrin inclusion complexes with lipids demonstrated the potential to replace lipoproteins in the process, they yielded inferior performance, characterized by early viability decline and reduced overall growth. Hence, this alternative did not prove to be a viable substitute for lipoprotein supplementation, highlighting the challenges that persist in achieving chemically defined lipid supplementation in SP2/0 cell cultures with performance comparable to lipoprotein supplementation. Various other compounds were explored to support cell growth, with Trolox emerging as a promising candidate, effectively mitigating oxidation-related issues. These results should help to further optimize the formulation of cell culture media and identify parameters that influence the growth and production performance of cells for recombinant antibody production.

1. INTRODUCTION

Mammalian cells are used to produce recombinant protein that are widely used in biotechnology, medicine, and research. The development of hybridoma technology, by fusion of a short-lived lymphocyte B and a myeloma cell line to form immortal antibody-secreting cell line derived (Milstein, 1999), allows the production and manipulation of monoclonal antibodies (mAb). Hybridoma cell lines have been used to manufacture recombinant mAb for over 25 years (Dhara, Naik, Majewska, & Betenbaugh, 2018), however they are eclipsed by CHO cells which have several advantages over hybridoma cells.

One of the major drawbacks of using hybridoma cells for mAb production is their lipid auxotrophy, their dependence on fatty acid (El Kouchni, 2011) and/or cholesterol supplementation (Sato, et al., 1988) for optimal growth. Historically, serum provided lipids to the cell culture, however, the use of serum is associated with process variability and poses a contamination risk when used in biopharmaceutical manufacturing (Van der Valk, et al., 2018). Replacement of serum by lipoprotein, a purified fraction of serum, has overcome several serum limitations but not all. Lipoprotein supplementation fulfils Sp2/0 hybridoma cell line lipid requirements, supports cell growth and enhances mAb production (Savonnière, et al., 1996). However, lipoprotein batch-to-batch variability, inherent to animal derived component was reported in the literature (Miles Inc. Diagnostics Division, 1991). The object of the present research is to understand and try to prevent the observed batch-to-batch variability in an upstream cell culture process, which had a significant impact on cell growth and process performance. Notably, some low-performing batches were identified during cell culture process without an identified root cause or detection method in place to this day to identify such poor performing batches.

1.1. Problem Statement

Challenges associated with lipoprotein supplementation of SP2/0 culture are similar to those associated with serum-based media. The composition of lipoprotein supplements for mammalian cell culture are likely to contain apolipoprotein, phospholipids, triglycerides, esterified and unesterified cholesterol, and trace of other serum compounds, but the effect of these components on the culture is not fully understood. Indeed, variation of lipoprotein composition triggers process performance variations. A better understanding of the correlation between lipoprotein components and process performance could increase process robustness. Moreover, identification of lipoprotein growth promoting or inhibiting compounds may enable lipoprotein batch selection in addition to batch supplementation with growth promoting compounds. Besides variation of lipoprotein composition, oxidation of lipoprotein could also alter batch performance; indeed oxidized lipids are able to trigger cell death mechanisms (Gaschler & Stockwell, 2017). For all these reasons, the development of a chemically-defined lipid formulation able to substitute to lipoprotein would be beneficial for process robustness. Fatty-acids complexed with rHSA (El Kouchni, 2011) and inclusions of methyl-β-

cyclodextrin with cholesterol (Gorfien, et al., 2000) were reported to substitute for animal derived components in serum-free culture of hybridoma cells.

1.2. Hypothesis statement

Associating physico-chemical characterization of several lipoprotein supplements batches and evaluation of their performance in cell culture tests, can allow to correlate lipoprotein composition with process performance, leading to the identification of growth promoting and limiting compounds. Thus, increasing understanding of the correlation between lipoprotein composition and batch performance would enable lipoprotein supplement manufacturing process improvement, lipoprotein supplement batch selection or supplementation with growth promoting compounds. Evaluation of lipoprotein susceptibility to oxidation and its impact on process performance could lead to manufacturing, packaging, and storage recommendations.

Lastly, identification of lipoprotein essential components for the culture would be a starting point for the formulation of a lipoprotein alternative lipid supplement. Fatty-acids complexed with rHSA (El Kouchni, 2011) and inclusion complexes of methyl-β-cyclodextrin (mbCD) with cholesterol (Gorfien, et al., 2000) were reported to substitute for animal derived components in serum-free culture of hybridoma cells. Inclusion complexes of mbCD and fatty acids is a potential solution to fulfill SP2/0 fatty acids requirement. However, attention should be given to the fatty acid formulation and the balance between fatty acids and mbCD.

1.3. Research Objectives

The primary objectives of this research can be described as follows:

1. Characterize the chemical composition and evaluate the performance in cell culture experiments of a representative sample of lipoprotein supplement batches. Then correlate lipoprotein composition with batch performance in order to identify growth promoting and limiting compounds.

2. Evaluate lipoprotein susceptibility to oxidation and its impact on process performance, if it is relevant, propose mitigation measures.

3. Based on the correlation between lipoprotein composition and batch performance, propose lipoprotein supplement manufacturing process improvement; evaluate lipoprotein supplementation with growth-promoting compounds to mitigate lipoprotein batch-to-batch variability and increase process performance; propose fingerprinting methods to detect low-performing batches.

4. Formulate a lipoprotein alternative lipid supplement to replace lipoprotein in SP2/0 cell culture based on the mbCD ability to solubilize lipids in aqueous solution and to deliver lipids to cells.

To answer the research questions, we treat a case study dealing with a particular lipoprotein supplement reference. For business reasons we cannot give the exact reference of the product, so we will simply refer to it as "the lipoprotein supplement" throughout this manuscript.

2. LITERATURE REVIEW

2.1. Overview of biopharmaceutical recombinant proteins production

Since the purification of penicillin from the mold Penicillium in the 1910s and the first transfer of genetic material in the 1970s, biotechnology has been applied to discover and produce pharmaceutical drugs leading to an uninterrupted growth of the pharmaceutical industry. The commercial production of recombinant proteins for therapeutic applications has started in the early 1980s, with a monoclonal antibody (mAb) to prevent kidney transplant rejection. Therapeutic proteins are high-molecular mass substances with varying biological properties useful for therapeutic applications in oncology (Zhang, Medeiros, & Young, 2018 ; Modest, et al., 2018), rheumatology, hematology, vaccination (Atanackovic, et al., 2019), for the treatment of autoimmune diseases (Tripathi & Shrivastava, 2019), viral infection (Saphire, et al., 2018) and were tested for SARS-COVID-19 infection (Chen, et al., 2021). The production of a wide variety of recombinant proteins was made possible thanks to recent progress in biological engineering and advances in recombinant DNA technologies. Despite a wide variety of forms and applications most of the approved biopharmaceutical proteins belong to the class of mAbs. Nowadays, with more than 100 mAbs approved in the United States (Kaplon, Chenoweth, Crescioli, & Reichert, 2022), the market of therapeutic antibodies represents 70 % of the global recombinant proteins market with a value of 163 billion of dollars and it is going to expand within the next years with more than 1200 monoclonal antibody candidates currently in development (Ecker, Dawn, Jones, & Levine, 2015). Massive progress made in the field of oncology, especially on the use of mAb in cancer therapy, were spotlighted when cancer immunotherapy was chosen as Science's 2013 Breakthrough of the Year (Coontz, 2013).

2.1.1. Therapeutic antibodies

Antibodies, also known as immunoglobulins, are used by the immune system of mammals for recognition and neutralization of foreign bodies. Antibodies work by binding to their target molecules or cells and triggering an immune response that participate in neutralizing and eliminating the targets.

Antibodies are Y shape proteins (Figure 1) with two edges able to bind specific antigen and the other edge triggering the immune response.

2.1.1.1. Structure of therapeutic antibodies

Antibodies are about 150 kDa large proteins with a typical Y shape that can be fragmented either in functional or structural subassemblies. Disulfide bonds between free thiol groups on mAbs structure and noncovalent interactions maintain the three-dimensional structure of mAbs.

Structural mAb subassemblies

A monoclonal antibody contains two heavy chains around 50 kDa each (H) and two light chains around 25 kDa each (L). Heavy chains are composed of a variable domain (V_H) and three constant domains

(C_H1,C_H2 and C_H3). Similarly, light chains are composed of a variable domain (V_L) and a constant domain (C_L). Both heavy and light chains have a constant (C) and a variable (V) region. Both heavy chains are connected by disulfide bonds in the hinge region, located between C_H1 and C_H2, and non-covalent interactions in the C_H3 region. Each heavy chain is attached to a light chain by disulfide bonds.

Functional mAb subassemblies

A monoclonal antibody can be divided in two fragment antigen binding (Fab) regions, each comprising a light chain, the C_H1 and the V_H domains and one fragment crystalline (Fc) region formed by the C_H2 and C_H3 domains. The Fab regions are responsible for the antigen binding and the Fc region is able to bind effector molecules and trigger an immune response.

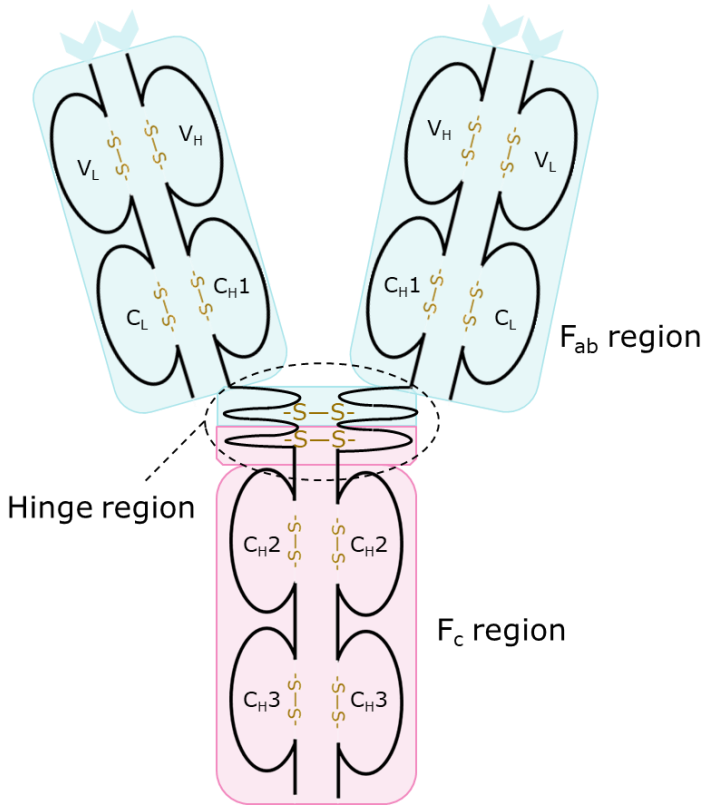


Figure 1: Antibody is composed of two heavy (H) and light (L) chains. The heavy chains contain a variable domain (V_H) and three constant domains (C_H1, C_H2 and C_H3). The light chains contain one variable domain (V_L) and one constant domain (C_L). Both heavy chains are connected by inter-chain disulfide bonds in the hinge region, located between C_H1 and C_H2, and non-covalent interactions in the C_H3 region. Each heavy chain is attached to a light chain by inter-chain disulfide bonds. The fragment antigen binding (Fab) regions (in blue) comprise a light chain, the C_H1 and the V_H domains and the fragment crystalline (Fc) region (in pink) is formed by the C_H2 and C_H3 domains.

2.1.1.2. Quality attributes of therapeutic antibodies

Drug product quality attributes are physical, chemical, biological or microbiological property or characteristic that should be within an appropriate limit, range or distribution to ensure the desired product quality (ICH Q8(R2) Pharmaceutical Development, 2009), *i.e.*, to ensure patient safety and treatment efficiency (ICH Q6B Specifications: Test procedures and acceptance criteria for

biotechnological/biological products, 1999). Quality attributes for a mAb include (i) bioactivity attributes such as effector function and activity, (ii) product variants such as aggregates, fragments, charge-variants also known as charge isotypes (acidic and basic forms) and post-translational modifications, especially glycosylation, non-enzymatic modifications including oxidation, deamidation and truncation, (iii) process-related impurities such as host cell proteins and DNA ; microbial contaminant like endotoxin, viral contaminant or prion, an (iv, excipient levels, pH, osmolality, appearance including turbidity, color and subvisible particles. Like many proteins, antibodies are subject to post-translational modification (PTM). PTM are modification that took place following the translation of a mRNA sequence into a polypeptide chain. Some PTMs are part of the protein biosynthesis and contribute to the broad range of mature antibodies (Magdelaine-Beuzelin, et al., 2007). On the other hand, some PTMs, like protein carbonylation are detrimental to the protein activity (Hauck & Bernlohr, 2016). Because PTMs involving addition of functional groups such as glycosylation, amidation, phosphorylation or disulfide linkages occur before antibody secretion (Walsh, 2010), most of the critical PTMs for mAb maturation take place during the cell culture part of the biopharmaceutical manufacturing process. Thus, the effect of cell culture conditions (temperature, pH, oxidative stress) must be carefully considered to ensure the production of mAb that meet quality attributes specifications (Li, et al., 2015). Meanwhile, avoiding deleterious modifications like protein aggregation, oxidation and deamidation during the purification part of the biopharmaceutical manufacturing process is critical to ensure high quality standards (Jenkins, Murphy, & Tyther, 2008).

2.1.2. Cell lines as expression systems

Recombinant proteins are industrially produced by a variety of expression systems including bacterial, yeast, insect, and mammalian cells. However, only mammalian cells can achieve appropriate post-translational modifications, especially N-linked glycosylation (Walsh & Jefferis, 2006), making mammalian cells the most used expression system used for monoclonal antibody production. Among mAb products approved, chinese Hamster Ovary (CHO) is by far the most common mammalian cell line in use for production. Remaining mAb are produced by hybridoma expression systems, mainly NS0 and SP2/0 cell lines (Walsh, 2018).

2.1.2.1. Hybridoma technology

An easy method to produce monoclonal antibodies that bind to a specific antigen is the hybridoma technology. A mammal, usually mouse, or rabbit, is immunized by exposition to an antigen, consequently its B cells will express an associated antibody. Then the B cell are recovered and fused with a parental myeloma cell line such as NS0 or SP2/0 cells. Polyethylene glycol is used to fuse adjacent membranes (Yang & Shen, 2006) of B cell and parental cells leading to hybrid fuse cells called hybridoma. Thanks to a selective medium only the fused cells can grow. The hybridoma clones are grown from single parent cells in the selective medium and the best clone is selected based on the ability of the antibody to bind to the antigen, the productivity, and the stability of the clone. Because

the fusion has occurred with a myeloma cell, hybridoma cells are immortal and so are suitable for long-term culture and large-scale manufacturing. Among parental cell lines used to make hybridoma cell lines, NS0 murine myeloma line is the most used fusion partner for producing antibodies at manufacturing scale (Walsh, 2018). NS1 cell line only secrets IgG light chain (NS stands for non-secreting) and NS0 cell line does not secrete either light or heavy chains. Both cell lines are derived from the P3K myeloma cell line by multiple passages and subcloning steps. Besides NS0, SP2/0 is the second most used hybridoma cell line. SP2/0-Ag14, also called SP2/0, is derived from SP2/HLGK, a hybrid between BALB/c spleen cells and the myeloma cell line P3-X63-Ag8, itself deriving from B lymphocyte (Shulman, Wilde, & Köhler, 1978). SP2/0 was used as a fusion partner for the recombinant expression of antibody because SP2/0 cell line was characterized by a high level of monoclonal antibody production (Melixetian, et al., 2003). SP2/0 cell line has lost the ability to secrete antibodies, because it lacks the chromosomes 6 and 12 that contain the genes coding for the mouse immunoglobulin light and heavy chains, respectively (Melixetian, et al., 2003). Thus, SP2/0 cell line can be fused with B cells and secrete only the specific antibody encoded by the cell parent. Thanks to this feature, SP2/0 cell line became one of the best fusion partners for making hybridoma cell lines.

2.1.3. Manufacturing process

Biopharmaceutical manufacturing includes two successive steps, the drug substance (DS) and the drug product (DP) manufacturing. The drug substance represents the active pharmaceutical ingredient, free of impurities, before transformation into a usable drug. The drug substance is formulated with excipient then divided into individual doses according to the finished form of the drug. The finished dosage form is named drug product. Lastly, the drug product is used in medical treatment by medical professionals or patients themselves. mAb DS manufacturing is composed of two distinct parts, the up-stream process (USP) and the down-stream process (DSP). The USP includes the cell expansion steps from thawing of a vial containing cells, usually called working cell bank vial, to the harvest of the production bioreactor. Cell expansion steps aims at increasing working volume from 1 ml to several thousand liters (depending on the production scale). These steps can take place in plastic flasks, roller bottles, spinners, wave-bags or bioreactors. The production step of the manufacturing process takes place in a larger bioreactor. Depending on the production mode, the harvest is collected though the culture (perfusion mode) or at the end of the culture (batch or fed-batch modes). The DSP includes at least one capture chromatography step, one or several polishing chromatography steps, ultrafiltration steps to concentrate the product or change the buffer. Several steps are designed to eliminate potential viral contaminants, such as viral inactivation at low pH, and nanofiltration. Chromatography steps can also be used for viral clearance purpose. The DSP process is completed by a final filtration before filling DS containers in order to ensure the absence of microbial contaminants in the DS. Lastly, several support activities are required to perform USP and DSP manufacturing processes. These activities include but are not limited to (i) the production of purified water (like water for injection), (ii) application of heating, ventilation and air conditioning (HVAC) technologies to ensure indoor air

quality, (iii) production of media for cell culture and buffers for purification steps, (iv) production and storage of cell banks (v) quality control, quality assurance and regulatory activities. All these activities are conducted according to good manufacturing practices (GMP).

2.2. Cell culture medium

A cell culture medium (CCM) is a solution formulated to provide cell nutrients, ensure suitable physicochemical and physiological conditions to support cell growth and maintenance, while enhancing recombinant protein production. Because CCM composition is critical for highly productive and cost-efficient cell culture processes, CCM improvement has appeared as one of the most important improvements ever in cell culture technology.

2.2.1. Classification of cell culture media

CCM composition differs greatly depending on the cell type, the origin (animal species), and the purpose of the culturing (Yao & Asayama, 2017). Eagle initiated the development of modern cell culture media (CCM) with the formulation of the Minimal Essential Medium (MEM) (Eagle, 1955). MEM contains only the minimum necessary components: inorganic salts, amino acids, vitamins, glucose and phenol red, thus MEM must be supplemented with serum to support mammalian cell lines. Dulbecco's Modified Essential Medium (DMEM or DME) (Dulbecco & Freeman, 1959) is a modified version of MEM, it is an enhanced supplementary formulation, enriched in amino acid and vitamins. DMEM is suitable for cell and tissue culture but still requires serum supplementation. By contrast with MEM and DMEM, Ham's F-12 medium (F-12) was the first basal medium able to support serum-free culture of some mammalian cell lines (Ham, 1965). A notable step in the development of serum-free media was the identification of insulin, transferrin, selenium and ethanolamine as lacking elements in DMEM /F-12 basal media to support long-term cultivation of mammalian cells in serum-free media (Murakami H., et al., 1982).

While there has been some interest in replacement of serum and alternatives to animal derived components in the literature since 1980, a concerted effort of the pharmaceutical industry only began at the beginning of the 21th century. In that time, there have been a large variety of CCM developed that support high-density mammalian cell culture. Nowadays, most serum-free culture basal media for CHO, hybridoma and stem cells are derived from a combination of DMEM and F-12 media, supplemented with chemical compounds. Based on the nature of their constituents, CCM can be divided in distinct categories (Table 1).

Classification	Description
Serum-based media	Serum-based media are supplemented with serum of animal or human origin
Serum-free media	Serum-free media do not require supplementation with serum but may contain discrete proteins or bulk protein fractions (e.g., animal tissue or plant extracts) and are thus regarded as chemically undefined.
Animal component-free media	Animal component-free media contain no components of animal or human origin.
Protein-free media	Protein-free media do not contain high molecular weight proteins or protein fractions, but may contain peptide fractions (protein hydrolysates), and are thus not chemically defined.
Chemically defined media	Chemically defined media do not contain proteins, hydrolysates, or any other components of unknown composition. Highly purified hormones or growth factors added can be of either animal or plant origin, or are supplemented as recombinant products.

Table 1: Classification of CCM based on the nature of their constituents. The categories are not exclusive and a CCM can be part of several categories. Adapted from van der Valk, et al. (2010).

2.2.2. Cell culture media components

Mammalian CCM contain at least, a carbohydrates source, amino acids, vitamins, inorganic salts, trace elements, reductants, ethanolamine or a derivate, and a protective additive. Some of these constituents could be provided to the CCM by serum. Moreover, hormones, pressure selection compounds and other components can be added to the CCM to improve cell growth or process productivity. The nature and the role of these CCM constituents that are pertinent for the presented research work is presented in sections 2.2.2.1 to 2.2.2.6.

2.2.2.1. Serum

Theodore Puck introduced the fetal bovine serum (FBS) to stimulate cellular growth in cell and tissue culture (Puck, Cieciura,, & Robinson, 1958). Since Puck's discovery, FBS has been used as a universal supplement in CCM for human and animal cells in research, biotechnology, and biopharmaceutical manufacturing (Van der Valk, et al., 2018). Some sera of alternative origin are also available such as calf serum, adult bovine serum and horse serum. Serum provides essential components for cell proliferation and maintenance such as lipids, hormones, vitamins, binding and transport proteins, trace elements, spreading and growth factors (Maurer, 1986 ; Brunner, et al., 2010 ; Glassy, Tharakan, & Chau, 1988 ; Hyclone Laboratories, 1984). Basal medium supplemented with serum supports growth of variety of cell types. However, serum-based media are sensitive to serum batch-to-batch variability making the culture results less reproductible and posing a contamination risk when used in

biopharmaceutical manufacturing. FBS is the most widely used bovine serum for mammalian cell culture (Van der Valk, et al., 2018) because it contains a low level of gamma globulins in comparison with other bovine sera (Gstraunthaler & Lindl, 2013). FBS low amount of gamma globulins makes purification of mAbs easier and cheaper (Even, Sandusky, & Barnard, 2006 ; Leist, Meyer, & Fiechter, 1990). However, the use of serum is associated with a number of challenges : non chemically defined composition and batch-to-batch variability (Baker, 2016), potential safety concerns in terms of endotoxins, mycoplasma, viral contaminants (Wessman & Levings, 1999 ; Hawkes, 2015), transmissible spongiform encephalopathies (Dormont, 1999) and other adventitious agents, and also ethical concerns about fetus distress during FBS collection (van der Valk, et al., 2004) and finally the risk of shortages in global supply. An important concern regarding the use of serum is the impact of batch-to-batch variability on cell culture performance. Even if the global composition and relative quantities of most components remain the same (Glassy, Tharakan, & Chau, 1988), the variation of serum composition according to the source, the seasonality, or the batch (Boone, 1973 ; Spector, Mathur, & Kaduce, 1980) may impact cell culture performance (Van der Valk, et al., 2018 ; Shah, 1999). The variation of lipid composition (supplementary Table 19) (Spector, Mathur, & Kaduce, 1980) can partially explain this phenomenom. Moreover, hormones are known to exert profound effects at concentrations in the picogram and nanogram range, thus the presence of these regulatory factors in serum may have a significant impact (Honn, Singley, & Chavin, 1975).

To overcome serum limitations, serum-reduced and serum-free media have been developed. In addition to the increased process robustness associate with the use of serum-free media, a lower glycan microheterogeneity in the antibody product was reported when serum-based medium was replaced by serum-free chemically defined CCM for hybridoma culture (Antonia Serrato, Hernandez, Estrada-Mondaca, Palomares, & Ramirez, 2007). The development of a serum-free medium is a challenging work because each cell line has specific requirements for growth promoting compounds and nutrients. Different approaches based on reduced-serum medium supplemented with proteins and essential components (Guilbert & Iscove, 1976), dialyzed serum (Klinman & McKearn, 1981), medium concentrates (Bibila, Ranucci, Glazomitsky, Buckland, & Aunins, 1994) and plant hydrolysates (Ranganathan, Patel, Pasupuleti, & Meganathan, 2010) have been proposed to supplement serum-free media. Nowadays, most cell culture media are composed of chemical compounds and cell specific supplements, such media are called chemically defined media. The formulation of chemically defined CCM with only pure chemical compounds avoids impurities present in undefined supplements, batch-to-batch variability inherent to plant- or animal-derived supplements. Today, more than 450 serum-free culture media are commercially available (van der Valk, et al., 2010), these media are formulated using serum substitutes. Most of the serum-free media used for the production of mAb using CHO or hybridoma are based on a mixture of basal medium (DMEM, F12) enriched with cell line-specific compounds (Andersson & Melchers, 1978 ; Iscove & Melchers, 1978 ; Bibila & Robinson, 1995 ; Murakami H. , et al., 1982 ; Kawamoto, Sato, Le, McClure, & Sato, 1983 ; Kovár & Franek, 1984 ; Kovár & Franěk, 1986 ; Chang, Steplewski, & Koprowski, 1980 ; Hayashi & Sato, 1976 ; Hutchings & Sato,

1978). Some of the CCM components commonly used to replace serum are presented in sections 2.2.2.4 to 2.2.2.6.

2.2.2.2. Carrier proteins

Crude protein fractions, such as bovine serum albumin (BSA) fraction V, or partially purified proteins like transferrin were identified as growth promoting fractions of serum due to their ability to transport lipids, metal cations and prevent oxidative damages. Thus, animal-derived proteins or recombinant proteins could be added in serum-free media.

Albumin

Albumin is a family of transport proteins that binds ligands and has a protection effect for the cells. Albumin binds ligands such as lipids (El Kouchni, 2011 ; Halliwell, 1988), hormones, vitamins (Kratzer, et al., 2009), peptide, metal ions (Iscove & Melchers, 1978) and free radicals (Roche, Rondeau, Singh, Tarnus, & Bourdon, 2008 ; Halliwell, 1988). Most of the time, bovine serum albumin (BSA) is used in mammalian cell culture because it is cheapest than recombinant human serum albumin (rHSA). Even if albumin is not essential for the culture of hybridomas and SP2/0 cell lines (Murakami H. , et al., 1982 ; Shacter, 1987 ; Tharakan, Lucas, & Chau, 1986), albumin supplementation of hybridoma culture increases cell proliferation (Kovár & Franěk, 1986) and antibody secretion (Sharath, Rinderknecht, & Weiler, 1984 ; Cole, Vreeken, & Order, 1985 ; Kobayashi, M., et al., 1994).

Transferrin

Transferrin is an iron-binding glycoprotein serving as an iron carrier in vertebrate (Octave, Schneider, Trouet, & Crichton, 1983) with growth promoting action reported in vitro (de Jong, van Dijk, & van Eijk, 1990). At first, transferrin was identified as an essential component for cell growth in serum-free media (Kawamoto, Sato, Le, McClure, & Sato, 1983 ; Kovar & Franek, 1985 ; Ekblom, Thesleff, Saxen, Miettinen, & Timpl, 1983) because its omission has led to severe inhibition of cell growth (Titeux, et al., 1984 ; Kovár & Franek, 1984) but it was later replaced by iron salts and chelating agents (Brock & Stevensen, 1987 ; Kovar & Franek, 1987). Nowadays, transferrin is substituted by iron chelate in chemically defined CCM.

2.2.2.3. Hormones

Several hormones were used for mammalian cell culture before their adaptation to hormone-independence. Hydrocortisone was reported to support growth of SP2/0 hybridoma and parental myeloma cell lines (X63-Ag8.653) (Kovár & Franek, 1984). Similarly, insulin was reported to support growth of several mammalian cell lines (Gey & Thalhimer, 1924 ; van der Valk, et al., 2010 ; Kovár & Franěk, 1986) however insulin is non-essential for B cells and some SP2/0 derivatives (Iscove & Melchers, 1978 ; Shacter, 1987). These hormones are naturally present at small concentration in serum and should be added in serum-free media for the culture of hormone dependent cell lines.

2.2.2.4. Vitamins

Vitamins are small organic molecules that are essential for mammal. They can be divided in two categories: fat-soluble vitamins (A, D, E and K) and water-soluble vitamins (B and C). A vitamin is a family of compounds that share similar biochemical functions. Vitamins have diverse biochemical functions, such as cofactors or cofactor precursors, promoter or regulator of cell growth, precursor of complex molecule like phosphatidylcholine. Moreover, some vitamins have antioxidant properties. In the following, a brief overview of three vitamins relevant to this work will be given: vitamins C and E because of their antioxidant properties, and choline due to its key role as phosphatidylcholine precursor. Vitamin C, or ascorbic acid, is a water-soluble vitamin with several functions in enzyme activation (Schlueter & Johnston, 2011) and oxidative stress reduction. Indeed, vitamin C protects cells from peroxyl radicals and restores the antioxidant properties of vitamin E (Bendich, Machlin, Scandurra, Burton, & Wayner, 1986). Vitamin C was reported to support growth of hybridoma and parental myeloma cell lines among them SP2/0-Ag14 and X63-Ag8.653 (Kovár & Franek, 1984 ; Kovár & Franěk, 1986).Vitamin E is a family of fat-soluble molecules that includes 4 stereoisomers of tocopherol and 4 stereoisomers of tocotrienol. Vitamin E exert antioxidant properties and protect cell from free radical damages (Azzi, 2007). Reaction of vitamin E with a free radical generates a tocopheryl radical that can be reduced by vitamin C (Traber & Stevens, 2011).

The classification of choline as a vitamin is subject to debate. Choline serves above all as a precursor of choline phospholipids like phosphatidylcholines, structural components of cell membranes (Zeisel, 2006), sphingomyelin, betaine and signaling molecules like acetylcholine. Phosphatidylcholine is the main constituent of very-low density lipoprotein (VLDL) and is the main component of lipoprotein membranes (Yao & Vance, 1988).

2.2.2.5. Lipoprotein

Lipoproteins are spherical particles composed of an hydrophilic membrane (Figure 2) consisting of phospholipids, unesterified cholesterol and apolipoprotein surrounding a hydrophobic core of triglycerides and cholesteryl esters (Havel, 1975). Lipoproteins are able to transport hydrophobic lipid molecules in aqueous extracellular fluids such as blood where they are prone to oxidation and glycation (More, et al., 1999), two modifications that can alter their properties and function. However, antioxidants like α-tocopherol (Behrens, Thompson, & Madère, 1982 ; Bowry & Ingold, 1999) and carotenoids (Bjornson, Kayden, Miller, & Moshell, 1976) are present in lipoproteins are prevent or delay their oxidation.

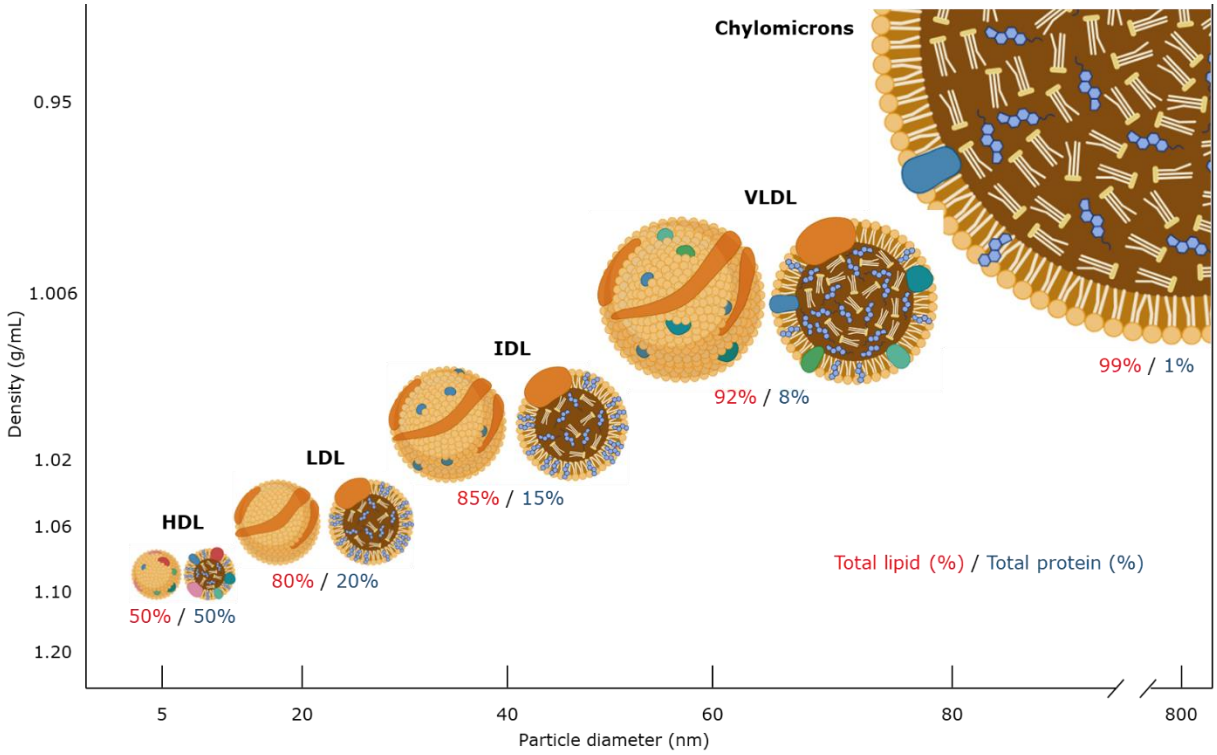


Figure 2: Lipoprotein classes distributed according to their diameter and density, adapted from Jairam, Uchida, & Narayanaswami (2012).

Lipoproteins are conventionally classified by their densities after ultracentrifugation: high-density lipoprotein (HDL), low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), very low-density lipoprotein (VLDL) and sometimes chylomicrons. The lipid content of a lipoprotein class is inversely related to its density (Figure 2). These distinct families of lipoproteins play defined roles in lipid transport, contain characteristic apolipoproteins, and have distinctive lipid compositions (Havel, 1975).

LDL composition has been widely studied due to its role in cholesterol transport and atherogenesis, a pathology that can lead to coronary artery disease. In human, in absence of hypercholesterolemia or diabetes, the core of a LDL particle contains on average 1600 molecules of cholesteryl ester, 100 of triglycerides and few hydrophobic antioxidants. Whereas the outer monolayer contains a single apolipoprotein B-100, 600 molecules of cholesterol, 700 of phospholipids and 6 tocopherol molecules (Bowry & Ingold, 1999). HDL composition is similar to LDL composition, but they contain equal proportion of lipids and apolipoproteins. HDL facilitate efflux of cholesterol from tissues to plasma lipoproteins for clearance, preventing cells from oxidative damages (Brewer, 2004 ; Bowry, Stanley, & Stocker, 1992 ; Tall, 2008).

Apolipoproteins

Apolipoproteins are proteins that bind lipids to form lipoproteins. Apolipoproteins stabilize the structure of lipoprotein particles (Havel, 1975) and improve lipid solubilization. Apolipoproteins are involved in lipoprotein interaction with cell-surface receptors, they are critical for lipoprotein transport

and uptake (Mathews, van Holde, & Ahrern, 1999 ; Havel, 1975). Moreover, few apolipoproteins have specific biochemical activities other than their roles as passive carriers of lipid from one tissue to another. For instance, apolipoprotein C-II is a co-factor of triacylglycerol hydrolysis by lipoprotein lipase (Fojo & Brewer, 1992). Nine major apolipoproteins are found in human lipoproteins, their nomenclature and properties are summarized in Table 2. The stoichiometry of apolipoproteins on different lipoprotein classes is susceptible to vary (von Zychlinski, Williams, McCormick, & Kleffmann, 2014).

Apoprotein	Characteristics
A-I	Major protein in HDL, it activates lecithin:cholesterol acyltransferase
A-II	Major protein in HDL
B-48	Found exclusively in chylomicrons
B-100	Major protein in LDL
C-I	Found in chylomicrons, it activates lecithin:cholesterol acyltransferase and lipoprotein lipase
C-II	Found primary in VLDL, it activates lipoprotein lipase
C-III	Found primarily in chylomicrons, VLDL, and HDL, it inhibits lipoprotein lipase
D	HDL protein, also called cholesterol ester transfer protein
E	Found in VLDL, LDL, and HDL

Table 2: Apolipoproteins of the human plasma lipoproteins.

2.2.2.6. Protein hydrolysates

Protein hydrolysates are derived from animals, microorganism, or plants. Protein hydrolysates are sources of amino acids and oligopeptides, carbohydrates, inorganic salts, and trace elements (Djemal, von Hagen, Kolmar, & Deparis, 2021), they are a potential alternative to serum. Due to safety concerns associated with the use of animal-derived raw-materials in biopharmaceutical manufacturing, plant hydrolysates are favored over animal hydrolysates (Siemensma, Babcock, Wilcox, & Huttinga, 2010). In serum-free media, plant hydrolysates improve cell growth of mammalian cells (Alireza, Farnaz, & Fatemeh, 2014 ; Obaidi, Mota, Quigley, & Butler, 2021) and enhance recombinant protein production (Siemensma, Babcock, Wilcox, & Huttinga, 2010 ; Lobo-Alfonso, Price, & Jayme, 2010). On the other hand, such non-chemically defined raw-materials are often subject to of batch-to-batch variability sometimes associated with productivity losses (Djemal, von Hagen, Kolmar, & Deparis, 2021).Historically, lipid auxotroph cell lines requirements were fulfilled by lipids contained in serum but in serum-free media lipids must be added to the CCM. Due to its central role in this research work, lipid supplementation is treated in a dedicated section.

2.3. Lipids

Lipids are a broad group of hydrophobic molecules often erroneously reduced to “fat” despite a wide variety of forms and functions that make them essential constituents of all living cells. Phospholipids and cholesterol are the main components of cell membranes, not only the plasma membrane but also the nuclear membrane, mitochondrion membrane, Golgi apparatus membrane and endoplasmic

reticulum membrane. These membranes are essential for cell survival and function, they define enclosed spaces but are also interface where take place many interactions. Lipids also serve as metabolic fuel for the cell, fatty acids are one of the main oxidative substrates whereas triglycerides serve as a storage form of fatty acid within the cell. Finally, bioactive lipids such as hormones derived from sterols, sphingolipids and prostaglandins play a crucial role in signaling and cell-cell recognition.

This section aims to analyze lipid delivery to the cells and their metabolism in order to identify challenges associated with lipid supplementation of hybridoma cell culture. First section introduces fundamentals regarding cholesterol and fatty acids uptake and metabolism. The second section examines the different approaches to overcome lipid solubilization challenges and synthetizes the current status on SP2/0 lipid supplementation. Lastly, the third section focus on lipid oxidation and its role in cell death. This section dedicated to lipids will end my literature review and provide the foundation for my investigation into enhancing lipid supplementation for hybridoma cell culture.

2.3.1. Lipid uptake and metabolism in mammalian cells

This section will introduce the uptake mechanisms of cholesterol and fatty acids that serve as building blocks for membrane components and complex lipids.

2.3.1.1. Cholesterol

Cholesterol is the principal sterol synthetized by all animals. It composes about 30-40% of all animal cell plasma membranes. Cholesterol is required to build and maintain membranes (Ohvo-Rekilä, Ramstedt, Leppimäki, & Slotte, 2002), modulates membrane fluidity over the range of physiological temperatures (Brown & London, 2000) and reduces the permeability of the plasma membrane to neutral solutes (Ohvo-Rekilä, H., Ramstedt, B., Leppimäki, P., Slotte, J.P., 2002; Yeagle, P.L., 1991) and ions (Haines, 2001). Moreover, cholesterol serves as a precursor for the biosynthesis of many molecules among steroid hormones, progestins, glucocorticoids, androgens and estrogens (Payne & Hales, 2004) as well as vitamin D (Rosenheim & Webster, 1927). Cholesterol is found in cell in two forms, free cholesterol and esterified cholesterol, esterified cholesterol only known function is to serve as storage from of free cholesterol.

Cholesterol uptake

Cholesterol and cholesterol esters are too hydrophobic to cross cell membranes by themselves. In the mid-1970, Michael Brown and Joseph Goldstein have showed that cholesterol uptake by cells is a receptor-mediated process based on the low-density lipoprotein (LDL) receptor (Brown & Goldstein, 1986). LDLs bind to LDL receptors present on the cell surface through recognition of the B-100 apolipoprotein by the receptor. Subsequently, the entire LDL molecule is taken into the cell within a lysosome. Cholesteryl ester hydrolase hydrolyzes the large quantity of cholesteryl esters present in the LDL, such hydrolysis of cholesteryl ester provides cholesterol for membrane production or the synthesis of cholesterol derivatives (Spector, Mathur, & Kaduce, 1980). This mechanism named

receptor-mediated endocytosis allows the cells to take up large molecules from the extracellular environment.

Regulation of cholesterol metabolism

Because cholesterol overload can be detrimental to the cell, uptake and de novo synthesis of cholesterol are strictly regulated. There are 3 mechanisms that regulate the cellular cholesterol concentration:

- Cellular cholesterol concentration down regulates the hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase enzyme activity, the rate-limiting enzyme of de novo cholesterol synthesis pathway.
    - Short-term regulation: cholesterol inhibits HMG-CoA activity by competitive inhibition, allosteric interactions, or covalent modification by reversible phosphorylation.
    - Long-term regulation: cholesterol accelerates HMG-CoA enzyme degradation and suppresses transcription of the gene for this enzyme, this biosynthesis speed modulation let to the diminution of the enzyme concentration.
- Up taken cholesterol down regulates synthesis of the LDL receptor involved in the cholesterol catchment, decreased synthesis of the receptor ensures that cholesterol will not be taken into the cell in excess of the cell's needs. Thus, LDL receptors quantity on the cell surface is subject to regulation by cholesterol content in the cell. High intracellular cholesterol concentration stops the LDL receptors synthesis while low concentration stimulates it. This occurs when cholesteryl esters are associated with LDL, these complexes are absorbed by the cell thanks to a receptor associated endocytose mechanism as described previously. Cholesteryl ester is hydrolyzed inside the cell to form cholesterol.
- Reversible phosphorylation of acyl-CoA cholesterol acyltransferase enzyme (ACAT), the enzyme that catalyze intracellular esterification of cholesterol, down regulates the kinetic of cholesterol esterification.

These mechanisms regulate cholesterol uptake and intracellular cholesterol concentration in order to fulfill cell requirement but without excessive cholesterol accumulation that can be detrimental to the cell.

2.3.1.2. Fatty acids

Fatty acids are aliphatic chains containing 4 to 28 carbon atoms, either saturated or unsaturated, with a carboxylic group. Usually, they are found in organisms as triglycerides, phospholipids, cholesteryl esters or more complex lipids but rarely as free fatty acids. Fatty acids can be used either to produce energy through β-oxidation, for membrane lipids or bioactive lipids synthesis (Spector, Mathur, & Kaduce, 1980). Unsaturated fatty acids, fatty acids with at least one C=C double bound, are divided

into classes based on the number of carbon atoms from the methyl terminus to where the first double bond is located. In this nomenclature, the unsaturated classes are linolenic (ω -3), linoleic (ω -6), palmitoleic (ω-7) and oleic (ω -9). Most vertebrate cells are able to synthetize de novo the ω-7 and ω-9 classes of unsaturated fatty acids. None, however, can synthetize the ω-6 and ω-3 classes, thus, they have been termed essential fatty acids. However, despite the term "essential", cultured cell growth in the complete absence of essential polyunsaturates fatty acids was reported (Geyer, Bennett, & Rohr, 1962). Fatty acids incorporated into lipoproteins are up taken thanks to the LDL receptor in a similar way to cholesterol.

Regulation of fatty acid metabolism

Like cholesterol, *de novo* synthesis of fatty acids in cell is regulated by free fatty acid concentration in the cells through two regulation mechanisms, short-term and long-term controls:

- Short-term control: free fatty acids down regulate the acetyl coenzyme A (acetyl-CoA) carboxylase enzyme activity, the rate-limiting enzyme in the de novo fatty acid pathway (McGee & Spector, 1974 ; McGee & Spector, 1975 ; Volpe & Marasa, 1975 ; Nilsson, Sundler, & Kesson, 1973). The acetyl-CoA carboxylase enzyme concentration remains constant, the down regulation is due to a modification of the acetyl-CoA carboxylase composition (McGee & Spector, 1975).

- Long-term control: free fatty acids inhibit fatty acid synthetases like malate dehydrogenase and citrate lyase that generates acetyl-CoA (Volpe & Marasa, 1975 ; Doi, Doi, Schroeder, Alberts, & Vagelos, 1978 ; Goodridge, 1975).

Similarly, to the inhibition induced by high concentration of free fatty acids, de novo synthesis of fatty acids is inhibited by uptake of triglycerides and phospholipids from lipoproteins (McGee, Brennenman, & Spector, 1977).

2.3.2. Lipid supplementation of hybridoma cell culture

Because most cultured cells are able to synthetize all the lipids that they require from water-soluble compounds present in the medium, most of commonly used CCM contain negligible lipid content or no lipid source at all. The most common starting point for hybridoma media development, a combination of DMEM and Ham's F-12 (1:1), contains only linoleic acid 0.042 mg/L. Yet, several hydrophobic molecules, sometimes categorized as lipid, like vitamins and lipoic acid are also present in the DMEM/F12 medium. Nonetheless, these lipids are not enough to support the growth of lipid auxotroph cell lines without further lipid supplementation of the culture. However, lipid supplementation is challenging for two aspects, first lipids are poorly soluble in water and require a carrier, then, lipid requirements are cell line dependent and may also vary through the culture stages. This section reviews promising alternatives to serum for lipid supplementation of hybridoma culture followed by a benchmark of the lipid composition of the CCM and lipid supplement that were published in the literature.

2.3.2.1. Lipids in cell culture media: solubilization strategies

Following the emergence of serum-free media there has been interest in serum alternative for lipid delivery in CCM. Different approaches have been evaluated to supplement lipids to the culture in serum-free media, the main challenges of lipid supplementation are poor lipid solubility in water, clogging during sterile filtration, and control of cell uptake to avoid cytotoxicity. The pros and cons of some of the solutions proposed to overcome the lipid solubilization challenge in CCM are presented in this section.

Lipoprotein

Lipoproteins are a source of a large variety of lipids including fatty acids and cholesterol, but also antioxidant like α-tocopherol (Behrens, Thompson, & Madère, 1982 ; Bowry & Ingold, 1999), they are a polyvalent lipid source that fulfill hybridoma cell growth requirements. Moreover, controlled lipoprotein uptake by the cells through the LDL receptor protects cells from lipid overload and avoids lipotoxicity. Lipoprotein supplements for mammalian cell culture were originally conceived as a partial replacement for serum, which address many essential nutrients and growth factors that may be lacking in serum-free media. Lipoprotein supplements are aqueous solutions composed mostly of cholesterol, phospholipids, triglycerides, and free fatty acids. The lipids are water soluble by virtue of association with their native lipoprotein carriers. Lipoprotein supplements were reported to enhance the growth and productivity of a variety of diverse hybridoma cell lines including NS0 (Gorfien, et al., 2000) and SP2/0 (Savonnière, et al., 1996 ; El Kouchni, 2011) as well as other mammalian cell lines (Hewlettet, Duvinski, & Montalto, 1989) in serum-free media. Lipoprotein supplementation in serum-free hybridoma cell culture influences membrane cholesterol, phospholipid composition and immunoglobulin production (Savonnière, et al., 1996). However, in a similar way to serum, lipoprotein composition batch-to-batch variability was reported (Miles Incorporation, 1991).

Lipid emulsions and formation of liposome

Emulsion and liposome are both compartments enclosed by phospholipid membrane, however an emulsion contains only small droplets of one liquid dispersed in another with which it is immiscible whereas a liposome encloses an aqueous compartment.

Non-proteinaceous lipid emulsions (triglyceride, phospholipid, cholesterol and glycerol) were reported to replace lipoprotein supplementation for NS0 growth (Hansen, 1992 ; Seamans, Gould, DiStefano, Silberklang, & Robinson, 1994). Replacement of lipoprotein by a non-proteinaceous lipid emulsion was reported to moderately decrease the viable cell density (VCD) peak but increase the culture longevity. However, one drawback of emulsion preparation is the sonication step (Ginsburg, Smal, & Atkinson, 1982 ; Via, et al., 1982), not suitable for the preparation of large volumes at an industrial scale.

Liposomes composed of glycerophospholipids, and anionic- or neutral-PEG lipid were formed by extrusion, these liposomes were up taken by endocytosis by HeLa and J774 cells (Miller, Bondurant, McLean, McGovern, & O'Brien, 1998).

Albumin

Lipid binding properties of albumin are used to solubilize lipids in CCM (Iscove & Melchers, 1978 ; Kovár & Franek, 1984 ; El Kouchni, 2011). BSA and recombinant human serum albumin (rHSA) bound to palmitic, stearic, oleic and linoleic acids according to a molar ratio of 1:0,45:0,48:0,2:0,05 and 1:0,34:0,34:0,34:0,68 respectively were reported to support SP2/0 growth (El Kouchni, 2011). However, the use of BSA and rHSA is, moreover commercially available albumin may contain unknown contaminants like fatty acids and proteins (El Kouchni, 2011).

The impact of albumin supplementation in cell culture may be biased by the presence of ligands bound to albumin. According to the characterization results of a commercially available BSA made by El Kouchni, S. (2011), the BSA was already bound to fatty acids. The studied BSA contained 1,009 fatty acids mol/mol BSA where oleic acid (20%), linoleic acid (5%), palmitic acid (40%) and stearic acid (30%) accounted for 85% of the total. Myristic acid, pentadecanoic acid, margaric acid, linolenic acid, arachidonic acid, coagulation factors, IgG-3 chain C region, transferrin, Apolipoprotein A-I, Phosphatidylinositol-glycan specific phospholipase D, retinol-binding protein and vitamin D-binding protein were also detected in the BSA samples. Similarly, in a commercially available recombinant human serum albumin (rHSA), El Kouchni, S. (2011) reported the presence of 5,7 octanoic acid mol/mol of rHSA added for long-term storage of the albumin solution.

Synthetic low-density lipoprotein

Synthetic low-density lipoproteins (sLDL) were reported to support NS0 cellular proliferation in absence of another lipid source (Hayavi & Halbert, 2005). However, sLDL manufactured according to the Hayavi & Halbert's procedure were highly toxic for SP2/0 cells (El Kouchni, 2011).

Cyclodextrin

Cyclodextrins (CD) are a family of cyclic polymers of glucopyranose. Main types of cyclodextrins are composed of 6, 7 or 8 monomers of glucopyranose and are named alpha, beta and gamma cyclodextrin respectively (Figure 3). Cyclodextrins form rings in the shape of a cone which are hydrophilic on the outer part and hydrophobic on the inside (Figures 4 and 5). This hydrophobic cavity may be used to bind thus solubilize a range of hydrophobic molecules for targeted rug delivery or general lipid transport in aqueous media. Cyclodextrin properties can be manipulated by partial or full modification of the hydroxyl groups. Cyclodextrin derivatization modifies the interaction between cyclodextrin and the gust-molecule as well as stability and solubility of the inclusion complex (Huang & London, 2013).

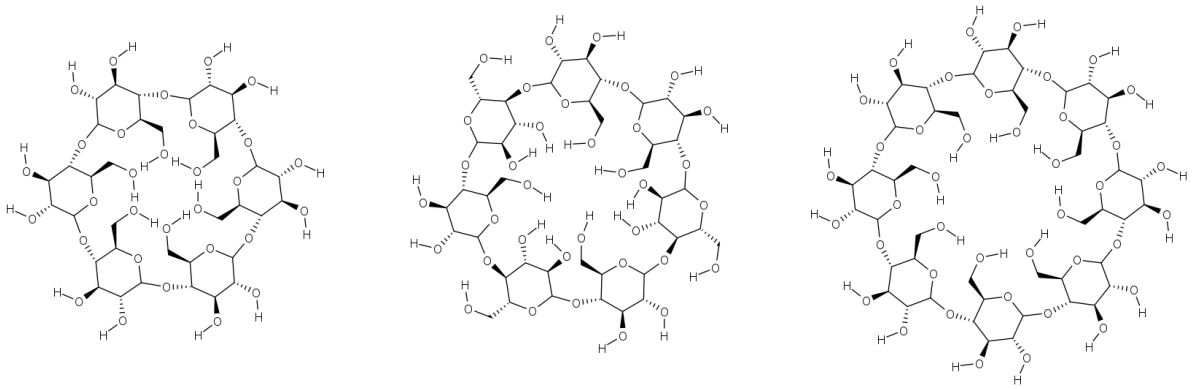


Figure 3: Chemical structures of, from left to right, α-CD, β-CD and γ-CD with 6, 7 and 8 glucopyranose monomers respectively.

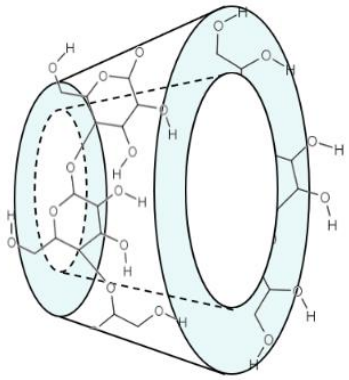


Figure 4: Tridimensional arrangement of cyclodextrin. The hydrophobic groups are oriented towards the central cavity of the structure whereas the hydrophilic groups are oriented to the outside.

CDs form with lipids CD inclusion complexes, illustrated in Figure 5, a lipophilic molecule or a lipophilic moiety of a poorly water-soluble molecule is stabilized inside the hydrophobic cavity of the CD molecule. Such complex formation is driven by electrostatic interactions, van der Waals contributions, hydrogen bonding (Li, Geng, Liu, Wang, & Liang, 2018), the release of conformational strain and charge-transfer interactions (Miao, et al., 2017 ; Brewster & Loftsson, 2007). CDs propensity to form inclusion complexes depends on their cavity size and functional modification of the hydroxyl groups.

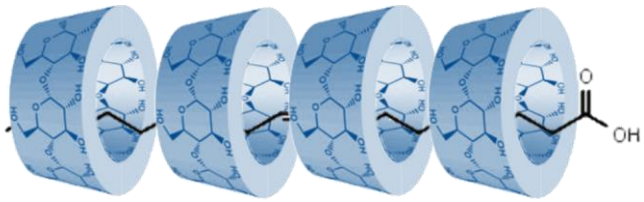


Figure 5: Illustration of fatty acid complexed with mbCD molecules. Note that only the hydrophobic part of the fatty acid is contained inside the cavity of mbCD molecules, the hydrophilic carboxylic group is free.

Thanks to their properties as poorly water-soluble molecule solubilizer, free cyclodextrins have been reported to be useful in cell membrane lipid depletion or exchanges as they bind easily to lipophilic

molecules like cholesterol and fatty acids (Christian, 1997). Moreover, CD inclusion complexes with lipids are efficient delivery systems in CCM (Gorfien, et al., 2000). mbCD inclusion complexes with lipids were able to support NS0 hybridoma growth in serum-and-protein free media, showing process performance similar to that of serum-containing media (Gorfien, et al., 2000 ; Walowitz, 2003). mbCD was more efficient than other CDS to solubilize phospholipids due to its adequate cavity size and the presence of methyl groups that interact with the acyl chains (Huang & London, 2013). Nonetheless, α -CD was shown to solubilize and transport fatty acids effectively in mammalian cells (Yamane, 1981). But CDs suffer drawbacks, among them the poor stability of CD inclusion complexes in aqueous solutions because of lipid precipitation, CD cytotoxicity and potential poor filtration yields showing sometimes low lipid recovery post-filtration. CD inclusion complexes formulations require an optimization of the ratio between CD and lipid to limit CD concentration in the CCM while avoiding lipid precipitation. Indeed, the optimal CD:lipid ratio depends on the lipid nature, different ratio lipid-mbCD have been reported in the literature, they are presented in supplementary Table 20. Moreover, CCM supplementation with CDs-cholesterol complexes in plastic bioreactors has shown to be problematic due to interactions of lipids with bioreactor components (Okonkowski, 2007 ; Tao, 2012). Lastly, β-cyclodextrin was reported to form inclusion complexes with three hydrophobic aminos acids: tyrosine, tryptophan and phenylalanine (Li, Geng, Liu, Wang, & Liang, 2018). It could be supposed that these amino acids may become less available for the cell in presence of free β-cyclodextrin in the culture. Even if nothing in the literature can attest of this phenomenon it should be considered because deprivation of these amino acids could lead to growth inhibition. Nonetheless, improvement of CD inclusions manufacturing technology was reported to improve lipid delivery efficiency (Walowitz, 2003). For example, electrosprayed cholesterol nanoparticle solubilized with mbCD 25 mg/mL and Pluronic F-68 1% (w/v) were reported to be even more efficient than inclusion complexes for cholesterol delivery to hybridoma (Wu, et al., 2011). Despite some drawbacks, CDs are a promising alternative to serum for providing lipids to cell culture.

