Regulatory Toxicology and Pharmacology 110 (2020) 104527

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Chemical compounds causing severe acute toxicity in heavy liquids used for intraocular surgery

Girish K. Srivastava[a,b,c], Cristina Andrés-Iglesias[a,*], Rosa M. Coco[a,c], Ivan Fernandez-Bueno[a,b,c], Jesús Medina[d], Juan García-Serna[e], Antonio Dueñas[a,f], Fernando Rull[d], J. Carlos Pastor[a,b,c,g]

[a] Instituto Universitario de Oftalmobiología Aplicada (IOBA), Eye Institute, Universidad de Valladolid, Valladolid, Spain
[b] Centro en Red de Medicina Regenerativa y Terapia Celular, Junta de Castilla y León, Valladolid, Spain
[c] Red Temática de Investigación Cooperativa en Salud (RETICS), OftaRed, Instituto de Salud Carlos III, Valladolid, Spain
[d] Department of Physics of Condensed Matter, Crystallography and Mineralogy, Universidad de Valladolid, Valladolid, Spain
[e] High Pressure Processes Group, Instituto de Bioeconomía, Departamento de Ingeniería Química y Tecnologías del Medio Ambiente, Universidad de Valladolid, Valladolid, Spain
[f] Toxicology Department, Universidad de Valladolid, Valladolid, Spain
[g] Department of Ophthalmology, Hospital Clínico Universitario de Valladolid, Valladolid, Spain

ARTICLE INFO

Keywords:
Cytotoxicity
Retina
Gas-chromatography
Mass spectrometry
FTIR
Perfluorooctanoic acid
ARPE-19
Perfluorooctane
Perfluorohexyl-octane
Tributyltin bromide

ABSTRACT

Perfluorocarbon liquids (PFCLs) have been considered safe for intraocular manipulation of the retina, but since 2013 many cases of acute eye toxicity cousing blindness have been reported in various countries when using various commercial PFCLs. All these PFCLs were CE marked (Conformité Européenne), which meant they had been subjected to evaluation complying with the International Organization for Standardization (ISO) guidelines. These dramatic events raised questions about the safety of PFCLs and the validity of some cytotoxicity tests performed under ISO guidelines. Samples from toxic batches were analyzed by gas chromatography-mass spectrometry combined with Raman and infrared spectrometry. Perfluorooctanoic acid, dodecafluoro-1-heptanol, ethylbenzene and tributyltin bromide were identified and evaluated by a direct contact cytotoxicity test using ARPE-19 cell line, patented by our group (EP 3467118 A1). Perfluorooctanoic acid at a concentration of > 0.06 mM and tributyltin bromide at a concentration of ≥ 0.016 mM were shown to be toxic, whereas the concentration found in the toxic samples reached 0.48 mM, and 0.111 mM, respectively.

These finding emphasized the idea that determination of partially fluorinated compounds are not enough to guarantee the safety of these medical devices.

1. Introduction

PFCLs have commonly been used as intraocular surgical tools since they were introduced in the 1990s by Chang (Chang et al., 1991), and they are considered bench-stable and well tolerated by the human eye, at least when used for short periods of time; if they remain inside the eye for long periods they may induce chronic intraocular inflammation (Bourke et al., 1996; Elsing et al., 2001; Figueroa and Casas, 2014: Yu et al., 2014). This is mainly why their removal is strongly recommended as soon as possible, and certainly before the end of surgery (Elsing et al., 2001). PFCLs for clinical use are regulated by the International Organization for Standardization (ISO) 16672:2015 "Ophthalmic Implants – Ocular Endotamponades", among others. According to the latter, manufacturers shall provide a description of each of the components in the finished product, and contaminants shall be quantified and identified whenever possible. Since 2013, serious episodes of acute toxicity related to the use of three commercial products, Merocta ne®, AlaOcta® and BioOctane Plus®, have occurred. All these products had the CE mark and were examined by different notify bodies according to the above-mentioned ISO guideline. These episodes have been reported in at least 125 patients in Spain and many more in other countries, and have resulted in cases of visual loss and even total blindness (Coco

Abbreviations: PFCL, perfluorocarbon liquids; ISO, International Organization for Standardization; CE, Conformité Européenne; PFO, perfluorooctane; ATR, attenuated total reflectance; GC-MS, gas chromatography-mass spectrometry; TBT-Br, Tributyltin bromide; PFOA, perfluorooctanoic acid; DFH, dodecafluoro-1-heptanol; ARPE-19, adult human retinal pigment epithelial cell line 19; FBS, fetal bovine serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; OD, optical density; FTIR, Fourier-transform infrared spectroscopy; IR, infrared spectroscopy; F6H8, perfluorohexyl-octane

* Corresponding author.
E-mail address: candresi@ioba.med.uva.es (C. Andrés-Iglesias).

https://doi.org/10.1016/j.yrtph.2019.104527
Received 26 July 2019; Received in revised form 5 November 2019; Accepted 8 November 2019
Available online 14 November 2019
0273-2300/

et al., 2018; Januschowski et al., 2018; Pastor et al., 2017). The episodes were acute events that appeared immediately after short periods of contact between PFCLs and the retina (< 15–20 min, in order to facilitate certain surgical manoeuvers). The signs and symptoms have been described (Coco et al., 2018; Januschowski et al., 2018; Pastor et al., 2017) and most of them resemble those that occurred many years ago after the use of intravitreal aminoglycosides to treat severe intraocular infections, which were attributed to the low pH of these drugs (Querques et al., 2017).

The pharmaceutical companies selling these products adhered to the European guidelines for cytotoxicity tests; however, some of the cytotoxicity evaluation methods used, despite complying with ISO 10993–5:2009 "Biological Evaluation of Medical Devices", failed to identify the toxic ones (Januschowski et al., 2018). In this regard, an indirect method was used for evaluating BioOctane Plus® and an extraction method for AlaOcta® (there is no information on the procedure of Meroctane®), and both failed to detect cytotoxicity probably because they did not consider the hydrophobic and volatile nature of PFCLs (Srivastava et al., 2018).

In an effort by the scientific community and producers to clarify these incidents, some manufacturers have claimed that the problems with these products were due to excessive concentrations of incompletely fluorinated compounds (those in which not all the C–H bonds have been replaced by C–F bonds) in the samples. Therefore, they have proposed limiting their presence in ocular endotamponades to10 ppm, as a criterion for determining that PFCLs are not cytotoxic (Menz et al., 2018). However, the method proposed to evaluate these types of impurities is under critical discussion (Srivastava et al., 2019; Menz et al., 2019-a), as not all partially fluorinated PFCLs will react to the mechanism proposed, which, according to Ruzza et al. (2019), requires the presence of a CHF-CF_2 moiety to achieve stoichiometry. Hence, other contaminants found in cytotoxic batches would not react or would react with a different stoichiometry, leading to an incorrect determination of the proposed method (Ruzza et al., 2019; Srivastava et al., 2019). Simple analytical methods could have been used to detect the presence of toxic impurities in the PFCL samples, such as gas chromatography-mass spectrometry or UV absorption at 220 nm–180 nm, which would detect the presence of impurities (Chang, 2017).

