Determination of Peptide Profile Consistency and Safety of Collagen Hydrolysates as Quality Attributes

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Abstract: Collagen hydrolysates are dietary supplements used for nutritional and medical purposes. They are complex mixtures of low-molecular-weight peptides obtained from the enzymatic hydrolysis of collagen, which provide intrinsic batch-to-batch heterogeneity. In consequence, the quality of these products, which is related to the reproducibility of their mass distribution pattern, should be addressed. Here, we propose an analytical approach to determine the peptide pattern as a quality attribute of Colagenart®, a product containing collagen hydrolysate. In addition, we evaluated the safety by measuring the viability of two cell lines exposed to the product. The consistency of peptide distribution was determined using Size Exclusion Chromatography (SEC), Mass Spectrometry coupled to a reversed phase UPLC system (MS-RP-UPLC), and Shaped-pulse off-resonance water-presaturation proton nuclear magnetic resonance spectrometry [^{1}Hwater_presat NMR]. The mass distribution pattern determined by SEC was in a range from 1.35 to 17 kDa, and from 2 to 14 kDa by MS-RP-UPLC. [^{1}Hwater_presat NMR] showed the detailed spin-systems of the collagen hydrolysates components by global assignment of backbone Hα and NH, as well as side-chain proton resonances. Additionally, short-range intraresidue connectivity pathways of identified spin-regions were obtained by a 2D homonuclear shift correlation Shaped-pulse solvent suppression COSY scheme. Safety analysis of Colagenart® was evaluated in CaCo-2 and HepG2 cells at 2.5 and 25 μg/mL and no negative effects were observed. The results demonstrated batch-to-batch reproducibility, which evinces the utility of this approach to establish the consistency of the quality attributes of collagen hydrolysates.

Keywords: collagen hydrolysates, ion mobility mass spectrometry, nuclear magnetic resonance, quality control, size exclusion chromatography

Practical Application: We propose state-of-the art analytical methodologies (SEC, MS, and NMR) to evaluate peptide profile and composition of collagen hydrolysates as quality attributes. These methodologies are suitable to be implemented for quality control purposes.

Introduction

According to health regulatory agencies, dietary supplements are oral products containing dietary ingredients (vitamins, minerals, herbs, amino acids, and/or enzymes) intended to provide nutritional benefits or physiological effects to the consumer (EFSA, 2018; FDA, 2018a). The U.S. Food and Drug Administration refers that these products are commonly used to increase, complement or supplement nutrients that are not provided by the regular food intake and cannot be used for the treatment of diseases (FDA, 2018b). However, they may contain ingredients that contribute to preserve health and even to enhance the effect of some therapeutic products in specific health disorders (Rawson, Miles, & Larson-Meyer, 2018). In this regard, various health specialists have reported that dietary supplements containing ingredients such as chondroitin, glucosamine, vitamin D3, hyaluronic acid, Omega-3, resveratrol, and polyphenols represent a viable alternative in helping to preserve people's health (Das & Das, 2007; do Carmo, Girotto, Marques, Granato, & Azevedo, 2018; Jerosch, 2011).

Different surveys indicate that three-quarters of the adults in the U.S. not only use dietary supplements to achieve a healthy life style (Dickinson & MacKay, 2014), but also to prevent different chronic and degenerative diseases before using medications (Mehta, Gardiner, Phillips, & McCarthy, 2008). Joint pain is one of the most common conditions that chronically affect the life quality of worldwide population, mainly elder people. This condition might be associated to a variety of primary causes such as sprains and strains resulting from physical activity, or due to degenerative diseases such as osteoarthritis and rheumatoid disorders (Grime, Richardson, & Ong, 2010; Paisley & Serpell, 2016).

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The treatment of joint disorders includes the use of steroidal and nonsteroidal anti-inflammatory drugs, analgesics, and narcotics. Although, these drugs reduce pain and inflammation at different levels while they are used, the long-term efficacy is compromised by side effects (for example, Cushing syndrome, gastritis, addiction) (Decani, Federighi, Baruzzi, Sardella, & Lodi, 2014; Kosten & George, 2002; Quinn, Bjarnason, & Price, 1993). In this sense, it has been reported that various dietary supplements represent a viable alternative to preserve joint health (Das & Das, 2007; Jerosch, 2011).

Collagen hydrolysates, also called peptide collagen or hydrolyzed collagen, are widely used in the nutraceutical and cosmetic industries due to their intrinsic properties and benefits for health. Although collagen hydrolysates are regarded as dietary supplement, these products have evinced healing properties in joint-related diseases, arthritis, and osteoarthritis (Bello & Oesser, 2006; Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011; Kumar, Sugihara, Suzuki, Inoue, & Venkateswarathirukumara, 2015; Moskowitz, 2000). Several brands and formulas with different quality and at different prices can be purchased. The global market of collagen hydrolysates was valuated at USD 3.71 billion in 2016 and it is expected to reach USD 6.63 billion by 2025 (Avila Rodriguez, Rodriguez Barroso, & Sanchez, 2018). Since the use of these products is intended to obtain health benefits, their quality must be guaranteed (Sarma, Giancaspro, & Venema, 2016).

Collagen hydrolysates are a low-molecular-weight (LMW) peptide mixture obtained from the enzymatic hydrolysis of collagen (Mohammad, Norhazwani, Abdul, & Jamaliah, 2014). Collagen is an insoluble-in-water high-molecular-weight protein (Shoulders & Raines, 2009). Conversely, the mixture of peptides derived from the digestion of collagen are highly soluble in water, and active peptides can be detected in blood stream and skin a few minutes after oral administration (Osawa et al., 2018; Yamamoto, Deguchi, Onuma, Numata, & Sakai, 2016; Yazaki et al., 2017). The manufacturing process of collagen hydrolysate requires two main steps: first, the soluble fraction is obtained by chemical (acidic or basic) or enzymatic hydrolysis, then LMW peptides are isolated by filtration or ultrafiltration procedures (Mohammad et al., 2014).

