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Limitation and detection of bis(2,4-di-tert-butylphenyl)phosphate (bDtBPP) from bioprocess container materials

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ECI conference, Single-Use Technologies 18-21 October 2015

Isabelle Uettwiller – Sartorius Stedim FMT SAS

Table of content

bDtBPP origin (1/6)
Interest of antioxidants in film material?

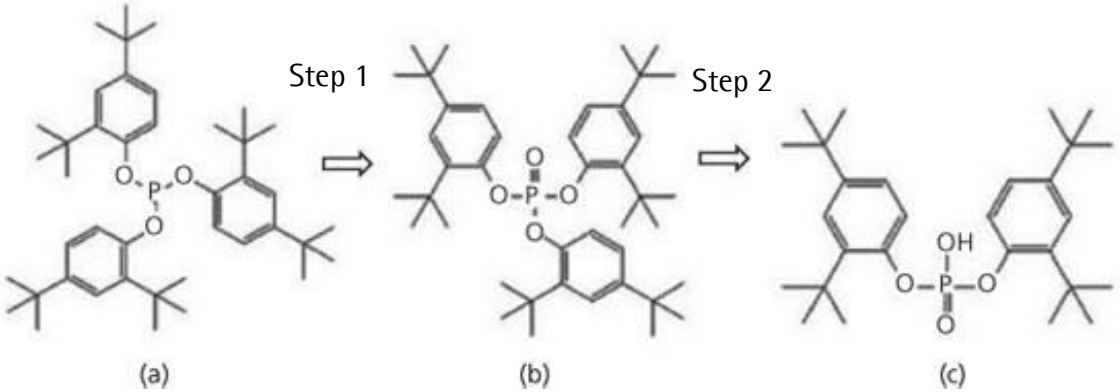
- Polymer Stabilizers such as organophosphite and stearically hindered phenols compounds are widely used to protect plastics from degradation by peroxide species
  - During the extrusion process (high temperature)
  - During sterilization (irradiation) due to chain scission or crosslinking
  - During the shelf life of the material
- Removal of antioxidants could lead to poor film properties
- TBPP: tris(2,4-di-tert-butylphenyl)phosphite (trend name: Irgafos 168) is a well-known organophosphite stabilizer described in Pharmacopeias

Single Use Bioprocess containers are used in Bioreactors

for media storage & cell growth applications

bDtBPP origin (2/6)
Degradation of organophosphite stabilizer

- Step 1: A large fraction of TBPP is converted into oxidized TBPP during the film **extrusion process,** the remaining TBPP is converted into oxidized TBPP during the sterilization process
- Step 2: After **irradiation,** further chemical breakdown occurs with the formation of bDtBPP and DtBP (2,4-di-tert-butylphenol) + other potential compounds



(a) tris(2,4-di-tert-butylphenyl)phosphite: TBPP (ex. of Trend name: Irgafos 168)
(b) Oxidized TBPP
(c) bis(2,4-di-tert-butylphenyl)phosphate: bDtBPP

bDtBPP origin (3/6)
Degradation product of antioxidant causing detrimental effect on cell growth

June 1, 2013

A Generic Growth Test Method for Improving Quality Control of Disposables in Industrial Cell Culture

By Brian Horvath,Valerie Liu Tsang,Weimin Lin,Xiao-Ping Dai, PhD,Kurt Kunas,Greg Frank

Independent data, using several different cell lines and growth media, reported g inhibition resulting from the use disposable bags and suggests a method that ca implemented for quality control at disposable-bag vendors.

ABSTRACT

Disposable bags are widely used in the biotechnology industry. The two main purposes a cell-culture media and to grow cells for inoculum or production. Several groups have reported growth inhibition resulting from the use of such products. This report shows independent data from four companies, using several different cell lines and growth media, and suggests a method that can be implemented for quality control at disposable-bag vendors.

Interlaboratory Test for Detection of Cytotoxic Leachables arising from Single-Use Bags

Nina Steiger, and Regine Eibl*

DOI: 10.1002/cite.201200171

An interlaboratory test for detection of cytotoxic leachables arising from single-use bags was established and performed. Results from cultivations with two bag materials indicate that leachables influencing cell growth and metabolism were secreted. For the other seven bag materials a migration of leachables can be excluded.

Identification of a Leachable Compound Detrimental to Cell Growth in Single-Use Bioprocess Containers

MATTHEW HAMMOND[1,*], HEATHER NUNN[1], GARY ROGERS[1], HANS LEE[1], ANATOLIA-LILIANA MARGHITOIU[1], LOURDES PEREZ[1], YASSER NASHED-SAMUEL[1], CARL ANDERSON[2], MICHAEL VANDIVER[2], and SALLY KLINE[1]

[1]Product Attribute Sciences; [1]Product Contact Assessment; [1]Materials Science, Amgen Inc., Thousand Oaks, CA; [1]Cell Sciences & Technology, Amgen Inc., Seattle, WA; and [2]Pilot Plant Operations, Amgen Inc., Bothell, WA

