

# Toxicity Threshold of Perfluorocarbon Liquids for Intraocular Use: Dose–Response Assessment of In Vitro Cytotoxicity of Possible Contaminants

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**Purpose:** This study assessed the cytotoxicity of the impurities detected in the perfluorooctane (PFO) batches for vitreoretinal surgery that were associated with serious adverse incidents of ocular toxicity, namely, the perfluorooctanoic acid (PFOA), 1H,1H,7H-dodecafluoro-1-heptanol (DFH), 1H-perfluorooctane (1H-PFO), ethylbenzene, anhydrous p-xylene, and perfluoro-2-butyltetrahydrofuran, and two additional substances 1H,1H,1H,2H,2H-perfluorooctane (5H-PFO) and hexafluoro-1,2,3,4-tetrachlorobutane.

**Methods:** Serial dilutions were tested by in vitro direct contact cytotoxicity test, validated in accordance with the ISO 10993-5:2009 standard using BALB3T3 and ARPE-19 cell lines, after sample application for 24 hours.

**Results:** Six of the eight tested substances were cytotoxic according to the above-mentioned ISO standard. Anhydrous p-xylene, ethylbenzene, and PFOA were the most cytotoxic impurities as traces 1.55 ppm, 1.06 ppm, and 28.4 ppm reached the cytotoxicity limit, respectively. Hexafluoro-1,2,3,4-tetrachlorobutane, DFH, and 1H-PFO were cytotoxic at 980, 22,500, and 123,000 ppm, respectively. Both 5H-PFO and perfluoro-2-butyltetrahydrofuran were non-cytotoxic at the highest available concentrations ( $\geq 970,000$  ppm). The dose-response curves allowed to calculate the cytotoxic concentration (CC<sub>30</sub>) for each tested substance that would reduce 30% of cell viability and corresponding to the cytotoxicity threshold according to ISO 10993-5.

**Conclusions:** Our study determined the in vitro cytotoxicity of several impurities in PFO associated with serious adverse incidents in retinal surgery patients.

**Translational Relevance:** Severe cytotoxicity of some impurities previously found in toxic perfluorocarbon liquids was confirmed. The cytotoxicity test validated according to the ISO 10993-5:2009 standard is a sensible and fast method for reliable detection of the cytotoxicity in perfluorocarbon liquids to guarantee maximal safety for the patients.

## Introduction

The perfluorocarbon liquids (PFCLs) are considered to be a valuable tool as vitreous tamponade in the management of vitreoretinal diseases such as complex retinal detachments.<sup>1,2</sup> The rationale for using

these compounds in vitreoretinal surgery lies in their highly specific gravity, optical clarity, and immiscibility with water.<sup>3,4</sup> One of the most largely used PFCLs is the fully fluorinated perfluoro-n-octane (PFO), whose safety profile has been extensively proved.<sup>4–7</sup>

Nonetheless, cases of ocular toxicity leading to visual loss, retinal necrosis and fibrosis, retinal

vascular occlusion, and retinal or optic nerve atrophy after the use of the commercially available AlaOcta PFO (Alamedics, Germany) were reported since 2013 in Spain and throughout Europe.<sup>8–10</sup> Such severe adverse incidents were demonstrated to be associated with the use of PFCL batches whose toxicity was not identified by the manufacturer before release of the medical device on the market.<sup>8,11</sup> The origin of PFCL toxic effects was thought to be due to a combination of impurities, chemical effects, and mechanical compression of PFCL.<sup>9</sup> However, the manufacturer claimed that the product had complied with the requirements of the Health Agencies and International Organization for Standardization (ISO) that are accepted worldwide.<sup>12,13</sup> In addition to the purity of raw material, the safety of PFO was assessed using the cytotoxicity test in accordance with the ISO 10993-5<sup>12</sup> and the extract dilution method on mouse fibroblast L929 cell line.<sup>8,11</sup> The test failed to detect the cytotoxicity of the medical device, probably because of the incorrect selection of the method that does not consider the hydrophobic and volatile characteristics of the PFCLs<sup>11,14</sup> and lack of evaluation of its critical steps.<sup>15</sup>

Accurate analyses of the AlaOcta batches revealed that they were contaminated with hazardous compounds including the benzene derivatives: 1,4-dimethylbenzene (*p*-xylene) and ethylbenzene, which are leachable and might be released from the primary container materials, and two hydroxyl compounds: perfluorooctanoic acid (PFOA) and 1H,1H,7H-dodecafluoro-1-heptanol (DFH), which could have resulted from degradation of the raw material during storage of the toxic batches.<sup>8</sup> Subsequently, 1H-perfluorooctane (1H-PFO) and perfluoro-2-n-butyltetrahydrofuran were also found as impurities in the toxic batches by other investigators.<sup>16,17</sup>