Two main quality attributes for collagen hydrolysates have been defined: peptide profile and safety. Peptide profile (peptide polydispersity) and peptide size evince consistency of the manufacturing process and could predict oral absorption (Watanabe-Kamiyama et al., 2010; Yazaki et al., 2017). In consequence, it is necessary to count on the most appropriate analytical techniques to assess this quality attribute. Size exclusion chromatography (SEC) is a well-known technique widely used to determine the polydispersity of collagen hydrolysates as a quality attribute (Khiari, Ndagijimana, & Betti, 2014). Notwithstanding, state-of-the-art techniques, such as mass spectrometry (MS) and nuclear magnetic resonance (NMR), are more sensitive and highly proficient methodologies to determine composition and identity of the components present in complex mixtures (Finehout & Lee, 2004; Wishart, 2005). However, to the best of our knowledge, they have not been fully explored for the analysis of collagen hydrolysates. On the other hand, safety ensures human consumption of dietary supplements (Dwyer, Coates, & Smith, 2018). Despite products containing collagen hydrolysates have been used for many years without reporting severe adverse events, it is worth to notice that different formulations of collagen hydrolysates are marketed and they could exhibit a dissimilar impact on safety (Kroes et al., 2007; Kruger & Mann, 2003). In this sense, several routine analyses used to evaluate safety in the pharmaceutical industry, such as viability test of cells exposed to the product and their formulation, can be extrapolated to assess dietary supplements (Angelis & Turco, 2011; Miret, De Groene, & Klaffke, 2006).

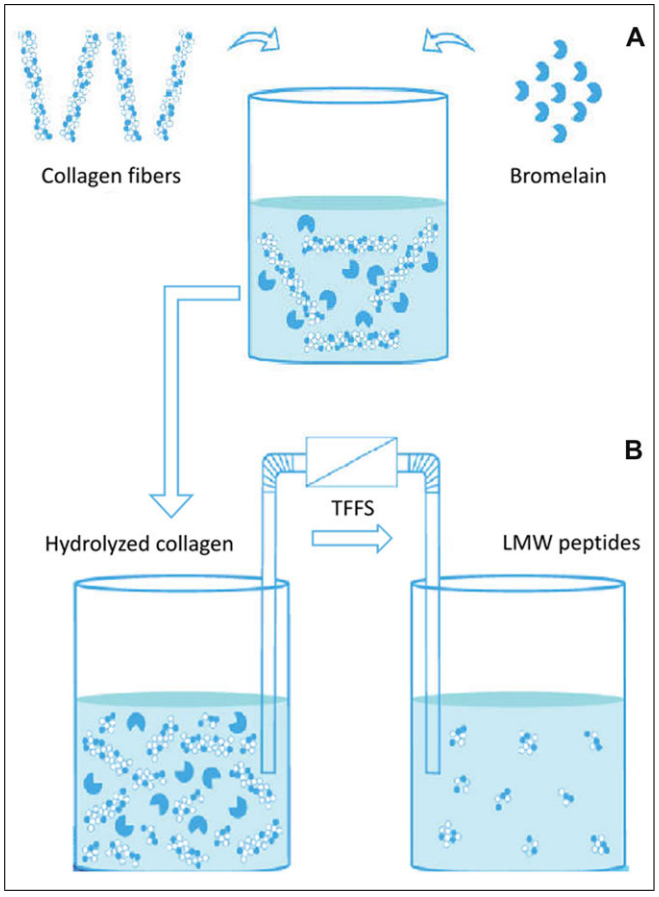

Figure 1–Scheme of the manufacturing process of Colagenart®. (A) Obtaining of soluble fractions from collagen fibers by enzymatic hydrolysis with bromelain. (B) Separation of low molecular weight (LMW) collagen peptides trough a 17 kDa tangential flow filtration system (TFFS).

Considering that techniques such as SEC, MS, and NMR have been successfully used to characterize complex mixtures of peptides (for example, glatiramer acetate) (Anderson et al., 2015; Rogstad et al., 2015), we determined the peptide profile of Colagenart®, a product containing collagen hydrolysate (Figure 1), and its consistency among batches by SEC with ultraviolet detection (SEC-UV, hereinafter referred only as SEC), reverse phase chromatography-coupled to electrospray ionization-ion mobility-quadrupole time of flight spectrometer (RP-HPLC-ESI-IMS-QTOF, hereinafter referred as MS), and shaped-pulse water-suppression one (1D)- and two-dimensional (2D) proton NMR spectra (hereinafter referred as NMR). In addition, safety was assessed *in vitro* using CaCo-2 and HepG2 cell lines, which are routinely used in the pharmaceutical industry to test drugs toxicity.

Materials and Methods

Analytical samples and reagents

Three batches of Colagenart® (HC018-01, HC018-02, and HC018-03) were provided by LEMAR S.A.P.I. de C.V. (Mexico City, Mexico). Sodium chloride, and monobasic, dibasic sodium phosphate, and sodium 3-(trimethylsilyl) tetradeuteriopropionate

