AN *IN VITRO* ALTERNATIVE ASSAY TO PREDICT THE HUMAN EYE IRRITATION POTENTIAL OF PROTIC IONIC LIQUIDS USED AS TEXTILE DYEING MEDIUM

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The HET-CAM (Hen's Egg Chorioallantoic Membrane) assay is a qualitative alternative method to the *in vivo* Draize Rabbit Eye test to assess the irritancy potential of chemicals. In this work, for the first time, the ophthalmic irritation of 13 different protic ionic liquids has been evaluated using ImageJ and PhotoScape image processing programs to analyze the results of the HET-CAM assay. The irritation potential of a substance can be semi-quantified using these computational tools by observing blood vessel changes, such as lysis, hemorrhage and coagulation. In conclusion, the modification of the established HET-CAM assay made it possible to determine the damage to minute blood vessels, highlighting the low-irritant profile of some studied protic ionic liquids to the ocular tissues. These results should guide their use as solvents or additives of safe human use, in agreement with the predictions based on their designed chemical structure.

Keywords: protic ionic liquids, ocular irritation, HET-CAM assay

INTRODUCTION

Ionic liquids (ILs) are innovative chemical compounds, which, due to their specific physico-chemical behavior, have been studied as alternative sustainable solvents in many areas of modern science, as well as in the industrial fields of pharmaceuticals[1-9] – including as potential tools to reformulate antiviral drugs for the treatment of COVID-19 into safer and more bioavailable forms[10-11] –, biotechnology, food and bioproducts,[12-18] textile processing[19-22] and many others. Due to their probable large-scale use in the near future – especially as cotton dyeing medium – a thorough analysis in terms of human and environmental safety has been attracting significant attention in the last few years.[23-30]

Precociously reported as "safe materials", due to their non-volatility and high thermal stability, many ionic liquids have been found to be resistant to biodegradation and photodegradation, and toxic towards cells and living organisms.[31-36] Despite the technical advantages of these compounds, these potential safety risks make it necessary to find better alternative solvents with a more human-friendly character. Once these ionic liquids can be modified or tailored by specific variations into the ionic structure, a new family of these molten salts has been proposed, avoiding complex functional groups hardly biodegradable and potential hazardous molecular groups (halogens, heterocycles, *etc*.). This new family –

protic ionic liquids (PILs) – is being increasingly studied and physico-chemically characterized,[37-47] and recent scientific research has suggested its strong environmentally friendly profile[48-54] and safety to specific kinds of cells, such as HaCat (human epidermal keratinocytes), HepG2 (human liver carcinoma cells) and IPC-81 (rat leukemia cells).[55-58]

Probably in the next few years, the real importance of protic ionic liquids to the industry and the society of the 21st century should be defined. The past century is full of technological advances (plastic industry, cold generation, global transportation based on combustion engines, *etc*.) that have translated into progress and humankind well-being, but also into heavy environmental footprints and collective health problems still unsolved (Pacific trash vortex, planet ozone depletion and global warming by greenhouse gas emissions). It is clear that the same historical errors that have already been made can be repeated if our research effort is focused only on characterization and technological uses of PILs, centering the global development of the economy as the keyword and considering aspects of environmental impact or potential effects on human health, as secondary. A simple bibliographic review expresses the exponential progression in the last years of the number of scientific publications of industrial profile in the scope of PILs (see Fig. 1, red stripes) *versus* a weak and shy start of works focused on environment (same figure, yellow and green stripes, 9 articles on ecotoxicity and 8 papers on cytotoxicity in the last decade, respectively).

Part of this biosafety analysis process is to elucidate the eye irritation potential of these new materials. In the regulatory context, the term 'eye irritation' is generally defined as the development of undesirable changes in the eye after the application of a test substance to the anterior surface of the eye, which are reversible within twenty-one days of treatment.[59] The Organisation for Economic Co-operation and Development (OECD) defined the substances that cause reversible tissue changes to the eye as ocular irritants and the severity of the irritancy depends on the change/damage caused to the eye by the substance. The changes that are not reversible over the mentioned period are classified as ocular corrosives.[60]

Regarding ocular toxicity assessment, *in vivo* eye toxicity evaluation used to be performed based on the Draize test, where damage to the cornea, conjunctiva, and iris of a rabbit was scored to classify the eye irritation/corrosion potential of a chemical.[61] However, it is worth mentioning that this test was not submitted to an official validation process and has been shown to be incapable of predicting the eye toxicity potential of chemicals for humans.[62-64] Regulatory agencies as European Chemicals Agency (ECHA) in the European Union, Environmental Protection Agency (EPA) in United States and the United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals have recently published advice on using new or revised OECD test guidelines related to serious eye damage/eye irritation and skin corrosion/irritation, recommending non-animal testing as a default approach to gather such information.[65-67]

In Brazil, until 2019, in order to evaluate these toxicological endpoints, most research used exclusively *in vivo* methods, which were accepted/recommended by the Brazilian Health Surveillance Agency (Agência Nacional de Vigilância Sanitária, ANVISA). However, recently, the Brazilian legislation was updated and the new ANVISA norm includes acceptance of alternatives to animal testing recognized by the OECD and adoption of the GHS criteria for eye toxicity categorizations, and the deadline for adoption of this new methodology is 2021.[68]

One alternative for the commonly applied *in vivo* Draize rabbit eye assay is the hen's egg test using a chorioallantoic membrane (HET-CAM test), developed by Luepke (1985),[69] a cheap and successful test that has shown good correlation to ophthalmic irritation in the *vivo* situation.[70-78] The HET-CAM test sits between *in vivo* and *in vitro* techniques, using fertile white leghorn chicken eggs instead of rabbits. The hen's egg chorioallantoic membrane (CAM) separates the embryo from the internal space and is an immunodeficient tissue containing arteries, veins and capillaries, which responds to injury with an inflammatory process, similar to what one would observe in the conjunctival tissue of a rabbit's eye. Its well-developed vascularization provides an ideal model for ocular irritation studies.[79]

A sequential approach was used by the authors to assess the bio-safety of these 13 protic ionic liquids by combining physico-chemical characterization, terrestrial ecotoxicity analysis and two *in vitro* methodologies, which were selected based on the applicability domain of each one (Fig. 2). Firstly, as published earlier, properties such as density, ultrasonic velocity,

pH and viscosity have been measured.[21,38,41-44] As it happens with other thermodynamic properties, there is an enormous gap of information in open literature in terms physicochemical data of protic ionic liquids.

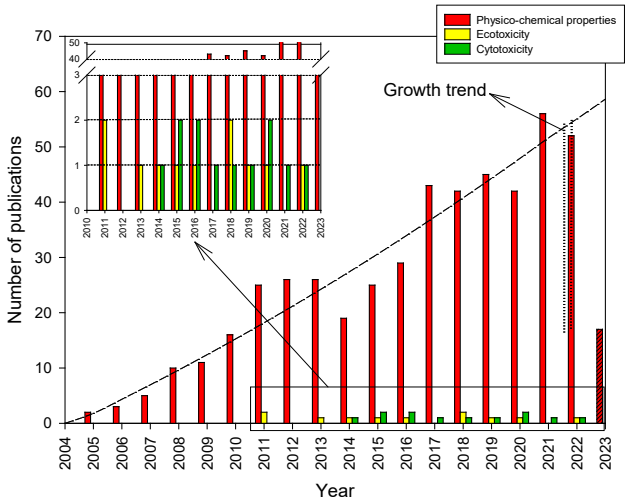


Figure 1: Evolution of number of scientific works *versus* timeline on different research paths related to protic ionic liquids

These properties are important for both the design of future cleaner technological processes and for understanding the interactions in such compounds. As a second step, the terrestrial ecotoxicity of representatives of this new family of PILs was analyzed by performing different bioassays with plants (onion, grass, and radish) and soil microorganisms involved in the most important biogeochemical cycles, such as carbon and nitrogen mineralization of organic matter.[48-50] As a result, PILs showed a potential for biodegradation in soil, while aprotic ILs exhibited inhibitory effects towards the carbon transforming microbiota. These findings indicate that protic ILs can be considered as less toxic and safer for the terrestrial environment than the aprotic ILs. Then, the PILs were tested for their *in vitro* toxic activities on two human cell lines (normal keratinocytes HaCaT and hepatocytes HepG2), using the well-known MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay.[56] These two cell lines were used aiming to detect any damage caused by the studied PIL or its metabolites, and to determine the IC_{50}, the PIL concentration required for achieving 50% inhibition of the cell culture. The studied PILs cytotoxicity evaluated using the MTT assay revealed higher IC_{50} values (lower toxicological profile) when compared to imidazolium derivative ionic liquids assessed on similar cell types.

