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Interaction of leachable model compounds and their impact on Chinese hamster ovary cell cultivation

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Abstract

The presence of leachables in biopharmaceutical processes using single-use technologies (SUT) is well known. For the detection and quantification of the latter, extractable studies of SUT are very common nowadays. Although a mixture of compounds is regularly found in extractable studies, research has only been carried out regarding the effect of individual compounds on cell culture and the cumulative effect of a mix of leachables has not been investigated yet. In this study, a set of leachable model compounds (LMCs) was chosen and the effect of the LMCs on a Chinese hamster ovary DG44 cell line producing an IgG antibody was investigated concerning cell growth, cell cycle distribution and productivity. It was shown that even if worst-case concentrations were used, the LMCs solely impact cell growth. Additionally, interaction studies revealed that the inhibiting effect of the mix is lower than the expected cumulative effect. A strong antagonism between the antioxidant butylated hydroxytoluene and the plasticizer Tris(2-ethylhexyl)trimellitate was found using an isobologram analysis.

KEYWORDS
antibody production, cytotoxicity, extractables and leachables, isobologram analysis, single-use systems

1 | INTRODUCTION

The fast growing market of biopharmaceuticals has increased the pressure to develop cost-effective and flexible production processes. Single-use (SU) technologies meet these demands and have become well established in the biopharmaceutical industry. However, concerns have arisen that potentially toxic substances migrate out of SU material into the process fluid and perturb the tightly regulated manufacturing process. To assess a possible impact of these compounds on the process, risk analysis are carried out and the migrated substances are identified and quantified.[1,2] Furthermore, in 2019 a tool was developed that simplifies the decision between the use of disposables versus conventional multi-use equipment.[3] One of the most prominent leachables with growth inhibiting effects on cell cultures is bis(2,4-di-*tert*-butylphenyl)phosphate (bDtBPP), a degradation product from the antioxidant Irgafos® 168, found in gamma-irradiated polyethylene bioreactor bags.[4] Since then, many efforts have been made to develop test systems for the early identification of potential critical leachables from SU material. Consequently, biocompatibility testing recommendations for SU material have been published,[5,6] which enabled effective optimization of polymeric film formulations without any negative impact on cell growth[7,8] and productivity.[9] Apart from potential leachable induced negative effects on upstream processes, a possible harmful impact on the drug product has to be examined to ensure patient safety. A recent study by Hauk et al[10] demonstrates that SU components used in downstream processes can be sinks of leachables. Moreover, Paudel et al[11] describe that leachables can be removed from the process fluid upon contact

with Chinese hamster ovary (CHO) cells, too, indicating an interaction mechanism between the latter and the leachables. Even if the impact of well-known leachables on cell cultures is described,[12,13] little is known about the effect of leachables on the cells' constitution and productivity. The leachable 3,5-Dinitro-bisphenol A, found in extracts of polycarbonate flasks, is known to cause a cell cycle arrest in CHO-S cells.[14] Since the cell cycle distribution of a culture can impact the productivity,[15] cell cycle arresting substances can have an impact on the production performance. Interestingly, Kelly et al[16] describe that even if the cell cycle distribution of CHO cells is not altered upon treatment with the leachable bD*t*BPP, a reduced IgG productivity was observed. However, based on the data presented in the publication, it can be assumed that the reduction in productivity may be attributed to a decreased viable cell density (VCD) and the cell-specific productivity appears to be not impaired.

A bioreactor assembly usually consists of different polymeric materials. Thus, it can be anticipated that a mixture of different leachables can be found in the culture broth. For this reason, it is of utmost importance to study the combined effect of a set of leachables on production processes. In this study, we have examined the impact of a set of leachables on an IgG production process that potentially migrate out of different SU materials. The leachables used in this study are called leachable model compounds (LMCs), each of them representing an own class of organic additive (e.g., antioxidants, plasticizers). First, literature research was carried out to define concentrations of the LMCs from extractable analysis, representing a realistic worst-case approach with exaggerated extraction conditions. The toxicity of the LMC-mixture (LMC-mix) was then assessed in order to estimate whether these concentrations are suited for a long-term study. Thereafter, a fed-batch cultivation was performed in shake flasks and the effect of the LMC-mix on cell growth, viability and productivity was examined. Furthermore, the characterization of apoptosis and cell cycle distribution was carried out. Additionally, toxicity of each individual compound was assessed in batch experiments. To gain a deeper understanding of how leachables interfere with each other, possibly resulting in synergistic or antagonistic effects, toxicity studies with different leachable combinations were performed.

1.1 | Selection of LMCs

For this study, a subset of leachables was chosen as model compounds that represent typical substances commonly detected in extractable analysis of polymeric material and are commercially available. These selected LMCs (Table 1) are different in terms of chemical characteristics (i.e., molecule size and lipophilicity expressed as the log of the partition in an octanol/water system, log $K_{O/W}$). The concentrations of the LMCs were found under exaggerated extraction conditions and thus, represent a worst-case scenario in this study. bD*t*BPP is built during gamma-irradiation of polyethylene-based films of SU bioprocessing materials.[4] Recently, we have described that degradation products from other antioxidants used in SU bioprocessing material can be built by the same degradation pathway and have a

TABLE 1 Overview of the selected leachable model compounds and chemical characteristics

