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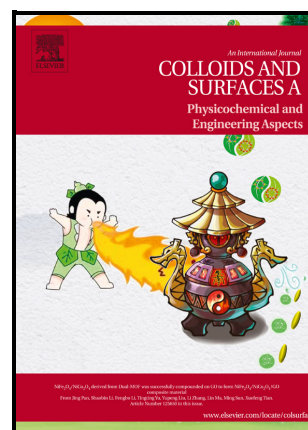
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Physical Characterization of Genipin Complexes coupled with Multivariate Data Analysis to Assess Formed Pigments

Ana Mayela Ramos-de-la-Peña*^a, Alfredo Santana-Reynoso^b, Catherine M.G.C. Renard^c, Juan Carlos Contreras-Esquivel^c, José Rodríguez-Rodríguez^a, Oscar Aguilar^a, José González-Valdez^a, Cuauhtémoc Licona-Cassani^a

^a Tecnológico de Monterrey, School of Engineering and Science, Av. Eugenio Garza Sada 2501 Sur, Monterrey, NL 64849, Mexico

^b Tecnológico de Monterrey, School of Engineering and Science, Epigmenio Gonzalez 500, Fracc. San Pablo, Queretaro, Qro 76130, Mexico

^c INRAE, TRANSFORM, F-84000, Nantes, France

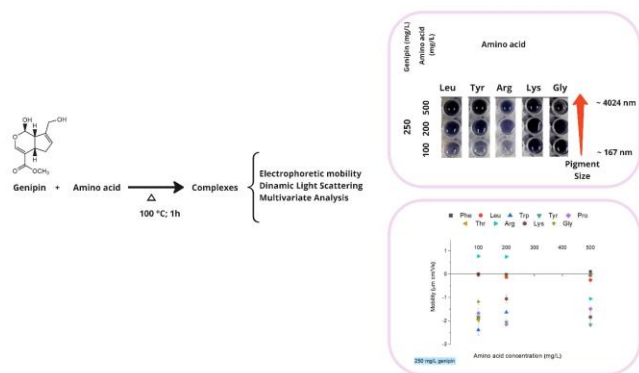
^d Laboratory of Applied Glycobiotechnology, Food Research Department, School of Chemistry, Universidad Autonoma de Coahuila, Boulevard Venustiano Carranza, Saltillo, Coahuila 25280, Mexico

Corresponding author: ramos.amay@tec.mx

Abstract

The potential of electrophoretic mobility and dynamic light scattering (DLS) was investigated to assess pigment formation in aqueous solution after the genipin reaction with different amino acids by separate. Absorbance, mobility, hydrodynamic size, polydispersity index, and conductivity of the aqueous solutions were measured after the reaction. Only complexes derived from genipin and leucine, tyrosine, arginine, lysine, and glycine developed dark blue colors. From all measured variables, complex size was correlated with their blue color intensity, and darkest blue complexes showed the highest hydrodynamic size (1073-4179 nm). Moreover, size pigments below 1 μm were obtained for all amino acids at different concentrations of genipin and amino acid, except for arginine. A partial least squares (PLS) model for size was built and exhibited a good ability to describe the distribution in blue complexes formed after the reaction of genipin and excess of amino acid (mM): ($R^2_{\text{adj}}=0.88$; RMSE=0.94). Electrophoretic mobility and DLS coupled with multivariate analysis is a useful tool as it can give reliable assessment of the physical properties of aqueous solutions of genipin pigments.

Graphical abstract



Keywords:

Gardenia blue; jagua blue; iridoid; cross-linking; electrophoretic mobility

1. Introduction

Genipin is an iridoid contained in the genipap fruit (*Genipa americana L.*) and *Gardenia jasminoides*, which can react with primary amine groups of amino acids, peptides, proteins, chitosan, and other molecules to give blue pigments [1,2]. It has been demonstrated that genipin has choleric, hepatoprotective, anti-inflammatory, anticancer, antithrombotic, antibacterial, antidiabetic, neuroprotective effects, among others [3-7].

Genipin derivatives are widely used in Eastern Asia as colorants in beverages, juices, nectars, desserts, gels, etc. It has been reported that these blue pigments in food products do not represent a significant genotoxic concern for humans [8]. However, more detailed characterization in the context of risk assessment of the blue pigments needs to be provided to achieve its approval as food colorant by the Food and Drug Administration of the US (FDA) and the European Food Safety Authority (EFSA), as well as positive feedback from the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) [8-10].

Besides food colorant, genipin can be used in hair, cotton, wool, and leather dyeing [11-13]. Additionally, since its cytotoxicity is $\sim 5,000 - 10,000$ times lower than glutaraldehyde, it has

demonstrated to be a cross-linking agent of biopolymers. For instance, genipin has been used in cartilage substitutes, in drug/biological compounds/living cells delivery systems such as ocular, buccal, oral, anti-inflammatory, antibiotic, antifungal, etc., in wound treatment in animals and humans, etc. [14-17]. Nonetheless, as is the case for the food industry, clinical trials must be performed to leverage its use as alternative non-toxic cross-linking agent in pharmaceutical applications [18,19].

The genipin reaction with primary amine groups results in a complex mixture of polymers, which is also influenced by the reaction conditions [20], and it has been found that several intermediate pigments are produced before the blue color formation [21]. Moreover, the formation mechanisms of these polymers have been investigated [22], their physicochemical characteristics have been reported [23], as well as their physical stability as function of pH, temperature, and light [24]. In addition, several studies have been conducted on the structure of the blue pigments derived from genipin, for instance, the complex formed by genipin and glycine (blue dimeric adduct 1:1), named genipocyanin, was determined by H-NMR spectroscopy [25], and the structural characterization of gardenia or genipap fruit blue components has been the subject of several works [21, 22, 26]. More recently, Tsutsumiuchi et al. [26] characterized the blue pigments of genipin and different amino acids (glycine, alanine, valine, leucine, and isoleucine) by liquid and solid-state NMR measurements and found that the complex was composed of an alternating copolymer of methyne and 5H-2-pyridine, which was subjected to amino acid substitution at N atom and linked with methyne.