2.3.2.2. Lipid supplementation of SP2/0 culture in serum-free media

History of lipid supplementation of mammalian cells

Since the complete replacement of serum by albumin, transferrin and soybean lipid for mammalian cell culture (Iscove & Melchers, 1978) a large variety of lipids source were used in CCM to fulfill cell requirements. Combination of lipoprotein supplement, bovine serum albumin or recombinant albumin, plant-derived phospholipids, animal-, plant-derived or synthetic sterols, defined mixture of fatty acids, vitamins, antioxidants and hormone-containing solutions in reduced serum or serum-free media were used in hybridoma CCM (Table 3) (Linstead, 1981 ; Maiorella, Inlow, Shauger, & Harano, 1988 ; Wilkie, Stockdale, & Pirt, 1980 ; Spens & Häggstrém, 2007 ; Spens & Häggström, 2005).

Novadays, all-in-one ccommercial lipid formulations are available from different manufacturers (Bovine cholesterol concentrate, ICN Biomedicals, Costa Mesa, California, USA; CPSR-1 and CPSR-2, Sigma, St Louis, Missouri, USA ; EX-CYTE®, Miles, Kankakee, Illinois ; ProNS0 Lipid Supplement –

Chemically Defined, Lonza, Cologne, Germany; Lipid Mixture 1, Chemically Defined, Sigma-Aldrich, St Louis, Missouri, USA; Chemically Defined Lipoprotein supplement, Creative Bioarray, Shirley, New-York, USA). However, despite solving the main challenge of lipid supplementation, the poor solubility of lipids, these supplements are not cell line specific and can not be adapted to the variations of cell lipid requirements through the culture.

Lipid auxotrophy of SP2/0 cells

Even if most of cultured cell lines are capable of synthetizing all the lipids that they require, the most used mouse myeloma parental cell lines NS0 (Sato, J.D., Gao, H., Kayada, Y. et al, 1988), NS1 (Sato, J. D., Kawamoto, T., McClure, D. B., & Sato, G. H., 1984) and X63 (Sato, J. D., Kawamoto, T., & Okamoto, T., 1987) are auxotrophs due to a deficiency in 3-ketosteroid reductase activity. Consequently, exogenous cholesterol is essential for their optimal growth (Birch, J.R., Boraston, R.C., Metcalfe, H. et al., 1994). In contrast with myeloma cells derived from NS-0/NS1 cell lines, SP2/0 cells are not cholesterol auxotroph (Sato, J. D., Kawamoto, T., & Okamoto, T., 1987 ; Sato, Kawamoto, McClure, & Sato, 1984).However, lymphocytes B cells proliferation requires exogenous lipids (Farrant, Newton, North, Weyman, & Brenner, 1984), thus cell lines derived from a fusion with a lymphocytes B like NS0 and SP2/0 hybridoma are known to require lipid supplementation for optimal growth and antibody secretion. Indeed, growth stimulation has been reported on several hybridoma cell lines treated with concentrations as low as 25-50 μM of oleic acid, linoleic acid or combinations of both, when complexed with bovine serum albumin (Butler & Huzel, 1995 ; Butler, Huzel, Barnabé, Gray, & Bajno, 1999 ; Jäger, Lehmann, & Friedl, 1988 ; Kovár & Franek, 1984 ; Kobayashi, Kato, Omasa, Shioya, & Suga, 1994 ; Schneider, 1989). Despite not being cholesterol auxotroph, SP2/0 cells were reported to stop growing after 5 passages in absence of lipoprotein supplement and fatty acids-BSA complexes indicating that lipids are essential for SP2/0 growth (El Kouchni, 2011). Moreover, fatty acids 25 – 100 µM, linoleic, oleic, palmitic and stearic acids distributed at a ratio 2:1:1:1, bound to 1 g/L BSA were able to support SP2/0 growth in a similar manner to lipoprotein supplement (El Kouchni, 2011). Taken together these results demonstrate that SP2/0 are fatty acids auxotrophs but do not dependent on LDL or cholesterol for growth.

Fatty acid supplementation of SP2/0 cells

The effect of adding fatty acids to a culture medium depends on the cell line, suggesting that different cell lines are not using fatty acids in the same way. Consequently, each cell line should be considered and studied independently when studying fatty acid supplementation. For example, linoleic acid promotes the growth of the mouse hybridoma 3A21 cell line without affecting antibody production (Kobayashi, Kato, Omasa, Shioya, & Suga, 1994) but significantly increases antibody production of the CC9C10 hybridoma cell line (Butler & Huzel, 1995). Thus it is not possible to extrapolate the lipid supplementation knowledge from a cell line to another (Savonnière, et al., 1996), with one exception, when cells require exogenous fatty acids supplementation the CCM should generally supply at least a precursor of the essential fatty acids of the ω-6 series : linoleic or alpha-linolenic acids.

Even if fatty acids supplementation increases cell growth and recombinant protein production (Butler & Huzel, 1995), the effect of fatty acid supplementation is dependent on the state of the culture, it is susceptible to vary if the cells are either proliferating or dying. Indeed, during rapid cell proliferation of PFU83 rat/mouse hybridoma, linoleic acid complexed with BSA was growth promoting at 25 and 50 µM whereas above 50 µM an increase in cell death through apoptosis was observed (Kisztelinski, et al., 2006). However, for stressed apoptotic cells linoleic acid caused partial growth inhibition at 25 µM and arrest of cell proliferation in the G(2)/M phase at 50 µM (Kisztelinski, et al., 2006).

Most of the time only linoleic and oleic acids are added to hybridoma CCM (Table 3) suggesting that only these two, or at least one of these two, fatty acids are required for optimal growth. However, the presence of fatty acids impurities in BSA have been demonstrated (El Kouchni, 2011). Moreover, depending on the media preparation, media storage and culture vessel nature, fatty acid leachable not present in the CCM composition may be released into the CCM or the culture. Indeed, stearic and palmitic acids have been identified as bioprocess bag leachable (Wakankar, et al., 2010). Thus, even in absence of fatty acid supplementation other than linoleic and oleic acids, other fatty acids are susceptible to be present in the culture fulfilling cell requirement for these fatty acids.

Lastly, not all fatty acids are the same when it comes to fatty acid supplementation, α-linolenic (ALA) and eicosapentaenoic (EPA) acids were reported to exert cytotoxic effects against SP2/0 cells at concentration as low as 18 µM (Kumar & Das, 1995). The cytotoxicity of ALA and EPA is due to the generation of free radicals and lipid peroxides in SP2/0 cells through lipid peroxidation. However, another PUFA prone to peroxidation, DHA 0.1 – 10 µM was reported to increase mAb production in M3C5 hybridoma in serum-containing medium, the positive effect being increased when co-treated with trans-retinoic acid (Rokni, et al., 2018).

Destabilization of the cellular fatty acid balance by supplementation

While in culture, mammalian cells are able to intake extracellular free fatty acids (Spector, 1968), triglycerides (Brenneman & Spector, 1974 ; Mackenzie, Meckenzie, & Reiss, 1974) and phospholipids (Peterson & Rubin, 1969). Meanwhile, when fatty acids are available in the CCM, de novo biosynthesis is inhibited. Consequently, the fatty acid composition of the cell membrane phospholipids reflected the composition of the lipids contained in the CCM (Geyer, Bennett, & Rohr, 1962). Thus, when supplemented in CCM, fatty acids should match cell requirement to not disturb the membrane phospholipids fatty acids balance. Indeed, imbalance of supplemented fatty acids can alter the cell membrane composition and robustness (Butler, Huzel, Barnabé, Gray, & Bajno, 1999).

Cell line	Basal medium	Lipid (mg/L)	Lipid carrier (g/L)	Reference	Comment
F0 Ag8.653	RPMI	Linoleic acid 5.0	FAF-BSA 1.0	(Kovár & Franek, 1984)	Lipids in serum-free media were provided from non-chemically defined raw material like soybean lipids and lipoproteins In parallel, BSA was used to solubilize free fatty acids
MPC11	IMDM:F12	Soybean lipids 0.5 – 5.0 Linoleic acid 0.04		(Murakami, Edamoto, Shinohara, & Omura, 1983)	
SP2/0 and NS1	IMDM	Soybean lipids 50 - 100 Cholesterol 0 - 8	BSA 0.5	(Iscove & Melchers, 1978)	
SP2/0	RPMI	Soybean lipids 20	BSA 0.5 LDL 2.0	(Shacter, 1987)	
NS1	RPMI:F12:DMEM (2:1:1)	Oleic acid 4.0	FAF-BSA 0.5 LDL*	(Kawamoto, Sato, Le, McClure, & Sato, 1983)	
SP2/0	F12:DMEM	Linoleic acid 0.04		(Murakami H. , et al., 1982)	Only free fatty acids, sometimes bound to BSA to facilitate solubilization
NS1	IMDM:F12	Oleic acid* bound to BSA (concentration not specified) Linoleic acid 0.04	BSA 0.5 – 1.0	(Jäger, Lehmann, & Friedl, 1988)	
NS0	RITM01	PC 6.7 Linoleic acid 4.08 Cholesterol 4.5	β-cyclodextrin*	(Spens & Häggstrém, 2007)	Protein-free medium with BSA being replaced by β-cyclodextrin

Table 3: Lipid composition of serum-free media for hybridoma cell culture. * Concentration not specified.

2.3.3. Lipid oxidation

Lipid oxidation is the transfer of electrons from electron-rich lipids, usually C=C double bound, to an electron acceptor, also called oxidant or oxidizing agent, most of the time dioxygen, hydrogen peroxide or radical. Lipid oxidation mechanisms can be divided in three categories, enzyme catalyzed oxidation, free radical oxidation and photo-oxidation (Huang & Ahn, 2019). An unbalance between oxidants and antioxidants is called oxidative stress (Sies, 1997). Severe cell oxidative stress leads to alteration of membrane properties, protein oxidation, DNA damages and apoptosis if these damages are not repaired. Irrespective of the nature of the medium, serum-based or serum-free medium, lipids are prone to oxidation.

2.3.3.1. Lipid peroxidation

Lipid peroxidation: a free radical mechanism

ROS and free radicals are natural products of cell metabolism, especially respiration processes that took place in mitochondria. ROS initiate lipid peroxidation (Figure 6) by subtracting a hydrogen atom from a lipid to form a water molecule and a lipid alkoxyl radical. Because lipid alkoxy radicals are unstable species, they react quickly with dioxygen to form lipid peroxy radicals (Marnett & Wilcox, 1995).

The radical chain reaction propagates with reaction between a radical and a non-radical that produces another radical. In lipid peroxidation, lipid peroxy radicals subtract a hydrogen atom from lipids leading to the formation of a new lipid alkoxyl radical that will continue the chain reaction, and a more stable lipid hydroperoxide (Figure 6). Following hydrogen abstraction, rearrangement of double bonds on the PUFA side chain occurs, leading to the formation of more stable conjugated dienes, notably conjugated dienes hydroperoxide.

Lipid peroxidation kinetic decreases as radical compound increases because reaction between two radicals gives a non-radical specie. The end of the radical chain reaction mechanism is called termination and it is caused by either consumption of a large portion of lipid peroxidation substrates have been oxidized into stable peroxidation derivatives (aldehydes, ketones, alcohols, carboxylic acids…).

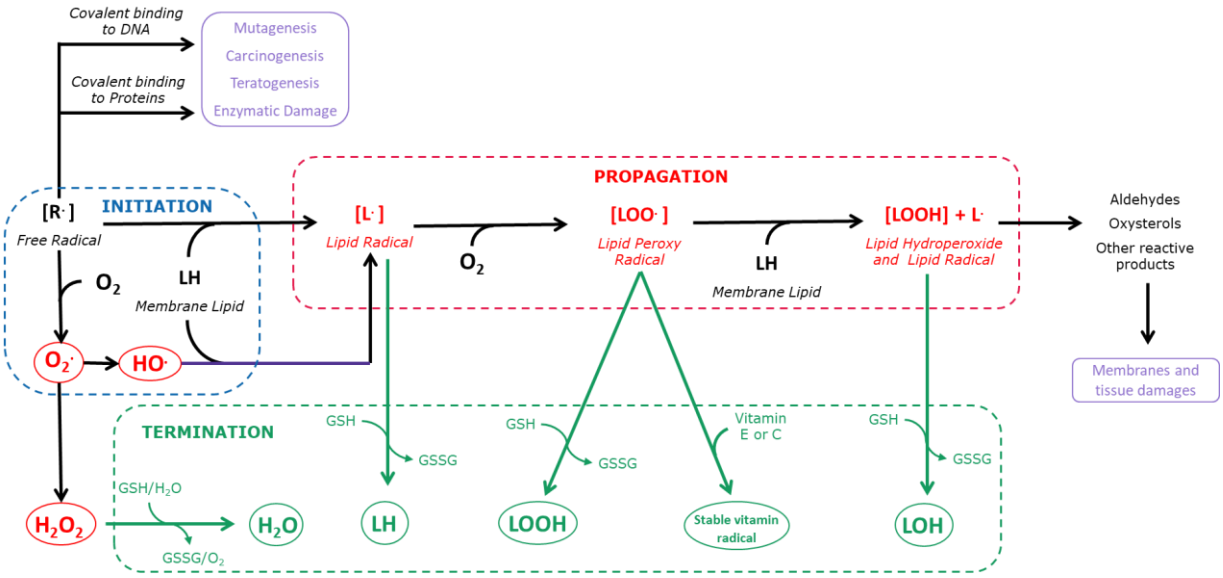


Figure 6: Mechanisms of lipid peroxidation, from free radical to cell damages (purple). Free radicals react with membrane lipids or O_2 initiating lipid peroxidation (blue), the radical mechanism propagates by further reacting with membrane lipids or O_2 (red) until the formation of stable products. Reduction of radicals by GPX4 or vitamins ends lipid peroxidation (green).

Factors that influence lipid peroxidation

Because the reaction product of a radical and a radical scavenger like an antioxidant is stable and do not propagate lipid peroxidation, antioxidants, like vitamins C and E, are able to delay or even block lipid peroxidation depending on the relative concentration of antioxidants compared to radical generation. Moreover, cells have detoxification tools able to neutralize ROS and radicals, protecting cells from oxidative damages. The cystine/glutamate antiporter, glutathione peroxidase 4 (GPX4) and glutathione (GSH) system for example is able to reduce lipid hydroperoxide and protect cells from ferroptosis, a regulated cell death caused by imbalance between oxidative stress and antioxidant systems leading to accumulation of lipid hydroperoxides (Li, et al., 2022). By contrast with antioxidants that block lipid peroxidation, low-valence-state ions like ferrous or cupric ions (Cu_{2+}) promote it (Ontko, 1970; Gardner, 1989). Lipid hydroperoxides are intermediates of lipid peroxidation relatively stable compared to radicals, however in presence of copper ions (Cu_{1+} and Cu_{2+}), lipid peroxides are decomposed into peroxyl and alkoxyl radicals through a redox-reaction, thus, further propagating lipid peroxidation (Burkitt, 2001 ; Thomas & Jackson, 1991 ; Minotti & Aust, 1992 ; Furono, Suetsuga, & Sugihara, 1996). From an experimental perspective, lipoprotein dialysis for 24h against $CuSO_4$ (6 µM) produces oxidized lipoprotein containing conjugated dienes, lipid hydroperoxides and oxysterols (Gerry & Leake, 2008). Such lipoprotein oxidation in presence of copper is named copper-mediated lipoprotein oxidation.

Primary products of lipid peroxidation

Free radical peroxidation of long chain polyunsaturated fatty acids (LCPUFA) produces prostaglandin-like compounds: peroxidation of eicosapentaenoic acid (EPA), arachidonic acid (AA) and γ-linoleic acid

produce isoprostanes (IsoPs) (Figure 7) (Janssen, 2001 ; Cracowski, Durand, & Bessard, 2002 ; Morrow, et al., 1990), while peroxidation of docosahexaenoic acid (DHA) produces neuroprostanes (NeuroPs) (Reich, et al., 2000).

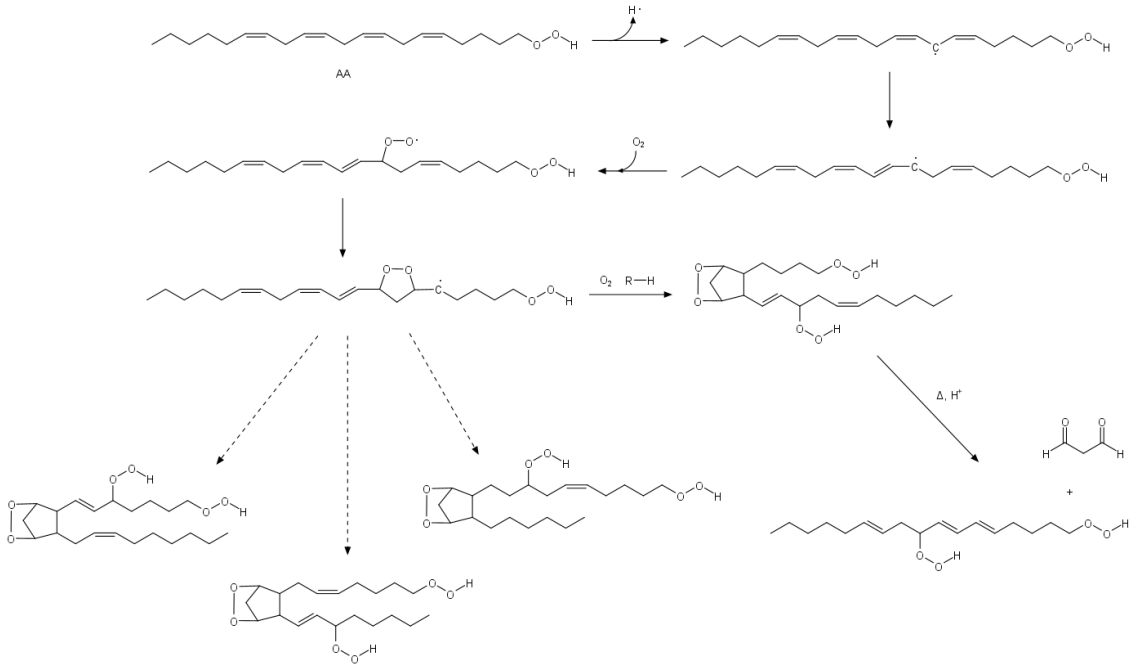


Figure 7: Peroxidation of arachidonic acid through formation of a conjugated dienes lipid hydroxy radical intermediate. Cyclization leads to the formation of isoprostane, depending on the fatty acid oxidized different isoprostanes can be produced (dotted arrows). Cleavage of the isoprostane molecule yields an hydroperoxide and MDA.

Free radical peroxidation of linoleic acid produces a mixture of 9-OOH and 13-OOH, these hydroperoxide decompose in the presence of divalent metal ions to form alkoxy radicals then react with an H-donor to yield the corresponding hydroxyoctadecadienoic acids (LOH) or oxodienoic acids (Loidl-Stahlhofen, Hannemann, & Spiteller, 1994) (Figure 8). Alternatively, cleavage between the alkoxyl radical and an adjacent C-C single bound produces aldehydes and secondary radicals, MDA and 2-hydroxyheptanal are the major aldehydic products of lipid peroxidation (Loidl-Stahlhofen, Hannemann, & Spiteller, 1994).

Low-valence-state ions like ferrous and copper ions induce lipid peroxidation in alpha-linolenic acid-loaded cultured rat hepatocytes leading to MDA accumulation and cell injury (Furono, Suetsuga, & Sugihara, 1996).

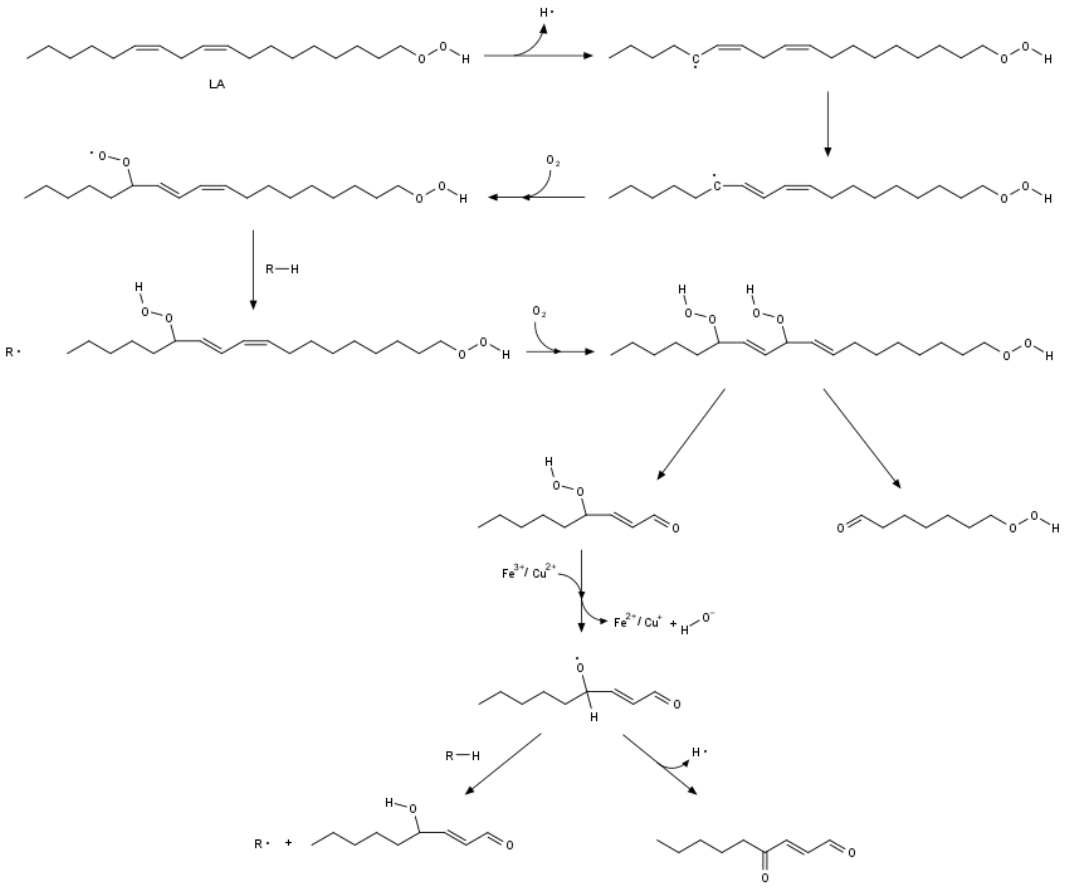


Figure 8: Peroxidation of linoleic acid, two mechanisms with and without divalent metal ions catalysis are presented. In presence of divalent metal ions, 4-hydroxy-2-alkenals are formed.

Secondary products of lipid peroxidation

Following lipid peroxidation, cleavage of lipid hydroperoxides produces a large variety of aldehydes of various carbon lengths and a shortened fatty acid. Notably, the level of unsaturation of the lipid species oxidized defines the nature of the aldehyde: ω-3 PUFA oxidation produces malonaldehyde (MDA), ω-6 PUFA oxidation produces hydroxy hexenal and ω-9 PUFA oxidation hydroxy nonenal (4-HNE) (Vigo-Pelfrey, 1990 ; Benedetti, Comporti, & Esterbauer, 1980).

2.3.3.2. Oxysterols

Oxysterols are a family of 27-carbon oxidized cholesterol derivatives that contain a second oxygen atom as a carbonyl, hydroxyl or epoxide group generated either through enzymatic or non-enzymatic oxidation. Up to 70 oxysterols have been identified (Schroepfer, 2000), the abbreviations and nomenclature of some oxysterols are presented in Table 4. Oxysterols act as intermediate in cholesterol catabolism and regulators of lipid metabolism. They affect de novo sterol biosynthesis, DNA synthesis, plasma membrane structure and properties, cellular functions, and cellular growth and proliferation (Smith & Johnson, 1989 ; Lordan, Mackrill, & O'Brien, 2009).

Abbreviation	Trivial name	Systematic name
Triol	Cholestanetriol	5α-Cholestane-3β,5,6β-triol
4α-OH	4α-Hydroxycholesterol	Cholest-5-ene-3β,4α-diol
4β-OH	4β-Hydroxycholesterol	Cholest-5-ene-3β,4β-diol
6β-OH	6β-Hydroxycholesterol	Cholest-4-ene-3β,6β-diol
7α-OH	7α-Hydroxycholesterol	Cholest-5-ene-3β,7α-diol
7β-OH	7β-Hydroxycholesterol	Cholest-5-ene-3β,7β-diol
22-OH	22-Hydroxycholesterol	Cholest-5-en-3β,22-diol
24-OH	24-Hydroxycholesterol	Cholest-5-en-3β,24(S)-diol
25-OH	25-Hydroxycholesterol	Cholest-5-en-3β,25-diol
26-OH	26-Hydroxycholesterol	25(R)-Cholest-5-en-3β,26-diol
27-OH	27-Hydroxycholesterol	25(R)-Cholest-5-en-3β,27-diol
α-epoxide	Cholesterol-5α,6α-epoxide	5,6α-Epoxy-5α-cholestan-3β-ol
β-epoxide	Cholesterol-5β,6β-epoxide	5,6β-Epoxy-5β-cholestan-3β-ol
7-keto	7-Ketocholesterol	3β-Hydroxycholest-5-en-7-one
7-keto-diene	7-Ketocholestadiene	Cholesta-3,5-diene-7-one
7α-OOH	7α-Hydroperoxycholesterol	3β-Hydroxycholest-5-ene-7α-hydroperoxide
7β-OOH	7β-Hydroperoxycholesterol	3β-Hydroxycholest-5-ene-7β-hydroperoxide

Table 4: Oxysterols abbreviations and nomenclatures adapted from (Brown & Jessup, 1999 ; Morrissey & Kiely, 2006 ; Lordan, Mackrill, & O'Brien, 2009).

Because cholesterol is critical for cell membrane structure, oxidation of membrane cholesterol, the second oxygen atom of oxysterol can disturb the structure of the membrane modifying its physicochemical properties (Massey, 2006). Moreover, oxysterols were reported to increase intracellular levels of reactive oxygen species (ROS) (Ong, et al., 2003), induce modification of cell proteins, alter various signaling pathways and gene expression (Morrissey P.A. & Kiely M., 2006).

Oxysterols are involved in cardiovascular diseases including atherosclerosis (Brown & Jessup, 1999 ; Zmyslowski & Szterk, 2019), and are suspected to be involved in various degenerative diseases such as Alzheimer's disease (Vaya & Schipper, 2007), osteoporosis and age-related macular degeneration (Vejux, Malvitte, & Lizard, 2008). Notably, oxidized low density lipoprotein (oxLDL) are rich in oxysterols (Vine, Mamo, Beilin, Mori, & Croft, 1998), oxysterols were reported to be for a large part responsible for oxLDL cytotoxicity and its detrimental effect on health (Colles, Maxson, Carlson, & Chisolm, 2001).

Oxysterol formation

Due to its C=C double bound, the cholesterol molecule is susceptible to autoxidation leading to the formation of oxysterols. Side chain oxidation, carbons C-22 to C-27 (Figure 9), is not observed in

autoxidation carried out in solution (Maerker, 1987), therefore 22- to 27-hydroxycholesterols are products of enzymatic oxidation only. The carbon C-7 is the most suitable for abstraction of a hydrogen atom, the intermediate of the C-7 oxidation is 7-hydroperoxide that is thermally instable (Bergström & Wintersteiner, 1942), the final oxidation products are 7-hydroxycholesterol and 7-ketocholesterol.

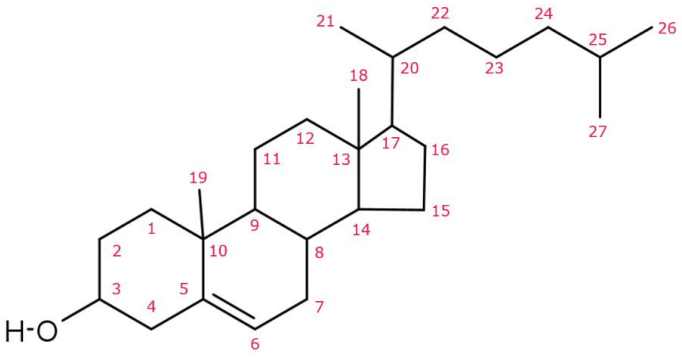


Figure 9: Structure of cholesterol with the numbering of the carbon atoms.

Oxysterol-binding proteins and oxysterol receptors

Oxysterols have the ability to interfere with cell metabolism through interaction with oxysterol-binding proteins (OSBP), also known as oxysterol-related proteins. Oxysterol-binding proteins display distinct oxysterol selectivity profiles and subcellular locations, consequently they are involved in various cellular functions: lipid metabolism, vesicular and non-vesicular sterol transport, signaling, and regulation of cell death (Ngo & Ridgway, 2009 ; Ngo, Colbourne, & Ridgway, 2010 ; Weber-Boyvat, Zhong, Yan, & Olkkonen, 2013).

Oxysterols also bind to nuclear receptors that promote expression of target genes, these receptors possess distinct selectivity for cholesterol and different oxysterols. Genes expressed in this way may encode proteins that facilitate the efflux of sterols (Makishima, 2005) Moreover, sterols can inhibit genes involved in the accumulation of sterols like those encoding for LDL receptors (Raghow, Yellaturu, Deng, Park, & Elam, 2008). Some differences observed between cell types regarding the effects of individual oxysterols on cell metabolism, especially metabolic pathway involving sterols, may be explained by expression profiles of OSBP and proteins encoded by genes expressed or repressed by nuclear receptors that bind to oxysterols.

2.3.3.3. Oxidized lipoprotein

It is important to highlight that most of the knowledge on oxidized lipoprotein comes from the study of their role in atherosclerosis and not on the effect of oxidized lipoprotein on mammalian cell culture for recombinant protein production.

Lipoprotein oxidation depicts a progressive transformation from a native lipoprotein to a minimally or partially oxidized lipoprotein, to end in an oxidized lipoprotein. Through lipoprotein oxidation, several lipoprotein constituents like phospholipids, cholesteryl esters, tocopherols and apolipoproteins are oxidized leading to the generation of new species. Oxidized lipoproteins properties differ from native

lipoproteins, which results in a loss of function. Dysfunctional oxidized lipoproteins failure to eliminate metabolism by-products and ensure correct lipid transport are detrimental for the maintenance of biological functions.

The role of lipid peroxidation in lipoprotein oxidation was confirmed by consumption of unsaturated lipids, production of conjugated dienes (Chang, Abdalla, & Sevanian, 1997). Moreover, the involvement of free radicals was demonstrated by blocking oxidation with the free radical scavenger butylated hydroxytoluene (Hessler, Morel, Lewis, & Chisolm, 1983). However, enzymatic oxidation may occur, like with lipoxygenase, able to oxidize lipoprotein cholesterol into oxysterols (Dzeletovic, Babiker, Lund, & Diczfaluzy, 1995). Lastly, lipid peroxidation by-products such as malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE) cause apolipoprotein carbonylation, further increasing the degree of oxidation of lipoproteins. These mechanisms are not incompatible; indeed, lipid peroxidation causes generation of short-chain aldehydes.

Lipoprotein oxidation

Based on the lipid composition of lipoprotein, C=C double bonds of PUFA, and the two abstractable hydrogens atoms on the carbon C7 of the cholesterol molecule are the most favorable sites for oxidation. However, the structure of the lipoprotein particle makes lipids present in the outer monolayer, phospholipids and unesterified cholesterol, more accessible for oxidation by external ROS. In this sense, oxysterol levels were reported to increase dramatically when the relative electrophoretic mobility of lipoprotein particle went above a threshold (Patel, Diczfalusy, Dzeletovic, Wilson, & Darley-Usmar, 1996 ; Carpenter, et al., 1994). A shift between oxidation of free cholesterol, present in the outer monolayer of the lipoprotein, to oxidation of esterified cholesterol present in the neutral core of the lipoprotein may explain this phenomenon (Patel, Diczfalusy, Dzeletovic, Wilson, & Darley-Usmar, 1996).

Moreover, a rapid increase of conjugated dienes concentration during the first hours of copper-mediated oxidation of lipoprotein was followed by a slight increase whereas oxysterol formation was detected at a later-time point: after 30 min conjugated dienes have reached 27% of their maximum concentration while oxysterols have reached only 1.9% (Dzeletovic, Babiker, Lund, & Diczfaluzy, 1995).

Taken together all these results confirm that lipoprotein oxidation is a progressive mechanism that includes several steps. First oxidation of the outer monolayer characterized by consumption of PUFA and formation of conjugated dienes. Then oxidation of the inner core of the lipoprotein, characterized by oxidation of esterified cholesterol into oxysterols. Overall, in copper-mediated oxidation, esterified cholesterol with long chain and multiple double bonds is oxidized preferably (Dzeletovic, Babiker, Lund, & Diczfaluzy, 1995 ; Brown, Dean, & Jessup, 1996). Lastly, arachidonic and linoleic acids were oxidized preferably compared to oleic acid (Dzeletovic, Babiker, Lund, & Diczfaluzy, 1995).

Lipoprotein oxidation does not concern only lipids, amino acids from the apolipoprotein peptide chain are also oxidation target. This phenomenon, called protein carbonylation, involves aldehydes formed

by cleavage of lipid hydroperoxides that react with cysteine, histidine, and lysine residues resulting in alteration of apolipoproteins structure and properties (Witz, 1989 ; Sayre, Lin, Yuan, zhu, & Tang, 2006 ; Hauck & Bernlohr, 2016 ; Bootorabi, Jänis, & Valjakka, 2008).

Copper-mediated oxidation of lipoprotein was reported to considerably decrease the number of accessible lysine residues (-65%), increase the negative charge, slightly increase the size of the apolipoprotein but not impacted the secondary structure (Vanderyse, et al., 1992). Reaction of MDA, 4-HNE and ROS with positively charged residues of apolipoprotein participate in the increasing negative charge of lipoprotein during oxidation (Vanderyse, et al., 1992). Even if protein carbonylation is not the only reaction that increases the amount of negative charge, these results are concordant with electrophoretic mobility changes observed by laser Doppler electrophoresis, the major population of lipoprotein were becoming more negatively charger during lipoprotein oxidation (Arrio, Bonnefont-Rousselot, Catudioc, & Packer, 1993).

Factors that influence lipoprotein oxidation

Lipoprotein composition, especially the degree of unsaturation of fatty acids and cholesteryl esters, highly influences lipoprotein oxidation. Indeed, the rate of oxidation is correlated to the degree on unsaturation (Soupas, Juntunen, Lampi, & Piironen, 2004 ; Brown, Dean, & Jessup, 1996 ; Porter, Caldwell, & Mills, 1995). Moreover, because antioxidants like vitamins C and E, and low-valence-state ions like ferrous or cupric ions influence lipid peroxidation, the presence and concentration of these compounds also influence lipoprotein oxidation.

The nature of the oxidation mechanism was reported to influence PUFA peroxidation intermediates and so by-products. Linoleic acid oxidation produced four PUFA hydroperoxides in about equal yields, two conjugated dienes, 9-OOH and 13-OOH, and two non-conjugated dienes 10-OOH and 12-OOH (Thomas & Pryor, 1980). Because, free-radical peroxidation of linoleic acid produced only the two conjugated dienes, 9-OOH and 13-OOH, therefore, the presence of 10-OOH and 12-OOH is an evidence that lipid peroxidation is not the only oxidation mechanism that occurred.

Because the kinetics of lipid peroxidation propagation and lipid hydroperoxide decomposition increase with temperature, temperature influences the kinetics of lipoprotein oxidation. More interestingly, depending in the oxidation temperature, 4°C or 37°C, oxidized lipoproteins are either hydroperoxide-rich oxidized lipoprotein (4°C) or oxysterol-rich oxidized lipoprotein (37°C) (Gerry & Leake, 2008). The lack of kinetic energy to allow copper to break down lipid hydroperoxides to lipid radicals at 4°C may explain this phenomenon (Gerry & Leake, 2008).

Also, the surrounding lipid matrix influences lipoprotein oxidation, especially sterol oxidation. Interaction between the lipid matrix and temperature were reported to have major effects on sterol oxidation, notably on the total oxysterol content (Soupas, Juntunen, Lampi, & Piironen, 2004) and on distribution of the secondary oxidation products (Li, Ohshima, Shozen, Ushio, & Koizumi, 1994 ; Li, Cherian, Ahn, Hardin, & Sim, 1996). At high temperatures (>140°C) sterols are more stable in

unsaturated than saturated fatty acids matrix, whereas as at lower temperature (<140°C) the opposite is true (Soupas, Juntunen, Lampi, & Piironen, 2004). At high temperatures, the unsaturated fatty acids matrix may be more readily oxidizable than sterols, protecting them from reacting.

Oxidation temperature was also reported to affect the secondary sterol oxidation products. The proportion of 7-ketones decreased during heating at low temperatures (<140°C) in unsaturated lipid matrix but remained stable in saturated lipid matrix (Soupas, Juntunen, Lampi, & Piironen, 2004). Similarly, after 22h of heating at 110°C (considered as low temperature), 7-ketones remained the main oxides in saturated palm oil whereas 7-ketones were replaced by 5b,6b-epoxides in unsaturated flax oil (Li, Cherian, Ahn, Hardin, & Sim, 1996) (Li, Ohshima, Shozen, Ushio, & Koizumi, 1994). Lastly, 7-ketocholesterol was replaced by 7b-hydroxides and 5b,6b-epoxides during oxidation of unsaturated sardine oil at 25°C (Li, Cherian, Ahn, Hardin, & Sim, 1996). These concordant results expose how the interactions between the lipid matrix and the oxidation temperature influence the nature of the final products of lipid oxidation (Table 5). In conclusion, the oxidized lipoprotein composition depends on both the lipoprotein composition and the oxidation conditions.

	< 140°C	**> 140°C**
Unsaturated lipid matrix	Sterols are less stable than the fatty acids matrix and tend to be oxidized 5b,6b-epoxides are the main sterol oxidation products	Sterols are more stable than the fatty acids matrix, matrix oxidation prevents sterol oxidation
Saturated lipid matrix	Sterols are more stable than the fatty acids matrix, matrix oxidation prevents sterol oxidation 7-ketones are the main sterol oxidation products	Sterols are less stable than the fatty acids matrix and tend to be oxidized

Table 5: Summary of thermal stability of sterols in lipid matrix from the research of Soupas, L., et al., (2004) ; Li, N., et al., (1994) and Li, S. X., et al., (1996).

Lastly, in vivo data showed that dietary oxysterols are incorporated in plasma lipoproteins increasing their susceptibility to oxidation (Vine, Mamo, Beilin, Mori, & Croft, 1998).

Oxidized lipoprotein composition

Lipoproteins are complex structures containing a large variety of compounds, their oxidation produces an even larger variety of molecules. During lipoprotein oxidation, formation free and esterified oxysterols was reported (Warner, Addis, Emanuel, & Wolfbauer, 1990 ; Bhadra, et al., 1991 ; Tanaka & Kanamaru, 1993 ; Carpenter, et al., 1994 ; Mori, Croft, Puddey, & Beilin, 1996), notably 7-ketocholesterol is the predominant cholesterol oxidation product (Dzeletovic, Babiker, Lund, & Diczfaluzy, 1995 ; Chang, Abdalla, & Sevanian, 1997 ; Rodriguez, Alam, & Lee, 2004 ; Gerry & Leake, 2008 ; Mori, Croft, Puddey, & Beilin, 1996), the other major products being oxysterols made by oxidation on the C7 carbon (~80%) 7-a- and 7-b-hydroxycholesterol (Rodriguez, Alam, & Lee, 2004), lastly, 5,6a- and 5,6b-epoxycholesterol are formed in significant amount (Patel, Diczfalusy, Dzeletovic,

Wilson, & Darley-Usmar, 1996 ; Brown, Dean, & Jessup, 1996), 25-hydroxycholesterol (Rodriguez, Alam, & Lee, 2004).

Despite oxysterols, conjugated and non-conjugated PUFA hydroperoxides including 9-OOH, 10-OOH, 12-OOH and 13-OOH (Thomas & Pryor, 1980), oxidized PUFA such as 9-HODE, 13-HODE and 15-HETE (Jira, Spiteller, Carson, & Schramm, 1998 ; Kitano, Yoshida, Kawano, Hibi, & Niki, 2007) and other chain-shortened PUFA derivative (Subbanagounder, Watson, & Berliner, 2000), aldehydes such as MDA, 4-HNE, acrolein, hexanal (Chang, Abdalla, & Sevanian, 1997) were formed during lipoprotein oxidation.

No matter what kind of oxidant used, peroxynitrite, myoglobin and copper, little or no production of 3,5,6-triol, 24, 25, 27-hydroxycholesterols was observed during lipoprotein oxidation confirming that oxysterol nature are independent of the oxidant (Patel, Diczfalusy, Dzeletovic, Wilson, & Darley-Usmar, 1996).

Oxidized lipoprotein uptake

Lipoprotein and oxidized lipoprotein uptake occur through the receptor-mediated pathway also known as mediated endocytosis. Some of these receptors are specific to a ligand or a few ligands with a similar structure, on the other hand scavenger receptors are able to interact with a large variety of structurally different ligands. Since the discovery of the cholesterol uptake mechanism based on the LDL receptor pathway involving recognition of the B-100 apolipoprotein (Brown & Goldstein, 1986), several cell surface receptors able to bind to both lipoproteins and oxidized lipoproteins have been discovered (Steinbrecher, 1999): The ApoB/E receptor is involved in the LDL receptor pathway and recognizes ApoB of LDL and ApoE of both VLDL and chylomicrons (Nykjaer & Wllnow, 2002). Because mildly oxLDL present ApoB with a structure similar to LDL they are still taken up by the cell through recognition by a apolipoprotein receptor in contrary to extensively oxLDL that are no more recognized by ApoB/E receptor. The lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) is a scavenger receptor that primarily binds and regulates oxLDL (Reiss, Anwar, & Wirkowski, 2009). LOX-1 receptor expression is upregulated by oxidative stress, shear stress, and oxLDL (Mehta, Chen, Hermonat, Romeo, & Novelli, 2006). LOX-1 receptor participates in oxLDL uptake, it is suspected to be involved in apoptotic pathway and to contribute to atherogenesis through formation of foam cells. Lastly, the scavenger receptor B family, including the cluster differentiation 36 (CD36) also known as fatty acid translocase, are scavenger receptor that binds to HDL, LDL, VLDL (Calvo, Gomez-Coronado, Suarez, Lasuncion, & Vega, 1998) as well as oxLDL (Endemann, et al., 1993). Notably, CD36 contributes to oxidized lipoprotein uptake and triggering of atherogenesis (Zhao, et al., 2005).

Because apolipoproteins are critical for lipoprotein uptake (Mathews, van Holde, & Ahrern, 1999 ; Havel, 1975), any structural or conformational change is susceptible to alter lipoprotein uptake mechanisms. Indeed, when incubated for 24h in presence of native lipoprotein, J774 macrophages were reported to take up 47 µg/mg cell protein compared to 92 µg/mg cell protein in presence of copper-mediated oxidized lipoprotein (Vanderyse, et al., 1992). The difference in uptake between

lipoprotein and oxidized lipoprotein suggests that apolipoprotein alteration during lipoprotein oxidation affects uptake mechanisms. Apolipoprotein alteration during lipoprotein oxidation seems to be a progressive process. Indeed, affinity of highly oxidized lipoproteins, with a lot of apolipoprotein alterations for apolipoprotein receptors and scavenger receptors differs from affinity of mildly oxidized lipoproteins with minor alterations of apolipoproteins (Steinberg, Parthasarathy, Carew, Khoo, & Witztum, 1989). Thus, lipoprotein uptake depends on the level of lipoprotein oxidation and lipoprotein uptake rate increases as the degree of oxidation of lipoprotein increases. By altering cysteine, histidine, and lysine residues of apolipoprotein, protein carbonylation participates in apolipoprotein alteration. Because amino acids alteration in apolipoprotein affect its ability to bind to extracellular matrix components (Chait & Wight, 2000), apolipoprotein carbonylation may disturb oxidized lipoprotein interactions with lipoprotein receptors.

Once a lipoprotein is bound to a lipoprotein receptor, internalization occurs through an endocytosis process. The lipoprotein is delivered to the endosome, there the lipoprotein receptor and the lipoprotein separate, then the lipoprotein is transported to the lysosome where esterified cholesterols and triglycerides are hydrolyzed. Following internalization, lipoproteins are degraded by lysosomal enzymes. However, lysosomal enzymes are inactivated by oxLDL compounds (Hoppe, O'Neil, & Hoff, 1994), consequently oxidized lipoproteins, especially oxLDL, are poorly degraded by lysosomal enzymes and accumulate in lysosomes. Due to their higher residential time, intra-lysosomal reactions are more likely to occur with oxidized lipoprotein than native lipoprotein.

Both lipoproteins and iron are taken up via endocytosis. Interestingly, iron-mediated intra-lysosomal oxidative reaction was reported to elicit lysosome destabilization, cause lysosome rupture and trigger apoptosis (Li, Yuan, & Brunk, 1998). Moreover, release of lysosomal proteolytic enzymes in the cytosol, degrading the cell from within was reported to initiate cell death (Fossel, Zanella, Fletcher, & Hui, Cell death induced by peroxidized low-density lipoprotein: endopepsis, 1994). Lastly, excessive lipoprotein uptake and accumulation of cholesterol above cell's need can lead to formation of foam cells. Thus, control of the cholesterol metabolism is crucial because regulation of the LDL receptor expression, and other receptors involved in lipoprotein uptake are dependent of the cytoplasmic cholesterol concentration.

Because the lipid fraction of oxidized lipoprotein contains the toxic compounds, internalization of oxidized lipoproteins is required to trigger the adverse effects of oxidized lipoprotein on the cell. Cytotoxic effects of oxidized lipoprotein components will be described in section § 2.3.4.3 Cytotoxicity of oxidized lipoproteins.

2.3.4. The role of lipid oxidation in cell death

2.3.4.1. Cell death mechanisms

Cell death is defined as the irreversible degeneration of vital cellular functions culminating in the loss of cellular integrity (Galluzzi, Vitale, Aaronson, & al., 2018). By contrast with necrosis, which is an uncontrolled and non-physiological process, programmed cell death mechanisms are highly regulated and genetically controlled processes. Although distinct, as these mechanisms progress and reach an advanced stage, they can induce further cell death pathways, leading to a cascade effect.

This section introduces cell death mechanism and will highlight how lipid supplementation and lipid oxidation, if not controlled, can trigger cell death during mammalian cell culture.

Caspases: main actors of programmed cell death

Cysteine-aspartic proteases, or cysteine-dependent aspartate-directed protease, shortened in "Caspases", are involved in apoptosis, necroptosis and proptosis triggering (Nirmala & Lopus, 2020) and inflammatory response to cell death (Rock & Kono, 2008).

Caspases are initially synthesized in an inactive form (procaspase) and are activated by other caspases or upon unbinding to inhibitors-of-apoptosis proteins (IAPs) (Kumar, Herbert, & Warrens, 2005). Caspases can be divided in 3 categories: caspase activators activate initiator caspases (Caspase 2, 8, 9, 10, 11 and 12) that will activate by proteolytic cleavage effector or executioner caspases (Caspases 3, 6 and 7) (Shi, 2004) and inflammatory caspases (Caspases 1, 4 and 5).

Necrosis

Necrosis, or necrotic cell death, occurs when cells lose their ability to maintain structural and functional integrity of their membranes due to external factors. In cultured cells, necrosis can be caused by changes in temperature, accumulation of toxic metabolites and viral or bacterial infections. Whereas in vivo tissue trauma, toxic substances and inflammation can cause necrosis. Loss of membrane integrity leads to its rupture and release of intracellular content into the extracellular space.

Apoptosis

Apoptosis, or Type I cell-death, is a programmed cell death mechanism initiated in response to a stress like nutrient deprivation, viral infection, increased intracellular concentration of free fatty acids or calcium, radiation, or heat. Apoptosis is characterized by phosphatidylserine translocation, morphological changes including cell volume decrease, chromatin condensation followed by nuclear fragmentation, DNA membrane blebbing and cell shrinkage (Kerr, Wyllie, & Currie, 1972) (Elmore, 2007). Apoptosis is induced through two major pathways: the mitochondrial pathway (Figure 10), also called the intrinsic pathway, and the death receptor-dependent pathway, also called the extrinsic pathway. Both mitochondrial and death receptor pathways share the final stages of apoptosis. Once mitochondrial membrane permeabilization has occurred, caspase activators such as cytochrome C, second mitochondria-derived activator of caspases and apoptotic inducing factor are released from

the mitochondria (Susin, et al., 1996; Susin, Lorenzo, & Zamzami, 1999). caspase activators activate initiator caspases that will activate effector caspases (Elmore, 2007). Effector caspases are involved in last steps of apoptosis: degradation of organelles, mRNA degradation (Thomas, et al., 2015), DNA fragmentation, membrane blebbing and thus cell death (Elmore, 2007).

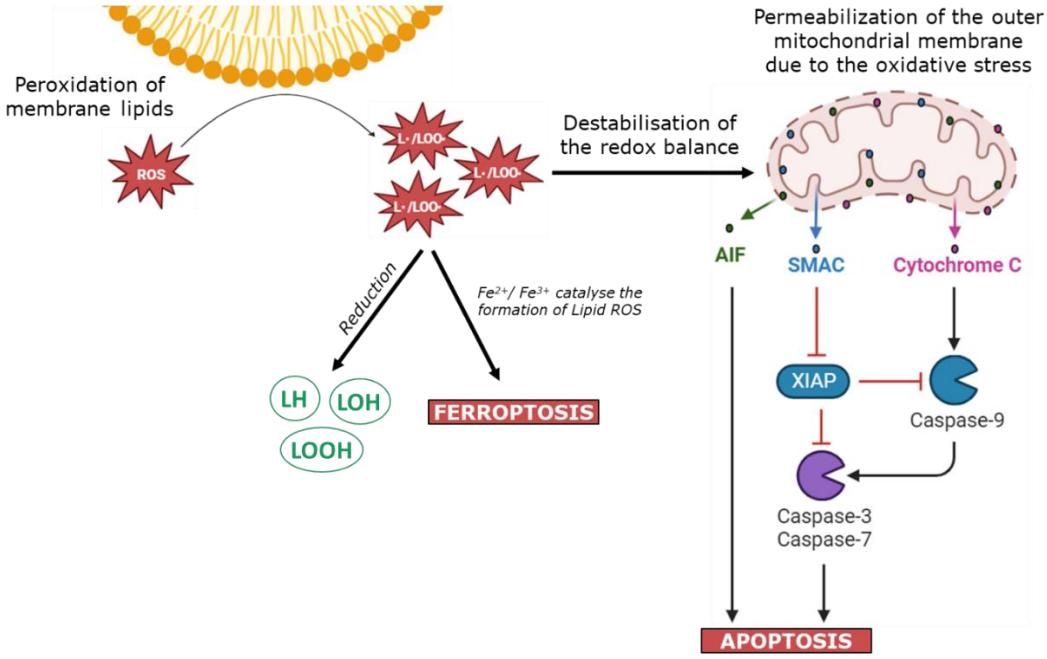


Figure 10: Mitochondrial apoptosis pathway and ferroptosis triggered by the peroxidation of membrane lipids getting out of control. ⟶ activation; ⊣ inhibition; AIF: apoptosis inducing factor; SMAC: second mitochondria-derived activator of caspase (also known as diablo homolog); XIAP: X-linked inhibitor of apoptosis protein.

Ferroptosis

Ferroptosis is an iron-dependent programmed cell death mechanism characterized by the accumulation of intracellular ROS and lipid peroxides leading to oxidative damage, membrane disruption (Cao, et al., 2020) and protein aggregation (Iuchi, Takai, & Hisatomi, 2021). Ferroptosis can be caused by a combination of dysregulation of iron metabolism and redox imbalance leading to uncontrolled lipid peroxidation (Figure 10). Because the glutathione peroxidase 4 (GPX4) catalyzes the reduction of lipid peroxides, its inhibition is known to cause ferroptosis (Yang & Stockwell, 2016). Moreover, GPX4 requires the cofactor glutathione to reduce lipid peroxides, consequently depletion of glutathione also causes ferroptosis.

Endopepsis

Endopepsis is not, strictly speaking, a cell death mechanism but an alternative pathway that leads to apoptosis. Endopepsis resembles certain aspects of the broad definition of apoptosis but differs because its critical step, disruption of lysosome and release of its content (Fossel, Zanella, Fletcher, & Hui, 1994), is neither part of the mitochondrial nor the death receptor-dependent pathways.

Endopepsis is triggered by either peroxidized low-density lipoprotein (Fossel, Zanella, Fletcher, & Hui, 1994), oxLDL (Yuan, Li, Olsson, & Brunk, 1997), acetaldehyde and MDA (Willis, Klassen, Carlson, Brouse, & Thiele, 2004). The characteristic step of endopepsis is disruption of lysosomal integrity by integrated oxidized lipoprotein leading to release of its content. Loss of lysosomal integrity was followed by foam cell formation (Yuan, Li, Olsson, & Brunk, 1997), caspase activation and apoptosis (Willis, Klassen, Carlson, Brouse, & Thiele, 2004).

2.3.4.2. Accumulation of intracellular lipid and lipotoxicity

Most of the mammalian cells, even those that are not lipid auxotroph, are able to take up lipids contained in the culture medium and use them as source of energy and building blocks. However, many mammalian cell lines excessively accumulate lipid when they are exposed to lipid-rich medium. Indeed, mammalian cells do not have a regulatory mechanism to control the uptake of extracellular lipids, especially free fatty acids, leading to a lipid loaded state which is detrimental for the cells. Such lipid accumulation leads to a phenomenon called lipotoxicity, most of the time synonym of cell death.

Intracellular lipid accumulation may occur when too much fatty acids (Doi, Doi, Schroeder, Alberts, & Vagelos, 1978 ; Spector, Kiser, Denning, Koh, & DeBault, 1979 ; Listenberger, et al., 2003), triglycerides (Howard, Howard, De la Llera, & Kefalides, 1976), cholesterol and cholesteryl-ester (Nikkari, Pietilä, & Salo, 1976 ; Rothblat, Arbogast, Krichevsky, & Naftulin, 1976) and LDL (Stein, Vanderhoeak, & Stein, 1977) are available in the culture medium. Lipotoxicity can appear as cytoplasmic inclusions named cytoplasmic lipid droplets composed either of triglycerides (Schneeberger, Lynch, & Geyer, 1971) or cholesteryl esters (Rothblat, Rosen, Insull, Yau, & Small, 1977), trigger ceramide synthesis (Listenberger, et al., 2003) and DNA laddering, a marker of apoptotic cell death (Listenberger, et al., 2003). Lipotoxicity should not be confused with phospholipidosis, another cellular lipid storage disorder, sometimes induced by drugs, and characterized by the excess accumulation of phospholipids in lysosome (Shayman & Abe, 2013).

Interestingly, lipotoxic cell death is highly dependent on the molecular species of fatty acid to which cells are exposed. CHO cells culture in presence of palmitate (500 mM) was associated with cell death, however co-supplementation with oleate (100 – 500 mM) was preventing palmitate-induced cell death and associated morphological changes (Listenberger, et al., 2003). Because palmitate is a precursor for de novo ceramide synthesis, compounds that amplify signals for palmitate-induced apoptosis, supplementation of CHO cells with palmitate was associated with higher levels of ceramides (Listenberger, et al., 2003). It was hypothesized that supplementation with oleate either prevents intermediate generation and ceramide synthesis or results in scavenging of both these molecular species, no decrease in palmitate (500 mM) uptake was observed in the presence of oleate (200 µM). Supplementation of both palmitate (500 µM) and oleate (200 µM) was associated with neutral lipid accumulation, i.e. triglyceride storage, whereas supplementation with palmitate only even at higher concentration (700 mM) was not associated with neutral lipid storage. Thus, the resistance to lipid-

induced apoptosis observed with oleate co-supplementation correlates with increased capacity for accumulation of neutral lipid (Listenberger, et al., 2003). Thus, balance between fatty acids is as important as fatty acid concentration to avoid lipotoxicity.

