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H-Content Is Not Predictive of Perfluorocarbon Ocular Endotamponade Cytotoxicity in Vitro

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Supporting Information

Is low H-content in PFCLs predictive parameter of product safety?

TESTED COMPOUNDS	Concentration (ppm)	H- content qNMR analysis (ppm)	Cytotoxicity test *in vitro* in ARPE-19 and BALB 3T3 cells
PFOA (purity 99%)	28	0.07	CYTOTOXIC
1H, 1H, 1H, 2H, 2H - PFO (purity 97%)	970000	2808	NOT CYTOTOXIC

Compound with ***low H-content was*** CYTOTOXIC, while ***compound with high H-content*** was NOT CYTOTOXIC

Low H-content in PFCLs is NOT predictive of safety

ABSTRACT: In recent years, cases of retinal toxicity occurred in some European, Middle Eastern, and South American countries following the use of perfluorocarbon liquids (PFCLs) on vitreoretinal surgeries owing to impurities in the product. Moreover, Spanish ophthalmologists reported several toxic cases on the use of perfluoro-*n*-octane Ala Octa (Alamedics, Dornstadt, Germany), raising the necessity of reviewing the current validated methods used for assessing the safety of PFCLs. We proved that in samples of PFCLs contaminated on purpose with impurities previously detected in Ala Octa devices, the determination of the so-called H-content using a ^{1}H NMR quantitative assay implemented with the electronic reference to access in vivo concentrations 2 technology failed to demonstrate a correlation between the H-content and in vitro cytotoxicity test in ARPE-19 and BALB 3T3 cell lines. Therefore, direct information on the safety of PFCLs was provided only by the cytotoxicity test in vitro validated according to ISO 10993-5, and the H-content was not predictive of perfluorocarbon ocular endotamponade cytotoxicity in vitro.

INTRODUCTION

Perfluorocarbon liquids (PFCLs) have been used effectively as an intraoperative tool during vitreous surgery for complicated retinal detachments since the 1980s.[1] In 1990, Sparrow et al.[2] showed that the presence of not fully fluorinated compounds at a concentration higher than 10% in PFCLs induced cytotoxicity in the fibroblast cell culture. Recently, Pastor et al.[3] reported 117 cases of acute retinal toxicity with severe visual loss, which is mostly characterized by retinal necrosis and vascular occlusion after intraoperative use of perfluoro-*n*-octane (PFO) Ala Octa (Alamedics, Dornstadt, Germany). Such severe adverse events raised an animated discussion within the scientific and medical community about the safety of PFCL medical devices,[4] particularly on the validity of the methods used for their safety assessment (ISO 10993-5 and ISO 16672),[5,6] and emphasized the necessity to review the standardized in vitro cytotoxicity test and the development of chemical analytical methods able to detect the presence of potentially cytotoxic compounds. To deal with the latter, Menz et al.[7] developed an analytical method based on the electrochemical determination of the fluorine produced via the reaction of partially hydrogenated perfluoroalkanes with diamine. Moreover, they introduced the so-called H-value, defined as the ppm content of partially hydrogenated perfluoroalkanes as a PFCL safety criterion, and attributed an H-value less than 10 ppm as the safety threshold.[7] However, the specificity of the chemical reaction leading to the "H-value" determination included a very narrow range of impurities, excluding a range of other potentially high cytotoxic contaminants, among which the impurities were identified by Pastor et al.[3] in Ala Octa PFO.

To determine the hydrogen content (H-content) of a wider range of partially hydrogenated compounds in PFCLs, we evaluated the possibility to use ^{1}H NMR spectroscopy. This is a powerful technique for elucidating the structure of organic compounds. Furthermore, ^{1}H NMR can be applied as a