In this work, as a continuation of this sequential testing strategy, these PILs were evaluated using the HET-CAM method, which allowed their classification based on eye vascular alterations. The outcomes of this study could provide a rationale for the usefulness of the PILs in industrial applications, in which there may be direct or indirect interaction of these compounds with humans.

EXPERIMENTAL

Preparation of protic ionic liquids

A three-necked glass flask was used, equipped with a reflux condenser, a PT-100 temperature sensor, and a dropping funnel. Mono and Diethanolamine (>99%, Merck Synthesis) was placed in the flask, and then it was placed in an ice bath. Under stirring with a magnetic bar, we added dropwise the organic acids (>99%, Merck Synthesis) to the flask. With the aim of obtaining a homogeneous and viscous yellowish liquid, stirring was continued for 24 h at room temperature. No solid crystals or precipitation was noted during storage or purification. The reaction is a Bronsted reaction, creating a salt of mono- or diethanolamine, namely 2-hydroxy ethanolammonium formate (2-HEAF), 2-hydroxy ethanolammonium acetate (2-HEAA), 2-hydroxy diethanolammonium acetate (2-HDEAA), 2-hydroxy ethanolammonium propionate (2-HEAPr), 2-hydroxy ethanolammonium lactate (2-HEAL), 2-

hydroxy diethanolammonium lactate (2-HDEAL), 2-hydroxy diethanolammonium benzoate (2-HDEABe), 2-hydroxy diethanolammonium salicylate (2-HDEASa), 2-hydroxy diethanolammonium maleate (2-HDEAMa), 2-hydroxy ethanolammonium adipate (2-HEAAd), 2-hydroxy diethanolammonium adipate (2-HEAAd), 2-hydroxy ethanolammonium citrate (2-HEACi) and 2-hydroxy diethanolammonium citrate (2-HDEACi). This collection of PILs was designed attending to three main objectives, analyze the effect of a progressively greater anion (from formate to salicylate), the effect of the cation (mono or diethanolamine) and the effect of different number of active sites in the ions (from 2-HEAF to 2-HEACi).

The complete synthesis protocol and spectrometric analysis of the protic ionic liquids used in our experiments have been reported earlier.[21] The chemical structure and the full and abbreviated names are listed in Figure 3. The molar mass (MM) and open literature physico-chemical data under standard conditions[21,39,41,44,56,80-88] for the studied protic ionic liquids are shown in Table 1.

HET-CAM assay

For each PIL tested, four fresh fertile Leghorn eggs were used. The eggs were incubated at 37 ± 0.5°C with a relative humidity of 60 ± 2% for 10 days in the vertical position to ensure the correct positioning of the embryo (away from the chorioallantoic membrane). They were manually rotated 180º two times a day for the duration of the test, to ensure correct development and viability of the embryo. On the tenth day, the eggs were removed individually from the incubator, and placed in a holder with the larger end upwards. The shell and the inner membrane were carefully scratched off with a cutter and then pared off with a fine forceps, exposing the chorioallantoic membrane. After verify visually if the CAM was suitable to test, 0.2 mL of each PIL was placed on the membrane surface. The same volume of 1M sodium hydroxide and phosphate buffered saline (PBS, pH = 7.2) solution was also added directly onto the CAM to serve as positive and negative controls, respectively.

Any lysis, haemorrhaging and/or coagulation was observed and photographs were taken at different times (0 s, 30 s, 120 s and 300 s) to record qualitative data.

A semi-qualitative analysis was performed using the photographs, where the severity of any haemorrhage was graded on a scale from 0 (no reaction) to 3 (strong reaction) using the method developed by Gupta *et al.*[89] (Table 2). The vascular effects were scored according to the criteria described in the Protocol ICCVAM HET-CAM test method (Table 3), *i.e.* 0–0.99 corresponding to non-irritant; 1.00–4.99 corresponding to slightly irritant; 5.00–8.99 corresponding to moderately irritant (MI); and 9.00–21.00 corresponding to severely irritant to the ocular tissue (SI).[90]

HET-CAM test: quantification using Photoscape and ImageJ

Following the methodology proposed by Mackenzie *et al*.,[75] the photographs were examined using Photoscape and ImageJ softwares (available as freeware from http://rsb.info.nih.gov/ij/) to quantify the vascular damage, allowing a more detailed and robust analysis of the PILs to be made.

The images of the chorioallantoic membranes were loaded onto Photoscape and converted to greyscale. To quantify the extent of any hemorrhaging, hyperemia and coagulation, the files formed using Photoscape were loaded into ImageJ. To analyze the greyscale values of the pixels over a standardised length of membrane, a 25 in^2 square was carefully selected in an appropriate area to exclude the shell and any out of focus regions. The profile of the average grey pixel value along the rectangular area was plotted by pressing 'Analyze > Plot Profile'. Once the square has been adjusted to the standardized length, the menu option 'List' was selected and the values plotted using SigmaPlot.

RESULTS AND DISCUSSION

The tested PILs were semi-quantitatively graded in terms of their eye irritation potential using the method developed by Gupta *et al.* This scoring method is partially subjective and dependent on the visual capacity of the observer, but does allow a score to be assigned to each tested sample. The obtained scores for protic ionic liquids and for negative and positive controls have been computed according to Tables 2 and 3, and are presented in Figure 4. The photographic response is presented in Figure 5. Taking into account the subjectivity of this assay, and the high probability of the observer not perceiving mild changes in blood vessels, and in order to quantify the photographic results, the images were subjected to software analysis using ImageJ and PhotoScape (Fig. 6). Modifications in the methodology proposed by Mackenzie *et al*.[75] were done in order to improve the sensibility of the analysis. The data was analyzed and processed as 'grey values' (Figs. 7A-7C – as examples of non-irritating, moderately and severe-irritating PILs – and Figs. 8A-8L), where lower values ('darker grey') correlate to hemorrhage or hyperemia and higher values ('lighter grey') correlate to coagulation.