Logically, accumulation of triglycerides, emergence of cytoplasmic lipid droplets and toxicity associated with free fatty acid accumulation can be avoided by supplementing the culture with fatty acid at a constant rate rather than by adding the entire amount at the beginning of the culture period (Mulligan, Lunch, Schneeberger, & Geyer, 1977).

2.3.4.3. Cytotoxicity of oxidized lipoproteins

Oxidized lipoproteins have been reported to be toxic towards a wide variety of cell type (Rusinol, et al., 2000 ; Sugawa, Ikeda, Kushima, Takashima, & Cynshi, 1997 ; Escargueil-Blanc, Salvayre, & Nègre-Salvayre, 1994 ; Escargueil-Blanc, et al., 1997 ; Hughes, Matthews, Lenz, & Guyton, 1994 ; Draczynska-Lusiak, Chen, & Sun, 1998 ; Alireza, Farnaz, & Fatemeh, 2014) (supplementary Table 21). Notably, cytotoxic of oxLDL towards arterial smooth muscle cells (SMC) has been extensively studied due to its contribution to atherosclerotic plaque formation causing atherosclerosis chronic inflammation (Pirillo, Norata, & Catapano, 2013).

Oxidized lipoproteins induce typical morphological changes, DNA fragmentation and increase of caspase activity, all characteristic of apoptosis (Bjorkerud & Bjorkerud, 1996 ; Bjorkerud & Bjorkerud, 1996 ; Harada-Shiba, Kinoshita, Kamido, & Shimokado, 1998 ; Escargueil-Blanc, Salvayre, & Nègre-Salvayre, 1994 ; Escargueil-Blanc, et al., 1997).

In endothelial, smooth muscle and macrophages cells, several scavenger receptors are expressed on their surface mediate the cellular effects of oxidized lipoproteins. Unregulated uptake of oxidized lipoproteins by macrophage scavenger receptors resulted in pro-atherogenic effects (Goldstein & Brown, 1977). Because SP2/0 hybridoma are fused with B cell, and B cell were reported to uptake oxidized lipoproteins (Waseem, et al., 2017 ; Waseem, Keeter, Moriarty, Fernandez-Hernando, & Galkina, 2019), SP2/0 should logically take up oxidized lipoproteins. Dysregulated uptake of oxidized lipoprotein can cause fatty acid accumulation, which is known to be toxic, potentially triggering apoptosis (Cnop, Hannaert, Hoorens, Eizirik, & Pipeleers, 2001 ; Listenberger, et al., 2003).

Toxic compounds of oxidized lipoproteins were extracted with an organic solvent (Hessler, Morel, Lewis, & Chisolm, 1983), thus the protein moiety of oxidized lipoprotein is not required for cytotoxicity. Among liposoluble compounds present in oxidized lipoprotein, many of them may contribute to the oxidized lipoprotein cytotoxicity. Oxidized phospholipids are suspected to play a role in toxicity because pre-treatment of oxLDL by phospholipase reduced oxLDL toxicity (Schmitt, Nègre-Salvayre, Troly, Valdiguié, & Salvayre, 1995). Aldehydes, by-products of lipid peroxidation, like MDA (Balcavage & Alvager, 1982) and 4-HNE (Benedetti, Comporti, & Esterbauer, 1980) have known adverse cellular effects. Oxidation of inner mitochondrial membrane component cardiolipin produces cardiolipin

hydroperoxides which can result in the conformation change of the lipid. The oxidized CL transfers from the inner membrane to the outer membrane, and then helps to form a permeable pore which release cytochrome C (Belikova, et al., 2006).

Cholesterol oxidation by-products such as oxysterols negatively affect cellular growth and proliferation (Smith & Johnson, 1989 ; Rodriguez, Alam, & Lee, 2004). Moreover, various studies have identified oxysterols as apoptosis inducers in mammalian cell lines (supplementary Table 22). Oxidized lipoprotein uptake produced a sterol composition in the cell similar to the oxidized lipoprotein composition itself (Brown, Dean, & Jessup, 1996). Oxysterols vary in their ability to induce apoptosis, the relation between oxysterol structure and their ability to trigger apoptosis, as well as the mechanisms by which oxysterols induce apoptosis are molecule and cell line dependents. Indeed, 7-ketocholesterol activates the tumor necrosis factor receptor (TNFR)-mediated death pathway in human aortic smooth muscle cells (Lee & Chau, 2001), on the contrary in human endothelial cells TNF-α secretion was not detected during 7-ketocholesterol induced apoptosis (Lemaire, et al., 1998). Even if oxysterol-induced apoptosis pathway may vary from one cell line to another, cell lines sharing common characteristics such as species, tissue of origin or cell type are more susceptible to share common pathway activated during oxysterol-induced apoptosis due to their similar metabolisms.

Partition of oxysterols present in oxLDL revealed that of the oxysterols tested, 7-ketocholesterol was the most cytotoxic towards retinal pigment epithelium cells (Rodriguez, Alam, & Lee, 2004). However, several oxidized lipids are present in oxidized lipoprotein, their association and interaction make it difficult to identify the responsibility of each in the toxic effect of oxidized lipoprotein.

Because some results on oxidized lipoproteins are contradictory, two aspects of the oxidized lipoproteins toxicity studies should be highlighted: oxidized lipoprotein cytotoxicity depends on the cell line studied and the type of oxidized lipoprotein (formation conditions, mildly- or extensively-oxidized lipoprotein). Identification of the respective role of each individual compound of oxidized lipoprotein is difficult due to the differences in their cell entry, metabolism, and molecular targets between when they are associated with lipoprotein and when they are added pure to the culture. To illustrate this, oxysterol-rich LDL were more toxic than hydroperoxide-rich LDL towards J774 monocyte-macrophages (Gerry & Leake, 2008), whereas the opposite was true for smooth muscle cells (Siow, Richards, Pedley, Leake, & Mann, 1999) indicating that the cytotoxic effects of oxidized lipoproteins is cell line dependent. Lastly, copper-mediated oxidation of lipoprotein increased oxidized lipoprotein cytotoxicity (Rodriguez, Alam, & Lee, 2004).

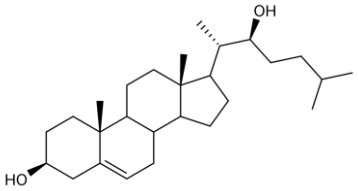
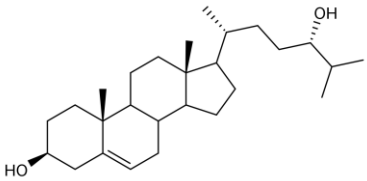
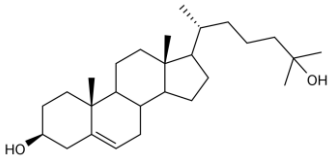
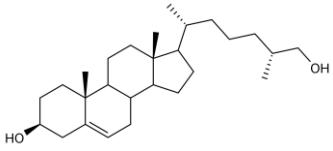
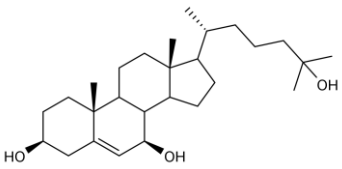
3. MATERIALS AND METHODS

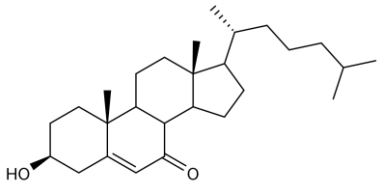
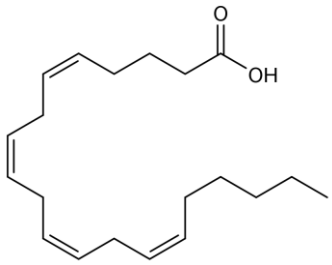
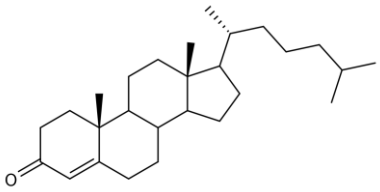
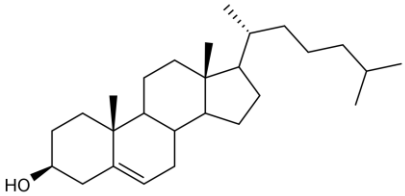
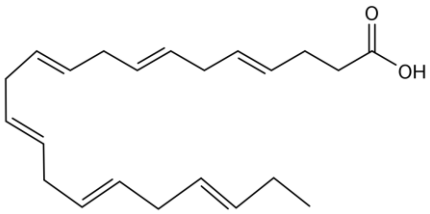
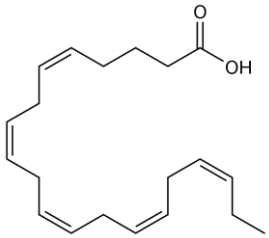
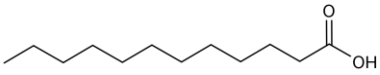
3.1. Materials

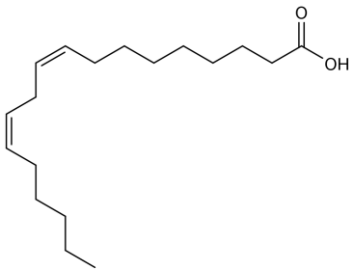
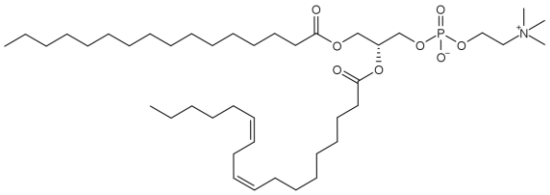
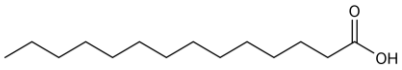
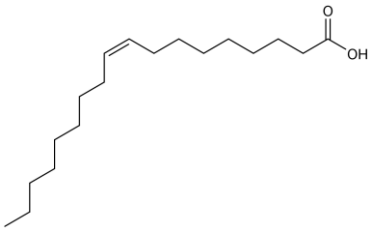
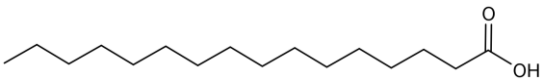
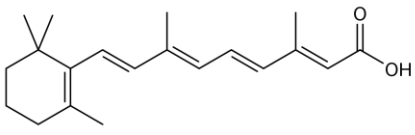
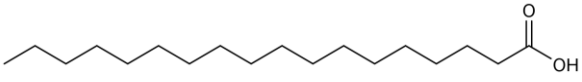
3.1.1. General chemicals and reagents

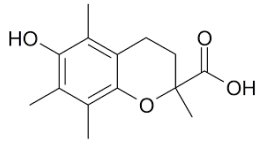
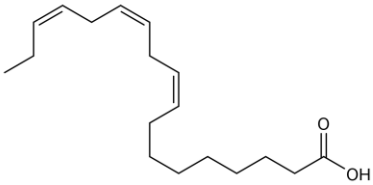
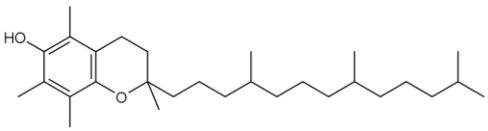
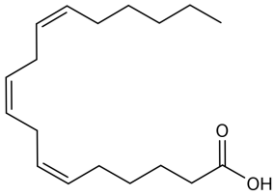
General chemicals and reagents	Supplier	Product Reference
Hydrochloric acid (HCl) solution 5 M	Sigma Aldrich	258148
Phosphate-buffered saline (PBS) powder	Sigma Aldrich	P3813
Sodium hydroxide (NaOH) pellets (anhydrous)	Sigma Aldrich	S5881

3.1.2. Lipids with chemical structures

Lipids with chemical structures	Supplier	Product Reference
22(S)-hydroxycholesterol 	Sigma Aldrich	H5884
24(S)-hydroxycholesterol 	Sigma Aldrich	SML1648
25-hydroxycholesterol 	Sigma Aldrich	H1015
27-hydroxycholesterol 	Sigma Aldrich	SML2042
7b,25-hydroxycholesterol 	Avanti Polar Lipids	700081P

7-ketocholesterol 	Avanti Polar Lipids	700015P
Arachidonic acid 	Sigma Aldrich	10931
Cholestenone 	Merck	188174
Cholesterol 	Sigma Aldrich	C3045
CIS-4,7,10,13,16,19-docohexaenoiacid (DHA) 	Sigma Aldrich	D2534
CIS-5,8,11,14,17-eicosapentaenoic acid (EPA) 	Sigma Aldrich	E2011
Lauric acid 	Sigma Aldrich	W261408

Linoleic acid 	Sigma Aldrich	L1012
Linoleic acid – Water Soluble 3% linoleic acid and 97% mbCD (w/w)	Sigma Aldrich	L5900
L-α-phosphatidylcholine 	Sigma Aldrich	P3556
Myristic acid 	Sigma Aldrich	M3128
Oleic acid 	Sigma Aldrich	O1008
Oleic acid – Water Soluble 3% oleic acid and 97% mbCD (w/w)	Sigma Aldrich	O1257
Palmitic acid 	Sigma Aldrich	P0500
Retinoic acid 	Sigma Aldrich	R2625
Soy phospholipids	Sigma Aldrich	11145
Stearic acid 	Sigma Aldrich	S4751

Trolox 	Sigma Aldrich	238813
α-linolenic acid 	Sigma Aldrich	L2376
α-tocopherol 	Sigma Aldrich	258024
γ-linolenic acid 	Sigma Aldrich	L2378

Chemical structures were drawn with MarvinSketch 18.22.2 software (ChemAxon).

3.1.3. Chemicals for cell culture and fed-batch experiments

Chemicals for cell culture and fed-batch experiments	**Supplier**	**Product Reference**
Chemically Defined Lipid Mixture 1	Sigma Aldrich	L0288
Cholesterol Lipid Concentrate	Thermo-Fisher Scientific	12531018
Choline Chloride	Sigma Aldrich	C7527
Dextran sulfate sodium salt	Sigma Aldrich	31395
D-Glucose	Merck	137048
Ethanolamine hydrochloride	Sigma Aldrich	E6133
Lipoprotein supplement for mammalian cell culture		
HyPep1510	Kerry	PHBT20
InSolution caspase-3 Inhibitor I, Cell-Permeable	Millipore	235427
Iron Chelate B	Gibco	01-0013

L-Glutamine	Merck	100286
Methotrexate	Pfizer	F000000758
Methyl-β-cyclodextrin (mbCD)	Sigma Aldrich Chemie GmbH	C4555
Myo-inositol	Sigma Aldrich	I7508
Proprietary Basal Medium		
ProYield Soy	Friesland Campina	SE50MAF-UF
Radioimmunoprecipitation assay (RIPA) Buffer	Sigma Aldrich	R0278
Sodium Bicarbonate	Merck	106323
SyntheChol® NS0 supplement	Sigma Aldrich Chemie GmbH	S5442
Trace elements 1000X	Invitrogen	ME110242L1

3.1.4. Chemicals used for analytics

Chemicals used for analytics	**Supplier**	**Product Reference**
2-aminobenzamide	Sigma Aldrich	A89804
37-component fatty acid methyl esters mix	Supelco	CRM47885
Acetonitrile (ACN)	Sigma Aldrich	34851
Ammonium bicarbonate	Sigma Aldrich	A6141
Citric acid monohydrate	SAFC	1.00242
di-Sodium hydrogen phosphate anhydrous	SAFC	1.06585
Indium II standard solution	Supelco	119504
Nitric acid	Supelco	16355
Sodium chloride	SAFC	1.06400
Sodium dihydrogen phosphate monohydrate	SAFC	1.06349

3.1.5. Consumables

Consumables	**Supplier**
Acquity ultra-performance liquid chromatography (UPLC) BEH glycan 1.7 µm column	Waters
Centrifuge tubes, Falcon (50 ml)	Corning Life Science
Combitips® advanced (several sizes)	Eppendorf
Corning® 125 mL Polycarbonate Erlenmeyer Flask with vent cap	Corning
Corning® 96-well ultraviolet (UV)-transparent microplates	Corning
Corning® mini bioreactor (50 ml)	Corning

Eksigent HALO Fused-Core Phenyl Hexyl column	Sciex
Eppendorf serological pipettes (2-100 ml)	Eppendorf
Eppendorf tubes® (1.5 ml and 2 ml)	Eppendorf
Hitrap Mabselect Sure 1mL prepacked column	GE Healthcare
Millex® Syringe filters 0.2 µm pore size, 33 mm diameter, polyethersulfone (PES) membrane	Millipore
Mono S® 5/50 GL ion exchange column	Amersham Biosciences
Pipette tips (20-2000 ml)	Eppendorf
ProntoSIL 120 C18H column	Dr.Maisch GmbH
Protein A PhyTips® columns (20-160 µl)	PhyNexus
SP®-2380 capillary GC (30 mm x 0.25 mm, 0.20 µm thickness)	Supelco
Stericup® sterile vacuum filtration systems (250 ml and 1L)	Merck
Steriflip® filter units (50 ml)	Merck
Synergi HydroRP-C18 column	Phenomenex
TubeSpin® Bioreactor (50 ml)	TPP

3.1.6. Equipment

Equipment	Supplier
780 pH meter	Metrohm
Manual multichannel pipette (several sizes)	Eppendorf
Manual single channel pipette (several sizes)	Eppendorf
Milli-Q® Water Filtration System	Millipore
Model 3320 osmometer	Advanced Instruments
Multifuge 3 S-R	Heraeus
Multipette® E3x	Eppendorf
Multitron incubator	Infors HT
Pipetboy 2	Integra
SevenExcellence pH/Cond meter S470	Mettler Toledo
SKAN Biohazard Silverline, class 2 safety cabinet	Kojair Blue Series Technology
Vortex Shaker	VWR

3.1.7. Software

Software	Supplier
Analyst software 1.6.2	Sciex
Eksigent Control Software 4.1	Sciex

Empower™ 3 Software 7.30	Waters
Image J	National Institute of Health
iQue Forecyt® Enterprise Client Edition 8.1 (R3)	Sartorius
Jump 16.2	JMP Statistical Discovery LLC
MarvinSketch 18.22.2	ChemAxon
Microsoft Office 365	Microsoft Corporation
OPUS 7.5	Brüker
SIMCA 16, 17 and 18	Sartorius
Unicorn 7	Cytiva
Vi-Cell™ XR 2.06.3	Beckman Coulter

3.2. Methods

The chemical composition of a lipoprotein supplement was partially characterized, analytical methods used for the physico-chemical characterization are presented in section 3.2.1 and the spectroscopic tools applied to fingerprinting are presented in section 3.2.2.

In parallel with the lipoprotein characterization, cell culture experiments were conducted, media and feed preparation protocols are presented in section 3.2.3 and cell culture procedure in section 3.2.4. At the end of cell culture experiments antibody quantification and purification were made with methods presented in 3.2.5. The role of apoptosis and lipoprotein oxidation was assessed during cell culture experiments based on experimental protocols presented in sections 3.2.6 and 3.2.7, respectively. Lastly, design of experiments (DOE) was applied during this work and results from experiments were analyzed with statistical tools, all these concepts are presented in section 3.2.8.

3.2.1. Physicochemical characterization

3.2.1.1. Fatty acid

Characterization of the fatty acid composition of the lipoprotein supplement was made by the St. Louis Biologics Midwest Process Development and Technology team from Millipore Sigma (Saint Louis, MO, USA).

Sample preparation

The lipid fraction of the lipoprotein supplement was extracted using a 40:10:1 mixture of 2-propanol, hexane, and 1.0 N H_2SO_4. Next, 0.1 N H_2SO_4 and hexane were added, and the resulting organic layer was transferred first into 0.01 N H_2SO_4 and then into a blank tube for subsequent evaporation under N_2. This extraction process was repeated in the same tubes with another aliquot of hexanes, and the organic layer was transferred and finally evaporated. The resulting extraction residue was then derivatized with 1 ml BF_3-methanol at 100-105 °C for 5 minutes to form fatty acid methyl esters

(FAMEs). After heating, a 3:1 mixture of saturated NaCl solution and hexane was added, mixed, and centrifuged, and the organic layer was separated for further analysis.

GC conditions

For analysis, 1 µL of the organic phase containing FAMEs was injected into the GC system (splitless). Detection was performed using a flame ionization detector (FID), and quantification was achieved with a certified 37-component FAME standard (Supelco, CRM47885) using the internal standard approach. The capillary GC column used was SP®-2380, with dimensions of 30 m x 0.25 mm and a thickness of 0.20 µm (Supelco, 24110-U). The stationary phase of the column consisted of stabilized poly (90% biscyanopropyl and 10% cyanopropylphenyl siloxane). The GC temperature program was as follows: 1) 50 °C for 3 minutes; 2) increase to 80 °C in 1.5 minutes (at 20 °C/min); 3) increase to 130 °C in 5 minutes (at 10 °C/min); 4) increase to 190 °C in 60 minutes (at 1 °C/min); 5) increase to 240 °C in 2.5 minutes (at 20 °C/min); and 6) hold at 240 °C for 3 minutes, resulting in a total program time of 75 minutes.

The results were calculated relative to the FAME standard and expressed in µg/mL lipoprotein supplement.

3.2.1.2. Proteomic

Characterization of the proteomic composition of the lipoprotein supplement was made by the Biomolecular Analytical R&D team from Millipore Sigma (Saint Louis, MO, USA). Following trypsin digestion, lipoprotein supplement samples were analyzed by tandem liquid chromatography-mass spectroscopy (LC-MS).

Trypsin digestion

First, 50 µg samples of the lipoprotein supplement were aliquoted and mixed with 50 mM ammonium bicarbonate and 10% SDS to achieve a 1% SDS concentration. The samples were then loaded onto Microcon-30kDa filters (Millipore, MRCF0R030) and centrifuged at 14,000 x g for 2 minutes. Next, 100 µL of 1% SDS in 50 mM ammonium bicarbonate was added to each sample, followed by another centrifugation step at 14,000 x g for 5 minutes. To remove the SDS from the filters, 200 µL of 12.5 M urea was added to the samples, and they were centrifuged at 14,000 x g for 10 minutes. This washing step was repeated twice more. To prevent amino acid modifications caused by urea during the reduction step, 200 µL of 50 mM ammonium bicarbonate was added to each sample, and they were centrifuged at 14,000 x g for 10 minutes. This step was repeated once more. The processed samples were then stored at -20°C until further spike-in testing was conducted.

Sample preparation

Initially, spike-in test was conducted to determine appropriate concentrations of selected heavy ApoB-100 peptides. The heavy peptides LIGLTDNAL[K] and VLLDQL[R] were utilized for spike-in purposes. Replicates of a sample were spiked with these peptides at concentrations of 1 ng spike-in peptide per 1 µg of the lipoprotein supplement and 10 ng spike-in peptide per 1 µg lipoprotein supplement. After

the replicates were dried using a speed vacuum, they were reconstituted with 50 µL of 0.1% formic acid.

LC conditions

First 10 µL of each replicate was injected into the QExactive (Thermo-Fischer) for LC-MS/MS analysis. The results of the replicates indicated that the spike-in concentration of 1 ng spike-in peptide per 1 µL lipoprotein supplement was appropriate.

After the spike-in testing, the digested lipoprotein supplement samples were thawed and spiked with a total of 50 ng of each spike-in peptide. The samples were then dried using a speed vacuum. After drying, the samples were reconstituted to a concentration of 1 µg/µL in 0.1% formic acid, and 1 µL of each sample was injected for LC-MS/MS analysis. The LC system employed an Acquity UPLC (Waters, Netherlands) with a Peptide BEH C18 nanoAcquity 100 µm I.D. x 10cm column (Waters) with a flow rate of 500 nL/min. The mobile phases used were A: 0.1% formic acid in water and B: ACN. The gradient program was as follows: 1) 1% mobile phase B for 1 minute; 2) 8% mobile phase B for 120 minutes; 3) 33% mobile phase B for 1 minute; 4) 90% mobile phase B for 7 minutes; 5) 90% mobile phase B for 1 minute; 6) 1% mobile phase B for 20 minutes, resulting in a total program time of 150 minutes.

MS/MS conditions

The MS instrument was a Fusion instrument (Thermo Fisher, MA, USA) equipped with PicoView Spray Source (New Objective, MA, USA) and the polarity was ESI positive.

Data Processing and Database Search

The raw data from the LC-MS/MS analysis were submitted and compared to existing data from the Swiss Prot Bovine and Contaminant databases. The results were then processed using the Scaffold software suite, and database searches were performed using X-Tandem. Scaffold analysis provided the number of peptides and sequence coverage percentages with the following parameter settings: Protein Threshold: ≤2% FDR, Peptide Threshold: ≤1% FDR, and Minimum # of unique peptides: 2. Common contaminants introduced during sample handling, such as keratins and trypsin, were removed from all Scaffold searches.

Estimation of Protein Abundance

To estimate the percent abundance of the detected proteins in the samples, the quantitative value was determined by multiplying the quantitative value of the normalized total ion chromatogram of the given protein by its molecular weight. The converted quantitative value of all identified proteins within the sample was used to determine the estimated percent abundance. Only proteins with a 95% probability rating and a minimum of two identified peptides were included in these calculations.

The results were expressed as a relative proportion (%) of the total protein content in the lipoprotein supplement.

3.2.1.3. Trace element

The analysis of trace elements was performed by the Element Analytics team from Merck Life Science (Darmstadt, Germany). The method employed for trace elements analysis involved the use of Inductively Coupled-Plasma Mass Spectrometry (ICP-MS). Two distinct methods were utilized during the analysis: a semi-quantitative overview analysis using quadrupole ICP-MS (Q-ICP-MS) and a quantitative re-measurement using high-resolution ICP-MS (HR-ICP-MS).

To elaborate, the trace elements standard (ICP multi-element solution VI, Merck) used in this study contained only 30 of the elements that needed to be determined. To account for the missing masses in the calibration solution, calibration lines were generated using sensitivities stored in the device software. However, it is important to note that certain elements experienced interference or overlapping masses, which could potentially impact the accuracy of the results. For this reason, masses that were significantly affected by interferences or posed challenges for determination using Q-ICP-MS were re-measured using HR-ICP-MS, which provides higher resolution and accuracy in such cases. By employing this approach, we aimed to ensure the reliability and precision of the trace elements analysis in our study.

Sample preparation

The sample preparation process involved taking 50 mg of the lipoprotein supplement and mixing it with 2 mL of nitric acid (Supelco, 16355) along with 1 mL of purified water. The mixture was then decomposed using a microwave digestion system. Following this, 50 µL of indium II standard solution (Supelco, 119504) was added to the samples, and they were diluted with purified water to achieve a final volume of 50 mL.

Semi-quantitative Q-ICP-MS

The semi-quantitative overview analysis of 67 elements was performed using a quadrupole ICP-MS Elan 6000 instrument (Totalquant, PerkinElmer). During ICP-MS analysis, the samples were atomized and broken down into individual atoms in an argon plasma at very high temperatures (> 5000 K). These ions were then separated based on their individual atomic masses within a mass spectrometer and detected with high sensitivity using a detector. Calibration lines were generated using blank values and a multi-element standard, against which the samples were measured.

High sensitivity HR-ICP-MS

HR-ICP-MS, which offers higher sensitivity compared to Q-ICP-MS, was employed in this study. Due to its increased resolution, HR-ICP-MS allows for better separation of interferences from the analyte signal. Unlike Q-ICP-MS, which relies on calibration lines, the element concentrations in HR-ICP-MS were determined quantitatively using an external calibration based on standard solutions containing all elements (Na, Mg, K, Ca, Sc, Ti, V, Cr, Fe, Ni, Cu, Zn, As, Se, and Ba) that needed to be re-measured.

The results obtained from both methods, Q-ICP-MS and HR-ICP-MS, were expressed in µg/g lipoprotein supplement.

3.2.1.4. Steroid

The steroid characterization of the lipoprotein supplement was conducted by the Biomolecular Analytical R&D team from Millipore Sigma (Saint Louis, MO, USA). To establish the workflow for steroid hormone analysis in the lipoprotein supplement, a method based on steroid analysis in human plasma (Marta, Bobaly, Fekete, & al., 2018) was adopted.

Sample Preparation

Ethyl acetate was added to the lipoprotein supplement samples under agitation, and after 10 minutes, the supernatant was separated and evaporated to dryness under a flow of nitrogen. The resulting residues were then reconstituted using a 90/10 (v/v) mixture of water and ACN.

HPLC conditions

For chromatographic separation, 90 µl of the reconstituted samples were injected into an Eksigent MicroLC 200 Plus UHPLC System (Sciex, MA, USA) equipped with an autosampler (PAL system, Switzerland). A HPLC analysis was performed using an Eksigent HALO Fused-Core Phenyl Hexyl 50 m × 0.5 mm, 2.7 µm column (Sciex) in combination with a ProntoSIL 120 C18H trap 10 m × 0.5 mm, 5 µm column (Dr.Maisch GmbH). The gradient started at 90% eluent A (0.1% formic acid in water) and 10% eluent B (0.1% formic acid in ACN), held for 0.2 minutes. This was followed by a linear gradient up to 90% B, and an isocratic elution step was performed for 1.2 minutes. The total gradient time was 1.8 minutes, and the initial eluent conditions were then set and held for 0.9 minutes. The flow rate was maintained at 40 µl/min, and the column temperature was set at 25°C.

MS conditions

The UHPLC system was coupled with a QTRAP 6500 triple quadrupole linear ion trap mass spectrometer (Sciex, MA, USA), equipped with a Turbo V Source (Sciex, MA, USA) in electrospray mode. The mass spectrometer was operated in both positive and negative electrospray ionization (ESI) modes, with the following interface parameters: curtain gas at 20 AU (arbitrary unit), ionspray voltage at 5000 V for positive ESI mode and -4000 V for negative ESI mode, and a probe temperature of 200°C. Nebulizer gas (GS1) and drying gas (GS2) were set at 15 AU and 20 AU, respectively. The diameter of the electrode in the electrospray probe was 65 µm. An optimized multiple reaction monitoring (MRM) mode was utilized with a dwell time of 10 milliseconds for each transition, using nitrogen as the collision gas.

Data Acquisition and Processing

Data acquisition and processing were performed using Analyst software 1.6.2 (Sciex) and Eksigent Control Software 4.1 (Sciex). These software tools allowed for the analysis and interpretation of the data obtained from the LC-MS/MS analysis of the steroid samples in the lipoprotein supplement.

3.2.1.5. Sterol

The characterization of sterols present in the lipoprotein supplement was conducted by Creative Proteomics (Shirley, NY, USA) using an LC-MS platform comprising a Prominence Modular HPLC (Shimadzu, Japan) coupled to a LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher, MA, USA).

Sample preparation

Prior to analysis, samples were diluted 50-fold in methanol. The samples were thawed on ice, vortexed, and briefly centrifuged. Then, 50 µL aliquots of the samples were spiked with D6-cholesterol (Avanti polar lipids, 700172) at a concentration of 250 pmol/µL plasma, serving as an internal standard for sterol quantification. The samples were further diluted in 300 µL of HPLC-grade methanol, and 1.0 mL of methyl-tert-butyl ether (MTBE) was added. After 1 hour of vortexing, 200 µL of HPLC-grade water were added, and the samples were vortexed for an additional 10 minutes. Then, the samples were centrifuged for ten minutes at 3000 x g, and the supernatant was collected into a new tube. The remaining aqueous phase and proteins were re-extracted, and the extracts were combined with the previous extract from each sample. The extraction solvent was then evaporated under vacuum in a speed vacuum centrifuge. The dried lipid extracts were resuspended in ethanolic 0.5 N potassium hydroxide and subjected to alkaline hydrolysis of sterol esters at room temperature overnight. The liberated sterols were extracted twice with chloroform, dried under vacuum, and finally resuspended in 100 µL of methanol containing 0.01% butylated hydroxytoluene (an antioxidant).

HPLC conditions

The LC system included two LC20AD pumps, a vacuum degassing system, an autosampler, and a column oven. The HPLC column used was a Synergi HydroRP-C18 column (2.0 mm x 150 mm, 4 micron, 80 Angstrom pore size, Phenomenex) equipped with a guard cartridge of the same column chemistry. Solvent A consisted of water containing 0.1% formic acid, and solvent B was methanol containing 0.1% formic acid. The flow rate was set to 250 µL per minute, and the column oven temperature was maintained at 50 degrees Celsius. The autosampler temperature was held at 15 degrees Celsius, and 10 µL of each sample was injected. The gradient conditions used were as follows: 85% solvent B from 0 to 2 minutes, linear increase to 100% solvent B between 2.0 and 16 minutes, holding at 100% solvent B for 9 minutes, and finally returning to 85% solvent B to re-equilibrate the column.

MS conditions

The column eluent was introduced to a LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher, MA, USA) through a heated electrospray ionization source. The mass spectrometer operated in positive ion mode at 60,000 resolution, and full scan MS data were collected from 300-700 m/z. Data-dependent product ion spectra were collected on the four most abundant ions at 30,000 resolution using the FT analyzer. The electrospray ionization source maintained a spray voltage of 4.5 kV, with sheath gas at 30 (arbitrary units) and auxiliary gas at 10 (arbitrary units). The inlet of the mass spectrometer was held at 350 degrees Celsius, and the S-lense was set to 50%. The heated electrospray ionization source

was maintained at 300 degrees Celsius. Sterol and oxysterol species were identified based on their [M-H2O+H]+, [M-2H2O+H]+, [M+H]+, [M+Na]+, and [M+K]+ ions under the employed conditions.

Peak Finding, Identification, and Quantitation

Chromatographic alignment, isotope correction, peak identification, and peak area calculations were performed using MAVEN 3.9 software. The concentrations of each analyte were determined based on the peak area of the internal standard, D-6 cholesterol (Avanti polar lipids, 700172). Sterols were reported as the sum of each identified ion type (e.g., [M-H2O+H]+, [M-2H2O+H]+, [M+H]+, [M+Na]+, and [M+K]+ ions).

The results were expressed in pmol/mL lipoprotein supplement.

3.2.1.6. Malonaldehyde

The quantification of malonaldehyde (MDA) present in the lipoprotein supplement was performed by OXIProteomics (Créteil, France) using the Lipid Peroxidation (MDA) Assay Kit (Colorimetric) assay kit (Abcam, 233471) following the manufacturer's instructions. This assay kit is based on the thiobarbituric acid reactive substances (TBARS) assay without the heating step.

Sample Preparation

Serial dilutions of MDA standard and lipoprotein supplement samples were prepared in dilution buffer. Then, 50 µL of each diluted sample was plated in duplicate on a 96-well plate. Dilution buffer was used as a blank for background correction.

MDA Assay Procedure

After plating the samples, 10 µL of MDA color reagent solution was added into each well, and the plate was incubated at room temperature for 20 minutes on an orbital shaker. Following this, 40 µL of reaction solution was added to each well, and the plate was further incubated at room temperature for 45 minutes on an orbital shaker.

Absorbance Measurement

The absorbance of MDA in the samples was measured at 695 nm using a Varioskan LUX Multimode Microplate Reader (Thermo Fisher, MA, USA).

Calculation of MDA concentration

The absorbance readings from the blank wells served as controls and were subtracted from the values obtained from the standards and test samples. The concentrations of MDA in the lipoprotein supplement samples were calculated by referencing the MDA standard curve. The obtained results were expressed in µM.

3.2.1.7. 4-hydroxynonenal

The quantification of 4-hydroxynonenal (4-HNE) present in the lipoprotein supplement was conducted by OXIProteomics (Créteil, France) using the Lipid Peroxidation (4-HNE) Assay Kit (Colorimetric) assay kit (Abcam, 238538) following the manufacturer's instructions. This assay kit is based on the enzyme-linked immunosorbent assay (ELISA) method.

Sample preparation

One day before the assay, 4-HNE conjugate coated plates were prepared. Serial dilutions of 4-HNE-BSA standard and lipoprotein supplement samples were prepared in dilution buffer. Then, 50 µL of each diluted sample was plated in duplicate on a 96-well plate. The plate was incubated at room temperature for 10 minutes on an orbital shaker.

4-HNE Assay Procedure

After plating the samples, 50 µL of the diluted anti-4-HNE antibody was added to each well, and the plate was incubated at room temperature for 1 hour on an orbital shaker. Following this, the plate was washed three times with wash buffer to remove any unbound substances. Then, 100 µL of the secondary antibody-HRP (horseradish peroxidase) conjugate was added to all wells, and the plate was incubated at room temperature for 1 hour on an orbital shaker. After incubation, the plate was washed three times with wash buffer to remove any excess secondary antibody-HRP conjugate.

Lastly, 100 µL of the substrate solution was added to each well, and the plate was incubated at room temperature for 10 minutes on an orbital shaker to initiate the enzymatic reaction. The enzyme reaction was stopped by adding 100 µL of stop solution.

Absorbance Measurement

The absorbance of 4-HNE in the samples was measured at 450 nm using a Varioskan LUX Multimode Microplate Reader (Thermo Fisher, MA, USA).

Calculation of 4-HNE concentration

The absorbance readings from the blank wells served as controls and were subtracted from the values obtained from the standards and test samples. The concentrations of 4-HNE in the lipoprotein supplement samples were calculated by referencing the 4-HNE-BSA standard curve. The obtained results were expressed in µg/mL lipoprotein supplement.

3.2.1.8. Protein carbonylation

The quantification of protein carbonylation in the lipoprotein supplement was conducted by OXIProteomics (Créteil, France).

Sample preparation

First, the total protein content in the lipoprotein supplement samples was quantified using the Bradford method (Bradford, 1976). Proteins were evenly distributed across all samples for subsequent

analysis. Carbonylated proteins were labeled with 2,4-dinitrophenylhydrazine (DNPH) to form specific protein-DNPH derivatives.

SDS-PAGE conditions

The labeled carbonylated proteins and total proteins were separated by high-resolution electrophoresis using a 4%-20% gradient SDS-PAGE gel with a DNPH fluorescent probe (Baraibar, Ladouce, & Friguet, 2013).

Data acquisition and processing

Images of the carbonylated proteins and total proteins on the gel were acquired using the iBright system (Thermo Fisher). Densitometric analyses of the specific fluorescence signals from the labeled carbonylated proteins were performed using the Image J software. Carbonylation levels were calculated by normalizing the specific fluorescence signal to the amount of loaded proteins on the gel (4 µg). The results were reported as relative values compared to the sample showing the lowest levels of carbonylation (set at 100% as a reference). Independent evaluations were performed on different molecular weight sectors due to the heterogeneity of signals among the samples.

Carbonylation level calculation

The obtained results were expressed in relative fluorescence units (RFU) per µg of protein in the lipoprotein supplement (RFU/µg lipoprotein supplement). The result values were provided for proteins with molecular weights <20 kDa, 20-50 kDa, and 50-100 kDa, respectively. This characterization offers valuable information about the carbonylation status of proteins in the sample at different molecular weight ranges.

3.2.2. Spectroscopic methods applied to fingerprinting

3.2.2.1. Near-infrared spectroscopy

All lipoprotein supplement batches arriving at the site warehouse are analyzed with near-infrared (NIR) spectroscopy to confirm their nature. These spectra were gathered for analysis.

NIR acquisition

Spectral acquisition was performed in diffuse reflectance mode using a FT-NIR Multi-Purpose Analyzer (Brüker, Germany) with 8 scans and 8 cm-1 resolution. The spectral range covered from 4,000 cm-1 to 12,493 cm-1. Acquisition was performed using OPUS software (Bruker Optics, USA).

Preprocessing

Preprocessing facilitates analysis and comparison of the spectra. The spectra were preprocessed in SIMCA 17 software (Sartorius). A first derivative with 15 data points was applied, then spectra were normalized by subtracting the mean and dividing by the standard deviation of the spectrum.

3.2.2.2. UV-visible spectroscopy

Sample preparation

First, lipoprotein supplement samples were 0.22 µm filtered, then diluted 100X with purified water. These diluted samples were then added in duplicate to a UV-transparent 96-well microplate (Corning, 8404) to prevent any interference from microplate absorption in the UV region. Purified water was used as a blank, except in the case of copper-mediated oxidized lipoprotein samples where purified water containing CuSO4 was used in parallel with purified water blanks.

Absorbance measurement

The lipoprotein supplement absorbance was measured from 230 to 1000 nm using a SpectraMax i3 instrument (Molecular Devices, CA, USA). The absorbance readings from the blank wells served as controls and were subtracted from the values obtained from the samples.

3.2.3. Cell culture media and feed preparation

All preparations described in this section were weighted with a NewClassic MF ML54/01 balance (0 – 1000 g) (Mettler Toledo, Switzerland), a X2002S balance (1 – 10 kg) (Mettler Toledo, Switzerland) and a SR32001 DeltaRange balance (>10 kg) (Mettler Toledo, Switzerland) all controlled by a ID7 balance dispersed terminal (Mettler Toledo, Switzerland).

3.2.3.1. Stock solution preparation

All stock solutions used for cell culture media supplementation were prepared in either purified water or absolute ethanol (Alcosuisse AG) at ambient temperature with appropriate stirring to ensure full dissolution. Stock solutions were not pH equilibrated. Upon preparation, they were either promptly utilized for the CCM preparation or 0.22 µm filtered aseptically then stored at 2-8°C protected from light.

Stock solutions were stored 30 days at most then new stock solution batches were prepared if needed. If precipitation in the stock solution was observed another stock solution batch was prepared. Stock solutions were vortexed prior to their utilization.

Fatty acid stock solutions

Stock solutions of fatty acids in absolute ethanol were prepared as follows: linoleic acid 5.0 mg/mL, stearic acid 5.0 mg/mL, oleic acid 2.5 mg/mL, palmitic acid 2.5 mg/mL, and α-linolenic acid 0.75 mg/mL, γ-linolenic acid 0.75 mg/mL, arachidonic acid 0.40 mg/mL, myristic acid 0.40 mg/mL, EPA 0.40 mg/mL and DHA 0.40 mg/mL.

Stock solutions of mbCD inclusion complexes with fatty acids were prepared in purified water as follows: linoleic acid 2.5 mg/mL and oleic acid 2.5 mg/mL both containing 77 mbCD mg/mL. These solutions were prepared by dissolving water soluble-linoleic acid (Sigma Aldrich, L5900) and water-soluble oleic acid (Sigma Aldrich, O1257) both containing fatty acid 30 mg/g (balance with mbCD).

Sterol stock solutions

Stock solutions of oxysterols in absolute ethanol were prepared as follows: 22-hydroxycholesterol, 24-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, 7b,25-dihydroxycholesterol and 7-ketocholesterol at an oxysterol concentration of 6.0 mg/mL.

Trolox stock solution

Stock solutions of Trolox in purified water were prepared at 900 and 1800 mg/L.

Methyl-beta-cyclodextrin stock solution

Stock solutions of mbCD in purified water were prepared at 125 mg/mL.

Copper (II) sulfate stock solution

Stock solutions of Copper (II) Sulfate (Sigma Aldrich, 451657) in purified water were prepared at 3mM.

Caspase-3 inhibitor stock solution

The cell permeable caspase-3 inhibitor solution, 5mM in dimethyl sulfoxide (DMSO), was further diluted in purified water to achieve a final concentration of 20 nM.

Dextran sulfate stock solution

A stock solution of dextran sulfate sodium-salt in purified water was prepared at 200 mg/mL.

3.2.3.2. Preparation of custom lipid formulations

The custom lipid formulations were prepared by the Upstream R&D Laboratory Technology Transfer team at Merck Life Science (Darmstadt, Germany). However, specific details concerning the lipid:mbCD ratio and the preparation protocol for water-soluble powder or aqueous solutions of mbCD inclusion complexes with lipids are intentionally withheld to maintain confidentiality.

In summary, mbCD (Sigma Aldrich, C4555) was dissolved in purified water, followed by sequential addition of lipids (refer to section 3.1.2 for details) under agitation. Solid lipids were added directly, while liquid lipids were diluted in absolute ethanol before addition. Continuous agitation was applied until complete dissolution of the lipids, and the solution was protected under a nitrogen atmosphere throughout the dissolution process.

For the liquid formulation, the solution was aseptically filtered through a 0.22 µm filter and stored at 2-8°C, protected from light. As for the powder formulations, the solutions were transferred to a rotary evaporator to remove ethanol, followed by crystallization using an Advantage Plus EL-85 freeze dryer (VirTis Bio Labs, India).

The final lipid concentration in both the liquid and dissolved 1X solid formulations ranged between 2.5 and 25 g/L.

3.2.3.3. Cell culture media and feed preparation

Cell Culture Media

A proprietary basal medium, derived from DMEM/F12 medium, was dissolved in purified water at 30-35°C under agitation for 25 minutes. Sodium bicarbonate (Merck, 106323), peptone hydrolysate, either HyPep1510 (Kerry, PHBT20) or ProYield Soy (Friesland Campina, SE50-MAF-UF), Iron Chelate B (Gibco, 01-0013), 5.0 g/L of the lipoprotein supplement or another lipid supplement, and MTX (Pfizer, F000000758) were sequentially added with 5-10 minutes of stirring between each addition. The pH was adjusted to 7.15 ± 0.25, and osmolality was measured to ensure its conformity. CCM were 0.22 µm filtered aseptically and then stored at 2-8°C, protected from light. A few hours before use, CCM was incubated at 37°C with 5% CO_2 in an HeraCell™ VIOS 160i (Thermo Fisher, MA, USA).

Main Feed

Glucose, amino acids, and trace elements (their nature is kept confidential) were added sequentially in purified water at 30-35°C, with 5-10 minutes of stirring between each addition. The pH was adjusted to 5.60 ± 0.10, and osmolality was measured to ensure its conformity. The main feed was 0.22 µm filtered aseptically and then stored at 2-8°C, protected from light.

Peptone Hydrolysate Feed

Peptone hydrolysate, either HyPep1510 (Kerry, PHBT20) or ProYield Soy (Friesland Campina, SE50-MAF-UF), was dissolved in purified water at 30-35°C and stirred for 60 minutes. The pH and osmolality were measured to ensure they fell within acceptable ranges: 6.90 ± 0.10 pH units and 1385 ± 25 mOsmol/kg, respectively. The peptone hydrolysate feed was 0.22 µm filtered aseptically and then stored at 2-8°C, protected from light.

Lipoprotein supplement feed

The lipoprotein supplement solution was 0.22 µm filtered aseptically and then stored at 2-8°C, protected from light.

Alternative lipid supplement feed

The custom lipid supplement, prepared by encapsulating lipids in mbCD according to section 3.2.4.2, along with water-soluble fatty acids, cholesterol, and mbCD were added sequentially in purified water at 30-35°C, with 5-10 minutes of stirring between each addition. After the last addition, the solution was agitated until complete dissolution. The lipid feed was 0.22 µm filtered aseptically and then stored at 2-8°C, protected from light.

Commercial lipid supplements and solutions of mbCD inclusion complexes with lipids, prepared according to the procedure detailed in section 3.2.4.2, were directly added to the culture and not mixed with the other components of the lipid feed to avoid lipid loss during a potential second filtration since they have already been filtered previously.

3.2.4. Cell culture

Cell culture experiments were conducted into a SKAN Biohazard Silverline, class 2 safety cabinet (Kojair Blue Series Technology) periodically cleaned with Dec-Clean cleaning solution (Veltek Associates, Inc.) and Dec-Quat cleaning solution (Veltek Associates, Inc.) according to Merck's cleaning procedures. Everything that entered the cabinet was first cleaning with denatured Ethanol 70% (B Braun).

3.2.4.1. Cell line

Cell culture experiments were performed with a SP2/0 murine hybridoma cell line producing a recombinant IgG1. The underlying selection principle of the cell line was based on dihydrofolate reductase (DHFR) overexpression allowing the growth of successfully transfected cells in DHFR inhibitor MTX-containing medium. DHFR converts dihydrofolate intro tetrahydrofolate, a compound required for de novo synthesis of thymidylic acid, a DNA building block. DHFR-lacking cells were used as a host so that only recombinant cells co-transfected with DHFR gene, and the IgG1 coding sequence, were able to survive in thymidine-lacking medium upon selection process. Moreover, supplementation of this medium with methotrexate, a competitive inhibitor of DHFR, increase selection pressure on cells expressing the highest levels of DHFR, and thus, allow the selection of the top recombinant protein producers (Say Kong, 2012).

3.2.4.2. Cultivation conditions

Cell cultures were maintained at 37°C, 90% humidity and 10% CO_2 in a Multitron incubator shaker (Infors). When culture took place Corning® 125 mL flask with cent cap the agitation was set on 130 rpm and the working volume was 40 mL, whereas when culture took place in Corning® mini bioreactor (50 mL) or TubeSpin® Bioreactor (50 ml) the agitation was set on 320 rpm and the working volume was 30 mL. Shake-flasks (SF) and shake-tubes (ST) were fitted with a vent cap for gas exchange.

3.2.4.3. Process description

Laboratory work was carried out using a small-scale model of a commercial process. The experiments were halted either after clarification when only titer results were needed, or after the capture chromatography step when quality results were also required.

The up-stream process (USP) consisted of two phases: the cell expansion phase and the production phase. The expansion phase began with the thawing of a working cell bank vial. The cells were then added to cell expansion medium, initiating a cell expansion process that lasted at least 28 days and occurred in 50 mL shaked-tubes. During cell expansion, cells were passaged with cell expansion medium at a seeding viable cell density of 2.0×10^5 viable cells/mL every two days, or at a seeding density of 1.2×10^5 viable cells/mL every three days. Since the cell expansion procedure was highly similar between the laboratory and the commercial manufacturing department, cell expansion samples were sometimes transferred from the commercial department to the laboratory at day 21 to

avoid the initial three weeks of cell expansion. On day 28, the cell expansion culture was used to inoculate the production phase culture.

The second phase was the production phase, which involved cell growth and the production of the mAb without resuspension of cells in fresh media. The production phase lasted 11 days and was performed in shaked-tubes. Production cultures were inoculated at a seeding density of 2.8×10^5 viable cells/mL. Each condition was tested in duplicate during screening experiments and in triplicate during confirmation experiments. Once the viable cell density (VCD) reached 1.0×10^6 viable cells/mL, usually on day 2 of the production phase, the lipoprotein supplement feed at 16.3 µL/mL of culture and main feed at 105.4 µL/mL of culture were added. If the lipoprotein supplement feed was replaced by an alternative lipid supplement feed, the volume of feed added by volume of culture was susceptible to vary depending on the experimental design. Once the VCD reached 2.0×10^6 viable cells/mL, usually on day 4 of the production phase, peptone feed at 9.88 µL/mL of culture was added. The culture was terminated, harvested, and clarified on day 11 of the production phase.

An illustration of the major steps of the process can be seen in Figure 11.

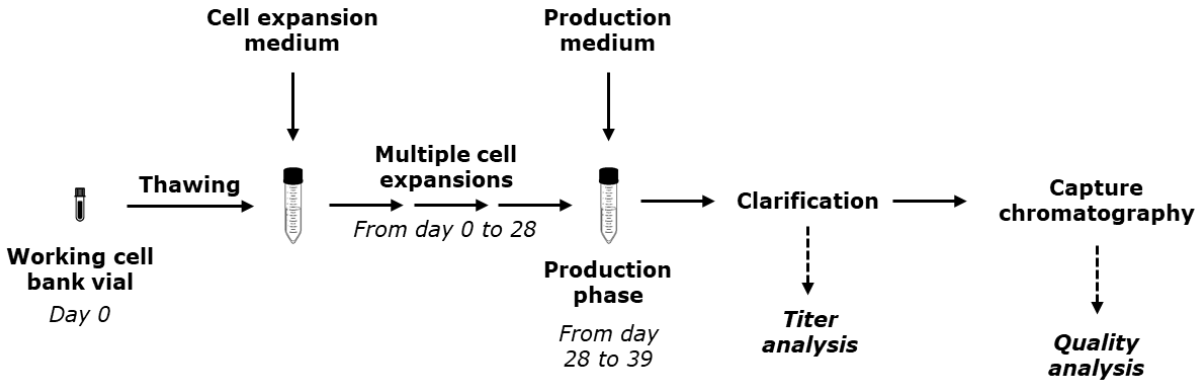


Figure 11: Simplified process illustration from the thawing of a cell bank vial to the capture chromatography step. Conducting all these activities represents what is called a "batch" or a "run".

3.2.4.4. Process monitoring

Sampling from the cultures occurred every two days, with 1.2 mL of sample collected each time for the monitoring of cell concentration, cell viability, and metabolite concentrations. Cell concentration and viability were assessed using trypan blue dye exclusion with a Vi-Cell™ XR analyzer (Beckman Coulter, CA, USA). For the analysis of glucose, lactate, glutamine, glutamate, ammonium concentration, and osmolality, a BioProfile™ Flex 2 (Nova Biomedical, MA, USA) was utilized.

3.2.5. Antibody quantification, purification and CQA analyses

3.2.5.1. Harvesting

On day 11 of the production phase, cultures were clarified through centrifugation at 1000 x g for 10 minutes, followed by filtration using Millex® Syringe filters with a pore size of 0.22 µm. The clarified samples were subsequently stored at -80°C for further analysis of mAb titer or purification via capture chromatography.

3.2.5.2. Antibody quantification

mAb quantification was conducted using a Protein A high-performance liquid chromatography (PA-HPLC) method with an Alliance 2690 HPLC separations module (Waters, Netherlands). To determine the normalized volumetric productivity for each condition, the mAb titer was compared with the control condition present in every experiment.

3.2.5.3. Capture chromatography

The capture chromatography step was conducted using a 1 mL prepacked protein A (PA) column, the Hitrap MabSelect Sure (GE Healthcare, 11003493), and operated with an AKTAxpress chromatography system (GE Healthcare, UK). The PA column was initially rinsed with purified water, followed by equilibration with a 10 mM NaH_2PO_4, 145 mM NaCl buffer at pH 7.2. Approximately 25 mL of the clarified sample was then injected into the system through the PA column. The column was washed with the equilibration buffer, and the elution was performed using 75 mM acetic acid at pH 3.0, with the eluate being collected.

After collection, the eluate fraction was neutralized by adding 1.0 M Tris HCl at pH 11.0 until the pH reached 7.0 ± 0.5. The neutralized eluate was then stored at -80°C. Post-capture samples were sent for quality analysis.

After elution, the column was stripped with 1.0 M acetic acid at pH 2.3, rinsed with purified water, sanitized with 0.1 M NaOH, rinsed again with purified water, sanitized once more with 1.0 M acetic acid, and finally stored in a 20% ethanol solution.

3.2.5.4. Charge-variants analysis

Charge-variants analysis was performed by CEX-HPLC cation exchange high performance liquid chromatography (CEX-HPLC) using Alliance 2690 HPLC separations module (Waters, Netherlands) and Mono S® 5/50 GL ion exchange columns. The relative amount of acidic forms was determined as the sum of the peaks area eluted before the main peaks over the sum of all peaks area and was normalized.

Charge-variants analysis was carried out using cation exchange high-performance liquid chromatography (CEX-HPLC) with an Alliance 2690 HPLC separations module (Waters, Netherlands) and Mono S® 5/50 GL ion exchange columns (GE Healthcare, 17516801). The assessment of relative

acidic forms was determined by calculating the sum of peak areas eluted before the main peaks over the sum of all peak areas, which was subsequently normalized.

3.2.6. Apoptosis detection and inhibition

3.2.6.1. Measurement of apoptosis markers

The impact of the lipoprotein supplement feed addition on apoptosis was evaluated by measuring apoptosis markers both before and 24 hours after the addition of the feed. This approach allowed for a specific assessment of the effect of the lipoprotein supplement feed on apoptosis triggering.

Measurement of caspase-3 activity

Caspase-3 activity in cell lysate was measured using a caspase-3 Assay kit (Sigma Aldrich, MAK457).

The assay procedure was adapted from the alternative assay procedure presented in the caspase-3 Assay kit Technical Bulletin. One million cells (1.0 ± 0.3 mL of culture) were centrifuged at 500 x g for 5 minutes, and the cell pellets were then lysed by treatment with 600 µL of RIPA buffer and shaken for 30 minutes at 4°C. The lysed suspensions were centrifuged at 2500 x g for 10 minutes at 4°C. Next, 50 µL of the supernatant and 100 µL of working reagent were added in duplicate to a black flat-bottom 96-well plate. Blank samples were prepared using 50 µL of purified water and 100 µL of working reagent. The plate was incubated at 37°C for 60 minutes in the dark, and the fluorescence intensity was measured at λ_{Ex} = 400 nm / λ_{Em} = 490 nm. The absorbance in blank wells was used as a control and was subtracted from the values of test samples.