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quantitative analytical technique because the intensity of the NMR signal is proportional to the number of nuclei and consequently to the amount of analytes.[8,9] Quantitative NMR (qNMR) gives accurate and precise results when internal standards are used[10,11] because the concentration of the analytes is directly determined via the integral value ratio. Owing to the properties of qNMR, this technique has been used to determine the impurities of pharmaceutical products[12] and to quantify analytes to prepare calibration solution for instrumental analysis.[13] Previously, Chang et al.[14] used 1,4-bis(trichloromethyl)benzene (TCMB) in deuterated benzene as an external proton standard. Indeed, its use as the internal standard was precluded because of the physicochemical properties of PFCLs.[15] To improve the accuracy and precision of qNMR assays, we evaluated the feasibility of the use of pulse length-based concentration (PULCON) determination methodology,[16] which correlates the absolute intensities in two 1D-NMR spectra via the principle of reciprocity,[17] to determine the protonated impurity in commercially available PFCL medical devices. This methodology was recently implemented in Bruker TopSpin software under the electronic reference to access in vivo concentration 2 (ERETIC2)[18] commercial name. Despite the usefulness of this method, only few studies on qNMR have been reported.[19−22]

In order to investigate the relation between H-content and cytotoxicity, samples of PFO were contaminated with different concentrations of perfluorooctanoic acid (PFOA) corresponding to the compound with very low H-content and two partially hydrogenated PFO analogues, HPFO and 5HPFO, corresponding to the compounds with high H-content. Both PFOA and HPFO were previously described as toxic PFCL manufacturing residues.[3] In addition to PFO-contaminated samples, 15 commercially available PFO or perfluorodecalin (PFD) medical devices, routinely used during vitreous surgery, were analyzed, and the H-content expressed as ppm of H was determined. In PFO-contaminated samples and 15 commercial medical devices, the correlation between H-content and the in vitro cytotoxicity test in ARPE-19 and BALB 3T3 cell lines performed according to ISO 10993-5[5] was investigated in order to evaluate the relevance of H-content value as a safety criterion for PFCL medical devices intended for intraocular use.

■ RESULTS

Performance Tests of the ERETIC2 Method with Different Analytes. Two partially hydrogenated PFO analogs, HPFO and 5HPFO, were initially analyzed. Their ^{1}H NMR spectra in PFO are reported in Figures S1 and S2 in the Supporting Information, respectively. The ^{1}H NMR spectrum of HPFO is characterized by a triplet of triplets (Figure S1) centered at 5.97 ppm with a coupling constant J of 52.3 and 4.0 Hz owing to the coupling of the H nucleus with the geminal and vicinal fluorine atoms, respectively. The ^{1}H NMR spectrum of 5HPFO exhibits a $-CH_3$ triplet centered at 1.22 ppm (J 7.5 Hz) and a $-CH_2$ multiplet (doublet of quartets) centered at 2.16 ppm, containing the contribution of both H−H and H−F couplings.

The two PFO analyte solutions were quantified by the ERETIC2 method using the well-separated signals at 5.97 and 2.16 ppm for HPFO and 5HPFO, respectively, and a 1.26 mM HPFO solution in PFO as a reference. The determined concentration (average of three replicated measurements ± S.D.) of analytes gave sufficient recovery (Table 1). The

Table 1. Comparison between the Concentration of Analytes in PFO Determined via Gravimetric, ERETIC2, and External Standard Methods

	concentration (mM)		
analytes	gravimetric	ERETIC2 ± S.D. (recovery %)	external standard[a] ± S.D. (recovery %)
HPFO	2.51	2.43 ± 0.02 (96.8)	2.32 ± 0.25 (92.3)
	1.25	1.22 ± 0.04 (97.6)	1.21 ± 0.12 (96.4)
	0.63	0.66 ± 0.04 (104.7)	0.66 ± 0.05 (105.7)
average recovery (S.D.)		99.7 (4.8)	97.9 (6.5)
5HPFO	2.95	3.01 ± 0.01 (102.0)	3.03 ± 0.39 (102.6)
	1.48	1.53 ± 0.06 (103.3)	1.42 ± 0.16 (96.1)
	0.74	0.70 ± 0.01 (94.6)	0.67 ± 0.09 (91.5)
average recovery (S.D.)		99.9 (5.0)	96.4 (5.6)

[a]TCMB was used as the external reference compound.[14]

average recovery was 99.7 and 99.9% for HPFO and 5HPFO, respectively, indicating that the ERETIC2 method allows the quantification of partially hydrogenated perfluoroalkyl analogs in the perfluorinated solvent. The relation between the concentration determined by gravimetric and ERETIC2 methods was plotted in Figure S3. The linear regression analysis ($y = 0.960x$, $R^2 = 0.9995$ and $y = 1.019x$, $R^2 = 0.9996$, for HPFO and 5HPFO, respectively) indicated that ERETIC2 gives better linearity and accuracy than the gravimetric method according to the data obtained by Frank et al.[19] in the quantification of benzoic acid, caffeine, and L-tyrosine.