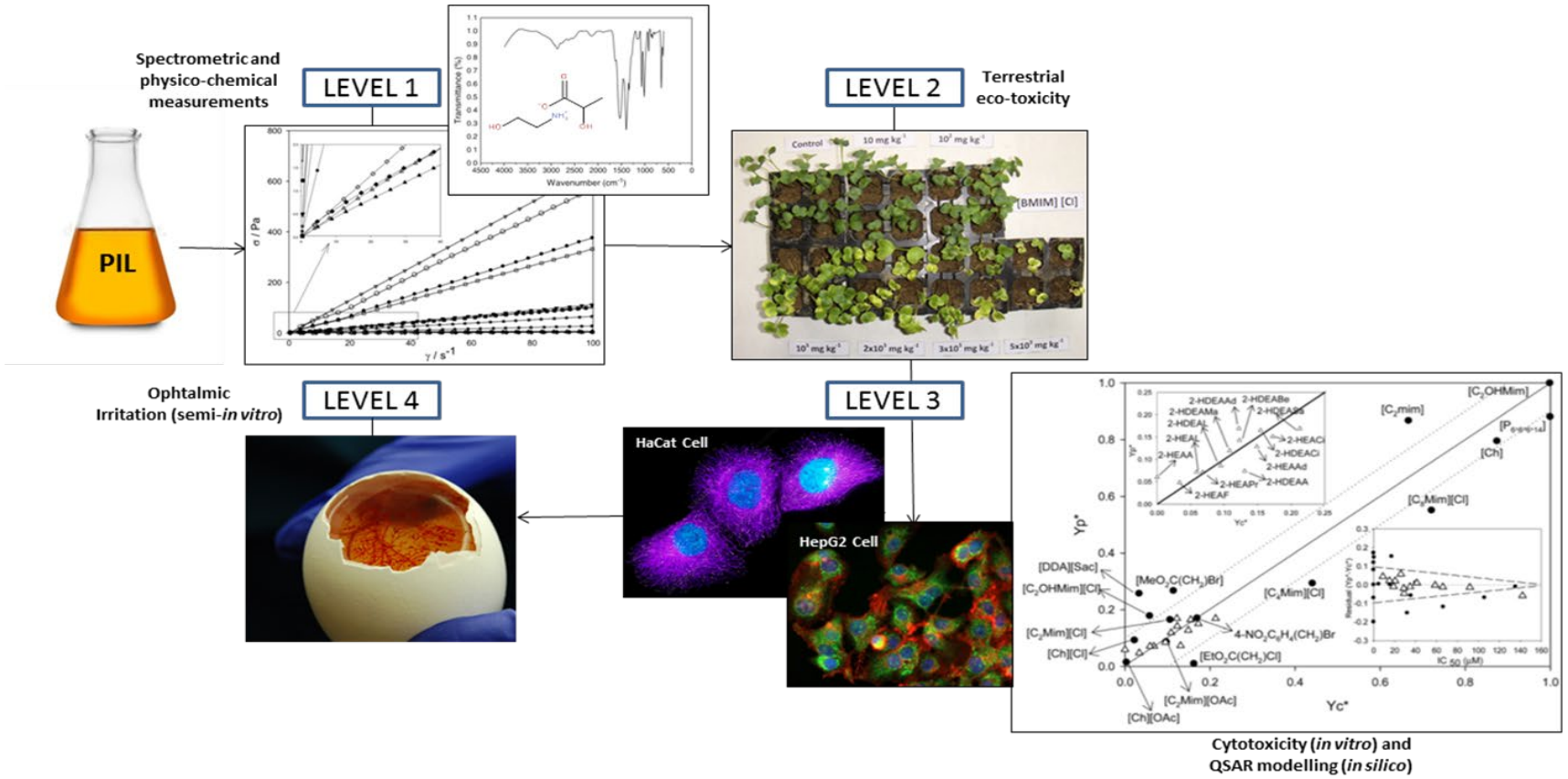
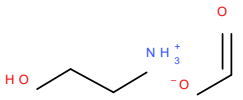
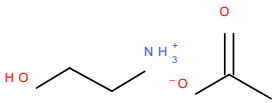


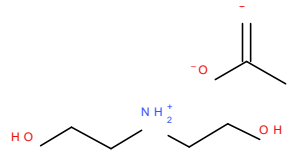
Figure 2: Sequential testing analysis applied by the authors



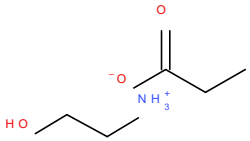
2-hydroxy ethanolammonium formate (2-HEAF)



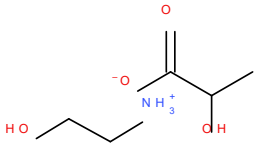
2-hydroxy ethanolammonium acetate (2-HEAA)



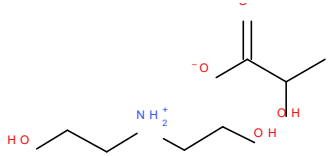
2-hydroxy diethanolammonium acetate (2-HDEAA)



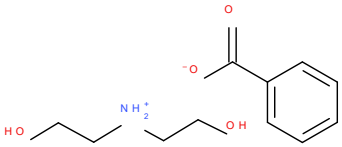
2-hydroxy ethanolammonium propionate (2-HEAPr)



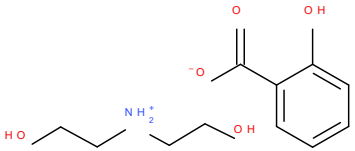
2-hydroxy ethanolammonium lactate (2-HEAL)



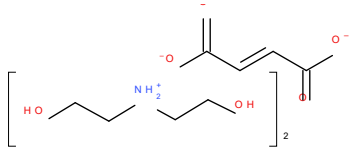
2-hydroxy diethanolammonium lactate (2-HDEAL)



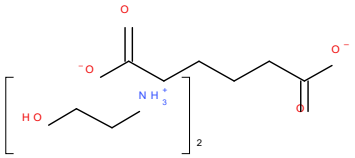
2-hydroxy diethanolammonium benzoate (2-HDEABe)



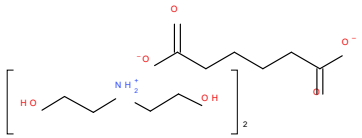
2-hydroxy diethanolammonium salicylate (2-HDEASa)



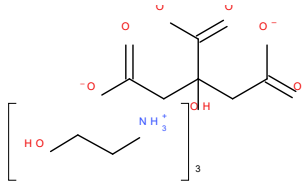
2-hydroxy diethanolammonium maleate (2-HDEAMa)



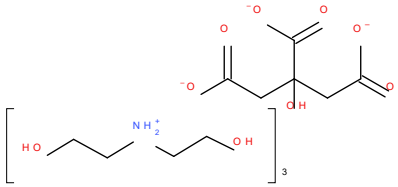
2-hydroxy ethanolammonium adipate (2-HEAAd)



2-hydroxy diethanolammonium adipate (2-HDEAAd)



2-hydroxy ethanolammonium citrate (2-HEACi)



2-hydroxy diethanolammonium citrate (2-HDEACi)

Figure 3: Molecular structures of PILs

Table 1
Literature data for 13 PILs at 298.15 K

Protic ionic liquid (PIL)	Molar mass ($gmol^{-1}$)	Lit. Density (gcm^{-3})		Lit. Ultrasonic velocities (ms^{-1})
2-HEAF	107.110	1.17817[a] 1.1771[b] 1.2105[c]	1.1762[d] 1.1929[e] 1.0335[f] 1.1510[g]	1840.94[a] 1719.59[b] 1782.59[d] 1782.87[e]
2-HEAA	121.137	1.14836[a] 1.1490[h] 1.0177[i]	1.1535[j*]	1790.94[a] 1790.73[h]
2-HDEAA	165.190	1.16748[a] 1.1675[e] 1.1755[k*]		1863.35[a] 1863.35[e]
2-HEAPr	135.163	1.09259[a] 1.1211[l*]		1636.90[a] 1570.01[l*]
2-HEAL	151.163	1.21361[a] 1.2016[m]		1877.02[a] 1865.53[m]
2-HDEAL	195.216	1.21361[a] 1.2187[n]		1877.02[a] 1877.64[n]
2-HDEABe	227.260	1.19931[a]		1878.58[a]
2-HDEASa	243.260	1.26421[a]		2017.06[a]
2-HDEAMa	326.347	1.28084[a]		2101.14[a]
2-HEAAd	268.311	1.19411[a]		2013.74[a]
2-HDEAAd	356.417	1.22170[a]		2010.54[a]
2-HEACi	375.377	1.32449[a]		2200.72[a]
2-HDEACi	507.536	1.29154[a]		2113.36[a]

[a] Zanoni *et al.*[56]; [b] Iglesias *et al.*[80]; [c] Ghatee *et al.*[81]; [d] Bharmoria *et al.*[82]; [e] Andrade *et al.*[21]; [f] Nazet *et al.*[83]; [g] Hosseini *et al.*[84]; [h] Alvarez *et al.*[85]; [i] Hou *et al.*[39]; [j] Pentilla *et al.*[86]; [k] Santos *et al.*[87]; [l] Sarabando *et al.*[88]; [m] Barros *et al.*[44]; [n] Kulhavy *et al.*[41]; * fitted data