Furthermore, manufacturers are obliged to meet the requirements of ISO 14971 on risk management for medical devices and EU regulations regarding the CE mark, such as Medical Device Directive CE 93/42/CEE, which since 2017 has been replaced by Medical Device Regulation (2017)/745. Accordingly, a complete set of specification parameters must be specifically defined, and these need to be routinely checked in each manufactured batch. However, the companies of these toxic products also failed to meet such requirements.

The aim of this study has been to identify contaminants in toxic batches of PFCLs by means of different analytical techniques, and to evaluate the cytotoxic concentrations of these compounds by a direct contact method based on ARPE 19 cells, taking into consideration the physical characteristics of the PFO (hydrophobicity and volatility).

2. Materials and methods

Gas chromatography-mass spectrometry (GC-MS) and vibrational spectroscopy were used to evaluate the differences between non-toxic and toxic batches of PFCL samples (AlaOcta® and Bio Octane Plus®). AlaOcta® samples were analyzed using Raman and Fourier-transform infrared spectroscopy (FTIR) transmission and attenuated total reflectance (ATR). Raman was used to structurally characterize perfluorooctane (PFO) compounds, examining the vibrational mode characteristics of the PFO molecule.

2.1. Samples

Non-toxic PFO samples currently marketed by three companies (the names of the companies are undisclosed in this study due to confidentiality agreements), and repeatedly shown to be non-cytotoxic by direct contact methods, were used as controls. In addition, a non-toxic sample of Bio Octane Plus® (a mixture of 90% PFO and 10% F6H8 [perfluorohexyl-octane]), batch number 1406119 (Biotech Vision Care, Gujarat, India), was used (Coco et al., 2018; Januschowski et al., 2018; Pastor et al., 2017).

Cytotoxic samples of PFO batches of AlaOcta® (Alamedics, Dornstadt, Germany), numbers 171214 (99% cell death), 061014 (99% cell death), 070714 (47% cell death) and 050514 (50% cell death), and samples of Bio Octane Plus® batch number 1605148 (100% cell death), were used (the names are identified as this is public information released by health authorities) (Coco et al., 2018; Januschowski et al., 2018; Pastor et al., 2017).

2.2. Vibrational spectroscopy

AlaOcta® samples were analyzed by vibrational spectroscopy: (1) Raman Spectrometer Kaiser OSI HoloSpec f/1.8i, with a spectral range of 100–3800 cm^{-1} and spectral resolution of 5 cm^{-1}, illuminated with a Laser Research Electro-Optics LSRP-3501, He–Ne at 632.8 nm. The optical head was a Raman Horiba Jovin-Yvon Superhead coupled to a Nikon Eclipse E600 microscope, and using a Nikon 100x long focal lens. The detector was a CCD Andor DV420A-OE-130 working at −40 °C. In most cases, and especially at the beginning of the experiments, the Raman spectra were taken inside the closed vials through the cover glass; (2) Infrared Perkin Elmer Spectrum 100 FT-IR spectrometer, working in the range of 450–4000 cm^{-1} with a spectral resolution of 4 cm^{-1} and 16 scans per spectrum. The transmission cell was a Perkin Elmer cell for liquid analysis with variable separation and ZnSe windows. The thickness of the cell is variable in the range of 1–4 mm so as to optimize the intensity of the spectral region of interest to about 60–80% of instrument response before saturation, whilst maximizing the signal to noise ratio (SRN) that will allow suitable differential spectra to be obtained. In all cases, the spectra to be compared were acquired in the same experimental conditions (the same optimized thickness and acquisition time), and each spectrum was repeated 3 times. The ATR device was a Universal ATR (UATR) single reflection diamond/ZnSe accessory for solids and liquids, and once again from Perkin Elmer.

2.3. Gas chromatography-mass spectrometry

One non-toxic (batch 1406119) and three toxic samples from various vials (batch 1605148) of Bio Octane Plus® were analyzed by GC-MS. Samples were stored in their original packages at room temperature and accessed only by study investigators. On the day of the analysis, 1.5 mL of each sample was placed in a 2 mL vial for GC-MS. Non-toxic samples were used as controls to evaluate possible differential toxic compounds, and all the samples were analyzed in triplicate.

One μL of each sample was injected into a 7890B GC system gas chromatography (Agilent Technologies, USA), coupled to a 5977A MSD (Agilent Technologies, USA) single quadrupole mass spectrometer. For gas chromatographic separation an HP-5ms capillary column of 30 m × 0.25 mm x 0.25 μm (Agilent Technologies, USA) was used. Helium at a constant flow of 0.7 mL/min was employed as the carrier gas. Splitless injection at 250 °C was used and the chromatography oven was programmed as follows: the initial temperature was set at 35 °C and was raised by 3 °C/min to 165 °C, maintained for 2 min, and then raised by 10 °C/min to 270 °C and maintained for 5 min.

The mass spectrometer was operated at 70 eV. The MS source temperature was kept at 230 °C and the MS quadrupole temperature at 150 °C. Detection and data acquisition were performed in scan mode

from 20 to 600 Da. Data analysis was performed using MassHunter Data Acquisition software (Agilent Technologies, USA).

2.4. Preparation of concentrations of tested contaminants for cytotoxicity evaluation

For biological analysis, serial concentrations of the identified contaminants (by GC-MS and vibrational spectrometry) in the toxic samples of Bio Octane Plus® (TBT-Br) and AlaOcta® (PFOA and DFH), were prepared by mixing each synthetic standard with non-toxic PFCL samples used as controls, as described below:

For tributyltin bromide (TBT-Br), serial concentrations of 1.622, 0.162 and 0.016 mM were prepared by mixing synthetic TBT-Br (Sigma Aldrich, reference 257893) with the non-toxic sample of Bio Octane Plus® batch number 1406119. Since all the concentrations were highly cytotoxic, lower concentrations of 0.0162, 0.0016 and 0.0002 mM, were prepared by mixing synthetic TBT-Br with a non-toxic PFCL sample used as control.

For perfluorooctanoic acid (PFOA), serial concentrations of 0.60, 0.24, 0.12 and 0.06 mM were prepared by mixing synthetic PFOA (Acros Organics, reference 173960250) with a non-toxic PFO control sample. For dodecafluoro-1-heptanol (DFH), serial concentrations of 264.99, 52.99, 26.50 and 4.48 mM were prepared by mixing synthetic DFH (Alfa Aesar, reference B20144) with a non-toxic PFO sample used as control.