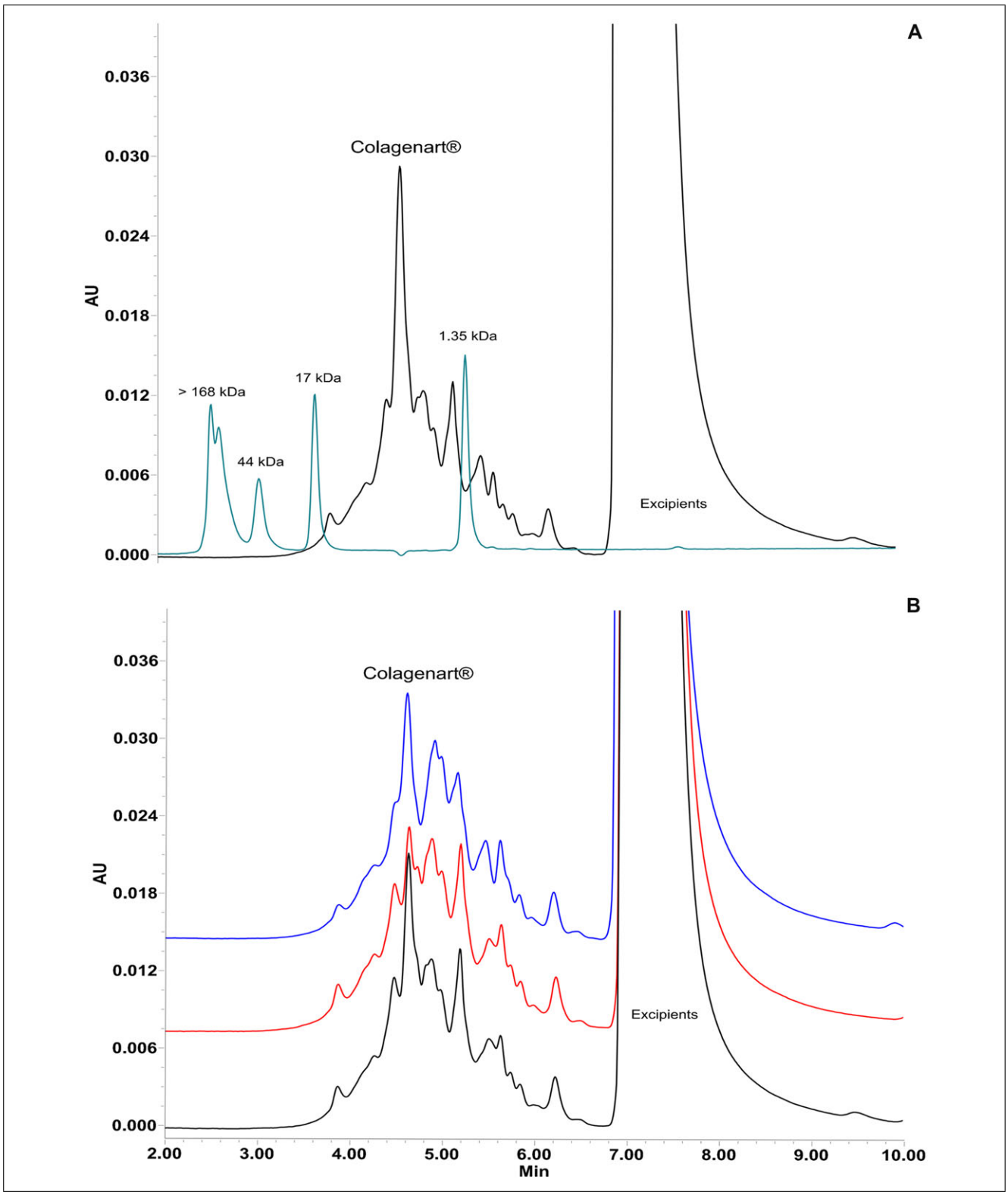

Figure 2–Peptide polydispertion of Colagenart® by size exclusion chromatography. (A) Molecular weight of Colagenart® peptides (black) in a range from 1.35 to 17 kDa according to the molecular weight standards (cyan) thyroglobulin/gamma-globulin (>80 kDa, RT 2.60 min), ovalbumin (44.2 kDa, RT 3.10 min), equine myoglobin (17.0 kDa, RT 3.75 min), and B12 vitamin (1.35 kDa, RT 5.38 min). (B) Consistency of size distribution profiles among three batches of Colagenart® (HC018-01 Black, HC018-02 Red, and HC018-03 Blue).

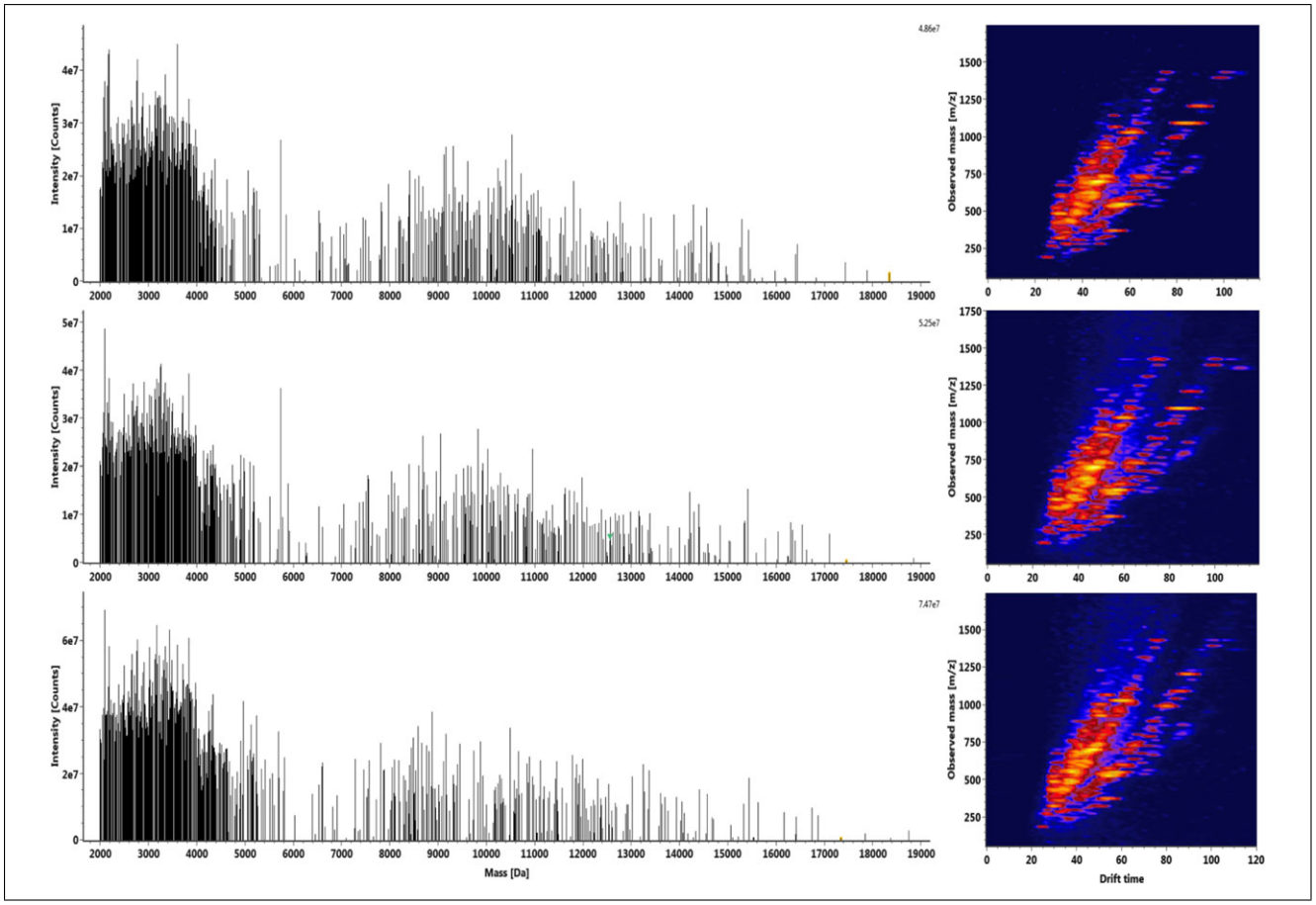

Figure 3–Results from MS analyses of three batches of Colagenart® (from top to the bottom HC018-03, HC018-02, and HC018-01). Exact mass analysis (left) show the peptide distribution according to their *m/z*. Striated patterns of heat maps obtained from ion mobility (right) correspond to the various peptides from the enzymatic hydrolysis the most intense peaks are shown in red and yellow. Consistency of peptide composition among batches is observed.