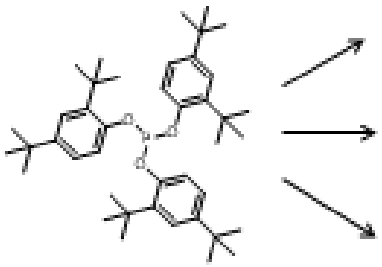
ABSTRACT: Out of the plethora of chemical species extractable at low levels from the materials of construction of single-use bioprocess containers, we have identified one particularly conspicuous compound and shown it to be highly detrimental to cell growth. The compound, *bis*(2,4-di-*tert*-butylphenyl)phosphate (bDtBPP), is derived from the breakdown of *tris*(2,4-di-*tert*-butylphenyl)phosphite (trade name Irgafos 168®), a common antioxidant additive present in many formulations of polyethylene (one of the polymers commonly used as the material contacting process fluids in bioprocess containers). Cell growth experiments using several mammalian cell lines and growth media spiked with bDtBPP show harmful effects at concentrations well below the parts-per-million range. Cellular response to bDtBPP is rapid, and results in a significant decrease in mitochondrial membrane potential. The migration of bDtBPP from polyethylene-based films is shown to be time- and temperature-dependent. Further, experiments suggest that exposure of oxidized Irgafos 168 to ionizing radiation (such as gamma irradiation) is an important condition for the generation of significant amounts of leachable bDtBPP.

Since 2013, several publications cover this subject …

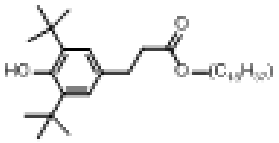
bDtBPP origin (4/6)
Degradation products - impact on cell growth

The leached compound is antioxidant-related

Leachable/extractables experiments turn up a list of compounds related to anti-oxidants…



Most used antioxidants



γ

#	Extractable compound
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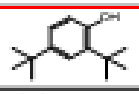
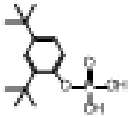
2	
3	
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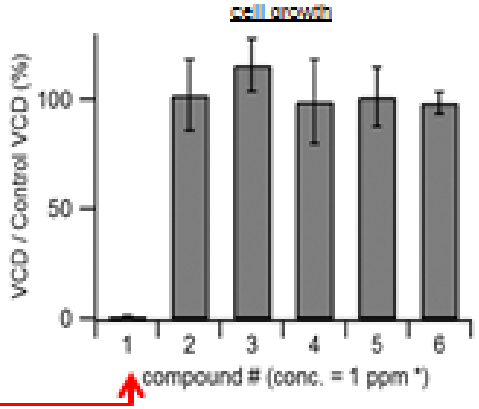
5	
6	



…*one* of which ("bDtBPP") severely impacts cell growth even at very low concentration

bDtBPP = Bis-(2,4-di-tert-butylphenyl) phosphate

AMGEN

Partnership approach to control SU variability for sustainable use in biopharmaceuticals production, Sally Kline, Magali Barbaroux, BPI Boston, October 22, 2014

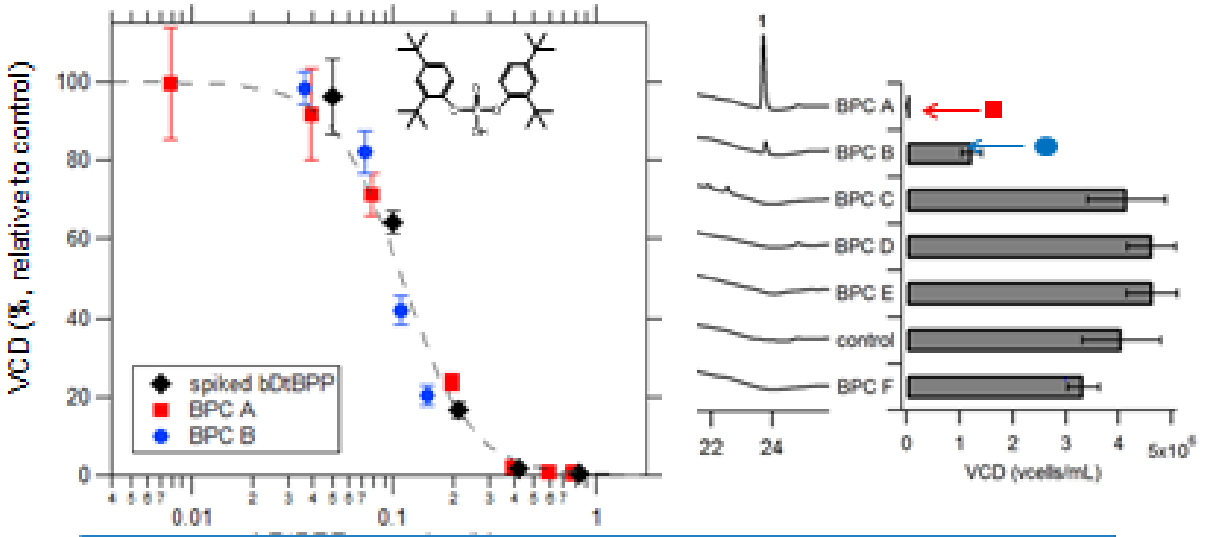
VCD: Viable cell density

bDtBPP origin (5/6)
bDtBPP leaching into the medium cause cell growth variability