Measurement of Annexin V binding to phosphatidylserine

Annexin V binding to phosphatidylserine was measured using the Annexin V-Cy3TM Apoptosis Detection Kit (Sigma Aldrich, APOAC). The assay procedure was adapted from the Annexin V-Cy3TM Apoptosis Detection Kit Technical Bulletin. One million cells (1.0 ± 0.3 mL) were suspended in PBS, centrifuged at 200 x g for 5 minutes, and the supernatant was removed. The washing step with PBS was repeated once more. After the second wash, the cell pellet was resuspended in 1 mL of PBS. Subsequently, 200 µL of the cell suspension was mixed with 200 µL of the double label staining solution (AnnCy3 and 6-CFDA) and incubated for 10 minutes at room temperature. Stained cells were then centrifuged at 200 x g for 5 minutes, and the supernatant was removed. The cells were washed with binding buffer and suspended in 100 µL of binding buffer. The samples were plated in duplicate on a 384-cell plate. The plate was transferred to an IntelliCyt® iQue Screener PLUS (Sartorius) and subjected to shaking at 3000 rpm for 15 seconds, followed by an additional 4 seconds at 3000 rpm after every 12 wells. Flow cytometry measurements were conducted using the following conditions: Sip Time 0:1.0; additional up time 0.500; pump 29 rpm. Three fluorescence measurements were taken: λ_{Ex} = 495 nm / λ_{Em} = 517 nm, λ_{Ex} = 532 nm / λ_{Em} = 568 nm, and λ_{Ex} = 543 nm / λ_{Em} = 570 nm. The equipment was controlled with the iQue Forecyt® Enterprise Client Edition 8.1 software (Sartorius).

3.2.6.2. Evaluation of oxidative stress and apoptosis in cell viability decline

To investigate the role of oxidative stress and apoptosis in the premature cell viability decline observed during fed-batch experiments, two compounds, Trolox and a caspase-3 inhibitor, were introduced into the culture.

Caspase-3 inhibitor spiking

For the inhibition of caspase-3 during cell culture, the InSolution caspase-3 Inhibitor I from Calbiochem (Sigma-Aldrich, 235427) was utilized. This solution contains a 16-amino acid peptide linked to the N-terminal sequence of the hydrophobic region of the signal peptide of Kaposi fibroblast growth factor, providing cell-permeability to the peptide. As specified by the caspase-3 inhibitor supplier, 200 pM of this compound inhibits 50% of the PARP cleavage by caspase in cultured human osteosarcoma cell extracts (InSolution Caspase-3 Inhibitor I, Cell-Permeable - Calbiochem, 2022). To assess the effect of the caspase-3 inhibitor, concentrations ranging from 2 to 20000 pM were tested. Half of the inhibitor was added at the time of inoculation, and the other half was introduced along with the lipoprotein supplement feed.

Trolox spiking

To explore the impact of oxidative stress on premature cell viability decline, Trolox, a well-known antioxidant (van den Berg, Haenen, van den Berg, & Bast, 1999), was introduced into the culture. A Trolox concentration ranging from 10 to 25 mg/L was added, with half at the time of inoculation and the other half during the lipoprotein supplement feed addition.

3.2.7. Control and monitoring of lipoprotein oxidation

3.2.7.1. Controlled lipoprotein oxidation

$CuSO_4$ was employed as a catalyst to facilitate lipoprotein oxidation following a previously published protocol (Gerry & Leake, 2008). The presence of cupric ion serves as a catalyst for lipid peroxidation reactions. Lipoprotein supplement samples were aseptically aliquoted into TubeSpin® Bioreactor (50 ml). To study the impact of different parameters on lipoprotein oxidation and oxidized lipoprotein toxicity, $CuSO_4$ was added to the solution at concentrations ranging from 0 to 375 µM using the stock solutions previously (refer to section 3.2.3.1 for more details). The samples were then incubated over varying time periods, ranging from 0 to 72 hours, at different temperatures 4°C, 20°C, and 37°C in a Multitron incubator (Infors).

3.2.7.2. Monitoring of lipoprotein oxidation

The oxidation of the lipoprotein supplement was monitored by measuring the absorbance at 276 nm. The measurements were conducted in accordance with the procedure presented in section 3.2.2.2.

3.2.7.3. Effect of lipoprotein oxidation on cell culture

Following copper-mediated lipoprotein oxidation, cell culture media and lipoprotein supplement feeds were prepared using oxidized lipoprotein samples. The cells were adapted to the culture media containing oxidized lipoprotein during the cell expansion phase, then fed-batch cultures were conducted. As oxidized lipoprotein contains $CuSO_4$, $CuSO_4$ control conditions were made in parallel by using unoxidized lipoprotein supplemented with $CuSO_4$ at the time of inoculation.

3.2.8. Design of experiments, data curation and analysis

3.2.8.1. Data curation and analysis

The physicochemical characterization results, UV-visible measurements, CCM composition, cell culture experiments, mAb titer, and quality data were gathered in Microsoft Excel 2018 (Microsoft Corporation). The NIR spectra were collected using SIMCA® multivariate analysis software (Sartorius). Multivariate data analysis of UV-visible and NIR spectra was conducted using SIMCA®. To establish correlations between CCM composition and process indicators and analyze physicochemical characterization results JMP® 16.2 statistical software (JMP Statistical Discovery LLC) was used.

In this research work, p-test analysis was employed to compare groups. Unless otherwise specified, p values are considered at $\alpha = 95\%$.

3.2.8.2. Design of experiments

The Design of Experiments (DOE) approach was used to reduce the number of laboratory experiments conducted to describe the relation between CCM composition and cell culture process performance indicators. Two DOE methods were used, the Plackett-Burman design for identification of main factors and Box-Behnken design was used when taking into account two-factor interactions was necessary. Experiment plans were generated using JMP® 16.2 (JMP Statistical Discovery LLC).

One-factor-at-a-time and two-factor response surface methods

In situations where the number of factors influencing the response was low, we also utilized the one-factor-at-a-time (OFAT) and two-factor response surface (RS) methods, although they are not strictly classified as DOE methods. OFAT involves varying a single factor while keeping the others constant to assess its individual impact. Similarly, RS consists of varying two factors simultaneously to evaluate their combined effects on the response.

Plackett-Burman design

The Plackett-Burman design enables to identify main factors by focusing on those with the most significant effects on the responses within the 2-level design spaces (Figure 12). In the Plackett-Burman design, the interactions between factors are assumed to be negligible.

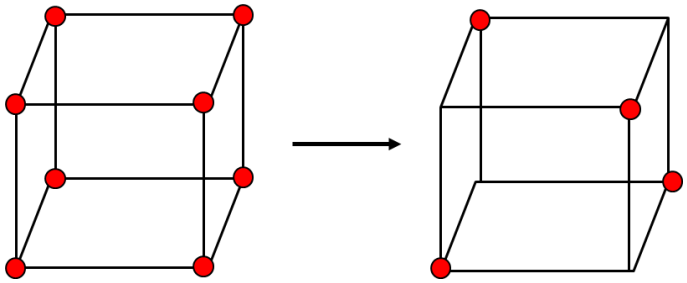


Figure 12: Illustration of a 3-factor 2-level design with 8 conditions reduced to a 4 condition Plackett-Burman design.

Box-Behnken design

The Box-Behnken design enables to construct a response surface using a second-degree polynomial model that incorporates essential factors such as linear terms, squared terms, and products of two factors into the model. The Box-Behnken design takes into account interactions between factors and their influence on the response while reducing the necessary number of experiments (Figure 13).

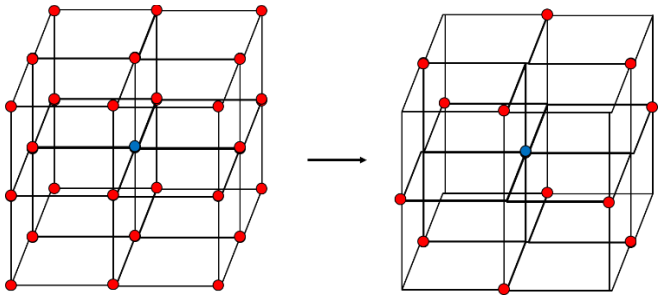


Figure 13: Illustration of a 3-factor 3-level factorial design with 27 conditions reduced to a 13 conditions Box-Behnken design, both designs include a center point (blue).

3.2.8.3. Cell growth comparison tools

To obtain a comprehensive view of cell culture process performance and enable comparison between conditions, not only were viable cell density (VCD), total cell density (TCD), and cell viability utilized, but also population doubling level (PDL) and integral viable cell density (IVC) were also calculated based on the Vi-Cell™ results.

The PDL was employed for comparing different cultures during cell expansion, while IVC was utilized for comparing different cultures during the production phase.

Population doubling level

PDL is an estimation of the number of times the cells in a cultured population have undergone doubling over a specific period. Calculating PDL facilitates unbiased comparisons of cumulative cell expansion passages, eliminating any biases arising from varying initial and final cell concentrations. During cell expansion, the VCD is measured before and after each dilution of the culture in fresh media. The PDL is calculated as follows:

$$PDL = PDL0 + 3.322 \times (logCf - logCi)$$

Ci and Cf are the viable cell concentration at the beginning and the end of each cell expansion passage, respectively.

Integral Viable Cell Density

The IVC quantifies the effective working time for dynamic viable cell concentration within a defined timeframe. In our case it was approximated using the trapezoid approximation method between consecutive VCD measurements.

$$IVC = \int_{culture\ start}^{culture\ end} VCD$$

3.2.8.4. Modelling

The relationship between lipoprotein supplement components and cell growth or productivity was modelled using three distinct analytical approaches: bivariate analysis, linear models, and response-surface models. Bivariate analysis was employed to investigate the relationship between two variables as expressed in equation (1). Linear models, on the other hand, employed multiple variables (X_1,X_2,X_3) to predict variations in a single response variable (Y), as described by equation (2). Only first-order correlations are used in linear models. In contrast, response-surface models, as described in equation (3), consider both first and second-order interactions.

$$(1)\quad Y = \beta_0 + \beta_1 X_1$$

$$(2)\quad Y = \beta_0 + \sum_{i=1}^{K} \beta_i X_i$$

$$(3)\quad Y = \beta_0 + \sum_{i=1}^{K} \beta_i X_i + \sum_{i=1}^{K} \beta_{ii} X_i^2 + \sum_{i=1}^{K-1} \sum_{j=i+1}^{K} \beta_{ij} X_i X_j$$

4. RESULTS AND DISCUSSION

4.1. Role of the lipoprotein supplement in the process

The starting point of this research work was to assess the contribution of the lipoprotein supplement during fed batch fermentation of SP2/0 cells for antibody production to the overall performance of the standard process. Because the up-stream process is divided in two distinct parts, the cell expansion and the production phases, the role of the lipoprotein supplement was studied in these two phases distinctly. First, the role of the lipoprotein supplement during cell expansion, where cells are passaged every 2-3 days in fresh expansion CCM was evaluated. Then, the role of the lipoprotein supplement in the production CCM and the role of the lipoprotein supplement feed addition at day 2 were evaluated. Only the lipoprotein supplement reference batch (batch 7) was used during the experiments presented in sections 4.1.1 and 4.1.2.

4.1.1. Evaluation of the role of the lipoprotein supplement during cell expansion

The role of lipoprotein during cell expansion was evaluated by removing the supplement from the expansion medium. Expansion and lipoprotein-free expansion media, containing 0 and 5 g/L lipoprotein supplement, respectively, were prepared. With these media, cells were passaged every 2-3 days for 3 weeks. The population doubling level (PDL), an indicator of cell growth during cell expansion, are presented in Figure 14. Even if cell growth was slightly higher in expansion medium containing lipoprotein, lipoprotein-free expansion medium was able to support cell growth during cell expansion. Thus, the lipoprotein supplement during expansion is growth promoting and not ess ential.

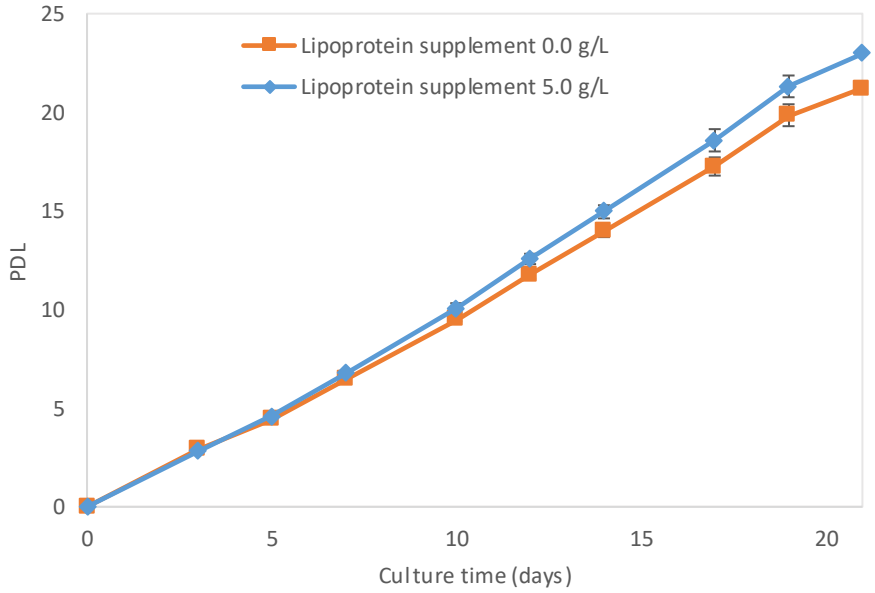


Figure 14: The effect of the removal of the lipoprotein supplement during the expansion phase. Cells were subcultured every two to three days to 0.20 or 0.12 x10^6 viable cells/mL, respectively, in the

expansion medium or same medium deprived from lipoprotein. Mean ± standard deviation from three independent cultures.

When studying lipid supplementation another basal medium component should not be neglected: bovine serum albumin (BSA). Indeed, 1 g/L of BSA is present in the expansion and production media. This BSA is complexed with 1,009 fatty acids mol/mol BSA with oleic acid (20%), linoleic acid (5%), palmitic acid (40%) and stearic acid (30%). Hence, with BSA 1 g/L to the culture are delivered approximately to the culture palmitic acid 1.600 mg/L, stearic acid, 1.180 mg/L, oleic acid 0.840 mg/L and linoleic acid 0.250 mg/L as well as traces of α-linolenic, myristic, pentadecanoic, magaric and arachidonic acids. Thus, the CCM contains fatty acids even if the lipoprotein supplement is removed. The small amount of fatty acids provided by BSA in lipoprotein-free CCM could potentially be sufficient to support cell growth when the CCM is replaced frequently, like during cell expansion.

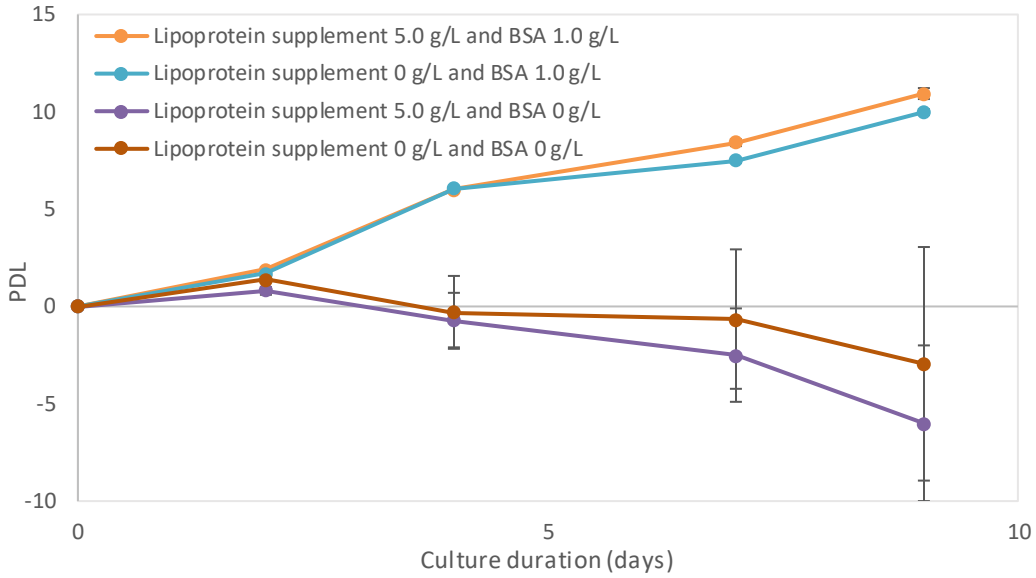


Figure 15: Effect of lipoprotein and BSA removal on cell growth during cell expansion. The lipoprotein supplement and BSA were removed from the expansion medium containing the lipoprotein supplement 5.0 g/L and BSA 1.0 g/L. BSA is necessary for cell growth, by contrast lipoprotein is growth promoting in presence of BSA and becomes growth limiting when BSA is removed from the CCM. Mean ± standard deviation from three independent cultures.

BSA is essential in the CCM because during cell expansion, cells were able to grow in lipoprotein-free medium but not in lipoprotein-free and BSA-free CCM (Figure 15). However, in lipoprotein-free medium, the use of defatted BSA, was reported to significantly decrease cell growth (El Kouchni, 2011). Moreover, complexation of delipidated BSA with its corresponding lipid fraction was able to restore partially its growth promoting ability. These results confirm that the lipid fraction complexed to BSA fulfill fatty acids cell requirements in fatty acids-free medium.

Taken together these results suggest that in the studied process lipid supplementation relies on a combination of multiple raw materials, namely the lipoprotein supplement and BSA. Consequently, it is crucial to investigate their individual contributions while also considering any possible interactions between them.

4.1.2. Evaluation of the role of the lipoprotein supplement during the production phase

The role of lipoprotein during the production phase was evaluated by removing the lipoprotein supplement from the production medium and removing the lipoprotein feed addition at day 2. Standard production medium and lipoprotein-free production medium, containing 5 and 0 g/L lipoprotein supplement, respectively, were prepared. Three production cultures were inoculated, each one in triplicate, one with lipoprotein in the production medium and lipoprotein feed addition (standard process), one with lipoprotein in the production medium but without lipoprotein feed addition and one with neither lipoprotein in the production medium nor lipoprotein feed addition. The integral viable cell density (IVC), an indicator of cell growth during fed-batch experiments, and titer obtained with these conditions are compared in Figure 16. By contrast with its role in cell expansion, the lipoprotein supplement is essential during the production phase, indeed, process performance indicators of the lipoprotein-free process were extremely low compared to other conditions. Addition of lipoprotein in the production medium doubled both IVC and titer. Moreover, combination of production medium containing lipoprotein and lipoprotein feed addition to the culture contain gave the best results, lipoprotein feed addition increased both IVC (+125%) and titer (+80%).

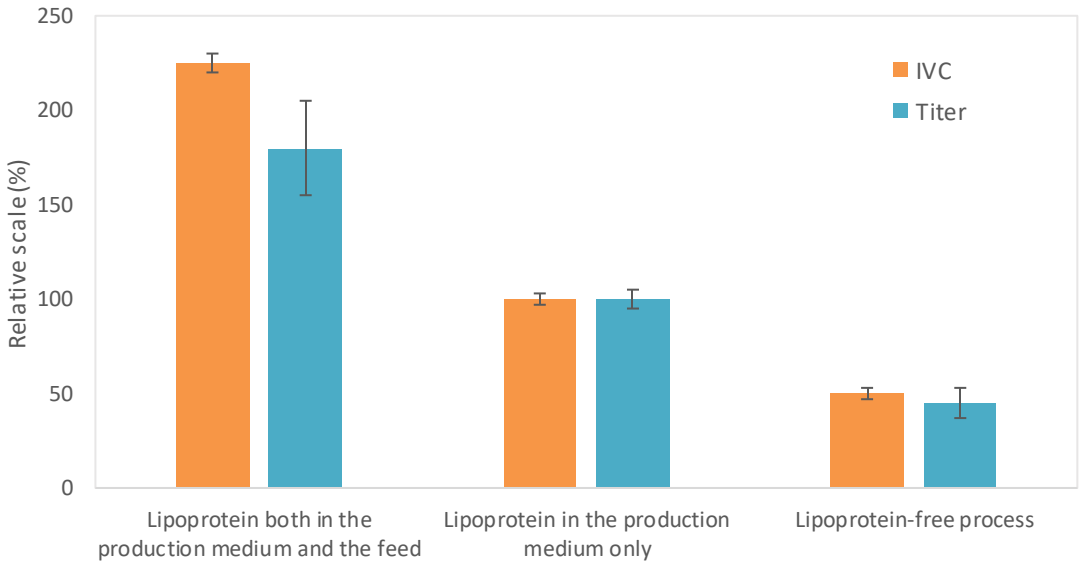


Figure 16: Effects of lipoprotein feed addition and withdrawal of lipoprotein from the CCM on the production process. Results expressed as the percentage of IVC and titer obtained with the CCM containing lipoprotein but without lipoprotein feed addition. Mean ± standard deviation from three independent cultures.

4.1.3. Discussion

The lipoprotein supplement is used to fulfill lipid requirements of the SP2/0 cells and enhance mAb production. During cell expansion the growth-promoting effect of lipoprotein was observed but it is not indispensable to support cell growth. By contrast, removing BSA from the CCM stopped cell growth no matter of the presence of lipoprotein. Because BSA is bound to fatty acids (El Kouchni, 2011), resuspension of the cells in fresh media (containing BSA) every 2-3 days during cell expansion must provide enough fatty acids to fulfill SP2/0 cells requirements. However, when both lipoprotein and BSA are removed from the media, the SP2/0 cells lack the fatty acids their require for maintenance and optimal growth. Furthermore, BSA serves a dual role by supplying fatty acids and exhibiting antioxidant as well as ion-chelating capabilities. Because BSA not only provides lipids but also exerts other functions, removing BSA from the CCM is detrimental to the culture regardless of the presence of lipoprotein.

Nonetheless, addition of lipoprotein both in the CCM and through feed addition was indispensable to ensure robust growth and high productivity during the production phase. Indeed, the fatty acids bound to BSA are not sufficient to fulfill cells requirement during the 11-day production phase. Thus, addition of lipoprotein in the CCM and supplementation of the culture through feed addition is essential to fulfill fatty acid requirements of the SP2/0 cells. Despite not being essential during cell expansion, the lipoprotein supplement is critical during the production phase to ensure high process performance. Consequently, any variation of the lipoprotein supplement composition or alteration of its components may affect process robustness.

4.2. Impacts of the lipoprotein supplement batch variations on process performance

The effect of batch-to-batch variations on process performance was investigated using 36 lipoprotein supplement batches from the same supplier. 22 lipoprotein supplement batches manufactured with bovine serum produced in New Zealand, batches 1 to 22 and 14 lipoprotein supplement batches manufactured with bovine serum produced in the United States, batches 23 to 36, were used. Impacts of batch-to-batch variability of the lipoprotein supplement, also known as inter-batch variation, on cell culture process performance indicators like cell growth, metabolite concentration and final titer were evaluated.

4.2.1. Volumetric productivity

Cell cultures conducted to assess the impacts of batch-to-batch variability of the lipoprotein supplement were harvested at day 11 and the mAb titer was measured. Figure 17 shows the average volumetric productivity for 36 batches relative to the chosen reference batch (batch 7). The productivity varied from 15% to 111% compared to the reference batch. A significant difference between US and NZ batches was observed, some NZ batches were associated with extremely low

volumetric productivity, only 15 to 40% compared the reference batch, these batches are low-performing ones. Whereas no US batch was causing productivity drops below 79%.

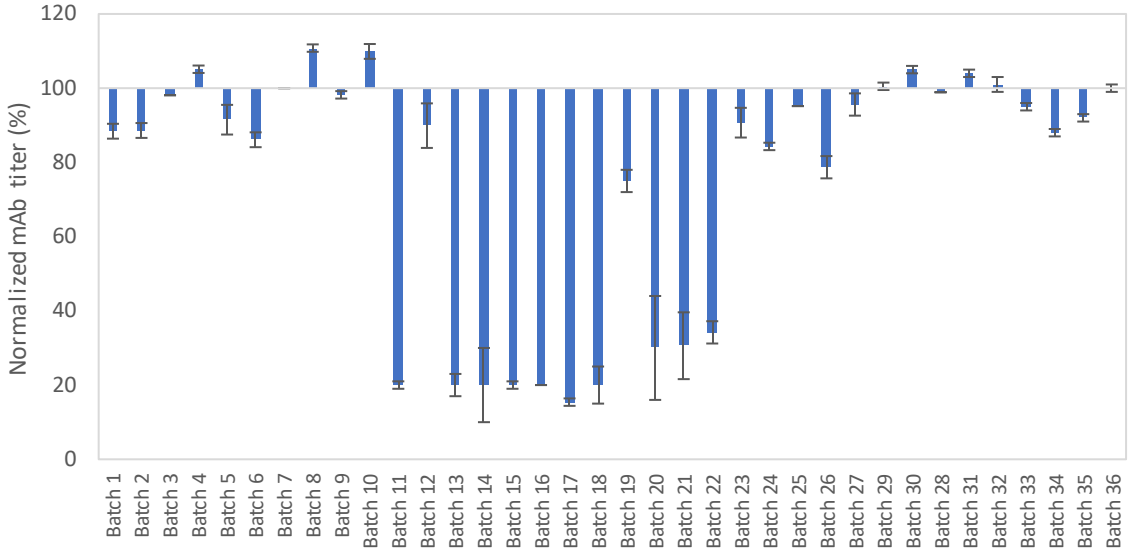


Figure 17: Impact of batch-to-batch variability of the lipoprotein supplement on mAb titer. 36 batches from the same vendor were compared to a given reference batch for which the mAb titer was fixed at 100%. Mean ± standard deviation from three independent cultures.

Lipoprotein supplement batches with a productivity below 50% compared to the reference batch were categorized as low-performing batches. None of the US batches is part of the low-performing, batches, only NZ batches. Thus, a batch performance issue seems to be related to the NZ origin.

4.2.2. Cell growth and cell metabolism

Different indicators could be measured to check, whether a change of lipoprotein batch could impact the cell culture such as the VCD and the cell viability, presented in Figures 18 and 19. The consumption of glucose and glutamine, and the accumulation of lactate, glutamate, and ammonia, presented in Figures 20 to 24, also reflect the metabolic activities of cultured cells. Samples were taken throughout the culture to monitor process indicators. Among the 36 batches tested, 5 are presented in Figures 18 to 24 in order to make the figures easier to read. Among the 5 batches presented, 2 are from US origin and 3 from NZ origin with 2 low-performing batches.

Lipoprotein supplement inter-batch variations had an impact on VCD (Figure 18) and cell viability (Figure 19) beyond day 2 of the production phase. Due to inter-batch variation, lipoprotein batches can be divided in two groups, first the batches with a VCD peak comprised between 3.5 – 4.0 x10^6 viable cells/mL and a cell viability that start to decrease from day 7, then the batches with a VCD peak at 2.5 x10^6 viable cells/mL and a cell viability that start to decrease from day 4.

Lipoprotein supplement inter-batch variations was found not to impact lactate (Figure 21) and glutamate (Figure 23) production during the cell culture. However, the batches that were negatively

affecting cell growth also affected glucose consumption (Figure 20), glutamine consumption (Figure 22) and ammonium production (Figure 24). The observed low glucose consumption, glutamine consumption and ammonium production could be related to the effect of the lipoprotein supplement batches on cell density.

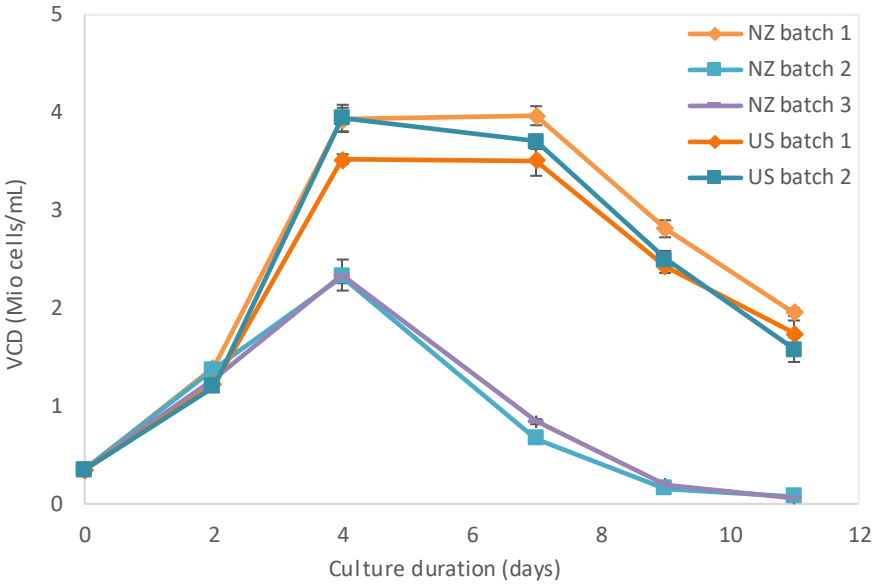


Figure 18: Impact of the lipoprotein supplement batches on VCD. For each condition the same lipoprotein supplement batch was used to prepared both the CCM and the lipoprotein feed. Two batches among the five presented have a negative impact on cell growth after feed addition at day 2. Mean ± standard deviation from three independent cultures.

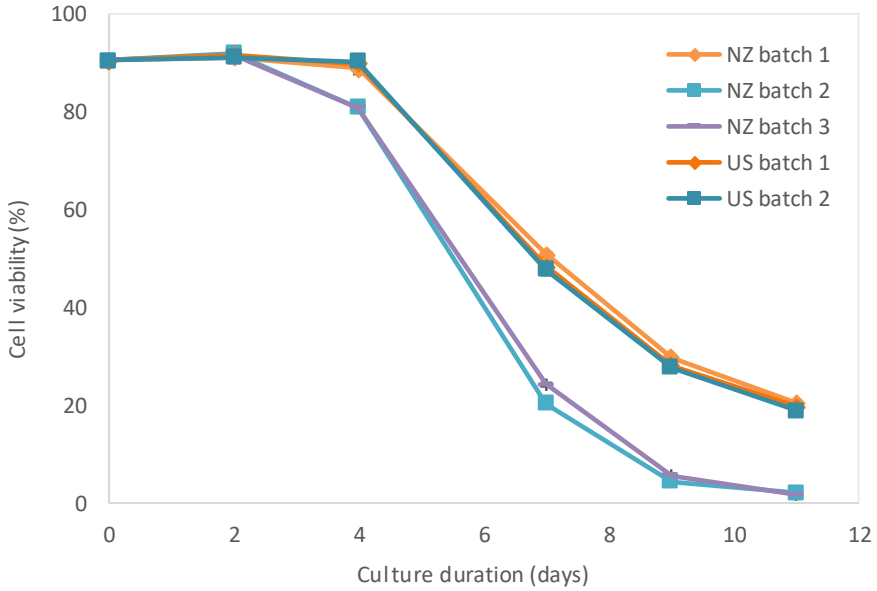


Figure 19: Impact of the lipoprotein supplement batches on cell viability. For each condition the same lipoprotein supplement batch was used to prepared both the CCM and the lipoprotein feed.

Two batches among the five presented have a negative impact on cell viability after feed addition at day 2. Mean ± standard deviation from three independent cultures.

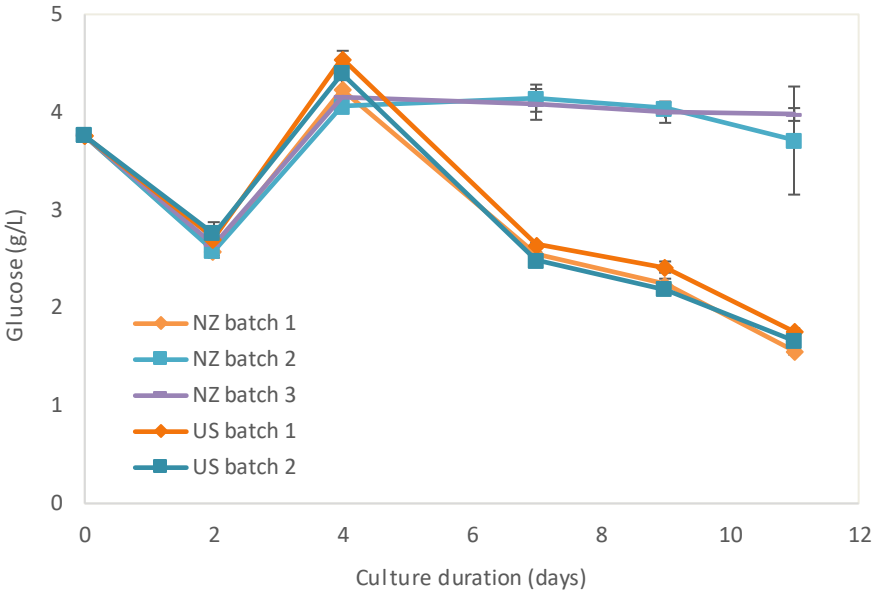


Figure 20: Impact of the lipoprotein supplement batches on glucose. For each condition the same lipoprotein supplement batch was used to prepared both the CCM and the lipoprotein feed. Two batches among the five presented have a glucose concentration that does not decrease, indicator of a low specific glucose consumption by the cells or a lower cell density. Mean ± standard deviation from three independent cultures.

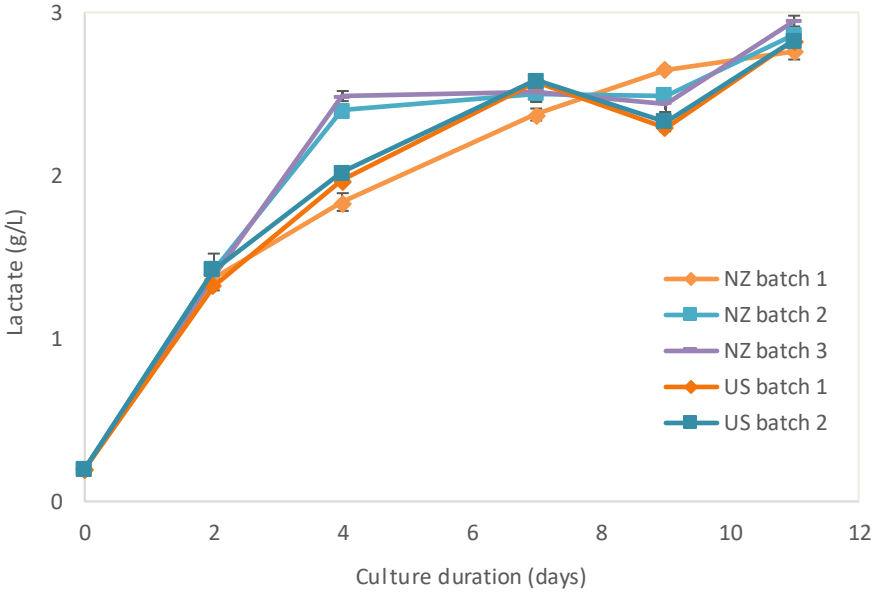


Figure 21: Impact of the lipoprotein supplement batches on lactate. For each condition the same lipoprotein supplement batch was used to prepared both the CCM and the lipoprotein feed. All 5 batches presented have a similar evolution of lactate concentration during the culture. Mean ± standard deviation from three independent cultures.

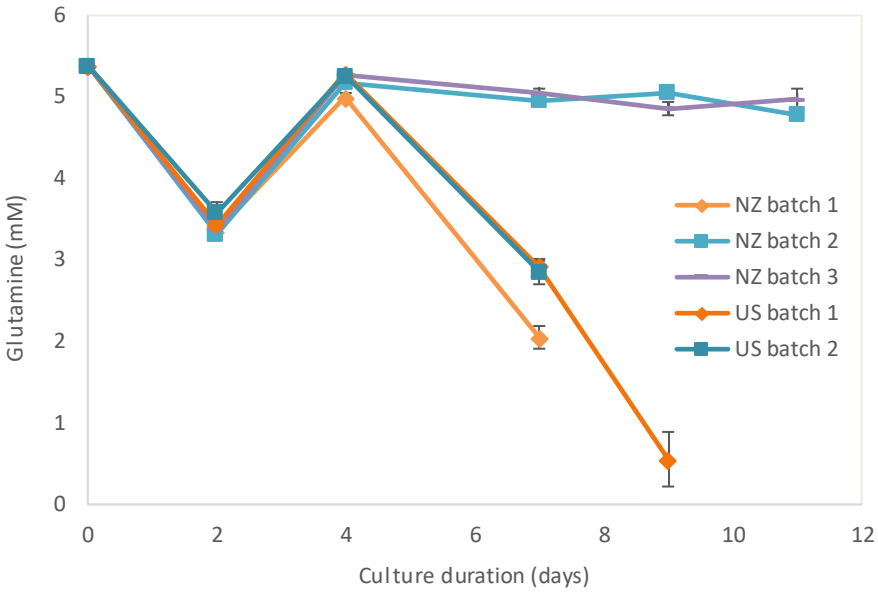


Figure 22: Impact of the lipoprotein supplement batches on glutamine. For each condition the same lipoprotein supplement batch was used to prepared both the CCM and the lipoprotein feed. Two batches among the five presented have a glutamine concentration that does not decrease, indicator of a low specific glutamine consumption or a lower cell density. Glutamine concentration was below the limit of detection after days 9 and 11 for batches 7 and 30, and only after day 11 for batch 29. Mean ± standard deviation from three independent cultures.

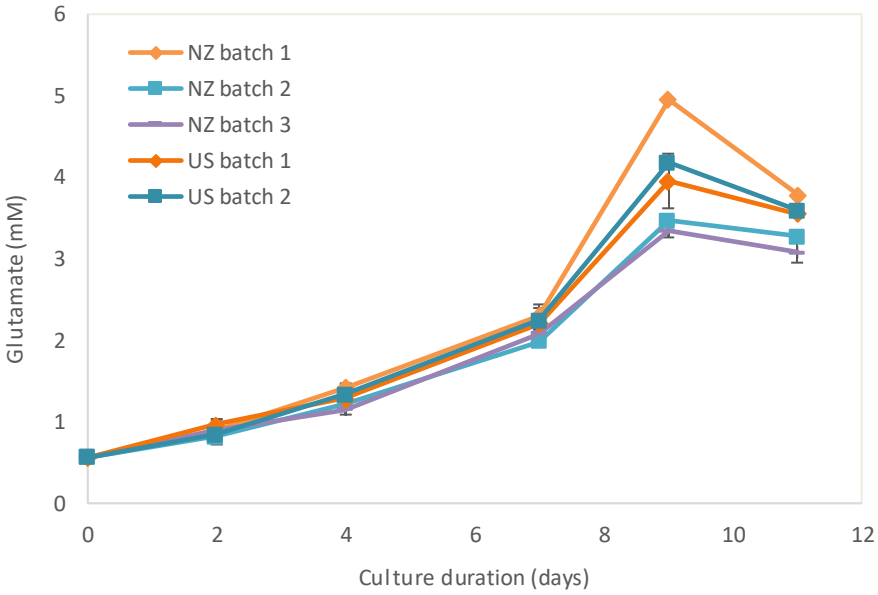


Figure 23: Impact of the lipoprotein supplement batches on glutamate. For each condition the same lipoprotein supplement batch was used to prepared both the CCM and the lipoprotein feed. All 5 batches presented have a similar evolution of glutamate concentration during the culture. Mean ± standard deviation from three independent cultures.

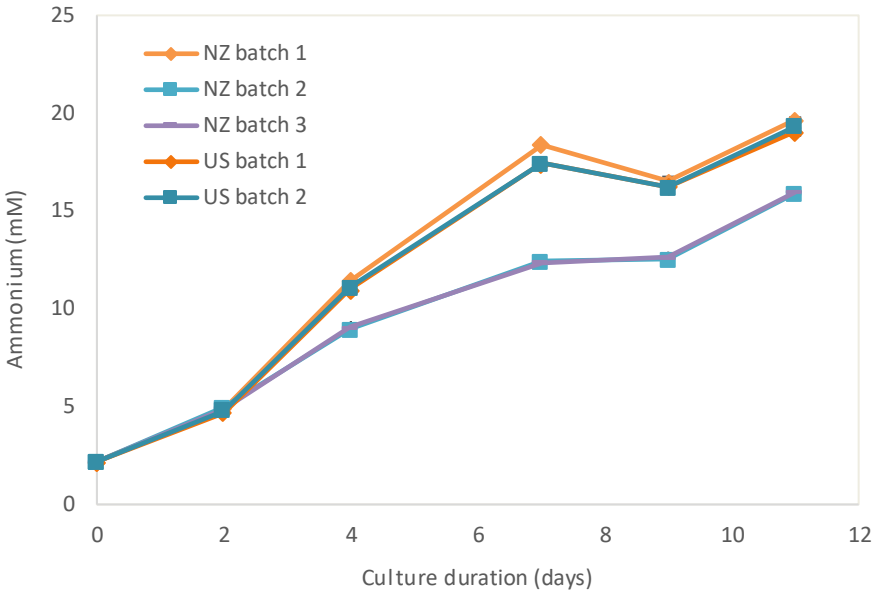


Figure 24: Impact of the lipoprotein supplement batches on ammonium. For each condition the same lipoprotein supplement batch was used to prepared both the CCM and the lipoprotein feed. Note that batches Two batches 21 and 22 are superposed. Two batches among the five presented have an ammonium concentration that increase slowly, indicator of a lower specific ammonium production or a lower cell density. Mean ± standard deviation from three independent cultures.

4.2.3. Correlating cell growth and productivity

IVC, a cell growth indicator through the process, was calculated from day 0 to 11 of the production phase, then IVC values were normalized by a comparison with the reference batch. The relation between the normalized mAb titer and the normalized IVC at the end of the culture for each batch is presented in Figure 25. Low-performing batches are characterized by a low IVC, i.e. low cell growth. The productivity drops associated with low-performing batches are due to the negative impact of these batches on cell growth.

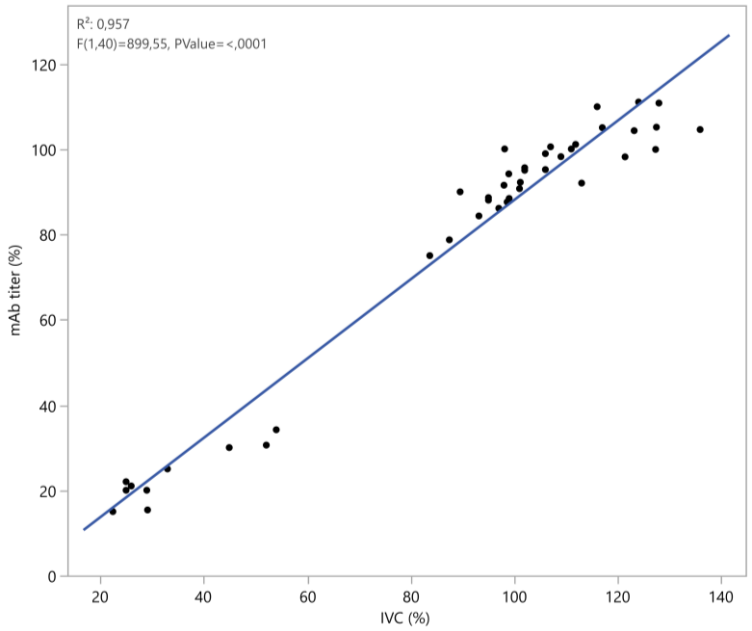


Figure 25: Fit of normalized mAb titer by normalized IVC at the end of the culture for 36 lipoprotein supplement batches. IVC and mAb titer data were gathered from distinct series, consequently IVC and mAb titer were normalized by comparison to the lipoprotein supplement reference batch (batch 7) present in every series. The correlation between normalized mAb titer and normalized IVC is statistically significant ($p < 0.0001$) with $R^2 = 0.957$. Mean from three independent cultures.

4.2.4. Dose-response relation between the lipoprotein concentration and cell growth

To investigate why low-performing batches were negatively impacting cell growth, two hypotheses were stated: 1) low-performing batches lack one or several essential compounds to support SP2/0 cell growth or 2) low-performing batches contain one or several growth-limiting compounds. With the aim to reject one of these hypotheses, cell expansions were conducted at various lipoprotein concentration with two lipoprotein supplement batches : the reference batch 7 (relative productivity 0%) and a low-performing batch, batch 11 (relative productivity -80%).

The effect of lipoprotein concentration on cell growth was investigated through 2-3 days passages. Because the lipoprotein concentration increases throughout the production phase from 5.0 g/L in the CCM to 21.0 g/L after feed addition, several concentrations from 5.0 to 21.0 g/L were tested. The PDL of the 4 passages are presented in Figure 26.

First, at 5.0 g/L the two batches supported growth, the PDL are similar. Consequently, at low concentration the low-performing batch was not negatively affecting PDL compared to the reference batch. Varying lipoprotein concentration from 5.0 to 21.0 g/L was not affecting the PDL when the reference batch was used. By contrast, even if the low-performing batch concentration had no impact on the PDL between 5.0 – 11.4 g/L, above 11.4 g/L this batch was correlated with a decreasing PDL. Thus, at low concentration the low-performing batch was not affecting cell growth, however above a threshold it became detrimental to cell growth.

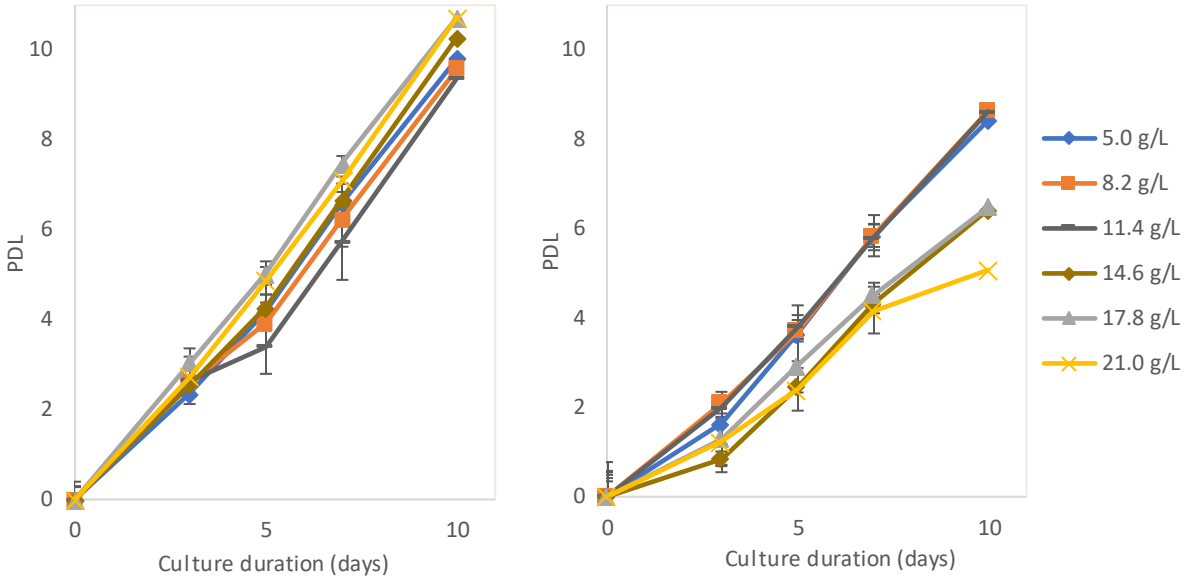


Figure 26: Effect of lipoprotein concentration, from 5.0 to 21.0 g/L, on PDL for 2-3 days cell expansions using the lipoprotein supplement reference batch (left) and a low-performing batch (right). From 5.0 to 21.0 g/L, the reference batch has no impact on PDL. Whereas, above 11.4 g/L the low-performing lipoprotein solution batch decreases PDL as the concentration increases. Mean ± standard deviation from three independent cultures.

The difference between the reference batch and the low-performing batch appeared at high concentration, above 11.4 g/L, and not at low concentration. Therefore, the low-performing batch does not lack one or several growth promoting compounds, but it contains one or several toxic compounds.

These results are coherent with what was observed during the production phase, before day 2 and the lipoprotein feed addition that increased lipoprotein concentration from 5.0 to 21.0 g/L, low-performing batches have no effect on VCD (Figure 18) and cell viability (Figure 19) compared to other batches.

4.2.5. Discussion

Testing at small-scale of the lipoprotein supplement batches prior to their use at manufacturing scale has demonstrated that batch-to-batch variability of the supplement was affecting cell growth, cell viability and process productivity. Moreover, evolution of metabolism indicators in the culture such as glucose and lactate concentrations were affected by the variations of growth profile induced by the lipoprotein supplement. These results confirm that the lipoprotein batch variations affect process performance. Indeed, a lipoprotein supplement is a complex non-chemically defined raw-material that must contain growth promoting and growth limiting compounds, variations of their respective concentration should affect cell growth leading to process performance variations.

Besides slight productivity variations inherent to non-chemically defined raw-materials, some lipoprotein batches, exclusively from NZ origin, were causing productivity drops up to -50% compared

to the reference batch. These batches were categorized as low-performing batches. Low-performing batches were causing important viability decline after feed addition at WD02, decreasing the productivity of the process. The correlation between volumetric productivity and IVC confirmed that the limited growth caused by low-performing batches was causing the productivity issues.

The limited growth observed with low-performing batches can be explained by either, the inadequate fulfillment of SP2/0 lipid requirement, or the presence of growth limiting compounds. The dose-response relationship observed between a low-performing lipoprotein batch and cell growth confirmed the presence of growth limiting compounds.

With low-performing batches a decreasing viability was observed when the concentration reaches 21 g/L after feed addition. At this lipoprotein concentration, the concentration of the growth inhibitory compounds must have surpassed their toxicity threshold towards SP2/0 cells. However other lipoprotein batches may also contain these growth inhibitory compounds in lower amount, thus never surpassing the toxicity threshold.

4.3. Characterization of the lipoprotein supplement

Among the lipoprotein supplement batches tested, some low-performing batches, characterized by early viability decline subsequent to lipoprotein feed addition were identified. Characterization of the lipoprotein supplement composition was conducted in order to identify the compounds that were detrimental to the cells.

First, main lipoprotein components were characterized to correlate composition and functionality of the lipoprotein supplement with the aim to increase understanding on how lipoprotein batch-to-batch variability affects process performance. In parallel, cytotoxic compounds generally present in oxidized lipoprotein according to the literature were characterized. Lastly, fingerprinting methods were evaluated to detect low-performing batches prior to their utilization.

4.3.1. Characterization of the lipoprotein supplement composition

4.3.1.1. Fatty acids

The fatty acid composition of 35 lipoprotein supplement batches was characterized, variations of total fatty acid content are presented in Table 6. An overview of the fatty acid profile of the lipoprotein supplement is presented in Figure 27, while the distinct profiles of US and NZ batches are presented in supplementary Figures 77 and 78, respectively. Determination of fatty acid content was performed by GC using the internal standard approach as described in section 3.2.1.1.

	Lipoprotein supplement (n=35)	US batches (n=24)	NZ batches (n=11)	Low-performing NZ batches (n=8)	Other NZ batches (n=3)
Fatty acid content (g/L)	3.39 ± 0.71	3.55 ± 0.76	3.04 ± 0.42	3.06 ± 0.48	3.00 ± 0.35

Table 6: Variations of the total fatty acid content of the lipoprotein supplement. US batches contain more fatty acid than NZ batches but there is not significative difference between NZ low-performing batches and other NZ batches.

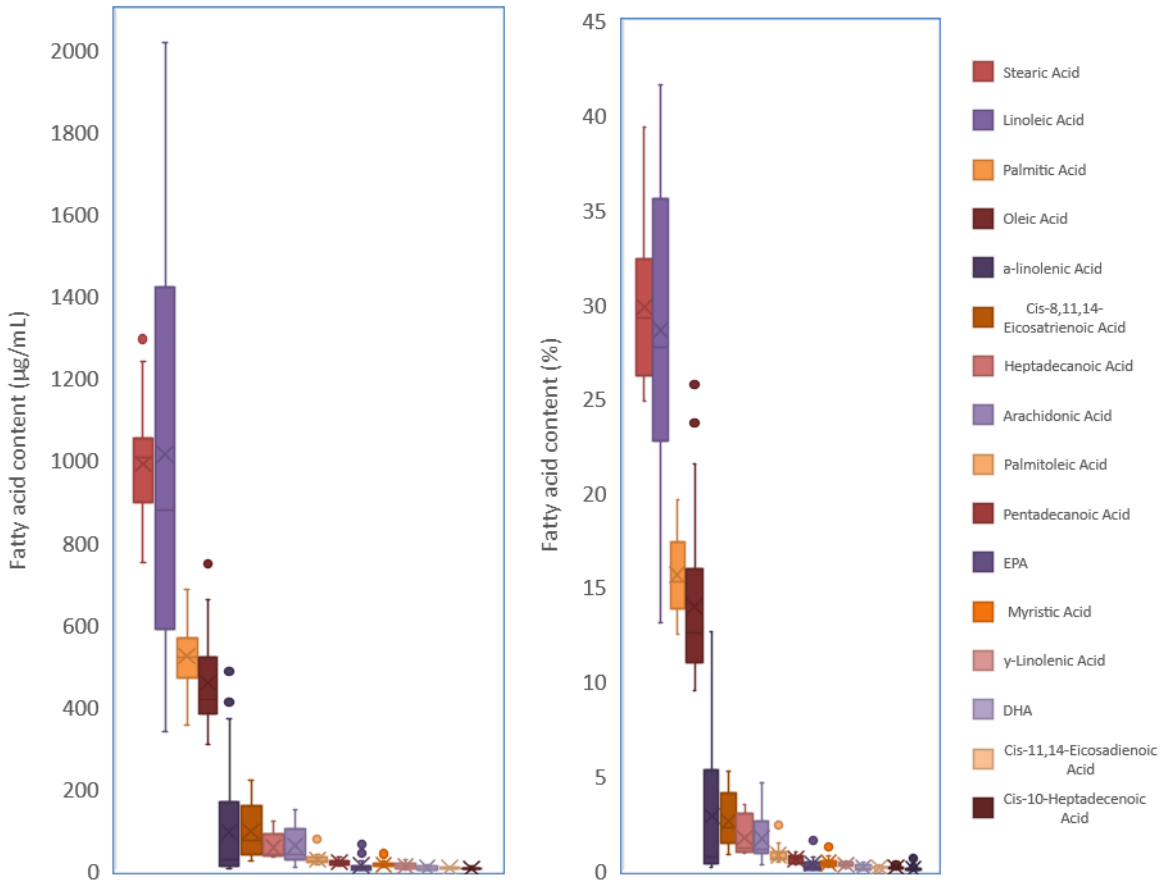


Figure 27: Fatty acid profile of the lipoprotein supplement. Mean ± standard deviation (n=35).

The fatty acid content is significantly higher in US batches than NZ batches (one-way ANOVA, $p = 0.0475$), US batches contain on average 16.8% more fatty acids than NZ batches (Table 6). In addition, the fatty acid compositions of US and NZ batches differ. The main differences are the higher levels of stearic, linoleic, palmitic, and arachidonic acids in US batches whereas NZ batches contain more oleic and α-linolenic acids. Moreover, the relative proportion of PUFA is significantly higher in NZ batches than US batches (one-way ANOVA, $p < 0.0001$), 11.7% and 6.2%, respectively. This difference is mainly due to the high content of α-linolenic acid in NZ batches, 254 ± 121 mg/L, compared to US batches, 18 ± 11 mg/L.

Despite important batch-to-batch variations in terms of fatty acid composition among batches from the same origin (supplementary Figures 77 and 78), no significant differences in fatty acid composition were found between the low-performing batches and the rest other NZ batches.

The fatty acid composition of the lipoprotein supplement displays significant variations among various sources and batches. Given that the supplement constitutes the primary source of fatty acids during the production phase, these variances are likely to have an impact on process robustness.

4.3.1.2. Proteomic

The proteomic composition of 31 lipoprotein supplement batches was characterized by LC-MS/MS after trypsin digestion as described in section 3.2.1.2. Variations of total protein content are presented in Table 7. An overview of the proteomic composition of the lipoprotein supplement is presented in Figure 28. No significant variations in protein content were observed among the batches.