(TSP) (analytical grade) were acquired from J. T. Baker; (NY, USA). Water, formic acid, and acetonitrile (MS grade) and dimethyl sulfoxide (DMSO) (analytical grade) were acquired from Sigma-Aldrich (MO, USA). Note that 99.98% deuterated water solution was acquired from Cambridge Isotope Laboratories, Inc. (MA, USA). HepG2 cell line (ATCC® HB-8065; VA, USA) and CaCo-2 cell line (ATCC® HTB-37) were acquired from ATCC (VA, USA). Fetal bovine serum and DMEM medium were obtained from Thermo Fisher Scientific (MA, USA). CellTiter 96® AQ_{ueous} reagent was acquired from Promega (WI, USA).

SEC

The size exclusion analysis was performed using ultraperformance liquid chromatography (SE-UPLC) as previously described for the characterization of LMW dialyzable leucocyte extracts (Medina-Rivero et al., 2014) Briefly, 10 μL of collagen hydrolysate (1 mg/mL) were directly injected into an Acquity UPLC Class-H system (Waters; MA, USA) using a Waters® BEH 125 SEC column (1.7 μm, 4.6 × 150 mm) at 30 °C and phosphate buffer solution (50 mM monobasic/dibasic sodium phosphate + 150 mM NaCl, pH 6.8) as mobile phase. The data were acquired at 280 nm and processed using the software Empower® (Waters®).

MS

Intact mass of Colagenart® was determined on a Vion® ESI-IMS-Q-TOF spectrometer (Waters®) coupled to an Acquity® UPLC Class-I system. Note that 2 μL of hydrolyzed collagen (1 mg/mL) were directly injected into an Acquity® UPLC CSH C18 Column (1.7 μm, 2.1 × 150 mm) (Waters) with a mobile phase (water + 0.1% formic acid / acetonitrile + 0.1% formic acid) at a flow rate of 0.2 mL/min. An elution gradient was performed as follows: 0% of organic solution from 0 to 2 min; 0% to 25% of organic solution from 2 to 90 min; and 25% to 0% of organic solution from 90 to 100 min. The analyses were performed in positive/resolution mode, which was tuned to determine the optimal acquisition parameters: 2.50 kV capillary voltage, 110 °C source temperature, 500 °C desolvation temperature, 0 L/h cone gas flow, and 600 desolvation gas flow L/hr. The data were acquired in the range 50 m/z to 2000 m/z using the ion mobility spectrometry (IMS) mode, and processed using the software UNIFI™ (Waters®).

NMR

Each NMR sample was prepared by dissolving the peptide in 500 μL phosphate buffer (Na_2HPO_4/NaH_2PO_4) at pH 5.8 with a 9:1 H_2O:D_2O ratio. The pH 5.8 Sørensen phosphate buffer was prepared with 92 mL of 0.2 M NaH_2PO_4 solution and 8 mL of 0.2 M Na_2HPO_4 solution using deuterated water (D_2O) as solvent for both cases (Meissner & OW, 2000). Solution pH values were checked using a pH electrode (Spintrode, Hamilton, Bonaduz, Switzerland) direct into the NMR tube. The spectra were internally referenced at 0 ppm to the singlet resonance of

1.0 mM TSP. The spectra were recorded on a Bruker 600 AVANCE III (Bruker BioSpin, DE, USA) equipped with a 5 mm 1H/D TXI probehead with z-gradient. All the spectra were recorded using a 1.6 mM hydrolyzed collagen sample at 300 K. The following set of NMR experiments were conducted: [$^1H_{water\_presat}$ NMR] 1D single pulse NOESY experiment with a home-made excitation sculpting shaped-pulse water presaturation during both, relaxation delay (2 s) and mixing time (100 ms), with a 0.000115 W power irradiation level and 2D homonuclear shift correlation shaped-pulse solvent suppression (0.000115 W power level irradiation during evolution t_1 period) COrrelation SpectroscopY [$COSY_{water\_presat}$ NMR], with homospoil gradient recovery. For 1D experiments, 64 transients (16 dummy scans) were collected into 24 K data points over a 20 ppm spectral width, with acquisition times of 1 s, producing experimental times of 7 min per sample. No apodization function was used during Fourier transformation (FT) and data were zero-filled to 24 K data points prior to FT. In turn, 2D-$COSY_{water\_presat}$ NMR experiments were carried out with 512 points in the indirect dimension, 16 transients per F1 point, 16 dummy scans, 2 s of relaxation delay, and 1.2 s of acquisition time, giving though experimental times of 9 hr per batch. Double Fourier transform was carried out in the absolute mode in the indirect dimension, with a sine bell apodization function for both F1 and F2 dimensions and a zero filling of 2 K in both F1 and F2.

In vitro assays

Safety of Colagenart® was assessed through *in vitro* assays using CaCo2 and HepG2 cells. The cells (6×10^3 HepG2 cells/well and 2.5×10^4 CaCo-2 cells/well) were placed in 96-well plates using DMEM medium supplemented with fetal bovine serum (10% and 20%, respectively). Both cell cultures were stimulated with Colagenart® at 2.5 and 25 μg/mL, which were the higher concentrations that do not compromise the properties of the culture medium, such as osmolarity; Colagenart® dilutions were prepared in culture media. The following controls were prepared in parallel: cells treated with mirror dilutions of vehicle, cells treated with 20% DMSO (added 20 min before evaluating survival) as positive control of cellular death, and untreated cells as negative control of cellular death. The cells were incubated at 37 °C under a 5% CO_2 atmosphere. Survival was evaluated by the MTS assay at 0, 24, 48, and 72 hr of incubation. In brief, 20 μL of CellTiter 96® AQ_{ueous} reagent were added to each sample and were incubated at 37 °C and 5% CO_2 during 2 hr. Optical density (OD) of the samples were determined in an Epoch® spectrophotometer (BioTek®; VT, USA) at 490 nm using the GEN5® software (BioTek®). Cell survival per sample was determined using the following equation:

$$Cell\ survival\ = \left[\frac{OD\ treated\ cells}{OD\ untreated\ cells}\right] \text{x } 100$$

Data were analyzed using the Prism software (v.6.0 for Windows, GraphPad, La Jolla, CA, USA). A Browne-Forsythe test was used to test homogeneity of variances, followed by a one-way analysis of variance (ANOVA) with a Dunnett's post hoc to compare differences between means. Statistical significance was set at 0.05. The results are presented as means ± standard deviation (SD).