Leachable	Compound class	Log $K_{O/W}$	Molecular weight (g/mol)	Concentration found and material
bis(2,4-di-*tert*-butylphenyl) phosphate (bDtBPP)	Degradation product of the secondary antioxidant Irgafos® 168	<3.32[11]	474.6[26]	0.1 mg/L, polyethylene SU bioreactor bags[13]
Butylated Hydroxytoluene (BHT)	Antioxidant	5.1[27]	220.35[27]	20 mg/L, pharmaceutical chlorinated butylrubber[28]
Acetophenone	Solvent	1.63[27]	120.15[27]	30 mg/L, medical polystyrene[21]
Tris(2-ethylhexyl) trimellitate (TOTM)	Plasticizer	5.94[27]	546.78[27]	100 mg/L, PVC tubing material (internal analysis)

comparable effect on cell growth.[17] Butylated hydroxytoluene (BHT) is a very common phenolic antioxidant used in different fields of application, for example, for food packaging material or pharmaceuticals.[18-20] Acetophenone is a solvent used in coating, ink and adhesives and found as a volatile after gamma irradiation of medical polymeric material.[21] Tris(2-ethylhexyl)trimellitate (TOTM) is a plasticizer used in polyvinyl chloride (PVC) material and a potential substitute to the most common plasticizer bis(2-ethylhexyl)phthalate (DEHP). The endocrine disrupting activity of DEHP is well described[22-24] and therefore, the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) recommends to replace DEHP if possible.[25]

2 | EXPERIMENTAL

2.1 | Cell cultivation

2.1.1 | Pre culture

An in-house IgG1 producing CHO DG44 cell line was used throughout all experiments. Pre cultures were thawed at passage number 10 and cultivated in chemically defined Seed Medium (Sartorius Stedim Cellca, Germany). In fed-batch cultivations, 20 nM methotrexate (CAS 59-05-2, Merck KGaA, Germany) was added to passage 10–12. Cultivations were performed in 500 ml non-baffled Erlenmeyer flasks (Corning, USA).

2.1.2 | Cultivation conditions and media used

Experiments were carried out in CHO production medium (PM). Additionally, two feed media (feed medium A, FMA, and feed medium B, FMB) were added in the fed-batch cultivation experiments. All media used are chemically defined and belong to the CellcaCHO Media System (Sartorius Stedim Cellca, Germany). Both batch and fed-batch cultivations were performed in 100 ml baffled glass shake flasks with vented cap (Glasgerätebau Ochs, Germany) in a Certomat® CT plus (Sartorius Stedim Biotech GmbH, Germany) at 7.5% CO_2 and 36.8°C. Shaking speed was adjusted to 120 rpm with an orbital diameter of 50 mm in 80% humidified atmosphere. Cultures were seeded at 0.2×10^6 cells/ml unless stated otherwise.

2.2 | Preparation of LMC mixture

Butylated hydroxytoluene (BHT, CAS 128-37-0, Merck KGaA) and Bis (2,4-di-*tert*-butylphenyl)phosphate (bD*t*BPP, CAS 69284-93-1, ARC Scientific, USA) were each predissolved in DMSO (CAS 67-68-5, Merck KGaA) and mixed in a glass vial. TOTM (CAS 3319-31-1, Merck KGaA) and acetophenone (CAS 98-86-2, Merck KGaA) were added directly to the mix without pre-dissolving.

2.3 | Cell growth and flow cytometry analysis

VCD and cell viability were determined using a Cedex HiRes Analyzer (Roche, DE) based on trypan blue exclusion. Flow cytometric analysis was performed with an iQue™ Screener Plus (IntelliCyt® Corporation, USA). Data were processed and analyzed using the Forecyt® software edition 6.2 (IntelliCyt® Corporation).

For characterization of apoptosis, the Multicyt® 4-Plex Apoptosis Kit was used (IntelliCyt® Corporation) according to the manufacturer's instructions. This multi parameter assay allows 4 different apoptosis measurements per sample (caspase 3/7 activity, surface expression of phosphatidylserine [PS] by annexin V binding, mitochondrial membrane polarization and viability). Cell cycle distribution analysis was assessed using propidium iodide (PI) (Thermo Fisher Scientific Inc., USA) that intercalates into DNA, yielding a fluorescence intensity proportional to the DNA amount per cell.

2.4 | IgG quantification

After certain time intervals, 1 ml samples of the fed-batch experiment were centrifuged at 10,000g for 10 min and the supernatant was stored at −18°C until IgG analysis (concentration and binding capacity). Supernatant was filtered through a 0.2 μm Minisart (Sartorius, Germany) and transferred into glass vials. Overall IgG concentration in the cell broth was determined by size exclusion on an Ultimate 3000 RS (Thermo Fisher Scientific Inc.), using a Yarra 3 μm SEC 3000 column (Phenomenx Inc., USA). The system was primed with running buffer (0.1 M Na_2SO_4, 0.05 M NaH_2PO_4, 0.05 M Na_2HPO_4) at a flow rate of 5 ml/min. Five microliters of each sample was injected into the system. Separation flow rate was set to 1 ml/min and a pressure limit of 180 bar was chosen. Samples were measured at 220, 260 and 280 nm. Antibody concentration was determined by automatic peak integration. For estimation of antibody binding capacity, an indirect enzyme-linked immunosorbent assay (ELISA) detecting IgG1 molecules (Abcam, USA) was carried out according to the manufacturer's specifications. The cell-specific productivity q_P was calculated according to Equation (1), with Δc being the concentration difference between two time points and the IVCC the integral viable cell concentration between these two time points.[29]

$$q_P = \frac{\Delta c}{\mathrm{IVCC}} \quad (1)$$

3 | METHODS

3.1 | Dose–response and spiking experiments

As a pretest, the toxicity of the LMC-mixture was estimated by executing a dose–response experiment with five different concentrations of the LMC-mix. The published concentration of the respective LMC (Table 1) was chosen as the center point. Two concentrations below

and above were tested as well. In order to classify the toxicity of the LMC-mix compared to the individual compounds, CHO DG44 cultures were spiked with bDtBPP, acetophenone, BHT and TOTM each, in the respective concentrations of the mix (Figure 1, bottom). A reference without any substance spiked and a positive control for reduced cell growth (2% DMSO, vol/vol) was included in both experiments. After spiking, cells were cultivated for 3 days. Samples for analysis were taken every day. The specific growth rate μ was calculated according to formula (2) for each condition in the exponential growth phase and normalized to the reference.

$$\mu = \frac{\ln(N_1) - \ln(N_0)}{t_1 - t_0}, \quad (2)$$

where N (cells/ml) is the VCD and t is the cultivation time, indices indicating different time points.