	Lipoprotein supplement (n=31)	**US batches** (n=11)	**NZ batches** (n=20)	**Low-performing NZ batches** (n=9)	**Other NZ batches** (n=11)
Protein content (g/L)	6.1 ± 0.3	6.2 ± 0.1	6.0 ± 0.4	6.1 ± 0.3	5.8 ± 0.5

Table 7: Variations of the protein content of the lipoprotein supplement. No significant differences among the batches were observed.

Regarding the proteomic composition, as expected for a lipoprotein solution, the supplement contains mainly apolipoprotein: ApoA-1 (48%), ApoB (22%), ApoA-4 (3%), ApoA-2 (1.5%) as well as ApoC, ApoD, ApoF and ApoM to a lesser extent. No significant variations in protein composition were observed between US and NZ batches.

However, low-performing batches contain more ApoB than other NZ batches (one-way ANOVA, $p < 0.0150$), 24.0% and 19.1% respectively, whereas the ApoA-1 levels are identical. Low-performing batches contain a higher proportion of LDL than other NZ batches. However, this LDL level is not abnormal as US batches contain 24.4% of ApoB.

Although there is a difference in ApoB, the multivariate analysis of the proteomic composition revealed no correlation between the proteomics and the performance of lipoprotein supplement batches.

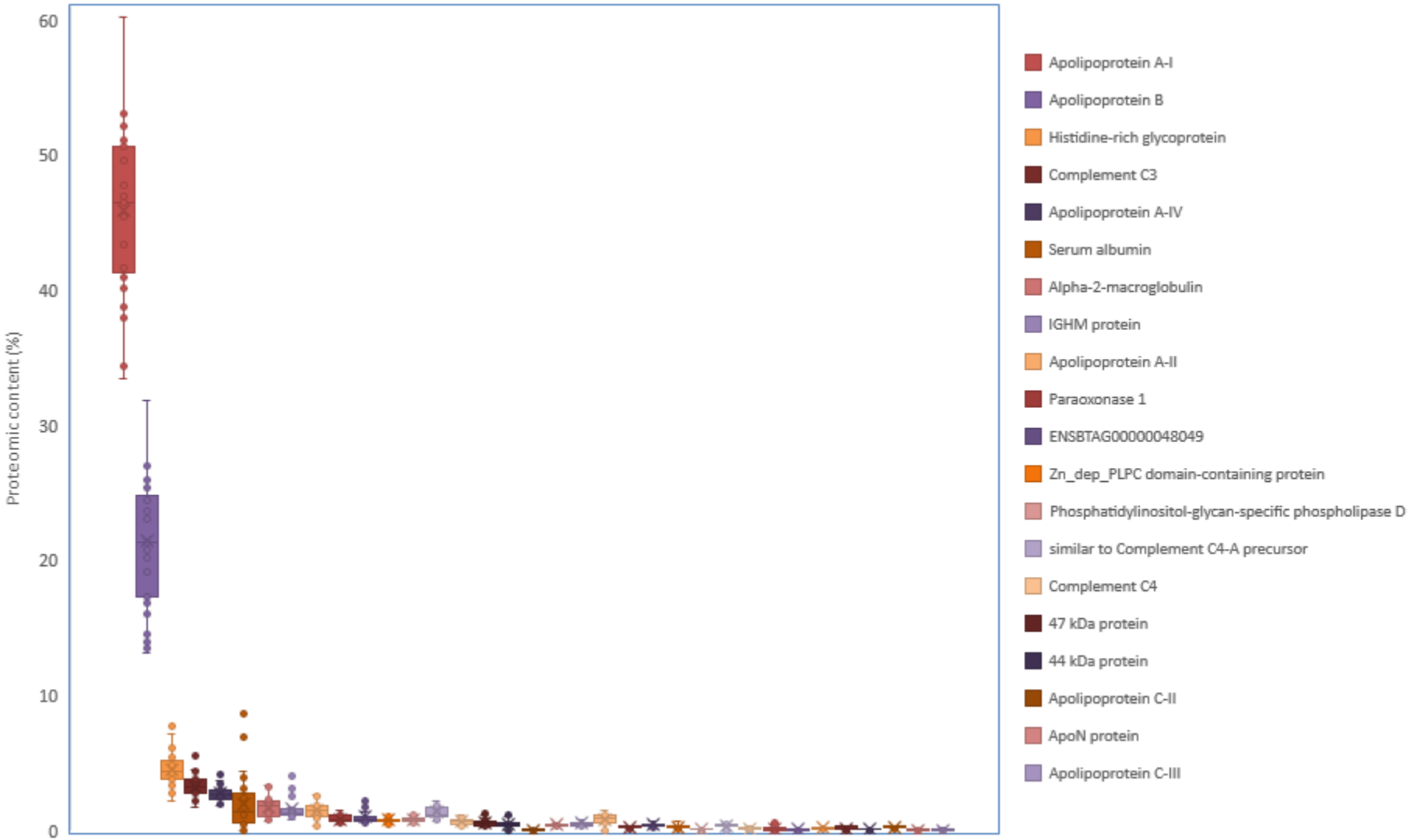


Figure 28: Variations of proteomic composition of the lipoprotein supplement. A large variety of proteins was detected, however apolipoproteins are preponderant in the supplement (>70%), as expected for a lipoprotein solution. Mean ± standard deviation (n=31).

Introduction of ketone and aldehyde groups in protein upon oxidation of side-chain amino acids is called protein carbonylation. Cysteine, histidine, and lysine residues react with ROS to form ketones and aldehydes, resulting in alterations of protein structure and properties. Protein carbonylation was measured by labeling with 2,4-dinitrophenylhydrazine (DNPH) to form specific protein-DNPH derivatives. The labelled proteins were separated according to their molecular weight by SDS-PAGE, lastly the quantification was performed with a DNPH fluorescent probe. The protocol for quantification of protein carbonylation is described in section 3.2.1.8.

The degree of protein carbonylation of 20 lipoprotein supplement batches was characterized, the results are presented in Table 8. Prior to the protein carbonylation measurement, the SDS-PAGE allowed for protein separation based on their molecular weight (MW).

Protein molecular weight (kDa)	**Lipoprotein supplement** (n=20)	**US batches** (n=9)	**NZ batches** (n=11)	**Low-performing batches** (n=7)	**Other NZ batches** (n=4)
< 20	4725 ± 1341	4775 ± 1359	4685 ± 1390	4381 ± 1089	5217 ± 1866
20 – 50	3303 ± 810	3660 ± 1040	3012 ± 415	3181 ± 295	2715 ± 464
50 - 100	2979 ± 787	3103 ± 814	2878 ± 789	2736 ± 600	3126 ± 1107

Table 8: Variations of protein carbonylation in lipoprotein supplement by protein molecular weight. The carbonylation result is expressed in fluorescence units per µg of protein injected.

The multivariate analysis of the protein carbonylation results revealed no significative correlation between protein carbonylation levels and either the origin of the lipoprotein supplement or batch performance. Notably, the degree of protein carbonylation in low-performing batches is similar to that of other batches.

However, the protein carbonylation method used in the lipoprotein supplement analysis was limited to proteins with MW less than 100 kDa. Notably, ApoB-100, the predominant protein, possesses a molecular mass of approximately 515 kDa, which exceeds the size range measured during the protein carbonylation analysis (< 100 kDa). As a consequence, the carbonylation level of ApoB-100 will be absent of the protein carbonylation results obtained from the analysis.

Although it has been confirmed that the lipoprotein supplement contains mainly apolipoproteins and that there is some level of protein carbonylation, the proteomic analysis of the lipoprotein supplement did not provide a more comprehensive understanding of the chemical compounds that could cause the premature viability decline observed with low-performing batches.

4.3.1.3. Sterols

The sterol composition of 12 lipoprotein supplement batches was characterized by LC-MS using the internal standard approach with D-6 cholesterol as described in section 3.2.1.5. Variations of sterol composition are presented in Table 9. An overview of the sterol profile of the lipoprotein supplement is presented in Figure 29, while the distinct profiles of US, NZ and low-performing batches are presented in supplementary Figures 79 to 81. NZ batches contain less sterol than US batches, however this difference is mainly due to the low-performing batches that contain significantly less sterol than all the other batches.

	Lipoprotein supplement (n=12)	**US batches** (n=3)	**NZ batches** (n=9)	**Low-performing NZ batches** (n=6)	**Other NZ batches** (n=3)
Sterol content (g/L)	12.3 ± 4.9	16.0 ± 2.4	11.1 ± 2.6	9.8 ± 0.7	13.7 ± 1.0

Table 9: Variations of sterol content in 12 lipoprotein supplement batches. No significant differences among the batches were observed.

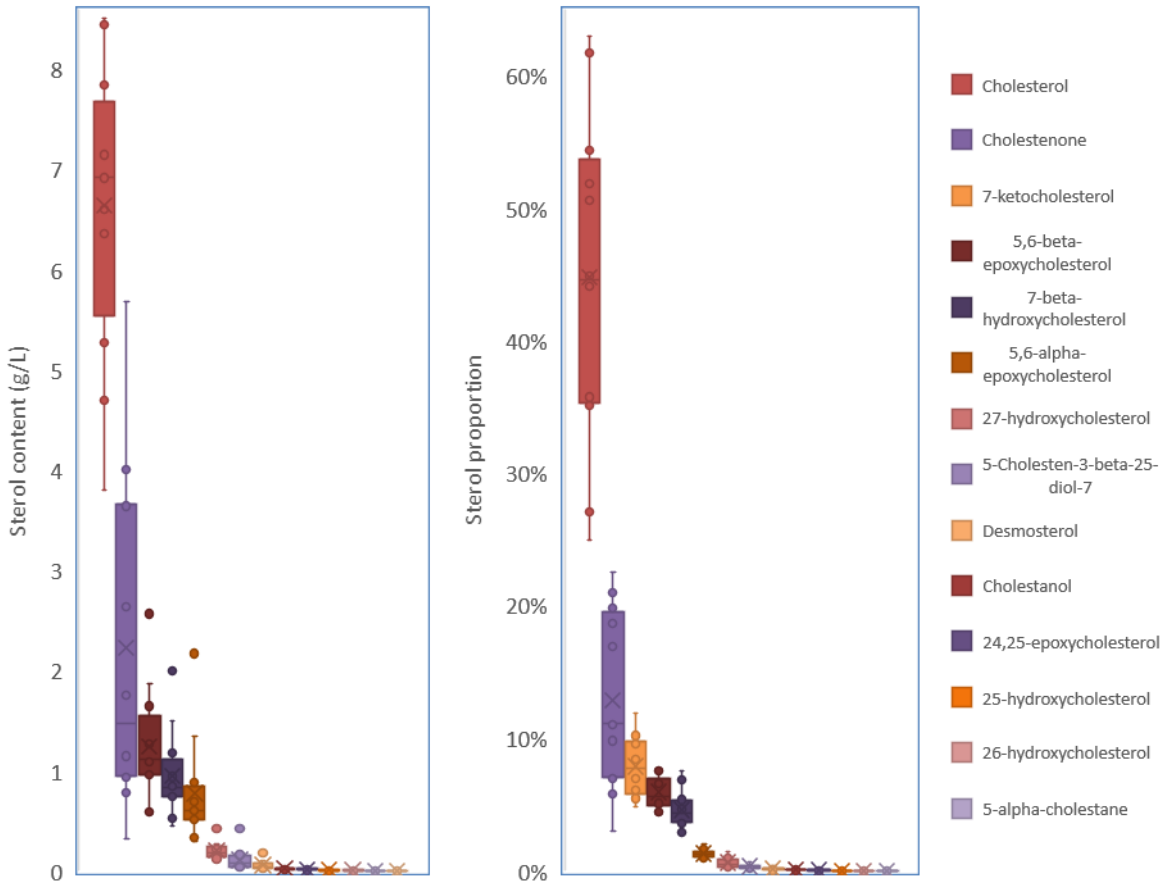


Figure 29: Variations of sterol composition of the lipoprotein supplement. Mean ± standard deviation (n=12).

Regarding the sterol composition, the lipoprotein supplement contains not only cholesterol but also, cholesterol precursors and oxidized cholesterol derivatives. Oxidized cholesterol derivatives present in

the supplement can be divided into 2 categories, oxysterols, cholesterol derivatives by addition of one or two oxygen atoms and oxidized forms of cholesterol without oxygen addition like cholestenone and cholestanol. Cholesterol is the primary sterol in the lipoprotein supplement, followed by cholestenone and oxysterols, notably 7-keto, 5,6-β-epoxy and 7-β-OH that are present in significative amount.

Despite low-performing batches having comparable cholesterol levels to the other batches, they contain less oxidative cholesterol derivatives. Specifically, low-performing batches contain significantly less cholestenone (one-way ANOVA, $p < 0.0001$), 0.90 ± 0.31 g/L, compared to the 3.44 ± 0.71 g/L found in other NZ batches. Similarly, low-performing batches contain significantly less 26- and 27-hydroxycholesterol, 24,25-epoxycholesterol, and 5-cholesten-3-beta-25-diol-7 than other NZ batches.

The sterol characterization has demonstrated that a significant proportion of the cholesterol present in the lipoprotein supplement has been oxidized. Intriguingly, the oxidation cholesterol derivatives content in low-performing batches, 3.2 ± 0.7 g/L, is lower than in other batches, 7.9 ± 3.2 g/L.

4.3.1.4. Lipid peroxidation by-products

Because the proteomic and sterol characterization of the lipoprotein supplement have confirmed that lipoprotein components are oxidized, two lipid peroxidation by-products, MDA and 4-HNE, were characterized in 20 lipoprotein supplement batches. The variations of MDA and 4-HNE content are presented in Table 10. Both MDA and 4-HNE were detected in every tested batch of the lipoprotein supplement, confirming the occurrence of peroxidation of lipoprotein components.

	Lipoprotein supplement (n=20)	**US batches** (n=9)	**NZ batches** (n=11)	**Low-performing NZ batches** (n=7)	**Other NZ batches** (n=4)
MDA (µM)	18.9 ± 4.7	16.9 ± 2.7	20.5 ± 5.4	20.9 ± 6.4	20.0 ± 3.5
4-HNE (µM)	304.8 ± 285.2	531.1 ± 281.2	119.7 ± 86.9	82.2 ± 35.7	185.2 ± 116.8

Table 10: Variations of MDA and 4-HNE content in the lipoprotein supplement.

In contrast to MDA, which remains consistent across all batches, the content of 4-HNE is significantly lower in US batches than NZ batches. Furthermore, low-performing batches contain significantly less 4-HNE than other batches.

Due to the detrimental effects of both MDA and 4-HNE on cell growth (Balcavage & Alvager, 1982 ; Benedetti, Comporti, & Esterbauer, 1980), the variations in MDA and 4-HNE levels from batch to batch have the potential to significantly impact process robustness.

4.3.1.5. Other components characterized

No steroids were detected in the lipoprotein supplement, and no batch variations were observed for trace elements (supplementary Figure 82).

4.3.2. Correlation of the lipoprotein supplement composition with its functionality

The lipoprotein supplement characterization confirmed that fatty acid and sterol compositions vary among batches, these variations may affect process performance. Therefore, in this section the correlation between the lipoprotein supplement composition and the process performance is investigated.

4.3.2.1. Effects of the fatty acid composition on process performance

Because the fatty acid composition of US and NZ batches are different, they were analyzed separately. Moreover, due to the high number of low-performing NZ batches tested we were lacking fatty acid composition of NZ batches with an acceptable productivity, consequently only US batches were used in this analysis of the effect of fatty acid composition on process performance.

Bivariate analysis, linear and response-surface models were used to investigate the correlation between the fatty acid composition of lipoprotein supplements and productivity. The theorical foundation of these models is described in section 3.2.8.4.

In bivariate analysis, a single response variable, typically a growth indicator or productivity, is plotted against varying concentration of a lipoprotein supplement component to assess its impact. Linear models include multiple variables to capture the variations in the response variable but are restricted to first-order correlations. In contrast, response-surface models offer a more comprehensive insight, capturing the response variable's variations through second-order correlations among several variables, here several lipoprotein supplement components.

No bivariate relationship between fatty acid concentration or fatty acid proportion and productivity was observed. Hence variations of a single lipoprotein component were not sufficient to describe productivity variations observed. Thus, a response surface between fatty acids and productivity was constructed as this model considers interactions among the different fatty acids contained in the lipoprotein supplement. First, every fatty acid was considered, but parameters with no or low effect on the productivity (LogWorth<2) were removed one at a time with a step-by-step approach until were remaining only significative parameters (LogWorth>2) and parameters interacting with other parameters in a significant way. However, if the removal of a non-significative parameter was decreasing the accuracy of the prediction, *i.e.* increasing Prob > ¦t¦, this parameter was added back to the model.

Effects of the fatty acid content of the lipoprotein supplement on productivity

First, a linear model without interaction that predicts US batch productivity based on the fatty acid content was built to identify the fatty acids with the higher impact on productivity (Figure 30), the effect of each fatty acid content on the model is presented in Table 11.

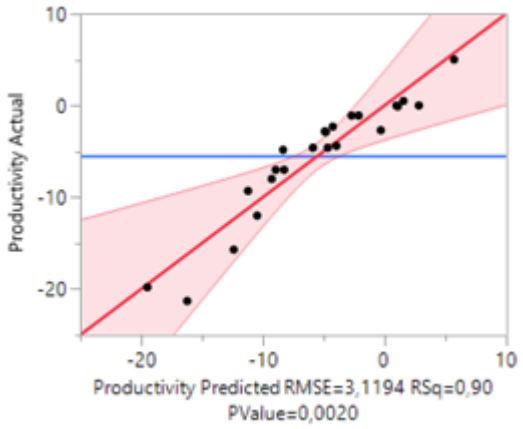


Figure 30: Comparison of US batch productivity measured experimentally, and the productivity predicted by a linear model based on the fatty acid content (n=23).

The 4 most present fatty acids in the lipoprotein supplement, stearic linoleic, palmitic and oleic acids as well as α-linolenic, γ-linolenic, pentadecanoic and arachidonic acids have a significative impact on productivity. Interestingly the total, unsaturated and the polyunsaturated fatty acid contents, as well as the unsaturated/saturated ratio are significative. Because these values are mostly determined by the 4 most present fatty acids in the supplement, and thus can be replaced by a combination of their concentrations, these 4 fatty acids seem to interact to influence the productivity.

Source	LogWorth	PValue
Unsaturated	3.979	0.00010
Pentadecanoic Acid	3.739	0.00018
Unsaturated/Saturated Ratio	3.720	0.00019
Total	3.331	0.00047
a-linolenic Acid	3.168	0.00068
Palmitic Acid	3.102	0.00079
Polyunsaturated (≥3C=C)	3.088	0.00082
Stearic Acid	3.075	0.00084
Linoleic Acid	3.062	0.00087
Oleic Acid	2.756	0.00175
y-Linolenic Acid	2.135	0.00732
Arachidonic Acid	1.968	0.01076

Table 11: Effect summary of a linear model that predicts US batch productivity based on the fatty acid content (n=23). Note that arachidonic acid is not a significative parameter (LogWorth<2) but its removal decreased the accuracy of the prediction, so it was maintained in the model.

Then, a mixture surface-response model that predicts US batch productivity based on its fatty acid content was built (Figure 31). This model aims to identify the fatty acids with the higher impact on

productivity, this model has a better batch productivity prediction than the precedent one. The effect of each fatty acid content on the model is presented in Table 12.

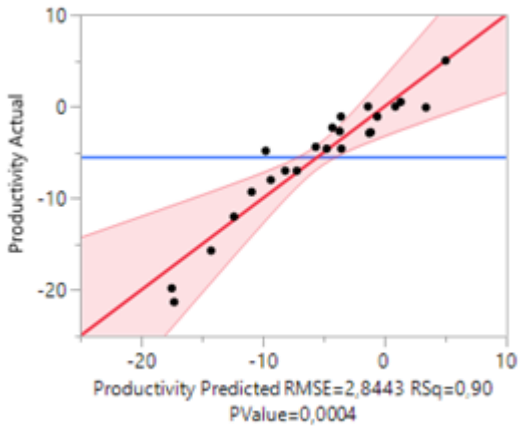


Figure 31: Comparison between US batch productivity measured experimentally, and the productivity predicted by a mixture surface-response model based on the fatty acid content (n=23).

Source	LogWorth	PValue	
a-linolenic Acid*Arachidonic Acid	3.025	0.00094	
Oleic Acid*Arachidonic Acid	2.934	0.00116	
Linoleic Acid*a-linolenic Acid	2.801	0.00158	
a-linolenic Acid	2.521	0.00301	^
Linoleic Acid*Palmitic Acid	1.653	0.02223	
Linoleic Acid	1.457	0.03493	^
Oleic Acid	1.420	0.03805	^
Palmitic Acid*a-linolenic Acid	1.314	0.04854	
Pentadecanoic Acid	1.259	0.05506	
Palmitic Acid	0.811	0.15469	^
Arachidonic Acid	0.049	0.89298	^

Table 12 : Effect summary of the mixture surface-response model that predicts US batch productivity based on the fatty acid content (n=23). Note that several fatty acid or fatty acid interaction are not a significative parameter (LogWorth<2) but their removal decreased the accuracy of the prediction.

According to the interaction profiles of the surface-response model presented in Figure 32, the respective effect of fatty acids on productivity often depends on other fatty acids concentration.

Overall, US batches with a high productivity contain high levels of linoleic and palmitic acids as well as low levels of α-linolenic acids. The effects of oleic and arachidonic acids were inter-dependent, but the higher productivity was obtained with high level of oleic acid and low level of arachidonic. Thus, according to the model, the best fatty acids composition of an US lipoprotein supplement batch to reach the highest productivity is linoleic acid >1500 µg/ml, palmitic acid >600 µg/ml, oleic acid >450 µg/ml, α-linolenic acid <15 µg/ml, arachidonic acid <50 µg/mL and pentadecanoic acid <15 µg/ml.

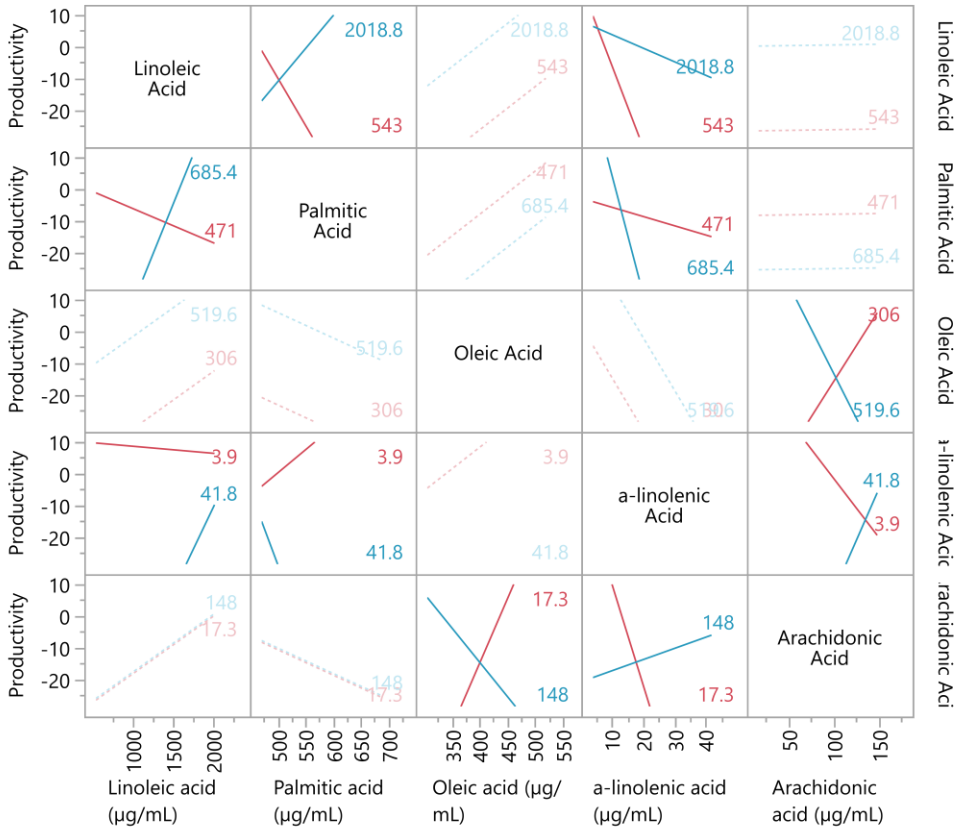


Figure 32: Interaction profiles of the mixture surface-response model that predicts US batch productivity based on the fatty acid content (n=23).

Confirmation of the optimal fatty acid composition to enhance the productivity

With the aim to test the optimal fatty acid composition of the lipoprotein supplement according to the model presented in Figure 31 and Table 12, an US lipoprotein supplement batch (batch 31) was spiked with fatty acids to reach the optimal concentrations.

According to the model, linoleic, palmitic and oleic acids optimal concentrations should exceed 1500, 600 and 450 µg/mL respectively. The reference batch does not fulfill these conditions, so these fatty acids were spiked to evaluate the impact of an increased concentration. Linoleic, palmitic and oleic acids were supplemented individually to reach the optimal concentration limit and beyond, moreover these three fatty acids were also supplemented together to reach the optimum.

According to the model, α-linolenic, arachidonic and pentadecanoic acids optimal concentrations should be maintained below 15, 50 and 15 µg/mL respectively. The lipoprotein supplement batch tested fulfills all these conditions. However, low-performing batches contain high levels of α-linolenic acid, consequently, to evaluate the impact of exceeding the limit, α-linolenic concentration was increased in one condition.

Moreover, to assess the effect of the nature of the fatty acid solubilizer, fatty acids were added using either a stock solution in ethanol for all fatty acids tested, or a water-soluble solution of cyclodextrin

(mbCD) solubilized lipids, inclusion complexes with linoleic acid or oleic acid. These fatty acids were spiked into the lipoprotein solution.

The supplemented lipoprotein supplement samples were used to carry out a fed-batch experiment preceded by a 1-week cell expansion. At the end of the production phase the IVC were compared to the lipoprotein supplement not supplemented (Table 13).

Because the fatty acid supplementation with stock solution in ethanol or mbCD inclusion complexes caused the presence of ethanol and mbCD in the culture, control conditions containing ethanol and mbCD but without fatty acid were tested too. Supplementation of fatty acid dissolved in ethanol never increased the ethanol concentration in the culture above 0.50%, such concentration had no impact on cell growth and product yield (data not shown). However, supplementation of mbCD inclusion complexes with fatty acid increased the mbCD concentration in the culture up to 400 mg/L. Two conditions with mbCD 100 and 400 mg/L were added as control, they negatively impacted the IVC. The IVC compared to the reference without mbCD were 98.3 and 93.0% for mbCD 100 and 400 mg/L respectively.

Only addition of mbCD inclusion complexes with oleic acid to the lipoprotein supplement had a positive impact on the IVC compared to the condition without supplementation (Figure 33). Addition of linoleic acid either with stock solution in ethanol or mbCD inclusion complexes was detrimental to the culture. Similarly, addition of palmitic and oleic acids with ethanol stock solution or addition of a combination of linoleic, palmitic and oleic acid were detrimental to the culture. Increase α-linolenic acid above the concentration limit of the model had a negative effect as suspected. Regarding supplementation of fatty acids dissolved in ethanol, their negative impact was not due to the presence of ethanol because addition of ethanol only had no impact on cell growth. Interestingly, addition of free-mbCD negatively affected cell growth (relative IVC from 93.0 - 98.3%) but mbCD inclusion complexes with oleic had a positive impact on cell growth (relative IVC 102.4 -102.8% compared to the reference) indicating a positive impact of oleic acid on cell growth with solubilized by mbCD. By contrast, supplementation of oleic acid dissolved in ethanol was detrimental to the culture (relative IVC 87.4 – 80.0% compared to the reference).

Overall, the effect of fatty acid addition with ethanol was always worse than addition with mbCD inclusion complexes. This highlights the importance of the nature of the fatty acid solubilizer. Indeed, the solubilized may impact fatty acid stability and availability for the cells.

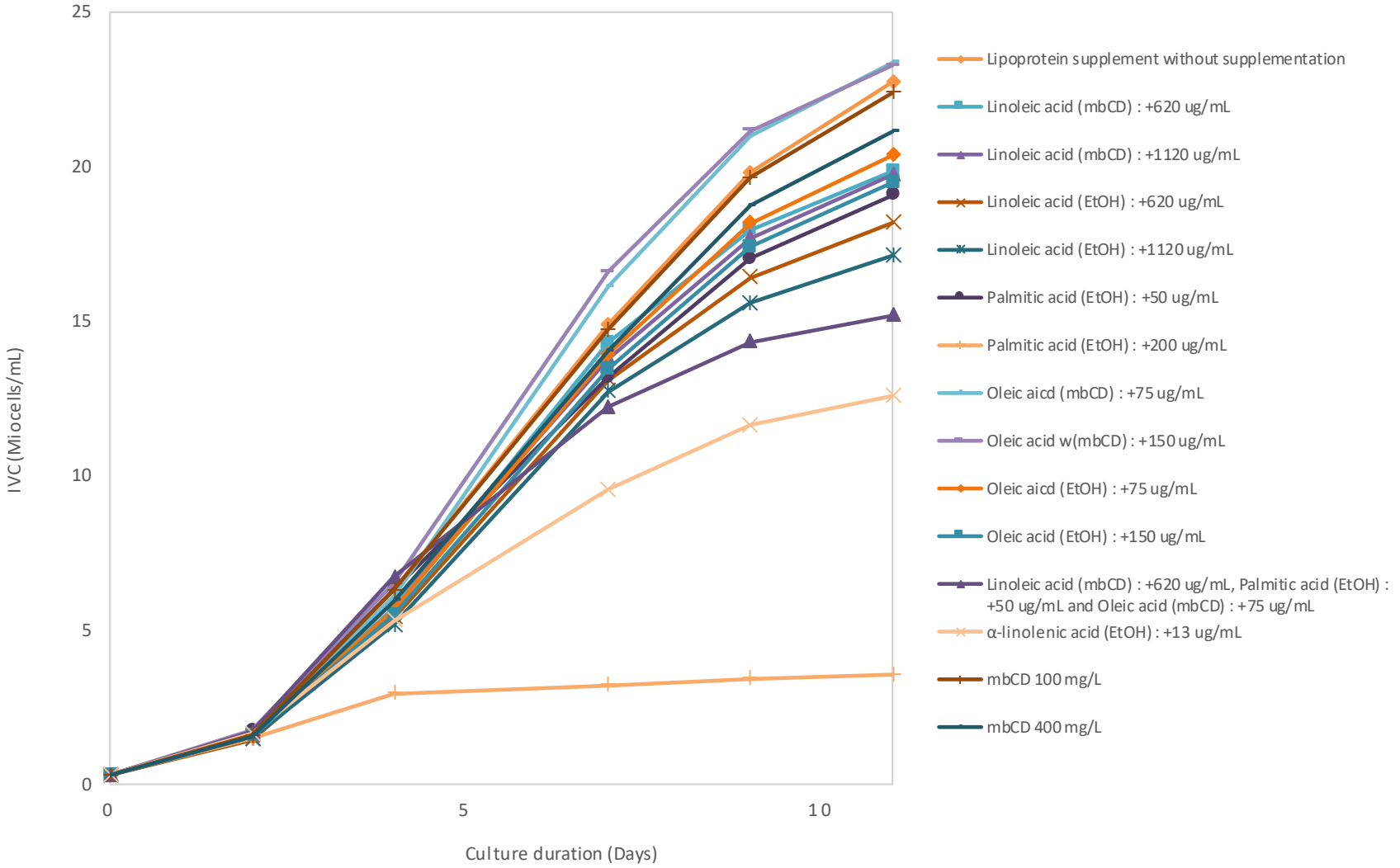


Figure 33: Effect of the fatty acid supplementation of the lipoprotein supplement on the IVC. The fatty acids were added to reach the optimal fatty acid composition of the lipoprotein supplement modeled in section 4.3.2.1. Only oleic acid supplementation showed a positive impact on cell growth compared to the reference without fatty acid supplementation. Mean of two independent cultures.

	Lipoprotein supplement batch 31 (µg/mL)	Optimal concentration according to the model (µg/mL)	Concentration after spiking (µg/mL)	Relative IVC compared to the lipoprotein supplement without fatty acid spiking (%)	Relative titer compared to the lipoprotein supplement without fatty acid spiking (%)	%EtOH (v/v)	mbCD (mg/L)
Linoleic acid	879	>1500	1500	87.3	91.8	-	430
				79.9	87.2	0.26	-
			2000	86.8	98.4	-	776
				75.3	92.1	0.47	-
Palmitic acid	543	>600	600	83.9	82.4	0.07	-
			750	15.6	8.3	0.28	-
Oleic acid	376	>450	450	102.8	91.7	-	52
				89.6	78.4	0.11	-
			525	102.4	95.9	-	104
				85.7	80.0	0.21	-
α-linolenic acid	12	<15	25	55.3	57.3	0.01	-
mbCD control	-	-	-	98.3	-	-	100
	-	-	-	93.0	-	-	400

Table 13: Effect of varying the fatty acid composition of a lipoprotein supplement on the IVC. Regarding linoleic and oleic acids supplementation, two delivery methods were compared, stock solution in ethanol and mbCD inclusions complexes. The relative titer and IVC results are an average of 3 independent cultures.

4.3.2.2. Effects of the sterol fraction on process performance

The sterol characterization of 12 lipoprotein supplement batches showed that US batches contain more sterol, and more oxidized cholesterol derivatives, than NZ batches. Despite the lower content of cholestenone and higher content of desmosterol in low-performing batches, there is no correlation between the sterol composition and batch performance.

Low-performing batches contain more desmosterol than other batches. If desmosterol has a negative impact on SP2/0 cell growth it could partially explain the low growth observed with low-performing batches. Indeed, desmosterol promotes the proliferation of SP2/0 cells at low concentrations but exhibits growth-inhibitory effects when concentrations exceed 10 µM (Sato, et al., 1988). However, desmosterol concentration in the lipoprotein supplement is extremely low, desmosterol concentration in the culture never exceeds 2 µM. Consequently, higher level of desmosterol in low-performing batches is not sufficient to explain growth issues related to these batches.

An important batch-to-batch variability of oxysterol content was observed among the batches tested. In order to determine, whether oxysterol concentration variations are susceptible to cause process performance variations, the effect of 3 oxysterols on process performance was assessed. The two most prevalent oxysterols in the lipoprotein supplement were selected, 7-ketocholesterol and 7-β-hydroxycholesterol, along with 25-hydroxycholesterol present in smaller quantities, yet representative of 24-, 26-, and 27-hydroxycholesterol. The lipoprotein supplement reference batch (batch 7) was supplemented with oxysterol from 50 to 1500 mg/L using oxysterol stock solutions.

Cultures were conducted in CCM containing the lipoprotein supplement enriched in oxysterol. In each culture the final ethanol concentration was adjusted at 0.1% (v/v) including the reference without oxysterol spiked. At the end of the production phase the IVC of each condition was compared to the IVC of the reference, results are presented in Figure 34.

The 3 oxysterols tested have a negative impact on cell growth on the concentration range tested but their degree of toxicity differ. The toxic effect of 7-ketocholesterol started from 550 mg/L lipoprotein supplement, however every lipoprotein supplement batch tested contained more than 300 mg/L of 7-ketocholesterol. The toxic effect of 7-β-hydroxycholesterol started from 150 mg/L lipoprotein supplement, similarly to 7-ketocholesterol, every lipoprotein supplement batch tested contained more than 150 mg/L 7-β-hydroxycholesterol.

Lastly, the toxic effect of 25-hydroxycholesterol started from 150 mg/L lipoprotein supplement, however the range of concentration tested during the spiking study is higher than to the 25-hydroxycholesterol concentration in the lipoprotein supplement. It is not possible to conclude on the impact of 25-hydrocholesterol on cell growth with the results of this experiment.

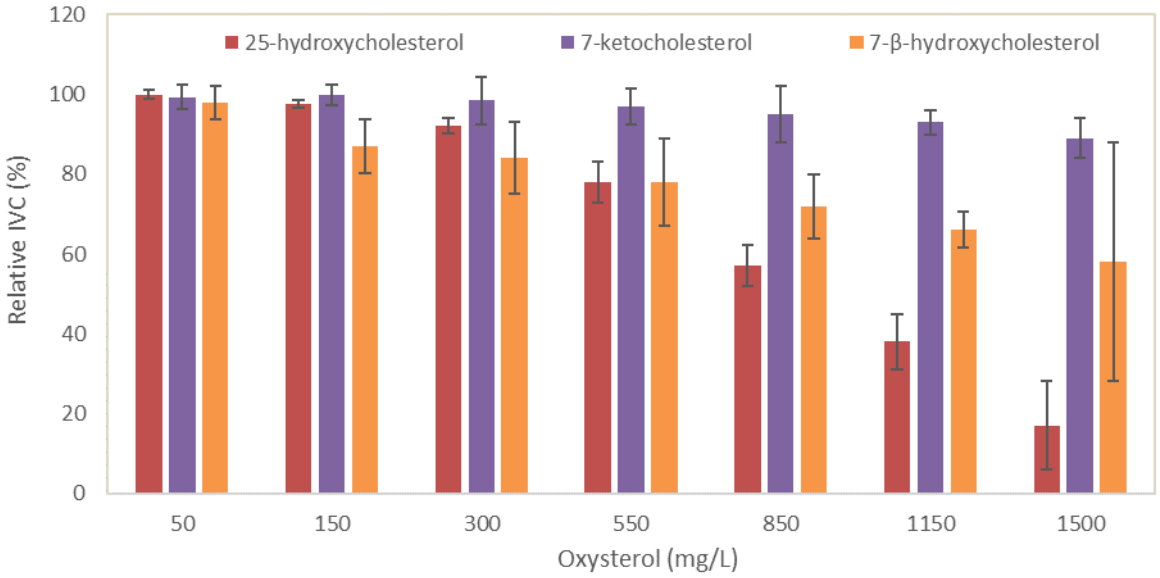


Figure 34: Effect of oxysterol concentration on cell growth after 11 days of production. IVC of each condition is compared to the IV of the reference without oxysterol spiked and expressed as the relative IVC. Mean ± standard deviation of three independent cultures.

In the light of the oxysterol spiking study, oxysterol concentrations in the lipoprotein supplement should be sufficient to impact cell growth and process performance. However, spiking of oxysterol with a stock solution in ethanol may not be fully reproductible of oxysterol assimilation by the cells when oxysterols are part of lipoproteins as for the lipoprotein supplement. Indeed, oxysterol might be less toxic in the range of concentration tested if they are associated with a lipoprotein.

4.3.3. Evaluation of fingerprinting methods

Characterization of the lipoprotein supplement composition has confirmed the presence of oxidation derivatives that are potentially detrimental to cell growth and process performance. Moreover, some lipoprotein components have been identified as growth-promoting or growth-limiting compounds. However, complete characterization of each lipoprotein supplement batch prior to its purchase is near impossible. In this section, inexpensive fingerprinting methods are evaluated with the aim to detect low-performing batches with a single spectroscopic measurement.

4.3.3.1. Near Infrared

Near infrared (NIR) transmittance measurement was conducted on lipoprotein supplement batches. This method allows for the identification of chemical compounds due to their absorption in certain regions. Spectra from 12493 to 4000 cm-1 of 42 NZ batches and 232 US batches were generated during raw-material incoming testing by quality control department on batches used for large scale manufacturing. In this spectral range, functional groups have distinct region of absorbance that allows the identification and quantification of chemical compounds. However, due to the non-chemically

defined nature of the lipoprotein supplement identification of a specific compound is impossible. Instead the complete spectra were compared using a multivariate data analysis tool, the principal component analysis (PCA).

First, spectra were preprocessed to reduce artifacts and instrument variations (refer to section 3.2.2.1). Covariance matrices were calculated to describe how each wavelength relates to all others. These covariance matrices were then decomposed to find eigenvalues and eigenvectors. The eigenvectors were sorted, and those that best describe the variance in the data were selected as principal components. Principal components represent patterns in the data, with each component being a linear combination of the original variables. This first part of the process is known as eigendecomposition. Then the original spectroscopic data are projected onto the selected principal components, reducing dimensionality, although at the cost of losing some variance in the process. The resulting reduced-dimension dataset can be used for visualization, clustering, outlier identification, and further analysis.

A PCA of the 274 NIR spectra is presented in Figure 35. Based on a visual analysis of the PCA, no significative difference was observed between US and NZ batches. Moreover, group-to-group comparisons between low-performing and other NZ batches was not able to identify a region that discriminate low-performing batches. Consequently, NIR spectroscopy is not a fingerprinting method able to detect low-performing batches.

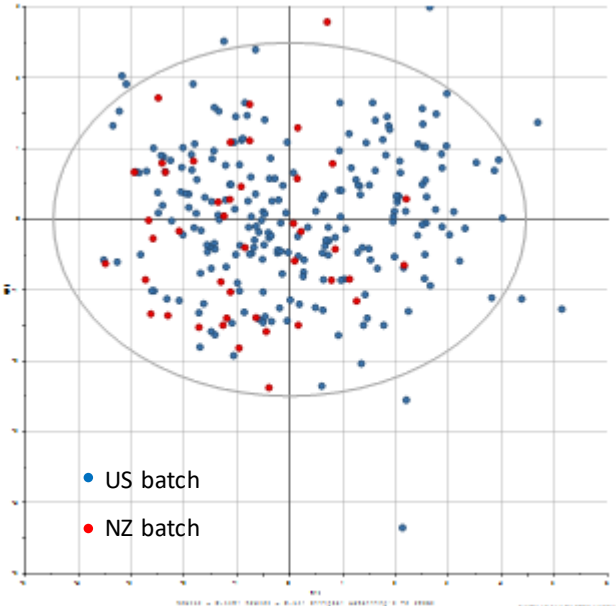


Figure 35: PCA score plot of the NIR analysis of lipoprotein supplement batches. US and NZ batches are not separated in two distinct clusters, their NIR spectra are undifferentiable.

4.3.3.2. UV-absorbance

UV-visible absorbance spectra of 16 NZ and 17 US batches were generated, the spectra are presented in Figure 36. The lipoprotein supplement UV-absorbance spectra are dominated by two peaks centered at about 230 and 276 nm. NZ batches absorb more than US batches from 230 to 290 nm (Figure 37).

Interestingly, low-performing batches absorb more than other NZ batches in the same region, especially at around 230 and 276 nm (Figure 37).

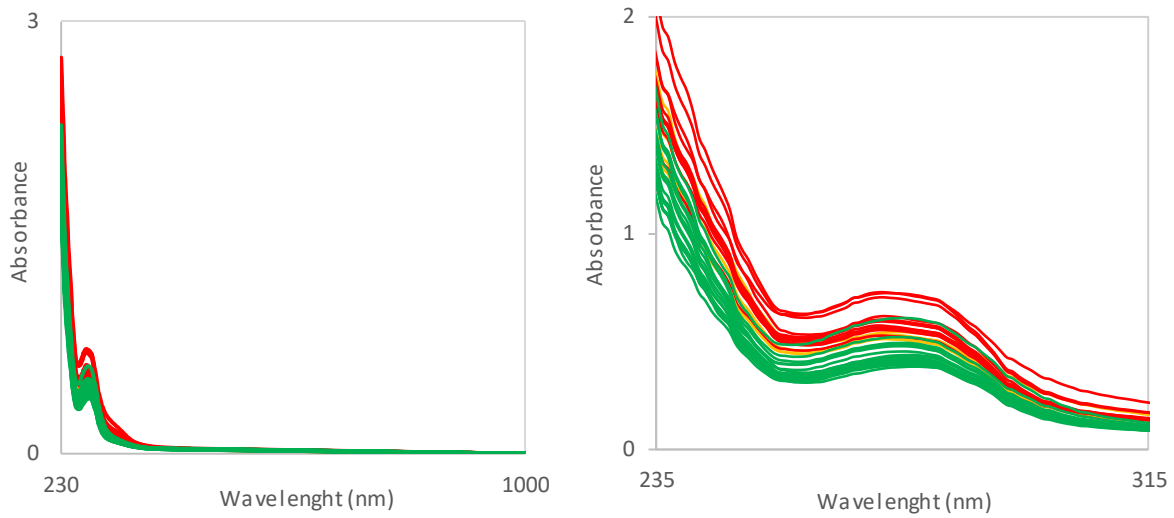


Figure 36: UV-visible absorbance spectra of 33 lipoprotein supplement batches diluted 100X in purified water. 17 US batches are in green, 11 NZ low-performing batches in red and 5 other NZ batches in orange. Step between each measurement: 1 nm. Average of two spectra.

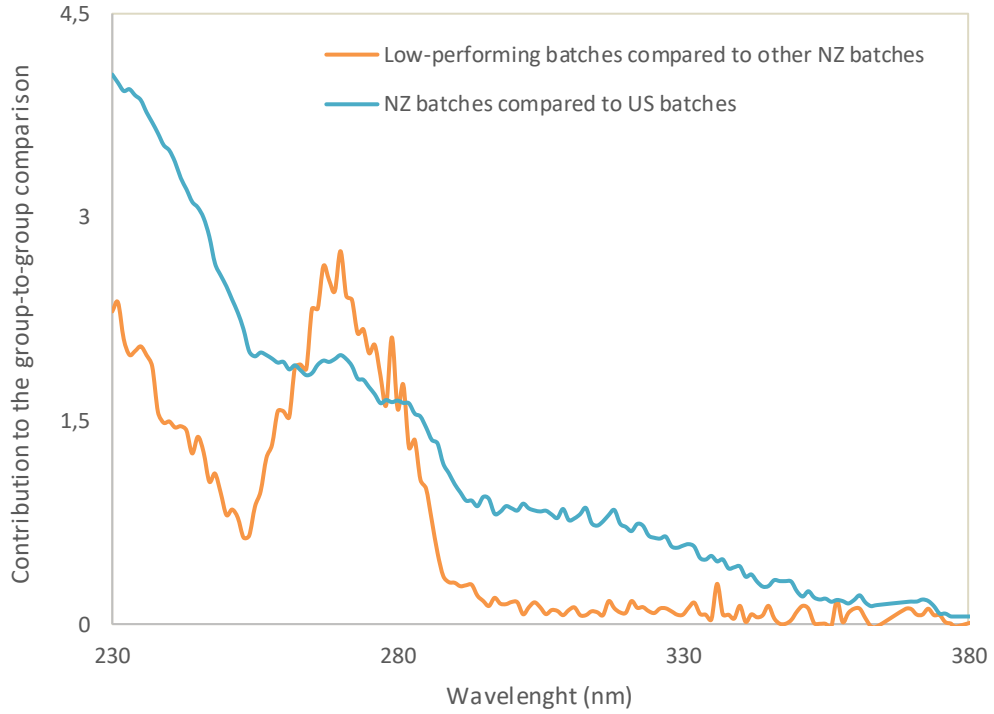


Figure 37: Group-to-group comparison of the spectra region contributing to the PCA between 16 NZ and 17 US batches (blue) and between 11 low-performing and 5 other NZ batches (orange). NZ batches absorb more than US batches in the UV region (200-380 nm). Low-performing batches absorb more than other NZ batches only at around 230 and 275 nm.

The distribution of Abs 276 nm of 33 lipoprotein supplement batches was analyzed in order to establish a threshold capable of discriminating low-performing batches from other NZ batches (Figure 38).

Distribution of Abs 276 nm of low-performing and other NZ batches indicated that the lower 95% mean of the low-performing batches and the upper 95% mean of other NZ batches converged at an absorbance value of 0.56. Hence, with a confidence level of 95%, we can affirm that lipoprotein supplement batches, when diluted 100X in purified water, exhibiting an Abs 276 nm exceeding 0.56, are low-performing batches. Among the 33 batches for those absorbance was measured, only one US batch and one NZ batch are not in accordance with this limit, they would have been detected as false positive and false negative respectively.

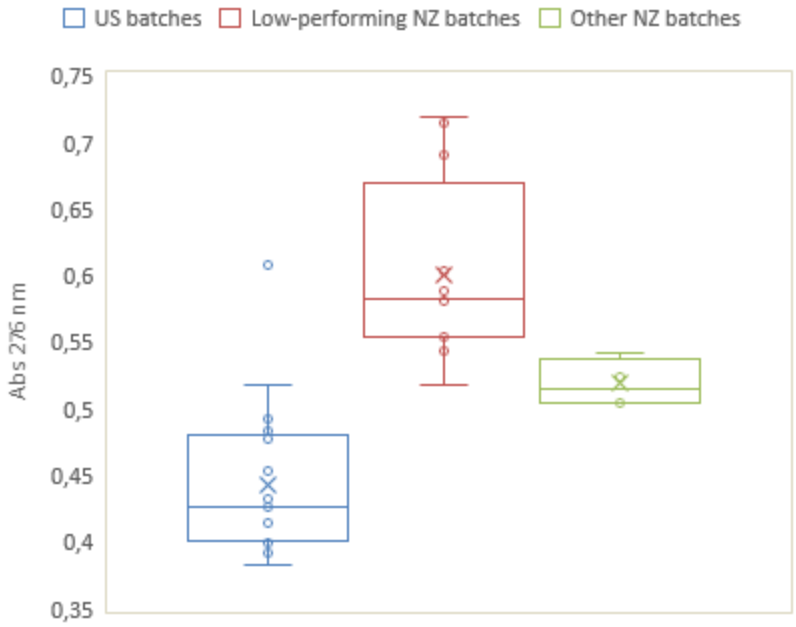


Figure 38: Distribution of the Abs 276 nm of US, low-performing and other NZ batches. Absorbance was corrected by subtracting blank absorbance that contain purified water. Measurements were made in duplicate (US batches n = 17 ; low-performing NZ batches n = 12 ; other NZ batches n = 4).

4.3.4. Discussion

The lipoprotein supplement characterization gave a better overview of its composition (Figure 39) and the magnitude of its batch variability. The characterization confirmed the lipoprotein nature of the supplement, especially its proteomic composition, indeed the proteins are mostly Apo A-1 and Apo B, two components of HDL and LDL. The lipoprotein supplement contains 15.8 g/L of lipids and 5.9 g/L of proteins, the lipid:protein ratio is 2.7, between those of HDL and LDL, 1 and 4 respectively. These results are concordant with the apolipoprotein composition of the supplement, mainly Apo A-1 and Apo B. The lipoprotein supplement must contain mostly HDL and LDL, the two lipoproteins commonly found in serum.

According to the Sterol:Fatty Acid and Lipid:Protein ratios, the lipoprotein composition in low-performing batches differs from that of other NZ batches (Figure 40). Low-performing batches appear to contain a higher proportion of HDL and a lower amount of LDL compared to other NZ batches based on these ratios. However, these results are inconsistent with the proteomics characterization, which

indicates that low-performing batches actually contain more ApoB than other NZ batches, at 24.0% and 19.1%, respectively. Consequently, low-performing batches should theoretically contain more LDL than other NZ batches, as ApoB is the primary apolipoprotein of LDL and is absent in HDL. This inconsistency in the lipoprotein composition of low-performing batches between the proteomics data and the Sterol:Fatty Acid and Lipid:Protein ratios could be attributed either to potential alterations in the lipid composition of low-performing batches or an abnormal lipid composition of the lipoproteins in low-performing batches.

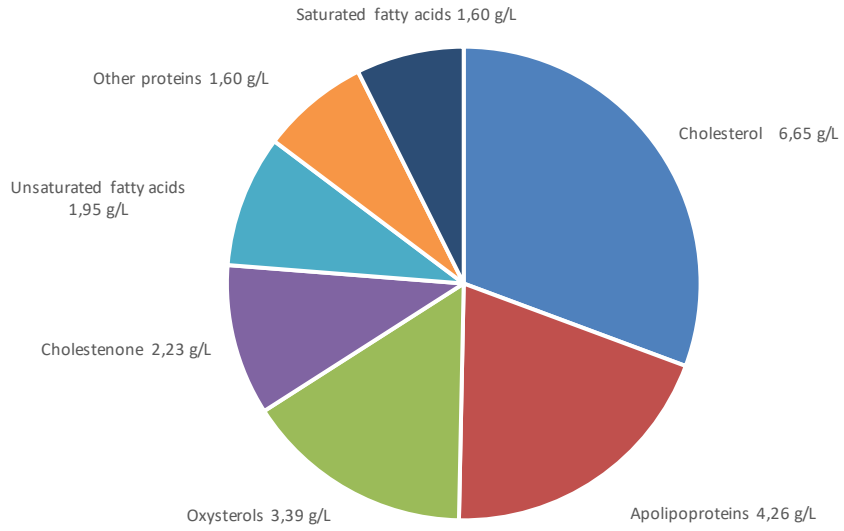


Figure 39 : Simplified composition of the lipoprotein supplement. Minor components like trace elements, tocopherol, 4-HNE and MDA are not displayed. Non-exhaustive list based on the compounds tested during the characterization.

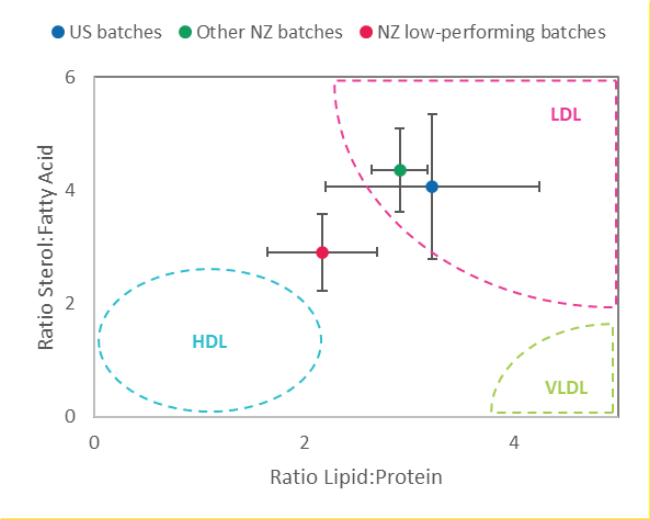


Figure 40: Plot of the lipoprotein supplements characterized on a 2D map of lipoprotein classes according to their Lipid:Protein and Sterol:Fatty Acid ratios. Mean ± standard deviation.

The characterization also confirmed an important batch-to-batch variability of the lipoprotein supplement. While such variations are inherent to raw materials derived from animals, they pose a

challenge to process robustness. Indeed, significant variations of several fatty acids, oxidized cholesterol derivatives and lipid peroxidation byproducts are likely to affect cell growth and so process performance. Moreover, the characterization has revealed that apart from the inter-batch variations, lipoprotein supplements originating from the US and NZ have two distinct compositions. As a result, supplementing the culture with an equivalent amount of either US or NZ supplement will lead in significantly different lipid concentrations within the culture. In light of this, these two references should be treated as separate raw materials that are not interchangeable.

Correlating the fatty acid composition of 23 US batches with their respective product yield allowed to identify both productivity-enhancing and productivity-limiting fatty acids, along with their optimal concentration ranges. This correlation enabled the identification of an optimal fatty acid composition for the lipoprotein supplement. However, attempts to enhance batch productivity by spiking fatty acids dissolved in ethanol or complexed with mbCD into the supplement to meet the optimal concentration proved unsuccessful. The differences in solubility, stability, and cellular uptake between fatty acid supplementation via lipoproteins versus ethanol or mbCD suggest that results from experiments conducted with these different methods might not be comparable. The same phenomenon was observed during the oxysterol spiking study. While most lipoprotein supplements contain over 1000 mg/L of oxysterols without significantly affecting cell growth, the addition of 1000 mg/L of oxysterols dissolved in ethanol had adverse effects on the culture for all three tested oxysterols. These outcomes emphasize the importance of not only considering the concentration but also the delivery method when studying lipid supplementation.

Significant quantities of oxidized cholesterol derivatives were detected, alongside lipid peroxidation byproducts and carbonylated proteins. It confirms that a substantial fraction of the lipoprotein supplement is oxidized. High levels of cholestenone interfere with cell membrane functionality (Neuvonen, et al., 2014) while oxysterols induce apoptosis (supplementary Table 22). As a result, lipoprotein supplement batches with an elevated concentration of oxidized cholesterol derivatives should have a detrimental effects on cell growth. However, the low-performing batches contained the lowest levels of cholestenone and oxysterols among all the batches tested. Because the carbonyl group makes cholestenone more oxidizable than cholesterol, cholestenone molecules present in the low-performing batches might have been consumed. Given these considerations, it is plausible that oxidized cholesterol derivatives contribute in part to the inherent variability in batch performance of the lipoprotein supplement. Nevertheless, oxidized cholesterol derivatives, lipid peroxidation byproducts and protein carbonylation is likely to contribute in part to the inherent variability in batch performance of the lipoprotein supplement but do not explain the viability decline associated with low-performing batches.