Results and Discussion

SEC

SEC, or gel filtration chromatography coupled to HPLC, has been widely used to determine the peptide populations present in collagen hydrolysates (Osawa et al., 2018; Yamamoto et al., 2016; Yazaki et al., 2017). Here, we performed a size exclusion analysis using an UPLC system, which improved the sensitivity and resolution of the analysis owing to (i) the lower diffusion index of the sample components within the chromatographic system and (ii) a higher performance of small-particle-size chromatographic columns (<3 μm). This is relevant when analyzing complex samples, because UPLC is capable to describe complex peptide mixtures in a more detailed fashion with respect to HPLC.

The proposed nondenaturing SEC conditions allowed obtaining the characteristic size peptide profile (polydispersion) of Colagenart®, which ranges from 1.35 to 17 kDa (Figure 2A) and corresponds to the theoretical value (≤17 kDa). In addition, as previously explained, UPLC analysis resolved particular peptide populations at 3.9, 4.6, 5.2, 5.5, and 6.2 min. A detailed SEC profile allows identifying slight differences, and their acceptable variation range among batches as evidence of manufacturing process consistency. In this sense, the peptide patterns among Colagenart® batches were similar, indicating that solubilization and enzymatic hydrolysis are controlled during the manufacture process (Figure 2B). Consistency of this step is essential for the product quality, in particular because it allows the enrichment of the peptide mixture with Gly-Pro-Hyp tripeptide in the final formulation, which increases the absorption in plasma after oral ingestion as previously reported (Yazaki et al., 2017).

MS

Different MS techniques have been employed to quantitate collagen-derived peptides in *ex vivo* samples and to characterize the peptide pattern of collagen hydrolysates products; this latter using MALDI-TOF spectrometric systems. Nevertheless, MALDI-TOF analyses have determined a low peptide complexity of collagen hydrolysates than the observed by chromatographic methods using spectrophotometric detectors (Chen et al., 2017; Schadow et al., 2013). Our ESI-IMS-Q-TOF analysis, which was designed to determine the mass distribution of Colagenart®, evinced a higher resolution index and sensitivity than previously reported by MALDI-TOF (Figure 3). Exact mass analysis of three Colagenart® batches exhibited a polydispersion ranging from 2 to 14 kDa (Figure 3), which correspond to that obtained by SEC. In addition, MS Colagenart® profile exhibits two peptide populations: from 2 to 5 kDa (the most abundant) and from 7 to 14 kDa. These results are valuable, because it has been reported that high amounts of collagen hydrolysates enriched in 3 kDa fractions reach quantitative concentrations in skin after oral administration (Yazaki et al., 2017). Thus, it is expected that Colagenart® peptides are absorbed and distributed to exert a biological activity after being orally administered.

Three criteria were established to determine the similarity between the intact mass profiles of Colagenart® batches: range of intact mass, signal intensity, and sequence coverage. As observed in Figure 3, the intact mass profile of the Colagenart® batches exhibited two well-defined peptide populations with a signal greater than $3e^7$ for LMW population (ranging from 2000 to 5000 Da) and greater than $2e^7$ for the high-molecular-weight population (ranging from 7000 to 14000 Da). On the other hand, the peptide sequencing of the three analyzed batches allowed