This study focused on the assessment of the cytotoxicity of *p*-xylene, ethylbenzene, PFOA, DFH, 1H-PFO and perfluoro-2-n-butyltetrahydrofuran, which were previously detected in the toxic AlaOcta batches.<sup>8,16,17</sup> Moreover, we tested the cytotoxicity of the partially hydrogenated PFO analogue 1H,1H,1H,2H,2H-perfluorooctane (5H-PFO)<sup>18</sup> and hexafluoro-1,2,3,4-tetrachlorobutane, a solvent used for fluorination processes.<sup>19</sup> In particular, by using an in vitro direct contact cytotoxicity test, validated in accordance with the ISO 10993-5 standard,<sup>11,15</sup> we conducted a dose-response study to determine the cytotoxicity concentration threshold (CC<sub>30</sub>) for each tested substance that, according to the above-mentioned ISO, is the concentration that reduced the in vitro cell viability by 30%.

## Material and Methods

### Preparation of Impurity Samples

Serial dilutions of the PFOA (CAS 335-67-1, Sigma Aldrich, Italy, purity: 98.8%), DHF (CAS 335-99-9; Santa Cruz Biotechnology, Dallas, TX, USA; purity: 98%), hexafluoro-1,2,3,4-tetrachlorobutane (CAS 375-45-1; Santa Cruz Biotechnology; purity: 90%), 1H-PFO (CAS 335-65-9; Fluorochem, Glossop, UK; purity: 98.4%), 5H-PFO (CAS 80793-17-5; Apollo Scientific, Cheshire, UK; purity: 97%), and perfluoro-2-butyltetrahydrofuran (CAS 335-36-4; Fluorochem; purity: 99.71%) stock solutions were prepared by adding PFO (F2 Chemicals Ltd, Preston, UK; purity > 99%) as a diluent and continuously stirring for 30 minutes at room temperature (RT). The PFOA, 1H-PFO, 5H-PFO, hexafluoro-1,2,3,4-tetrachlorobutane, and perfluoro-2-butyltetrahydrofuran solutions were also tested as undiluted samples. The concentrations of each tested compound are shown in Table 1. Anhydrous *p*-xylene (CAS 106-42-3; Sigma-Aldrich Corp., St. Louis, MO, USA; purity: 99.8%) and anhydrous ethylbenzene (CAS 100-41-4; Sigma-Aldrich Corp; purity: 99.8%) are insoluble in PFO. The samples containing these compounds were prepared by mixing 0.05 g of each stock solution with 5 g of PFO (F2 Chemicals Ltd; purity > 99%) and were stirred at RT for two hours. After the phase separation, the PFO solution was withdrawn, diluted, and analyzed by cytotoxicity test in vitro and ultra-high-pressure liquid chromatography (UHPLC).

## Cytotoxicity Test In Vitro

### Cell Cultures

The ISO 10993-5 reference cell line, murine fibroblast cells BALB3T3 clone A31 (ATCC CCL163), and the human retinal pigment epithelial cell line ARPE-19 (ATCC CRL-2302) were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) and used as previously described.<sup>15,18</sup> In particular, after thawing, BALB3T3 and ARPE-19 cells were grown as a monolayer in Dulbecco's modified Eagle medium (DMEM) with high glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate (Sigma-Aldrich Corp) and Dulbecco's modified Eagle medium/Nutrient Mixture F-12 medium with L-glutamine without 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Gibco,

**Table 1.** Dose-Response Values Corresponding to Different Concentrations of Eight Tested Substances and Their Effect on % of Mortality Rate  $\pm$  SEM in ARPE-19 e BALB3T3 Cell Lines