Comparison of Analyte Concentrations between the ERETIC2 and External Standard Method. The two analytes—HPFO and 5HPFO—were alternatively quantified using TCMB as an external standard[14] (Table 1, and see Supporting Information for an exhaustive description of the procedure).

The relation between the average concentrations calculated using TCMB as the external reference compound and ERETIC2 method was plotted (Figure S4). The R^2 values were 0.9995 and 0.9996 with a slope of 0.967 and 0.961 in the analysis of HPFO and 5HPFO, respectively, indicating that ERETIC2 gives better linearity and accuracy than the external standard method.

Cytotoxicity Analysis of PFO Contaminated with Compounds Having Different H-Contents. The cytotoxicity of PFO solutions contaminated with either some manufacturing contaminants described by Pastor et al.[3] (PFOA and HPFO) or 5HPFO has been evaluated, according to ISO 10993-5,[5] and compared with their H-content (Table 2).

The PFO sample contaminated with 57 ppm of PFOA (0.0057%), corresponding to an H-content of 0.13 ppm, was strongly cytotoxic and showed 42 and 35% reduction of cell viability in ARPE-19 and BALB 3T3 cells after 24 h of treatment, respectively. The cytotoxicity test demonstrated that 28 ppm PFOA (H-content of 0.07 ppm) induced cytotoxicity in ARPE-19 cells (but not in BALB-3T3 cells) with a reduction of cell viability of 38% (Table 2), suggesting different sensibility of these cell lines towards PFOA. To obtain a similar reduction of cell viability of ARPE-19 cells (44%) by the HPFO contaminant, a 12.3000% solution of HPFO in PFO (H-content of 295.13 ppm) was necessary. In these conditions, BALB 3T3 cells showed a reduction of cell

Table 2. Correlation of Cytotoxicity at 24 h, Expressed as Reduction of Cell Viability, with H-Content of Some PFO Contaminants[a]

sample	% in PFO	reduction of cell viability (%)				H-content (ppm)
		ARPE-19		BALB 3T3		
PFOA	0.0057	42	cytotoxic	35	cytotoxic	0.13
	0.0028	38	cytotoxic	25	not cytotoxic	0.07
	0.0006	12	not cytotoxic	13	not cytotoxic	0.01
HPFO	12.3000	44	cytotoxic	48	cytotoxic	295.13
	6.1500	28	not cytotoxic	21	not cytotoxic	147.56
	3.0750	17	not cytotoxic	9	not cytotoxic	73.78
5HPFO	97.0000	26	not cytotoxic	13	not cytotoxic	2808.65
	48.5000	24	not cytotoxic	12	not cytotoxic	1346.41
	24.2500	17	not cytotoxic	8	not cytotoxic	702.16

[a]According to the direct contact test (ISO 10993-5), a sample was cytotoxic if cell viability reduction was greater than 30%. Contaminants were dissolved in PFO (purity 99.8%, AL.CHI.MI.A S.r.l, Italy). ND: not determined.

viability of 48%, resulting in more sensitivity to contaminant. At lower concentrations of HPFO (6.1500 and 3.0750%, corresponding to H-content values of 147.56 and 73.78 ppm), BALB 3T3 cell lines confirmed its greater resistance to the cytotoxic effects of this contaminant. 5HPFO solutions showed high H-content from 2808.65 to 702.16 ppm and resulted in noncytotoxicity at all tested concentrations (Table 2).

