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NT-II™ Undenatured Type II Collagen: Comprehensive *In Vitro* Characterization of Bioaccessibility, Epitope Integrity, and Collagen Typing

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ABSTRACT

NT-II™ is a nutraceutical compound derived from Atlantic salmon bone, offering a sustainable source of type II collagen. It holds therapeutic potential in maintaining joint integrity and modulating immune responses. We conducted a detailed analysis of NT-II™ to evaluate its collagen content, digestion behavior, and molecular composition under conditions that mimic human gastrointestinal physiology. Simulated gastrointestinal digestion was conducted using the standardized INFOGEST 2.0 model and a time-resolved pepsin-only protocol. Bioaccessible undenatured type II collagen was quantified in post-digestion supernatants via ELISA targeting native epitopes, while total collagen was determined by hydroxyproline analysis. Collagen type distribution was evaluated using DEAE–Sepharose chromatography and SDS–PAGE. NT-II™ yielded a total collagen content of the bone powder of 28.5% by weight, with 78% classified as type II collagen. Vertebral disk–enriched fractions contained 28.1–64.5% undenatured type II collagen by ELISA. Epitope release increased progressively with digestion time, reaching 48.6 mg/g after 90 minutes of pepsin treatment and 70.9 mg/g under full INFOGEST digestion. Epitope recovery averaged 7.1% (range: 6.3–7.8%) relative to total collagen content. ELISA signal intensity varied modestly relative to UC-II®, likely due to partial shielding of the salmon bone collagen by the hydroxyapatite matrix. The concentration of native epitopes released under simulated digestive conditions corresponds to levels shown to induce immune tolerance in prior UC-II® studies. These results demonstrate that NT-II™ delivers bioaccessible levels of native type II collagen epitopes during digestion, matching concentrations associated with oral tolerance and efficacy demonstrated in UC-II® trials.

Keywords: Undenatured Collagen; Type II Collagen, NT-II; Salmon Bone; Marine-Derived; ELISA; Hydroxyproline; SDS-PAGE, Oral Tolerance; Joint Health

Abbreviations: UC-II: Undenatured Type II Collagen (chicken-derived): NT-II™: Natural Type II Collagen (Derived from Salmon Bone); HYP: Hydroxyproline; ELISA: Enzyme-Linked Immunosorbent Assay; GI: Gastrointestinal; SDS-PAGE: Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis; FDA: Food and Drug Administration; GRAS: Generally Recognized as Safe; HPLC: High-Performance Liquid Chromatography

Introduction

Fish processing in Norwegian aquaculture, particularly of Atlantic salmon (Salmo salar), produces substantial volumes of side streams, including collagen-rich tissues such as bone and cartilage [1]. During filleting, approximately a third of the fish remains as by-products, composed of bones, heads, scales, skin, fins, and residual muscle tissue. Aquaculture processing by-products are increasingly recognized as valuable side-streams or secondary raw materials rich in high-value macro- and micronutrients, that, given appropriate processing, can be rendered suitable and indeed confer health-promoting properties for humans as nutraceutical products [2,3]. Valorization processes employing enzymatic hydrolysis can turn fish by-products into nutrient-rich ingredients—yielding bioactive peptides and hydrolysates, mineral-dense protein-hydroxyapatite complexes, and full-spectrum fish oils [3]. Fish bones are rich in calcium, phosphate and zinc, and

type II collagen and small amounts of growth factors. In isolation, fish bone shows significant promise as a highly bioavailable and underutilized calcium source. [4]. Historically, collagen has come chiefly from bovine, porcine, and avian sources, but the industry is now increasingly embracing collagen extracted from marine organisms. Marine collagens are increasingly recognized as a sustainable, cost-effective, and efficacious alternative to mammalian-derived collagens. They offer superior bioavailability, distinctive functional properties, and a favorable safety profile—most notably, the absence of prion disease transmission risk [5].

Supporting this, the U.S. Food and Drug Administration (FDA) has classified marine-sourced collagen as Generally Recognized as Safe (GRAS), affirming its safety for human use and its lack of disease transmission risk [6]. Marine-derived collagens are attractive supplements due to their effective processing, functional versatility, and rich nutritional and bioactive profiles. Their sustainability and safety advantages have helped establish marine collagen as a high-quality option in therapeutic, biomedical, and nutraceutical markets [7]. Owing to its diverse functional properties, marine collagen has garnered significant interest in both clinical and preclinical research. Studies have explored its therapeutic potential across a range of health domains, including wound healing [8], even collagen-based bioink applications [9], alleviation of joint pain and stiffness [10,11], preservation of bone mineral density in postmenopausal women [12], support for muscle protein synthesis alongside exercise [13], and the improvement of skin and hair health [14]. Particular attention has been directed toward the bioactive properties of marine collagen, which are actively being investigated in both preclinical and clinical studies [15,16]. Atlantic salmon (Salmo salar) stands out not only for its global popularity but also for its exceptional nutritional profile. As the leading producer of Atlantic salmon, Norway plays a pivotal role in aquaculture, with its salmon industry serving as a benchmark for sustainable and high-yield aquaculture practices [17,18].