Tested Substance, Molecular Formula, (Purity (%), CAS Number)	Tested Concentrations (ppm)	Mortality Rate $\pm$ SEM (%)	
		% ARPE-19 Cells	BALB3T3 Cells
<i>p</i> -Xylene, anhydrous, C <sub>8</sub> H <sub>10</sub> , (99.8%, CAS 106-42-3)	1.71	98 $\pm$ 1	99 $\pm$ 1
	<b>1.55</b>	<b>70 <math>\pm</math> 2</b>	<b>75 <math>\pm</math> 2</b>
	1.07	8 $\pm$ 2	11 $\pm$ 1
Ethylbenzene, anhydrous, C <sub>8</sub> H <sub>10</sub> , (99.87%, CAS 100-41-4)	8.50	98 $\pm$ 1	99 $\pm$ 1
	<b>7.63</b>	<b>78 <math>\pm</math> 1</b>	<b>77 <math>\pm</math> 1</b>
	0.05	22 $\pm$ 6	14 $\pm$ 6
Perfluorooctanoic acid, C <sub>8</sub> HF <sub>15</sub> O <sub>2</sub> (PFOA, 98.8%, CAS 335-67-1)	568	87 $\pm$ 1	96 $\pm$ 1
	284	71 $\pm$ 1	67 $\pm$ 3
	142	58 $\pm$ 1	52 $\pm$ 2
	56.80	43 $\pm$ 1	36 $\pm$ 1
	<b>28.40</b>	<b>38 <math>\pm</math> 2</b>	<b>25 <math>\pm</math> 2</b>
	5.68	12 $\pm$ 2	13 $\pm$ 2
1H,1H,7H-dodecafluoro-1-heptanol, C <sub>7</sub> H <sub>4</sub> F <sub>12</sub> O (DFH, 98%, CAS 335-99-9)	2.84	9 $\pm$ 3	5 $\pm$ 1
	18,375	99 $\pm$ 1	97 $\pm$ 1
	9800	96 $\pm$ 1	97 $\pm$ 1
	9114	99 $\pm$ 1	97 $\pm$ 1
	<b>980</b>	<b>28 <math>\pm</math> 2</b>	<b>8 <math>\pm</math> 1</b>
	98	20 $\pm$ 1	7 $\pm$ 1
	49	20 $\pm$ 1	5 $\pm$ 1
	24.50	16 $\pm$ 1	5 $\pm$ 1
	12.25	15 $\pm$ 1	3 $\pm$ 1
	6.13	13 $\pm$ 1	2 $\pm$ 1
Hexafluoro-1,2,3,4-tetrachlorobutane, C <sub>4</sub> Cl <sub>4</sub> F <sub>6</sub> (90%, CAS 375-45-1)	3.06	9 $\pm$ 2	2 $\pm$ 1
	90,000	90 $\pm$ 2	84 $\pm$ 3
	45,000	52 $\pm$ 3	44 $\pm$ 2
	<b>22,500</b>	<b>37 <math>\pm</math> 2</b>	<b>31 <math>\pm</math> 1</b>
	9000	17 $\pm$ 1	16 $\pm$ 1
	900	12 $\pm$ 1	10 $\pm$ 1
	450	9 $\pm$ 1	6 $\pm$ 1
	225	9 $\pm$ 1	6 $\pm$ 1
1H-perfluorooctane, CF <sub>3</sub> (CF <sub>2</sub> ) <sub>7</sub> H (1H-PFO, 98.4%, CAS 335-65-9)	112.5	5 $\pm$ 2	3 $\pm$ 1
	984,000	98 $\pm$ 1	94 $\pm$ 2
	492,000	ND	97 $\pm$ 2
	246,000	60 $\pm$ 1	78 $\pm$ 2
	<b>123,000</b>	<b>44 <math>\pm</math> 1</b>	<b>48 <math>\pm</math> 2</b>
	61,500	28 $\pm$ 2	21 $\pm$ 1
	30,750	17 $\pm$ 2	9 $\pm$ 1
1H,1H,1H,2H,2H-perfluorooctane, C <sub>8</sub> H <sub>5</sub> F <sub>13</sub> (5H-PFO, 97%, CAS 80793-17-5)	15,350	7 $\pm$ 2	1 $\pm$ 1
	970,000	26 $\pm$ 2	13 $\pm$ 1
	485,000	24 $\pm$ 1	12 $\pm$ 1
	242,500	16 $\pm$ 1	8 $\pm$ 1
	121,250	11 $\pm$ 2	5 $\pm$ 1
	60,625	8 $\pm$ 2	6 $\pm$ 1
	30,312	8 $\pm$ 2	5 $\pm$ 1
	15,156	4 $\pm$ 2	5 $\pm$ 1
	7578	2 $\pm$ 2	3 $\pm$ 1
	3789	-3 $\pm$ 2	4 $\pm$ 1
Perfluoro-2-butyltetrahydrofuran, C <sub>8</sub> F <sub>16</sub> O (99.71%, CAS 335-36-4)	1894	-6 $\pm$ 1	4 $\pm$ 1
	997,100	12 $\pm$ 1	5 $\pm$ 1
	498,550	12 $\pm$ 1	5 $\pm$ 1
	249,275	11 $\pm$ 1	3 $\pm$ 1
	124,637	9 $\pm$ 2	0 $\pm$ 1
	62,318	6 $\pm$ 1	3 $\pm$ 1
	31,159	-1 $\pm$ 1	4 $\pm$ 1

In bold: Minimal concentration (ppm) resulting in cytotoxicity in at least one cell line.  
 ND, not determined.