Table 2
Scoring scale for the HET-CAM test

Effect	Time (min)		
	≤ 0.5	0.5 < t ≤ 2	2 < t ≤ 5
Hyperaemia	5	3	1
Haemorrhage	7	5	3
Coagulation	9	7	5

Table 3
Cumulative scores for assessment of ocular irritation potential

Substance score	Category of irritation score classification
0.0 to 0.9	Non-irritant or practically none
1.0 to 4.9	Weak or slight irritation
5.0 to 8.9	Moderate irritation
9.0 to 21.0	Strong or severe irritation

Protocol ICCVAM HET-CAM test method[90]

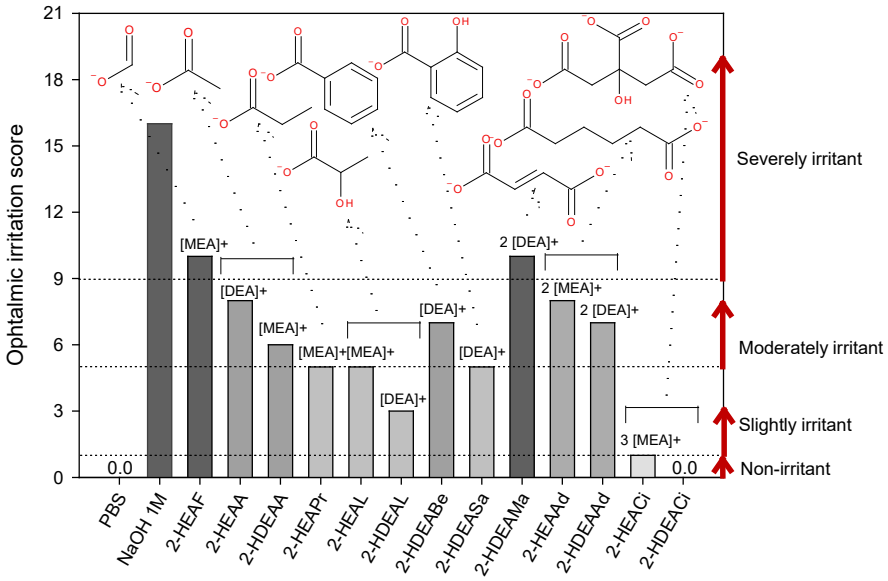
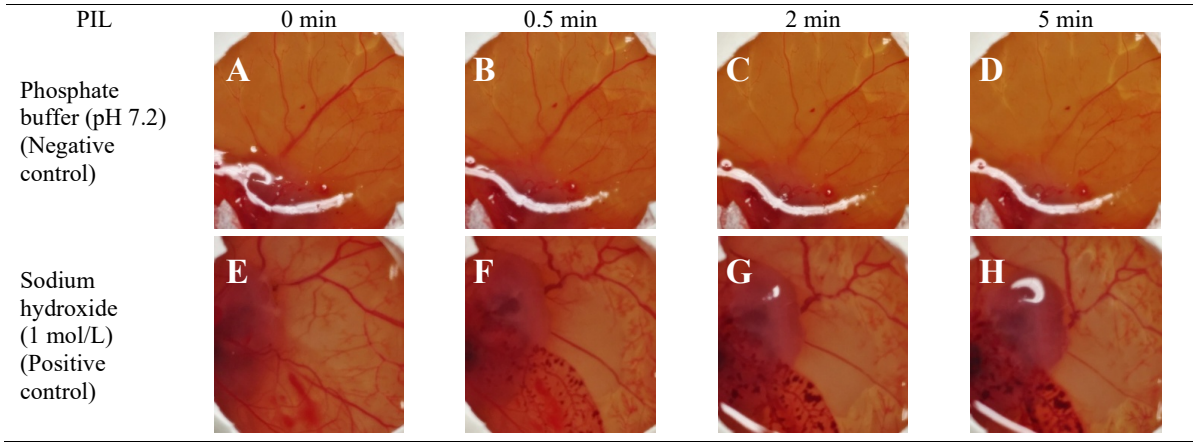
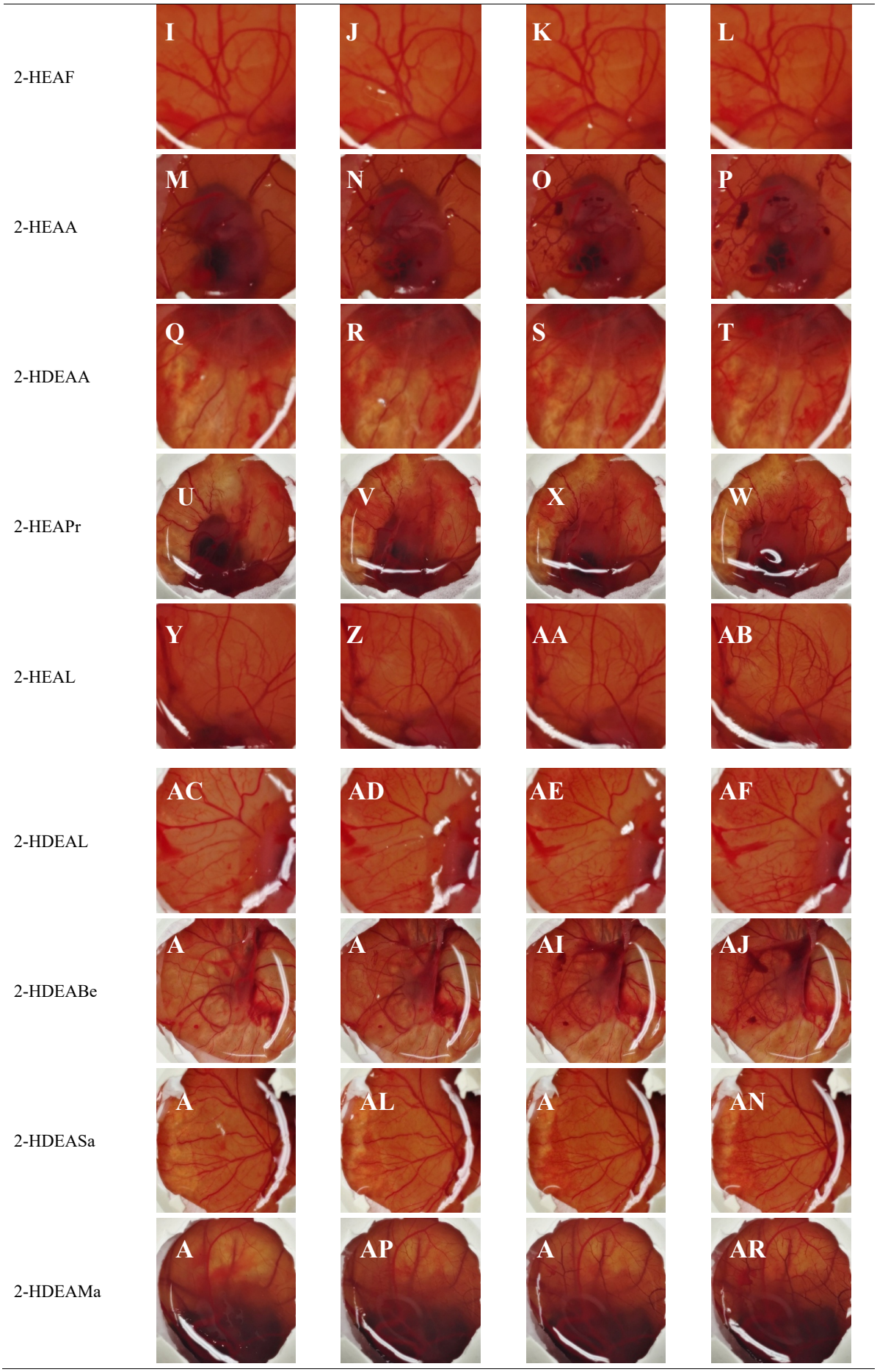