Salmon is a rich source of high-quality protein, omega fatty acids, essential minerals such as selenium, and vitamins such as B12 and D, all of which contribute to cardiovascular health and overall physiological well-being. The potent antioxidant and carotenoid astaxanthin confers the vibrant pink-orange pigmentation to salmon muscle tissue, recognized to have anti-inflammatory and antioxidant properties [19]. Processing side streams from salmon, particularly the skin, bones, joint cartilage and scales—constitute a rich source of collagen, with collagen proteins accounting for up to 30% of their total dry weight. Notably, salmon-derived collagen has demonstrated superior digestibility (~73%) in in vitro models compared to other fish sources, underscoring its potential for functional and nutritional applications [20]. The term 'collagen' is a generic term referring to a superfamily of structural proteins belonging to the extracellular matrix of tissues and that share similar structural features. To date, 28 types of collagen have been described called type I to type XXVIII based on the chronological order of their discovery [21]. This ropelike protein is each composed of three left-handed polypeptide chains (α-helices), which assemble to form right-handed triple-helix structures, linked by hydrogen bonds [22]. One α-helix is composed of about 1000 amino acid residues, characterized by repeating glycine, proline and hydroxyproline residues. The high concentration of hydroxyproline residues is unique to type I collagen, and thus is commonly used for quantification of particularly this specific protein [23].

Fish skin contains the highest concentration of collagen (type I) per gram. Still, salmon bones present a distinct compositional advantage as a source of both type I and type II collagen, particularly in the vertebrae and cranial regions [24]. Compared to skin, bones are also available in greater quantities as a by-product of fish processing, making them an abundant, underutilized, and ethically sustainable raw material with significant potential for the extraction of type II collagen for use in nutraceuticals specifically targeting joint health [24]. Structurally, the collagen in fish bones is embedded within a dense hydroxyapatite matrix and must first be liberated through demineralization, typically with exposure to a strong acid or alkaline solution. Undenatured type II collagen—used in Lonza's UC-II® and derived from chicken sternum cartilage—retains its native triple-helix conformation and has demonstrated efficacy in reducing osteoarthritis symptoms across both clinical and preclinical studies [11,25,26]. Its efficacy is linked to the induction of oral tolerance—an immune-regulatory mechanism triggered by the presentation of intact collagen epitopes in the gut-associated lymphoid tissue (GALT), as evidenced by experimental models [27]. The preserved tertiary structure is essential, enabling resistance to gastric digestion and effective immune recognition in Peyer's patches [15,17]. Repeated oral exposure promotes reprogramming of regulatory T cells downregulating systemic T-cell–mediated attacks and inflammatory signaling impacting cartilage integrity.

Despite the recognized value of marine by-products, value-added products are infrequently characterized and rarely advanced to clinical trials. Native type II collagen has shown therapeutic potential for rheumatoid arthritis and osteoarthritis, though most studies have focused on terrestrial sources. Research on marine-derived undenatured type II collagen remains limited. The present investigation focuses on NT-II™—a naturally occurring undenatured type II collagen embedded in salmon bone powder. In this matrix, the triple-helix conformation of collagen appears structurally protected by the calcium-rich hydroxyapatite scaffold of bone. We hypothesize that salmon-derived NT-II™ may exert comparable immunomodulatory effects to UC-II® by engaging oral tolerance mechanisms under physiologically relevant digestive conditions. To date, no salmon bone–derived type II collagen preparations have been systematically evaluated under physiologically relevant digestion conditions with quantitative epitope assessment, particularly in direct comparison to established undenatured type II collagen reference materials such as UC-II®. To

investigate this, we assessed the stability and solubility of NT-II™ epitopes using validated in vitro digestion models—including both pepsin-only and INFOGEST 2.0 protocols—followed by ELISA-based quantification of antigenic integrity. To our knowledge, few marine collagen preparations have been studied in this way, particularly with matched comparisons to terrestrial counterparts. The study also examines time-dependent solubilization kinetics, collagen type distribution within the salmon bone matrix, and benchmarks NT-II™ against a chicken sternum undenatured collagen (UC-II®) reference. Together, these analyses aim to establish the functional equivalence of NT-II™ and support its development as a sustainable, marine-derived alternative for joint health applications.