Impact on cell growth detected for bDtBPP concentrations in the range of 0,04 – 0,05 µg/mL[1]

Leached bDtBPP from different films Causes Poor Growth in Cell Culture

Results of spiking bDtBPP at various concentrations + results of progressive dilution of held media samples:



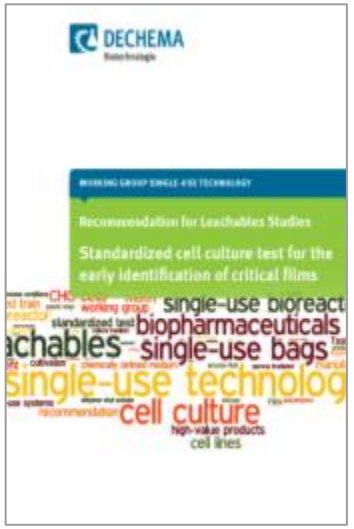
Variability in Raw Materials and Film Processing can affect lot-to-lot cell grown

AMGEN

[1]Hammond M, et al. A Cytotoxic Leachable Compound from Single-Use Bioprocess Equipment That Causes Poor Cell Growth Performance. Biotechnol. Prog., 2014, Vol. 30, No. 2, p332

bDtBPP origin (6/6)
Dechema interlaboratory test*

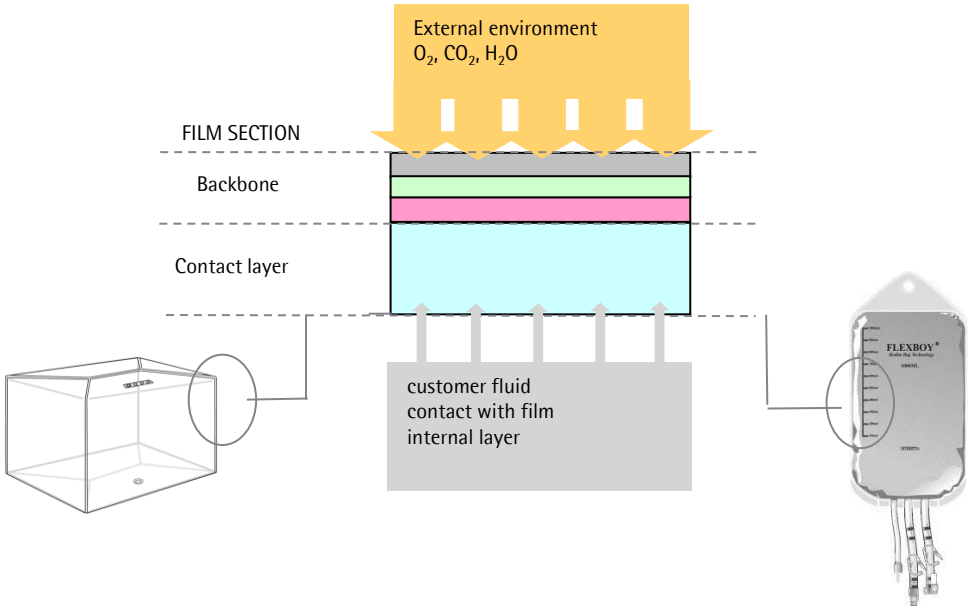
- Bag suppliers send bags to the Zurich University of Applied Sciences (ZHAW)
- Bags are subjected to media extractions and WFI extractions (analogue to experiments describes herein). Positive control: borosilicate glass
- 11 films including negative control from different suppliers
- Sartorius Stedim supplied S71 (EVA) and S80 (PE) films
- 4 users (3 industry, 1 academia)
- 8 different cell lines
- 7 different CD media

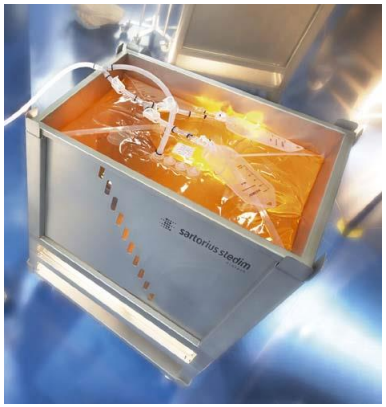


*Eibl et al: Standardized cell culture test for the early identification of critical films for CHO cell lines in chemically defined culture media Dechema: ISBN: 978-3-89746-149-9

Table of content

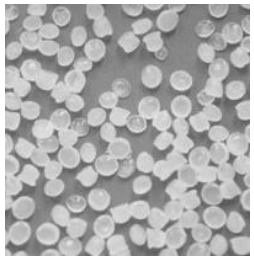
Film is the Single Use (SU) component with the highest contact to surface ratio and highest contact time

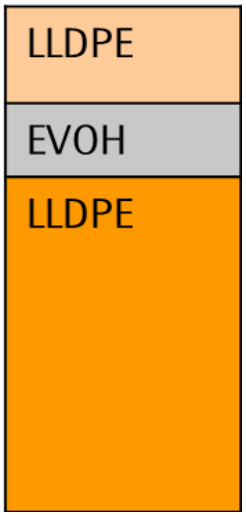




➔ and therefore considered as the most critical one.