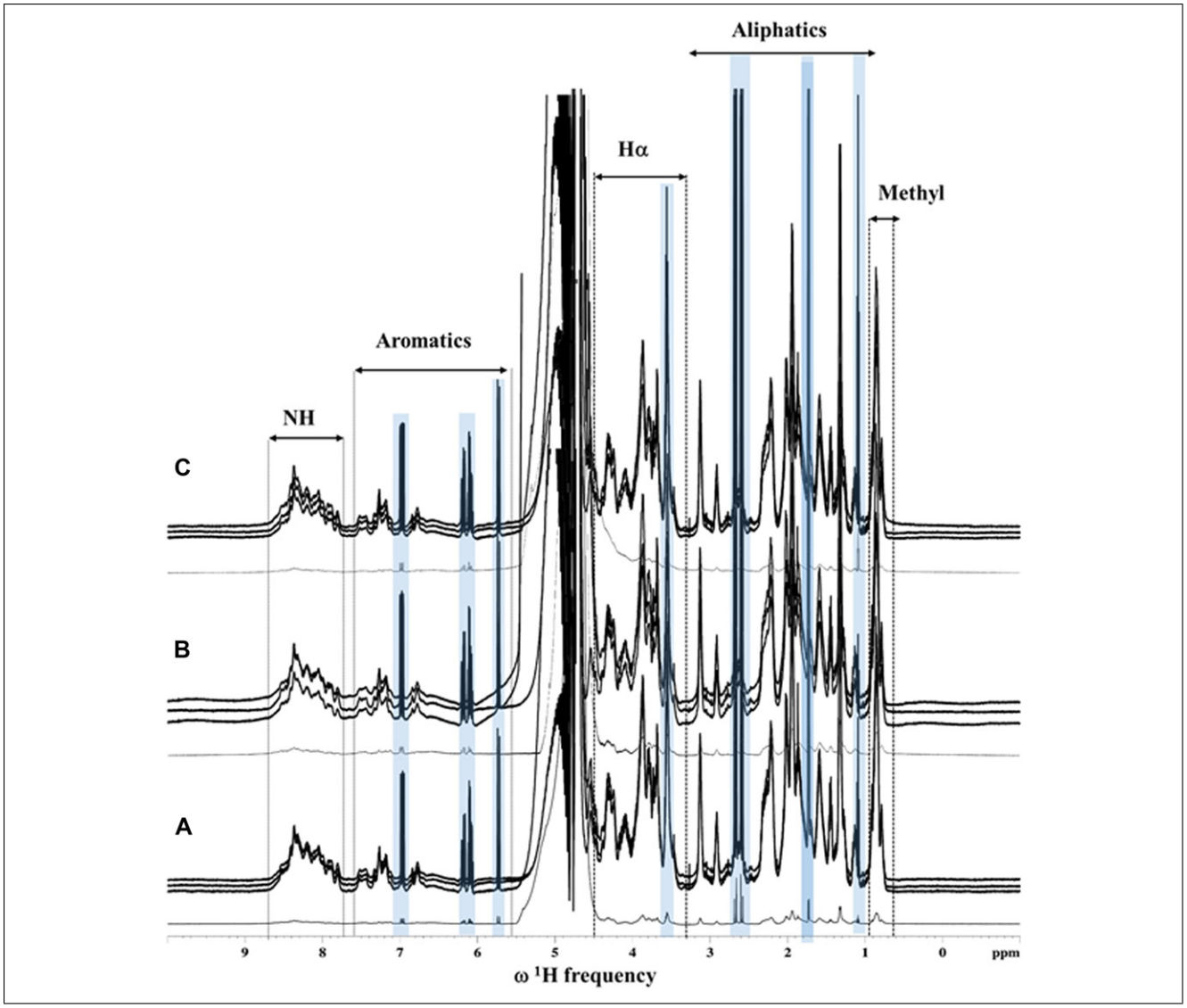

Figure 4–1D NOESY shaped-pulse solvent presaturation proton spectra [$^{1}H_{water\_presat}$ NMR] of Colagenart® batches (A: HC018-01, B: HC018-02, and C: HC018-03). Each batch has been nonconsecutively analyzed by triplicate (stacked plots). At the bottom of each stacked triad, it is depicted the standard ^{1}H spectra (no solvent suppression) of every batch, whereas it is shown that the sensitivity of Colagenart® spin-systems is severely penalized by the presence of water signal (4.7 ppm) when standard experiments were used. A local chemical shift dispersion of Colagenart® spin systems has been defined by means of standard proteins' ^{1}H chemical shift regions (Jerosch, 2011). Spin systems of samples' matrix (excipients) are highlighted in blue.

determining a sequence coverage >95% (Supplementary Material). Overall, these criteria indicate that the mass intact profile of the Colagenart® batches is reproducible. In addition, the sequencing analysis suggests that bathes with similar peptide species are obtained from the manufacturing process of Colagenart®.

To the best of our knowledge, this is the first time that the peptide profile of a collagen hydrolysate product is investigated using an IMS analysis. This technique is based on the "determination of mobilities in electric fields of gas phase ions derived from constituents in a sample," and, owing to its capability to differentiate peptide conformers, has been successfully employed to characterize complex peptide-derived drugs (Borsdorf & Eiceman, 2006). The obtained DriftScope images of Colagenart® batches showed that its peptide components have a reproducible m/z—drift time correlation pattern with no significant differences among them (Figure 3). The results observed in SEC and MS analyses, allowed determining the molecular weight of peptides present in Colagenart® (<17 kDa) and established this parameter as a quality attribute for the approval of commercial batches.

NMR

The advantage of NMR is its capability to study complex samples from a holistic perspective (Wishart, 2005). In this sense, this technique has been widely used for the quality analysis of complex products such as plant extracts and beverages in the food industry (Godelmann et al., 2013; Jahangir, Kim, Choi, & Verpoorte, 2008; Le Gall, Colquhoun, & Defernez, 2004; Ursem, Tikunov, Bovy, van Berloo, & van Eeuwijk, 2008). Although, NMR has been previously used for collagen hydrolysate characterization (Schadow et al., 2013), we performed an in-depth nonstandard analysis with quality control purposes. Series of 1D- and 2D-proton NMR experiments were carried out to complement and support the results observed by SEC and MS. The main interest in performing NMR is to propose a noninvasive physicochemical technique that allows

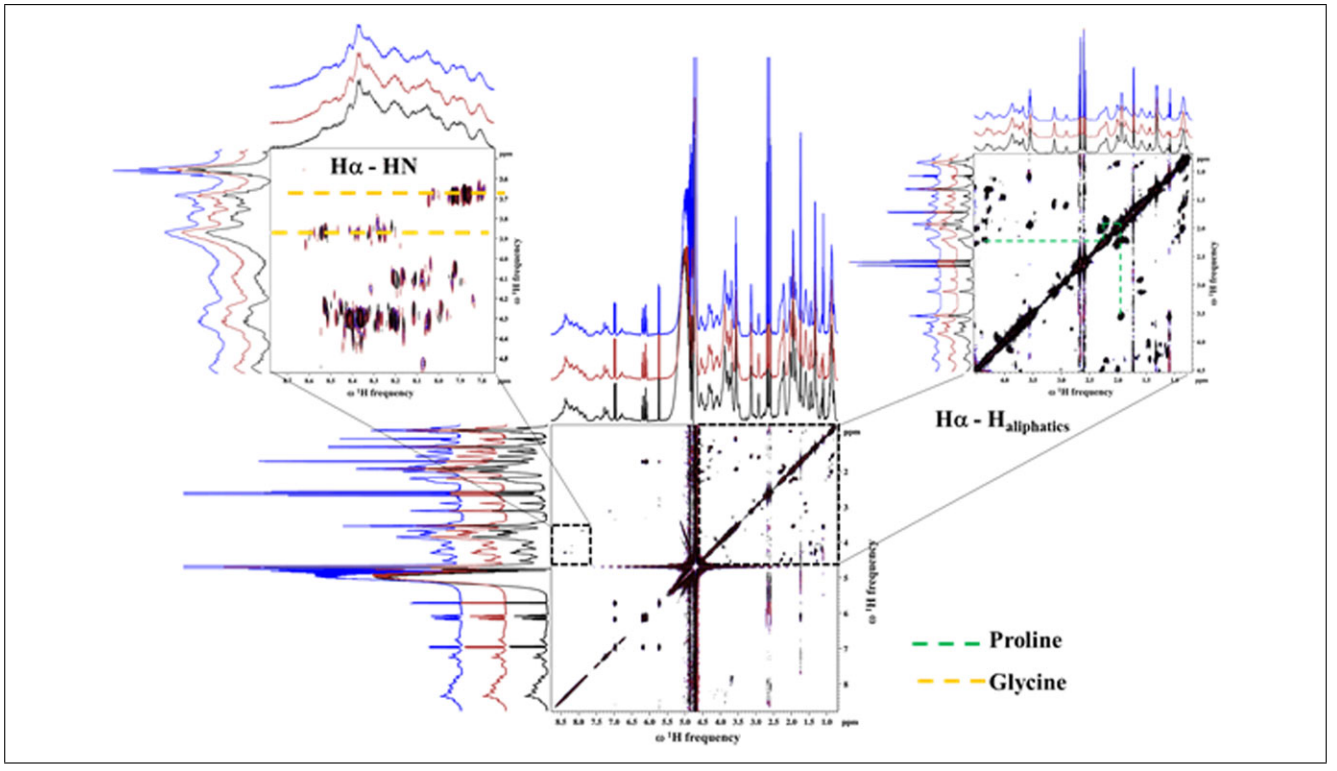

Figure 5–2D homonuclear shift correlation shaped-pulse solvent suppression correlation spectroscopy [$COSY_{water\_presat}$ NMR] overlay of batches HC018-01 (Black), HC018-02 (Red), and HC018-03 (Blue) with expansions of Ha-HN and Hα-H aliphatic regions.