However, further research is needed to satisfy the current regulatory standards in the context of safety, more structural information about the pigments formed and fundamental research on the reaction mechanism needs to be performed for the approval of genipin as food colorant, and as a cross-linker in pharmaceuticals [20]. With this in mind, this work pursued three aims: to investigate, for the first time, the physical characteristics such as electrophoretic mobility, hydrodynamic size, polydispersity index, and conductivity of formed genipin-amino acid complex aqueous solutions. Second, to examine whether the size of the formed pigments influenced color intensity of these pigments in aqueous solutions; and third, to predict the physical characteristics of the formed pigments by means of models built using the partial least squares (PLS) technique.

2. Materials and methods

2.1 Materials

Genipin was purchased from Chengdu King-tiger Pharm-chem. Tech. Co., Ltd (Chengdu, China). Different amino acids were used as models for complex formation: L-phenylalanine (Phe), L-leucine (Leu), L-tryptophan (Trp), L-tyrosine (Tyr), L-proline (Pro), L-threonine (Thr), L-arginine (Arg), L-lysine (Lys), and L-glycine (Gly) were procured from Merck KGaA (Darmstadt, Germany). All reagents, genipin and α -amino acids were at least 99% pure.

2.2 Methods

2.2.1 Genipin-amino acid reaction

An aliquot of 1.25 mL of 500 or 250 mg/L of genipin was placed in test tubes. Afterwards, 1.25 mL of 100, 200 or 500 mg/L of each amino acid was added to the genipin containing test tubes, without any quenching agent. Genipin and amino acid concentration was varied to evaluate their effect on the physicochemical characteristics of the aqueous solutions of the formed pigments. Subsequently, the mixtures were boiled in a water bath (VWR, Radnor, PA, USA) for 1 h. Despite high temperatures in cross-linking reactions are avoided due to proteins denaturation and Maillard reaction, it was not the case in the present study due to sugars absence and to degradation temperatures of amino acids > 150 °C was not surpassed [27-32]. In addition, high temperatures enhance the reaction of genipin with primary amino groups [33-34].

After heating, the samples were cooled at room temperature (25 °C) for 15 min. To avoid batch-to-batch variation, measurements were conducted from a single batch, and measurements were carried out in triplicate. Water was selected as reaction medium [33-37] to avoid molecular interactions that could alter the physical properties of the formed complexes [38]. Aliquots (200 μ L) of the aqueous solutions of the mixture of genipin with different amino acids after heating at 100 °C for 1 h were transferred to a microplate, photographs were taken (F 2.4, 1/60 s, 4.30 mm, ISO 125, white balance mode: automatic, without flash) and pH was measured with a litmus test strip roll.

2.2.2 Spectral analysis

The UV-Vis spectra from 200 to 980 nm of reaction solutions were recorded on a microplate spectrophotometer (Epoch, Biotek Instruments – Agilent, Santa Clara, CA, USA) at 25 °C. Principal Component Analysis (PCA) was applied to the spectral data to identify the most contributing wavelengths. In addition, genipin, glycine, and the formed complex (precipitate) were analyzed by infrared attenuated total reflectance (FTIR/ATR) spectroscopy, performed by a spectrometer Perkin Elmer Frontier (Waltham, MA, USA) equipment operating at 4 cm⁻¹ resolution and 16 scans. Spectra were collected from 4000 to 400 cm⁻¹ in the transmittance mode.

2.2.3 *Electrophoretic mobility and DLS measurements*

Electrophoretic mobility and conductivity were measured using a DTS 1070 folded capillary cell (750 µL sample), and hydrodynamic diameter, and polydispersity index measurements were performed using a DTS 0012 cuvette (1 mL sample), with a Zetasizer Nano ZSP Instrument (Malvern, Worcestershire, United Kingdom) at 25 °C, in triplicate.

2.2.4 *Statistical and multivariate analyses*

The normal distribution of the datasets was demonstrated after a Shapiro-Wilk test ($\alpha=0.05$), the results were presented as mean values of triplicates, and the data dispersion within the experimental dataset was expressed as standard deviation. A one-way analysis of variance (ANOVA) was carried out to determine the significant differences ($p < 0.05$) due to genipin and amino acid concentration on each dependent variable: electrophoretic mobility, hydrodynamic diameter, particle size, polydispersity index, and conductivity distribution of aqueous solutions containing the complexes using Minitab version 17 (Minitab, State College, LLC, PA, USA). The pairwise comparison between means was performed using Tukey's test at the 95 % certainty level ($p < 0.05$). Principal Component Analysis (PCA) and partial least squares (PLS) regression were performed with Matlab version R2022b (Mathworks, Inc. Natick, Natick, MA, USA) using the SAISIR package [39] and Minitab version 17 (Minitab, LLC, PA, USA). Model performance was assessed using the determination coefficient (R^2_{adj}), and the root mean square error (RMSE).

3 Results and discussions

3.1. Reaction of genipin with amino acids in aqueous solution

Figure 1 shows photographs of the aqueous solutions of genipin with different amino acid mixtures after heating at 100 °C for 1 h. A dark blue color was developed when leucine, tryptophan, tyrosine, arginine, lysine, and glycine were used. However, this was not the case for phenylalanine, which exhibited a brownish-yellowish color, and for proline, with a pale-yellow hue. In the case of threonine, only a light blue color was developed at the highest genipin and amino acid concentration. pH increased after heating the mixture of all amino acids and genipin, except when phenylalanine, proline, threonine, and lysine were used (Figure 1). Table 1 shows the Pantone® identifier of the formed genipin-amino acid complexes aqueous solutions. More intense colors were developed at 250 mg/L genipin and 500 mg/L amino acid (genipin 1: amino acids 3 – 8 mM), and 500 mg/L genipin and 500 mg/L amino acid (genipin 1: amino acids 1 – 4 mM). These results are consistent with previous reports, in which amino acids have been added in excess [23-24].

It is well known that the characteristic blue pigment forms due to the reaction of genipin and primary amines at different pH values [20]. For instance, in acidic and neutral reaction media, the amino groups carry out a nucleophilic substitution on the olefinic carbon of genipin at C-3, causing the opening of the dihydropyran ring, which reacts with the secondary amino group on the formed aldehyde group. On the other hand, under basic reaction medium, the genipin ring-opening reaction is carried out by means of a nucleophilic substitution by hydroxyl ions in aqueous solution to give intermediate aldehyde groups, which undergo aldol condensation. Finally, the terminal aldehyde groups on the polymerized genipin are subjected to a Schiff reaction with the amino groups [15, 40-42].