Waltham, MA, USA), respectively, each supplemented with 10% of newborn calf serum (Sigma-Aldrich Corp) and 1% penicillin–streptomycin (Sigma-Aldrich Corp), in 75 cm<sup>2</sup> culture flasks in an incubator (Memmert, Schwabach, Germany) at 37°C ± 1°C, 90% ± 10% humidity, 5.0% ± 1.0% CO<sub>2</sub>/air. The cultures were used after two passages.

### Direct Contact Cytotoxicity Test

As described by Romano et al.,<sup>15</sup> in vitro direct contact cytotoxicity test, validated in accordance with the ISO 10993-5 standards,<sup>12</sup> was performed. Briefly, BALB3T3 and ARPE-19 cell suspension containing 2.0 to 3.0 × 10<sup>5</sup> cells/mL were seeded into 96-well microtiter plates and incubated at 37°C ± 1°C, 90% ± 10% humidity, and 5.0% ± 1.0% CO<sub>2</sub>/air for 24 hours to reach 70% to 80% confluence. Afterward, the cells were washed one time with 150 µL of Dulbecco's phosphate-buffered saline solution before application of samples. The culture media used for BALB3T3 and ARPE-19 cell culture growth were the media used as respective vehicle. Ultrapure PFO, purity 99.8%, (AL.CHI.MI.A. S.r.l, Italy), and 1H-PFO with purity 98.8% (Fluorochem, Italy) were used as noncytotoxic (negative) and cytotoxic (positive) controls, respectively.

### Samples Application and Removal

To ensure the constant contact between the tested samples and the cell layer for the whole duration of the test, 50 µL of samples containing each tested compound, as well as either the negative or positive controls, was applied on the cells by immersing the pipette tip in the medium at two-thirds depth of the well containing 300 µL of the medium: this procedure created a bubble that deposited on the cell layer as a result of gravity. All samples were incubated for 24 hours. At the end of incubation, each PFCL bubble was removed along with the medium.

### MTT and Neutral Red Uptake Vitality Assays

In the absence of ISO 10993-5<sup>12</sup> indications for ARPE-19 cells, the MTT cytotoxicity test in these cells was performed using the TOX-1 In Vitro Toxicology Assay Kit, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-phenyltetrazolium bromide) (Sigma-Aldrich Corp) as previously described by Romano et al.<sup>15</sup> Briefly, the viable cells are metabolically reduced to a blue-violet insoluble formazan by the yellow water-soluble MTT; therefore the extent of cell viability correlates with the

intensity of the color that is determined by photometric measurement after dissolving the formazan in alcohol.

The TOX4 In Vitro Toxicology Assay Kit Neutral Red (NRU)-based (Sigma-Aldrich Corp) was used for the test on the BALB3T3 cells according to Annex 1 of the ISO 10993-5<sup>12</sup>. Neutral red stains the lysosomes in viable cells, and the quantity of released dye after washing, fixing, and dye extraction is used to determine the extent of viable cells. Values of >30% cell mortality were considered cytotoxic.<sup>12</sup>

### UHPLC Analysis

The content of anhydrous *p*-xylene and ethylbenzene in PFO was determined by UltiMate 3000 UHPLC (Dionex, Sunnyvale, CA, USA) equipped with ultraviolet detector set at 254 nm, poroshell 120 SB - C18 2.7µm (4.6 × 100 mm) column and Chromeleon 7.2.8 Software. *P*-xylene and ethylbenzene were extracted with methanol from the PFO, and the samples were analyzed by isocratic chromatography in a mobile phase consisting of water-methanol (30-70). The flow rate was set at 1 mL/min.

### Data Analysis and Statistics

At least six optical density (OD) values at 570 nm were acquired at each 96-well microplate for all samples (impurity, vehicle, positive control, and negative control) using the Absorbance Microplate Reader ELx 80 (Biotek Instruments, Winooski, VT, USA).