Film formulation answers technical and strategic needs

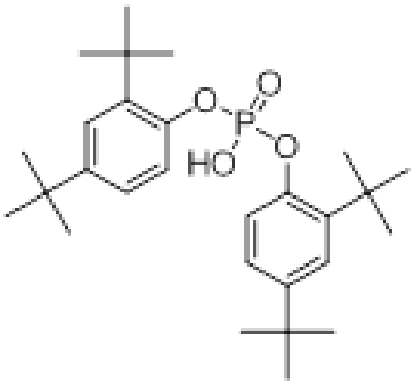




- Selection of basic type of polymer and molecular architecture according to the application
  - Physical properties: flexibility – robustness – gas barrier…
  - Compliance with Pharmacopoeias, Reach, TSE/BSE free
- Optimize additives in the formulation while keeping long term performances and resistance to gamma
  - Additive optimization; primary (long-term) and secondary (short-term) antiox. package, slipping agent removal…
  - Additives selection specified in Pharmacopoeias to ease tox. assessment
- Approved supplier and "block buster" polymer
  - Access to resin polymer formulation and additives by CAS number
  - Assurance of supply and change control are key factors

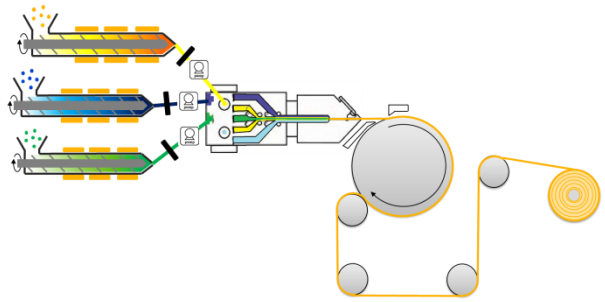
Film manufacturing process plays a key role in film quality

- Selection of film manufacturing process (cast versus blown extruder)
- Avoidance of water cooling to reduce endotoxin risks
- Removal of slipping agents and usage of mechanical rather than chemical antiblocking agents
- Potential release of bDtBPP reduced by different actions:
  - By reducing the quantity of TBPP (Irgafos 168)
  - By decreasing the oxidation effect: Process optimization
  - By using or increasing the quantity of other antioxidants



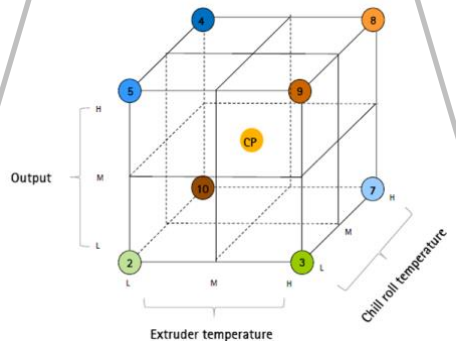
Film manufacturing process Design of Experiment (DOE)

DOE critical film extrusion process parameters



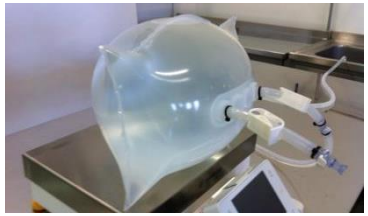
- 3 orthogonal parameters linked to residence time e.g. most critical
  - . Melting T°
  - . Cooling T°
  - . Extrusion Speed (output)

DOE Plan



- Full factorial 2^3 experiment
  - . 8 variations
  - . 3 center points

DOE response attributes



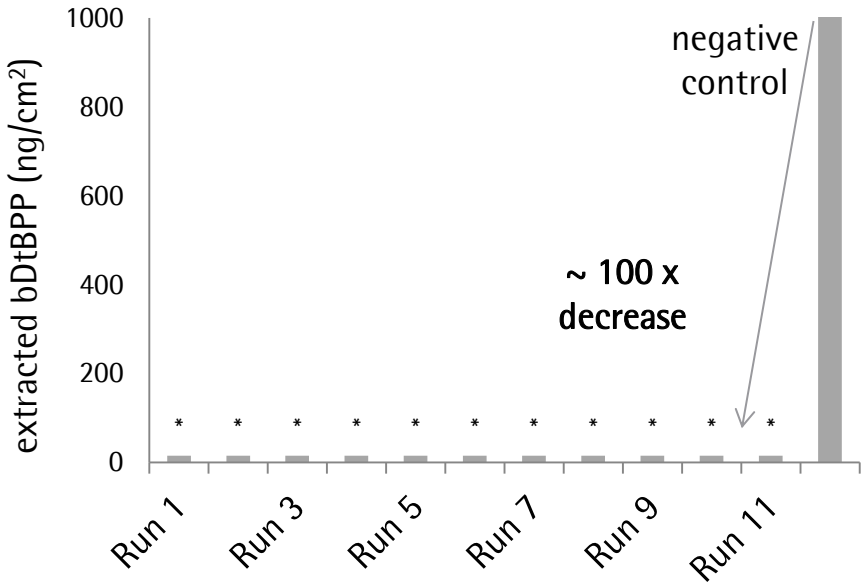
- Mechanical properties
- Cell growth assay
- Extractables (bDtBPP)