Figure 4: Ophtalmic irritation scores obtained from the HET-CAM test

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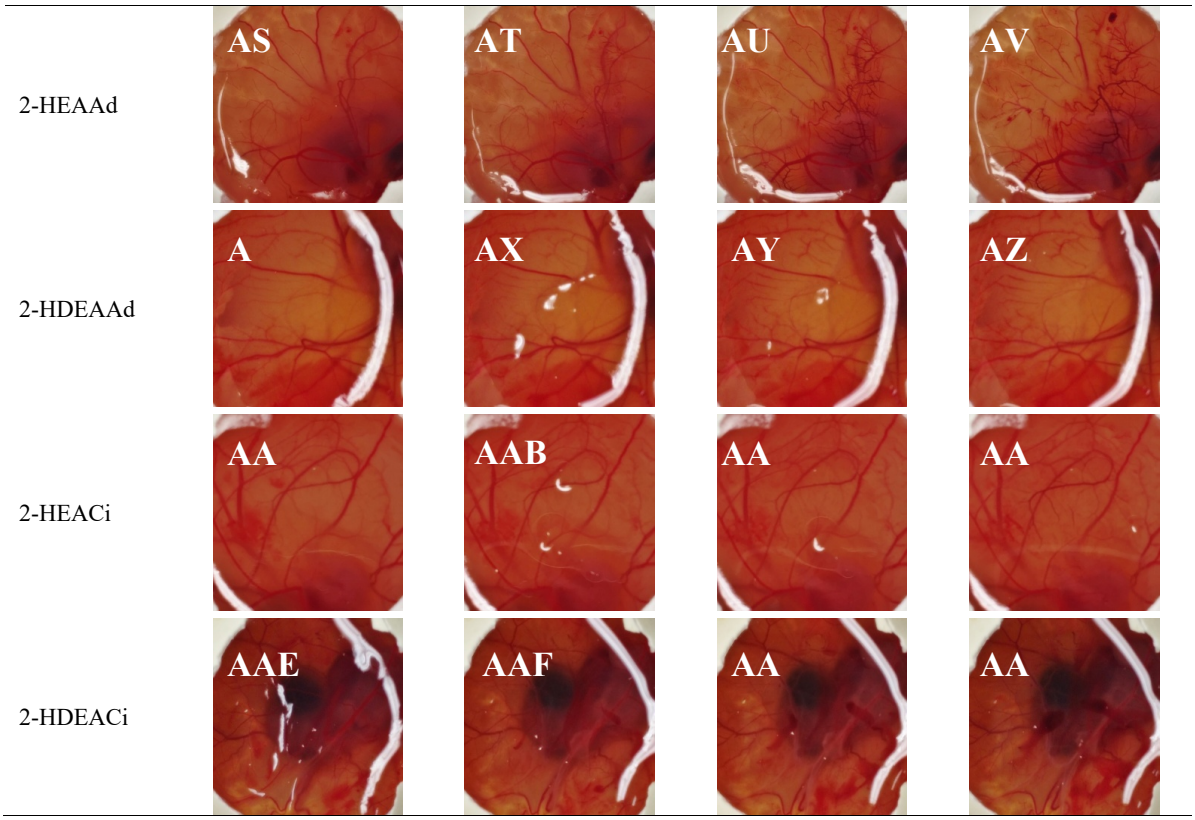


Figure 5: Sequence of photographs illustrating the effect of protic ionic liquids on the chorioallantoic membrane over a 300s period

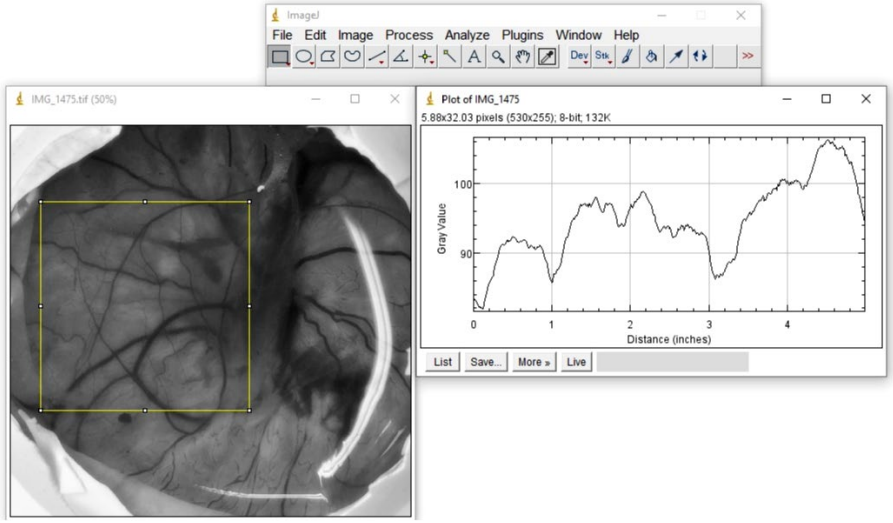


Figure 6: An example profile plot on ImageJ, highlighting the correlation between dark blood vessels and greyscale values

Since each egg has its own blood vessel network, the authors consider it inconsistent to use this quantification method to compare different samples. However, grey values of photographs taken over time for each tested sample were analyzed, which allowed for a more objective assessment of the physiological response as a function of the time of the chorioallantoic membrane when in contact with each PIL individually. As observed, the application of 0.2 mL of phosphate buffered saline (PBS) solution to the healthy membrane produced no visual response (cumulative score 0, Fig. 4) over the 5 min period (Figs. 5A-5D and 8A). On the other hand, the same volume of 1M sodium hydroxide produced a severe hemorrhage since the first moment (cumulative score 16, Fig. 4). Over five minutes its injurious capability has progressively increased (Figs.

5E-5H and 8B), grading this solution as a severe irritant to the ocular tissues.

According to the visual analysis (Figs. 5AAA-5AAD and 5AAE-5AAH), 2-HEACi and 2-HDEACi produced no relevant damage to the CAM, with a behavior similar to that of the negative control, resulting in a cumulative score 1 and 0, which classifies them as slightly-irritating and non-irritating PILs, respectively. Figures 7A and 8L confirm the expected, in which it is possible to notice that the grey values for times 0, 30, 120 and 300 s approximately overlap for 2-HDEACi, indicating absence of physiological response, and slightly decreases for 2-HEACi, indicating a mild hyperemia, as observed visually. In both cases, it is possible to perceive a slight coagulation at 30 s in a specific area (visually imperceptible, but clearly shown in the grey values graph), which, as observed, falls apart over time. Changes in blood vessels usually start quickly after injury or infection, but develop at varying speeds, depending on the nature and severity of the original inflammatory stimulus. It is common that before vasodilatation of the arterioles, resulting in increased blood flow and opening of the capillary beds (hyperemia), a slight transient vasoconstriction occurs as the first physiological response to the aggression.[91]

Citric acid has an extensive distribution among animals, and different from the other studied acids, its presence was demonstrated in the aqueous humor, vitreous body and bloom serum of various species of mammals and birds, as well as in the intraocular fluids of some fishes.[92] This fact could explain the lower irritating capacity of these PILs.

Similarly, 2-HDEAL was graded as slightly-irritant according to Gupta's method, presenting a cumulative score 3 due to a hyperemia detected between 30 and 120 s after the CAM's contact with the sample (Figs. 5AC-5AF). Figure 8G indicates, as observed for other PILs, a slight constriction/coagulation, not visually perceived, but observed in the first 30 s of the test by the increase in the gray scale values, followed by an expected progressive hyperemia (decreasing grey values). 2-HEAL presented hyperemia in the first 30 seconds of the test, with no further associated effects (Figs. 5Y-5AB), which theoretically graded it as a moderately-irritating liquid (cumulative score 5). However, Figure 8F indicates that after a slight initial reduction in gray values (darkening of the image, indicative of hyperemia), blood vessels tend to return to their original appearance, as evidenced by the superposition trend of the 0 s, 120 s and 300 s time curves.