Phenylalanine has developed blue colored polymers after cross-linking with genipin and heating at 70 °C for 5 h [23-24]. However, the amino acid concentration was up to 10 times higher than that of genipin. It is known that differences in color pigment can be observed depending on the type of the amino acid and the resulting pH [20, 23], and it has been demonstrated that genipin and amino acid concentration directly impacts the intensity of the formed blue colored complex [37].

The genipin and proline mixture did not develop the blue pigment after heating, since proline has a secondary amino group, that does not react with genipin [21], and the yellowish color of the solution is attributed to genipin auto-reaction, as seen in Figure 2 (a,b, c,d) [34,43]. It has been reported that proline has developed black shades with genipin [38]. However, the genipin used in this study comes from the genipap fruit juice (which contains some proteins) [34], and it was not in its pure form.

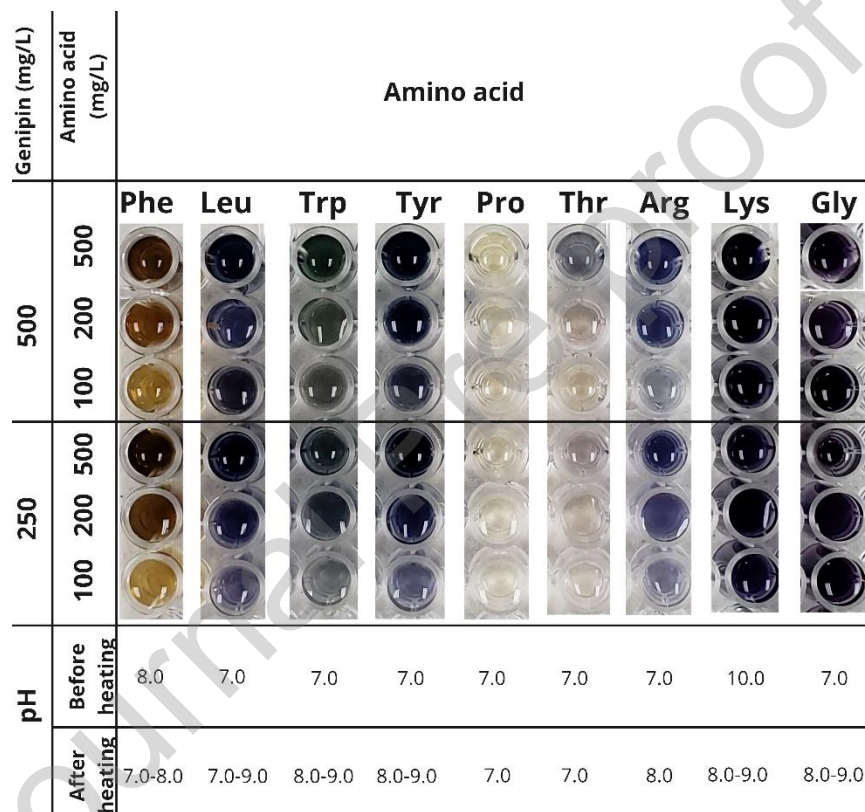


Figure 1. Photographs of genipin aqueous solutions after reaction with different amino acids.

Table 1. Pantone® identifiers of the formed genipin-amino acid complex aqueous solutions.

Genipin (mg/L)	Amino acid (mg/L)	Pantone®	Phe	Leu	Trp	Tyr	Pro	Thr	Arg	Lys	Gly
500	500	Pantone®	372516	0E131D	162420	0E1117	B7AA89	50515A	1C203E	090D10	020207
	200		59381B	27283E	2D342A	05090D	B4A78E	A6958D	181C39	090B0E	171422
	100		876C36	27232C	363634	131723	BEB39C	A79784	61646C	0B0C0E	090B0D
250	500	Pantone®	1F170F	08070D	101619	000102	AFA289	847576	0F1125	0F0F1A	111116
	200		402913	151323	161A24	0A0D1D	CCC4B1	B0A097	2B294A	0D0D15	181926
	100		866B39	594F67	4F5256	50516A	CBC4B3	C6BBB0	68677A	11101E	090A0F

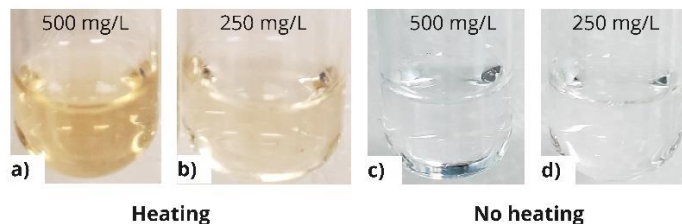


Figure 2. Genipin after heating at 100 °C for 1 h (a,b) and without heating (c,d).

3.2 Spectral analysis

After Principal Component Analysis of the UV-Vis spectra of the complexes formed in aqueous solutions of genipin and amino acids after reaction (Figure 3 a), all treatments included, the wavelengths around 590 - 610 nm were the main contributors on the principal component 1 (PC1). These wavelengths are consistent with absorption maxima of the pigments formed from genipin and amino acids, as shown in Figure 3 b. Moreover, these results are consistent with the visual inspection shown in Figure 1 and with Lee et al. [23], Lee et al. [45]; Paik et al. [24], Ramos-de-la-Peña et al. [34], and with Fujikawa et al. [25], who found a linear relationship between the amino acid molecular weight and the formed blue pigment λ_{\max} . Figure 3c shows FTIR spectrum of glycine and genipin standards, and of the formed precipitate after genipin and glycine reaction. Genipin standard exhibited a double band at 3169-3111 cm^{-1} which has been attributed to aromatic C-H and O-H vibration bands overlapping. Band at 2951 cm^{-1} is characteristic of C-H stretching vibration. On its part, the signal observed at 1680 cm^{-1} has been identified as stretching vibrations of carboxymethyl group C=O, and at 1620 cm^{-1} to C=C vibration of the olefin ring [46]. Glycine spectrum showed signals at 1418, 1333, 1125, 1033, and 916 cm^{-1} , which correspond to O-C=O symmetric stretching, C-H₂ wagging, N-H₃ rocking, C-N stretching, and CH₂ rocking, respectively [47]. On the other hand, formed pigment after genipin and glycine reaction showed a strong band at ~1628 cm^{-1} , attributed to C=O stretch in secondary amides, which resulted from the reaction of genipin ester and hydroxyl groups and amino groups [48].