3.2 | Interaction study

For studying interaction effects of the leachables on cytotoxicity, an interaction study was carried out. For this reason, 2 ml of a 1×10^6 cells/ml cell suspension was pipetted into each well of a 24-well plate (Greiner, Germany) and different combinations of the LMCs were spiked to the cells. The concentrations used for cell growth assay are stated in Table 1. Cell viability was analyzed 2 h after spiking, because a strong impact on viability was already observed at that point. During incubation, the plate was shaken at 350 rpm at room temperature to prevent sedimentation of the cells. Pretests have shown that a lack of CO_2-gassing and a lower temperature does not influence the viability of the cells during the incubation time (data not shown).

3.3 | Fed-batch cultivation

In order to observe the effect of the LMC-mix on CHO cell cultivation over a longer cultivation period, an 8-day fed-batch cultivation was carried out. The feeding started on Day 3, a glucose feed was applied from Day 5 on once the glucose level dropped below 5 g/L. The shake flasks were inoculated with 0.3×10^6 cells/ml. Samples for VCD and viability analysis were taken daily, samples for characterization of apoptosis, cell cycle distribution and IgG concentration were taken on Days 0, 3, 6 and 8. The specific growth rate of each condition was normalized to a reference fed-batch in parallel without leachables spiked.

3.4 | Flow cytometry analysis

3.4.1 | Apoptosis

For the investigation of the leachable induced apoptosis behavior of CHO DG44 cells, an apoptosis assay (Multicyt® 4-Plex Apoptosis Kit, IntelliCyt® Corporation) was performed. The preparation of the dye mixture was according to the manufacturer's instructions. Samples were taken from the cultivation at different time points. VCD was adjusted to 2×10^6 cells/ml and an equal amount of the dye mixture was added to each cell suspension resulting in a final VCD of 1×10^6 cells/ml. Samples were incubated for 1 h in 5% CO_2, at 37°C and 80% humidified atmosphere and afterwards transferred to U-bottom 96-well plates (Greiner, Germany) for analysis in the flow cytometer. For control of successful staining, a positive control of dead cells was incorporated by heat-inactivation at 95°C for 5 min. Additionally, an unstained sample was included to evaluate background fluorescence of the cells. Depending on the fluorescence signals, cells were classified as dead, late, early or non-apoptotic according to Vermes et al[30] (Table 2).

3.4.2 | Cell cycle distribution

In order to assess the cell cycle distribution by equimolar staining of DNA, PI staining was performed. Since this dye is not able to penetrate intact cell membranes, cells were fixed with ice cold 70% (vol/vol) ethanol for 30 min on ice. After two washing steps with PBS, cells were transferred to 1 µg/ml PI solution containing 110 Units of RNase A (AppliChem GmbH, Germany) for every 1 million cells. After thorough mixing, cells were incubated for 30 min in the dark before analysis was carried out on the iQue™ Screener Plus (IntelliCyt® Corporation).

3.5 | Isobologram analysis

For estimation of interaction effects between the LMCs based on graphical analysis, an isobologram evaluation was created. First, dose–response experiments were carried out and different EC_i-values were calculated after 3 days of incubation. The EC_i value indicates at which concentration the cell growth is inhibited to ith extent (%). Then, an isobologram was created as described by Berenbaum.[31] This graph allows to study the combined effects of substance mixtures and characterize them as additive, synergistic or antagonistic, as is frequently used in the field of pharmacology.[32] If the graph is a straight line, there is additivity between the two substances. If the curve is concave, the effect is called synergistic and a convex curve is called antagonistic.[31] However, in order to classify the interactions in a more

TABLE 2 Fluorescence marker used for apoptosis assay, the related cellular signals detected and affected phases of apoptosis

Marker in assay	Cellular signal	Phase of apoptosis
Viability	Permeabilized cell membrane	Dead
Annexin V	Presentation of phosphatidylserine on the cell surface	Late
Mitochondrial damage	Mitochondrial depolarization	Early
Caspase	Caspase activation	Early

Note: Classification was made according to Vermes et al.[30]

quantitative way, combination indices (CI) based on Chou et al were calculated (Equation 3).[33]

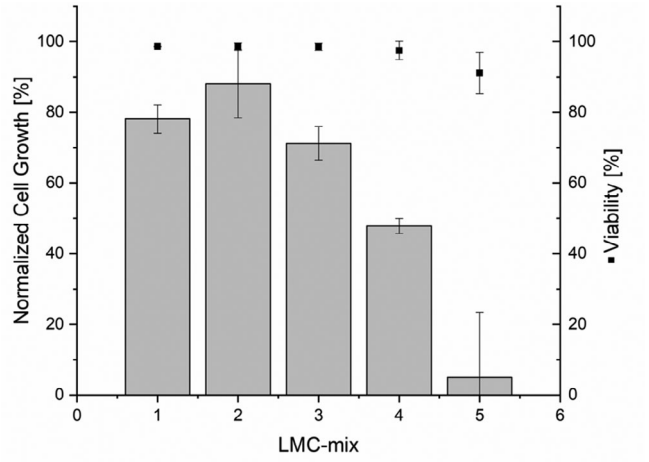
$$\mathrm{CI}\,(\mathrm{EC}_i) = \frac{\mathrm{EC}_i \text{ of substance A in combination}}{\mathrm{EC}_i \text{ of substance A}} + \frac{\mathrm{EC}_i \text{of substance B in combination}}{\mathrm{EC}_i \text{of substance B}} \quad (3)$$

In case of synergism, the CI is ≤0.9, for additivity 0.91 ≥ CI ≤ 1.09 and for antagonism CI ≥ 1.10.[33]

