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1 **Gelatine, present use and future applications: decryption of a**
2 **high-value multi-purpose by-product of the agro-food industry**

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21 **Keywords:** Bioactive peptides; Collagen-derived peptides; DPP-IV inhibitory peptides; Food
22 protein digestion; Gelatine hydrolysates; Glucose metabolism; Type 2 diabetes

23 **Running head:** Gelatine, a high-value by-product of industry
24
25

26 **Abstract**

27

28 According to a report from the United Nations on June 17th, 2019, the world population will
29 increase by 2 billion people by 2100, resulting in a global population of nearly 11 billion people.
30 How to achieve a sufficient and healthy diet while limiting the environmental impact of the
31 food industry? Certain solutions emerge such as the valorisation of the circular economy and
32 in particular the valorisation of collagen/gelatine-derived by-products. The novelty of this
33 review lies in the integrative nature of the presentation of collagen and its derivatives such as
34 gelatine and gelatine hydrolysates. It gathers critical insights into the subject that often do not
35 appear in this genre of literature. This review therefore includes the historical context of its use,
36 their physicochemical properties, the endogenous production of collagen. An entire chapter is
37 dedicated to the different extraction and analysis methods currently available. The last chapter
38 is devoted to the various scopes of application of bioactive peptides arising from collagen
39 hydrolysis, varying from anti-oxidant, anti-hypertensive uses, as well as the numerous
40 applications for the regulation of glucose metabolism.

41

42 **1 Introduction**

43

44 To meet the needs of the food and feed sectors, global demand for protein is expected to
45 increase by approximately 40% by 2030, representing a 7% annual increase [1]. To meet this
46 strong demand while limiting the economic and ecological impacts, alternatives such as the use
47 of by-products from the vegetables, fishing, or meat industry would constitute significant
48 protein resources that are presently still underexploited.

49 In the global functional food market and in light of societal developments and climatic change,
50 the demand is mainly for so-called natural, non-ultra-processed products, with low
51 environmental impacts and among these, collagen peptides have become a high priority must
52 for many consumers. According to a new report from Grand View Research, Inc., the global
53 collagen market is expected to reach 5.87 billion Euros by 2025, with a compound annual
54 growth rate (CAGR) of 6.5% over the forecast period. The interest in collagen peptides
55 generated from gelatine lies in their bioavailability and ease of digestion. They have many
56 applications in health care, such as incorporation in designed formulas or as a powder that is
57 to be diluted. Collagen peptides do not contain hormones, chemicals, or antibiotics. Their
58 innocuousness is also one of the arguments for their consumption, whether by people who are
59 ill or people concerned about their health. They are used by athletes for joint and tendon health
60 during their daily workouts [2]. The increasing prevalence of several diseases such as obesity
61 and diabetes [3], as well as a host of pathologies associated with metabolic syndrome, has led
62 to a surge in demand for natural food sources containing such bioactive peptides as an early
63 treatment option [4]. Bioactive peptides are used in a wide range of therapeutic areas, from
64 alteration of the immune system to the gastrointestinal system to antihypertensive uses [5].
65 Indeed, the market for bioactive peptides, worth USD 48.65 billion in 2020, continues to grow
66 and is expected to reach USD 95.71 billion by 2028, at a CAGR of 8.86% between 2021 and 2028
67 [6].

68
69 The novelty of this review lies in its propensity to discuss collagen under integrative aspects,
70 which have often been eluded to date. First of all, via its historical use in ancient Egypt, Chinese
71 traditional medicine, as well as in more contemporary times in Europe, to finally describe the
72 different uses in our current societies. Then, the fundamental aspects of its structure and

73 physicochemical properties, as well as the sources and methods of extraction, are addressed.
74 Additional novelty is added regarding the methods of identifying peptides generated by the
75 hydrolysis of food matrix to understand all the processes involved in the exploitation of such
76 products, which are often hard to identify exhaustively in complex mixtures. Finally, a section
77 on the bioactivities of collagen hydrolysates shows the versatility of their uses in today's and
78 tomorrow's health, with the significant added value of these products, which is often
79 overlooked in contemporary therapeutic arsenals, such as in glucose metabolism management
80 for instance.