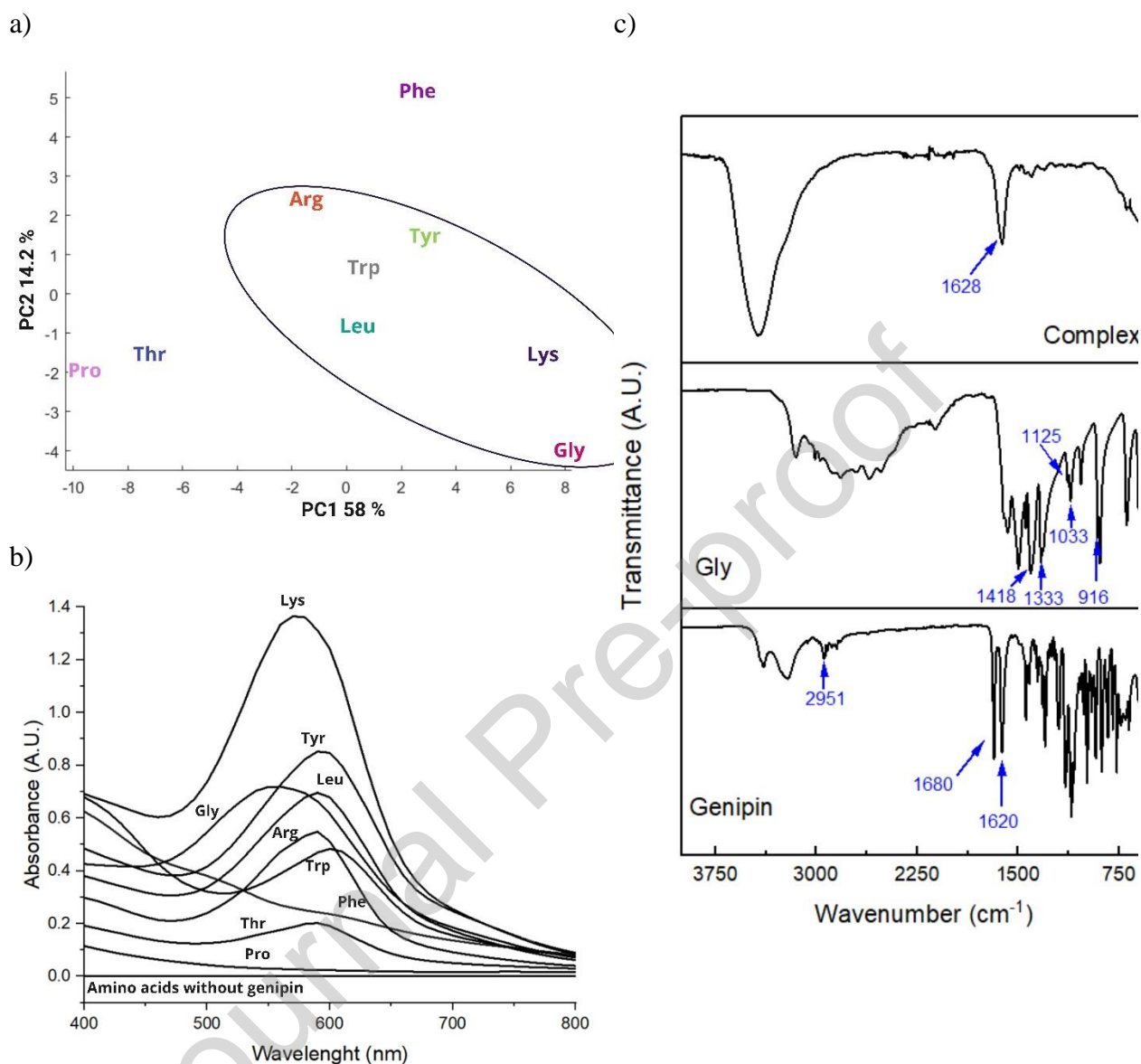


Figure 3. a) Principal Component Analysis of the UV/Vis spectra of all aqueous solutions after genipin-amino acid reaction between and 400 and 800 nm. b) UV/Vis spectra (dilution factor :3) and c) ATR-FTIR spectra of glycine and genipin standards, and formed complex after reaction.

3.3 Effect of amino acid and genipin concentration on electrophoretic mobility of genipin pigments

Figure 4 shows the effect of amino acid and genipin concentration on pigment electrophoretic mobility (a,b). Results for amino acids, water, and genipin without reaction (blanks) could not be obtained since their particle size was below the lower detection limit (≤ 3.8 nm) and because no

pigment was formed. At 250 mg/L genipin and 500 mg/L amino acid (genipin 1: amino acids 3–8 mM), the complexes which developed blue color exhibited a negative charge, except pigments formed between genipin and threonine, and glycine.

At all amino acid concentrations, no change in mobility values was observed for those complexes formed by genipin and arginine, and tyrosine. However, negative charges were observed at higher arginine concentration whereas positive charges at lower arginine concentration. On the other hand, mobility of pigments derived from genipin, leucine, and lysine exhibited a proportional behavior towards amino acid concentration.

At the highest genipin concentration level (500 mg/L) pigments from genipin and phenylalanine, tryptophan, arginine, glycine, and threonine (except at the highest threonine and glycine level) (genipin 1: amino acid 0.3 – 4 mM), mobility did not change as amino acid concentration increased. However, leucine and lysine (except at the highest lysine concentration level) showed a proportional behavior towards amino acid concentration.