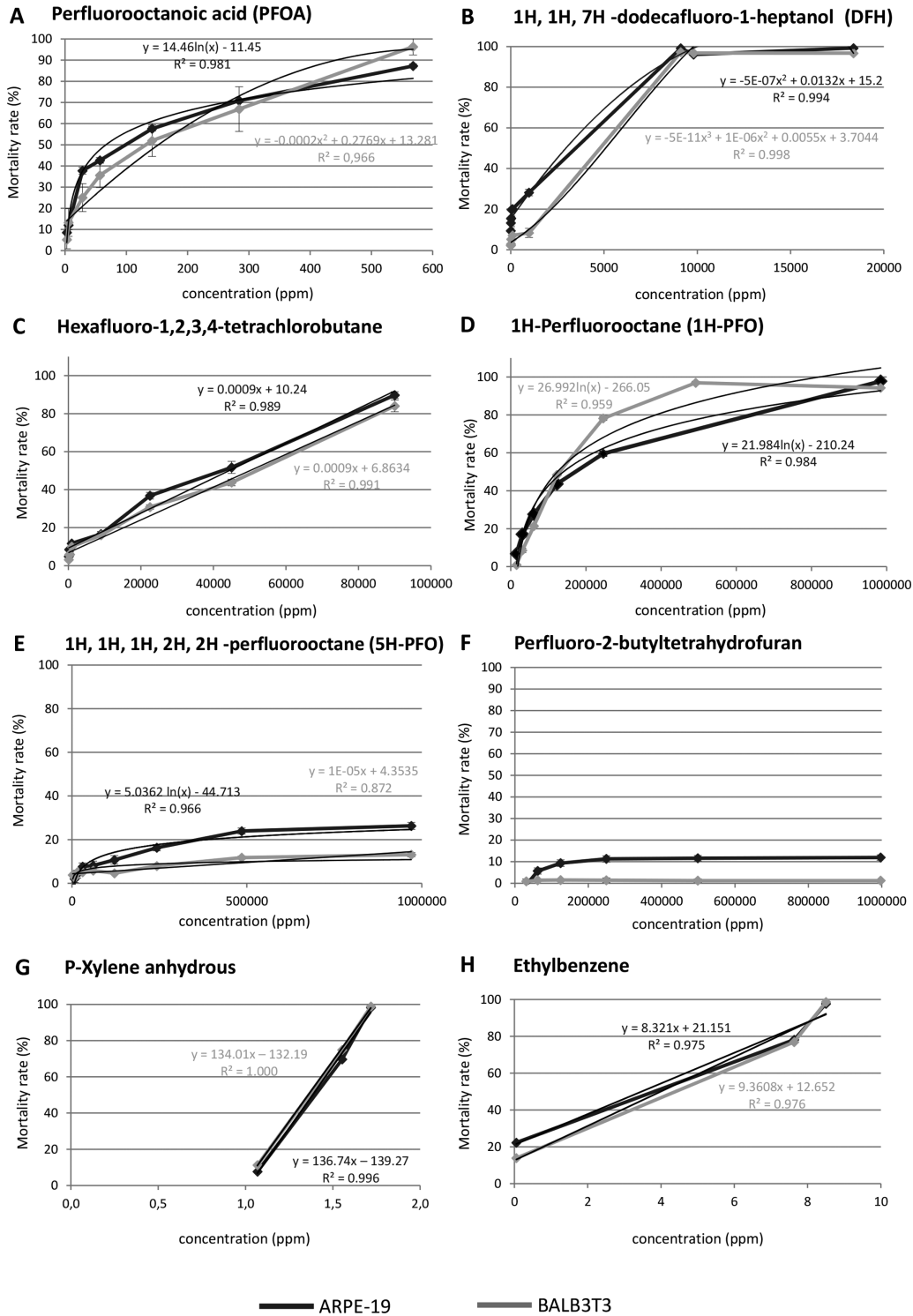
Mean percentage of reduction of cell viability was calculated as follows:

$$\text{Mean \% of reduction of cell viability} = \text{mean} \frac{\text{OD tested sample} - \text{OD blank \%}}{\text{OD vehicle} - \text{OD blank \%}}$$

(blank = vehicle with no cells + MMT or NRU + dissolving agent)

Mean percentage of reduction of cell viability of ARPE-19 and BALB3T3 cells and standard error of the mean (SEM) were calculated for each concentration of tested substances and both controls.

Theoretical values of cytotoxic concentration (CC<sub>30</sub>), corresponding to the concentrations that would reduce 30% of cell viability in ARPE-19 and BALB3T3 cells, for substances including *p*-xylene, ethylbenzene, PFOA, DFH, hexafluoro-1,2,3,4-tetrachlorobutane, 1-HPFO, 5H-PFO, and perfluoro-2-butyltetrahydrofuran were calculated from the fitted regressions reported in the Figure. The differences between cell lines in percent mortality were determined by using Student's *t*-test.



**Figure.** Graphic representation of the dose–response relationship of (A) PFOA, (B) DFH, (C) hexafluoro-1,2,3,4-tetrachlorobutane, (D) 1H-perfluorooctane, (E) 5H-PFO, (F) perfluoro-2-buthyltetrahydrofuran, (G) *p*-xylene, and (H) ethylbenzene.