Regarding fingerprinting outputs, NZ batches absorb more in the UV region than US batches. Among the NZ batches, the low-performing batches absorb more at around 230 and 275 nm, two regions associated with conjugated dienes. The presence of conjugated dienes, known end products of lipid

peroxidation (Chang, Abdalla, & Sevanian, 1997), is coherent with the presence of lipid peroxidation markers such as MDA, 4-HNE, oxidized cholesterol derivatives, and carbonylated proteins. Moreover, the analysis of the Abs 276 nm of 33 lipoprotein supplement batches confirmed that low-performing batches absorb significatively more at 276 nm. Based on the distribution of the Abs 276 nm a limit that separate low-performing batches from other batches using Abs 276 nm was established. If 100 µL of a lipoprotein supplement batch diluted 100X in purified water absorb more than 0.557 at 276 nm after measurement in Corning® 96-well UV-transparent microplate, then it is possible to state with 95% of confidence that it is a low-performing batch. Note that the supplement bottle must be opened right before the analysis, we observed that the Abs 276 nm value varies between analysis if the same bottle is used several times. Replacement of the inert atmosphere originally contained in the bottle by atmospheric air probably initiates oxidation which will increase Abs 276 nm. Because low-performing batches are challenging to detect otherwise than with cell culture experiments, the implementation of a fingerprinting method would contribute to increase the robustness of the processes that involve lipoprotein supplement.

Although various elements suggest that oxidation is the discriminant factor between low-performing and other batches, the outcomes derived from the lipoprotein supplement characterization present a paradox. Some elements indicate that the low-performing batches are oxidized to a lesser extent than other batches. On the other hand, the high levels of α-linolenic acid prone to lipid peroxidation and the signs of elevated conjugated dienes levels within the low-performing batches compared to other batches are consistent with a higher degree of oxidation.

4.4. Investigation of the root cause of low-performing batches

Because the characterization of the lipoprotein supplement proved unsuccessful to identify the compounds that were causing the viability decline associated with low-performing batches, further investigations were carried out to explore the relationship between low-performing batches and viability drops.

Because the lipoprotein supplement is manufactured from bovine serum, we started by investigating the presence of toxic compounds in the bovine serum batches used to manufacture low-performing lipoprotein supplement batches. Then we increased our understanding of the mechanisms underlying performance issues by examining the role of oxidative stress and apoptosis in the premature viability decline observed. Lastly, the effect of the lipoprotein supplement oxidation on process performance was evaluated, alongside its monitoring by UV-spectroscopy.

4.4.1. Bovine serum or lipoprotein supplement low-performing batches?

The possibility that the compounds in low-performing batches causing viability decline might already exist in bovine serum before its conversion into lipoprotein supplement was considered. To investigate this hypothesis, reserve samples of the bovine serum batches employed in the manufacturing of

lipoprotein supplement were utilized in cell culture experiments to evaluate the impact of serum batch variations on cell growth.

4.4.1.1. Development of a small-scale model where the supplement is replaced by bovine serum

The first step was to replace the lipoprotein supplement by bovine serum in the CCM in order to find conditions allowing to test the cell growth in the presence of serum. However, the concentration should be considered precisely, indeed the new media should support cell growth and show the effect of serum batch variations on cell growth. Several serum concentrations, from 5 to 30%, were tested in lipoprotein supplement-free CCM to identify which one fulfill the growth criteria. Cells were adapted to the new CCM then, one week of cell expansion was carried out in order to evaluate the effect of serum concentration on cell growth, after 7 days of cell expansion PDL were calculated (Figure 41).

Cell growth was supported in a comparable manner by lipoprotein supplement-free CCM containing serum 5% and 10% when compared to the reference medium containing lipoprotein supplement at 5 g/L. The medium containing 10% serum was chosen for further analysis as it demonstrated adequate support for cell growth, and the higher serum concentration, in contrast to 5% serum, was expected to better highlight the impact of serum batch variations on cell growth.

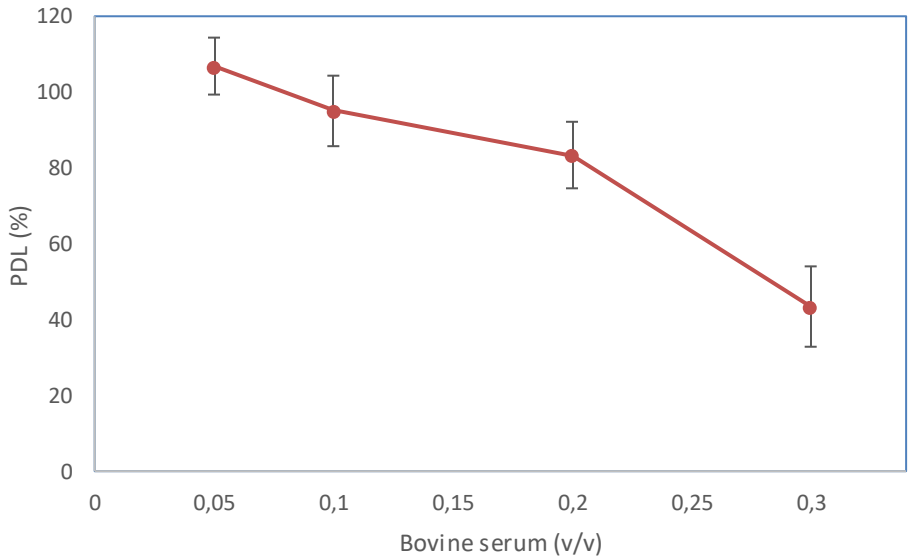


Figure 41 : Effect of serum concentration in lipoprotein supplement-free CCM on cell growth. Cell growth is expressed as the relative PDL after 7 days of cell expansion compared to the media containing lipoprotein supplement 5 g/L. Cells grow at the same rate in lipoprotein supplement-free CCM containing 5 or 10% serum and in the reference media. Mean ± standard deviation from three independent cultures.

4.4.1.2. Assessment of the impact of bovine serum batch variations on cell growth

The effect of bovine serum batch variations on cell growth was assessed using the lipoprotein supplement-free CCM containing serum 10%. A total of 9 bovine serum batches, which served as the starting material for lipoprotein supplement manufacturing, were compared (Figure 42). Among these serum batches, 2 originated from the US, and 7 originated from NZ. Among the 7 NZ bovine serum batches, 6 were associated with low-performing lipoprotein supplement batches (NZ bovine serum batches 2 to 7), while 1 was transformed into a lipoprotein supplement batch without any performance issues (NZ bovine serum batch 1).

The US bovine serum batches and the NZ bovine serum batch 1 supported cell growth while viability was maintained at around 80% during the 1-week cell expansion. On the other hand, the utilization of NZ bovine serum batches in the manufacturing of low-performing NZ batches resulted in rapid viability drops, with viability decreasing to below 30% by the end of the 1-week expansion period. These observed viability drops are likely attributed to the presence of toxic compounds present in these bovine serum batches, suggesting that bovine serum batches already contain toxic compounds before undergoing transformation into lipoprotein supplement.

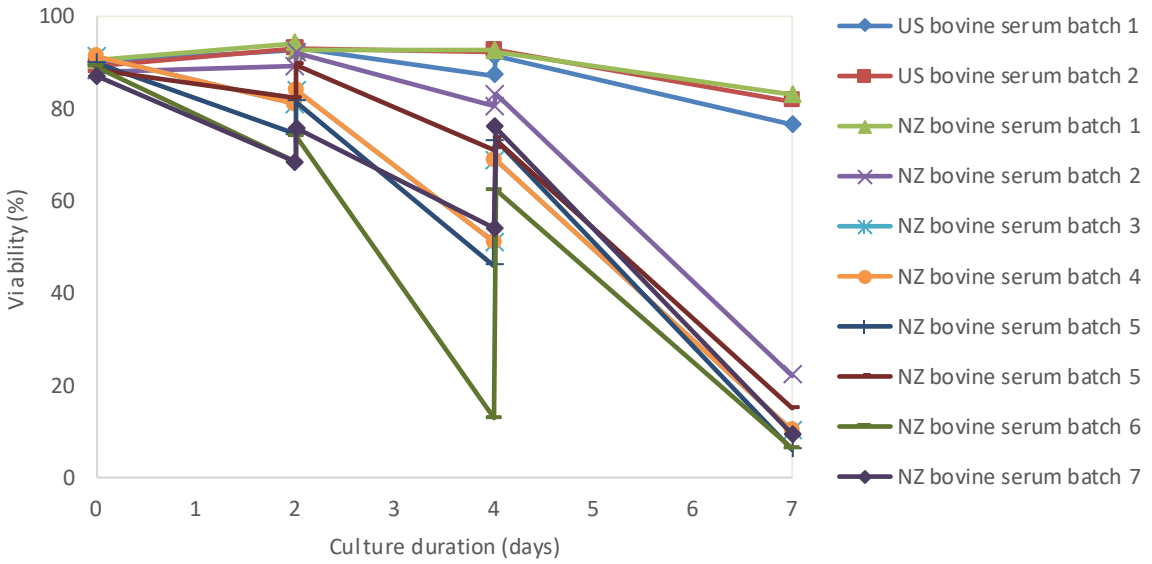


Figure 42: Viability trends during cell expansion using lipoprotein supplement-free expansion medium containing 10% bovine serum. Different bovine serum batches used to manufacture lipoprotein supplement batches were tested. Several NZ bovine serum batches were causing important viability drops, these batches were used to manufacture low-performing supplement batches. Mean of three independent cultures.

A hypothesis driven approach was applied to exclude the lipoprotein supplement manufacturing process as the root cause of the low-performing batches. By contrast with other batches, the bovine serum batches transformed in low-performing lipoprotein supplement batches were already causing growth issues prior to the isolation of their lipoprotein fraction. Thus, the compounds affecting SP2/0

cell viability were already present in the bovine serum and conserved in the low-performing lipoprotein supplement batches. The lipoprotein supplement manufacturing process was excluded as a root cause, the bovine serum batches used to manufacture the low-performing batches are likely the origin of the problem.

4.4.2. Evaluation of oxidative stress and apoptosis in cell viability drops

4.4.2.1. Measurement of cell death markers

An early decrease in viability was observed as the concentration of the lipoprotein supplement increased in the culture following the feed addition of low-performing batches. This led to the suspicion of a controlled cell death mechanism causing the premature viability decline. Consequently, an investigation was conducted to determine the presence of apoptosis markers.

During the production phase, apoptosis markers including Annexin V binding to phosphatidylserine, mitochondrial damage and caspase-3 activity were measured.

Measurement of cell death markers by flow-cytometry

The effect of low-performing batches on apoptosis markers during the production phase was assessed by comparing 4 low-performing batches to 2 US batches and 2 NZ batches without performance issues. The Annexin V-CY3 assay kit (Sigma Aldrich, APOAC) and IntelliCyt® iQue Screener PLUS (Sartorius) were utilized for this experiment. Samples were collected during the production phase at day 2, before lipoprotein feed addition, as well as 24 hours and 48 hours after lipoprotein feed addition.

Cells in the exponential growth phase (with cell viability > 90%) were used as the negative control of apoptosis, while cells in the exponential growth phase treated with ethanol 30% for 30 minutes served as the positive control of apoptosis. To evaluate the impact of medium compound fluorescence on the analysis, CCM was used.

After flow cytometry analysis, the first step was to identify single cells using forward scatter (FSC) versus side scatter (SSC) gating to isolate the single cell population. Then, caspase positive, annexin V positive, non-viable, and mitochondrial damage events were identified (Figure 43).

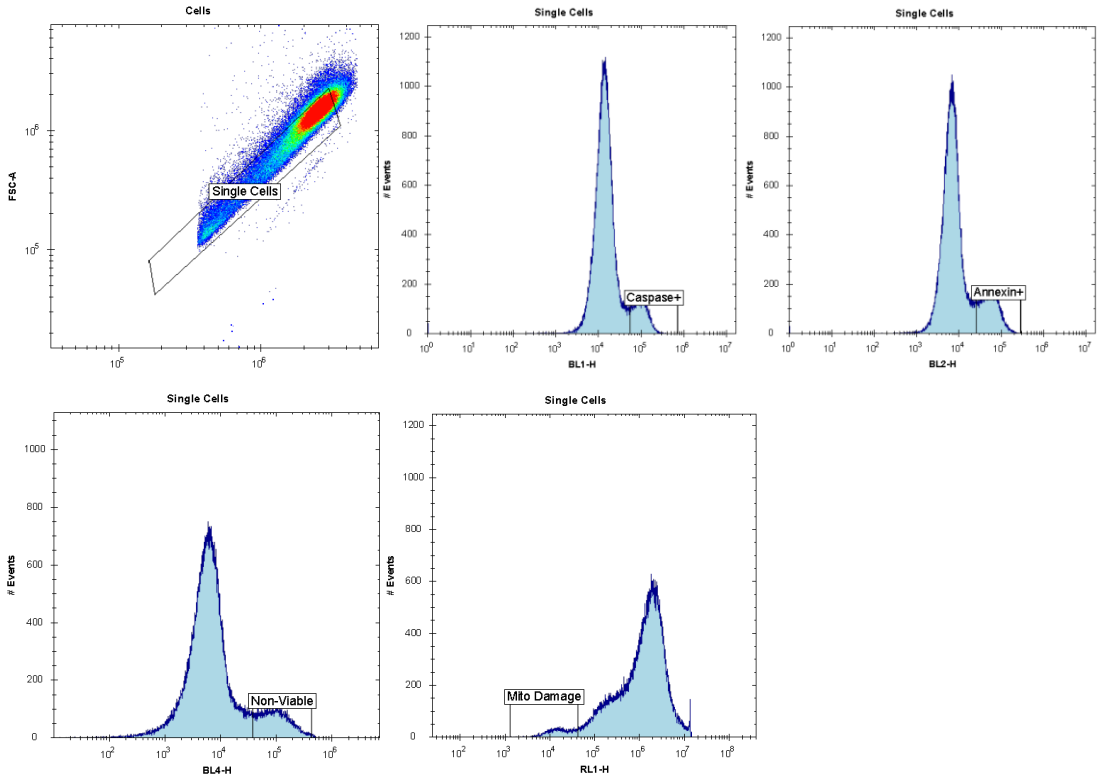


Figure 43: Identification of population by gating, from left to right, single cells, caspase positive, annexin V binding positive, non-viable cells and cells that have suffered mitochondrial damage.

No significant difference was observed between the positive and negative controls, nor between the low-performing and other batches. Various hypotheses may explain this outcome, including the possibility of an error in the experiment. The relatively low viability of the cells in expansion (90-95%), *i.e.*, the negative control, could have led to the release of PS and caspase. Lastly, SP2/0 cells are known for their sensitivity to shear-stress, and multiple centrifugation and vortex steps could have potentially caused significant damage to them.

Despite the inconclusive results, a substantial proportion of cells showed positive responses to caspase and Annexin V, both of which are markers of apoptosis. Consequently, the experiment was repeated using a different assay kit and a modified protocol that involved measuring caspase-3 activity through fluorometry.

Measurement of caspase-3 activity

Similar to the measurement of apoptosis markers by flow-cytometry, the role of apoptosis in cell viability drops observed subsequent to feed addition of low-performing batches was assessed by analysis of caspase-3 activity. Caspase-3 activity was measured at day 2, before feed addition, and 24h after feed addition for two lipoprotein supplement batches: the reference batch 7 (relative productivity 0%) and a low-performing batch, batch 11 (relative productivity -80%). Cell pellets were lysed, then a specific substrate from the caspase-3 assay kit (sigma Aldrich, MAK457) was added to the lysed suspension. The substrate was cleaved by caspase-3, forming a fluorescent product.

Fluorescence was measured using a SpectraMax i3 spectrophotometer UV-visible (Molecular Devices, CA, USA).

The average sample fluorescence intensity of every duplicate was calculated, then the sample fluorescence intensity value from the blank, working reagent, was subtracted to every values. For each condition, the relative caspase-3 activity before and after feed addition was calculated by comparison of the fluorescence intensities. The relative increase of caspase-3 activity following lipoprotein supplement feed addition is presented in Figure 44. In contrast to feed addition of the reference batch, which increased caspase-3 activity by +37%, feed addition of the low-performing batch led to a significant increase of +223% in caspase-3 activity. Only 24 hours after feed addition of the low-performing batch, caspase-3 activity within cells had notably risen, indicating the activation of the apoptosis pathway by certain compounds present in these batches, which were either absent or present in small amounts in the reference batch.

According to the literature, the triggering of apoptosis by lipoprotein and oxidized lipoprotein is sometimes caused by oxidative stress, lipid peroxidation, and other oxidation mechanisms (supplementary Table 21). Therefore, the effect of an antioxidant on the increase in caspase-3 activity caused by lipoprotein feed addition was evaluated. Trolox, a water-soluble analog of vitamin E known for its antioxidant properties (van den Berg, Haenen, van den Berg, & Bast, 1999), its prevention of lipid peroxidation (Lucio, et al., 2009), and its prevention of oxidative stress-induced apoptosis (Forrest, Kang, McClain, Robinson, & Ramakrishnan, 1994), was added to the culture at 25 mg/L. The effect of Trolox on the caspase-3 activity increase caused by lipoprotein feed addition is presented in Figure 44. Interestingly, the addition of Trolox at 25 mg/L to the culture reduced the effect of the low-performing batch feed addition on caspase-3 activity from +223% to +73%.

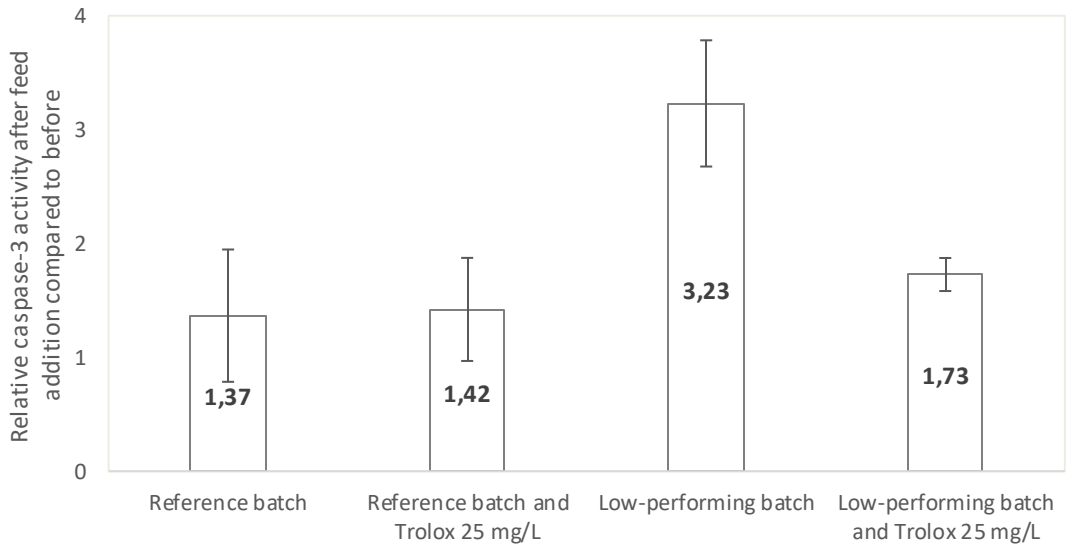


Figure 44: Effect of lipoprotein feed addition on caspase-3 activity in absence and presence of Trolox 25 mg/L. Relative caspase-3 activity was estimated by a comparison of the fluorescence intensity measured with a caspase-3 assay kit. Feed addition of the reference batch increased caspase-3

activity (+37%) by contrast with the low-performing batch (+223%). Mean ± standard deviation from three independent cultures measured in duplicate.

The addition of Trolox 25 mg/L to the culture did not show a statistically significant effect on the caspase-3 activity increase when the reference batch was used. However, in the presence of Trolox, the caspase-3 activity increase caused by the low-performing batch was significantly reduced from +223% to +73%. This difference proved to be statistically significant (p=0.0001). Hence, Trolox partially prevented the caspase-3 activity increase induced by the toxic compounds present in the lipoprotein supplement. These results indicate that low-performing batches contain toxic compounds that trigger apoptosis through a mechanism that can be partially inhibited by an antioxidant.

Interestingly, the addition of Trolox had no impact on the caspase-3 activity increase caused by the reference batch, suggesting that this increase is not caused by oxidative stress or lipid peroxidation.

With the aim of increasing the understanding of the relationship between compounds more present in low-performing batches, their effect on process performance, oxidative stress, caspase-3 activity, and viability drops, several compounds were spiked into the culture.

4.4.2.2. Prevention of cell death

To investigate the respective roles of oxidative stress and apoptosis in the premature viability decline caused by low-performing batches, the culture was supplemented with Trolox and a caspase-3 inhibitor.

Trolox

Early viability drops and an important increase in caspase-3 activity were associated with feed addition of low-performing batches compared to other batches. Because Trolox is known for its ability to prevent oxidative stress-induced apoptosis (Forrest, Kang, McClain, Robinson, & Ramakrishnan, 1994), its ability to mitigate adverse effects of low-performing batches was assessed. Two low-performing batches and two batches without performance issue (called reference batches) were utilized in this study. A comparison was made between conditions without Trolox and conditions with Trolox at 5 mg/L added in the medium and during lipoprotein feed addition, final Trolox concentration 10 mg/L. Additionally, for one reference batch, Trolox at 10 mg/L was added to the medium and during the feed, final Trolox concentration 20 mg/L. For the measurement of caspase-3 activity, cell lysates were obtained right before and 24 hours after lipoprotein feed addition.

As expected, the low-performing batches negatively impacted cell growth (Figures 45 and 46), however despite decreasing caspase-3 activity (Figure 47), the addition of Trolox did not significantly mitigate the adverse effects of these batches. Interestingly, a slight positive impact of Trolox on cell growth was observed for reference batches.

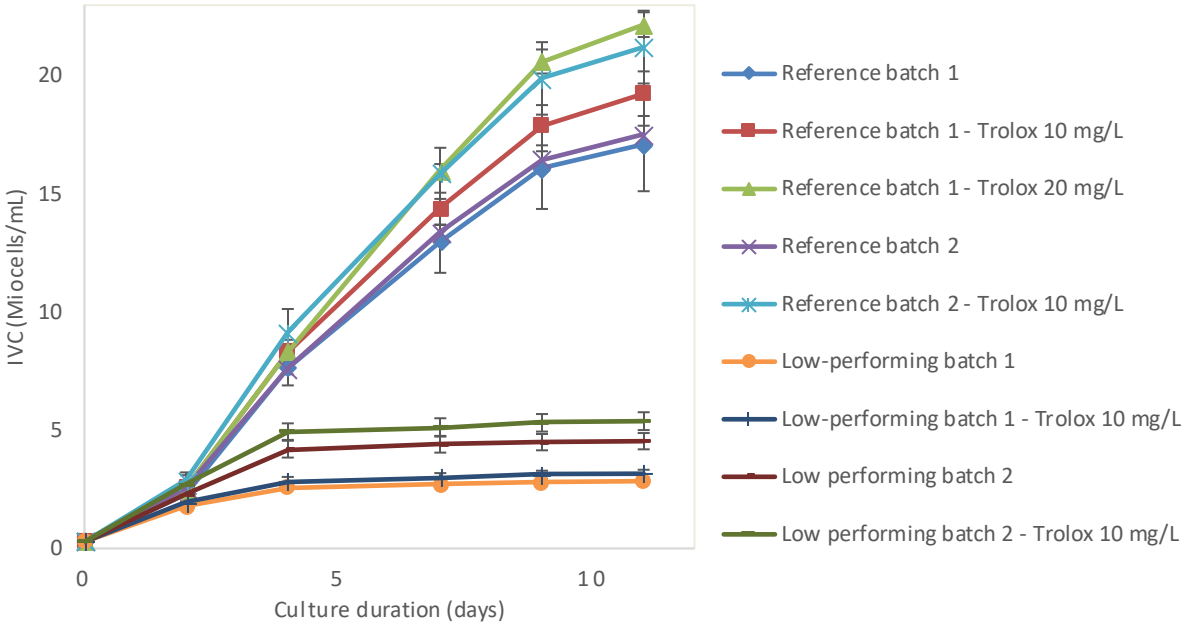


Figure 45: Effect of Trolox concentration and lipoprotein supplement batch variations on the evolution of IVC. Cell growth was definitively stopped 2-5 days after feed addition of low-performing batches, addition of Trolox had no effect on it. However, addition of Trolox to the reference batches had a positive impact on cell growth. Mean ± standard deviation from two independent cultures.

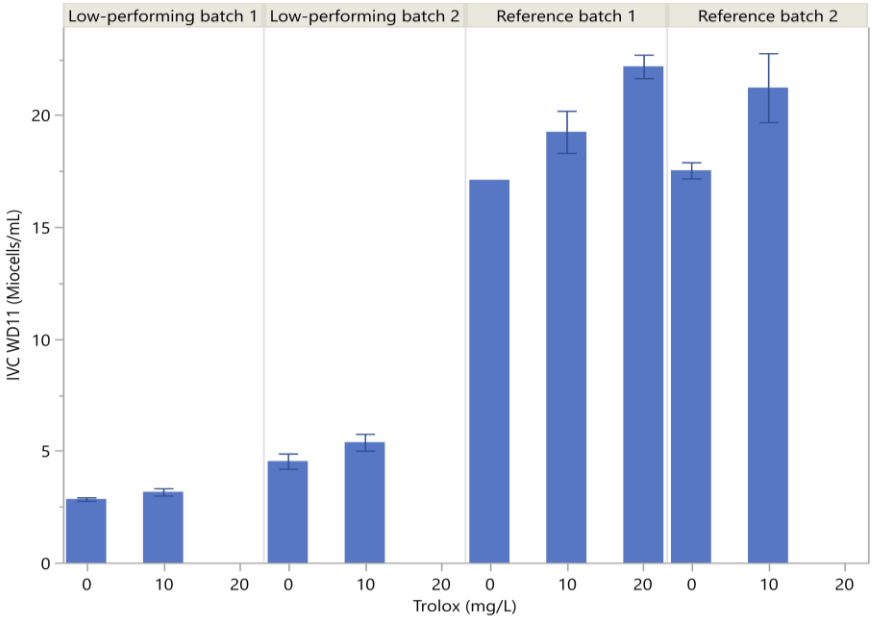


Figure 46: Effect of Trolox concentration and lipoprotein supplement batch variations on IVC at WD11. Cell growth was definitively stopped 2-5 days after feed addition of low-performing batches,

addition of Trolox had no effect on it. However, addition of Trolox to the reference batches had a positive impact on cell growth. Mean ± standard deviation from two independent cultures.

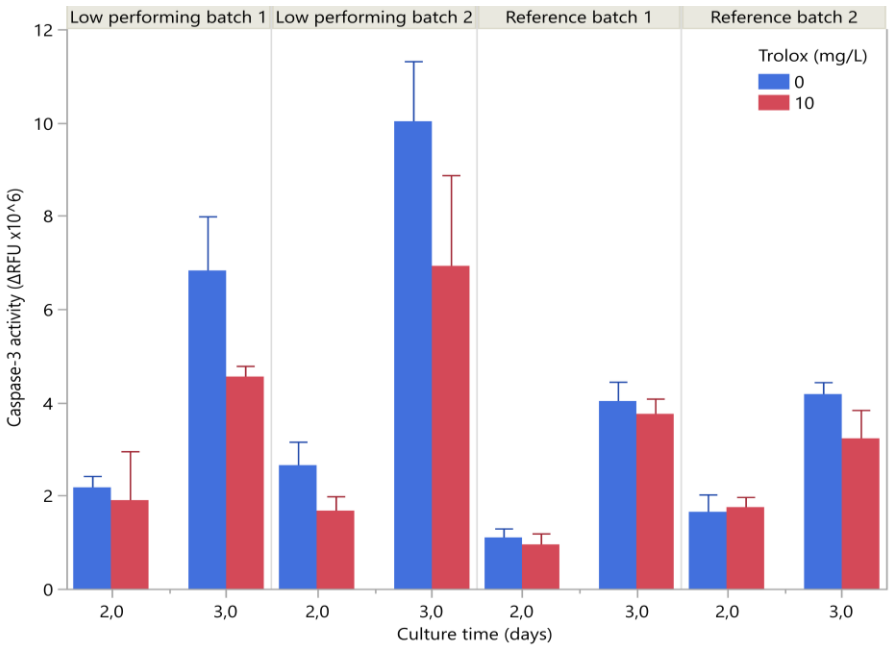


Figure 47: Effect of Trolox concentration on variations of caspase-3 activity during lipoprotein feed addition for several batches. Mean ± standard deviation of two measurements from two independent cultures.

Despite the reduction in caspase-3 activity, the addition of Trolox at 10 mg/L was unable to mitigate the adverse effects of low-performing batches on cell growth. This observation suggests a complex mechanism linking low-performing components to cell death, involving multiple pathways, some of which may not involve caspase-3 or may remain unaffected by the antioxidant properties of Trolox. Alternatively, it is possible that the metabolic cascade has progressed too far at the time of caspase-3 activation, making cell death inevitable despite the reduction in caspase-3 activity.

On the other hand, the addition of Trolox at 10-20 mg/L to the reference batch had a positive impact on cell growth. This finding confirms that reference batches, despite not being low-performing batches, negatively affect the culture due to their content of oxidative compounds, including oxysterols and lipid peroxidation products. The presence of these compounds was observed not only in low-performing batches but also in reference batches during the lipoprotein supplement characterization. This result indicates that cell culture process performance could be improved by the addition of an antioxidant. Furthermore, a decrease in caspase-3 activity in the presence of Trolox was observed for the reference batches, confirming the preventive effect of Trolox on apoptosis reported in the literature (Forrest, Kang, McClain, Robinson, & Ramakrishnan, 1994).

Because high caspase-3 activity and cell death were still observed after the addition of Trolox to the culture, the same experiment was repeated with a caspase-3 inhibitor.

Caspase-3 inhibitor

An increase in caspase-3 activity was observed when low-performing batches were utilized. To assess the effect of caspase-3 inhibition on the early viability drops caused by low-performing batches, a cell-permeable inhibitor of caspase-3 was employed. The peptide used as the inhibitor consists of a sequence that inhibits caspase-3 activity, coupled with a hydrophobic region that confers cell-permeability to the peptide.

The N-terminal sequence provides cell permeability, the 16 amino acids peptide sequence corresponds to the hydrophobic region of the signal peptide of Kaposi fibroblast growth factor and confers cell permeability to the peptide. The C-terminal sequence is a specific inhibitor of caspase-3 (K_i < 1 nM). The peptide sequence is Ac-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Asp-Glu-Val-Asp-CHO.

First, the range of caspase-3 inhibitor toxicity was evaluated. After two cell expansion passages, the production phase was inoculated with caspase-3 inhibitor concentrations in the medium ranging from 2.10^{-12} to 2.10^{-8} M. At the end of the production phase, the integral viable cell density (IVC) was compared to the best growing condition (Figure 48). As the caspase-3 inhibitor started decreasing cell growth above 2.10^{-11} M, the spiking study was conducted only on the concentration range from 10^{-12} to 10^{-10} M.

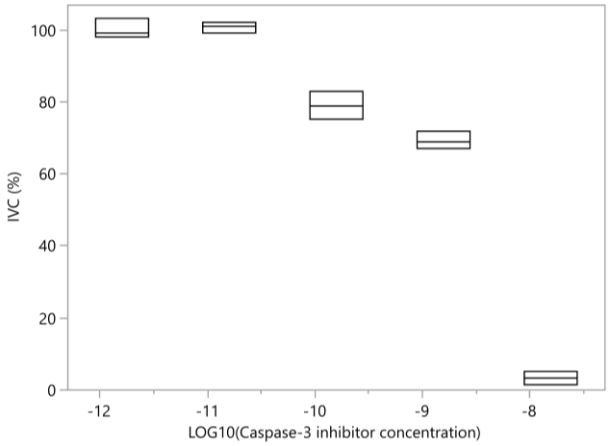


Figure 48: Effect of caspase-3 inhibitor concentration on cell growth. IVC was compared to the culture without caspase-3 inhibitor. From 2.10^{-12} to 2.10^{-11} M caspase-3 inhibitor had no impact on cell growth, above 2.10^{-11} M caspase-3 inhibitor starts to become growth limiting. Mean of three independent cultures.

The effect of addition of caspase-3 inhibitor, in a concentration range where it has no negative impact on cell growth, was evaluated on the reference batch (batch 7) and two low-performing batches. The VCD trends and the IVC at the end of the production phase are presented in Figures 49 and 50, respectively. Addition of caspase-3 inhibitor had no impact on the adverse effects of the two low-performing batches tested. Despite the addition of the caspase-3 inhibitor, viability started to drop after lipoprotein feed addition leading to a low IVC at the end of the culture. Interestingly, a slight positive impact of caspase-3 inhibitor on cell growth was observed when added to the reference batch.

These results are concordant with the cell growth enhancement reported after addition of apoptosis inhibitor to SP2/0 fed-batch culture (Wang, Liu, & Zhou, 2018).

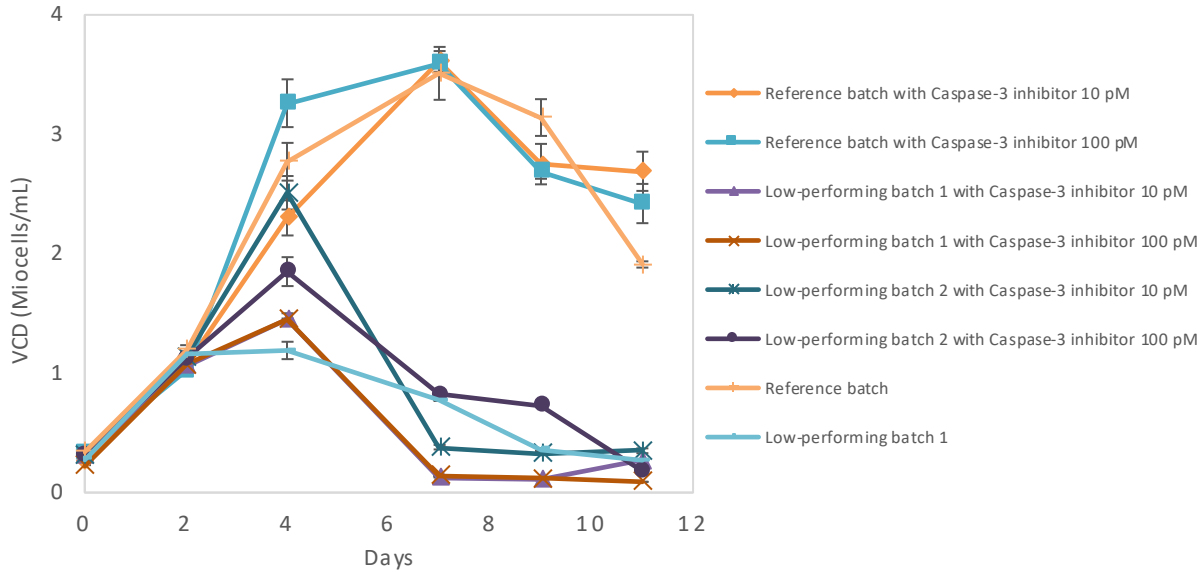


Figure 49: Effect of caspase-3 inhibitor concentration on the VCD for 3 lipoprotein supplement batches. Mean ± standard deviation from three independent cultures.

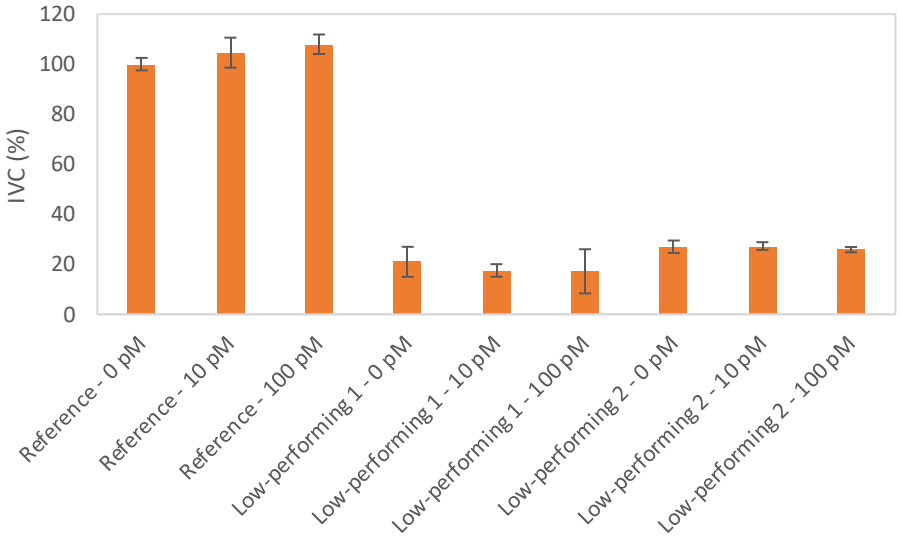


Figure 50: Effect of caspase-3 inhibitor concentration on the IVC at the end of the culture for 3 lipoprotein supplement batches. IVC are compared to the condition with the reference batch without addition of caspase-3 inhibitor. caspase-3 inhibitor did not mitigate the adverse effects of low-performing batches on cell growth. Mean ± standard deviation from three independent cultures.

Despite a slight positive impact on growth when the reference batch was used, the effects of the inhibition of caspase-3 activity were not observed when low-performing batches were used. Addition of caspase-3 inhibitor to the culture failed to prevent the adverse effects of low-performing batches on cell growth.

Regardless of the apoptosis pathway triggered, caspase-3 activation is central in the late stages of apoptosis. Indeed, caspase-3 activates and releases the endonuclease caspase activated DNAse 1 that initiates chromatin condensation, DNA degradation, fragmentation and finally cell death. Increasing caspase-3 activity was observed when low-performing batches were added to the culture, concomitant with the beginning of quick viability drops. However, addition of caspase-3 inhibitor to the culture did not attenuate the detrimental impact of low-performing batch feed addition on cell growth. caspase-3 activation being part of the last steps of apoptosis triggering, impact of disruptions caused at the earliest stages of the pathway could also have led to cell death despite caspase-3 inhibition.

Several hypotheses could explain why caspase-3 inhibition was not reducing viability drops and cell death. Mitochondrial damages caused by lipid peroxidation and ROS could have damaged the cell enough to end in death despite caspase-3 inhibition. Alternatively, incorporation of oxidized lipoprotein can cause disruption of lysosome and release of its content leading to cell death.

Combinations of Trolox and caspase-3 inhibitor were also tested and no impact on low performance batches was observed (data not shown).

Addition of Trolox partially inhibits the cell death mechanism highlighting the role of oxidative stress in the viability drops associated with low-performing batches. Consequently, the impact of the lipoprotein supplement oxidation on process performance was studied.

4.4.3. Impact of lipoprotein oxidation on process performance

Oxidation derivatives were detected in every lipoprotein supplement batches during characterization of its composition, these compounds are known for their adverse effects on cell growth and may negatively impact process performance. With the aim to investigate whether lipoprotein oxidation could cause the premature viability decline associated with low-performing batches, the impact of the lipoprotein supplement oxidation on batch performance was assessed. Controlled lipoprotein oxidation was conducted using $CuSO_4$ as a catalyst, this method is called Cu^{2+}-mediated oxidation (Gerry & Leake, 2008). Cu^{2+}-mediated oxidation of lipoprotein produces lipid hydroperoxide- or oxysterol-rich low-density lipoprotein depending on the oxidation temperature. The Cu^{2+}-mediated oxidation method was used because it allows the fatty acid production of oxidized lipoprotein that have a composition similar the lipoprotein oxidized in absence of a catalyst. The effects of oxidation duration (0 – 72h), oxidation temperature (4, 20 and 37°C) and $CuSO_4$ concentration (0 – 150 µM) on the lipoprotein supplement browning and cell growth were evaluated. For these experiments the reference batch (batch 7) was used. 30 oxidation conditions were tested. After oxidation, oxidized lipoprotein samples were used to prepare media and feeds, then cell culture experiments were conducted to evaluate the impact of oxidation on process performance.

4.4.3.1. Impact of oxidation on cell growth

First, lipoprotein supplement browning was observed during Cu^{2+}-mediated oxidation. Throughout oxidation the color of the solution has changed from yellow to orange and brown (Figure 51).

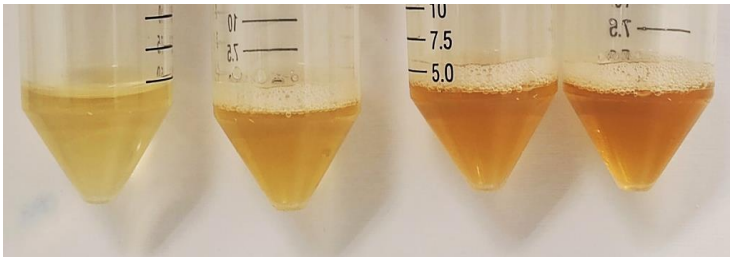


Figure 51: Lipoprotein supplement browning after 24h of Cu^{2+}-mediated oxidation at 37°C. The samples were oxidized with various $CuSO_4$ concentrations, from left to right 0, 50, 100 and 150 µM.

Lipoprotein supplement oxidation was correlated with a negative impact on cell growth and viability (Figure 52). Depending on the conditions of oxidation, and therefore the degree of oxidation, oxidized supplement either decreased cell growth but not viability, decreased cell growth and viability or rapidly decreased the viability of the cells. $CuSO_4$ itself was not causing decreased cell growth and viability drops, as controls with $CuSO_4$ addition in the culture (without pre incubation in the cell culture medium) did not show decreased performance (Figure 52: Reference batch - 8h at 37°C 0 µM $CuSO_4$ then addition of 150 µM $CuSO_4$ during inoculation).

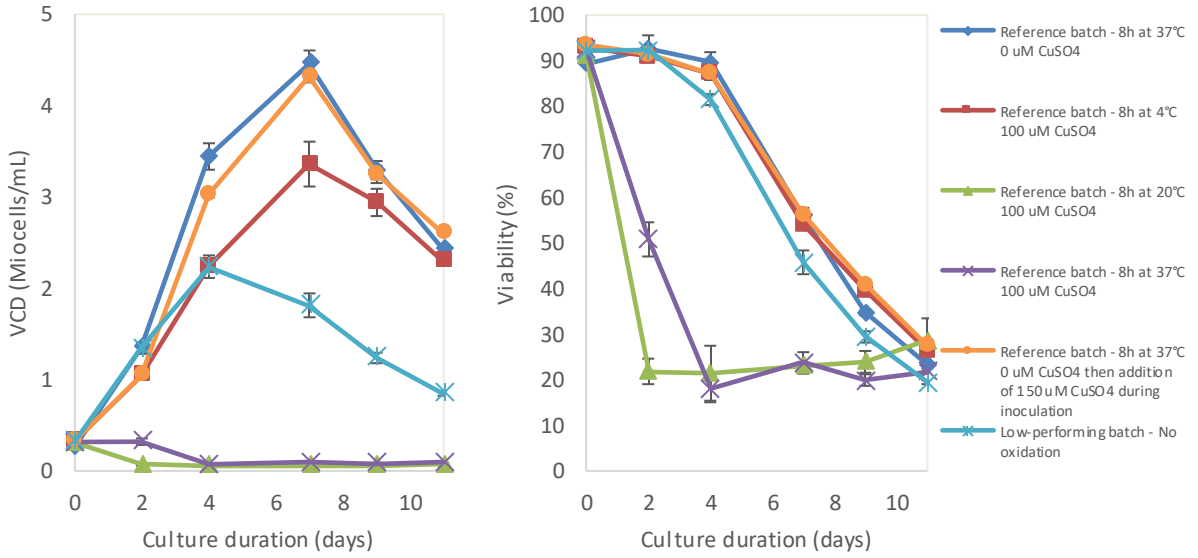


Figure 52: Effect of lipoprotein supplement oxidation on cell growth (left) and viability (right) during the production phase. Mean ± standard deviation from three independent cultures.

Supplement oxidation with more than 50 µM $CuSO_4$ at 20 or 37°C quickly decreased the viability of the culture every time. Oxidized supplements in these conditions were always worse than low-performing batches. Below 50 µM $CuSO_4$, the oxidation conditions were correlated to process performance (Figure 53 and Table 14).

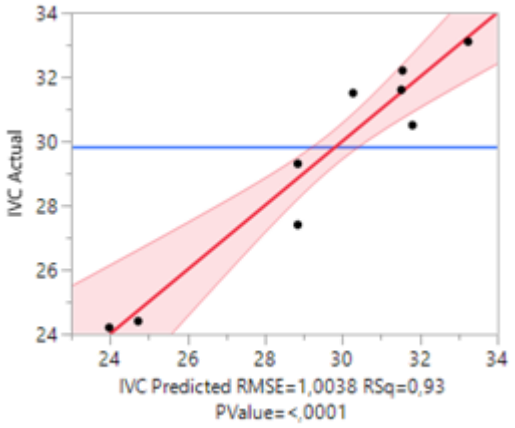


Figure 53: Comparison between the IVC measured experimentally and the IVC predicted by a surface-response model based on oxidation duration and CuSO4 concentration. Oxidation temperature : 37°C and CuSO4 concentration < 50 µM. Mean from three independent cultures.

Source	LogWorth	PValue
Oxidation duration (h)	5.445	0.00000
CuSO4 concentration (µM)	3.477	0.00033
CuSO4 concentration (µM)* CuSO4 concentration (µM)	1.885	0.01302
Oxidation duration (h)* CuSO4 concentration (µM)	0.426	0.37519
Oxidation duration (h)* Oxidation duration (h)	0.387	0.41041

Table 14: Effect summary of a surface-response model that predicts oxidized lipoprotein supplement IVC based on oxidation duration and CuSO4 concentration. Oxidation temperature : 37°C and $CuSO_4$ concentration < 50 µM.

The influence of divalent cations on the lipoprotein oxidation kinetic reported in the literature (Gerry & Leake, 2008; Ontko, 1970) was observed during our experiments. Indeed, the $CuSO_4$ concentration has a non negligeable impact on the lipoprotein supplement oxidation kinetic.

Nonetheless, we observed that the supplement oxidation also occurs in absence of a $CuSO_4$. Indeed, incubation at 37°C of the supplement without $CuSO_4$ for more than 24h led to a gradually decreasing process performance (Figure 54). Still addition of $CuSO_4$ 10 µM in the same conditions increased the kinetic of oxidation and so the negative impact on process performance represented here by the IVC.

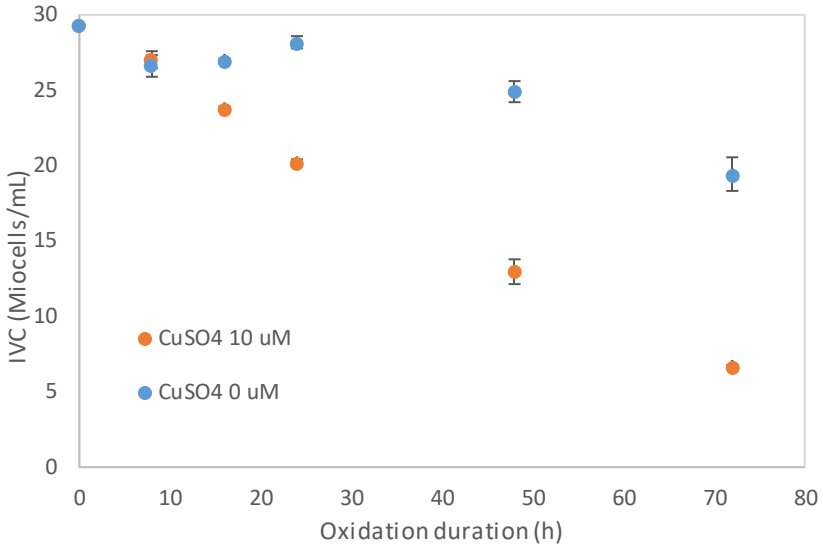


Figure 54: Effect of $CuSO_4$ concentration during lipoprotein supplement oxidation. Addition of $CuSO_4$ during lipoprotein supplement oxidation increased the kinetic of oxidation, the degree of oxidation and so the IVC at the end of the production phase. Note that lipoprotein supplement degradation occurred in absence of $CuSO_4$ leading to the gradual decrease in IVC. Mean ± standard deviation from three independent cultures.

Interestingly, oxidation of the lipoprotein supplement in absence of $CuSO_4$ for less than 24 hours had nearly no impact on the IVC whereas in presence of $CuSO_4$ 10 µM there is a linear correlation between decreasing IVC and oxidation duration starting from 0 hour. The presence of antioxidant in lipoprotein like α-tocopherol could explain this difference, they delay oxidation.

In absence of an oxidation catalyst like $CuSO_4$, the antioxidants may block oxidation, but once the antioxidants are consumed the oxidation kinetic returns to normal and we observe a linear correlation between oxidation duration and the negative impact on the culture. Thus, the antioxidants may delay the negative effect of oxidation on process performance. Whereas, in presence of an oxidation catalyst, here $CuSO_4$ 10 µM, the oxidation kinetic is more important, all the antioxidants are consumed nearly immediately. In this case, the oxidation duration and the negative impact on the culture are strongly correlated since the beginning of the oxidation.

The experiment was repeated with three US batches (Figure 55); after 24h of oxidation the decreasing IVC was correlated with oxidation duration. However, only two of the three batches were not impacted by oxidation during the first hours of oxidation. The ability of the antioxidants to delay oxidation could be dependent on the lipoprotein supplement batch. On the other hand, oxidation of three low-performing batches showed no impact on cell growth (Figure 56). The toxic compounds that negatively affect the culture appear to be already present in the supplement prior to oxidation, moreover the oxidation seems to have no impact on these batches.

Both Cu^{2+}-mediated oxidation and Cu-free oxidation were applied to study lipoprotein supplement oxidation. Regardless of the oxidation method employed, the greater the extent to which the

lipoprotein supplement undergoes oxidation, the more pronounced its negative impact on cell growth and process performance becomes.

Lipoprotein oxidation is affected by the presence of a catalyst, such as $CuSO_4$, oxidation duration and the temperature of oxidation. While $CuSO_4$ serves as a catalyst that modulates the kinetics of oxidation, the oxidation temperature exerts an influence on the composition of the oxidized lipoproteins produced. Indeed, formation of hydroperoxide-rich oxidized lipoprotein was reported during Cu^{2+}-mediated oxidation at 4°C, whereas oxysterol-rich oxidized lipoproteins were formed at 37°C (Gerry & Leake, 2008).

Considering that both bovine serum and the lipoprotein supplement are stored at 4°C, oxidation would likely produce hydroperoxide-rich oxidized lipoprotein. The hydroperoxides could decompose upon heating of the CCM prior to the culture. Lipid hydroperoxides analysis has not been conducted, thereby preventing us from definitively affirming or refuting this hypothesis. Regarding the oxidation kinetics, in absence of a catalyst we observed a time lag of a few hours before the beginning of the detrimental impacts of lipoprotein oxidation on process performance. Lipoprotein antioxidants, most likely α-tocopherol, could contribute to this delay in oxidation. However, not all batches exhibited uniform behavior in terms of their capacity to delay oxidation, in some batches the antioxidant reservoirs may be partially depleted. Although partially protected from oxidation by their antioxidants, controlled oxidation of the lipoprotein supplement confirmed its susceptibility to oxidation resulting in a decline in process performance. Oxidized batches behave like low-performing batches, but it is not sufficient to confirm that oxidation is the root cause of low-performing batches given they do not contain more oxysterols, MDA and 4-HNE than other batches.

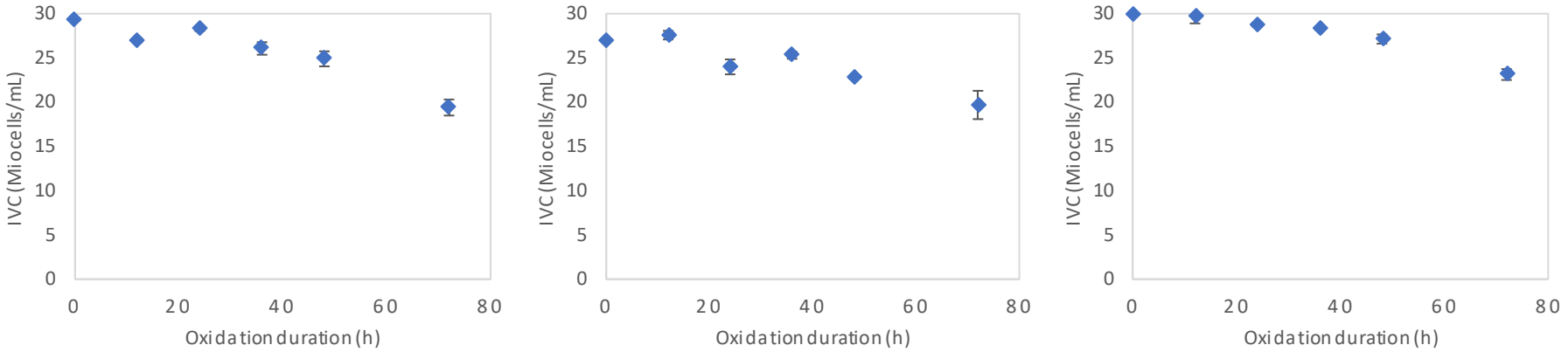


Figure 55: Effect of oxidation duration on 3 US batches in absence of $CuSO_4$. Mean ± standard deviation from three independent cultures.

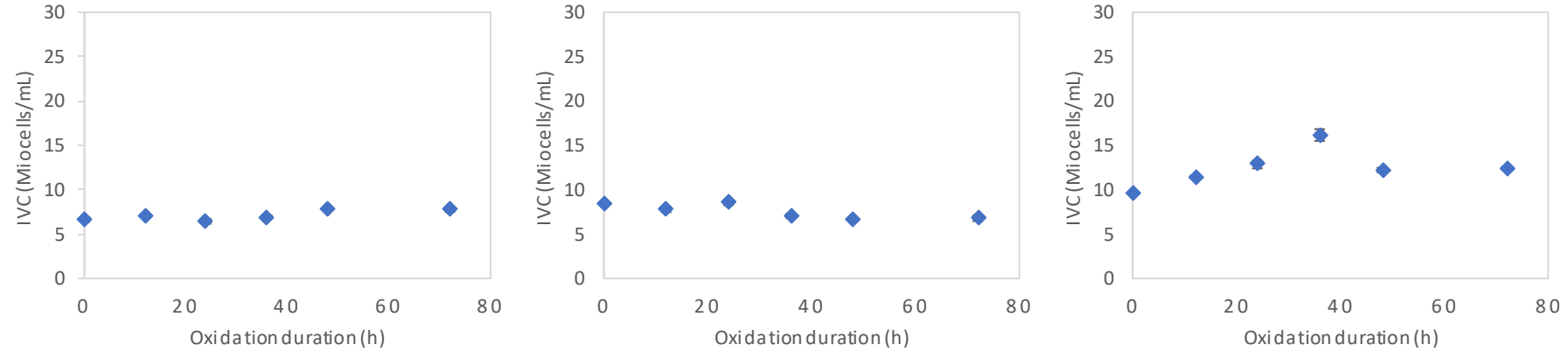


Figure 56: Effect of oxidation duration on 3 low-performing batches in absence of $CuSO_4$. Mean ± standard deviation from three independent cultures.

4.4.4. Monitoring of the lipoprotein supplement oxidation by UV spectroscopy

Because lipoprotein supplement oxidation was associated with solution browning and the absorbance in the UV-region was identified as a candidate fingerprinting method to detect low-performing batches (refer to section 4.3.3.2), UV spectroscopy was evaluated with the aim to monitor oxidation and potentially detect oxidized batches.

First, the reference batch (batch 7) was stored from 0 to 72 hours at 37°C in absence of $CuSO_4$ while absorbance was measured in the UV-region (Figure 57). The intensity of the two dominating peaks at about 230 and 275 nm was correlated with the resulting extent of oxidation. These peaks are potential markers of the degree of oxidation. In addition to the increasing absorbance a small drift of the peak at around 275 nm was observed from 277 nm at the beginning to 274 nm after 72 hours of incubation. Both peaks centered at about 230 and 275 nm have been identified as markers of oxidation, however, the peak at 230 nm is truncated, as a result the peak centered at about 275 nm was used to monitor oxidation.