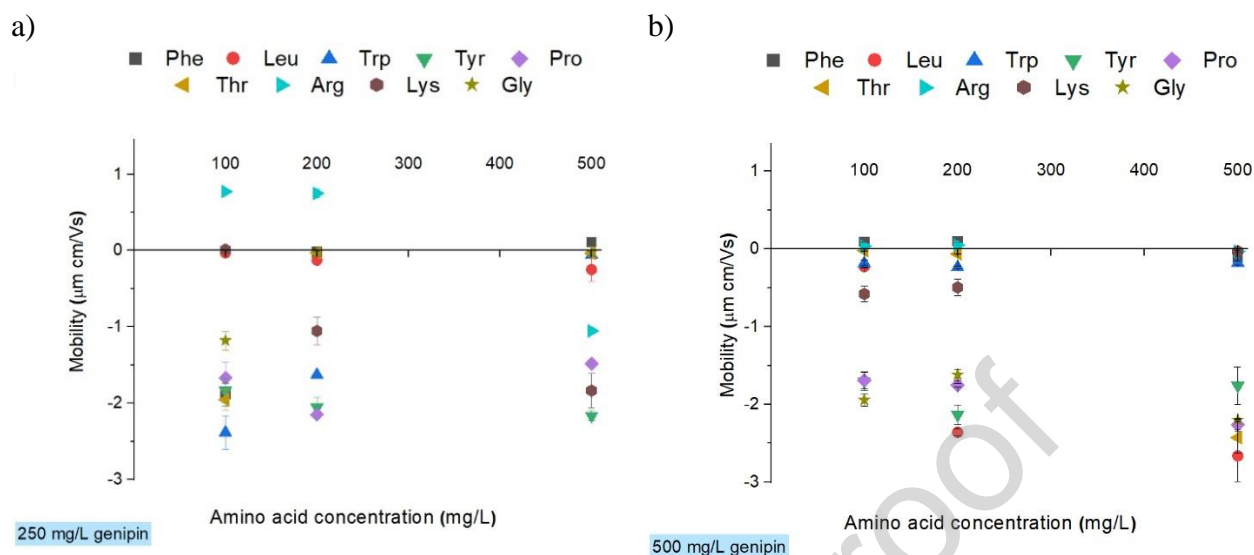


Figure 4. Effect of amino acid and genipin concentration on electrophoretic mobility (a,b) of aqueous solutions of pigments formed after reaction.

It is well known that charged solutes or particles suspended in an electrolyte are attracted towards the opposite charge electrode if an electric field is applied across a medium, and when equilibrium is reached between viscosity and movement, solutes move at constant velocity. Therefore, electrophoretic velocity is the migration distance divided by time, and electrophoretic mobility μ (m^2/Vs) is the observed electrophoretic velocity divided by an electric field strength [49].

It has been demonstrated that glycine, alanine, valine, leucine, and isoleucine have formed > 3 kDa blue pigments after their reaction with genipin, and that the pigments are substances with polymeric structures based on repeating units [26]. Since electrophoretic mobility determines how fast a particle moves under the influence of an electric field [50], complexes formed between genipin at 250 mg/L (genipin 1: amino acids 0.5 – 8 mM) and tryptophan, threonine, and glycine were slower in moving as amino acid concentration increased. Velocity did not change in complexes derived from genipin and arginine, and tyrosine (tyrosine was faster moving than arginine). However, pigments from genipin and leucine were faster in moving as amino acid concentration increased.

On their part, polymers from 500 mg/L genipin and phenylalanine, tryptophan, and arginine (genipin 1: amino acids 0.3 – 4 mM), were slow in moving, and this behavior was constant as amino acid ratio was raised. Genipin complex with glycine moved faster, and its behavior did not

change when amino acid concentration increased, except for the highest level of glycine. Threonine was slow in moving except at the highest threonine concentration (500 mg/L). Leucine was faster in moving as amino acid concentration increased, whereas lysine moved slower as amino acid concentration increased. The observed differences in genipin complex mobility could be attributed to their size, which is discussed in the next section.

3.4 Effect of amino acid and genipin on hydrodynamic size and polydispersity index (PDI) of pigments

Table 2 shows the effect of amino acid and genipin concentration on hydrodynamic size (a,b) of pigment aqueous solutions. All size values were > 0.3 nm and < 10 μ m, within measurement size range maximum of the instrument. However, values for amino acids, water, and genipin without reaction (blanks) could not be measured since their particle size was below the lower detection limit (≤ 0.3 nm) and because no pigment was formed.

At 500 mg/L genipin (genipin 1: amino acids 0.3 – 4 mM), the complex formed with phenylalanine and tryptophan (brown-yellow and green color, respectively) remained unchanged despite variations in amino acid concentration, from 48 to 57 nm and from 160-169 nm, respectively.

The pigment size of tyrosine, lysine, and glycine complexes increased proportionally with amino acid concentration (dark blue complexes, 339-2100 nm, 116-1690 nm, and 1073-2225 nm, respectively), whereas size of arginine pigment (light blue complexes) exhibited an inversely proportional behavior against amino acid concentration (4180-2324 nm). Moreover, size of leucine complexes decreased at 200 mg/L, and increased again at 500 mg/L of leucine.

At the lowest genipin level (250 mg/L) (genipin 1: amino acids 0.5 – 8 mM), complex size between genipin and phenylalanine and tryptophan showed low variation after increments in amino acid concentration (36-54 nm and 152-184 nm, respectively). The sizes of pigments derived from leucine, tyrosine, and glycine decreased at amino acid concentrations of 200 mg/L and increased again at concentrations of 500 mg/L. The arginine complex sizes increased as amino acid concentration raised, however it decreased at arginine concentrations of 500 mg/L. Pigment size of genipin and lysine increased as lysine concentration was raised (528-836nm).

As size increased, electromobility of genipin complexes for threonine and lysine decreased and *vice versa* at concentrations of 250 mg/L and 500 mg/L of genipin and amino acids, respectively. Touyama et al. [21] proposed a mechanism for the blue complex formation, consisting of degradation of the polymeric pigments and recombination of the degradation products. It is known that size and shape of particles in pigments influence their optical characteristics, such as hiding power, tinting strength, color, hue, brightness, and saturation [51]. All amino acids that developed blue color formed pigments below 1000 nm at different levels of genipin and amino acid concentration. Particle size < 1 μm enhances scattering power, opacity, and high gloss degree in pigments, which are desired characteristics for pigments, however, adhesion forces also are enhanced [51]. However, more research needs to be conducted to assess the effect of particle size on the optical characteristics of genipin pigments.