Materials and Methods

Samples and Materials

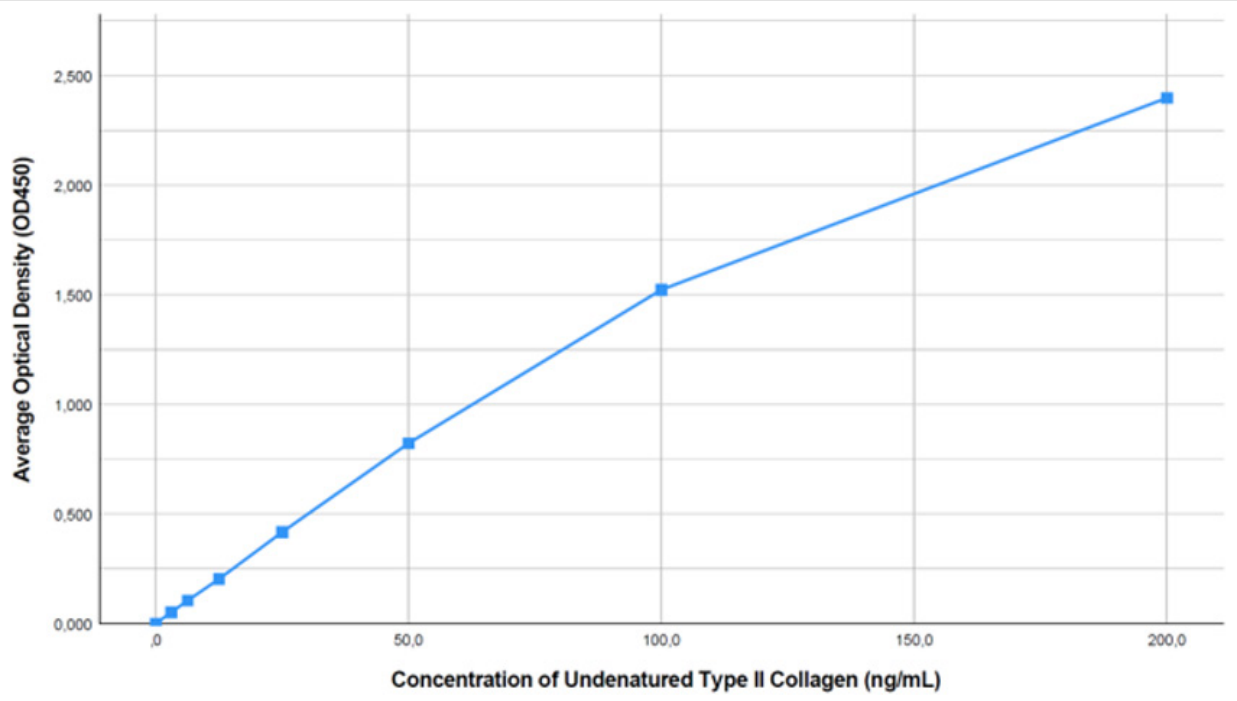
Two production batches of NT-II™ salmon bone powder (Batch #22007 and Batch #18001; Hofseth BioCare ASA, Ålesund, Norway) were analyzed. Both batches were derived from enzymatically cleaned salmon backbones and processed using a proprietary low-temperature hydrolysis method designed to avoid collagen denaturation and preserve the native triple-helical conformation of type II collagen and immunogenic epitopes. The resulting powder was milled and sieved to a uniform particle size (U.S. Mesh #3–18) and exhibited an off-white, water-insoluble, powdered appearance. Representative physiochemical and compositional characteristics of NT-II™, including total and undenatured type II collagen content, moisture, mineral composition, and safety profile, are summarized in Table 1. For comparative purposes, UC-II® capsules (Healthy Origins, USA)—each standardized to contain 40 mg of chicken sternum cartilage (providing ~10 mg of undenatured type II collagen)—were used as a reference benchmark. All test materials were air-dried, ground to a fine powder, and stored in desiccated containers at room temperature prior to use. Unless otherwise stated, all reagents, enzymes, and analytical-grade chemicals were procured from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Table 1: Key Physicochemical Characteristics of NT-II™ Salmon Bone Powder.

Parameter	Typical Value or Description	Analytical Method
Appearance	Off-white powder	Visual and sensory inspection
Solubility	Insoluble in water	Sensory test
Moisture Content	< 5%	Gravimetric analysis
Density (tapped/untapped)	0.5–0.9 g/cm³ (approximate range)	European Pharmacopoeia 2.9.34
Total Collagen Content	25–31% (w/w)	ISO 13903:2005
Undenatured Type II Collagen	19–24% of product (w/w)	ISO 13903:2005
Mineral Content	Present (notably calcium, phosphorus, zinc)	DS/EN 13805:2014; DS/EN ISO 11885/17294
Microbial Safety	Complies with food-grade safety limits	AFNOR microbiological standards
Heavy Metals (As, Pb, Cd, Hg)	Below detection or regulatory thresholds	ISO 17294; ASU L 25.06-1

Total Collagen Content of NT-II™ Powder via Hydroxyproline Quantification

Total collagen content in NT-II™ powder was determined by measuring hydroxyproline (Hyp) concentration via high-performance liquid chromatography (HPLC). This compositional assay establishes the baseline collagen content of the test article prior to digestion and provides a reference point for interpreting solubilization and epitope preservation data. Hydroxyproline was quantified following acid hydrolysis in 6N HCl at 110 °C for 24 hours under nitrogen. Derivatization and quantification were performed using reverse-phase HPLC with a validated internal standard in accordance with ISO 13903:2005 and EU Regulation 152/2009. This method has been validated for marine matrices with low elastin content, such as salmon bone, and provides a robust proxy for total collagen. To estimate collagen content, a conversion factor of 12.5 was applied, assuming Hyp comprises approximately 8% of total collagen by weight — consistent with published data for cold-water fish species. Results were expressed as grams of collagen per 100 grams of NT-II™ powder. All measurements were conducted in triplicate. Hydroxyproline content was determined across 37 production batches of NT-II™ from 2022 to 2024 (Figure 1; mean total collagen: 28.5% w/w; standard deviation: ±3.87%; range: 23.25%–42.75%).