getting a deeper insight of both: (1) The local chemical environment and structural homogeneity of the sample, and (2) the use of nuclear spin properties to define an alternative of quality control for collagen hydrolysate samples.

Due to the dilution conditions of Colagenart® with respect to the well-known sensitivity penalization of NMR techniques, it is imperative the use of nonstandard NMR experiments, whereas low power solvent suppression with shaped-pulses becomes fundamental for accurate quality controls. Figure 4 shows a series of 1D- [$^1H_{water\_presat}$] NMR experiments of three batches of Colagenart®, by triplicate, in comparison to the standard 1D-^{1}H NMR experiment for each batch. First, each nonconsecutive triad of [$^1H_{water\_presat}$] experiments per Colagenart® sample, confirms the batch-to-batch reproducibility. Stacked-plots of each batch, as well as comparison among batches, confirm the reproducibility of the sample, in terms of highly consistent frequency shifts, signal intensities, and half band widths of all experiments carried out at identical conditions.

It is important to highlight the importance of the use of solvent pre-saturation for the present NMR analysis, by comparing the 1D- [$^1H_{water\_presat}$] signal-to-noise ratio (c.a., 2000) with respect to the standard 1D-^{1}H scheme (c.a., 600) and by visually comparing both type of experiments that were carried out under identical conditions (Figure 4), wherein signal enhancement of 1D-[$^1H_{water\_presat}$] is increased by roughly a factor 8, with respect to the standard scheme. An accurate signal enhancement allows defining within the 1D spectra, different proteins' ^{1}H chemical shift regions, in the vicinity of the random coil shift values (Biological Magnetic Resonance Data Bank). Figure 4 shows the chemical shift dispersion of NH, $H_{aromatics}$, Hα and $H_{aliphatics}$ spin-systems of Colagenart® batches. 1D-^{1}H NMR standard experiments with equivalent dispersion of nonassigned collagen systems have been reported previously (Schreiber & Gaudet, 2012). To the best of our knowledge, the latter represents the first effort for assigning global patterns within the complex NMR fingerprint of collagen hydrolysates.

Deeper analyses of Colagenart® NMR proton profiles were carried out with the aid of 2D $COSY_{water\_presat}$ NMR schemes (Figure 5). First, the batch-to-batch consistency of Colagenart® batches is depicted within the overlay of $COSY_{water\_presat}$ NMR spectra. Hα-NH correlations show a pattern of several NH contacts concentrated in roughly four Hα chemical shift environments (4.2, 4.1, 3.8, and 3.6 ppm). For Hα in the range of 4.2 to 4.1 ppm, those spin-systems show respectively short intraresidue contacts with $H_{aliphatics}$ in the range of 2.2 to 1.8 ppm and 1.6 to 1.2 ppm, which might suggest the presence of Glu, Gln, Asp, and Asn amino acids within the formulation. In contrast, H chemical shift environments in the range of 3.8 and 3.6 ppm show no correlation with $H_{aliphatics}$ spin-systems, highly suggesting the presence of Gly within the Colagenart® formulation (yellow dotted lines, Figure 5). The connectivity pathways 3.55, 1.95, 2.22, and 4.35 ppm corresponding to Cδ, Cγ, Cβ, and Cα prospin system (green dotted line, Figure 5) has been assigned as well, within the Hα-$H_{aliphatics}$ region. Finally, spin connectivity between methyl and aliphatic moieties within the $COSY_{water\_presat}$ NMR experiments, suggest the presence of Ile, Val, and Leu within the Colagenart® formulation.

Although the full assignment of Colagenart® $COSY_{water\_presat}$ NMR experiments will be published elsewhere, combined with long-range contact schemes ($TOCSY_{water\_presat}$ and $NOESY_{water\_presat}$), NMR spectroscopy intends to explain an amino acid profile contained within the observed polypeptide dispersion of collagen hydrolysates and to prove consistency among batches in an alternative way.