The effect of amino acid and genipin concentration on the formed polymer polydispersity index (PDI) in aqueous solutions is shown in Table 2. At 250 mg/L genipin concentrations the lowest PDI was exhibited by phenylalanine and tryptophan (brown-yellow, and light blue complexes; 0.17 and 0.04, respectively) indicating uniform complexes in terms of particle size [52], whereas the highest PDI was shown by lysine, arginine, leucine, and tyrosine (dark blue complexes; 0.93, 0.84, 0.87, and 0.72, respectively), suggesting a high polydisperse complex with multiple size populations [53]. These values correspond to the highest particle size of polymers obtained for each amino acid, however, lower PDI values can be found in smaller size pigments, which is desired since a narrow particle size distribution enhances a uniform surface appearance.

At 500 mg/L genipin concentrations, results were like those obtained for the lowest genipin concentration level. Tryptophan had the lowest PDI values (green complexes; 0.04), whereas glycine, arginine, leucine, and lysine presented the highest (dark blue pigments; 1.0, 1.0, 0.84, and 0.83, respectively) at the highest particle size. These results suggest that dark blue pigments with the highest size are heterogeneous in polymer size distribution, whereas green, brown-yellow, dark blue, and light blue pigments with the lowest particle size are composed of more homogeneous complex populations.

Table 2. Effect of amino acid and genipin concentration on size (a,b), and polydispersity index (PDI) (c,d) of the obtained pigment aqueous solutions.

		Genipin concentration (mg/L)							
		500		250		500		250	
		Size (nm)				PDI (A.U.)			
Amino acid	mg/L	±		±		±		±	
Phe	100	48.19	0.34	35.92	0.71	0.30	0.02	0.23	0.06
	200	43.52	0.84	45.14	0.55	0.19	0.04	0.18	0.01
	500	57.42	0.98	53.87	1.69	0.16	0.03	0.17	0.04
Leu	100	1458.50	139.30	1535.00	261.63	0.84	0.18	0.14	0.02
	200	188.80	16.56	968.40	56.00	0.68	0.10	0.67	0.03
	500	477.70	66.89	2609.00	39.60	0.81	0.01	0.87	0.04
Trp	100	169.30	2.86	151.80	1.40	0.08	0.02	0.07	0.01
	200	168.63	1.70	183.60	1.32	0.08	0.02	0.08	0.01
	500	159.87	1.44	164.17	1.93	0.04	0.02	0.04	0.01
Tyr	100	338.90	14.78	703.60	30.41	0.30	0.06	0.18	0.05
	200	552.70	8.06	433.05	12.94	0.55	0.08	0.72	0.09
	500	2100.00	162.35	1975.50	241.12	0.43	0.04	0.62	0.02
Pro	100	42.77	0.54	55.86	17.41	0.10	0.01	0.40	0.00
	200	48.05	0.93	55.21	0.69	0.16	0.02	0.29	0.02
	500	66.33	1.10	53.22	2.28	0.28	0.01	0.26	0.15
Thr	100	95.36	0.05	2065.50	31.82	0.00	0.00	0.70	0.09
	200	207.85	115.19	0.00	0.00	0.27	0.10	0.00	0.00
	500	0.00	0.00	1489.50	67.18	0.06	0.01	0.58	0.26
Arg	100	4179.50	123.74	2697.50	229.81	0.78	0.32	0.47	0.06
	200	2467.50	560.74	3908.00	26.87	0.48	0.23	0.77	0.32
	500	2324.00	1646.14	1323.00	84.85	1.00	0.00	0.84	0.02
Lys	100	ND	ND	528.70	1.84	ND	ND	0.93	0.10
	200	116.12	27.27	836.10	2.40	0.83	0.15	0.47	0.03
	500	1690.00	104.06	39.04	16.34	0.23	0.03	0.53	0.01
Gly	100	ND	ND	609.60	115.97	ND	ND	0.19	0.12
	200	1073.00	15.56	166.65	7.99	0.65	0.10	0.22	0.02
	500	2224.67	0.11	4024.00	230.52	1.00	0.00	0.51	0.04

*ND: Not detected by the instrument.

3.5 Effect of amino acid and genipin on conductivity of pigments

Figure 5 (a,b) shows the effect of amino acid and genipin concentration on complex conductivity. An increase in conductivity was observed as amino acid and genipin concentration increased in phenylalanine reactions. For leucine, conductivity increased as amino acid concentration was increased at 250 mg/L genipin concentration. However, at the highest genipin level, when amino acid concentration was increased, conductivity decreased. Glycine exhibited

its highest conductivity value at 500 mg/L of genipin and 200 mg/L of amino acid (genipin 1: glycine 1.5 mM).

It was demonstrated that complexes formed between genipin and phenylalanine are electrically conductive between 250 and 500 mg/L of genipin and from 100 to 500 mg/L amino acid (genipin 1: amino acids 3 – 8 and genipin 1: amino acids 1 – 4 mM). This finding suggests that the conductive pigment derived from phenylalanine and genipin could be used for antistatic coatings with applications in electronic equipment, floor coverings, explosion-protected area surface finishings or primers for plastic painting [54].