Note: Average optical density (OD450) values were plotted against known concentrations of native type II collagen (ng/mL) using the Chondrex Type II Collagen Detection Kit. The resulting linear regression ($R^2 > 0.99$) confirmed assay sensitivity and range suitability for test sample interpolation.

Figure 1: ELISA calibration curve for undenatured type II collagen (UC-II™) standards.

In Vitro Digestion Models to Assess Collagen Solubility and Epitope Integrity

Two validated in vitro digestion protocols were employed to assess the release and preservation of undenatured type II collagen epitopes under physiologically relevant gastric and gastrointestinal conditions. The INFOGEST 2.0 static digestion model was used to simulate sequential oral–gastric–intestinal phases. A simplified pepsin-only gastric model was used in parallel to assess acid resistance and proteolytic stability of the undenatured collagen.

INFOGEST 2.0 Gastrointestinal Digestion Model: The standardized INFOGEST 2.0 static digestion model was employed to evaluate the digestive resilience and bioaccessibility of undenatured type II collagen under physiologically complete conditions. In this study, bioaccessibility refers to the extent to which collagen is solubilized and remains available for potential biological interaction after digestion. This internationally recognized protocol simulates sequential oral, gastric, and intestinal digestion in the fed state, using harmonized enzyme activities, electrolyte composition, pH, and transit times. In the context of NT-II™, this method enables assessment of

- The degree to which collagen becomes solubilized in gastrointestinal fluids,
- Whether bioactive epitopes are preserved following exposure to digestive enzymes and bile, and
- Whether epitope-bearing fragments can be recovered post-digestion for downstream quantification via ELISA.

i. Oral Phase: One gram of NT-II™ powder was combined with 0.1 mL simulated salivary fluid (SSF) containing α-amylase (75 U/mL) and 0.2 mL of 0.3 M $CaCl_2$. The mixture was vortexed to ensure even dispersion and interaction.

ii. Gastric Phase: The oral bolus was diluted 10:1 with simulated gastric fluid (SGF) containing 0.2 mL of 0.3 M $CaCl_2$ and rabbit gastric extract (lipase activity: 60 U/mL). The pH was adjusted to 3.0 using HCl, and the mixture was incubated at 37 °C for 30 minutes to simulate gastric digestion.

iii. Intestinal Phase: Following gastric treatment, the sample was further diluted 10:1 with simulated intestinal fluid (SIF) containing pancreatin (providing 100 U/mL trypsin activity), bile salts

(10 mM), and 0.1 mL of 0.3 M $CaCl_2$. The pH was adjusted to 7.0, and the mixture was incubated at 37 °C for 50 minutes. Enzymatic activity was terminated by heating to 90 °C for 20 minutes. Final digests were centrifuged at 10,000 rpm for 10 minutes, and the resulting supernatants were collected and stored at −20 °C for downstream quantification.

Pepsin-Only Simulated Gastric Digestion: To isolate the gastric-phase stability of NT-II™ and evaluate its resistance to acidic proteolysis, a simplified pepsin-only digestion model was employed. This model also enables time-dependent assessment of epitope preservation under simulated stomach conditions, complementing the full gastrointestinal simulation provided by the INFOGEST protocol. Specifically, 14.7 g of NT-II™ powder was suspended in 100 mL of pepsin solution (30 mg/L in 10 mM HCl, pH 2.3) and incubated at 32 °C. Aliquots were collected at 0, 15, 30, 60, and 90 minutes. After each time point, samples were centrifuged (10,000 rpm, 10 min), and supernatants were neutralized to pH 6.0 to halt enzymatic activity. All supernatants were stored at −20 °C for subsequent ELISA analysis to quantify undenatured type II collagen. This approach enables direct evaluation of epitope release kinetics and proteolytic resilience under gastric conditions alone. We opted for this time-resolved approach to simulate gastric phase exposure and monitor stability over physiologically relevant timeframes.

Comparative Digestion of Size-Fractionated NT-II™ Powders

To evaluate whether the physical granule size of NT-II™ powder influences the release of soluble, undenatured type II collagen, seven mesh-size fractions of CalGo® NT-II™ (U.S. Mesh sizes 3 to 18) and a manually separated vertebral disk fraction were subjected to a 90-minute pepsin digestion, following the same conditions described in Section 2.2.2. Supernatants were collected post-centrifugation and analyzed via ELISA to quantify and compare the relative yield of soluble undenatured collagen across particle sizes.