Cytotoxicity and qNMR Analysis of PFO and PFD Medical Devices. The H-content and cytotoxicity of 15 commercially available PFO and PFD medical devices were determined and compared. Proton resonances were detectable in the chemical shift region associated with hydrogen-containing fluorocarbons (2−6.5 ppm),[23] and at lower δ than the water signal, corresponding to the general hydrocarbon-type impurities (as example, the ^{1}H NMR spectrum of the PFO2 device is reported in Figure S5). The proton amount, excluding the residual proton of both deuterochloroform lock solvent and water, is reported in Table 3.

PFD medical devices showed an H-content in the range from 3.5 to 11.8 ppm, and all the samples were not cytotoxic in the two tested cell lines. Commercially available PFO devices showed an H-content in the range from 2.5 to 8.2 ppm with the exception of PFO1 and PFO2 that were characterized by an H-content of 67.97 and 54.11 ppm, respectively. Only these two PFO medical devices, analyzed by the direct contact cytotoxicity test, resulted in cytotoxicity with a reduction of cell viability of 56 and 31% in ARPE-19 cells for PFO1 and PFO2, respectively. PFO1 was cytotoxic also in BALB 3T3 cells, while no data were available for the PFO2 sample with this cell line. All other analyzed PFO medical devices did not significantly reduce the cell viability after 24 h of direct contact and were not cytotoxic (Table 3).

Correlation between Cell Toxicity and H-Content of PFCL Compounds. To evaluate any relation between the cytotoxicity data obtained in both cell lines for all the investigated PFCL compounds and the respective H-content determined by qNMR, a linear regression fit was performed. In the case of commercially available PFCL medical devices reported in Table 3, a significant correlation was found for the abovementioned parameters in both cell lines, being the calculated Pearson's correlation coefficient r = 0.8820 (p < 0.0001) for ARPE-19 cells and r = 0.9718 (p < 0.0001) for BALB-3T3 cells as shown in Figure 1. In contrast, using the same approach, no significant correlation was found between the H-content and cytotoxicity of PFO solutions containing PFOA, HPFO, or 5HPFO (Figure S6). Indeed, while PFOA is cytotoxic with a very low concentration (28 ppm) and HPFO is at a concentration of 12.30%, 5HPFO does not show any cytotoxicity in all the range of concentration examined and as a pure compound.

Moreover, the presence of a significant correlation only in commercially available PFCL medical devices was confirmed by a nonparametric evaluation of data; hierarchical clustering (Ward's method) of samples according to their cytotoxicity

Table 3. Correlation of Cytotoxicity at 24 h, Expressed as Reduction of Cell Viability, with H-Content of Some PFCL Medical Devices[a]

devices	manufacturer	reduction of cell viability (%)				H-content (ppm)
		ARPE-19		BALB 3T3		
PFD1	1	12	not cytotoxic	ND	ND	5.32
PFD2	2	7	not cytotoxic	ND	ND	4.45
PFD3	3	16	not cytotoxic	6	not cytotoxic	4.91
PFD4	4	21	not cytotoxic	6	not cytotoxic	3.57
PFD5	5	10	not cytotoxic	2	not cytotoxic	11.73
PFD6	6	2	not cytotoxic	4	not cytotoxic	4.73
PFD7	6	5	not cytotoxic	3	not cytotoxic	6.96
PFD8	7	0.7	not cytotoxic	ND	ND	4.03
PFD9	8	2.4	not cytotoxic	ND	ND	4.74
PFO1	1	56	cytotoxic	43	cytotoxic	67.97
PFO2	1	31	cytotoxic	ND	ND	54.11
PFO3	2	5	not cytotoxic	ND	ND	5.98
PFO4	3	13	not cytotoxic	5	not cytotoxic	2.48
PFO5	6	2	not cytotoxic	3	not cytotoxic	8.24
PFO6	6	7	not cytotoxic	2	not cytotoxic	6.13

[a]According to the direct contact test (ISO 10993-5), a sample was cytotoxic if cell viability reduction was greater than 30%. ND: not determined.