As important components in cosmetic products, lactate derivatives (including ammonium, potassium sodium, methyl, ethyl, lauryl, myristyl and cetyl lactates) have been previously tested for their potential for eye irritation in many *in vivo* studies.[93] To the best of our knowledge, all studied formulations were tested undiluted. For example, in a chorioallantoic membrane vascular assay (CAMVA), two eye cream formulations containing 1.18% of 85% aq. lactic acid, pHs 5.64 and 4.00, tested undiluted had RC_{50} (reactive concentration 50%, concentration of test chemical that reacts with 50% of the model chemical) values >100%.[93] These test samples were considered non-irritating to the eyes, in accordance with the authors' results.

Most of the studied PILs were categorized as moderately irritating. With a cumulative score 5 due to a slight progressive hyperemia since the first 30 s from the beginning of the test (Figs. 5U-5W and 8E), without other associated effects, 2-HEAPr showed a moderately irritant behavior equivalent to that of 2-HEAL, which was expected, since their structures are very similar and differ only by the presence of a hydroxyl group in the lactate compound.

2-HEAA (Figs. 5M-5P and 8C) and 2-HDEAA (Figs. 5Q-5T and 8D) had cumulative scores 8 and 6, respectively, due to the hemorrhagic response between times 120 and 300 s for both, associated with a hyperemia effect observed in the first seconds of testing for 2-HEAA, and between 30 and 120 s for 2-HDEAA. It is important to note that the sample dripped on the chorioallantoic membrane often flows in preferential ways, revealing a physiological response concentrated in a reduced area of contact, which hinders the visual perception of the changes. This fact is observed in the grey scale graphs – in both cases, there is a tendency for the curves to overlap (indicative of no response to the stimulus), except at the bottom of the images (see extreme right of Figs. 8C and 8D), where it is noticeable the reduction of values after the time of 30 s for

the 2-HEAA, and after the time of 120 s for the 2-HDEAA.

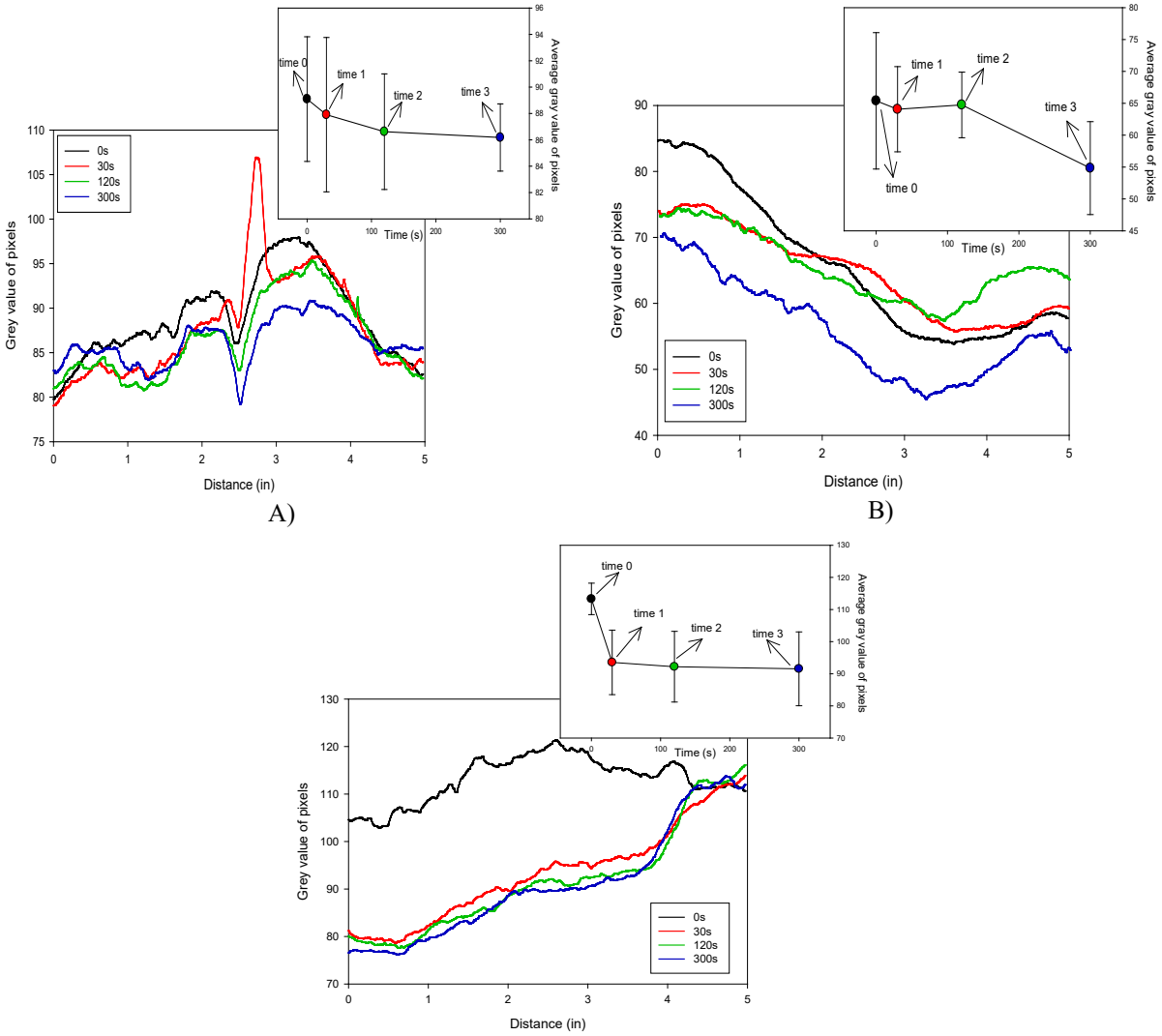
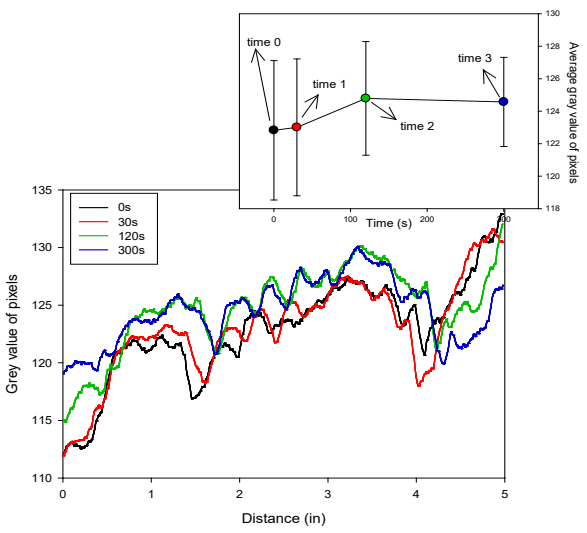
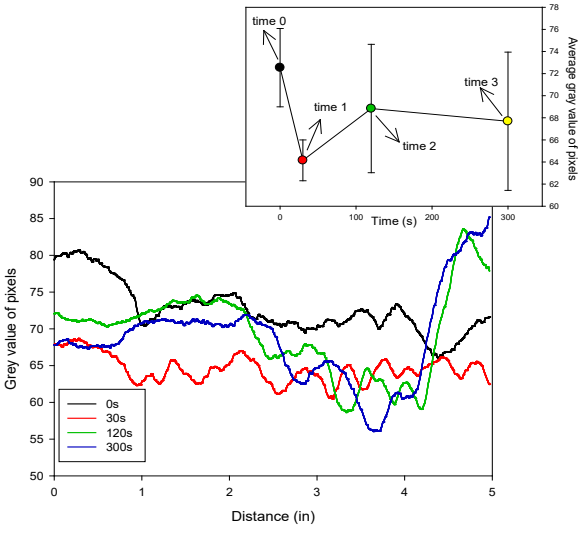