Quantification of Undenatured Type II Collagen Epitopes via ELISA

Digestion supernatants from the INFOGEST (Section 2.3.1) and pepsin-only (Section 2.3.2) models were analyzed to quantify soluble, undenatured type II collagen—the bioactive fraction that remains intact after digestion and is relevant to oral tolerance and clinical efficacy. This antigenically intact form, characterized by preserved triple-helical epitopes, was measured using the Chondrex Type II Collagen Detection ELISA Kit (Cat. No. 6018). Each sample was serially diluted (5 × 10-fold dilutions) and run in duplicate using a 24-well plate layout. Optical density values (OD) values were recorded and interpolated against a standard curve (0–200 ng/mL; slope ≈ 82.4). Final concentrations of undenatured type II collagen were calculated by converting ELISA OD values to ng/mL using the standard curve, then adjusting for dilution factors and a total digestate volume of 5 mL per gram of NT-II™ powder. Results were expressed as milligrams per gram (mg/g) of starting material.

Molecular Typing of Collagen by Anion-Exchange Chromatography and SDS–PAGE

To determine collagen type composition, pepsin-solubilized samples were subjected to anion-exchange chromatography using a DEAE–Sepharose CL-6B column (20 cm), equilibrated with 0.2 M NaCl in 0.05 M Tris–HCl buffer (pH 7.5). Bound proteins were eluted sequentially with 0.2 M and 1.0 M NaCl to separate collagen isoforms by charge. Elution was monitored by UV absorbance at 280 nm. Eluted fractions were analyzed by SDS–PAGE (Laemmli method) using a 5% stacking gel and 7.5% separating gel. Samples were mixed with Tris–HCl buffer containing SDS, β-mercaptoethanol, glycerol, and bromophenol blue, and stained with Coomassie Brilliant Blue R-250. Type II collagen was identified by the presence of α1(II) chains in the 100–140 kDa range, with a prominent band at ~120 kDa. Densitometric analysis indicated 78% of collagen was type II, consistent with expected banding profiles.

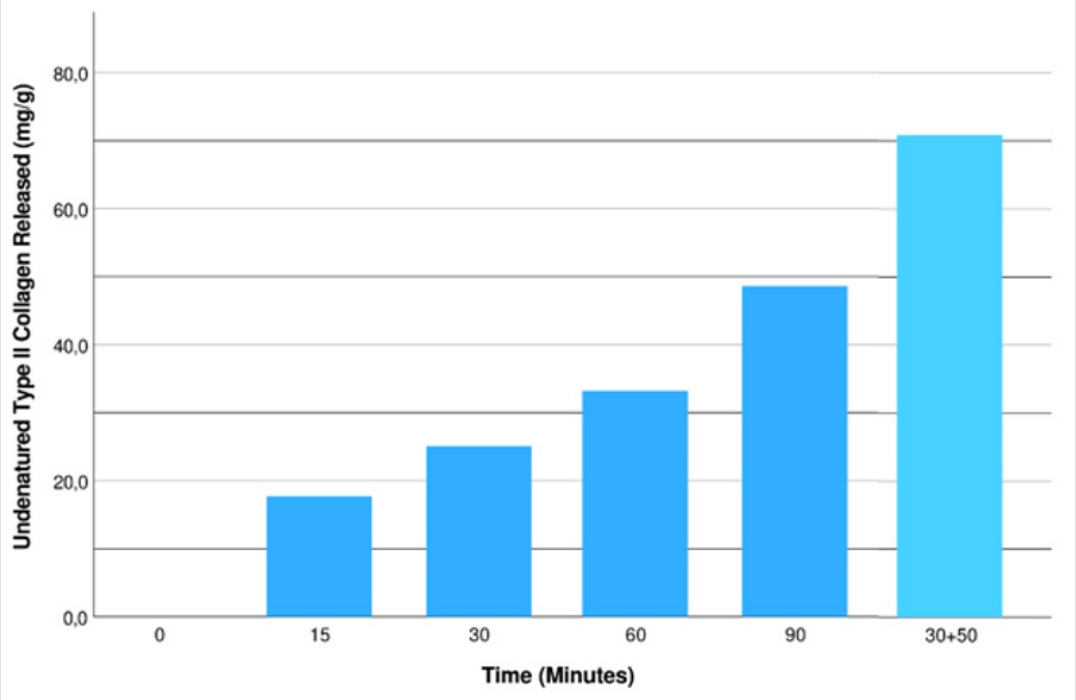
Statistical Analysis

All digestion samples were analyzed in triplicate. Data are reported as mean ± standard deviation (SD). Inter-assay variability was assessed using the coefficient of variation (CV%). Comparative analysis between CalGo® and UC-II® was conducted where applicable.

Results

Simulated Gastrointestinal Digestion of NT-II™

NT-II™ retained measurable levels of undenatured type II collagen following full simulated gastrointestinal digestion using the INFOGEST 2.0 protocol. ELISA analysis of three replicate samples (n = 3; UnDC-001, -002, -003) yielded OD values of 1.772, 1.964, and 1.580, corresponding to 141.72, 157.08, and 126.32 ng/mL, respectively. The mean epitope concentration was 141.71 ± 15.42 ng/mL, equivalent to 7.1% NT-II by weight (range: 6.3–7.8%). This conversion assumes 5 mL of supernatant recovered per gram of input powder, in accordance with the digestion protocol. The coefficient of variation (CV%) was 10.8%. This consistency across replicates affirms the reproducibility of the extraction and quantification protocol. The ELISA standard curve was generated using the Chondrex detection kit with average OD values plotted against known concentrations of type II collagen (ng/mL), yielding a calibration slope of 82.4 and confirming assay linearity across the tested range (Figure 2). These results demonstrate that NT-II™ retains measurable levels of native type II collagen epitopes following full gastrointestinal digestion, indicating stability under simulated physiological conditions.