2.5. Biological analysis

Direct contact cytotoxicity tests were performed as previously described by Coco et al. (2018), Pastor et al. (2017) and Srivastava et al. (2018). In brief, cultures of human retinal pigment epithelial cell line-19 (ARPE-19) cells were prepared in 96-well culture plates, followed by 24-h cell cycle synchronization in a FBS-free cell culture medium. Cultures were then exposed directly to the non-toxic PFCL mixed with contaminants (TBT-Br, PFOA and DFH) at different concentrations for 60 min. Our choice of 60 min exposure time was determined by our previous studies with other PFCL toxic samples, which enabled us to conclude that the selection of parameters such as exposure times and post-exposure growth periods are important to confirm toxicity (Srivastava et al., 2018). Following exposure, non-toxic PFCL mixed with contaminants and the culture medium were removed from each well. Cell cultures were washed to remove any remnants of contaminants and then incubated for 24 h for cell growth. Subsequently, the viability of cell cultures was measured by MTT assay. Experiments were performed in accordance with ISO guidelines for cell cytotoxicity tests and with Good Laboratory Practices certification. Values of < 70% viability were considered cytotoxic (ISO 10993-5).

Data were analyzed by calculating the optical density (OD) value of cell culture viability in each well, which was recorded with a SpectraMax® M5. All the data were analyzed by Microsoft Excel and a previously published formula (Srivastava et al., 2018). The percentage of viable cells, standard deviations (SD) and p values were calculated. The percentage of viable cells obtained for wells incubated with only the cell culture medium was set at 100%, and this was compared with the others so as to determine the possible cytotoxic effects of contaminants on cells.

3. Results

3.1. Chemical analysis

AlaOcta® samples (batches 171214, 061014. 050514 and 070714) were analyzed by FTIR and Raman vibrational spectroscopy techniques. The results were consistent with those expected for Raman active modes and no structural differences were observed between non-toxic and toxic PFO samples. FTIR in ATR mode was also used for structural verification in order to ascertain the IR active PFO vibrational modes. The toxicity observed for certain PFO compounds could not be assigned to structural modifications of the PFO molecule. In addition, spectra displaying slight differences in spectral regions non-characteristic of PFO vibrations were obtained. Previously-assigned intense bands were disregarded, as the lengthy integration times saturated the detectors and attention was focused on very weak bands other than those of PFO, with tiny differences being observed between non-toxic and toxic compounds.

A new methodology has been developed to confirm and analyze these differences in greater detail. FTIR in transmission mode was used with a special transmission cell adapted to observe the small bands. The results showed clear differences in the C–H, OH and C═O regions, and particularly noticeable were the C═O (bands at 1772, 1800 and

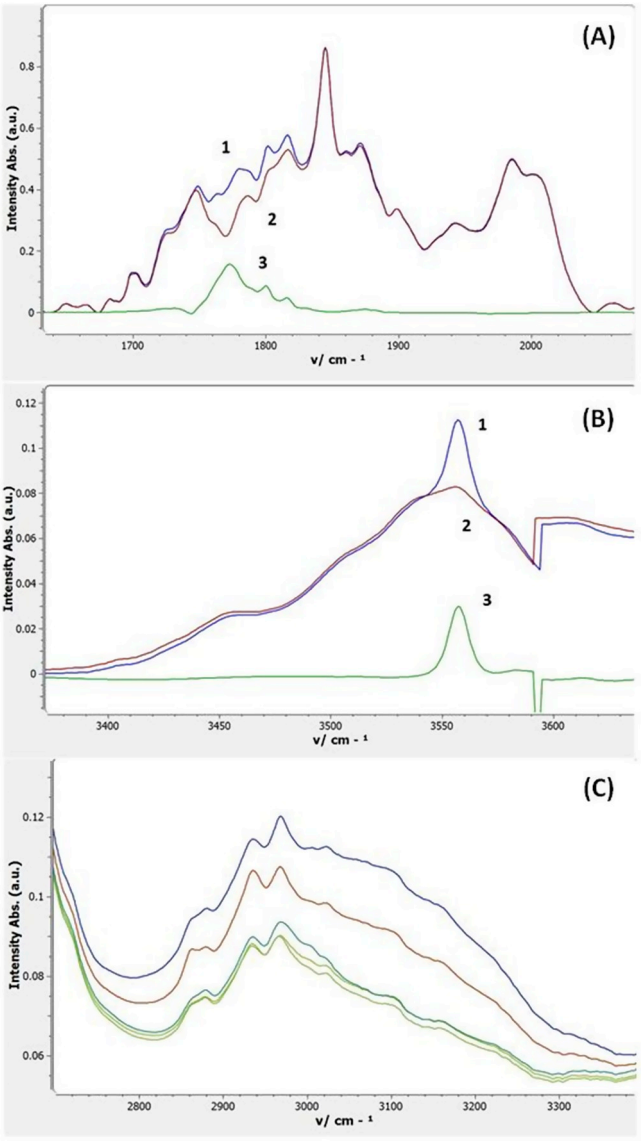

Fig. 1. Fourier-transform infrared spectroscopy (FT-IR) of (A) transmission spectra in the v(C═O) spectral region of PFO. Toxic sample (1), non-toxic (2) and the different spectrum after baseline correction (3). The main band was observed at 1772 cm^{-1}. (B) Transmission spectra in the v(O–H) spectral region of PFO. Toxic (1) and non-toxic (2) samples and the different spectrum after baseline correction (3). The main band was observed at 3557 cm^{-1}. (C) Transmission spectra of PFO in the v(C–H) spectral region. Toxicity increased from bottom (non-toxic) to top (highest toxicity). The number of different C–H species present in this region is noteworthy.

Table 1
OH band intensity, mean, standard deviation, error and concentration of perfluorooctanoic acid found in the toxic batches of AlaOcta®.

AlaOcta® samples	I (OH)			Mean	Standard deviation	Error (%)	Concentration (mM)
171214	0.510	0.503	0.506	0.506	0.004	0.69	0.48
061014	0.254	0.250	0.257	0.254	0.004	1.38	0.23
070714	0.070	0.060	0.064	0.065	0.005	7.78	0.06
050514	0.130	0.125	0.134	0.130	0.005	3.48	0.12

1816 cm^{-1}) and OH (3557 cm^{-1}) assigned to the COOH functional group (Fig. 1 A and B). To analyze these bands in detail, spectra treatment consisting of the following steps was used: precise baseline correction, intensity normalization by a common reference band and, finally, calculation of the differences between spectra from non-toxic and toxic compounds. This permitted a semi-quantitative estimate of the differences.