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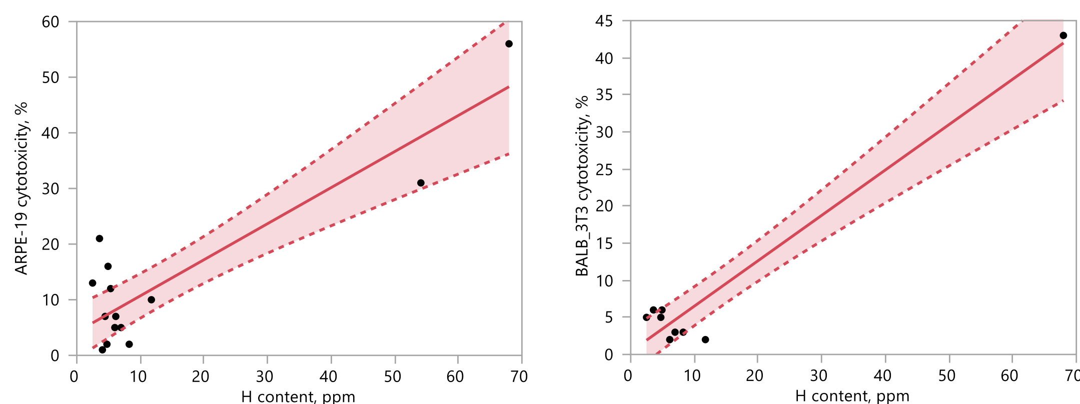

Figure 1. Correlation between H-content of analyzed PFO and PFD samples with cytotoxicity determined in ARPE-19 (left panel) and BALB-3T3 (right panel) cells. Shaded areas show the confidence intervals for the fitted lines. A significant correlation was found for both cell lines (for ARPE-19 cells: $r = 0.8820$, $p < 0.0001$; for BALB-3T3 cells: $r = 0.9718$, $p < 0.0001$).

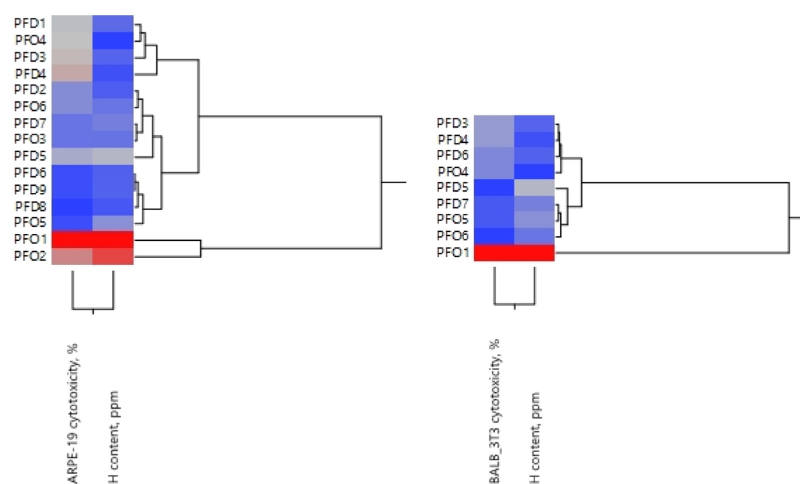

Figure 2. Dendrograms showing hierarchical clustering (Ward's method) of samples according to cytotoxicity and H-content in both cell lines (left panel: ARPE-19 cells; right panel: BALB-3T3 cells). Notably, for BALB-3T3 cells, some samples have not been tested (see Table 2).