## Results

According to the direct contact cytotoxicity test according to ISO 10993-5,<sup>12</sup> a sample is cytotoxic when it causes a reduction of cell viability greater than 30%. Table 1 illustrates the results of the cytotoxicity tests obtained with the tested dilutions of each substance.

Residues of *p*-xylene and ethylbenzene present in PFO were determined by UHPLC. The PFO samples containing *p*-xylene and ethylbenzene corresponding to 1.55 ppm and 7.63 ppm, respectively, induced cytotoxicity and cell mortality higher than 70% in both cell lines (Table 1).

PFOA induced the cytotoxicity above the 30% threshold when applied on ARPE-19 cells at 28.4 ppm, whereas the same concentration of PFOA induced mortality rate of  $25 \pm 2\%$  in BALB3T3, which was slightly below the cytotoxicity limit (Table 1). DHF applied at 980 ppm induced cytotoxicity in ARPE-19

cell line (mortality rate  $28 \pm 2\%$ ); the extent of cell mortality was  $8 \pm 1\%$  (Table 1) when the same concentration was applied on BALB3T3 cells. When 1H-PFO was applied at the concentration of 123,000 ppm, both BALB3T3 and ARPE-19 cell lines showed cell mortality greater than 30% (the cytotoxicity threshold); similarly, when hexafluoro-1,2,3,4-tetrachlorobutane was applied at 22,500 ppm, both cell lines showed cell mortality higher than 30%. Even when they were applied at the maximum available concentration of 970,000 ppm and 997,100 ppm, respectively, 5H-PFO and perfluoro-2-buthyltetrahydrofuran did not cause reduction of cell viability greater than 30% in ARPE-19 and in BALB3T3 cell lines (Table 1).

Table 2 shows the tested substances listed according to the decreasing order of cytotoxicity, along with the concentrations that reached the toxicity threshold in both cell lines (if applicable) and estimated CC30 values calculated based on the dose-response curves. Anhydrous ethylbenzene, *p*-xylene, and PFOA were the most cytotoxic among all tested substances, as they

**Table 2.** Tested Substances Listed in Order of Increasing CC<sub>30</sub> and Decreasing Cytotoxicity and Separated by the Definition of Toxicity/Nontoxicity

Tested Substance	Minimal Concentration Resulting in Cytotoxicity in Cell Lines (ppm)	CC <sub>30</sub> * (ppm)	
		ARPE-19 Cells	BALB3T3 Cells
<b>Cytotoxic<sup>†</sup></b>			
Ethylbenzene, anhydrous, C <sub>8</sub> H <sub>10</sub> , (99.87%, CAS 100-41-4)	7.63	1.06	1.85
<i>p</i> -Xylene, anhydrous, C <sub>8</sub> H <sub>10</sub> , (99.8%, CAS 106-42-3)	1.55	1.21	1.24
Perfluorooctanoic acid, C <sub>8</sub> HF <sub>15</sub> O <sub>2</sub> (PFOA, 98.8%, CAS 335-67-1)	28.4	17	63
1H,1H,7H-dodecafluoro-1-heptanol, C <sub>7</sub> H <sub>4</sub> F <sub>12</sub> O (DFH, 98%, CAS 335-99-9)	980	1173	3209
Hexafluoro-1,2,3,4-tetrachlorobutane, C <sub>4</sub> Cl <sub>4</sub> F <sub>6</sub> (90%, CAS 375-45-1)	22,500	21,956	25,707
1H-perfluorooctane, CF <sub>3</sub> (CF <sub>2</sub> ) <sub>7</sub> H (1-HPFO, 98.4%, CAS 335-65-9)	123,000	55,712	57,992
<b>Noncytotoxic<sup>†</sup></b>			
1H,1H,1H,2H,2H-perfluorooctane, C <sub>8</sub> H <sub>5</sub> F <sub>13</sub> (5H-PFO, 97%, CAS 80793-17-5)	970,000	2,772,322	2,564,455
Perfluoro-2-butyltetrahydrofuran, C <sub>8</sub> F <sub>16</sub> O (99.71%, CAS 335-36-4)	997,100	Undeterminable	Undeterminable

\*Theoretical values of cytotoxic concentration 30 (CC30) corresponding to the reduction of cell viability of 30% calculated from the fitted regressions.