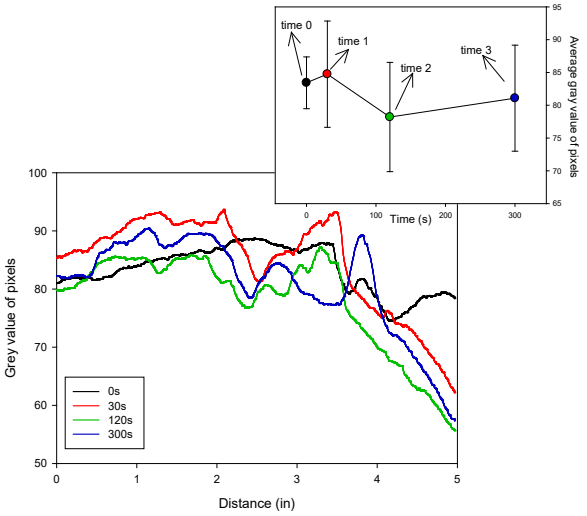
Figure 7: Plots of the grey value over distance for A) 2-HDEACi, a non-irritating PIL, B) 2-HEAAd, a moderately-irritating PIL, and C) 2-HEAF, a severe-irritating PIL


time 0
time 1
time 2
time 3
Average gray value of pixels
Time (s)
0s
30s
120s
300s
Grey value of pixels
Distance (in)

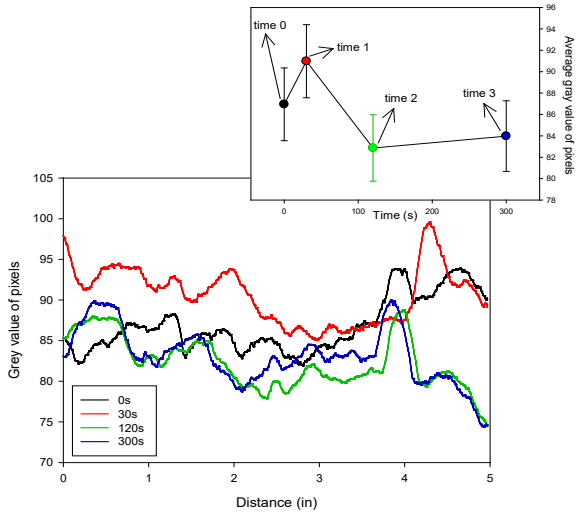
A)


time 0
time 1
time 2
time 3
Average gray value of pixels
Time (s)
0s
30s
120s
300s
Grey value of pixels
Distance (in)

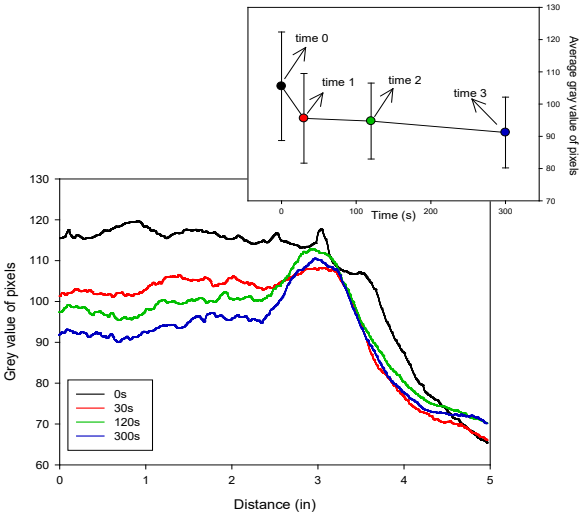
B)


time 0
time 1
time 2
time 3
Average gray value of pixels
Time (s)
0s
30s
120s
300s
Grey value of pixels
Distance (in)

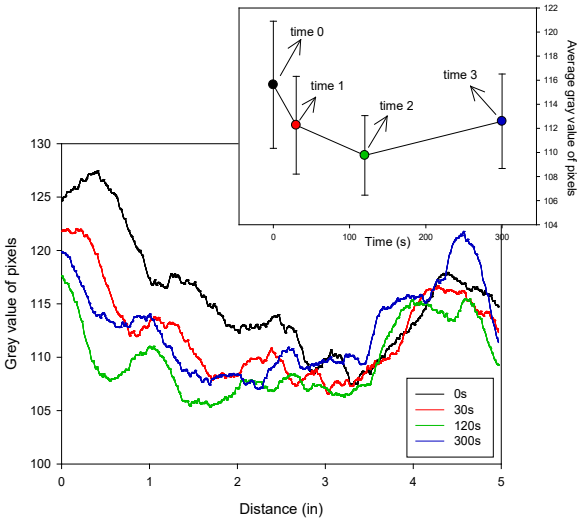
C)


time 0
time 1
time 2
time 3
Average gray value of pixels
Time (s)
0s
30s
120s
300s
Grey value of pixels
Distance (in)

D)


time 0
time 1
time 2
time 3
Average gray value of pixels
Time (s)
0s
30s
120s
300s
Grey value of pixels
Distance (in)

E)


time 0
time 1
time 2
time 3
Average gray value of pixels
Time (s)
0s
30s
120s
300s
Grey value of pixels
Distance (in)

F)