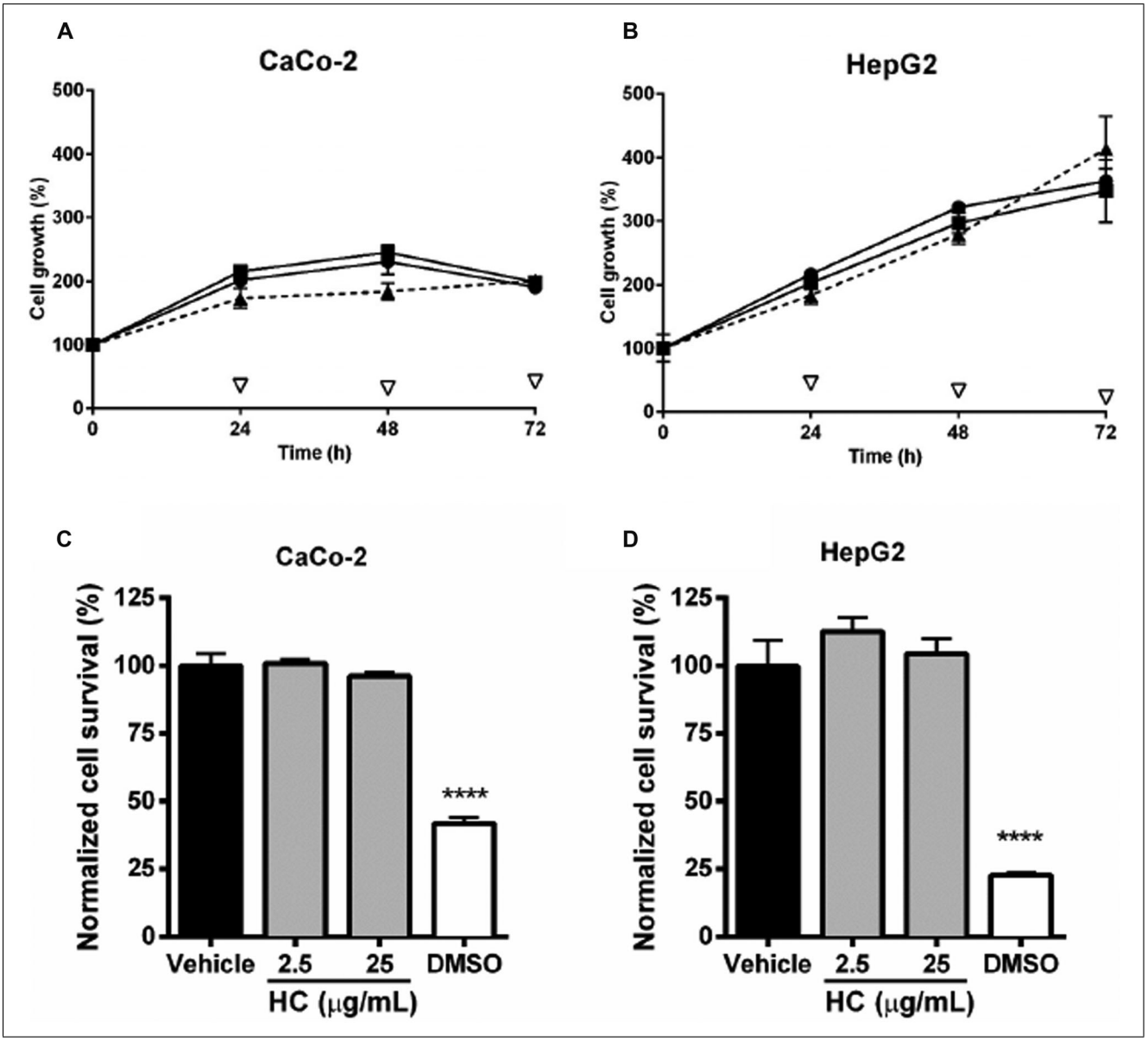

Figure 6–Survival of cells exposed to Colagenart®. (A and B) Proliferation throughout 72 hr of CaCo-2 and HepG2 cells after being exposed to Colagenart® (filled circles), vehicle (filled squares), untreated cells (filled triangles), and DMSO (clear triangles). (C and D) There are no significant differences in CaCo-2 and HepG2 cells survival at different doses of Colagenart® (2.5 and 25 μg/mL) or vehicle at 72 hr. Conversely, the differences were significant for the cellular death control (DMSO).

In vitro assays

Safety is another relevant quality attribute for dietary products. Nevertheless, it is often disregarded by some manufacturers under the assumption of the innocuousness of raw materials, without testing if the final combination is innocuous. Safety of Colagenart® was tested using two cell lines commonly employed in *in vitro* toxicity studies: HepG2 (derived from human liver) used to test liver metabolism and toxicity (Miret et al., 2006) and CaCo-2 cells (derived from human colorectal epithelium) used to test intestinal absorption and toxicity (Angelis & Turco, 2011). Unlike other dietary supplements under development, whose toxicity requires to be tested, peptides derived from collagen are not toxic at doses higher than 15 g/kg when orally administered in rats (Benjakul, Karnjanapratum, & Visessanguan, 2018; Parenteau-Bareil, Gauvin, & Berthod, 2010). Thus, our test focused on evaluating the effect of the whole formulation on the exposed cells. During the *in vitro* assay, we performed a visual inspection of HepG2 and CaCo-2 cells, and we did not observe any apparent change in those exposed to Colagenart® or vehicle with respect to the untreated cells, neither in morphology nor in density. On the other hand, the cells treated with 20% DMSO (cellular death control) showed a reduction in density and exhibited shrinkage. OD measurements from the MTS assay at different times revealed that cell lines exposed to Colagenart® followed the same trend in cell proliferation over time than untreated cells (Figure 6A and B). In the case of CaCo-2 cells, an increase in proliferation in cells exposed to Colagenart® and vehicle was observed at 24 and 48 hr with respect to untreated cells, nevertheless, it is not indicative of toxicity. Although this finding will be assessed later, it could be associated to the capability of CaCo-2 cells to absorb nutrients and to the environmental conditions that favored their biological activity at 24 and 48 hr. Therefore, the major proliferation of CaCo-2 cells

is congruent with an easier absorption of small peptides (<3 kDa). The statistical analyses confirmed that, among the treatments to which the cells were exposed, the differences were significant [$p < 0.0001$, α 0.05] only for the cellular death control (DMSO) in both cell lines (Figure 6C and D). These results indicate that neither Colagenart® nor the components of its formulation induce toxicity on the evaluated cell lines.

Conclusion

Collagen hydrolysates are composed of a myriad of peptides and their quality attributes should be addressed to ensure the expected benefits and their safety. We took advantage of the capabilities of the analytical techniques SEC, MS, and NMR to establish the characteristic peptide patterns as an identity test of collagen hydrolysates. The obtained results from these analytical techniques are congruent with the theoretical values of Colagenart®, and evinced their utility to determine batch-to-batch consistency, regardless of their sensitivity and the sample complexity. On the other hand, Colagenart® did not elicit toxic effects in HepG2 and CaCo-2 *in vitro* assays. In summary, collagen manufacturers to establish the quality of their products could implement the proposed analysis. In addition, these results support the manufacturing process and the quality of Colagenart®.

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Author Contributions

López-Morales and Medina-Rivero designed the study. López-Morales, Medina-Rivero, and Vallejo-Castillo wrote the manuscript. Vázquez-Leyva and Vallejo-Castillo performed SEC and MS analysis. Herbert-Pucheta and Zepeda-Vallejo designed and interpreted the NMR analysis. Carballo-Uicab and Muñoz-García performed the assays of in vitro toxicity. Velasco-Velázquez, Pavón, and Pérez-Tapia provided a critical advisory and improved the quality of this research. All authors revised and approved the manuscript.

Conflict of Interest

Authors Vázquez-Leyva, Velasco-Velázquez, Pavón, Pérez-Tapia, and Medina-Rivero are involved in the development and manufacturing of Colagenart®. All other authors declare no conflict of interest.

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