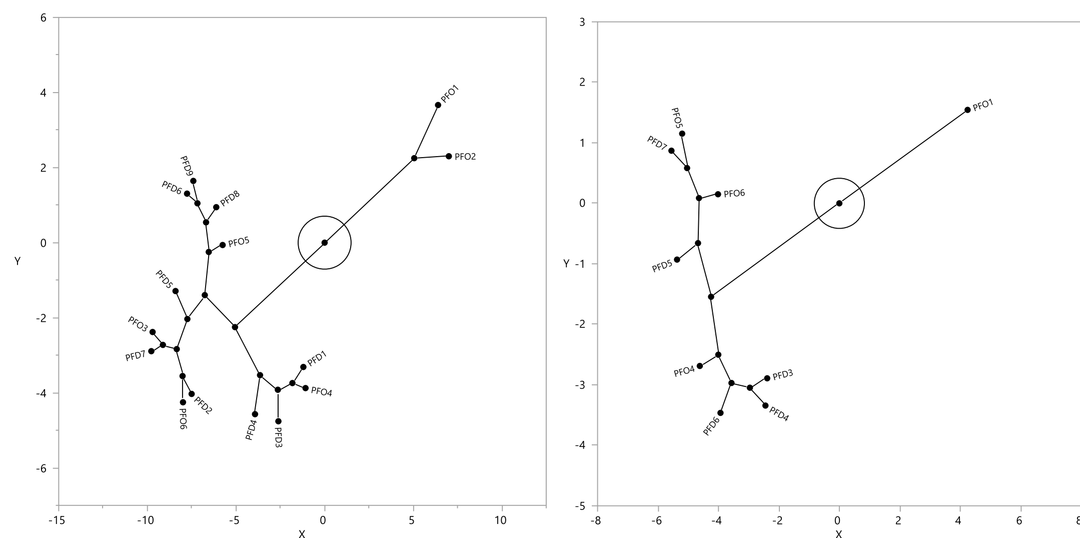

Figure 3. Constellation plot for both cell lines (left panel: ARPE-19 cells; right panel: BALB-3T3 cells). Constellation plot arranges each PFC sample according to their similarity herein because of both H-content and relative cytotoxicity. The length of the lines between points approximates the distance among clusters. Notably, for BALB-3T3 cells, some samples have not been tested (see Table 2).

and H-content gave consistent results in both cell lines (Figures 2 and 3). For the ARPE-19 cell line, four distinguishable clusters were detected; particularly, the cluster comprising PFO1 and PFO2, characterized by relevant cytotoxicity (>30%) according to ISO 10993-5, was well enucleated from the other clusters that presented a low H-

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content and moderate/low cytotoxicity. Similar behavior was found for BALB-3T3 cells, although the incomplete data set was available.

DISCUSSION AND CONCLUSIONS

Toxic effects correlated with prolonged use of PFCLs are very well known since the beginning of the nineties of the last century; however, these molecules are considered safe and well tolerated for a short time of use. Early studies demonstrated that PFO is not toxic after 48 h of treatment in the rabbit vitreous cavity.[14] Moreover, in vitro experiments using fibroblasts demonstrated that their reduction of attachment and growth rate was because of the presence of partially fluorinated impurities and not PFCLs themselves.[2] However, several studies claimed the possibility of retinal toxic effects.[14,24] Indeed, it is well known that the retained PFO at the vitreous cavity may activate inflammation,[24−26] even if the origin of this intolerance is unclear. Some authors correlate the chemical toxicity in animals to the impurities of PFCLs that can cause the formation of a protein film for epiretinal membrane formation in animals.[27,28] In recent years, some cases of adverse reactions of PFCLs and particularly of PFO have been reported. In 2013, toxic effects related to the use of PFO were reported in Chile and Spain, which were attributed to the careless manipulation of the product.[3] Recently, a case of PFO toxicity has been reported, and toxicological investigation demonstrated that in addition to organic contaminants, toxic polar compounds appeared during storage because of PFCL oxidation due to either incomplete purification of the synthesized product or inadequate storage.[2,27] The risk of the presence or appearance of toxic substances necessitates the development of analytical methods of PFCLs and related safety criteria for their clinical use.

Menz et al.[7] proposed the analysis of partially hydrogenated perfluoroalkanes correlating the fluorine ions released via the reaction with hexamethylenediamine of partially hydrogenated PCFLs to the content in hydrogenated compounds. Based on this method, they proposed a partially hydrogenated perfluoroalkane content of less than 10 ppm as a safety criterion for PFCL medical devices. Unfortunately, the mechanism of this reaction requires the presence of a CHF−CF_2 moiety to proceed with the stoichiometry proposed by Menz, whereas other partially hydrogenated PFCLs and contaminants found in cytotoxic PFO medical devices would not react or would react with a different stoichiometry; thus, it leads to an incorrect determination of the content of partially hydrogenated compounds.