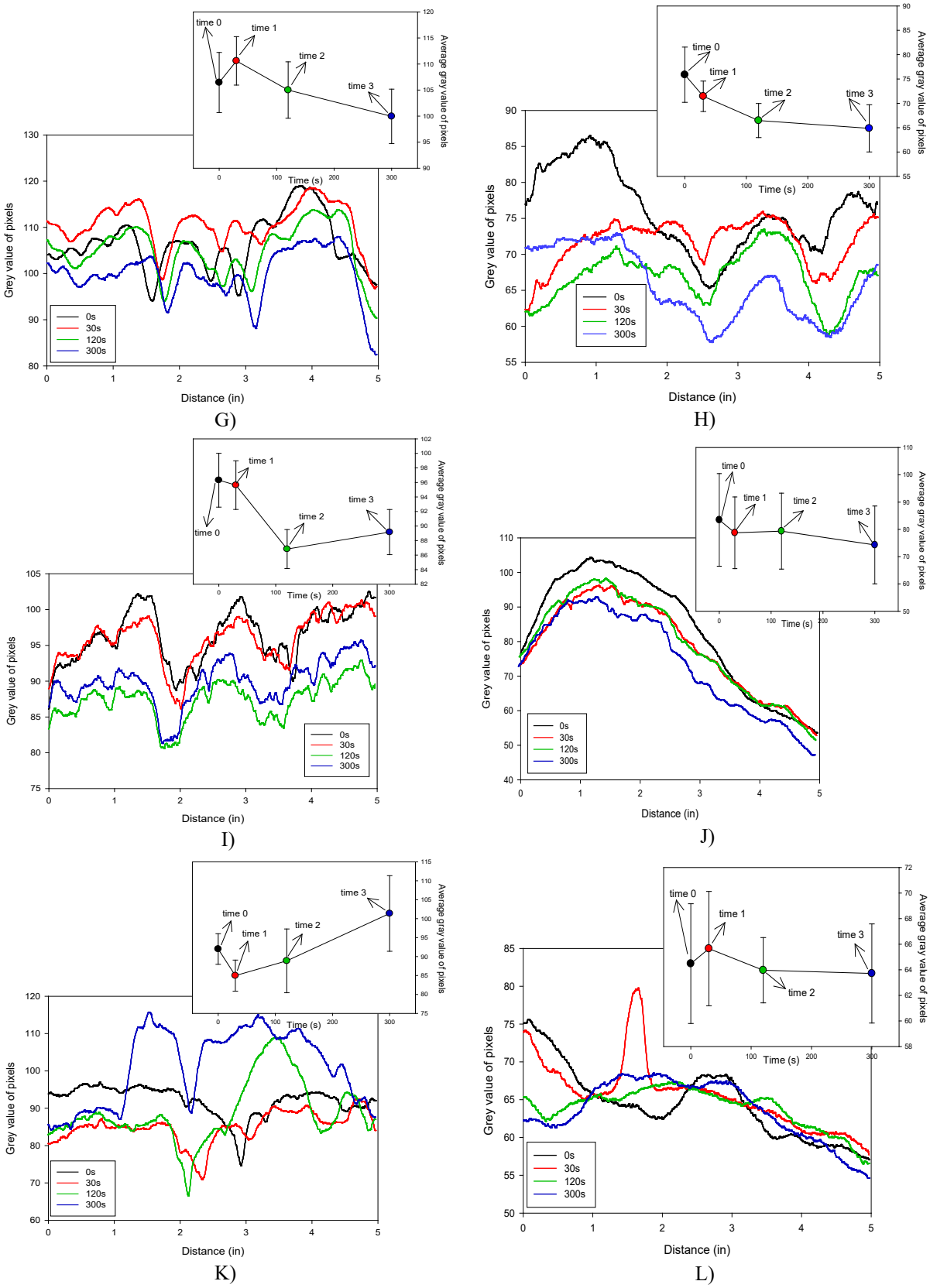


Figure 8: Plots of the grey value over distance for A) negative control, B) positive control, C) 2-HEAA, D) 2-HDEAA, E) 2-HEAPr, F) 2-HEAL, G) 2-HDEAL, H) 2-HDEABe, I) 2-HDEASa, J) 2-HDEAMa, K) 2-HDEAAd, L) 2-HEACi

2-HDEABe (Figs. 5AG-5AJ) and 2-HDEASa (Figs. 5AK-5AN), the only studied aromatic PILs, showed cumulative scores 7 and 5, respectively, due to a hemorrhage

observed in the first 30 s for the benzoate salt and between 30 s and 120 s for the salicylate salt. In both cases, no associated effects were visually observed. Figure 8H clearly shows the reduction over time of the grey values for the 2-HDEABe (see average grey value of pixels), indicating that the bleeding started in 30 s is not contained, but shows a progressive increase over time. On the other hand, Figure 8I reveals a tendency to contain the hemorrhage that started at 120 s, as evidenced by the reduction in the average grey values for the 300 s time curve.

In 2012, the Cosmetic Ingredient Review (CIR) Expert Panel issued a safety assessment on 2-HEABe and 2-HEASa, the respective salts of monoethanolamine (instead of the diethanolamine salts of benzoate and salicylate studied here).[94] In that report, the Panel concluded that these compounds, extensively used in cosmetics as preservatives, are safe as used when formulated to avoid skin irritation and when formulated to avoid increasing the skin's sun sensitivity. However, the report is limited to information on the potential for skin irritation; the ocular irritation potential is assessed in this report for monoethanolamine only.

2-HEAAd and 2-HDEAAd showed cumulative scores 8 and 7, respectively. The contact of the CAM with 2-HEAAd (Figs. 5AS-5AV) resulted in a slight hyperemia during the first contact (before 30 s), followed by a hemorrhage observed from the 300 s of the test (see average grey values of pixels, Fig. 7B). On the other hand, 2-HDEAAd allowed visual perception of a hemorrhagic effect since the first moment, without any other associated effect (Figs. 5AW-5AZ). In spite of this, Figure 8K reveals the reduction of grey values from the time of 120 s, indicative of a containment of the generated hemorrhage, and a progressive vasoconstriction over time.

2-HEAF (Figs. 5I-5L) and 2-HDEAMa (Figs. 5AO-5AR) were the only two studied PILs that had a severely irritating profile. With a cumulative score 10, the contact of both with the chorioallantoic membrane resulted in hyperemia in the first 30 s, followed by hemorrhage observed between 30 s and 120 s. Figures 7C and 8J show a progressive decreasing trend in grey values, an indicative result of progressive hyperemia/hemorrhage over time.

Although there is no information available in the literature on the eye irritation potential of 2-HEAF, formic acid and formate salts are widely used in the cosmetic industry as fragrance ingredients, preservatives, pH adjuster and preservative in cosmetic products, which is why some of these products have already had their eye irritation potential assessed.[95] To compare, the ocular irritation potential of sodium formate was evaluated *in vivo* using 6 New Zealand white rabbits (3 males, 3 females; at least 8 weeks old). Transient (moderate to severe) conjunctival irritation was observed in all 6 rabbits, and conjunctival necrosis was observed in 4 of the 6 rabbits. All reactions had cleared by day 17.[96]

In general terms, it is important to highlight that, for the same anion, the diethanolamine salts showed less irritating potential than the respective monoethanolamine salts (2-HEAA>2-HDEAA, 2-HEAL>2-HDEAL, 2-HEAAd>2-HDEAAd and 2-HEACi>2-HDEACi). Triethanolamine (TEA), Diethanolamine (DEA), and Monoethanolamine (MEA) are amino alcohols used in cosmetic formulations as emulsifiers, thickeners, wetting agents, detergents, and alkalizing agents,[97] and have been studied regarding their eye irritation potential in rabbits[98-104] and rhesus monkeys.[105] In high concentrations and with long contact time, TEA, DEA, and MEA are irritating to the rabbit eye at concentrations of 100%, 50%, and 5% m/m, respectively, *i.e.*, monoethanolamine has a greater irritating potential than diethanolamine, in accordance with the authors' results.

For the aliphatic and monoelectrolyte PILs, it is evident that the increasing anionic chain decreases the irritant potential of the compound (2-HEAF > 2-HEAA > 2-HEAPr and 2-HEAL; 2-HDEAA > 2-HDEAL). The same behavior is observed for aromatic (2-HDEABe > 2-HDEASa) and polyelectrolytic PILs (2-HEAAd > 2-HEACi and 2-HDEAMa > 2-HDEAAd > 2-HDEACi), which could be explained by the fact that complex structures with higher steric hindrance effect (high molar volumes) have greater resistance to passage across biological membranes, reducing their ocular irritating potential.[106]

Additionally, the recurring opacity effect observed in the greyscale graphs (although often not visually perceptible) may not

necessarily be due to an irritating character of the PILs, but rather to the hypotonic nature of the samples. This occurs when the cell contains a higher concentration of solutes than the solution, causing water to move into the cell and swelling of the ophthalmic tissues, as previously observed for saline compounds.[75]

Of the two analysis methods used in this paper, semi-quantitative (Gupta *et al*.) and quantitative (a modified method after Mackenzie *et al*.), neither fully describes the effects of all the test solutions. Therefore, it is useful to include both methods to allow for a full and accurate description of the damage caused to the CAM.

CONCLUSION

The eye irritation potential of 13 protic ionic liquids was determined using a HET-CAM test and a modified quantification method. In order to represent the eye conditions *in vivo*, the HET-CAM test is commonly used to evaluate substances that could be associated with ocular injuries such as haemorrhaging, hyperemia and coagulation. This assay produces a qualitative result determined by analyst's observation, which may fail or be inaccurate. Thus, in this research, a combination of two free software programs, ImageJ and Photoscape, was used to obtain a semi-quantitative result to reinforce the preliminary qualitative analysis.

Considering the results, it is evident that the increasing anionic chain decreases the irritant potential of the compound (2-HEAF > 2-HEAA > 2-HEAPr and 2-HEAL; 2-HDEAA > 2-HDEAL). The same behavior is observed for aromatic (2-HDEABe > 2-HDEASa) and polyelectrolytic PILs (2-HEAAd > 2-HEACi and 2-HDEAMa > 2-HDEAAd > 2-HDEACi), which could be explained by the fact that complex structures with higher steric hindrance effect (high molar volumes) have greater resistance to passage across biological membranes, which reduces their ocular irritating potential.

Regarding the analysis of PILs, our experience and the literature indicate that the toxicity profile may vary depending on the concentration and structural features. However, through rational design, the potential for irritation can be mitigated by establishing a structure–toxicity relationship. Considering the importance of performing toxicity assessments to fully confirm the green behavior of PILs, this modified method could be the key to providing predictive ability that guides the design of new, more environmentally friendly ILs for industrial applications.

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