Evaluation of quantity of bDtBPP and Cell growth experiment

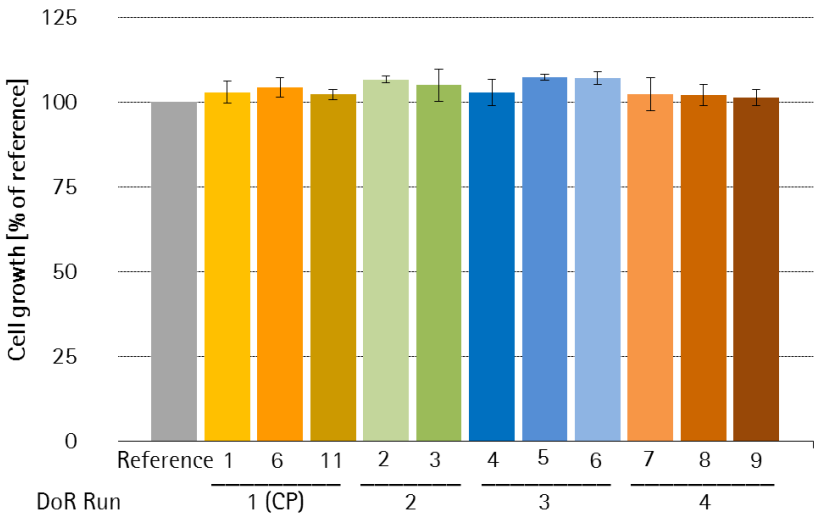
Film extrusion critical process parameter variations within design space do not impact cell growth performance

❶ Amount of bDtBPP un-quantifiable in AMGEN developed extractable assay[1]

(Performed by Amgen)



❷ Cell growth comparable to glass ref. throughout design space in SSB developed cell growth assay[2]



[1]Matthew Hammond, Heather Nunn, Gary Rogers, et al., Identification of a Leachable Compound Detrimental to Cell Growth in Single-Use Bioprocess Containers, *PDA J Pharm Sci and Tech* **2013**, *67* 123-134

[2]Jurkiewicz E, et al. Verification of a New Biocompatible Single-Use Film Formulation with Optimized Additive Content for Multiple Bioprocess Applications. Biotechnol. Progr., 2014, 30 (5), p985

Table of content

Quantification of bDtBPP (1/6)
Extraction conditions were designed to exaggerate real application conditions

Gamma-irradiated bags were filled with ethanol and incubated in following conditions:

Solvent :	100% ethanol (worst case extraction)
Surface to volume ratio :	1,5 cm^2/mL
Temperature :	40°C
Sterilisation status :	gamma-irradiation at 25-45kGy (routine dose)
Extraction time :	3, 21, 70, 120 days in static mode
Film materials:	9 different films with 2 from Sartorius Stedim are tested

Quantification of of bDtBPP (2/6)
HPLC-UV method of analysis is appropriate to detect bDtBPP

An in-house SSB analytical method was developed to quantify bDtBPP based on HPLC-UV

Column:		Nucleosil C18
Gradient:	A:	Acetonitrile
	B:	Water
Flow rate :		1 ml/min
Analysis time:		65min
Temperature:		40°C
Injection volume:		20µl

Detection : UV/Vis DAD-Detector wavelength 220nm

Quantification of of bDtBPP (3/6)
LOQ/LOD method validation by HPLC-UV

5	0,50	21,966
6	0,60	27,508
7	0,70	32,389
8	0,80	36,4928
9	0,90	41,313
10	1,00	45,626
11		
12		
13		
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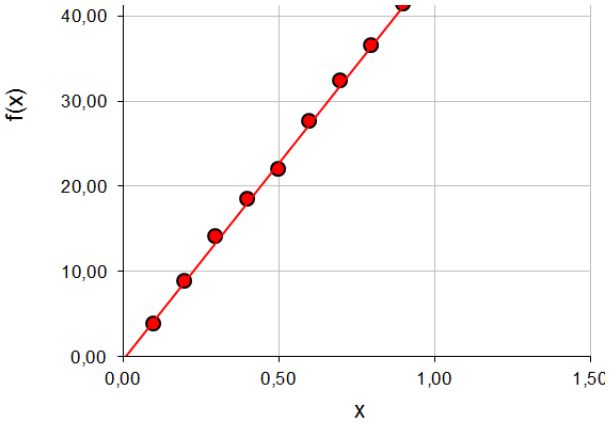
15		
16		