Note: Undenatured type II collagen (mg/g NT-II™) was quantified by ELISA following pepsin-only digestion (0–90 minutes) and full gastrointestinal simulation (30 min gastric + 50 min intestinal; INFOGEST 2.0). Epitope release increased over time, reaching 48.6 mg/g at 90 minutes and 70.9 mg/g under INFOGEST conditions. These results show progressive solubilization and digestion stability of native collagen, suggesting that NT-II™ remains bioaccessible throughout gastrointestinal transit.

Figure 2: Time-dependent release of undenatured type II collagen from NT-II™ under simulated digestion.

Time-Dependent Solubilization in Pepsin Digestion

Progressive solubilization of native type II collagen was observed during pepsin digestion. At 0 minutes, no detectable UC-II™ was present. Epitope concentrations increased over time, with ELISA OD values rising from 0.4245 at 15 minutes (35.48 ng/mL; 17.7 mg/g) to 1.1625 at 90 minutes (97.17 ng/mL; 48.6 mg/g). Intermediate values at 30 and 60 minutes were 50.11 ng/mL (25.1 mg/g) and 66.33 ng/mL (33.2 mg/g), respectively. All values represent the average of triplicate measurements. Native collagen epitopes in NT-II™ are progressively solubilized under acidic gastric conditions, while remaining ELISA-detectable, indicating epitope preservation and bioaccessibility for potential interaction with the gastrointestinal immune system.

Size-Fractionated Bone Analysis

Undenatured type II collagen content was further evaluated across size-sieved NT-II™ fractions. Following 90-minute pepsin digestion, vertebral disk material exhibited the highest epitope concentration (129.01 ng/mL; 64.5 mg/g), followed by Mesh #3 (120.78 ng/mL; 60.4 mg/g), Mesh #5 (107.82 ng/mL; 53.9 mg/g), Mesh #10 (95.15 ng/mL; 47.6 mg/g), and Mesh #14 (56.23 ng/mL; 28.1 mg/g). Results demonstrate a clear inverse relationship between particle fineness and UC-II™ yield, with coarser fractions and vertebral structures retaining higher levels. This trend highlights the structural protection afforded by denser fractions during proteolysis.

Benchmarking Against UC-II®

To contextualize NT-II™ undenatured type II collagen content, pepsin-digested NT-II™ was compared to a commercial UC-II® reference (Healthy Origins) under identical conditions. At the second serial dilution (Dil2), UC-II® yielded an average binding response of 13.1 mg/g, whereas NT-II™ yielded 2.7 mg/g. This represents a 4.8-fold difference in exposed native epitope concentration, supporting a preliminary dose equivalence of approximately 200–240 mg/day NT-II™ to match the typical 40 mg/day UC-II® dose. These findings provide an initial benchmark for bioequivalence scaling and support future dose-selection for clinical trials.

Total Collagen Content and Type Distribution

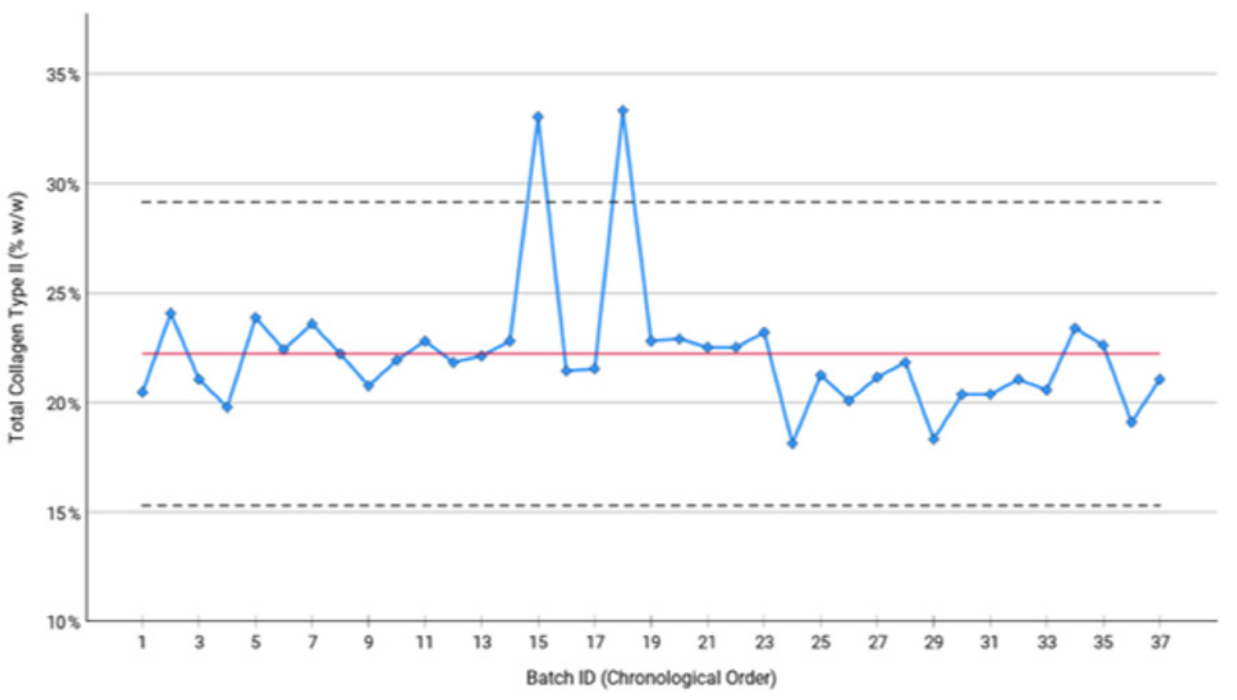
Hydroxyproline analysis of NT-II™ Batch #18001 yielded 2.59 g Hyp per 100 g powder, corresponding to a total collagen content of 32.3% by weight. Collagen type distribution, assessed via DEAE–Sepharose anion-exchange chromatography and SDS–PAGE, demonstrated a composition of approximately 78% type II, 19% type I, and 3% type III collagen.