The COOH group was identified as deriving from PFOA. To confirm identification and estimate the concentration of PFOA in the toxic batches of AlaOcta®, mixtures of synthetic standard PFOA and control (non-toxic) PFO compounds were prepared in concentrations similar to those observed in the spectra (0.483, 1.207 and 2.415 mM). These concentrations were used to construct the calibration curves by means of the integrated intensity of the characteristic bands of PFOA (OH and C═O) and the concentrations. The calibration curve of the OH vibrational band was used for more accurate semi-quantification of PFOA in the toxic samples. The results revealed the presence of 0.48, 0.23, 0.12 and 0.06 mM in toxic sample batches 171214, 061014, 050514 and 070714, respectively (Table 1). These results confirmed the previous assignment, and the estimated concentrations were used for cytotoxicity analysis.

In addition to the OH band at 3557 cm^{-1} assigned to the acidic species and, in this particular case, to PFOA, the IR spectra showed very slight differences in the OH region, at 3647 cm^{-1}. This was consistent with alcoholic species, but in this case the lack of clear characteristic features in other spectral regions made this assignment difficult. Nevertheless, results obtained in a previous work by GC-MS clearly identified DFH (Pastor et al., 2017). To corroborate these results, dilutions of DFH in control samples of PFO and spectral comparison with the toxic samples confirmed the presence of the alcohol by IR spectroscopy.

Moreover, spectra obtained by FTIR in transmission mode showed significant differences between control samples, and between control and toxic samples (Fig. 1 C). These bands were assigned to partially-fluorinated hydrocarbons, but the complexity of the spectral features do not appear to be consistent with this interpretation. In the case of the toxic samples, C–H bands at 2859, 2936 and 2968 cm^{-1} were assigned to ethylbenzene, as reported previously by Pastor et al. (2017).

In the case of Bio Octane Plus®, toxic and non-toxic batches 1605148 and 1406119, respectively, were analyzed by GC-MS. The data analysis demonstrated that the toxic samples showed a differential peak at 41.61 min, which was identified as TBT-Br. This toxic compound was previously identified and reported by Pastor et al. (2017). In this study, a highly accurate identification and quantification is presented. Identification was first based on the direct match with the NIST17 spectra library, with a Match and R. Match of 912 and 961, respectively. This identification was validated by comparison of MS spectra and retention time with the standard and the extracted ion chromatogram (EIC) of ion fragments 313, 199, 257 and 57 m/z (Fig. 2).

To determine the concentration of TBT-Br in the toxic batch samples, a calibration curve was constructed by adding different amounts of standard TBT-Br (0.5, 1, 2, 3, 5 and 7 µL, all of which were also weighed) to 1000 µL of the non-toxic sample of Bio Octane Plus®. Due to the tiny amount of sample available, the GC-MS split mode was used to ensure correct dilutions for the calibration curve. A split ratio of 1:50 was applied in samples and for the calibration curve, with the corresponding concentrations: 0.5 µL (0.0432 mM), 1 µL (0.0918 mM), 2 µL (0.1996 mM), 3 µL (0.2964 mM), 5 µL (0.3927 mM), and 7 µL (0.4402 mM). Analyses were performed in triplicate, and toxic samples were analyzed in the same sequence, also in triplicate. TBT-Br concentrations in the toxic samples were calculated by using the area of the characteristic ion fragment 313 m/z [$C_8H_{19}BrSn$] and the calibration curve. The results showed the presence of 0.1106 mM, 0.0960 mM and 0.0962 mM in the three toxic samples analyzed from different vials of the same batch (Table 2).

In summary, toxic samples of AlaOcta® contained PFOA at a concentration of 0.48 to 0.06 mM. Toxic samples of Bio Octane Plus® analyzed by GC-MS showed TBT-Br at a concentration of up to 0.111 mM (Table 3).

3.2. Biological analysis

As quality standards, cell cultures for biological analysis should respond to positive (phenol; ~1% viability) and negative (culture medium, PFCL; ~100% viability) control samples, with a number of homogeneous cells maintained (variation ≤ 15% viability) in each culture plate and > 0.2 OD. Cell cultures exposed to two non-toxic PFO samples (PFO* and PFO**) maintained viability at 95% (95 ± 3 and 95 ± 6, respectively) compared with 100% viable cell cultures incubated with cell culture medium.

To evaluate the toxicity of PFOA and DFH clearly identified and semi-quantified by FTIR and Raman in AlaOcta® toxic samples, different diluted concentrations of the synthetic PFOA and DFH in non-toxic PFO, described in section 2.4, were used in the cell cultures (Fig. 3). Similarly, for BioOctane Plus®, various diluted concentrations of synthetic TBT-Br in non-toxic control samples, also described in section 2.4, were employed for cytotoxicity tests (Fig. 4).

Cytotoxicity evaluation of PFOA showed that cell cultures exposed to 0.60, 0.24, and 0.12 mM concentrations of PFO mixed with PFOA reduced viability to 0%, 50% and 57%, respectively. Cell viability was at the limit of toxicity, at a concentration of 0.06 mM. Cytotoxicity evaluation of DFH showed that, in cell cultures exposed to 264.99, 52.99 and 26.50 mM concentrations of PFO mixed with DFH, viability was reduced to 0%, with the toxicity limit at 4.48 mM (Fig. 3).

In the case of TBT-Br, in cell cultures exposed to 1.622, 0.162 and 0.016 mM concentrations of non-toxic Bio Octane Plus® mixed with TBT-Br, viability was reduced to 3%, 3% and 2%, respectively; this showed that ≥0.016 mM concentrations are toxic. In further experiments, a non-cytotoxic PFO was used to prepare the mixture due to the tiny amount of non-toxic Bio Octane Plus® sample available. The experiments were performed for 0.0162, 0.0016 and 0.0002 mM of a mixture of non-cytotoxic PFO and synthetic TBT-Br. At these concentrations, the viability of the cell cultures was reduced to 1% for 0.0162 mM. However, viability was maintained at 101% and 100% for 0.0016 and 0.0002 mM, respectively (Fig. 4).

4. Discussion

Although sub-acute toxicity has been demonstrated for PFCLs if they remain in the eye (Pastor et al., 2017), these series of acute toxicity events are new in the literature.