4 | RESULTS AND DISCUSSION

4.1 | Growth inhibiting effect of the LMC-mix

A potential toxic effect of the LMC-mix on CHO DG44 cultures was assessed by performing a dose–response experiment. The specific growth rate μ normalized to the reference is affected by the LMC-mix in a dose-dependent manner (Figure 1). A growth inhibiting effect can be observed with increasing concentrations of the LMC-mix. Interestingly, after a first drop of cell growth at LMC-mix 1 to 78%, the growth seems to increase again with higher compound concentrations to 88% at LMC-mix 2. However, the error bar of LMC-mix 2 is quite high (10%) and the difference to LMC-mix 1 can be regarded as not significant. Viability measurements indicate that, even if cell growth is affected in a dose-dependent manner, the effect of the LMC-mix to the cells can be characterized rather cytostatic than cytotoxic. This is because cell growth is inhibited, while viability of treated samples is not impaired. The concentrations of the leachables in LMC-mix 3, which were actually found in extractables and leachables studies (Table 1), cause a decreased cell growth (71%), while the viability remains above 97%.



Compound [mg/L]	1	2	3	4	5
bD*t*BPP	0.025	0.05	0.1	0.2	0.4
TOTM	25	50	100	200	400
BHT	5	10	20	40	80
Acetophenone	7.5	15	30	60	120

FIGURE 1 Top: The dose–response dependent growth inhibiting effect of the leachable model compound mixture (LMC-mix) on CHO DG44 cells after 3 days of treatment (bars), viability measurements are shown as black squares. Error bars represent SD of three replicates. Bottom: Concentrations (mg/L) of the different LMC-mix

However, exposure time of cells to leachables in a bioreactor assembly is generally longer in industrial cultivation processes, for example, for the production of monoclonal antibodies.[34] To study possible long-term effects of LMCs on cells, a fed-batch process with LMC-mix 3 was performed (Figure 2a). After 8 days, the viability of the reference dropped below 70% (harvest criterion) and the cultivation was therefore stopped. During the cultivation run, it can be observed that cell growth of cultures treated with the LMC-mix is inhibited and the peak cell density reaches only about 50% of the reference. At the same time, viability is not affected by the LMCs. The data show the same trend observed in the spiking experiment (Figure 1) and the effect of LMC-mix 3 on CHO cell cultures can be described as cytostatic rather than cytotoxic, since only the cell growth is affected and the viability remains high.

To gain a better understanding of the mode of action of the LMC-mix on CHO cells, apoptosis was assessed after 3, 6 and 8 days of cultivation in the fed-batch process (Figure 2b). On Days 3 and 6, almost no apoptosis signal was measured in both the reference and the LMC sample. Since about 7% of the untreated reference sample are characterized apoptotic, it can be assumed that the LMC-mix does not induce apoptosis signals that are significantly different to the reference at the tested time points. The apoptosis induction in the reference can be attributed to culture-related stress factors such as substrate limitations and toxic metabolites.

4.2 | Productivity of LMC-treated cultures

As Kelly et al have demonstrated, 0.05 mg/L bD*t*BPP induces a significant decrease in IgG productivity in CHO-DP12 cells.[16] Therefore, productivity of CHO DG44 cultures after LMC-treatment was assessed using size-exclusion chromatography and ELISA. An IgG titer about 58% lower than the reference was observed for the LMC culture at Day 8 of the fed-batch run (Figure S1a). This is not surprising, because it can be anticipated that fewer cells produce less antibodies. Consequently, the cell-specific productivity was assessed, as this parameter contains more information regarding the single cell's ability to produce IgG in a given time interval. The time intervals of interest were chosen as the following: Day 0–Day 3 (lag phase and start of log phase), Day 3–Day 6 (log-phase) and Day 6–Day 8 (stationary phase and death phase). The comparison between reference and LMC sample shows that there is no significant difference in cell-specific productivity (q_P) in all three time intervals (Figure 3a). Hence, the lower IgG titer in the LMC culture can be exclusively attributed to a lower cell number producing the antibody, which is also indicated by the fact that the cell cycle distribution of treated cells is not much different to

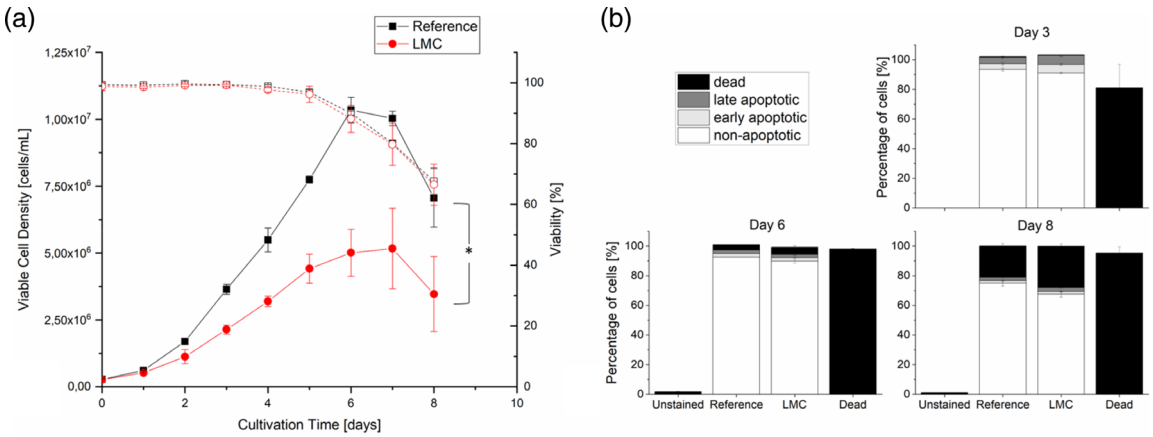


FIGURE 2 Fed-batch CHO DG44 cultivation spiked with the LMC-mix 3. (a) Growth of Reference (black squares) and leachable model compound (LMC)-spiked cultures (red circles) by means of viable cell density (cells/ml) and viability (%). Viability data are presented as dotted lines. (b) Apoptosis characterization 3, 6 and 8 days after spiking. Cells were defined as non-apoptotic (white), early (light gray), late apoptotic (dark gray) or dead (black) as described in Section 3. A positive control of dead cells was incorporated by heat-inactivation at 95°C for 5 min. Sample size was $n = 3$ each, error bars represent the SD (*$p < 0.05$, Student's t-test)