Solubilization Across Digestive Phases

Progressive solubilization of undenatured type II collagen was observed during time-resolved pepsin digestion and full INFOGEST 2.0 gastrointestinal simulation. In the pepsin-only model, collagen concentrations increased from 0 mg/g at baseline to 48.6 mg/g after 90 minutes. In contrast, INFOGEST digestion yielded 70.9 mg/g, representing a 46% higher recovery. These findings highlight the importance of intestinal-phase enzymes—bile salts and pancreatic proteases—in promoting the hydroxyapatite matrix breakdown and epitope release beyond acid-driven solubilization alone. The results support the digestive stability and bioaccessibility of NT-II™ under physiologically relevant conditions.

Collagen Content Across NT-II™ Production Batches

Hydroxyproline analysis was performed on 37 production batches of NT-II™ spanning 2022–2024 (Figure 3). Hydroxyproline content ranged from 1.86 to 3.42 g/100 g (mean: 2.28 g/100 g). Applying the validated conversion factor (×12.5), total collagen content averaged 28.5%, with calculated type II collagen content (based on SDS–PAGE and DEAE–Sepharose chromatography, which consistently showed 78% type II) averaging 22.2% of NT-II™. These values confirm production consistency and support the continued use of the 12.5 conversion factor in routine batch-level assessments. This batch data affirms consistency in collagen yield and validates the robustness of the quantification method used in this study. This reproducibility across multiple lots supports the natural consistency of NT-II™ and its potential for scalable clinical application.



Note: Type II collagen content was assessed in 39 production batches of NT-II™ using hydroxyproline quantification and a validated collagen conversion factor (×12.5), combined with established type II collagen enrichment (78%) from SDS–PAGE and DEAE–Sepharose chromatography. The chart displays individual batch values (blue line), the process mean (red line), and control limits (UCL = 29.16%, LCL = 15.31%; dashed lines). Two batches exceeded the UCL, while all remaining batches fell within control limits, supporting consistency of production and robust quantification methods.

Figure 3: Batch-Level Control Chart for Total Type II Collagen Content (% w/w) in NT-II™ Across 37 Production Lots (2022–2024).

Discussion

This study provides the first in-depth characterization of NT-II™, a calcium–collagen composite derived from Atlantic salmon bone, containing undenatured type II collagen, produced through gentle enzymatic hydrolysis of freshly filleted salmon, preserving the native structure and mineral-collagen matrix. Using validated digestion models and ELISA quantification, we demonstrate that NT-II™ retains native epitopes and releases them under physiologically relevant conditions. ELISA results show that NT-II™ delivers measurable levels of undenatured type II collagen epitopes, reaching 70.9 mg/g under INFOGEST conditions and 48.6 mg/g with pepsin alone. For full gastrointestinal digestion simulation (INFOGEST 2.0), this corresponds to 7.1% of NT-II by weight, confirming bioaccessible antigen release during digestion. Under gastric-only (pepsin) digestion, NT-II™ released up to 48.6 mg of soluble epitopes per gram of bone meal—the highest amount recoverable in this simplified setting. The higher yield observed following full INFOGEST digestion—approximately 46% greater—highlights an important role for intestinal enzymes in helping to free native collagen molecules from its mineralized hydroxyapatite structure.