81

82 **2 Collagen state of the art: History, structural, and physicochemical** 83 **properties, biosynthesis, industrial processes, and different sources**

84

85 **2.1 Historical use of collagen**

86

87 According to the literature, collagen and especially its hydrolysed form gelatine have been used
88 by humans for thousands of years. In ancient Egypt, gelatine was extracted by boiling animal
89 skins such as cowhides and bones [7]. This method produced glue that was then used for the
90 confection of clothes and supplies (Adams, 2021), but it has also been found in coloured wall
91 paintings dating back 4400 years (Walker, 2020). In addition to this use, the consumption of
92 gelatine extracted from bone broths was also a common practice in everyday life. The culinary
93 use of gelatine quickly became commonplace in medieval Baghdadi culture, being incorporated
94 into many preparations from the early 10th century [10]. In China, its use as an adhesive agent
95 can be traced back to 1980–1450 BCE [11]. From a medicinal point of view, oriental and

96 particularly traditional Chinese medicine describes the use of gelatine as far back as 771 BC
97 [12]. The use of gelatine in the medical field was for a long time confined to China, Japan, and
98 some East Asian countries.

99 It was not until the beginning of the 19th century that the extraction and use of gelatine were
100 introduced in the Western world. First of all, in France, Denis Papin invented a more efficient
101 method of extracting gelatine. Then, the English chemist Charles Hatchett carried out a further
102 study on the use of acids on bones in 1806. From there, he developed a patent for the
103 application, and it was used on a large scale from 1812 onward in Paris for food purposes. The
104 methodology used at the time was divided into six stages. Two acid macerations (the first with
105 hydrochloric acid) for 24 hours, then washing with cold water, followed by immersion in boiling
106 water, a sodium carbonate solution, and finally washing with cold water until neutral and drying
107 in an oven [13]. The first use of gelatine in the Western world for medical purposes was
108 documented in France in the 19th century, with the use of gelatine as an excipient in the oral
109 administration of bitter medicines such as syphilis treatments, pioneered by the pharmacist
110 François Mothes, who filed a patent for the invention of the gelatine capsule in 1833 [14]. The
111 dietary value of gelatine has been discussed at length by chemists, researchers, nutritionists,
112 and others. It was used during the siege of Paris in 1870 by the Prussians when the resulting
113 famine had to be contained. It was also prescribed in hospices to feed the most destitute and
114 the sick in hospitals [13]. Although the nutritional value of gelatine has been debated for almost
115 three centuries, at present, the recycling of various waste from the food industry [15]–[19] is
116 leading to study of its physiological effects in greater depth, beyond the purely nutritional
117 aspect.

118 Collagen and the gelatine derived from it are sources of very high added value that can be found
119 in several fields of application. In medical areas, collagen films are used as a drug delivery

120 system for infected corneal tissue [20], [21], as well as in tissue engineering for wound healing
121 [22] and cosmetology/dermatology [5], [23]. Collagen solutions have significant advantages,
122 notably their low production cost, and the large quantities indirectly produced by the food
123 industry, as well as their bioavailability, biocompatibility, and lack of adverse effects [24].