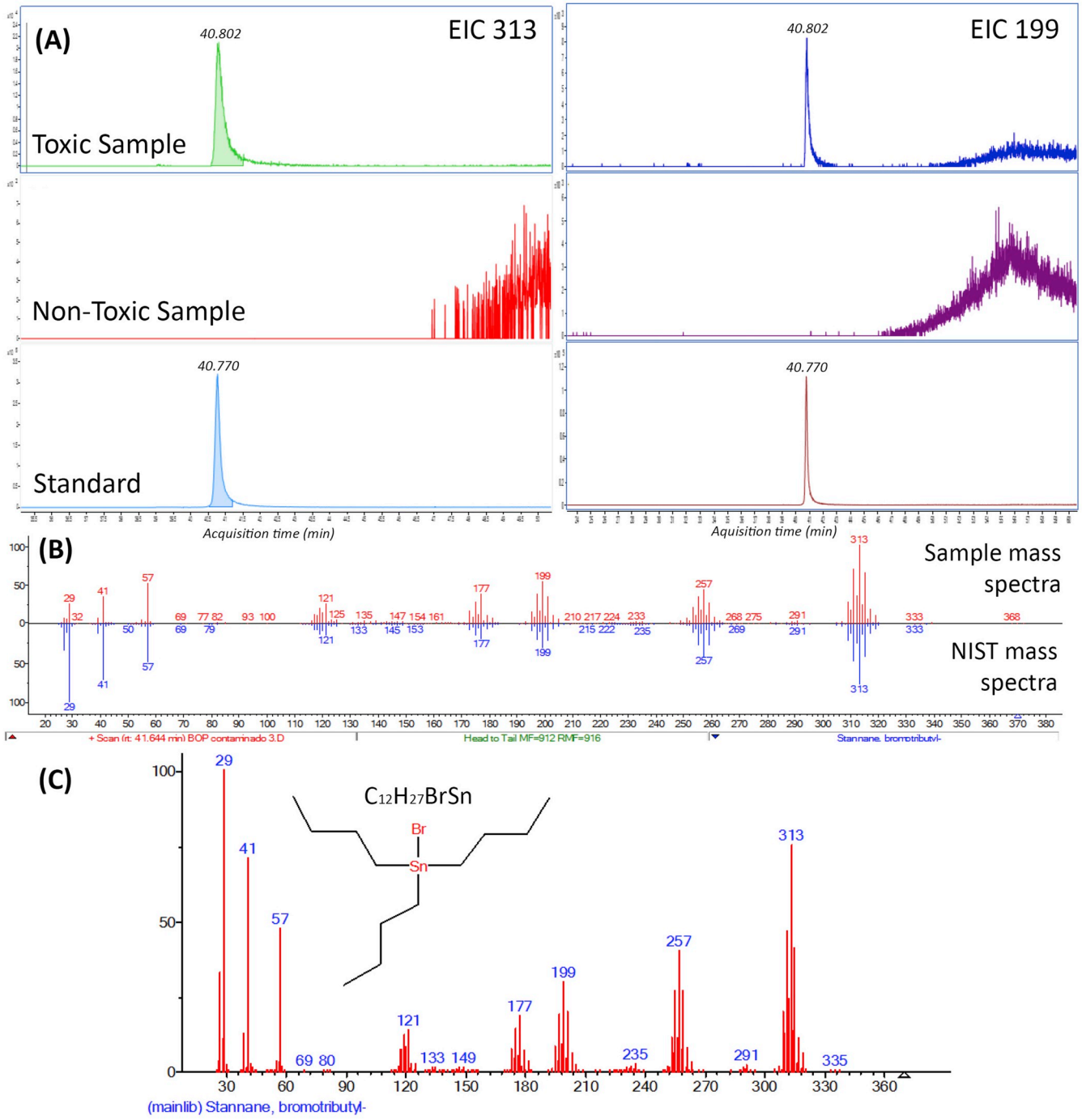

Fig. 2. (A) Comparison of the extracted ion chromatogram (EIC) in Bio Octane Plus® non-toxic (batch 1406119) and toxic (batch 1605148) samples and the standard of TBT-Br for ion fragments 313 and 119*m/z*. (B) Comparison of the sample mass spectra and the NIST mass spectra. (C) Mass spectra of TBT-Br.

Table 2
Sample data of the integrated area from the chromatogram; mean, standard deviation, error and concentration found in the toxic batch.

	EIC 313 [m/z]				Standard deviation	Error	Concentration (mM)
	Area 1	Area 2	Area 3	Mean			
Sample 1	3.39E+06	2.91E+06	3.31E+06	3.21E+06	2.54E+05	7.94%	0.1106
Sample 2	2.74E+06	2.85E+06	2.68E+06	2.76E+06	8.85E+04	3.21%	0.0960
Sample 3	2.67E+06	2.84E+06	2.78E+06	2.76E+06	8.65E+04	3.13%	0.0962

Table 3
Concentration of PFOA and TBT-Br in the AlaOcta® and Bio Octane Plus® samples.

PFCL sample	Toxic compounds	Analytical techniques	Batches	Concentration (mM)
Bio Octane Plus®	Tri-n-butyltinbromide	GC-MS	1605148	0.111–0.096 ± 0.05
AlaOcta®	Perfluorooctanoic acid	Vibrational spectroscopy (semi-quantitative)	171214	0.48 ± 0.004
			061014	0.23 ± 0.004
			050514	0.12 ± 0.005
			070714	0.06 ± 0.005

For AlaOcta® samples, concentrations were estimated by FTIR and prepared by means of a comparison with synthetic admixtures of the toxic products identified and the control batches. The AlaOcta® toxic samples showed PFOA at concentrations of 0.48, 0.23 and 0.12 mM, which reduced cell viability to < 70%; this confirmed that the concentration found in the commercial PFO samples was toxic for retinal cells. In the case of 0.06 mM, the acid concentration was at the limit of toxicity (Fig. 5 A). In this regard, the results of a comparison of cell death by using our direct contact method (Srivastava et al., 2018) in the different synthetic admixtures of PFO and PFOA, concentrations showed that the higher the concentration of acid in the batch, the greater the cytotoxicity (Fig. 5 A). In addition, the interaction of toxic compounds other than PFOA is clear in 2 of the analyzed batches; for instance, DFH, benzene derivatives and the underfluorinated impurities described in Menz et al. (2019-b). In batches 070714 and 061014, cell death increased from 30% to 47% and from 50% to 99%, respectively (Fig. 5 B).

Cell cultures confirmed that 264.99, 52.99 and 26.50 mM of DFH are cytotoxic for ARPE-19 cells (Fig. 3). Cell viability was at the limit of toxicity, at 4.818 mM. Chemical analysis showed 0.483 mM of DFH in toxic samples, suggesting DFH at this concentration might not be solely the cause of acute toxicity. In the case of Bio Octane Plus®, the biological results showed that cells died at a concentration of 0.016 mM of TBT-Br, and, therefore, all higher concentrations will inevitably be cytotoxic. The concentration of the samples analyzed by GC-MS, was 0.096–0.111 mM of TBT-Br in toxic samples, supporting the hypothesis that the acute toxicity was caused by TBT-Br.