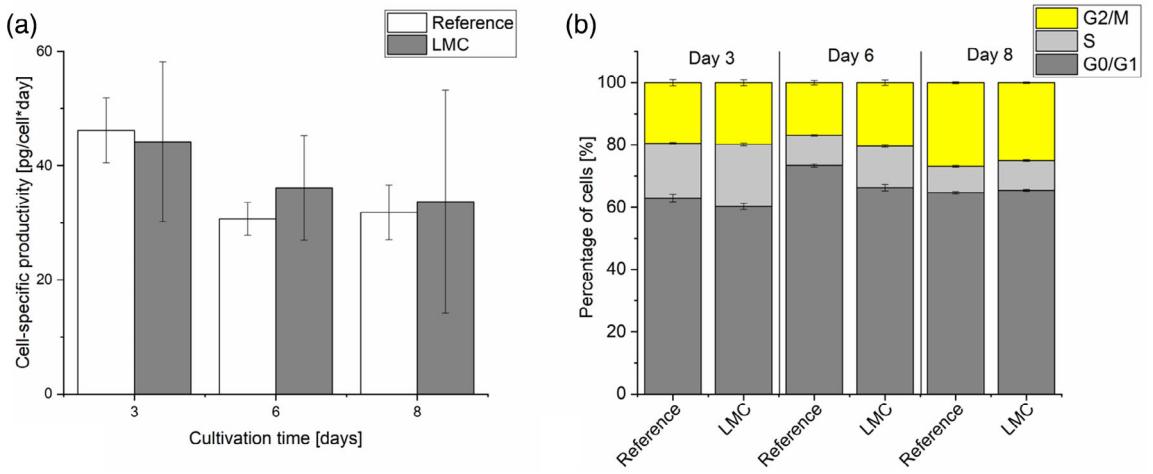


FIGURE 3 Influence of leachable model compound mixture (LMC-Mix) 3 on CHO DG44 cells. (a) Cell-specific IgG production (pg/cell × day) of the reference (white) and LMC-treated CHO DG44 cultures (gray) on Day 3, 6 and 8. (b) Cell cycle distribution on Days 3, 6 and 8 of the reference and LMC-treated cells assessed by flow cytometry. The share of cells in G_0/G_1-phase are illustrated in dark gray, cells in S-phase in light gray and G_2/M-cells are presented in yellow. Error bars represent the SD of three replicates

the reference (Figure 3b). Additionally, the binding affinity of the IgG does not change during the cultivation run and there is no effect of the LMC-mix on the binding affinity (Figure S1b).

4.3 | Interactions of LMCs

Cell growth in the presence of a toxic single leachable can have a different impact on cell growth than in the presence of a mix of leachables. Combinations of leachables, as used in this study, may exhibit synergistic or antagonistic effects on cell growth caused by interactions as described for multiple drug treatment.[35] To elucidate whether there is any interaction between the leachables, CHO DG44 cultures were spiked with each leachable individually and an interaction study was conducted with concentrations provided in Table 1. As illustrated in Figure 4, 0.1 mg/L of the leachable bDtBPP caused a reduced cell growth of only 65% ± 12%, while the viability was not affected. This observation is in line with a study by Kelly et al,[16] who report that the EC_{50}-value of bDtBPP has an impact on cell growth without apoptosis induction. It is assumed that bDtBPP causes oxidative stress. At higher concentrations this leads to apoptosis and consequently, lower viabilities. Until then, however, the cell can cope with the oxidative stress by overexpression of specific proteins, such as Hypoxia upregulated protein 1 (HYOU1).[16] Surprisingly, in contrast of bDtBPP, TOTM and acetophenone no living cells were detected in 20 mg/L BHT-treated cultures (Figure 4). TOTM and acetophenone did not impair cell growth to a great extent, while BHT showed the most severe effect. This observation is even more striking considering that the presence of BHT in the

LMC-mix did not cause such effects (Figure 1). These findings indicate that there is an antagonistic effect, that is, the toxicity of BHT is weakened in the presence of the other LMCs. The effect of bD*t*BPP on cell growth is nearly identical to the effect of the LMC-mix.

To assess which leachable may mediate the antagonistic effect for BHT, CHO cells were spiked with BHT in combination with bD*t*BPP, acetophenone and TOTM, respectively. Results are presented in Figure 4b by means of viability loss (%) 2 h after leachable