Characteristics

Slope a	46,315		Number of measurements n	1
Intercept b	-0,440		Standard error of estimate Sy	0,436
Correlation coefficient r	0,9996		Standard error of procedure Sx	0,009
Result uncertainty	33,33	%	Sum of squared deviations	0,825
Probability of error (alpha)	1,00	%	Quantile (one-sided)	2,896
			Quantile (two-sided)	3,355

Analytical limits according to DIN 32645

Limit of detection	0,033	mg/L	
Limit of quantitation	0,110	mg/L	(approximation)
	0,110	mg/L	(exact)

LOD/LOQ method validation according to DIN 32645 (equivalent ISO11843-2)

Quantification of bDtBPP (4/6)
Limit of Detection and Limit of Quantification validation

- LOD and LOQ validated according to DIN 32645
- At the time of the experiment the LOD is 0.03 µg/mL and the LOQ is 0.11 µg/mL
- LOD and LOQ allow to detect the lowest quantity of bDtBPP that can impact cell growth (i.e. between 0.04 – 0.05 µg/mL[1])
- Due to limited analytical experience with quantitation of bDtBPP, it has been decided to apply a LOD of 0.05 µg/mL and a reporting limit of 0.3 µg/mL

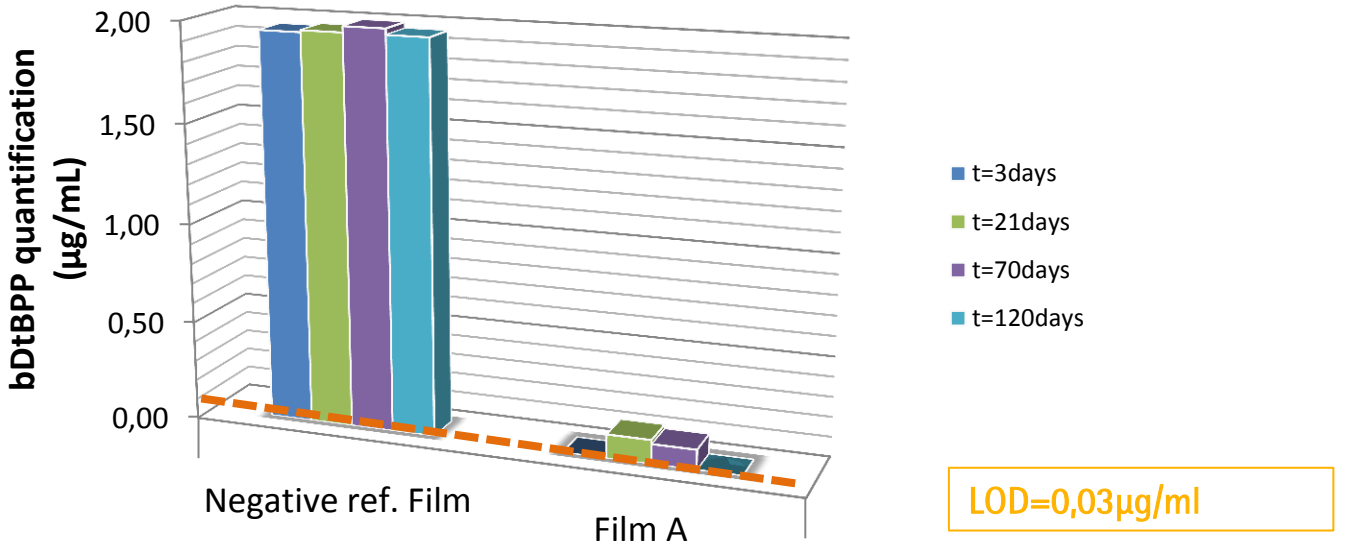
[1]Hammond M, et al. A Cytotoxic Leachable Compound from Single-Use Bioprocess Equipment That Causes Poor Cell Growth Performance. Biotechnol. Prog., 2014, Vol. 30, No. 2, p332

Chemical tests of bDtBPP (5/6)
bDtBPP concentrations detected in ethanol extracts from bags are close to LOD

Results show that bDtBPP level is close to the LOD in ethanol extracts which represent a worse case compared to media extraction

Film	bDtBPP quantification (µg/mL)			
	t=3days	t=21days	t=70days	t=120days
Film A	ND	Detected	Detected	ND
Negative ref. Film	1.95	1.96	1.99	1.96