In the production processes of organic fluoride compounds, the hydrogen atoms of alkanes may be partially or totally replaced by fluorine. Fully fluorinated alkanes (perfluoroalkanes), with an empirical formula of C_nF_{2n+2}, are perfluorocarbons (PFCs) when all the hydrogen atoms bonded to the carbon have been replaced by fluorine, whereas partially fluorinated alkanes are those in which not all the hydrogen atoms are replaced by fluorine (Sandford, 2003; Siegemund et al., 2016). In the case of the products in our study, perfluorooctane constitutes a fully fluorinated alkane, while perfluorohexyloctane (F6H8) is a partially fluorinated one.

Organic fluoride compounds can be produced by different processes: the substitution of hydrogen in hydrocarbons, halogen-fluorine exchange, the synthesis of higher molecular mass fluorine compounds from reactive fluorinated synthons, and the addition of fluorine, hydrogen fluoride or reactive nonmetal fluorides to unsaturated bonds (Siegemund et al., 2016). In the case of perfluoroalkane synthesis, the most common process is the direct replacement of all carbon-hydrogen bonds (C–H) with carbon-fluorine bonds (C–F) by two established methods: the direct substitution of hydrogen in hydrocarbons by means of fluorine or metal fluorides, or electrochemical fluorination (Sandford, 2003; Siegemund et al., 2016). However, current synthesis techniques cannot guarantee that organic fluoride compounds are 100% pure, and, therefore, purification is needed for medical

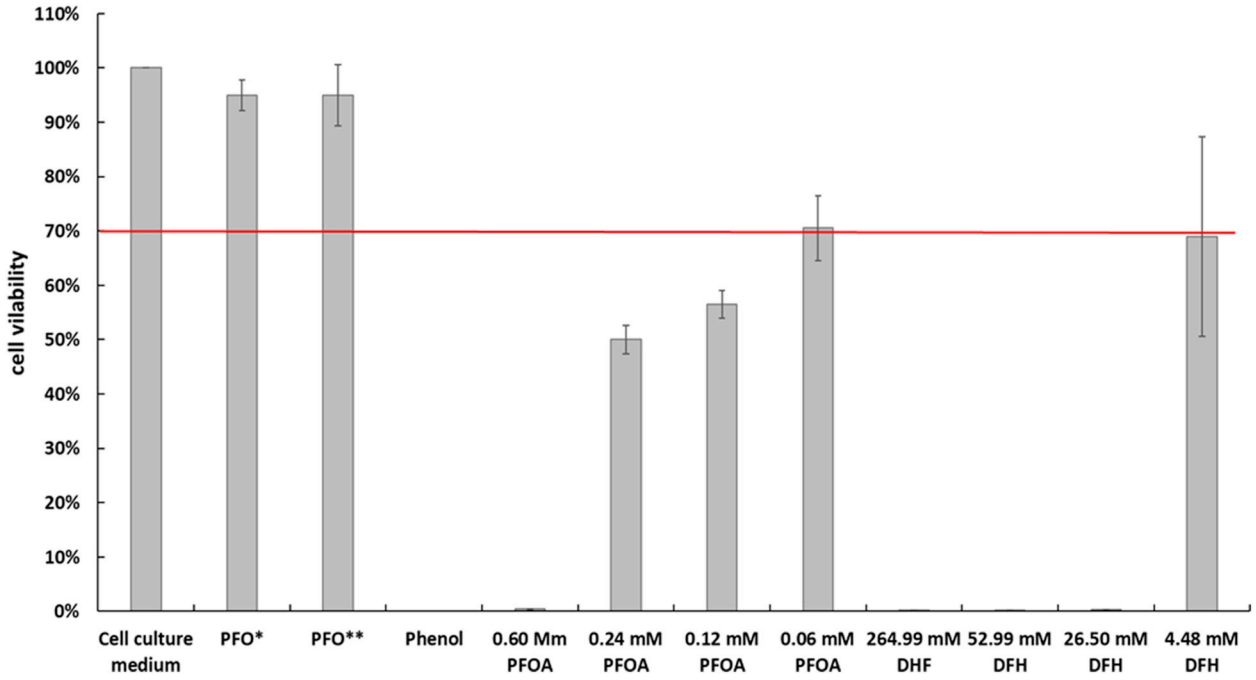

Fig. 3. Cytotoxicity of perfluorooctanoic acid (PFOA) and dodecafluoro-1-heptanol (DFH) ARPE-19 cell line. Cell cultures were exposed for 1 h to 0.60, 0.24, 0.12 and 0.06 mM concentrations of PFOA, and 264.99, 52.99, 26.50 and 4.48 mM concentrations of DFH, prepared by mixing in non-toxic PFO samples. After exposure, cell cultures were grown for 24 h. An MTT assay was performed to measure the viability of the cell cultures. The data obtained were compared by setting the mean percentage of the viability of the cell cultures exposed only to the cell culture medium at 100%. Test samples with ≤70% cell culture viability were considered cytotoxic, and those with ≥70% cell culture viability non-cytotoxic (as per ISO 10993–5:2009 norms).

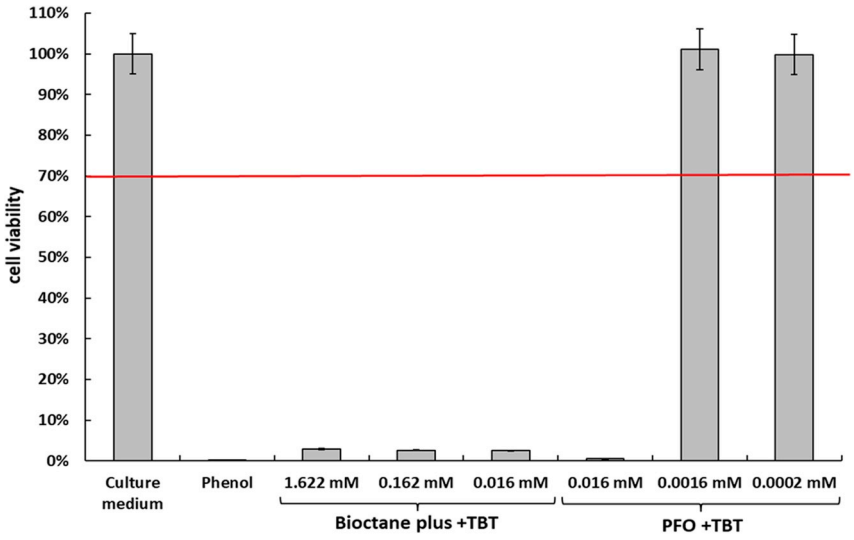

Fig. 4. Cytotoxicity assessment of tributyltin bromide (TBT-Br) in ARPE-19 cell line. Cell cultures were exposed for 1 h to 1.622, 0.162 and 0.016 mM of TBT-Br mixed with non-toxic Bio Octane Plus® batch number 1406119, and 0.0162, 0.0016 and 0.0002 mM of TBT-Br mixed with non-toxic PFCL. After exposure, cell cultures were grown for 24 h. An MTT assay was performed to measure the viability of the cell cultures. The data obtained were compared by setting at 100% the mean percentage of cell culture viability of the cells exposed only to the cell culture medium. Test samples with ≤70% cell culture viability were considered cytotoxic, while those with ≥70% viability were considered non-cytotoxic (as per ISO 10993–5:2009 norms).