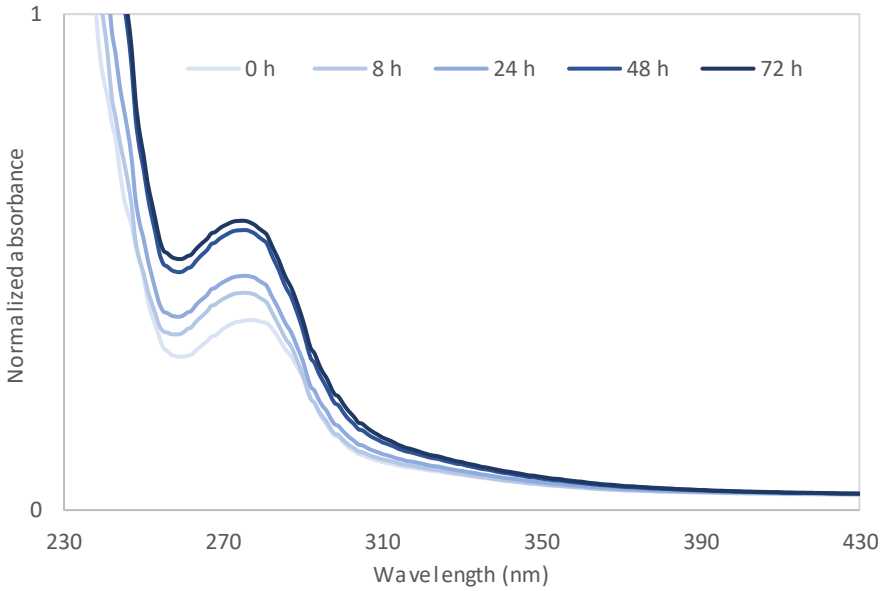


Figure 57: Evolution of UV absorbance of the 100X diluted batch 7 during incubation at 37°C in absence of $CuSO_4$. Step between each measurement: 1 nm. Mean of three independent measurements.

In absence of $CuSO_4$ the absorbance at 276 nm (noted Abs 276) was correlated to the oxidation duration and so the degree of oxidation (Figure 58). During Cu^{2+}-mediated oxidation, Abs 276 was also correlated to the degree of oxidation. Indeed, a response-surface model considering incubation duration and $CuSO_4$ concentration was able to explain 74% of the absorbance at 276 nm variations observed during lipoprotein supplement oxidation (Figure 59). Consequently, Abs 276 nm measurement is an accurate tool to monitor lipoprotein supplement oxidation.

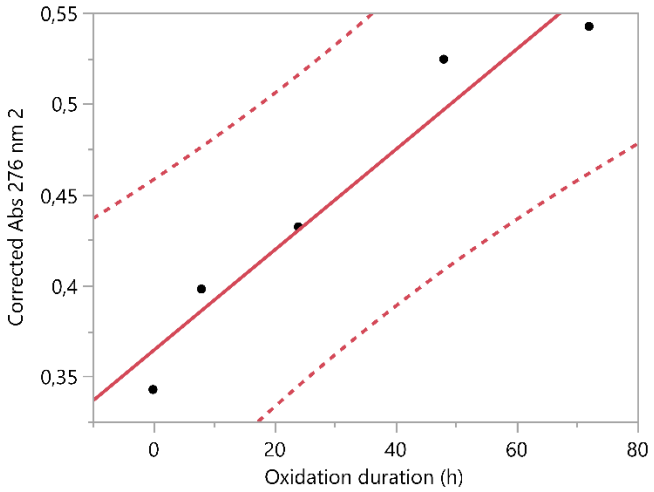


Figure 58: Fit of the corrected absorbance at 276 nm of oxidized lipoprotein supplement by the oxidation duration at 37°C in absence of $CuSO_4$. Corrected absorbance was calculated by subtraction of the blank absorbance to the sample at 276 nm. Confidence curve α = 0.05, p = 0.0068 and R^2 = 0.94. Mean of two independent measurements.

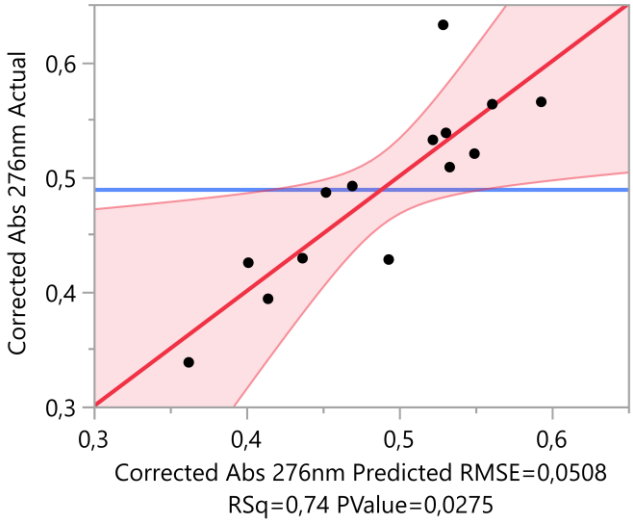


Figure 59: Comparison of the corrected absorbance at 276 nm with the predicted by an OPLS of oxidation duration (h) and $CuSO_4$ (µM). Confidence curve α = 0.05 and R^2 = 0.74. Mean of two independent measurements.

In light of the correlation observed between Abs 276 nm and the degree of oxidation of the lipoprotein supplement, as well as between the degree of oxidation and process performance, we plotted the IVC against Abs 276 nm over the course of oxidation (Figure 60). Because oxidation had no impact on low-performing batches, they were not used in this study, only 2 US batches and 1 NZ batch without performance issue were tested. As expected, the impact of oxidized lipoprotein supplement batch was correlated with its absorbance at 276 nm ($R > 0.70$). Consequently, Abs 276 emerges as a fingerprinting tool able to predict the performance of a given lipoprotein supplement batch, especially if it has been oxidized.

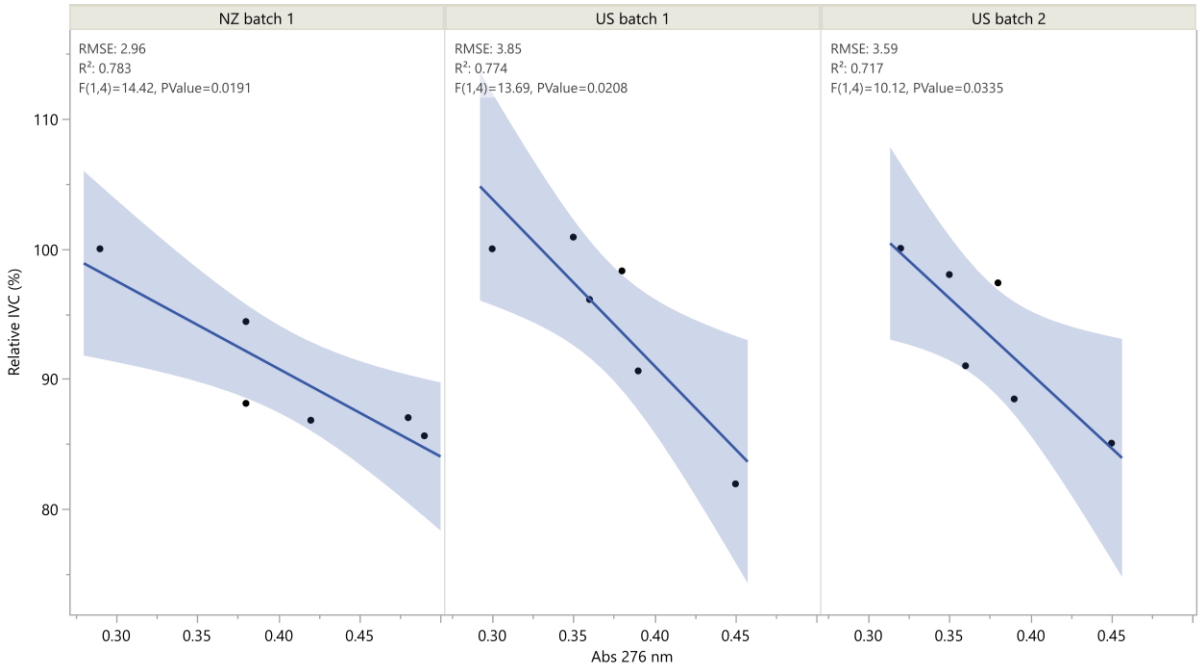


Figure 60: Correlation between increasing Abs 276 nm and decreasing IVC during oxidation of 3 lipoprotein supplement batches. Mean of three independent cultures and two absorbance measurements at 276 nm.

Absorbance measurement at 276 nm proved to be an effective method to monitor the oxidation of the lipoprotein supplement, exhibiting a direct correlation with the performance outcomes of a given batch. The compounds generated during oxidation, which absorb in the 276 nm region, are indicative of the degree of oxidation and the process performance of a given batch. The presence of these compounds derived from lipoprotein oxidation in substantial quantities aligns with viability drops, reduced growth, characteristics of low-performing batches. These results are concordant with the higher absorbance of the low-performing batches at 276 nm region observed in section 4.3.4.2.

Absorbance measurement at 276 nm has proven effective in monitoring the oxidation of the lipoprotein supplement. Moreover, the increasing Abs 276 nm during oxidation was correlated with decreasing process performance, demonstrating the ability of the absorbance measurement to identify oxidized batches that detrimentally impact cell growth. Additionally, an analysis of 33 lipoprotein supplement batches revealed that low-performing batches exhibited statistically higher absorption levels compared to the other batches. These findings establish Abs 276 nm as a robust fingerprinting method able to detect both oxidized and low-performing batches.

While this method might not provide a definitive solution to the challenge of low-performing batches, its implementation on incoming lipoprotein supplement batches could prevent their utilization in commercial manufacturing, thus increasing process robustness.

4.4.5. Discussion

An investigation was conducted to identify the root cause of the low-performing lipoprotein supplement batches. The aim of this investigation was to either confirm the exclusive presence of a contaminant in the low-performing batches, or to identify the growth-limiting compounds present in higher concentrations in the low-performing batches compared to the others. Regarding the nature of the contaminant we searched for a chemical compound, as the presence of a microbiological contaminant, mycoplasma or viral agent was excluded based on supplier testing.

Lipoprotein supplement feed addition increased caspase-3 activity irrespective of the batch used. However, caspase-3 activity was significantly higher after feed addition of low-performing batches compared to other batches. This observation confirms that the toxic compound present in low-performing batches and causing important viability drops could also be present in other batches but in a lower amount. Since caspase-3 is an executioner caspase, activated in the late stages of apoptosis, these observations strongly suggest that a controlled cell death mechanism, likely apoptosis, is triggered following feed addition of low-performing batches.

Oxidized lipoproteins induce typical morphological changes, DNA fragmentation and increase of caspase activity, all characteristic of apoptosis (Bjorkerud & Bjorkerud, 1996; Bjorkerud & Bjorkerud, 1996; Harada-Shiba, Kinoshita, Kamido, & Shimokado, 1998; Escargueil-Blanc, Salvayre, & Nègre-Salvayre, 1994; Escargueil-Blanc, et al., 1997). The ability of oxidized lipoprotein to exert oxidative-stress and induce cell death *in vivo* was also observed during mammalian cell culture (supplementary Table 21). Based on these observations oxidized lipoproteins were suspected to contain the toxic compounds causing the caspase-3 activity increase and viability drops observed after feed addition of low-performing batches. Oxidative stress-induced apoptosis being prevented by Trolox (Forrest, Kang, McClain, Robinson, & Ramakrishnan, 1994), the influence of Trolox on caspase-3 and cell growth activity following feed addition were evaluated. Cell growth stimulation by Trolox reduces oxidative stress and thereby could improve process performance (Chevallier, Andersen, & Malphettes, 2020). Regarding low-performing batches, Trolox 10 mg/L significantly decreased caspase-3 activity but with only a negligible impact on viability drops. Thus, Trolox did not mitigate the adverse effect of low-performing batches on cell growth.

Our results seem contradictory, increasing caspase-3 activity and viability drops were observed following feed addition of low-performing batches, however addition of Trolox mitigated the increasing caspase-3 activity still the cells were dying. The cell death mechanism activated following feed addition of low-performing batches may not be quantitatively correlated with caspase-3 activity, alternatively multiple pathways could be activated with at least one not including caspase-3 or not affected by the antioxidant properties of Trolox. Indeed, caspase-3 is an executioner caspase activated in the late stages of the apoptosis pathway and events that occurred earlier may end in cell death independently of caspase-3 activation. For example, in the mitochondrial apoptosis pathway,

mitochondrial damages lead to the translocation of the apoptosis-inducing factor ending in DNA fragmentation and apoptosis independently from caspase (Joza, et al., 2009). Alternatively, oxidized lipoproteins were reported to trigger endopepsis, a cell death mechanism where a lysosome is disrupted leading to the release of its content ending with caspase activation and apoptosis (Willis, Klassen, Carlson, Brouse, & Thiele, 2004). In this case release of the lysosome content may lead to cell death without caspase-3 activation. Lastly, MCF-7 human breast cancer cells were reported to undergo apoptosis through caspase activation despite not expressing caspase-3 (Liang, Yan, & Schor, 2001). Overall identification and characterization of cell death mechanisms was challenging because SP2/0 viability barely exceeded 90%, a small proportion of the cells are constantly dying. Moreover, in fed-batch cells start to die after few days depending on the growth kinetic.

Knowing the role of oxidative stress in the viability drops associated with low-performing batches, the impact of lipoprotein supplement oxidation on process performance was studied. Browning of the solution confirmed that the supplement is altered during oxidation. Oxidized supplement impacted cell viability and hindered cell growth like low-performing batches do. On the other hand, oxidation of low-performing batches did not modify their effect on cell growth; the degree of oxidation of these batches might be too high to further increase their detrimental effect on cell growth through additional oxidation. The presence of antioxidant in lipoprotein, most probably α-tocopherol, prevented oxidation at the beginning of supplement incubation at 37°C but had no effect during Cu^{2+}-mediated oxidation. It is tempting to speculate that after consumption of all the antioxidants, oxidation starts to produce lipid oxidation derivatives that will negatively affect process performance.

Because lipoprotein supplement oxidation was associated with solution browning, UV spectra were acquired throughout oxidation. Oxidation increased supplement absorbance from 230 to 350 nm, notably at about 230 and 276 nm. Absorbance at 276 nm was strongly correlated with oxidation duration, thus oxidation derivatives formed during lipoprotein oxidation absorb at 276 nm. Abs 276 nm was correlated with batch performance during supplement oxidation. The increasing concentration of oxidation derivatives formed during supplemented oxidation, measured thanks to their absorbance at 276 nm, is strongly correlated with the decreasing cell growth observed during oxidation. Thus, the oxidation derivatives that absorb at 276 nm are likely causing the process performance decrease associated with lipoprotein supplement oxidation.

Interestingly, comparison of the UV-spectra of the supplement during oxidation and the regions where low-performing batches absorb more than other NZ batches (Figure 61) shows clear similarities. According to the spectroscopic analysis, low-performing batches are more oxidized than other batches, however it was not confirmed upon characterization of the lipoprotein supplement composition. Low-performing batches do not contain more oxidized cholesterol derivatives, lipid peroxidation by-products (4-HNE and MDA) nor higher protein carbonylation levels. Interestingly, NZ batches contain more α-linolenic acid (274 ± 122 ug/mL) than US batches (25 ± 9 ug/mL). α-linolenic acid is known to exert cytotoxic effects on SP2/0 cells by generating free radicals and lipid peroxides during lipid

peroxidation (Kumar & Das, 1995). Given the fatty acid composition of NZ batches, it becomes more likely to induce cell death in SP2/0 cells through oxidation compared to US batches.

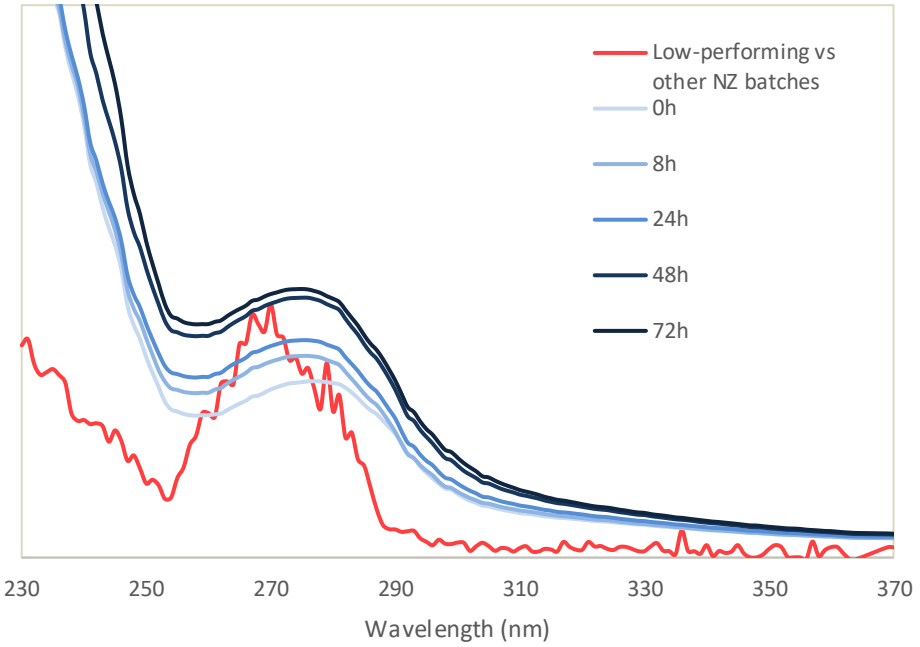


Figure 61: Overlay of the increasing absorbance of the lipoprotein supplement during oxidation (blue curves) and the regions where low-performing batches absorb more than other NZ batches (red curve). Low-performing batches absorb more in the UV region where absorbance increases during lipoprotein supplement oxidation.

Analysis of the Abs 276 nm of 33 lipoprotein supplement batches confirmed that low-performing batches absorb significatively more at 276 nm. Based on the distribution of the Abs 276 nm a limit that separate low-performing batches from other batches using Abs 276 nm was established. If 100 µL of a lipoprotein supplement batch diluted 100X in purified water absorb more than 0.55 at 276 nm after measurement in Corning® 96-well UV-transparent microplate, then it is possible to state with 95% of confidence that it is a low-performing batch. Notably, the supplement bottle must be opened right before the analysis. We observed that the Abs 276 nm value varies between analysis if the same bottle is used several times. Replacement of the inert atmosphere originally contained in the bottle by atmospheric air probably initiates oxidation which will increase Abs 276 nm. Because oxidized and low-performing batches are challenging to detect otherwise than with cell culture experiments, the implementation of a fingerprinting method would contribute to increase the robustness of the processes that involve lipoprotein supplement.

During this investigation, we acquired a more comprehensive understanding of the root cause of low-performing batches. First, the manufacturing process can be excluded as root cause, indeed the compounds causing viability drops were already present in the bovine serum batches used to manufacture low-performing batches. Low-performing batches induce cell death through multiple pathways, with at least one involving oxidative stress and caspase-3 activation. Reduction of the

oxidative stress by supplementation of the culture with Trolox partially mitigated caspase-3 activation and had a positive impact on cell growth, especially on batches without performance issue. However, addition of Trolox and/or of a caspase-3 inhibitor did not prevent cell death.

The lipoprotein supplement oxidation was correlated with decreasing process performance and increasing absorbance at 276 nm. Moreover, oxidized and low-performing batches share similar UV absorbance spectra. Notably, the Abs 276 nm was used to monitor precisely supplement oxidation and detect low-performing batches.

Because the studied lipoprotein supplement is a variable raw-material that affect process robustness, and the root cause of low-performing batches is still not fully understood the substitution of the supplement by an alternative lipid supplement was evaluated in the next section.

4.5. Substitution of the lipoprotein supplement by an alternative lipid supplement

Knowing challenges associated with the use of lipoprotein supplement in SP2/0 cell culture, its replacement by a chemically-defined alternative would present several advantages, the most important being an increase of process robustness. For this purpose, several water soluble-lipid supplements, based on the mbCD inclusion complex technology, were formulated. In parallel, lipid supplements for mammalian cell culture commercially available were evaluated. These potential lipoprotein supplement substitutes and their composition are presented in section 4.5.1. Screening of substitutes was conducted during cell expansion; then the most promising were used for production step, the results are presented in section 4.5.2.

4.5.1. Chemically defined lipid formulations

This section introduces the lipid sources evaluated to substitute for the lipoprotein supplement: commercially available lipid supplement and custom lipid formulations.

4.5.1.1. Commercial lipid supplements

Different lipid supplements commercially available were evaluated to substitute for the lipoprotein supplement, their compositions are presented in Table 16. All the commercial lipid supplements tested are animal component free (ACF), with the exception of the Chemically Defined Lipid Mixture 1 (CDLM) that contains cholesterol from New Zealand sheep's wool.

4.5.1.2. Custom lipid formulations

Besides commercially available products, custom lipid formulations were prepared with the aim to substitute for the lipoprotein supplement. A total of 4 alternative lipid supplements (ALS) were formulated, these four formulations are referred in the following as ALS1 to ALS4. Their compositions are presented in Table 16 and their preparation protocol in section 3.2.3.2. The ALS formulations are chemically-defined, and ACF. ALS1 and ALS2 compositions mimicked the lipoprotein supplement

composition, then these 2 initial ALS formulations were modified step-by-step in order to match at its best lipid requirements of SP2/0 cells.

ALS1/2: Reproduce the lipoprotein supplement composition

Characterization of the supplement composition has revealed that it contains mostly proteins, sterols and fatty acids. Main proteins, sterols and fatty acids of the supplement are presented in Table 15.

Two protein-free chemically defined lipid formulations were made based on the simplified lipoprotein supplement composition ALS1 and ALS2. Apolipoprotein were removed because their role of lipid carrier was replaced by mbCD, Histidine-rich glycoprotein and Complement component 3 are irrelevant to lipid transport. Lastly despite being present in the lipoprotein supplement and important for lipid transport, BSA was not added to the formulations. Indeed, the basal medium provides 100 times more BSA to the CCM than the supplement, thus BSA provided by the supplement is negligible for the process. Regarding sterols, only cholesterol and cholestenone were added to the formulations, all oxysterols were removed due to their potential detrimental impact on cell growth. Lastly, the 5 main fatty acids were added to the formulation.

Protein (g/L)	Sterol (g/L)	Fatty acids (g/L)
Apolipoprotein A-I	Cholesterol	Linoleic acid
7.75 ± 0.97	6.65 ± 1.43	1.00 ± 0.50
Apolipoprotein B	Cholestenone	Stearic acid
3.35 ± 0.76	2.23 ± 1.67	0.96 ± 0.14
Histidine-rich glycoprotein	7-ketocholesterol	Oleic acid
0.71 ± 0.18	1.24 ± 0.56	0.55 ± 0.15
Complement component 3	5,6-beta-epoxycholesterol	Palmitic acid
0.51 ± 0.13	0.95 ± 0.43	0.51 ± 0.08
Bovine Serum Albumin	7-beta-hydroxycholesterol	α-linolenic acid
0.30 ± 0.32	0.78 ± 0.52	0.16 ± 0.15

Table 15: Main proteins, sterols and fatty acids present in the lipoprotein supplement. Mean ± standard deviation (n protein = 31), (n sterol = 12) and (n fatty acid = 33).

ALS1 and 2 share a common lipid composition (Table 16) but they differ by their physical forms, ALS1 is a powder formulation whereas ALS2 is a liquid formulation. Consequently, ALS1 contains more mbCD (+15%) than ALS2 which rater contains ethanol. The mbCD:lipid ratios of the custom formulations are kept confidential.

ALS3: Split sterol and fatty acid sources to better match SP2/0 lipid requirements

Even if in the lipoprotein fatty acids and sterols are provided together to the cells, with a constant balance sterol:fatty acid, another approach was used to improve lipid supplementation. By contrast with ALS1 and ALS2, ALS3 and ALS4 contain only fatty acids, sterols were removed from the formulations. By combining two lipid sources, ALS3 or 4 for fatty acid supplementation and a cholesterol source, it becomes possible to adjust the sterol:fatty acid balance according to cell requirements and to not provide lipids to the culture as the lipoprotein supplement does.

Origin	Lipid Source	Physical state	Linoleic acid (mg/L)	Stearic acid (mg/L)	Oleic acid (mg/L)	Palmitic acid (mg/L)	α-linolenic acid (g/L)	Cholesterol (mg/L)	Cholestenone (mg/L)	Solubilizing agent
Custom formulation	**ALS1 (1X)**	Powder	5.0	4.8	2.8	2.6	0.8	33	11	mbCD
	ALS2 (1X)	Liquid	5.1	4.8	2.8	2.6	0.8	33	11	mbCD and EtOH
	ALS3 (1X)	Powder	5.1	5.1	2.6	2.5	1.0	-	-	mbCD
	ALS4 (1X)	Powder	5.1	5.1	2.6	2.5	-	-	-	mbCD
Commercial lipid supplement	**SyntheChol® (1X)**	Liquid	-	-	-	-	-	7*	-	At least mbCD
	CDLM 1:100 (v/v)	Liquid	10	10	10	10	10	220	-	Tween-80 and Pluronic F-68
	CLC	Liquid	?	?	?	?	?	yes	-	mbCD**
	Cholesterol-Water soluble	Powder	-	-	-	-	-	yes	-	mbCD 24 g per g of cholesterol
	Linoleic acid-Water soluble	Powder	yes	-	-	-	-	-	-	mbCD 32 g per g of linoleic acid
	Oleic acid-Water soluble	Powder	-	-	yes	-	-	-	-	mbCD 32 g per g of oleic acid

Table 16: Chemical composition of lipid source evaluated to replace the lipoprotein supplement. ALS1, ALS3 and ALS4 are powder, their constituent concentrations are given after a right dissolution to obtain a solution concentrated 1X. Note that ALS2 and SyntheChol® solutions were originally concentrated 200X and 250X respectively. CDLM contains also arachidonic acid 2.0 mg/L, myristic acid 10 mg/L and tocopherol acetate 70 mg/L. CLC contains fatty acids and solubilizing agent(s) but its exact composition is unknown.

SyntheChol® and CLC are confidential proprietary formulations, however information about their compositions are available in the literature:

* Cholesterol concentration in SyntheChol® 250X was titrated (Wu, et al., 2011).

** The presence of mbCD in CLC was affirmed but not demonstrated (Zhang & Robinson, 2005).

4.5.2. Lipoprotein-free cell culture

4.5.2.1. Substitution of the lipoprotein supplement during the expansion phase

With the aim to find a robust substitute for the lipoprotein supplement, several lipid sources were screened during cell expansion in lipoprotein-free CCM. Their impacts on cell growth are presented in Figures 62 to 65. Note that lipoprotein-free CCM is able to support cell growth during cell expansion (section 4.1.1).

Commercial lipid supplement

The effect of SyntheChol® (Sigma Aldrich, S5442), CDLM (Sigma Aldrich, L0288) and Cholesterol Lipid Concentrate (CLC) (Thermo-Fisher Scientific, 12531018) on growth during cell expansion was investigated (Figure 62). SyntheChol® addition to the culture demonstrated that cholesterol supplementation slightly enhance growth during cell expansion (PDL +8% after 2 weeks).

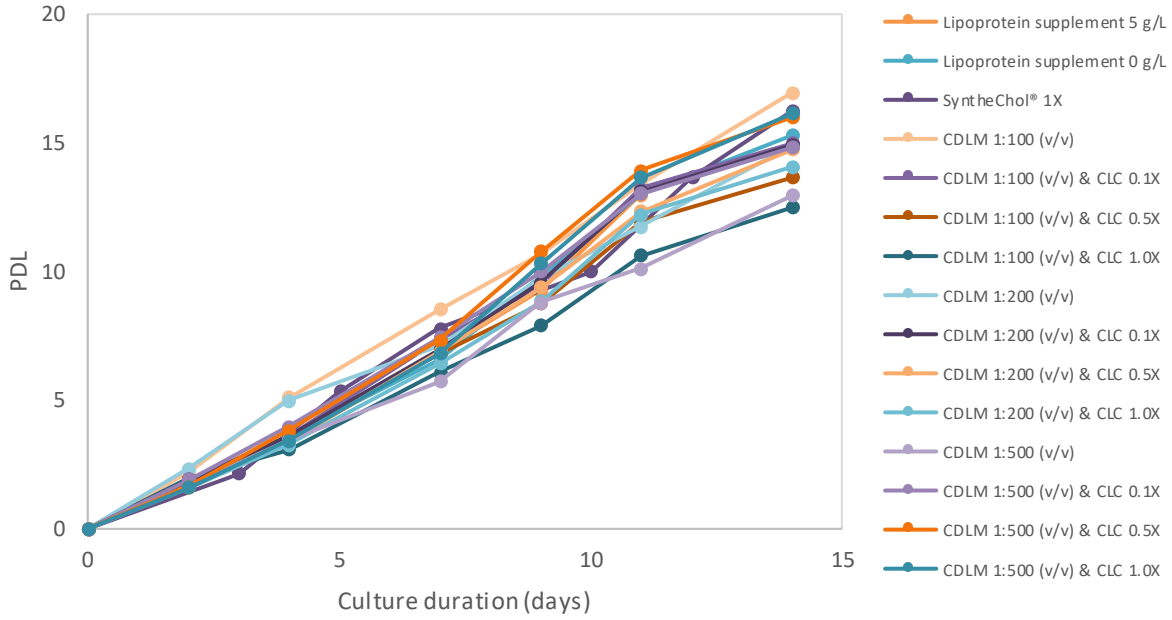


Figure 62: Effect of the lipoprotein supplement substitution by commercially available lipid supplement on the PDL during cell expansion. Mean from two independent cultures.

Regarding the combination of complex commercial lipid supplements, CDLM and CLC, the highest growth was obtained with a combination of CDLM 0.0 – 0.4% (v/v) and CLC 0.2 – 1.0X (Figure 63). Beyond the optimal concentration range, the lipid supplements became growth limiting. These results confirm the growth-promoting effect of lipid supplementation in the culture, moreover it highlights the narrow range of optimal concentrations.

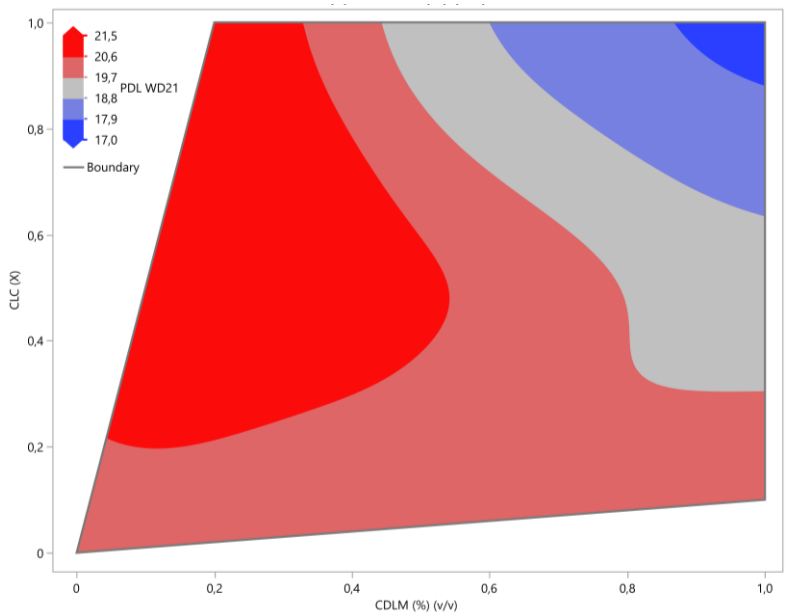


Figure 63: Effect of the couple CDLM and CLC added to the culture on growth during cell expansion. Growth is measured by the PDL after 21 days of cell expansion. 2-D surface model obtained by various combinations of CDLM and CLC added in the CCM.

Fatty acid dissolved in ethanol

The effect on cell growth of the 5 main fatty acids present in the lipoprotein supplement was investigated using fatty acid stock solution in ethanol (Figure 64). None of the fatty acid showed significant growth-promoting effects.

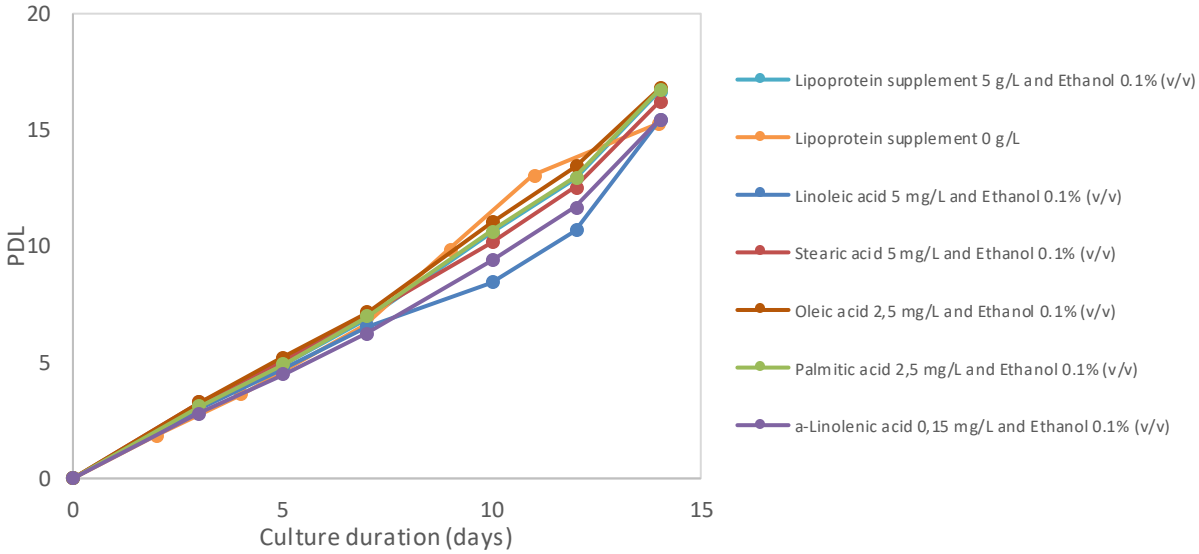


Figure 64: Effect of the lipoprotein supplement substitution by fatty acid dissolved in ethanol on the PDL during cell expansion. Mean from two independent cultures.

Fatty acid requirements of SP2/0 could be already fulfilled by the fatty acids bound to BSA present in the medium, however a growth promoting effect was observed with commercially available lipid supplements in the previous section. The presence of cholesterol in CDLM and CLC may enhance cell growth. On the other hand, the addition of stock solution of fatty acids in ethanol might not efficiently

deliver the fatty acids to the cells compared to lipoproteins, mbCD inclusion complexes, or lipid emulsions.

Consequently, ALS1 and ALS2 were tested, these lipid formulations provide fatty acids and sterols to the culture. Moreover, utilizing the mbCD inclusion complexes technology is expected to offer improved lipid delivery to the cells compared to lipid stock solution in ethanol.

Evaluation of ALS1 and ALS2

The effect of ALS 1 and 2 on growth during cell expansion was investigated (Figure 65), they both exhibit toxicity at above 0.5X (Figures 66 and 67). Moreover, ALS2 was more toxic than ALS1 at equal concentration. It could be explained by the presence of ethanol in ALS2. Indeed, ALS1 and 2 share a common lipid composition but differ by their physical form, ALS1 is a powder that contains fatty acids, sterols and mbCD whereas ALS2 is a liquid that contains ethanol as well.

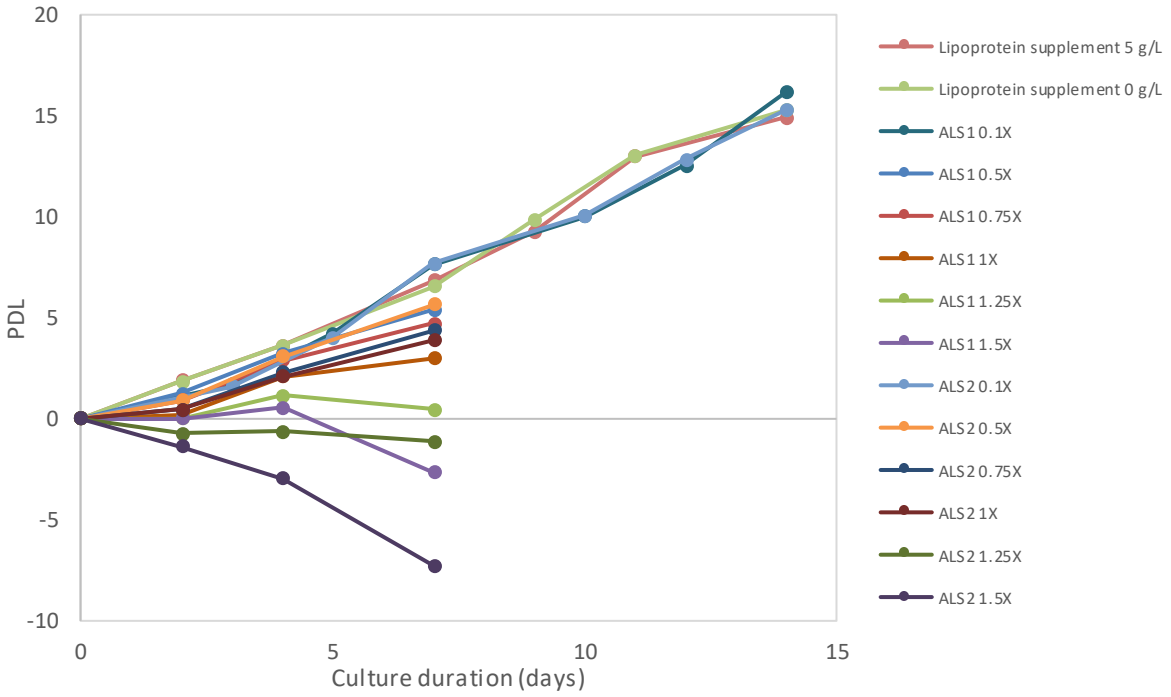


Figure 65: Effect of the lipoprotein supplement substitution by ALS1 and ALS2 on the PDL during cell expansion. Conditions with slow growth or low viability were stopped after 7 days. Mean from two independent cultures.

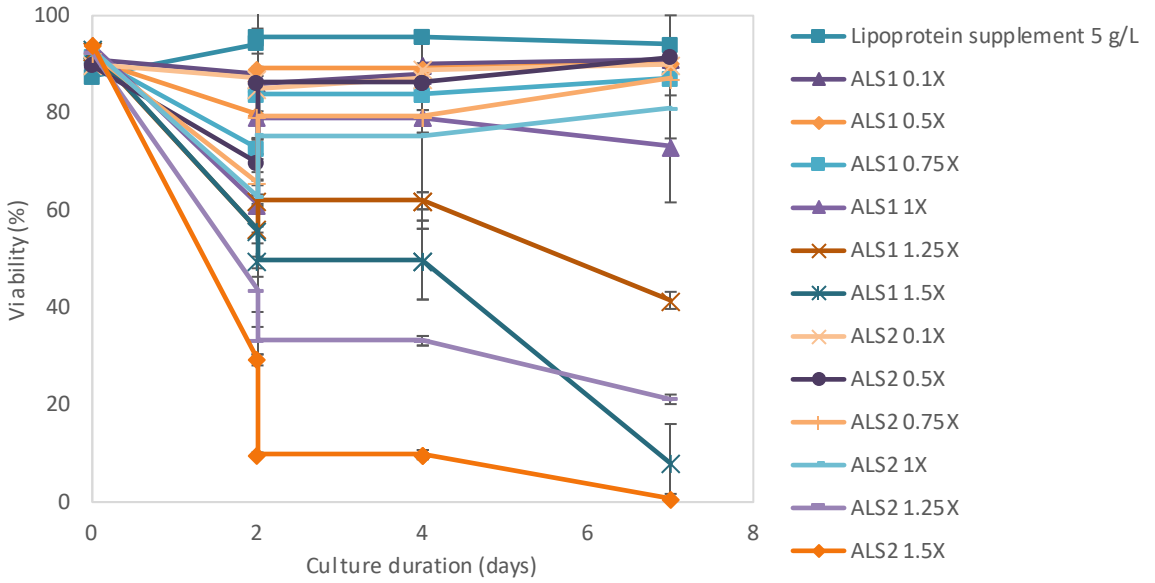


Figure 66: Evolution of cell viability during 1 week of cell expansion with the lipoprotein supplement being replaced by different concentration of ALS1 and 2 from 0.5X to 1.5X. Above concentration 1X ALS1 and 2 exert a severe negative impact on cell viability while between 0.5-1.0X a moderate negative impact was observed. Mean ± standard deviation of three distinct cultures.

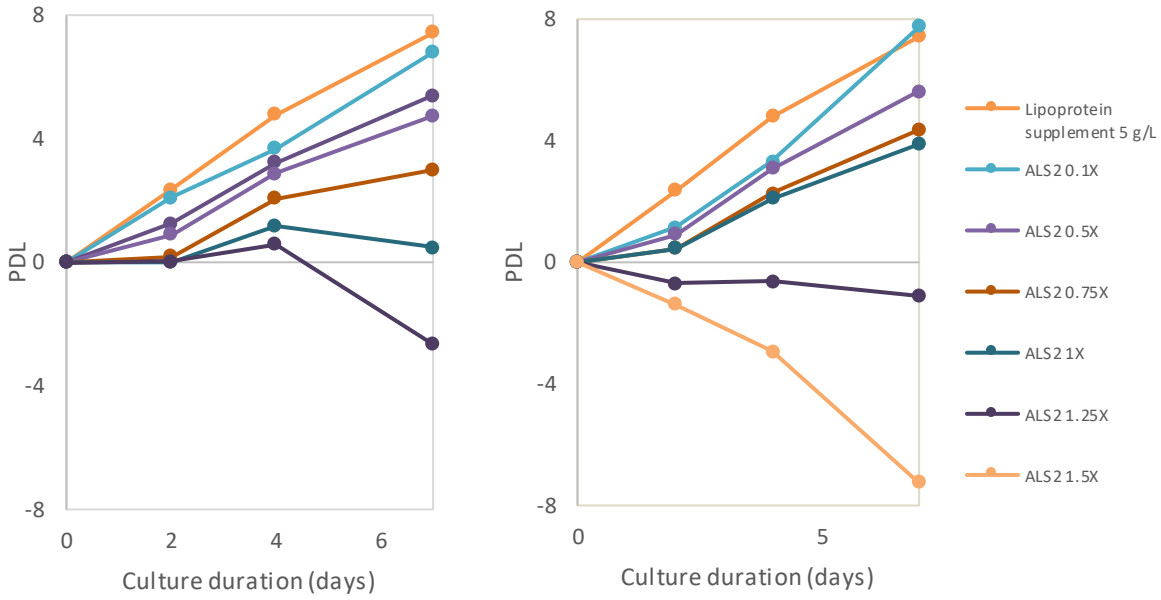


Figure 67: Evolution of PDL during 1 week of cell expansion with the lipoprotein supplement being replaced by different concentration of ALS1 (left) and ALS2 (right) from 0.1X to 1.5X. Mean of three distinct cultures.

Because during cell expansion the fatty acids bound to BSA fulfill fatty acid requirements of the SP2/0 cells in lipoprotein-free CCM, cell expansion is not suitable for the evaluation of lipoprotein supplement alternatives. Nonetheless, these experiments provided evidence that too much concentrated formulations (ALS1 and 2 1.0 – 1.5X) and to a less extent CDLM 1:100 (v/v) & CLC 1.0X cause viability drops and growth inhibition. At high concentration the growth limiting compound could be a lipid or a lipid solubilized. Lastly, both ALS1 (powder) and ALS2 (liquid), 0.1 – 0.5X, were able to support cell growth without significant difference between the two formulations. The physical form of

the formulation seems to have no impact on the process performance. Consequently, the next ALS formulations will be prepared in powder form because powders are more stable and easier to store than liquids.

After evaluation of lipoprotein supplement alternatives during cell expansion, the same work was conducted for the production phase.

4.5.2.2. Substitution of the lipoprotein supplement during the production phase

Evaluation of lipoprotein supplement alternatives during cell expansion concluded that all the commercially available lipid supplements tested, as well as ALS1 and 2 were potential substitutes. These alternatives were tested not only during the expansion but also during the production phase. First, the cells were adapted to the lipid supplement during one week of cell expansion, after which the production phase started. The lipid composition in both the expansion and production media remained identical. In addition to the lipid supplement present in the CCM, the culture was supplemented on day 2 through a lipid supplement feed addition. The specific ratio between the lipid contained in the medium and that provided through feed addition varied at times, and such variations are indicated. If no ratio indication is provided, it means that the feed provided to the culture three times more lipid than the medium (like it is the case for the lipoprotein supplement feed).

Combination of CDLM and CLC

Combinations of CDLM and CLC enhanced growth during cell expansion compared to lipoprotein-free CCM. Then, combinations of CDLM and CLC were tested during the production phase (Figure 68). None of the conditions tested was able to match the standard process where lipids are provided by the lipoprotein supplement. The best condition was CDLM 1.0 – 2.0% (v/v), and CLC 1X with a relative IVC of 40% compared to the standard process.

As expected for a supplement containing only cholesterol and no fatty acids, SyntheChol® 1X was unable to support cell growth, the relative IVC compared to the standard process was 27.3% (data not shown). Combinations of CDLM and CLC were more performant, but the relative IVC still not exceeded 40% of the standard process. These results are concordant with a precedent evaluation of commercial lipid supplements, replacement of the lipoprotein supplement by CLC, Chemically Defined Lipid Concentrate (Invitrogen), ProNS0 (Lonza) or SyntheChol® led to substantial IVC decreases from -50 to -60% when compared to the reference medium during 7-day cultures (El Kouchni, 2011). Despite supplier claims that they support “high densities” culture of hybridoma cells, commercial lipid supplements were not able to substitute for the lipoprotein supplement with comparable levels of growth. Perhaps the lipid composition of these supplements does not match SP2/0 lipid requirements, or solubilizing agents are toxic towards the cells. Consequently, in the next experiments fatty acids and cholesterol supplementation were split with the aim to better match cell requirements for fatty acids and cholesterol.

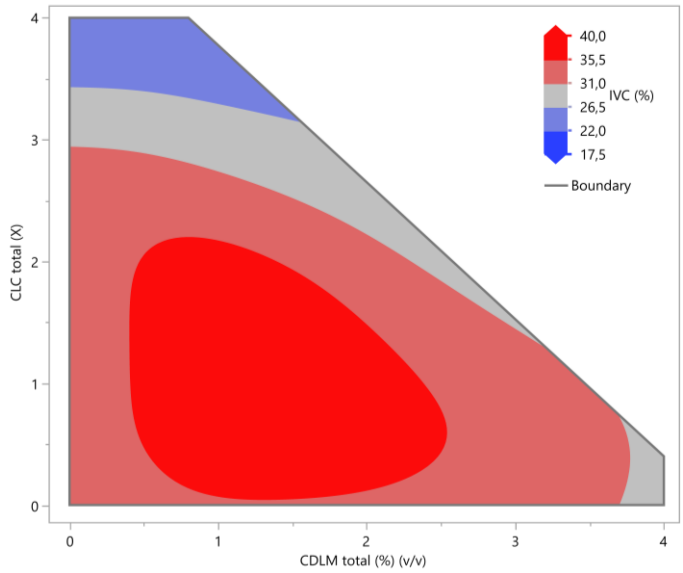


Figure 68: Effect of the couple CDLM and CLC added to the culture after feed addition on cell growth. 2-D surface model obtained by various combinations of CDLM and CLC added in the CCM and through feed addition at WD02. The IVC is expressed as a percentage relative to the standard process.

Evaluation of a combination of ALS3 and SyntheChol®

Due to the dramatic effect of ALS1 and 2 at high concentration (0.5 – 1.5X) during cell expansions, another custom formulation with less lipid was formulated: ALS3. ALS3 has a fatty acid composition identical to ALS1/2 but without sterols that represented ~75% of the lipids in ALS1/2. ALS3 was combined with SyntheChol® to provide both fatty acids and cholesterol to the culture (Figure 69). Moreover, the effect balancing ALS3 and SyntheChol® supplementation between the CCM and feed addition at WD02 was tested (Figure 70).

Like for commercially available supplements, none of the conditions tested was able to match the standard process where lipids are provided by lipoprotein. At high concentration, ALS3 and SyntheChol® were detrimental for cell growth and viability. The best condition, ALS3 0.5X in the CCM and 1.25X after feed addition combined to SyntheChol® 1X in the CCM and 2X after feed addition had a relative IVC of 70% compared to the standard process. It is still better than with the combination of CDLM and CLC (40%), however it is not robust enough to replace the lipoprotein supplement.

As expected for a supplement containing only cholesterol and no fatty acids, supplementation of the culture with only SyntheChol® 1X was unable to support cell growth, the relative IVC compared to the standard process was 27.3%.

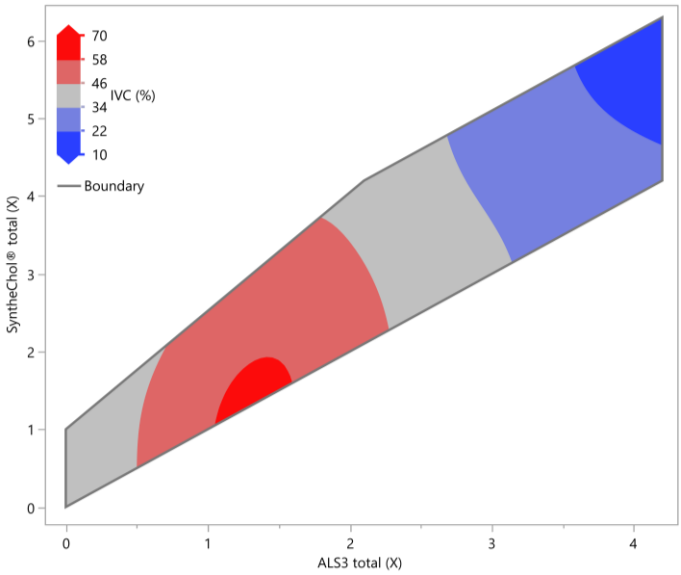


Figure 69: Effect of the couple ALS3 and SyntheChol® added to the culture on cell growth. 2-D surface model obtained by various combinations of ALS3 and SyntheChol® added in the CCM and through feed addition at WD02. The IVC is expressed as a percentage relative to the standard process.

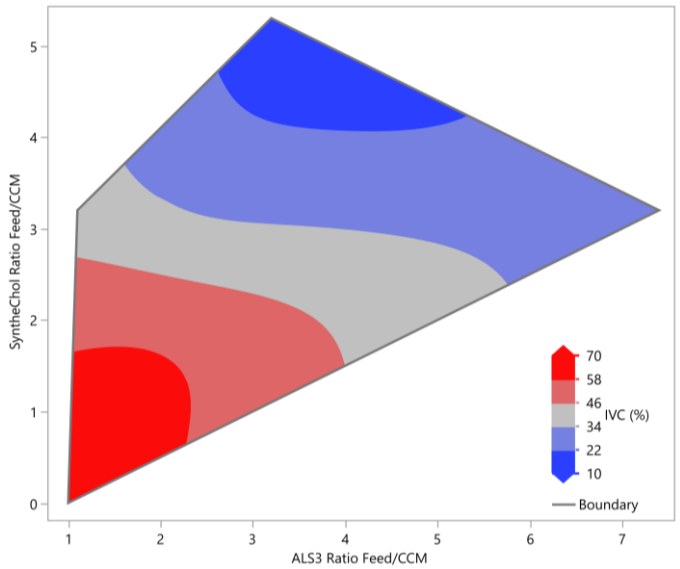


Figure 70: Effect of the couple ALS3 and SyntheChol® ratio variations between feed addition and CCM on cell growth. 2-D surface model obtained by balancing ALS3 and SyntheChol® supplementation between feed addition and the CCM. The IVC is expressed as a percentage relative to the standard process.

Overall, initial levels of ALS3 and SyntheChol® in the CCM had a slight effect on cell growth (Figure 71) compared to the impact of their concentration after feed addition where some of their components may become growth inhibitory as their concentration increases. ALS3 and SyntheChol® concentrations in the CCM are too low to be growth inhibitory. However, high levels of ALS3 and SyntheChol® after feed addition had a negative impact on cell growth. Lastly, a ratio of ALS3 or SyntheChol® added to the culture after feed addition compared to the amount added in the CCM over 3 was detrimental too.

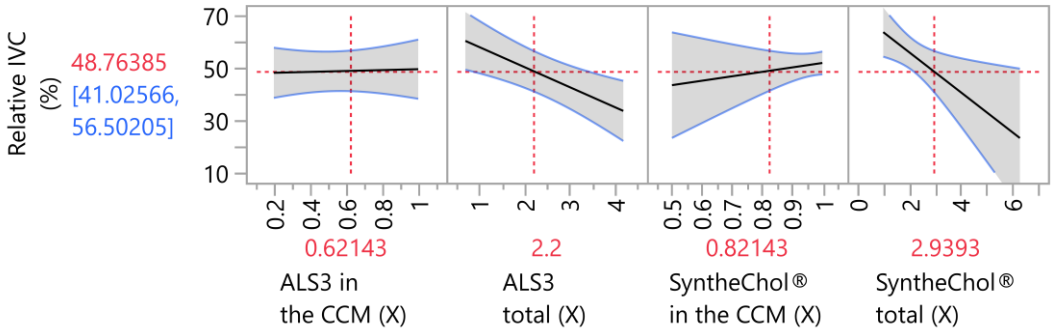


Figure 71: Effect of ALS3 and SyntheChol® content in the CCM and after feed addition on the relative IVC at the end of the culture compared to the standard process.

There are several non-exclusive hypotheses that could explain these observations:

- Lipid requirements of the cells are unmet thus limiting cell growth.
- Excessive uptake of lipids by the cells leads to lipid accumulation and cell death.
- One or more components found in the lipoprotein supplement are absent when a combination of ALS3 and SyntheChol® is used.
- One or more compounds present in ALS3 and/or SyntheChol® are toxic towards SP2/0 cells.
- The lipid combination provided by ALS3 and SyntheChol® does not match cell requirements, resulting in an imbalance that harms the cells.
- The uptake of lipids provided by mbCD inclusion complexes differs significantly from that of lipoproteins.

To confirm or reject these hypothesis, several experiments were conducted, they are presented in the next section.

4.5.3. Challenges associated with the replacement of the lipoprotein supplement

The combinations ALS3 and SyntheChol® tested failed to substitute for the supplement, several hypothesis could explain it. In this section, we present a series of experiments designed to confirm or reject several hypotheses that may explain the observed failure. Specifically we focused on an assessment of mbCD toxicity towards SP2/0 cells, the adaptation of fatty acid balance to better meet cell requirements, the improvement of the feeding strategy, and the evaluation of growth promoting compounds that may be lacking in the ALS3 and SyntheChol® combination.

4.5.3.1. Assessment of mbCD toxicity towards SP2/0 cells

mbCD inclusion complexes present in ALS3 and SyntheChol® transfer their cholesterol and fatty acid molecules to the cell membrane, then remains only free mbCD. However, free mbCD can deplete cell membrane lipids damaging the cell (Christian, 1997). Consequently, the total amount of mbCD added to the culture should be controlled to avoid mbCD toxicity. The effect of free mbCD concentration on SP2/0 cell growth was evaluated and not only the mbCD concentration but also the ratio between mbCD concentration and cell density was considered.

mbCD concentration varied from 0.0 to 2.0 g/L. Cells were passaged 5 times in CCM containing mbCD, at each passage cells were centrifuged 5 min at 200 g and resuspended in fresh medium. Two viable cell densities were considered, 0.28 and 1.00 Miocells/mL (10^6cells/mL), which are the VCD at the inoculation of the production phase and the minimum VCD to reach before addition of the lipid feed at WD02, respectively.

Significative viability drops (Figure 72) growth reductions (Figure 73) were observed for cultures inoculated at 1.00 Miocells/mL. Nutrient limitation and not the mbCD concentration is suspected to cause the observed growth limitation. Indeed, the cultures inoculated at 1.00 Miocells/mL reached VCD up to 4 Miocells/mL after 2-3 days and had already consumed the most limiting nutrients in the CCM, causing growth reduction and probably activation of controlled cell death pathways. The effect of nutrient limitation was more important than the effect of mbCD on cell growth, no matter of the mbCD concentration. Thus, the effect of mbCD concentration on cultures inoculated at 1.00 Miocells/mL was not observed in this experiment and these conditions are not usable to assess the effect of mbCD concentration on cell growth.