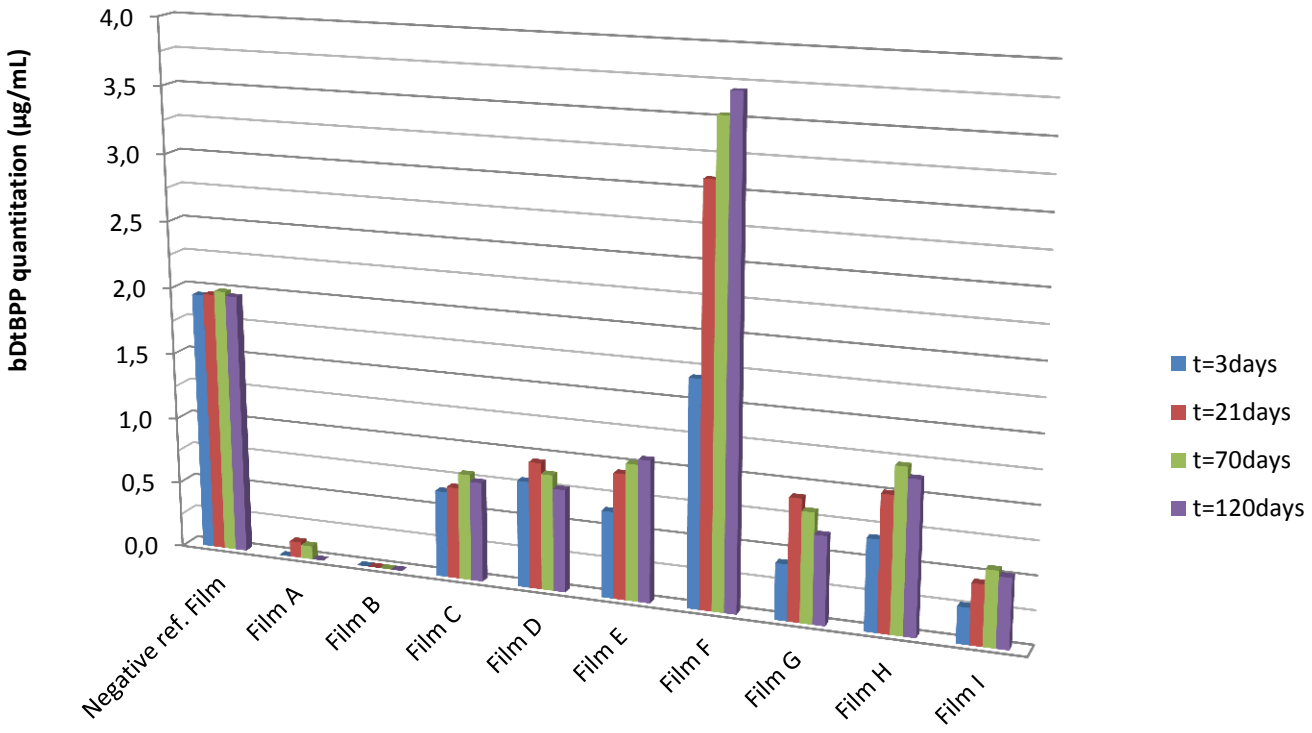
ND: Not detected



LOD=0,03µg/ml

Chemical tests of bDtBPP (6/6)
Comparison of bDtBPP levels between films from different suppliers

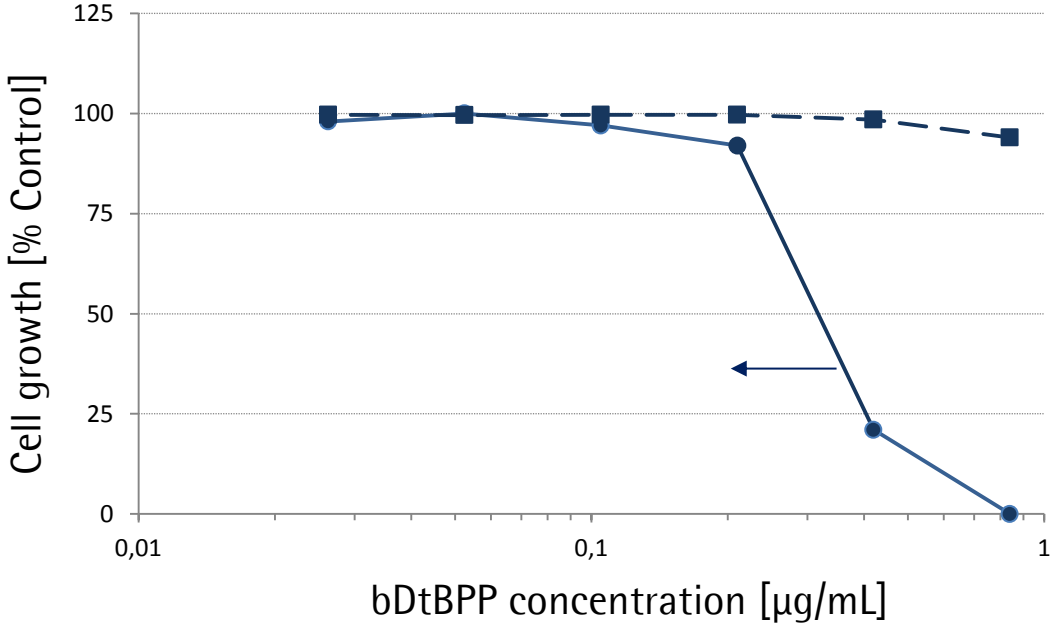
bDtBPP quantitation by HPLC-UV after EtOH extraction for t = 3, 21, 70, 120 days at 40°C



Cell growth testing (1/2)
CHO based cell growth assay and dose response to bDtBPP established