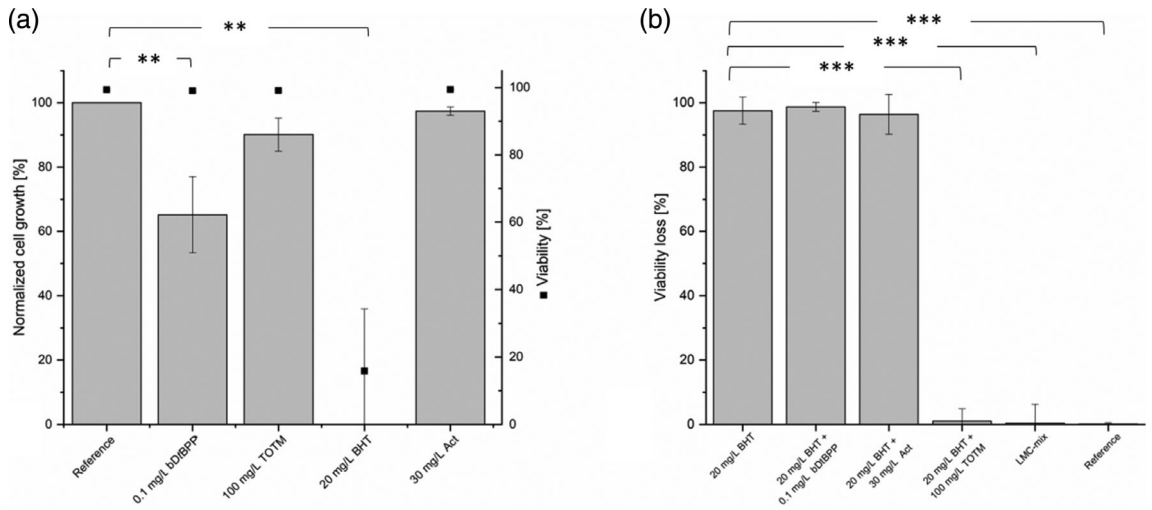
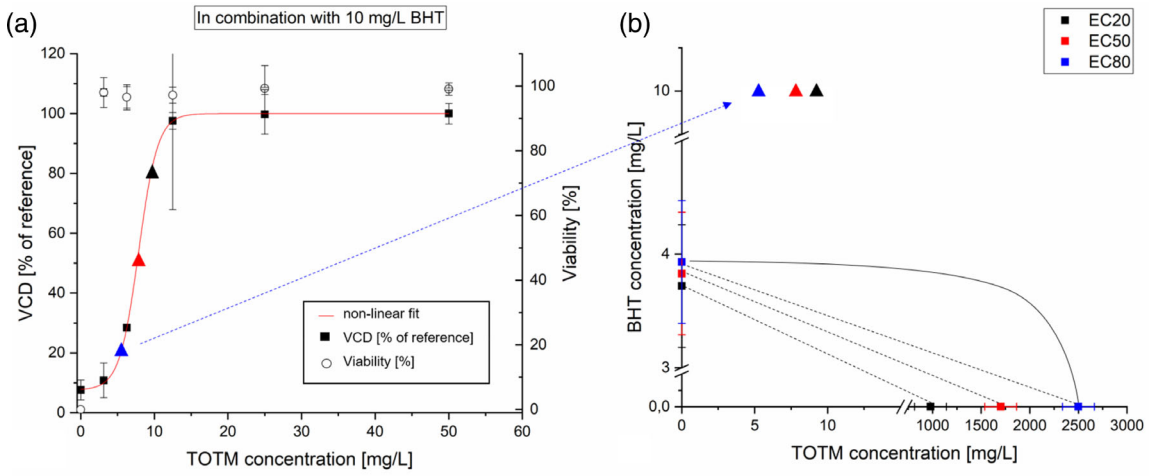


FIGURE 4 (a) Growth inhibiting effects of the individual leachable model compounds (LMCs) on CHO DG44 cultures after 3 days of cultivation presented as normalized cell growth (%) in gray bars and viability (%) in black squares. (b) Toxicity of BHT only and in combination with the other LMCs by means of viability loss (%) in CHO DG44 cultures 2 h after spiking. Error bars represent SD of triplicates. Act = acetophenone (**$p < 0.01$, ***$p < 0.001$, Student's *t*-test)



Effective Concentration (EC) [mg/L]	TOTM	BHT	TOTM in combination with 10 mg/L BHT	Combination Index (CI)
EC_{20}	978.77 ± 164.04	3.72 ± 0.54	9.53 ± 0.04	2.69
EC_{50}	1,700.55 ± 164.04	3.83 ± 0.54	7.66 ± 0.04	2.61
EC_{80}	2,498.88 ± 164.04	3.93 ± 0.54	5.46 ± 0.04	2.54

FIGURE 5 Combination effects of BHT and TOTM on CHO DG44 cells after 3 days of incubation. (a) Dose–response graph with 10 mg/L BHT and varying concentrations of TOTM. Viable cell density (VCD) is depicted as black squares and the viability as circles. EC_{20} (black), EC_{50} (red) and EC_{80} (blue) values, derived from a non-linear fit using the Hill equation (red line), are illustrated as triangles. (b) Isobologram for the interaction of BHT and TOTM. The dotted lines illustrate additive effects, the black line visualizes the shape of an exemplary antagonistic curve at the EC_{80} value. The EC values of combination effects from A are represented as triangles, as indicated in the example of the EC_{80} with the blue arrow. Bottom table: EC_{20}, EC_{50} and EC_{80} values were derived from the respective dose–response experiments with BHT and TOTM individually (Figure S2) and in combination. Error bars represent SD of $n = 3$ replicates

spike with respect to the initial viability. The plasticizer TOTM seems to abolish the toxic effect of the antioxidant BHT completely, whereas both combinations BHT and bD*t*BPP as well as BHT and acetophenone induce nearly 100% viability loss. Based on these data, an antagonistic effect between BHT and TOTM can be assumed. To further investigate this assumption, the toxicities of both substances were determined by calculating EC_{20}, EC_{50} and EC_{80} values from dose–response experiments (Figure S2). To study combination effects, 10 mg/L BHT and varying concentrations of TOTM were spiked to CHO DG44 cultures. Figure 5a shows the normalized VCD after 3 days of cultivation. Using these data, an isobologram (Figure 5b) was created as described in chapter 3.5. The dotted lines between the equi-effective concentrations of BHT and TOTM individually represent theoretically combined effects in the case of additivity. The shape of a typical antagonistic curve is depicted as a hyperbolic black line using the example of the EC_{80} value. It is obvious that the calculated equi-effective concentrations for 20%, 50% and 80% VCD of the combined effect (illustrated as triangles) is above the black line, which means that the inhibitive effect of BHT is completely compensated by TOTM. These results fit well to the previously reported findings (Figure 4). However, it must be highlighted that this issue only applies until TOTM causes a growth inhibition itself, which starts at a TOTM concentration of about 1000 mg/L. Based on this, it can be anticipated that the graph of the combination effect between BHT and TOTM would rather look like a parabola instead of the typical hyperbolic shape. The CI, calculated according to Equation (3), is >2.5 for all three EC values, which implies a strong antagonism. A possible explanation is that both substances compete for the same cellular binding sites. Future experiments, aiming at identification of cellular targets for BHT and TOTM, should be carried out to gain deeper insights into this finding.