These findings confirm that NT-II™ retains its antigenic structure through digestion, providing a biochemically stable source of native epitopes with potential to engage mucosal immune pathways upon oral administration. Although immune activation and mucosal uptake were not directly assessed, the detection of intact epitopes post-digestion aligns with the structural criteria proposed by Bagchi et al. (2022) as necessary for oral tolerance induction in undenatured type II collagen interventions [28]. These findings provide a rationale for the ongoing investigation into the immunomodulatory potential of NT-II™. Protein profiling indicated that approximately 78% of the collagen present in NT-II™ is type II, aligning with the expected distribution in salmon vertebral tissue [5,6]. Total collagen content was initially estimated at 32.3% by weight using hydroxyproline quantification and the conventional 12.5× conversion factor [29]. Although this factor is widely applied across collagen studies, recent insights suggest it may slightly underrepresent collagen levels in marine-based matrices. Hydroxyproline analysis across 39 independent production batches yielded a consistent mean collagen content of 28.5%, supporting the suitability and applicability of the conversion factor in this matrix and reinforcing the estimated type II collagen content of approximately 22.2%. The method of production plays a key role in preserving the structural integrity of undenatured type II collagen.

Processing techniques employed in certain previously studied undenatured collagen preparations have been shown—through electron microscopy and colorimetric analyses—to disrupt or modify native molecular structure [30]. NT-II™ is manufactured using low-temperature, enzyme-assisted hydrolysis specifically optimized to minimize thermal and chemical denaturation, thereby maintaining the native triple-helical conformation and preserving bioactive epitopes. In this process, type II collagen remains embedded in a mineralized calcium–hydroxyapatite scaffold, closely approximating its native bone environment. This matrix allows for gradual release of epitope-rich fragments during digestion while offering protection against premature degradation. Within this framework, ELISA serves both as a quantitative tool and a critical quality control measure, confirming the preservation of native epitopes essential for eliciting oral tolerance mechanisms.

The preservation of epitope integrity observed across in vitro digestion models reflects both the biochemical stability of NT-II™ under gastrointestinal conditions and the critical role of controlled processing parameters in maintaining functional collagen structure. The verification provided here substantiates functional equivalence between NT-II™ and established undenatured type II collagen sources. Importantly, the epitope concentrations recovered from NT-II™ exceed the 3% native content—the bioaccessible antigenic fraction—typically reported for UC-II® [28], placing it within a range that has previously demonstrated clinical efficacy. Although ELISA signal intensity was lower than UC-II® under matched conditions, this is likely due to matrix shielding rather than loss of antigenicity. The hydroxyapatite scaffold may obscure some epitope sites during early digestion, but may allow delayed exposure, potentially exhibiting a sustained-release behavior. Moreover, the hydroxyapatite matrix appears to enhance the thermal stability of NT-II™, offering increased flexibility in its formulation across diverse product applications. In addition to its immunological potential, NT-II™ provides a source of bioavailable calcium, phosphorus, and trace minerals such as zinc, supporting a classification as a dual-function ingredient with relevance to both skeletal health and immune modulation. Its marine origin offers a non-avian alternative to poultry-derived collagens, accommodating dietary restrictions and minimizing exposure to common food allergens, thereby enhancing its suitability for broader population use.

Based on the ELISA quantification of epitope release, we calculate that a daily intake of just under 200 mg NT-II™ yields antigen exposure equivalent to the 40 mg UC-II® dose validated in clinical trials. To accommodate inter-individual variability in digestion efficiency, bioaccessibility, and immune responsiveness, a recommended intake of 240 mg/day NT-II™ is proposed to ensure consistent epitope delivery across the target population. This estimate offers a biochemical rationale for functional comparability—a basis for dose-equivalence modeling in future investigations. This study is inherently limited by its exclusive use of in vitro digestive models. While such models are designed to approximate physiological conditions, they do not fully capture the complexity of human gastrointestinal processes, including absorption dynamics, enzymatic interactions, and microbiota influences. While in vitro digestion and ELISA offer strong biochemical validation, functional outcomes must be confirmed in future studies. Preliminary clinical data, including findings from a pilot study presented at the ICFSR conference, suggest early promise for NT-II™ in

supporting joint health, particularly in individuals with osteoarthritis [31]. However, randomized controlled clinical trials are necessary for clinical verification and to elucidate the compound's profile in the modulation of inflammatory pathways relevant to joint health [32].

Conclusion

NT-II™, a marine-derived calcium–collagen complex sourced from salmon, exhibits preservation of structural and antigenic integrity under physiologically relevant in vitro digestion conditions. Quantitative ELISA analysis confirmed consistent recovery of native type II collagen epitopes, yielding epitope levels above established thresholds linked to bioactivity in joint health intervention trials. This suggests that NT-II™ delivers antigen concentrations sufficient to elicit oral tolerance mechanisms for clinical benefit. Based on comparative benchmarking, a daily intake of 240 mg NT-II™ is proposed to provide an epitope load comparable to the standardized 40 mg UC-II® dose used in clinical studies. These findings offer a biochemical rationale to position NT-II™ as an immunologically relevant marine alternative to poultry-derived undenatured type II collagen, offering both functional stability and formulation versatility. Taken together, the results support further clinical investigation into NT-II™ for applications in joint health and immune modulation.

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Conflict of Interest

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