In the present study, we have developed and validated a method based on the quantitative analysis of ^{1}H NMR spectra of perfluorinated compounds to determine a large range of possible impurities present in PFCLs. The proposed method permitted to detect not only partially fluorinated compounds also detectable by the method proposed by Menz et al.[7] but also partially hydrogenated perfluoroalkanes and other cytotoxic compounds that would not react with hexamethylenediamine. Therefore, the present method would also detect PFOA, perfluoroalkyl alcohols, and benzene derivatives described by Pastor et al.[3] as contaminants of Ala Octa devices used in the patient adverse events. Such impurities may have been formed because of PFCL oxidation or may be residues derived from the PFCL manufacturing processes. The determination of the presence of different classes of hydrogenated molecules in PFCLs by a single analysis is the strength of this method. Moreover, this technique can provide useful information on the stability of PFCLs during storage. We analyzed 15 PFCL medical devices for clinical use in retinal surgery currently available on the market for both the H-content and cytotoxicity. Significant correlation demonstrated between the H-content and cytotoxicity of analyzed products allowed us to suppose that the H-content could be used to indirectly define the cytotoxicity of PFCL medical devices as previously suggested by Menz et al.[7] However, the hypothesis had to be strongly rejected when PFCL samples, contaminated with highly toxic impurities previously detected in Ala Octa devices,[3] were analyzed for both the H-content and cytotoxicity. A very high cytotoxicity was obtained in samples contaminated with PFOA, while showing extremely low H-content (0.13 and 0.07 ppm). Conversely, in the PFCL samples contaminated with 5HPFO showing a high H-content (up to 2800 ppm), the cytotoxicity was not detected. Hence a correlation between H-content and cytotoxicity could not be confirmed in these samples having low and high H-content. Therefore according to our study, H-content cannot be used as a safety criterion to determine PFCL safety.

Overall, these data indicated that the quantification of partially hydrogenated compounds is not sufficient to determine the safety of PFCLs. Despite the fact that the qNMR analysis, which is developed and validated in the present study, could reliably determine the presence of impurities in the tested samples and could warn about possible undesired contamination, it could not directly provide any information about the toxicity of such impurities. Therefore, the cytotoxicity test in vitro remains the only method that can directly assess the cytotoxicity of total impurities present in the samples and demonstrate the toxicity or safety of the product.

In conclusion, the toxicity of PFCL medical devices, related to chemical contaminants due to either an incomplete purification or unsuitable storage conditions of the raw materials, could be prevented by systematic cytotoxicity testing of PFCL medical device batches before their release for sale, in addition to physicochemical analyses of raw materials during manufacturing processes. We demonstrated that the H-content alone could not predict and guarantee the safety of the PFCL product. The direct information on the safety of the product in vitro can be provided only by the cytotoxicity test, provided the validation of the method considering the particular specifications and conditions of use of the PFCLs.

EXPERIMENTAL SECTION

Chemicals. PFOA, HPFO, and 5HPFO were obtained from Sigma-Aldrich (Italy). PFO and PFD medical devices intended for intraocular use were procured from local distributors (suppliers are indicated from 1 to 8). Deuterated chloroform (100 atom % D) was purchased from Armar AG (Switzerland). The reference compound TCMB was obtained from Acros Organics (Belgium).

qNMR Analysis. All NMR spectra were acquired via a Bruker AVANCE III HD spectrometer (1H: 400 MHz) with a BBI probe (Bruker BioSpin, Germany) using Wilmad coaxial NMR tubes (inner tube 2.97 mm × 1.96 mm, O.D. × I.D.) at 298 K. The data acquisition and data processing were performed using TopSpin 3.0 software (Bruker BioSpin). Prior to the data acquisition, the 90° pulse length was calibrated. Each sample was well tuned and matched manually. The acquisition parameters used for performance tests were set as follows: pulse sequence, zg; relaxation delay (D_1), 6 s;

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spectral width (SW), 14 ppm; data acquisition time (AQ), 3 s; dummy scans (DS), 8; number of scans (NS), 1700; spinning, OFF. RG was automatically set by TopSpin 3.0 software (RG: 203).