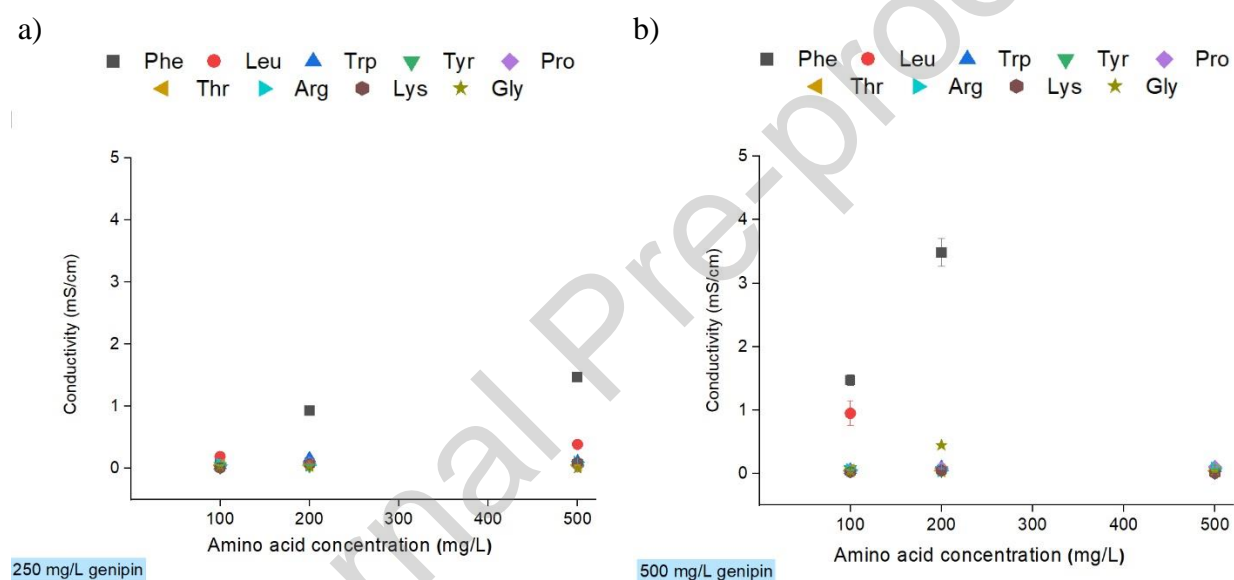


Figure 5. Effect of amino acid and genipin concentration on conductivity of pigments after reaction.

3.6 Multivariate analyses

The box plots of mobility, size, PDI, and conductivity are shown in Figure 6. The most intensive variations were observed in PDI, followed by mobility, and hydrodynamic size, indicating a large heterogeneity, and the lowest variations were exhibited by conductivity, showing a limited heterogeneity.

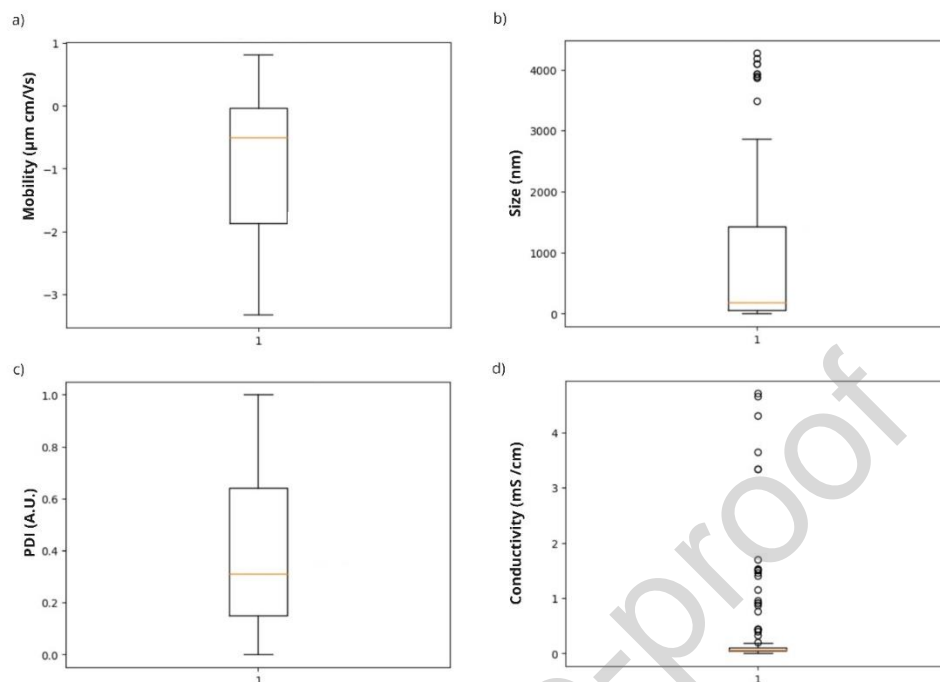


Figure 6. The boxplots of a) electrophoretic mobility, b) hydrodynamic size, c) PDI, and d) conductivity of pigments formed between genipin and amino acids.

3.6.1 Principal component analysis (PCA)

The first two principal components represented 68.4 % of the total variability (Figure 8 a,b). For all amino acids, a clear discrimination was shown along principal components 1 and 2 (PC1, PC2) The most contributing factors of PC1 and PC2 were size and conductivity, respectively. The first PC1 (attributed to size) discriminated complexes between genipin and arginine (dark blue color) on the right, and proline, threonine, and phenylalanine on the left (no blue color), and accounted 36.5 % of the total variability. Complexes with glycine, leucine, lysine, and tyrosine overlapped among them (dark blue color). On the other hand, PC2 (attributed to conductivity) discriminated pigments formed between phenylalanine from the rest of pigments.

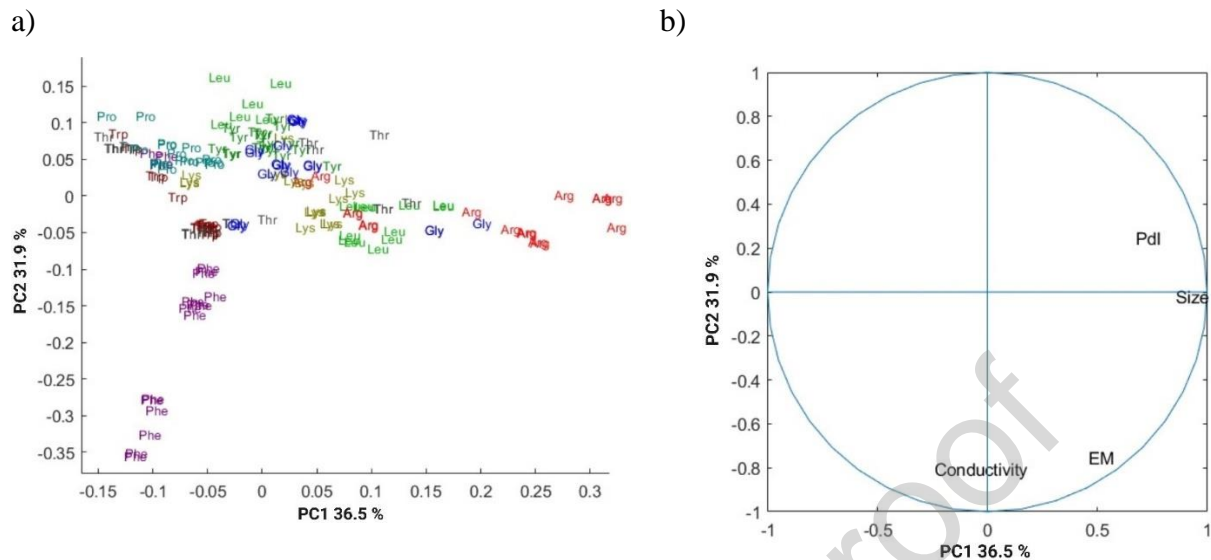


Figure 7. Principal Component Analysis (PCA) (a) and correlation plot performed on data acquired for genipin-amino acid pigments (b).