Standardized cell growth assay

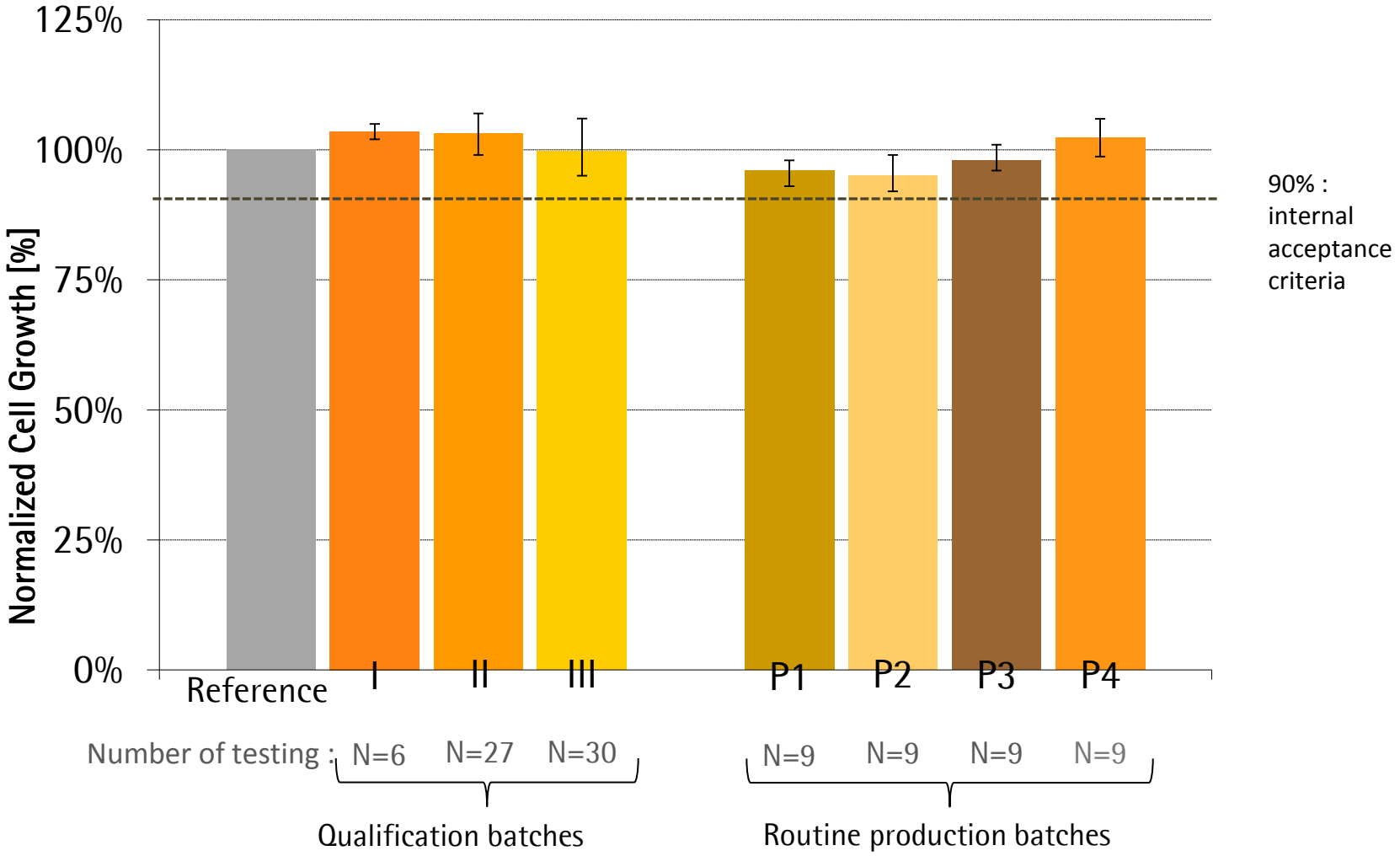
- rCHO DG44 cells grown in 6 well plates in protein free cell culture medium
- Medium incubated for 3 days, at 37°C in γ-irradiated sample bags at a volume-to-surface-ratio of 3cm2/ml
- Control: medium incubated 3 days, at 37°C in borosilicate bottle



bDtBPP dose response confirmed

Cell growth testing (2/2)

Routine cell growth testing of film batches shows consistent cell growth and therefore proves that the amount of bDtBPP is below the detrimental level.



Key take Home Messages

- The link between cell growth performance and quantity of leaching bDtBPP from plastic materials has been verified through various studies and publications
- Analytical method (HPLC-UV) has been implemented at Sartorius Stedim to verify the quantity of bDtBPP leaching compound with an acceptable Limit of detection
- Verification of Cell growth experiment on film material from Sartorius Stedim demonstrate very good performance of the film developed with formulation and process optimization

Film material with reduced quantity of Phosphite antioxidant and optimized extrusion parameters can lead to both polymer degradation protection and good cell growth performance

Variability of the film quality is limited with a strong control of your supplier on the resin formulation and the extrusion parameters

Acknowledgements

- Magali Barbaroux
- Samuel Dorey
- Elke Jurkiewicz
- Thomas Loewe
- Ina Pahl
- Roberto Menzel

And our external partners.....

Thank you very much for your attention!