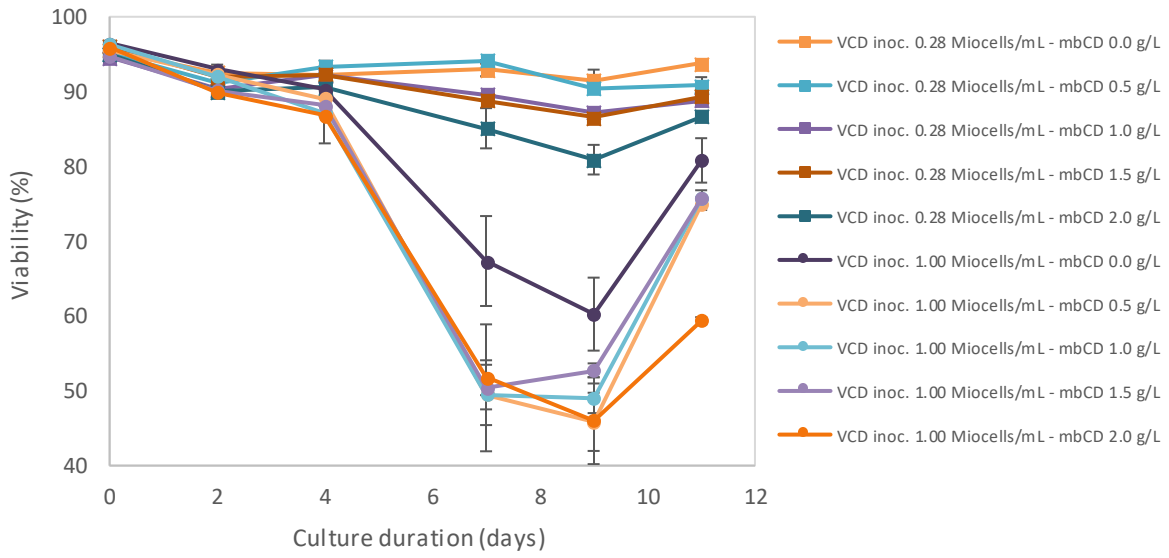


Figure 72: Evolution of cell viability during cell expansion in cell expansion medium containing the lipoprotein supplement, at two target VCD at the inoculation (0.28 and 1.00 Miocells/mL), and at

several mbCD concentration ranging from 0.0 to 2.0 g/L. Viability drops were observed for cultures inoculated at 1.00 Miocells/mL. Mean ± standard deviation from two independent cultures.

Regarding the cultures inoculated at 0.28 Miocells/mL, nutrient limitation is not expected to affect cell growth during 2-3 days cell expansions, consequently the slight viability drops (Figure 72) and growth reduction (Figure 73) observed are likely to be caused by the varying mbCD concentration in the medium.

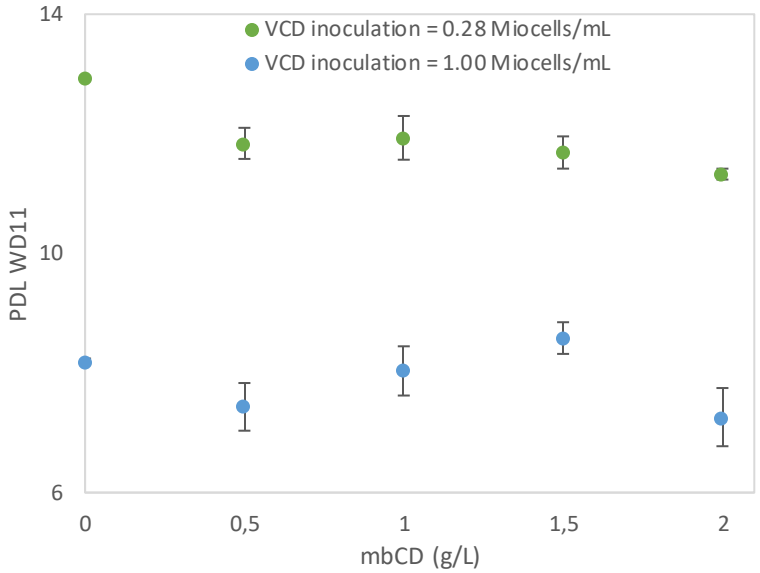


Figure 73: Evolution of PDL at WD11, after 5 cell expansion passages, with an increasing mbCD concentration in the medium for two inoculation VCD. Addition of mbCD to the medium has a negative impact on PDL, but no dose-response correlation was observed between 0.5-2.0 g/L for cultures inoculated at 0.28 Miocells/mL. No impact of mbCD concentration on PDL for the cultures inoculated at 1.00 Miocells/mL was observed. Mean ± standard deviation from two independent cultures.

The cytotoxic effect of mbCD is likely due to its ability to extract cholesterol from cell membranes, even when cholesterol concentration in the medium is high (17.5 µg/mL) (Wu, et al., 2011). Despite a high cholesterol concentration in the CCM supplemented the lipoprotein supplement, cholesterol ± 30 µg/mL, or in lipoprotein-free CCM supplemented with SyntheChol®, cholesterol 3.5 – 14.0 µg/mL, high concentration of mbCD may perturb cell membrane and slightly negatively impact cell growth. However, mbCD concentration varying from 0 - 1 g/L did not affect cell growth and viability, consequently, the mbCD concentration was maintained below this limit in further experiments.

The impact of mbCD on SP2/0 growth was confirmed, mbCD negatively affects viability and growth. However, the slight decreases in viability and growth observed when maintaining mbCD concentration below 1.0 g/L are insufficient to explain the disparity in growth performance between the ALS3 and SyntheChol® combination and the lipoprotein supplement.

4.5.3.2. Modification of the fatty acid balance

Fatty acid supplementation plays a crucial role in substituting lipoprotein as SP2/0 cells are fatty acid auxotrophs. Failure to fulfill the lipid requirements of the cells is prejudicial for the culture. Besides limiting cell growth, fatty acid imbalance can potentially alter cell membrane properties and destabilize them. Moreover, excessively high concentrations of fatty acids can be detrimental to cells. Addition of arachidonic acid and modification of the linoleic, oleic and α-linolenic acids balance were studied to improve the fatty acid composition of ALS and better meet the specific requirements of SP2/0 cells.

Effect of α-linolenic acid

α-linolenic acid was reported to exert cytotoxic effects against SP2/0 cells at concentration as low as 18 µM (~5 mg/L) (Kumar & Das, 1995). BSA provides α-linolenic acid 0.1 mg/L to the culture and addition of ALS3 increases α-linolenic acid culture content potentially leading to growth inhibition. Indeed, supplementation of the culture with ALS3 1X means addition of α-linolenic acid 0.75 mg/L. Consequently, α-linolenic acid toxicity was evaluated using stock solution of α-linolenic acid in ethanol. Culture containing only ethanol was used as control.

Addition of α-linolenic acid 1.0 mg/L to the CCM, increased at 4.0 mg/L after feed addition was fatal for the culture, with a relative IVC of 2% compared to condition without α-linolenic acid.

Addition of arachidonic acid

Addition of arachidonic, absent in ALS3, was evaluated using stock solution of arachidonic acid in ethanol. Culture containing only ethanol was used as control. Addition of arachidonic acid 0.30 mg/L to the CCM, increased at 1.20 mg/L after feed addition was detrimental to the culture, relative IVC 41% compared to condition without arachidonic acid.

Modification of the fatty acid balance

Combination of linoleic and oleic acids was reported to support the growth of fatty acid auxotroph hybridoma cell lines (Butler & Huzel, 1995 ; Butler, Huzel, Barnabé, Gray, & Bajno, 1999 ; Jäger, Lehmann, & Friedl, 1988 ; Kovár & Franek, 1984 ; Kobayashi, Kato, Omasa, Shioya, & Suga, 1994 ; Schneider, 1989). These fatty acid are critical to fulfill lipid requirements. Water-soluble linoleic acid and Water-soluble oleic acid were added to the ALS3 and SyntheChol® formulation to evaluate the influence of varying their concentrations. The effects of increasing linoleic or oleic acid concentration were very similar, making these fatty acids likely exchangeable (Figure 74). As expected, increasing their concentration too high was detrimental to the culture. The optimal concentration after feed addition was 10 – 20 mg/L for linoleic acid and 5 – 12 mg/L for oleic acid. Addition of ALS3 0.5X to the CCM, further increased at 1.25X after feed addition complies with the optimal range. Indeed, after feed addition linoleic acid reaches 11.25 mg/L, and oleic acid 5.63 mg/L.

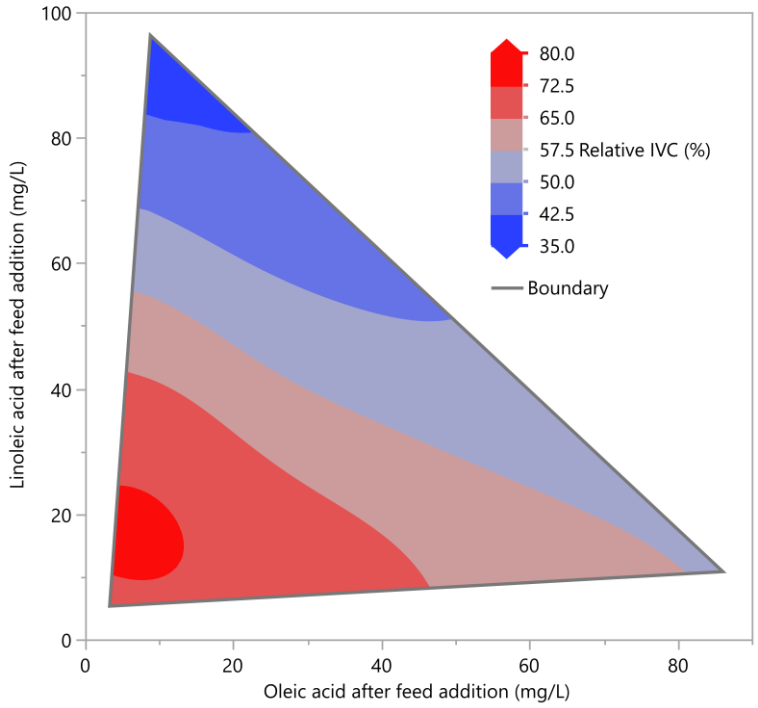


Figure 74: Effect of the couple linoleic and oleic acids added to the culture after feed addition on cell growth. 2-D surface model obtained by various combinations of water-soluble linoleic acid and water-soluble oleic acid in lipoprotein-free CCM containing ALS3 and SyntheChol® 1X. Note that the fatty acid provided by BSA are considered. The IVC is expressed as a percentage relative to the standard process.

The complete substitution of ALS3 with combination of mbCD inclusion complexes with linoleic or oleic acids did not perform better than ALS3 (data not shown). This highlights the importance of fatty acid diversity for the cells. Moreover, the 2:1:1:1 balance of linoleic, oleic, palmitic and stearic acids was reported to fulfill fatty acid requirements of SP2/0 cells in absence of lipoprotein (El Kouchni, 2011). Varying linoleic and oleic acids concentration led to the identification of an optimal range, which coincided with the optimal ALS3 supplementation identified previously. Therefore, the concentration of linoleic and oleic acids remained unchanged.

The addition of arachidonic and α-linolenic acids, when dissolved in ethanol, exhibited toxicity towards SP2/0 cells. It remains uncertain whether the fatty acids themselves are harmful to the cells or if the method of solubilizing them in ethanol increases their susceptibility to oxidation. Both arachidonic and α-linolenic acids are prone to lipid peroxidation (section 2.3.3.1), resulting in the generation of harmful by-products that negatively affect cell growth.

Arachidonic and α-linolenic acids may receive partial protection from oxidation when encapsulated within mbCD inclusion complexes or lipoprotein particles. However, it is not possible to make vary arachidonic or α-linolenic acids content of lipoprotein using water-soluble solution of cyclodextrin (mbCD) solubilized lipids as mbCD inclusion complexes with arachidonic or α-linolenic acids are not commercially available. Because of the arachidonic and α-linolenic acids toxicity observed in these

experiments, a formulation without α-linolenic acid, ALS4, was used in the next experiments, arachidonic was not added to this formulation.

Variation of the linoleic and oleic acids balance did not improve cell growth, suggesting that the ratio of these fatty acids in ALS3/ALS4 is already well balanced for SP2/0 cells. Lastly, combination of water-soluble linoleic and oleic acids failed to substitute for ALS in lipoprotein-free culture. It confirms that maintaining a diverse range of fatty acids is crucial for cell growth. Such diversity is assured by the fatty acid bound to BSA and the 4 fatty acids present in ALS4.

4.5.3.3. Enhancement of the formulation with growth promoting compounds

Despite optimization of the cholesterol and fatty acid supplementation to match cell requirements, the substitution of the lipoprotein supplement by a combination of SyntheChol® and ALS failed. The supplement is a complex raw-material primarily used to provide lipids to the culture, but it contains much more. Lipoproteins are not only a source of cholesterol and fatty acids but also of antioxidants like α-tocopherol, carotenes, retinoids, as well as phospholipid building blocks such as choline, ethanolamine, myo-inositol. Moreover, the lipoprotein supplement matrix could exert shear-stress protectant properties that are not caused by a specific compound. Consequently, substitution of the supplement by a combination of mbCD inclusion complexes with fatty acids and cholesterol could reduce the antioxidant and phospholipid building block content in the CCM.

In this section, addition of antioxidant, phospholipid components and shear stress protectant to a combination of ALS4 and SyntheChol® was evaluated.

Antioxidant

Lipoproteins contain not only fatty acids, sterols and proteins but also α-tocopherol. Based on the characterization results obtained previously, the α-tocopherol concentration can be estimated by calculation.

The α-tocopherol concentration in the lipoprotein supplement is estimated based on the proteomic characterization results, and the α-tocopherol molecules per LDL particle according to the bibliography. The Apo B-100 concentration measured was 3,35 ± 0,76 g/L, equivalent to 6,20 ± 1,41 µM. Apo B-100 molecular weight is 540 kDa.

LDL, IDL and VLDL contain one single Apo B-100 per particle (Gianazza, et al., 2021), thus the lipoprotein concentration in the lipoprotein supplement can be estimated equal to the Apo B-100 concentration. Lastly, assuming that each LDL particle contains 6 to 12 α-tocopherol molecules (Bowry & Ingold, 1999), the α-tocopherol concentration in the lipoprotein supplement can be estimated by calculation (Table 17).

Apo B-100 (g/L)	Apo B-100 (kDa)	Apo B-100 (µM)	α -tocopherol molecules per LDL particle	α -tocopherol (µM)
3.35 ± 0.76	540	6.20 ± 1.41	6 - 12	55.8 ± 12.7

Table 17: Estimation of the alpha-tocopherol in the lipoprotein supplement based on Apo B-100 concentration and α-tocopherol molecules per LDL particle.

The tocopherol delivered to the culture by the basal medium is negligible compared to that provided by the lipoprotein supplement. Thus the lipoprotein supplement substitution should include a source of antioxidant.

Due to its low solubility in water, α-tocopherol was replaced by Trolox. First, the effect of Trolox concentration on cell growth was evaluated during 2 weeks of cell expansion in lipoprotein-free CCM containing ALS4 0.5X and SyntheChol® 0.5X (Figure 75). Trolox started to be growth inhibitory above 10 mg/L during cell expansion. These results are consistent with the observations reported in the literature. Despite decreasing the ROS intracellular levels in HeLa cells from 1 to 5 mg/L, Trolox started to increase ROS intracellular levels and triggered apoptosis above 10 mg/L (Giordano, Caricato, & Lionetto, 2020). Indeed, Trolox exhibits prooxidant properties at high concentration (Giordano, Caricato, & Lionetto, 2020). Free radical attack on the hydrogen from the hydroxyl group of the chromanol ring leads to the formation of phenoxyl radical (Sharma & Buettner, 1993) which propagates lipid peroxidation in presence of PUFA (Ingold, Bowry, Stocker, & Walling, 1993).

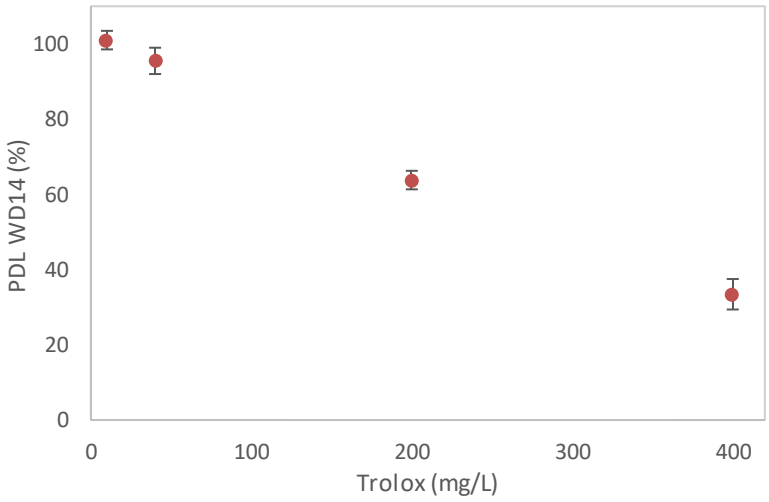


Figure 75: Effect of Trolox concentration on cell growth during 14 days of cell expansion. The PDL is expressed as a percentage relative to the condition without Trolox. Trolox starts to be growth above 40 mg/L. Mean ± standard deviation from three independent cultures.

Then addition of Trolox was evaluated during the production phase (Figure 76). The CCM contained ALS4 0.5X, SyntheChol® 0.5X, Trolox then ALS4 1X, SyntheChol® 0.5X and Trolox were added through feed addition at WD02. Addition of Trolox 10 and 25 mg/L enhanced cell growth. Whereas at 50 mg/L the beneficial effects of Trolox started to decrease, likely due to its prooxidant properties at high concentration.

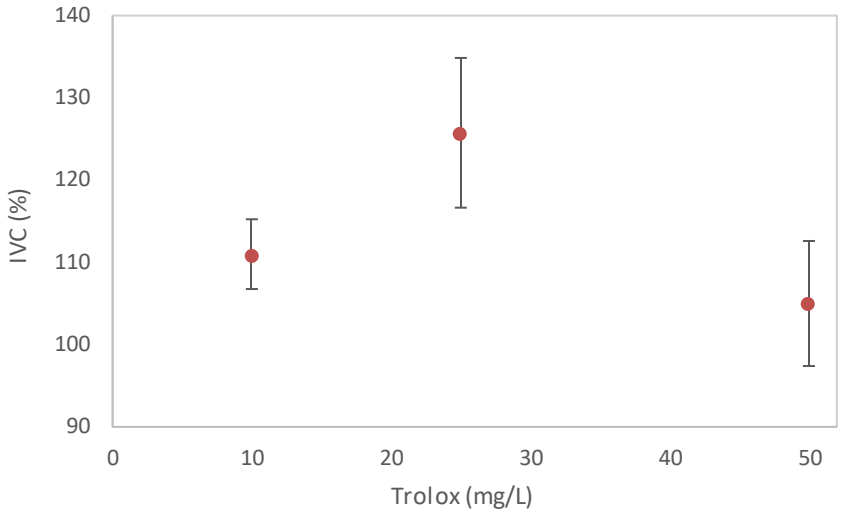


Figure 76: Effect of Trolox added to the culture after feed addition on cell growth. The PDL is expressed as a percentage relative to the condition without Trolox. A quarter of the Trolox was present in the CCM, the remaining Trolox was added with the feed at WD02. Mean ± standard deviation from three independent cultures.

These results showed that addition of an antioxidant to the combination of fatty acids and cholesterol enhances growth in lipoprotein-free cell culture. Substitution of the lipoprotein supplement by ALS4 0.5X, SyntheChol® 0.5X, Trolox 6.25 mg/L in the CCM, and addition at WD02 of ALS4 1X, SyntheChol® 0.5X and Trolox 18.75 mg/L led to a relative IVC of 73% compared to the standard process.

Shear-stress protectant

According to a patent, addition of β-D-xylopyranoses, substituted with phosphate, carboxyl and/or sulfate groups, in serum-free media was claimed to increase mammalian cell culture performance (Stockinger, H., 1991, Serum-free culture medium for mammalian cells, U.S. Patent No. 5,063,157). Notably, pentosan polysulphate with a molecular weight of from 4 to 6 kDa and concentration from 80 to 120 mg/L. Pentosan polysulphate being difficult to order, dextran polysulfate was used instead. Dextran sulfate and β-D-xylopyranoses sulfate have similar structures and properties (Parthasarathi & Jayakrishnan, 2011).

Addition of 50 – 200 mg/L of dextran sulfate with a molecular weight of 4 and 10 kDa did not enhance cell growth. The protective effect of the supplement matrix compounds against shear stress is likely to be negligible compared to the protective effect provided by the components of the basal medium.

Other compounds tested

Choline, ethanolamine and myo-inositol are building blocks of phospholipids, because lipoproteins contain phospholipids the supplement is a source of these compounds. Lipoprotein is also a source of retinoic acid (Li, Wongsiriroj, & Blaner, 2014), retinoic acid was reported to enhance mAb production in hybridoma (Inoue, et al., 2000). Like for Trolox, the effect of adding these compounds to lipoprotein-free culture was evaluated. However, it failed to improve process performance (data not shown).

The addition of Trolox to the combination of ALS and SyntheChol® resulted in improved cell growth. This suggests that the role of the lipoprotein supplement in the culture does not limit to providing fatty acids and cholesterol. However, despite increasing the IVC, addition of Trolox was unable to fully substitute for the lipoprotein supplement with comparable performance.

4.5.3.4. Modification of the feeding strategy to limit mbCD inclusion complexes toxicity

Modifying the composition of the lipoprotein supplement substitute increased its cell culture performances without reaching the one of the standard process. However, according to our knowledge on the lipid requirements of the SP2/0 cell line, few fatty acids should be enough to fulfill the cells requirement. Lipid uptake by the cells was suspected to differ between lipoproteins and mbCD inclusion complexes. In such a case, the optimal lipid composition of the lipoprotein supplement and the one of its substitute may be different. A unique bolus feed addition at WD02 may be appropriate for supplementing the culture with the supplement but inappropriate for a feed based on mbCD inclusion complexes. Indeed, lipoprotein uptake is a controlled mechanism. The receptor-mediated endocytosis of lipoproteins uses the LDL receptor which expression is regulated by intracellular level of cholesterol. Hence, even if the culture contains elevated levels of lipoprotein, the cells take up only the necessary amount. In contrast, lipid exchanges between mbCD inclusion complexes and the cell membrane are governed by an equilibrium between mbCD inclusion complexes, free mbCD and the cell membrane. Therefore, substantially increase mbCD inclusion complexes with lipid in the culture may suddenly increase the lipid content in cell membranes in an uncontrolled manner.

The feeding strategy, originally based on a unique bolus addition at WD02, was spread over several days. This modification prevents the mbCD inclusion complex concentration from rising too high. Based on the best conditions observed in previous experiments, substitution of the lipoprotein supplement by combinations of ALS4 and SyntheChol® with constant Trolox concentration (10 mg/L), in parallel with combinations of CDLM and CLC was evaluated. Lipid feed addition was spread on WD02 and WD04, or WD02, WD04 and WD07. Lipid concentration in the CCM and the balance between the lipid amount fed at WD02, WD04 and WD07 varied too.

However, spreading the lipid feed addition did not result in a significant improvement in cell growth. Furthermore, the viability still declined earlier compared to the standard process.

In conclusion, distribute lipid feed addition over several days better match lipid consumption rate of the cells slightly increased cell growth. It is worth noting that the lipid formulation used in these experiments was initially designed for a single bolus feed addition at WD02 and may not be entirely optimized for the multiple feed addition approach.

4.5.4. Discussion

To address the issues associated with the use of animal-derived raw materials, the most effective approach is to eliminate these raw materials from the process and replace them with chemically defined ACF substitutes. Successful replacement of lipoprotein and bovine serum albumin with recombinant human albumin bound to fatty acids in SP2/0 cell culture was reported (El Kouchni, 2011), providing evidence of the feasibility. Yet despite promising outcomes observed upon substituting the lipoprotein supplement during cell expansion, we were unable to replace lipoprotein supplementation while maintaining constant process performance during the production phase.

Despite supplier claims that they support "high densities" culture of hybridoma cells, commercial lipid supplements were not able to substitute for the lipoprotein supplement with comparable levels of growth. Because the composition of these supplements is fixed and can not be adjusted to meet the specific requirements of SP2/0 cells, we prepared custom formulations starting from the lipid composition of the lipoprotein supplement. The best custom formulation (Table 18) performed better than the best combination of commercial supplement, 73% and 40%, respectively, of the final IVC of the standard process. Several factors could contribute to this enhancement, such as the improved lipid composition, better alignment with SP2/0 cell requirements, the absence of toxic solubilizing agents, or the low level of mbCD.

Compound	Linoleic acid	Oleic, stearic and palmitic acids	Cholesterol	Trolox	mbCD
Cell culture media (mg/L)	5.00	2.50	3.50	10.0	-
Feed addition at WD02 (mg/L)	6.25	3.13	3.50	15.0	-
Total after feed addition (mg/L)	11.25	5.63	7.00	25.0	< 1000

Table 18: Chemical compounds to supplement to the culture for the lipoprotein supplement substitution. Fatty acids were provided by ALS4 and cholesterol by SyntheChol®.

There is still some room for improvement. First, the solubilizing agent, mbCD, has major drawbacks, especially its ability to exchange lipids with the cell membrane makes it cytotoxic as it is accumulating in the culture. Lipoprotein could be replaced not only by mbCD inclusion complexes but also by non-proteinaceous lipid emulsions, liposomes, or synthetic lipoproteins, whose individual components, even if they accumulate in the culture, are not toxic.

Beyond the solubilizing agent, we made another choice which is debatable. Even if SP2/0 are not cholesterol auxotroph (Sato, Kawamoto, McClure, & Sato, 1984), we conserved a cholesterol source as we observed that cholesterol supplementation of the culture significantly enhanced cell growth during cell expansion. However, in view of the limitations posed by the lipid and/or mbCD

accumulation in the culture, the cholesterol source should have been removed even at the cost of a slight loss of growth rate.

Some interaction between CCM components and lipoprotein might be beneficial for the culture . If so, some CCM components would lose their efficiency in absence of lipoprotein, thus another CCM optimization would become necessary after substitution. On the other hand, the lipoprotein alternatives may interact with CCM components. Like tyrosine, tryptophan and phenylalanine that form inclusion complexes with cyclodextrin (Li, Geng, Liu, Wang, & Liang, 2018), thus reducing their availability for the cells.

5. CONCLUSION AND FUTURE PERSPECTIVES

5.1. A better understanding of the lipoprotein supplementation of SP2/0 cultures

Batch-to-batch variability is inherent to non-chemically raw materials and causes process performance variations that may disrupt process robustness. Some lipoprotein supplement batches were found to cause early viability declines and drastic productivity decreases (from -60% to -85%). To address this challenge, we increased understanding of the relationship between batch variations and process variation to mitigate their impact on process robustness. Identification of the chemical compounds varying, through characterization of several batches, and identification of their respective impact on key process parameters is the initial step of the improvement of their control. Thus, the starting point of this project was to understand the role of the lipoprotein supplement in the manufacturing process in order to gain insights into how any variations of in its composition or alteration of its components might affect process robustness.

During cell expansion, lipoprotein supplementation of the culture is not essential since the fatty acids provided by BSA are sufficient to support cell growth when cells are frequently resuspended in fresh CCM. Indeed, both lipoprotein and BSA supply essential lipids for the survival of SP2/0 cells making them partially substitutable. However, the lipoprotein supplement is essential to ensure robust growth and high productivity during the production phase. Although BSA can provide sufficient fatty acids to sustain SP2/0 cells during 2-3-day passages during cell expansion, it cannot meet the lipid requirements during the 11-day production phase. After lipoprotein feed addition at WD02, the supplement becomes the primary lipid source in the culture.

Characterization of the supplement confirmed its lipoprotein nature, primarily composed of HDL and LDL. The lipoprotein supplement contains various components, including sterols (55% cholesterol, 27% cholestenone, and 18% oxysterols), fatty acids (45% saturated, 17% monounsaturated, and 38% polyunsaturated), proteins (72% apolipoproteins and 28% other proteins), lipid peroxidation end products as well as trace elements, tocopherol, and phospholipid building blocks in small quantity.

The diverse composition of lipoprotein makes them a versatile substance capable of supporting the growth of a wide range of cell lines in serum-free medium. However, this complexity also complicates the understanding of the relationship between variations in its components and process performance. During the characterization, it was observed that the lipoprotein supplement exhibits significant batch-to-batch variations in composition. This variation is not restricted between US and NZ EX-CYTE®, but also among batches from the same source. Another concern about the lipoprotein supplement composition is the oxidation of its components. Indeed, a non-negligeable proportion of sterol are cholesterol oxidation byproducts, cholestenone and oxysterols represent 45% of the sterol content. Moreover, lipid peroxidation end products (4-HNE and MDA) were found in every tested batch, along with evidence of protein carbonylation. In addition to the batch-to-batch variations observed in the

lipoprotein supplement composition, we also suspect variations in the degree of oxidation among different batches.

The fundamental role of the supplement to ensure robust growth and high productivity associated with its important batch-to-batch variability pose a threat to process robustness. Testing of 36 batches confirmed that the batch-to-batch variability is affecting cell growth, cell viability and process productivity.

5.2. Low-performing lipoprotein supplement batches

An investigation was conducted to identify the root cause of the low-performing lipoprotein supplement batches and the factors affecting process performance so severely. First, the supplement manufacturing process itself was excluded as the primary cause, as the serum batches used in manufacturing the low-performing batches already exhibited inhibitory effects on cell growth. Then, analysis of cell death markers revealed the activation of apoptosis when the low-performing batches were used. However, it seemed that multiple pathways were activated. Indeed, caspase-3 activity was modulated when the oxidative stress was mitigated but a caspase-3 inhibitor did not prevent the viability drops associated with the low-performing batches. The compound(s) causing the abnormal viability drops were not identified. Nevertheless, there is strong suspicion that oxidation plays a significant role in this phenomenon.

The addition of Trolox to the culture proved effective in reducing caspase-3 activation associated with the low-performing batches and also led to improved cell growth in other batches. Furthermore, during the characterization of the lipoprotein supplement, oxysterols, lipid peroxidation end products, and protein carbonylation were detected. There is evidence that the supplement contains not only HDL and LDL but likely oxLDL, which are known to induce apoptosis and exhibit toxicity towards various cell types. Lipoprotein supplement batches without issues were oxidized to produce oxysterol- and lipid hydroperoxide-rich oxLDL. In addition to causing solution browning, oxidized supplements exhibited a negative impact on cell growth proportional to the extent of oxidation.

UV-spectroscopy was used to monitor oxidation. Notably, the absorbance at 276 nm was strongly correlated with both the degree of oxidation and cell culture performance of oxidized supplements. The oxidation monitoring tool was successfully applied to every batch available. We observed that low-performing batches absorb more at 276 nm compared to other batches. Low-performing batches are likely containing high levels of conjugated dienes, end products of lipid peroxidation. However, this observation is contradictory with the characterization results, low-performing batches do not contain significantly higher levels of oxysterols, 4-HNE, or MDA compared to other batches. The oxidation products that absorb at 276 nm and are more abundant in low-performing batches might not have been detected during the initial characterization. To distinguish low-performing batches from others, a limit was established based on their absorbance at 276 nm. Implementation of this detection method

would avoid the use of low-performing batches in large-scale manufacturing. This approach could reduce the impact of the lipoprotein supplement batch variations on process robustness.

5.3. Substitution of the lipoprotein supplement by a chemically defined alternative

To address the issues associated with the use of lipoprotein supplement, the most effective approach is to eliminate these raw materials from the process and replace them with chemically defined and ACF substitutes. However, it is crucial that the substitute does not disrupt the process, maintaining a comparable productivity and product quality with the original process. Unfortunately ,our formulation of mbCD inclusion complexes with lipids did not perform as well as the original lipoprotein supplement.

Due to its cytotoxic effects at high concentrations and its interactions with CCM constituents, cyclodextrin is not the ideal solubilizing agent within the context of mammalian cell culture. Similarly, lipid emulsions, liposomes and synthetic lipoproteins also face limitations in the context of large-scale manufacturing implementation as they necessitate a sonication or extrusion step. The microemulsion and the solvent evaporation methods (Nikam, Chavan, & Sharma, 2014) however, deserve to be evaluated. In the microemulsion approach, a mixture of lipids, emulsifiers, and co-emulsifiers is heated then dispersed in cold water without sonication. The solvent evaporation method involves dissolving lipids in an organic solvent which is dispersed in an aqueous phase and eliminated via pressure reduction. The water-in-oil dispersions achieved through both of these methods lack stability, with the second method potentially resulting in the presence of residual solvent traces with in the final product. Yet they are easy to implement, easy to scale-up and cytotoxic components-free methods that could yield water soluble lipid solution for mammalian cell culture supplementation. Nonetheless, they are simple, scalable, and cytotoxicity-free approaches that hold the potential to produce water-soluble lipid solutions for mammalian cell culture supplementation.

A more disruptive approach to address the limitations caused by the lipid supplementation would be to overcome the fatty acids auxotrophy of SP2/0 cells like it was done for the cholesterol auxotrophy of NS0 cells. Both adaptation to cholesterol-free medium by multiple passages (Keen & Steward, 1995) and alteration of gene expression (Seth, Ozturk, & Hu, 2005) were reported to produce cholesterol-independent NS0 cells.

6. APPENDIX

6.1. Supplementary figures and tables

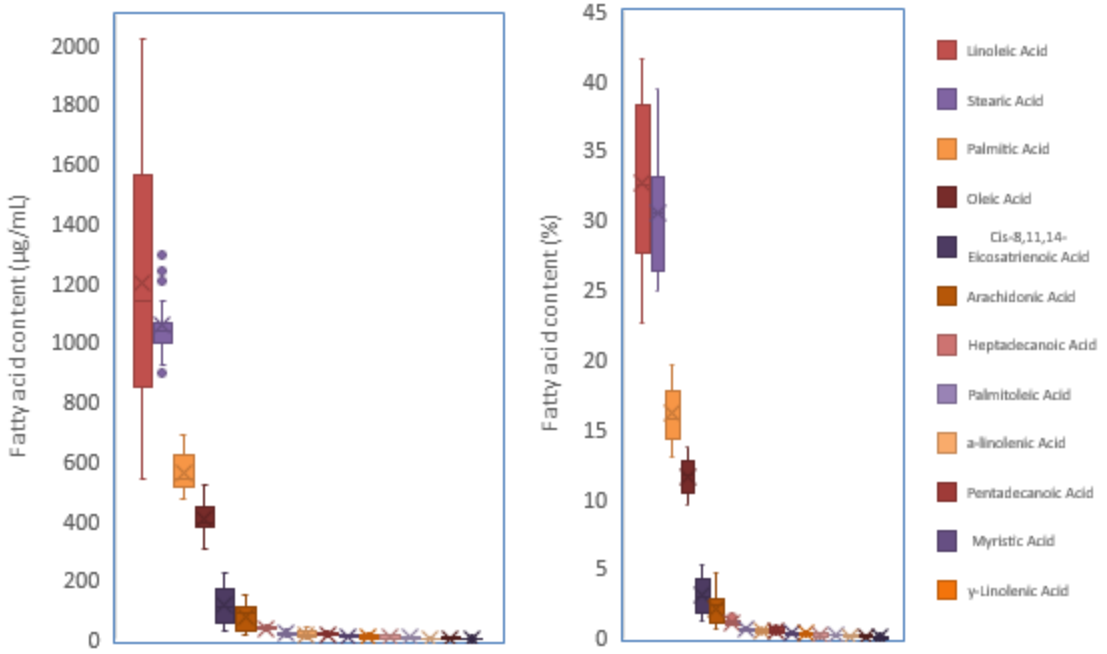


Figure 77: Variations of fatty acid composition of US batches. Important variations of linoleic acid among batches were observed whereas the concentration of other fatty acids remains stable. Mean ± standard deviation (n=24).

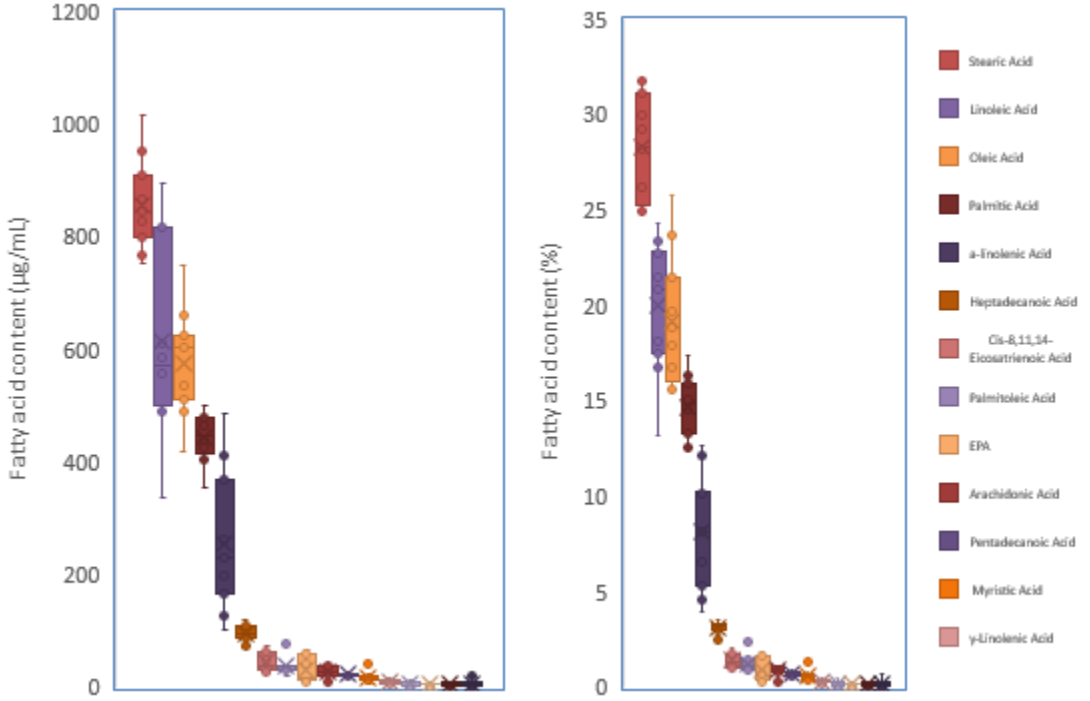


Figure 78: Variations of fatty acid composition of NZ batches. Mean ± standard deviation (n=11).

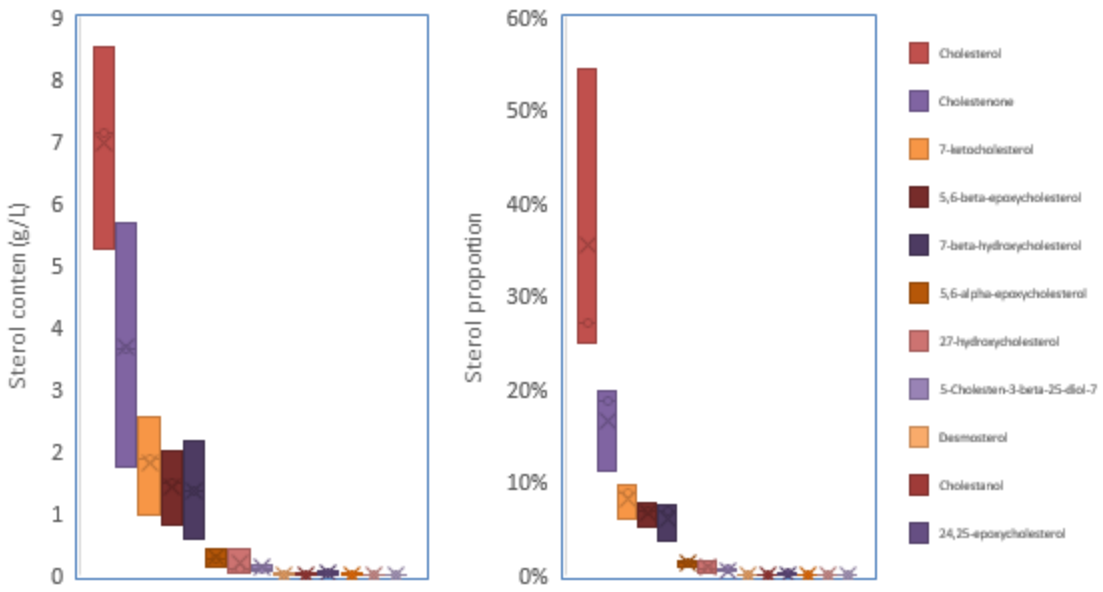


Figure 79: Variations of sterol composition of US batches. Mean ± standard deviation (n=3).

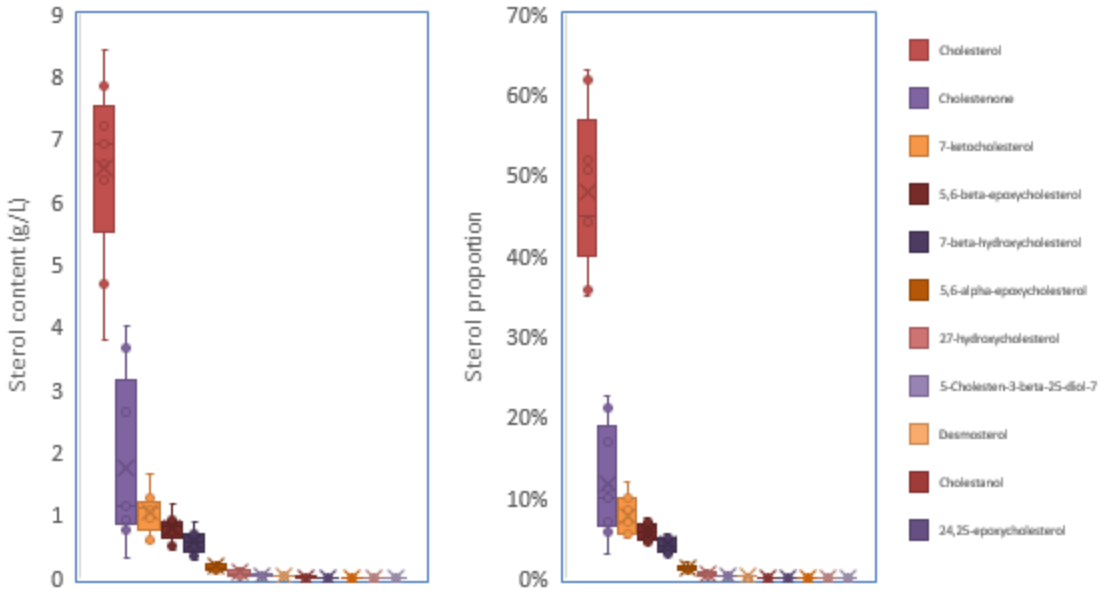


Figure 80: Variations of sterol composition of NZ batches. Mean ± standard deviation (n=9).

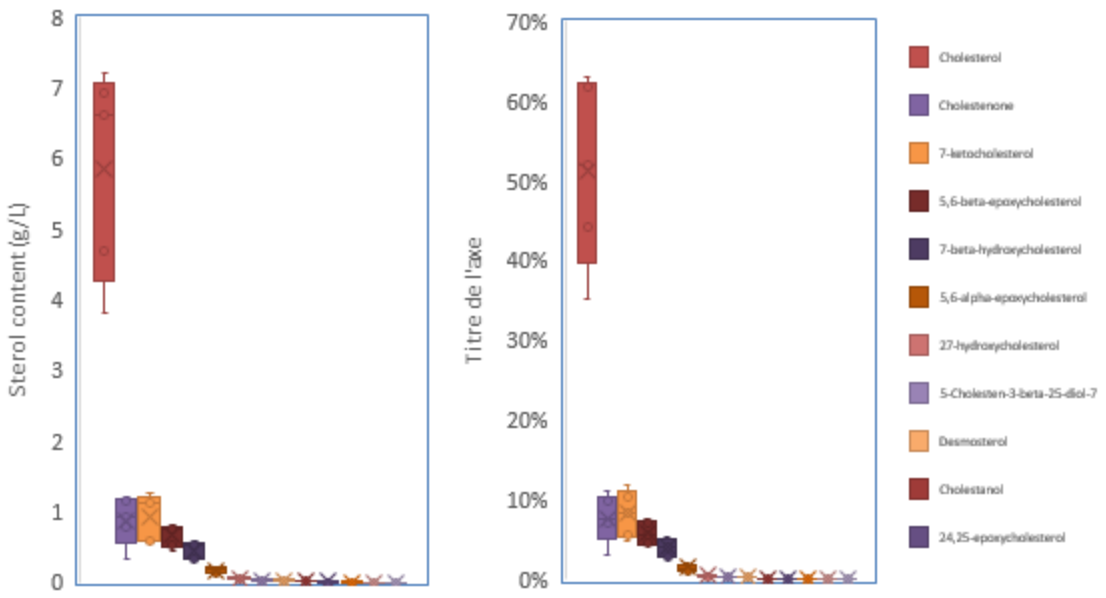


Figure 81: Variations of sterol composition of low-performing batches. Mean ± standard deviation (n=6).

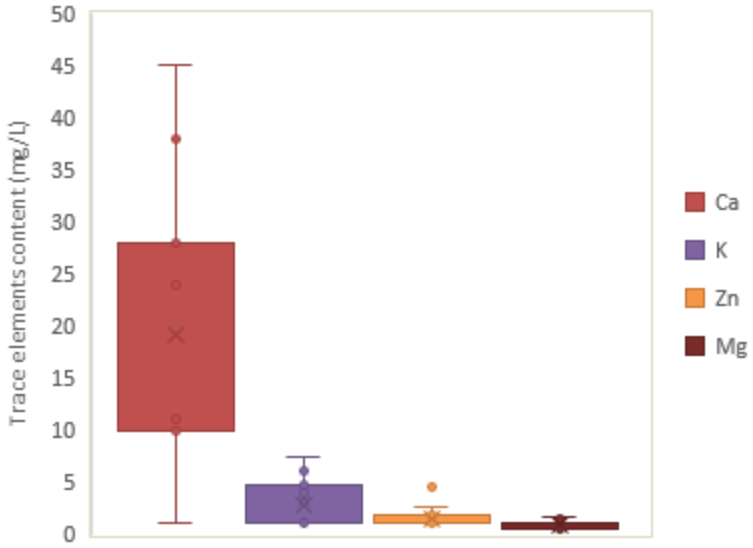


Figure 82: Variations of trace elements composition of the lipoprotein supplement. Other elements were below the limit of detection. Mean ± standard deviation (n=15).

	Phospholipids	Triglycerides	Free and esterified cholesterol	Free fatty acid
Total (mg/dL) / Fatty acid (%)	243 ± 12	22 ± 2	144 ± 7 (68% esterified)	0.35 (µmol/mL)
Classes				
Saturated (%)	47	42	32	39
Monoenoic (%)	27	27	43	28
Polyenoic (%)	25	23	22	29
Individual fatty acids				
Palmitic acid (%)	23.8	23.9	23.6	21.6
Palmitoleic acid (%)	1.5	2.4	10.2	3.1
Stearic acid (%)	20.8	13.5	3.9	14.3
Oleic acid (%)	23.9	24.3	32.4	24.5
Linoleic acid (%)	3.6	15	10.2	9.8
y-linolenic acid (%)	0.3	2.3	0.6	1.1
Arachidonic acid (%)	6.7	2.5	7.3	9.1
EPA (%)	5.2	1	0.6	1.9
DHA (%)	4.9	Not detected	1	3.3

Table 19: Lipid composition of a pool of commercially available FBS batches (Spector, Mathur, & Kaduce, 1980). Note that the total fatty acid composition of the serum may not be fully representative of serum performance because phospholipids, triglycerides, cholesteryl esters and free fatty acid have distinct functions. While free fatty acid and fatty acid from triglycerides provide cells with building block and oxidative substrate, phospholipids are components of lipoproteins and are susceptible to be exchanged or incorporated into the cell membrane. The composition of the individual lipid fractions differs one from another but their utilizations by the cells differ too.

Lipid	Molar ratio mbCD:lipid	Method used to determine the ratio	Reference
Cholesterol	1	Phase solubility diagram of cholesterol with different β-CD concentrations.	(Yunxiang, et al., 2022)
	2	UV-visible spectroscopy, 1H and 13C NMR, one-dimensional and rotating frame nuclear Overhauser enhancements.	(Ramaswamy & Soundar, 1998)
	3	X-ray diffraction, thermogravimetric analysis, differential scanning calorimetry and 13C NMR.	(Cloudy, et al., 1991)
Oleic acid	1	Calculations from UV-visible absorbance measurements of competitive inclusion system.	(Xue Feng, Ji Ju, & Rong Jun, 2011)
Linoleic acid	1 - 2	Thermogravimetry	(Tatsuji, Shuji, & Ryuichi, 1995)
	2	NMR one-dimensional nuclear Overhauser enhancement.	(Jyothirmayi, Ramadoss, & Divakar, 1991)
Arachidonic acid	2		
Eicosapentaenoic acid	3 - 4	Thermogravimetry	(Tatsuji, Shuji, & Ryuichi, 1995)
Docosahexaenoic acid	2 - 3	Quantities of CD determined by a phenol-sulfuric acid method. DHA analyzed by GC.	(Mikuni, Hara, Qiong, Hara, & Hashimoto, 1999)

Table 20: mbCD:lipid ratio reported in the literature. Note that the methods used to determine these ratios are different from one reference to another and could affect the result.

Oxidized lipoprotein concentration	Cell line	Effect on cells	Reference
10 µg/mL	CHO/CD36	Apoptosis	(Rusinol, et al., 2000)
10-100 µg/mL	Embyonic central nervous system cells	Necrosis and apoptosis	Sugawa, M., et al. (1997)
5 µg/mL	Human arising retinal pigment epithelia cell line	Decrease cell viability	Rodriguez, I. R., et al. (2004)
100-150 µg ApoB/mL	Human arterial smooth muscle cells	Apoptosis	(Bjorkerud & Bjorkerud, 1996)
100-150 µg ApoB/mL	Human fetal lung fibroblast cells	Apoptosis	(Bjorkerud & Bjorkerud, 1996)
200 µg ApoB/mL	Human umbilical vein EC line	50% of apoptosis after 24h	(Escargueil-Blanc, et al., 1997)
25 µg protein/mL	Human umbilical vein endothelial cells	18% apoptosis vs 5% without ox-LDL	(Harada-Shiba, Kinoshita, Kamido, & Shimokado, 1998)
200 µg ApoB/mL	Lymphoblastoid cell lines	Almost all cells died after 48 hours of incubation: 60-70% stained blue and 25-30% apoptotic cells	Escargueil-Blanc, I., et al. (1994)
200 µg ApoB/mL	Lymphoma cancer murine cell		Escargueil-Blanc, I., et al. (1994)
50-100 µg/mL	Rat adrenal pheochromocytoma cells	Apoptosis	(Draczynska-Lusiak, Chen, & Sun, 1998)

Table 21: Cytotoxicity of oxidized lipoprotein towards various cell lines.

Oxysterol	Concentration	Cell line	Reference
7β-hydroxycholesterol	5 µM	Lymphoma cancer murine cell	(Christ, Luu, Mejia, Moosbrugger, & Bischoff, 1993)
7β,25-dihydroxycholesterol	10-20 µM		
7-ketocholesterol	25 µM	Japanese white rabbit aortic smooth muscle cell line	(Nishio, Shinya, & Watanabe, 1996)
25-hydroxycholesterol	1 µM	T cell derived human leukemia lines	(Bansal, Houle, & Melnykovych, 1996)
25-hydroxycholesterol	20-40 nM	Human acute lymphoblastic leukemia cell line	(Bakos, Johnson, & Thompson, 1993)
20α-Hydroxycholesterol	30 nM		
7-ketocholesterol	450 nM		
7β-hydroxycholesterol	30 µM	Human monocytic cell lines	(Aupeix, et al., 1995)
25-hydroxycholesterol	30 µM		
25-hydroxycholesterol	12.5 µM	Murine thymocytes cell line	(Zhang, Xue, Jondal, & Sjövall, 1997)
27-hydroxycholesterol	12.5 µM		
25-hydroxycholesterol	3 µg/mL	CHO/K1	(Rusinol, et al., 2000)
7-ketocholesterol	40 µg/mL	Human monocytic THP-1 cells	Berthier, A.,et al., (2005)
7-ketocholesterol	100 µM	Promonocytic leukemia cells	Prunet, C., et al., (2005)
7β-hydroxycholesterol	50 µM		
25-hydroxycholesterol	5 µg/mL	Human aortic smooth muscle cells	Ares, M. P. S., et al., (1997)
25-hydroxycholesterol	300 nM	Human leukemic CEM cells	Ayala-Torres, S., et al., (1997)

Table 22: Induction of apoptosis by oxysterols on various cell lines.

6.2. List of abbreviations

13-HODE	13-hydroxyoctadecadienoic acid
15-HETE	15-hydroxyeicosatetraenoic acid
4-HNE	4-hydroxynonenal
9-HODE	9-hydroxyoctadecadienoic acid
ACN	Acetonitrile
ACF	Animal component-free
ALS	Alternative Lipid Supplement
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
CD	Cyclodextrin
CD 36	Cluster differentiation 36
CDLM	Chemically Defined Lipid Mixture 1
CHO	Chinese hamster ovarian cell
CLC	Cholesterol Lipid Concentrate
DHA	CIS-4,7,10,13,16,19-docohexaenoiacid
DME/DMEM	Dulbecco's Modified Essential Medium
DMSO	Dimethyl sulfoxide
DP	Drug product
DS	Drug substance
EPA	CIS-5,8,11,14,17-eicosapentaenoic acid
F12	Ham F12 medium
FA	Fatty acid
FBS	Fetal bovine serum
FFA	Free fatty acid
GPX4	Glutathione peroxidase 4
GSH	Glutathione
HDL	High Density Lipoprotein
IDL	Intermediate density lipoprotein
IMDM	Iscove's modified Dulbecco Medium
IsoPs	Isoprostanes
IVC	Integral Viable Cell Density
kDa	kilo Dalton
LDL	Low Density Lipoprotein
LOX-1	Lectin-like oxidized low-density lipoprotein receptor-1
mAb	Monoclonal antibody
mbCD	Methyl-β-cyclodextrin
MDA	Malonaldehyde
MEM	Minimal Essential Medium
Neuroprostanes	NeuroPs
OFAT	One-factor-at-a-time
OSBP	Oxysterol-binding proteins

oxLDL	Oxidized Low Density Lipoprotein
PBS	Phosphate-buffered saline
PCA	Principal Component Analysis
PDL	Population Doubling Level
PS	Phosphatidylserine
PUFA	Poly Unsaturated Fatty Acid
rHSA	Recombinant human serum albumin
ROS	Reactive oxygen species
RS	Response surface
sLDL	Synthetic low-density lipoprotein
ST	Shaked-tubes
TCD	Total Cell Density
TNF/TNFR	tumor necrosis factor/tumor necrosis factor receptor
UPLC	Ultra-performance liquid chromatography
USP	Up-stream process
UV	Ultraviolet
VCD	Viable Cell Density
VLDL	Very low-density lipoprotein

6.3. List of figures

6.4. List of tables

6.5. Acknowledgments

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AFFIRMATIONS

Erklärungen

§8 Abs. 1 lit. c der Promotionsordnung der TU Darmstadt

Ich versichere hiermit, dass die elektronische Version meiner Dissertation mit der schriftlichen Version übereinstimmt und für die Durchführung des Promotionsverfahrens vorliegt.

§8 Abs. 1 lit. d der Promotionsordnung der TU Darmstadt

Ich versichere hiermit, dass zu einem vorherrigen Zeitpunkt noch keine Promotion versucht wurde und zu keinem früheren Zeitpunkt an einer in- oder ausländischen Hochschule eingereicht wurde. In diesem Fall sind nähere Angaben über Zeitpunkt, Hochschule, Dissertationsthema und Ergebnis dieses Versuchs mitzuteilen.

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Die Arbeit hat bisher noch nicht zu Prüfungszwecken gedient.

Date: 21.09.2023

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Rémy Moisant

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