Figure 8 shows a schematic illustration with the physicochemical characteristics of the analyzed amino acids, and the resulting color of the formed pigment.

Amino acid	Molecular weight (g/mol)	R-group	Polarity	Isoelectric point (25°C)	+ 250 and 500 mg/L Genipin	Pigment	Hydrodynamic diameter (nm)
Threonine	101.11	hydroxyl-	polar	5.60	→		→ 208-2065
Tryptophan	186.21	aromatic	non-polar	5.88			
Phenylalanine	147.18	aromatic	non-polar	5.76			
Glycine	57.05	aliphatic	non-polar	6.06	→		→ 167 - 4024
Leucine	113.16	branched aliphatic	non-polar	6.04			
Tyrosine	163.18	aromatic	non-polar	5.63			
Lysine	128.17	basic	polar	9.47	→		→ 116 - 1690
Arginine	156.19	basic	polar	10.76			

Figure 8. Chart summarizing physicochemical characteristics of amino acids, and visual perception of the pigment. *Physicochemical characteristics were obtained from literature [55-57].

The darkest blue complexes showed highest hydrodynamic size. Complexes between genipin and tryptophan and phenylalanine did not develop blue color, and their size was ~23 times lower than those which developed dark blue color. These results are consistent with Cho et al. [58], in which glycine, tyrosine, and alanine were selected as the best reactants to produce blue pigments.

3.6.2 Modeling electrophoretic mobility, hydrodynamic size, PDI, and conductivity of pigments

The ability of DLS data coupled with PLS to predict mobility, hydrodynamic size, PDI, and conductivity of complexes formed between genipin and different amino acids was demonstrated. A good prediction was observed only for size ($R^2_{\text{adj}}=0.88$; $\text{RMSE}=0.94$), exhibiting a value of $R^2 > 0.8$ which indicates that the model accounts for over 80% of the variance [59]. The full-cross validation is shown in Figure 9. The results of model evaluation show that it is suitable for the hydrodynamic size prediction of exclusively blue pigments derived from genipin and amino acids [60-61].

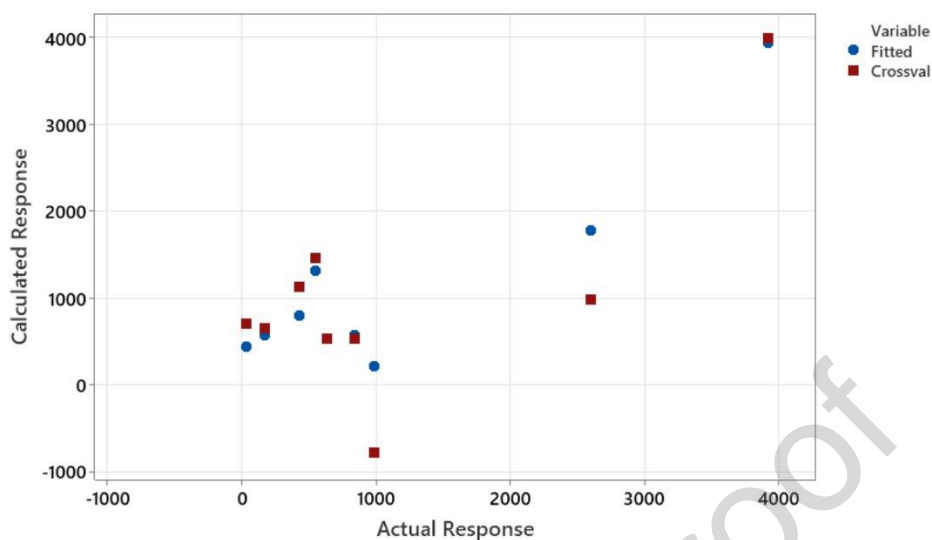


Figure 9. Full-cross validation of hydrodynamic size of resulting pigments after the reaction of genipin and different amino acids.

4. Conclusions

Electrophoretic mobility and DLS coupled with multivariate analysis are useful tools for the accurate assessment of the physical characteristics of pigments formed after genipin reaction with different amino acids. Regardless of the amino acids nature (basic or neutral), the formed blue pigments had a negative charge at the best reaction conditions (250 mg/L genipin and 500 mg/L amino acid (genipin 1: amino acids 3– 8 mM) and 500 mg/L genipin and 500 mg/L amino acid (genipin 1: amino acids 1 – 4 mM). The darkest blue complexes showed the highest hydrodynamic size (1073-4179 nm). Pigments with size below 1 μm were obtained for all amino acids at different concentrations of genipin and amino acid, except for arginine. PLS allowed to build a model which was able to describe the distribution of the hydrodynamic size in blue pigments formed between genipin and amino acid excess (mM). Such insights have promising applications in quality control of pigments derived from genipin, including optical and bulk properties. Moreover, electrophoretic mobility and conductivity of complexes can be applied to better design different micro, and nanoscale systems in which genipin acts as a cross-linker, with future perspectives in food and pharmaceutical industries.

CRedit authorship contribution statement

Ana Mayela Ramos-de-la-Peña: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing – review & editing, Visualization. Alfredo Santana-Reynoso: Visualization, Formal analysis, Data curation, Software, Resources. Catherine M.G.C. Renard: Methodology, Investigation, Validation, Writing - review & editing. Juan Carlos Contreras-Esquivel: Visualization, Formal analysis, Writing - review & editing. José Rodríguez-Rodríguez: Formal analysis, Resources, Methodology. Oscar Aguilar: Formal analysis. José González-Valdez: Visualization, Writing - review & editing, Funding acquisition. Cuauhtémoc Licon-Cassani: Formal analysis, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that there is not conflict of interest regarding the publication of this article.

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