5 | CONCLUSION

Former studies on leachables found in SU systems have focused on detrimental effects of these substances in biopharmaceutical processes. These studies, however, have only focused on one leachable individually, neglecting the fact that in a SU system a mix of different leachables migrates into the process fluid. Therefore, our study characterizes the effect of a mixture of selected LMCs, which are actually found in extractable analysis, on a CHO production process. Interestingly, even if we found growth inhibiting effects of the LMC mix in both the dose–response experiments and a fed-batch run, the cell-specific productivity was not impaired. These findings are contrary to the data presented by Kelly et al, who describe reduced productivity after bD*t*BPP treatment of CHO cultures.[16] Since worst-case approaches were applied for defining the LMC concentrations used in this study, it can be anticipated that under normal process conditions the impact of leachables on CHO cell cultures is even lower. Especially during a cultivation carried out in perfusion mode, the impact of leachables can be expected to be negligible due to lower leachable concentrations, as the cultivation medium is exchanged several times during a process. If any, we expect a possible impact of leachables at the beginning of a process, where the leachable concentration per cell is relatively high. Thus, the impact will likely decrease over time with growing cell numbers. Since no effect on cell cycle, apoptosis or cell viability was observed, long-term effects of LMCs on CHO cell cultivation are not anticipated. Surprisingly, we found that the inhibiting effect of LMC mix on a CHO cell culture can be lower than their expected cumulative effect and a strong antagonism is described for the combination of BHT and TOTM.

Although we have shown that one LMC can weaken the toxicity of another one, it must be stressed that this effect was only observed with one cell line and possible effects of cultivation medium were not considered. For a realistic assessment of the migration behavior of process-equipment related leachables, the solubility of the compound and the surface-to-volume ratio of the related SU material in the process must be taken into consideration. Therefore, the exposure estimation based on extractables and leachables data is of high importance for a thorough risk-analysis of leachables in a biopharmaceutical process.

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AUTHOR CONTRIBUTIONS

Dana Budde: Conceptualization; data curation; investigation; methodology; writing-original draft; writing-review & editing. **Gian-Luca Albano:** Formal analysis; methodology. **Thomas, Prof. Dr. Noll:** Writing-review & editing. **Elke Jurkiewicz:** Supervision.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the publication of this article.

DATA AVAILABILITY STATEMENT

Data available on request from the authors

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REFERENCES

1. Ding W. Determination of extractables and leachables from single-use systems. *Chem Ing Tech*. 2013;85(1–2):186-196. https://doi.org/10.1002/cite.201200113.
2. Gao Y, Allison N. Extractables and leachables issues with the application of single use technology in the biopharmaceutical industry. *J Chem Technol Biotechnol*. 2016;91(2):289-295. https://doi.org/10.1002/jctb.4824.
3. Shirahata H, Hirao M, Sugiyama H. Multiobjective decision-support tools for the choice between single-use and multi-use technologies in sterile filling of biopharmaceuticals. *Comput Chem Eng*. 2019;122:114-128. https://doi.org/10.1016/j.compchemeng.2018.06.001.
4. Hammond M, Nunn H, Rogers G, et al. Identification of a leachable compound detrimental to cell growth in single-use bioprocess