The obtained NMR spectra were processed by multiplying with exponential (0.3 Hz line broadening) and zero-filling. The phases were corrected manually; subsequently, the baseline was corrected by a fifth-order polynomial. Peak integration was manually selected. The slope and bias corrections of the integral were not used. The concentration of the analyte was quantified via the ERETIC2 method in TopSpin 3.0 software. Individual integral values, concentration, and the number of protons in the signal used for quantitation in the reference were entered in PULCON software; further, the concentration of the signal in the sample was automatically calculated.

Alternatively, the concentration was quantified via the external standard method by dissolving 87.5 mg of TCMB in 10 mL of $CDCl_3$ and then diluted until the final amount of the reference compound was 79.69 μM.

Direct Contact Cytotoxicity Test in Vitro. The human retinal pigment epithelial cell lines ARPE-19 (ATCC CRL-2302, Manassas VA, USA) and BALB 3T3 (ATCC CCL-163, Manassas VA, USA) were grown as a monolayer in a vehicle medium containing DMEM/nutrient mixture F-12 medium with L-glutamine and without HEPES (Gibco, Italy), each supplemented with 10% of iron-fortified bovine calf serum (Sigma-Aldrich) and 1% penicillin−streptomycin (Sigma-Aldrich). ARPE-19 cell suspension containing 2.0−3.0 × 10^5 cells/mL was seeded into 96-well microtiter plates, grown to 70−80% confluence, and washed once with 150 μL of Dulbecco's phosphate-buffered saline containing $MgCl_2$ and $CaCl_2$ (Sigma-Aldrich) before the application of test samples.

Ultrapure PFO, purity 99.8%, (AL.CHI.MI.A S.r.l, Italy), and HPFO with purity 98.8% (Fluorochem, Italy) were used as positive and negative controls, respectively. Contact time and area between the sample and cells corresponded to 24 h and 60% area (50 μL), respectively. The samples were deposited directly on the cells with the tip immersed in the medium at 2/3rd depth of the well to obtain constant contact with the cell layer.

TOX-1 in vitro toxicology assay kit, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-phenyltetrazolium bromide)-based (Sigma-Aldrich) vitality assay was used to quantify ARPE-19 cell viability. The cells were examined microscopically (Leica DM IL LED, Germany) by two independent operators before sample removal and after the phase of dye extraction and cell fixing in the MTT assay. The reactivity zone under and around the sample was graded according to ISO 10993-5.[5] Grade 4 was not applicable in our testing conditions. The achievement of a numerical grade greater than 2 was considered as a cytotoxic effect. At least six values were acquired at two different 96-well microplates for all samples (vehicle, positive control, and negative control). Mean % of cell mortality and standard error of the mean were calculated for each sample and experimental condition. Reduction in cell vitality greater than 30% was considered to be a cytotoxic effect.

Statistical Analysis. Statistical analysis was performed using JMP13 software for Windows (SAS Institute, Cary, NC, USA). Relations between pairs of variables were tested by least-squares linear regression. Pearson's correlation coefficient r was used to quantify the strength of the relations. A $p < 0.05$ was considered as statistically significant. To classify the investigated PFCL compounds according to the pattern of their H-content and cytotoxicity, a procedure of hierarchical unsupervised clustering was also used. The linkage rule was according to the Ward's method on standardized data, and the distance measure was determined by the standardized Euclidean distance.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b01793.

> NMR analysis, ^{1}H NMR spectrum of HPFO (600 ppm) in PFO recorded using a coaxial tube, ^{1}H NMR spectrum of 5HPFO (600 ppm) in PFO recorded using a coaxial tube, linear regression of concentration of either HPFO or 5HPFO determined by either gravimetric or ERETIC2 methods, linear regression of concentration of either HPFO or 5HPFO determined by either the external standard or ERETIC2 methods, ^{1}H NMR spectrum of PFO2 devices recorded using a coaxial tube, correlation between H-content and cytotoxicity of PFO solutions, and references (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

DMEM, Dulbecco's modified Eagle medium; ERETIC2, electronic reference to access in vivo concentrations 2; HPFO, 1H-perfluorooctane; 5HPFO, 1H,1H,1H,2H,2H-perfluorooctane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-phenyltetrazolium bromide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PFCL, perfluorocarbon liquid; PFOA, perfluoro-octanoic acid; PULCON, pulse length-based concentration; qNMR, quantitative NMR

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