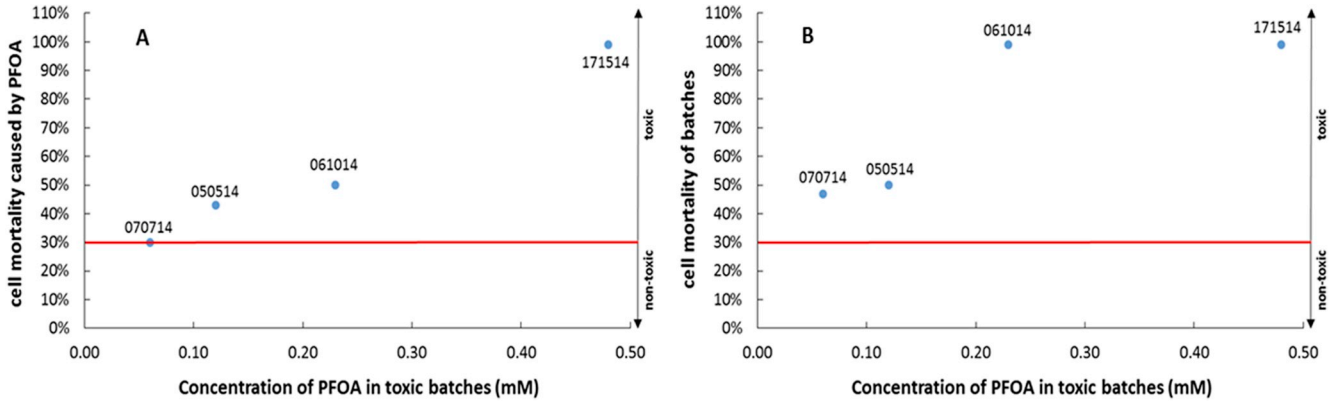

Fig. 5. (A) Cell death caused only by the concentrations of PFOA in the synthetic admixtures (B) Cell death of the AlaOcta® toxic batches.

applications.

Although PFCLs for ophthalmic surgery should contain no compounds other than highly-purified fluorinated alkanes (PFO and F6H8), our results confirmed the presence of contaminants - perfluorooctanoic acid, dodecafluoro-1-hepthanol, ethyl benzene and TBT-Br - in the toxic batches.

The production processes, the origin of the raw material and how PFCLs are later purified and managed, are crucial aspects to be considered for a proper understanding of and solution to the problem. Nevertheless, some manufacturers of the raw materials are located outside of the European Union and it is difficult to obtain this critical information.

Impurities such as PFOA and DFH have also been found in some toxic AlaOcta® batches in recent studies by Menz et al. (2019-b). Among possible hypotheses as to the source of these impurities, DFH might be used as regional preferred raw material for easy access to n-PFO and perfluoroalkanoic acids derivatives as residues of unreacted starting compounds (Menz et al., 2019-b), being as it is a very toxic substance (Agency for Toxic Substances and Disease Registry, 2018).

Acute short-term human exposure to ethylbenzene, which was also found in toxic AlaOcta® samples, causes throat irritation and chest constriction, irritation in the eyes, and neurological effects (Pubchem database, 2018). Ethylbenzene is a typical well-known leachable, and is used as an additive for polymers; it generally derives from plastic storage containers such as plastic syringes or rubber closures (Menz et al., 2019-b).

In the case of Bio Octane Plus®, toxicity could be explained by a single contaminant, TBT-Br. TBT-halides, such as TBT-I or TBT-Br, are potential side products during the manufacture of semiperfluoro alkanes, such as perfluorhexyl octane contained in Bio Octane Plus® (Pospiech and Jehnichen, 2014). The systemic toxicity of stannanes is well known, including effects on the retinal neurons of developing zebrafish (Dong et al., 2006; Nath, 2008), and studies in cancer drug candidates showed that TBT-Br caused caspase-3/7 dependent apoptosis (Hunakova and Brtko, 2017). This contaminant induces a high risk for patients and, in accordance with MDR and ISO-14971:2007, it should have played an essential role in the manufacturer's risk assessment. The complete removal or maximum reduction (as low as possible) of this contaminant (TBT-Br) in medical devices whose composition includes semiperfluorocarbons should be mandatory. As previously mentioned, some manufacturers claim that the toxicity was caused by high concentrations of incompletely fluorinated impurities (Menz et al., 2018), but on this basis contaminants such as those detected in our study could not be identified. Another recent study (Ruzza et al., 2019), proposed a different alternative method based on ^{1}H NMR to quantify not only partially fluorinated compounds, but in addition partially fluorinated perfluoroalkanes and other cytotoxic compounds including PFOA, perfluoroalkyl alcohols and benzene derivatives.

A critical issue is the method chosen to guarantee the biological safety of these medical devices. According to ISO 10993–5:2009, three cytotoxicity methods (indirect, direct and extractive) are allowed, but we have previously demonstrated that only the direct method takes into consideration two unique physical characteristics of PFO: hydrophobicity and volatility (Srivastava et al., 2018). It is necessary to incorporate validated chemical and biological analyses that consider the properties of the specific product in order to guarantee safety.

5. Conclusion

Our results show that toxicity of perfluorocarbon liquids for intraocular surgery was caused by specific chemical compounds, and that their presence can be quantitatively and qualitatively analyzed by using a set of developed chromatographic and spectroscopic techniques.

While the Bio Octane Plus® toxic batch source of toxicity is clearly due to a single impurity (TBT-Br), AlaOcta® toxic batches revealed the presence of different contaminants.

The PFCLs under examination had been assigned the CE mark, meaning they were subject to compliance with the ISO guidelines and UE Directives. Therefore, and as ISO international standard 16672 for ocular endotamponades is currently under revision, we suggest that the best procedure to guarantee the safety of PFCLs for ophthalmic use is a combination of appropriate analytical and biological methods which pay attention to the hydrophobic and volatile nature of the products. Any methods used to guarantee safety need to be validated, and all impurities that can cause toxicity should be removed or reduced as low as possible from the medical devices and should remain under risk management until the absence of significant contamination can be demonstrated and evaluated during the life cycle of the product.

Notes

Authors Girish K. Srivastava, Ivan Fernandez-Bueno, Rosa M. Coco and J. Carlos Pastor declare competing interests on a patent application publication number EP3467118: Method for Evaluating cytotoxicity of chemicals.