<sup>†</sup>According to the direct contact test (ISO 10993-5, 2009), a substance is cytotoxic if cell viability reduction is greater than 30%.

achieved the toxicity threshold at very low concentrations. They were followed by DHF, hexafluoro-1,2,3,4-tetrachlorobutane, and finally, 1H-PFO. Even when tested as pure compounds, 5H-PFO and perfluoro-2-butyltetrahydrofuran were noncytotoxic, because they did not reach the toxicity threshold (Table 2).

The dose-response curves of the tested substances are illustrated in the Figure. Different types of dose-response curves and fitted trend lines were obtained. Cytotoxic concentration 30 (CC<sub>30</sub>) that corresponds to the concentration that reduced cell viability of 30% was calculated from fitted regressions for both cell lines. Calculated CC<sub>30</sub> values confirmed that ethylbenzene, *p*-xylene and PFOA were the most toxic substances showing the lowest CC<sub>30</sub> values. Nearly flat curves are shown (Fig.) by 5H-PFO and perfluoro-2-butyltetrahydrofuran, which resulted as noncytotoxic.

## Discussion

It is now acknowledged that the toxicity induced by AlaOcta PFO batches was related to the chemical damage and direct contact of the toxic impurities with the retina, causing retinal irreversible damage.<sup>9</sup> The company was clearly negligent in their responsibility for selling untested AlaOcta PFO toxic batches. The risk of presence or appearance of potentially toxic substances in PFCL-based medical devices used as vitreous tamponades needs to be excluded by the manufacturers using accurate analytical methods and adequate safety criteria for their clinical use.<sup>6-8,11,18,21</sup>

The different physical-chemical methods such as gas chromatography coupled with mass spectroscopy, nuclear magnetic resonance (NMR), ultraviolet-spectrophotometry and ion selective potentiometry<sup>10,13,21</sup> may be used to detect and quantify the presence of specific potentially hazardous substances. However, these methods do not provide any information on their toxicity. According to the ISO standard<sup>13</sup> the concentration of the contaminants in PFCLs should be as low as possible; however, the acceptance limits for specific PFCLs impurities are not clearly described.

In this study the substances that were previously found as contaminants of the toxic batches of AlaOcta PFO<sup>8,11,17</sup> along with those of 5H-PFO and hexafluoro-1,2,3,4-tetrachlorobutan were tested by in vitro validated direct contact cytotoxicity test<sup>15</sup> to obtain dose-response curves. The dose-response curves allowed to the level of the cytotoxicity of each tested substance based on an estimated CC<sub>30</sub>

(cytotoxic concentration that would reduce cell viability of 30%) and the lowest tested concentration that reached or passed the cytotoxicity threshold as defined by the ISO 10993-5<sup>12</sup> to be determined. The CC<sub>30</sub> value allowed the classification of tested substances on the basis of the level of cytotoxicity. The calculation of CC<sub>30</sub> was based on the dose-response curves that were obtained experimentally by application of seven to 11 concentrations of each tested substances to two cell lines and the quantification of reduction of cell viability after 24 hours of application. The CC<sub>30</sub> values calculated according to the equations confirmed the order of cytotoxicity obtained experimentally for all tested substances. The CC<sub>30</sub> values calculated for ethylbenzene and *p*-xylene were similar and both very low, corresponding to 1.06 ppm and 1.21 ppm, respectively, indicating that both substances show very high cytotoxicity at ppm level in tested cell lines. Our findings showed that, among all tested substances, ethylbenzene and *p*-xylene were the most toxic, followed by PFOA and DFH; hexafluoro-1,2,3,4-tetrachlorobutane and 1H-PFO were less but still cytotoxic, and finally, 5H-PFO and perfluoro-2-butyltetrahydrofuran were definitely noncytotoxic, even when tested at the highest commercially available concentrations.

The toxicity of PFCLs is often caused by incomplete fluorination of hydrocarbons, the major impurities in PFCLs contain residual hydrogen-containing compounds and unsaturated carbon bonds.<sup>20-22</sup> Chang et al.<sup>7</sup> determined hydrogen content in PFO by NMR spectroscopy. Because the safety threshold of protonated impurity in PFCL for intraocular use was unknown, they sought to obtain liquid of the highest grade corresponding to protonated impurity content < 0.1 ppm and equivalent to the detection limit of the method.<sup>16</sup> The work of Sparrow et al.,<sup>22</sup> demonstrated the toxicity of 1H-PFO at the perfluorocarbon-fluid interface in tissue culture by a qualitative assessment. These studies<sup>7,22</sup> indicated the level of impurities that were used for approval of perfluoro-*n*-octane from the U.S. Food and Drug Administration.