containers. *PDA J Pharm Sci Technol*. 2013;67(2):123-134. https://doi.org/10.5731/pdajpst.2013.00905.
5. Eibl R, Steiger N, Fritz C, et al. Recommendation for leachables studies: standardized cell culture test for the early identification of critical films for CHO cell lines in chemically defined culture media. *Dechema*. 2014;1-22.
6. ASTM E3231-19. *Standard Guideline for Cell Culture Growth Assessment of Single-Use Material*. West Conshohocken, USA: ASTM International; 2019 www.astm.org.
7. Jurkiewicz E, Husemann U, Greller G, Barbaroux M, Fenge C. Verification of a new biocompatible single-use film formulation with optimized additive content for multiple bioprocess applications. *Biotechnol Prog*. 2014;30(5):1171-1176. https://doi.org/10.1002/btpr.1934.
8. Jachuck JR, Krishnathu SM, Landau JE, Ko HF, Bhatia R. Sensitivity of a PER.C6® cell line to bis(2,4-di-tert-butylphenyl)phosphate and evaluation of a new, biocompatible single-use film. *Biotechnol Prog*. 2020;36:e3060. https://doi.org/10.1002/btpr.3060.
9. Kelly PS, Dorival-García N, Paré S, et al. Improvements in single-use bioreactor film material composition leads to robust and reliable Chinese hamster ovary cell performance. *Biotechnol Prog*. 2019;35:e2824. https://doi.org/10.1002/btpr.2824.
10. Hauk A, Jurkiewicz E, Pahl I, Loewe T, Menzel R. Filtration membranes—scavengers for leachables? *Eur J Pharm Sci*. 2018;120(April):191-198. https://doi.org/10.1016/j.ejps.2018.04.044.
11. Paudel K, Hauk A, Maier TV, Menzel R. Quantitative characterization of leachables sinks in biopharmaceutical downstream processing. *Eur J Pharm Sci*. 2020;143:105069. https://doi.org/10.1016/j.ejps.2019.105069.
12. Kim JH. Analysis of the in vitro effects of di-(2-ethylhexyl) phthalate exposure on human uterine leiomyoma cells. *Exp Ther Med*. 2018;15(6):4972-4978. https://doi.org/10.3892/etm.2018.6040.
13. Hammond M, Marghitoiu L, Lee H, et al. A cytotoxic leachable compound from single-use bioprocess equipment that causes poor cell growth performance. *Biotechnol Prog*. 2014;30(2):332-337. https://doi.org/10.1002/btpr.1869.
14. Peng J, Zhao Y, Hong Y, et al. Chemical identity and mechanism of action and formation of a cell growth inhibitory compound from polycarbonate flasks. *Anal Chem*. 2018;90(7):4603-4610. https://doi.org/10.1021/acs.analchem.7b05102.
15. Ibarra N, Watanabe S, Bi J-X, Shuttleworth J, Al-Rubeai M. Modulation of cell cycle for enhancement of antibody productivity in perfusion culture of NS0 cells. *Biotechnol Prog*. 2003;19(1):224-228. https://doi.org/10.1021/bp025589f.
16. Kelly PS, McSweeney S, Coleman O, et al. Process-relevant concentrations of the leachable bDtBPP impact negatively on CHO cell production characteristics. *Biotechnol Prog*. 2016;32(6):1547-1558. https://doi.org/10.1002/btpr.2345.
17. Budde D, Maier TV, Jurkiewicz E, et al. Identification and evaluation of cell- growth-inhibiting bDtBPP-analogue degradation products from phosphite antioxidants used in polyolefin bioprocessing materials. *Anal Bioanal Chem*. 2020;412:4505-4518. https://doi.org/10.1007/s00216-020-02736-z.
18. Yehye WA, Rahman NA, Ariffin A, et al. Understanding the chemistry behind the antioxidant activities of butylated hydroxytoluene (BHT): a review. *Eur J Med Chem*. 2015;101:295-312. https://doi.org/10.1016/j.ejmech.2015.06.026.
19. Babich H. Butylated hydroxytoluene (BHT): a review. *Environ Res*. 1982;29(1):1-29. https://doi.org/10.1016/0013-9351(82)90002-0.
20. Ash M, Ash I. *Handbook of Plastic and Rubber Additives*. 2nd ed. Endicott, NY: Synapse Information Resources; 2005.
21. Buchalla R. Radiolysis products in gamma-irradiated plastics by thermal desorption–gas chromatography–mass spectrometry; 2000.
22. Nohynek GJ, Borgert CJ, Dietrich D, Rozman KK. Endocrine disruption: fact or urban legend? *Toxicol Lett*. 2013;223(3):295-305. https://doi.org/10.1016/J.TOXLET.2013.10.022.
23. Martinez-Arguelles DB, Culty M, Zirkin BR, Papadopoulos V. In utero exposure to di-(2-ethylhexyl) phthalate decreases mineralocorticoid receptor expression in the adult testis. *Endocrinology*. 2009;150(12):5575-5585. https://doi.org/10.1210/en.2009-0847.
24. Chen X, Xu S, Tan T, et al. Toxicity and estrogenic endocrine disrupting activity of phthalates and their mixtures. *Int J Environ Res Public Health*. 2014;11(3):3156. https://doi.org/10.3390/IJERPH110303156.
25. The safety of medical devices containing DEHP-plasticized PVC or other plasticizers on neonates and other groups possibly at risk (2015 Update); 2016. doi:https://doi.org/10.2772/45179
26. PubChem Database. https://pubchem.ncbi.nlm.nih.gov/compound/Bis_2_4-di-tert-butylphenylphosphate. Accessed June 26, 2020.
27. GESTIS-Stoffdatenbank. http://gestis.itrust.de/nxt/gateway.dll/gestis_de/000000.xml?f=templates&fn=default.htm&vid=gestisdeu:sdbdeu. Accessed June 26, 2020
28. Kaiser S. Bestimmung von Extraktiv- und Migrationsstoffen aus pharmazeutischen Kunststoffbehältnissen: vorgelegt von Susann Kaiser; 2010.
29. Acosta ML, Sánchez A, García F, Contreras A, Molina E. Analysis of kinetic, stoichiometry and regulation of glucose and glutamine metabolism in hybridoma batch cultures using logistic equations. *Cytotechnology*. 2007;54(3):189-200. https://doi.org/10.1007/s10616-007-9089-9.
30. Vermes I, Haanen C, Reutelingsperger C. Flow cytometry of apoptotic cell death. *J Immunol Methods*. 2000;243(1–2):167-190. https://doi.org/10.1016/S0022-1759(00)00233-7.
31. Berenbaum MC. Synergy, additivism and antagonism in immunosuppression. A critical review. *Clin Exp Immunol*. 1977;28(1):1-18. http://www.ncbi.nlm.nih.gov/pubmed/324671%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1540873.
32. Huang RY, Pei L, Liu Q, et al. Isobologram analysis: a comprehensive review of methodology and current research. *Front Pharmacol*. 2019;10:1-12. https://doi.org/10.3389/fphar.2019.01222.
33. Chou T-C, Talalay P. Analysis of combined drug effects: a new look at a very old problem. *Trends Pharmacol Sci*. 1983;4:450-454. https://doi.org/10.1016/0165-6147(83)90490-X.
34. Li F, Vijayasankaran N, Shen AY, Kiss R, Amanullah A. Cell culture processes for monoclonal antibody production. *MAbs*. 2010;2(5):466-479. https://doi.org/10.4161/mabs.2.5.12720.
35. Tan MT. Fang HB, Huang H, Yang Y. (2016) Design and Statistical Analysis of Multidrug Combinations in Preclinical Studies and Phase I Clinical Trials. In: Lin J., Wang B., Hu X., Chen K., Liu R. (eds) Statistical Applications from Clinical Trials and Personalized Medicine to Finance and Business Analytics. ICSA Book Series in Statistics. Springer, Cham. https://doi.org/10.1007/978-3-319-42568-9_18.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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