Funding

Authors declare that this project has been performed without specific funding for this issue.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank the Spanish Agency of Medicines and Medical Devices (AEMPS), Spanish Ministry of Health, and SERV (Spanish Vitreo Retina Society). We are grateful to M. Teresa García-Gutierrez from IOBA who assisted us in data acquisition in the cytotoxicity tests.

References

Agency for Toxic Substances and Disease Registry, toxicological profile for perfluoroalkyls, 2018. https://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=1117&tid=237 (accessed 19 July 2019).

Bourke, R.D., Simpson, R.N., Cooling, R.J., Sparrow, J.R., 1996. The stability of perfluoro-n-octane during vitreoretinal procedures. Arch. Ophthalmol.114 537–544.

Chang, S., Sparrow, J.R., Iwamoto, T., Gershbein, A., Ross, R., Ortiz, R., 1991. Experimental studies of tolerance to intravitreal perfluoro-n-octane liquid. Retina 11, 367–374.

Chang, S., 2017. Impure perfluorocarbon liquids: a preventable tragedy. Editor. Retin. 37, 1019–1020.

Coco, R.M., Srivastava, G.K., Andrés-Iglesias, C., Medina, J., Rull, F., Fernandez-Vega-Gonzalez, A., Fernandez-Bueno, I., Dueñas, A., Pastor, J.C., 2018. Acute retinal toxicity associated with a mixture of perfluorooctane and perfluorohexyloctane: failure of another indirect cytotoxicity analysis. Br. J. Ophthalmol. https://doi.org/10.1136/bjophthalmol-2017-311471.

Dong, W., Muramoto, W., Nagai, Y., Takehana, K., Stegeman, J.J., Teraoka, H., Hiraga, T., 2006. Retinal neuronal cell is a toxicological target of tributyltin in developing zebrafish. J. Vet. Med. Sci. 68, 573–579.

Elsing, S.H., Fekrat, S., Green, W.R., Chang, S., Wajer, S.D., Haller, J.A., 2001. Clinicopathologic findings in eyes with retained perfluoro-n-octane liquid. Ophthalmology 108, 45–48.

Figueroa, M.S., Casas, D.R., 2014. Inflammation induced by perfluorocarbon liquid: intra- and postoperative use. BioMed Res. Int., 907816.

Hunakova, L., Brtko, J., 2017. Sn- and Ge- triorganometallics exert different cytotoxicity and modulation of migration in triple-negative breast cancer cell line MDA-MB-231. Toxicol. Lett. 279, 16–21.

Januschowski, K., Irigoyen, C., Pastor, J.C., Srivastava, G.K., Romano, M.R., Heimann, H., Stalmans, P., Van Keer, K., Boden, K., Szurman, P., Spitzer, M.S., 2018. Retinal toxicity of medical devices used during vitreoretinal surgery: a critical overview. Ophthalmol. Times. https://doi.org/10.1159/000488504.

Letter to the EditorMenz, D.H., Feltgen, N., Lechner, T., Menz, H., Müller, B.K., Dresp, J., Hoerauf, H., 2019-a-a. Chemical considerations regarding the H-value methodology and its relation with toxicity determination. Investig. Ophthalmol. Vis. Sci. 60, 3365–3367.

Menz, D.H., Feltgen, N., Lechner, T., Menz, H., Müller, B.K., Dresp, J., Hoerauf, H., 2019-b-b. Hydrofluoric acid and other impurities in toxic perfluorooctane batches. Transl. Vis. Sci. Techn 8, 1–13.

Menz, D.H., Feltgen, N., Menz, H., Müller, B.K., Lechner, T., Dresp, J., Hoerauf, H., 2018. How to ward off retinal toxicity of perfluorooctane and other perfluorocarbon liquids? Investig. Ophthalmol. Vis. Sci. 59, 4841–4846.

Nath, M., 2008. Toxicity and the cardiovascular activity of organotin compounds: a review. Appl. Organomet. Chem. 22, 598–612.

Pastor, J.C., Coco, R.M., Fernandez-Bueno, I., Alonso-Alonso, M.L., Medina, J., Sanz-Arranz, A., Rull, F., Gayoso, M.J., Dueñas, A., Garcia-Gutierrez, M.T., Gonzalez-Buendia, L., Delgado-Tirado, S., Abecia, E., Ruiz-Miguel, M., Serrano, M.A., Ruiz-Moreno, J.M., Srivastava, G.K., 2017. Acute retinal damage after using a toxic perfluoro-octane for vitreo-retinal surgery. Retina 37, 1140–1151.

Pospiech, D., Jehnichen, D., 2014. Self-organizing semifluorinated methacrylate fluoropolymers. In: Smith, D.W., Iacono, S.T., Iyer, S.S. (Eds.), Handbook of Fluoropolymer Science and Technology. Wiley, New Yersey, pp. 235–290.

Pubchem open chemistry database, 2018. U. S. Natl. Libr. Med. https://pubchem.ncbi.nlm.nih.gov/compound/ethylbenzene#section=Top (accessed 19 July 2019).

Querques, L., Miserocchi, E., Modorati, G., Querques, G., Bandello, F., 2017. Hemorrhagic occlusive retinal vasculitis after inadvertent intraocular perforation with gentamycin injection. Eur. J. Ophthalmol. 27, 50–53.

Ruzza, P., Gatto, C., Ragazzi, E., Romano, M.R., Honisch, C., D'Amato Tóthová, J., 2019. H-content is not predictive of perfluorocarbon ocular endotamponade cytotoxicity in vitro. ACS Omega 4, 13481–13487.

Sandford, G., 2003. Perfluoroalkanes. Tetrahedron 59, 437–454.

Siegemund, G., Schwertfeger, W., Feiring, A., Smart, B., Behr, F., Vogel, H., McKusick, B., Kirsch, P., 2016. Ullmann's encyclopedia of industrial chemistry. In: Fluorine Compounds, Organic, seventh ed. .

Srivastava, G.K., Andrés-Iglesias, C., Coco, R.M., Fernandez-Bueno, I., Medina, J., García-Serna, J., Dueñas, A., Rull, F., Pastor, J.C., 2019. Letter to the Editor: chemical considerations regarding the H-value methodology and its relation with toxicity determination. Investig. Ophthalmol. Vis. Sci. 60, 3363–3364.

Srivastava, G.K., Alonso-Alonso, M.L., Fernandez-Bueno, I., Garcia-Gutierrez, M.T., Rull, F., Medina, J., Coco, R.M., Pastor, J.C., 2018. Comparison between direct contact and extract exposure methods for PFO cytotoxicity evaluation. Sci. Rep. 8, 1425.

Yu, Q., Liu, K., Su, L., Xia, X., Xu, X., 2014. Perfluorocarbon liquid: its application in vitreoretinal surgery and related ocular inflammation. BioMed Res. Int., 250323.