Menz et al.<sup>10</sup> used physical-chemical determination of partially hydrogenated perfluoroalkanes through the ion-selective potentiometry after digestion of perfluorocarbon liquid.<sup>13,21</sup> This analysis determines the so-called H-value, defined as the ppm content of reactive partially hydrogenated perfluoroalkanes and to which an H-value < 10 ppm was attributed as the safety threshold by Menz et al.<sup>10</sup> However, the ion-selective potentiometry does not detect toxic substances without a specific hydrogen-fluoride-containing compounds such as the hydroxyl impurities identified in the toxic batches of PFCL.<sup>8,11,17</sup>

Our study showed that in vitro cytotoxicity test can provide direct information on cytotoxicity of impurities contained in PFCLs, independently from substances identification and quantification.

The findings in this study are in agreement with the results obtained by Ruzza et al.,<sup>18</sup> who studied the extent of ARPE-19 and BALB3T3 cell mortality after the application of 1H-PFO, PFOA, and 5H-PFO at three of the concentrations tested in this study and Romano et al.'s.<sup>15</sup> The toxicity threshold assessed for PFOA and DFH in this study only slightly differed from those evaluated by Srivastava et al.,<sup>17</sup> who found that PFOA at 0.06 mM was close to the toxicity limit, which corresponded to 25 ppm versus CC<sub>30</sub> of 17 ppm determined in our study, and DFH was toxic at 4.48 mM (corresponding to 1488 ppm vs. 1173 ppm in our study). It is possible that the small differences between the two studies were related to the different testing conditions (e.g., sample size, contact time and number of replicates).

Consistent with the findings from other studies,<sup>14,15,17,21</sup> the extent of mortality of ARPE-19 and BALB3T3 cells in this study generally increased at each increasing concentration of all tested substances.

With respect to analyses of the contaminant profiles of the toxic AlaOcta batches conducted by Menz et al.,<sup>16</sup> we might affirm that the toxicity of those batches could not be solely related to the presence of 1H-PFO or DFH, because their concentrations, which differed between the 59 and 875 ppm for 1H-PFO and between 29 and 45 ppm for DFH, were well below the cytotoxicity limit (CC<sub>30</sub>) calculated in this study (CC<sub>30</sub>: 55712 ppm for 1H-PFO and 1173 ppm for DFH). Instead, the PFO batches containing PFOA at concentrations varying between 50 and 700 ppm and a mix of *p*-xylene isomers/ethylbenzene (82%:18% ratio) at 5 to 30 ppm<sup>16</sup> were surely toxic, because the quantity of these compounds was well above the cytotoxic limit found in this study (CC<sub>30</sub>: 17 ppm for PFOA; 1.21 ppm for *p*-xylene and 1.06 ppm for ethylbenzene). In addition, Srivastava et al.<sup>17</sup> also analyzed the contaminants in the toxic AlaOcta batches and found that DFH was contained at nontoxic concentration, whereas PFOA was detected at concentrations that reduced the ARPE-19 cell viability of > 70% and thus were inevitably cytotoxic. However, as also affirmed by the two groups of investigators,<sup>16,17</sup> the ocular toxicity of those AlaOcta batches were likely to be due to the combined effect of all contaminants rather than one specific substance; furthermore, the individual risk of each type of contaminant may be increased by the interaction with other contaminant groups.<sup>16</sup> In the presence of unknown contaminants, the cytotoxicity test represents a relatively fast and

simple quality control method because it detects the overall cytotoxicity of samples and can be used for monitoring of the cytotoxicity during manufacturing processes.

Similarly to the previous study,<sup>15</sup> close contact between the contaminant diluted in PFO and the cell layer for 24 hour was obtained, and the cell mortality induced by cytotoxic substances was mainly located in the contact area under the PFO bubble.

In addition to murine fibroblast cells BALB3T3 cell lines required by the ISO 10993-5 standard,<sup>12</sup> we used the human retina-derived ARPE-19 to imitate more closely what happens during vitreoretinal surgery. In line with previous studies,<sup>15,18</sup> we can assert that overall trend for higher sensitivity of ARPE-19 cells to contaminants was confirmed.

In summary, the results in this study indicate that the in vitro direct-contact cytotoxicity test was highly sensitive to hazardous contaminants even when they were tested at very low concentrations, and the dose-response curves obtained with this test allowed the cytotoxicity concentration of the tested substances to be determined. The in vitro direct contact cytotoxicity test may represent an additional tool to assess the safety of the PFCLs batch. Finally, the manufacturing processes of PFCLs should ensure the absence of impurities, and the safety evaluation should comprise whole range of validated methods including NMR spectroscopy, gas chromatography coupled with mass spectroscopy, in vitro cytotoxicity testing by direct contact, and animal model testing in the design phase, before the product can be considered for clinical use.

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