124

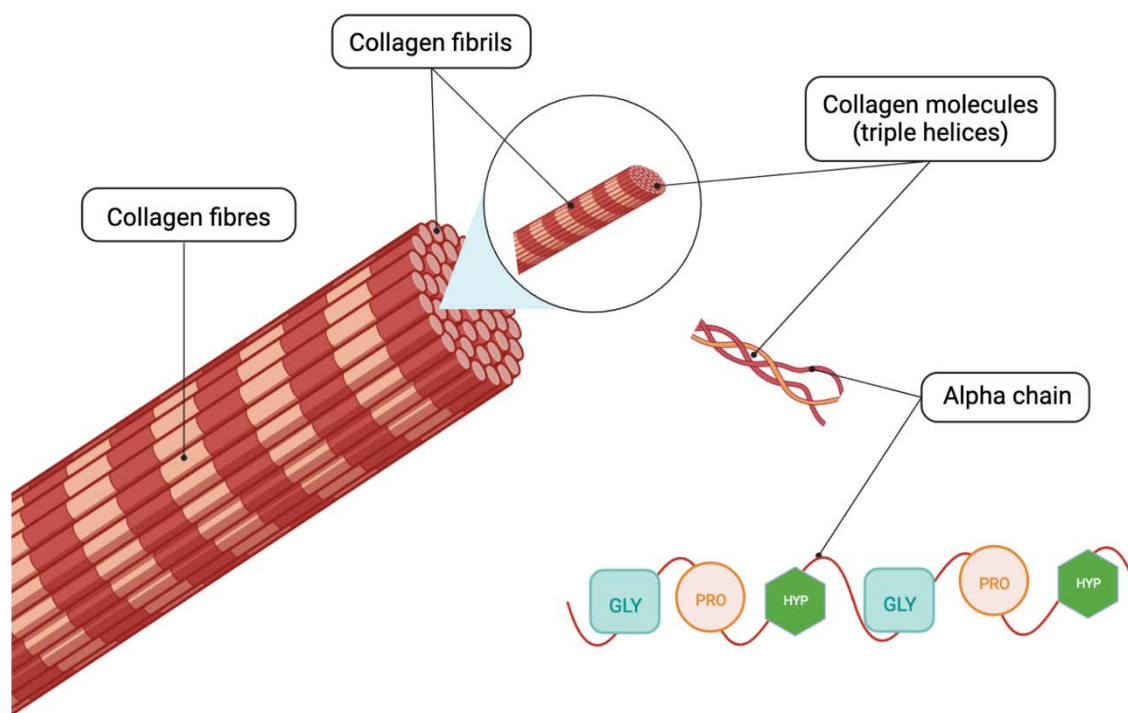
125 2.2 Structural description

126

127 Collagens are water-soluble proteins present in connective tissues, skin, tendons, and bones.
128 They are the most abundant fibrillar structural proteins, as they constitute up to 25% to 35% of
129 the total proteins of the body [25]. The amino acid sequences of collagens and their structures
130 are highly conserved in mammals. There are different types of collagen, and 29 are currently
131 enumerated, of which the most abundant is type I collagen, which is the predominant form in
132 the human body [26]. Up to now, the 29 types of collagens in vertebrates have been classified
133 by their function and domain homology, as represented in **Table 1**. They can be divided into
134 fibrils, networks, beaded filaments, anchoring fibrils, and collagen associated with interrupted
135 triple helix (FACIT) fibrils. Of these, the most common form is fibrillar collagen, which is present
136 in most connective tissues [27].

137 Collagen conformation can be divided into four structures: the amino acid triplet Gly-Pro-Hyp,
138 forming the primary structure; the secondary structure (the alpha-chain); the tertiary structure
139 (triple helix conformation formed by two alpha-1 and one alpha-2 collagen chains); and the
140 quaternary structure (fibrils) forming collagen fibres [28]; as illustrated in **Figure 1**.

141



142

143 *Figure 1. Schematic of the four-level structure of type I collagen fibres.*

144 *The conformation of collagen can be divided into four structures: the amino acid triplet Gly-Pro-*
 145 *Hyp, which forms the primary structure; the secondary structure (the alpha chain); the tertiary*
 146 *structure (triple helix conformation consisting of two alpha-1 and one alpha-2 collagen chains;*
 147 *and the quaternary structure (fibrils), which form the collagen fibres. GLY: glycine, PRO: proline*
 148 *and HYP: hydroxyproline. Illustration created with BioRender.com*

149

150 The structure of the collagen alpha chain is determined by the amino acid sequence. Out of the
 151 20 proteinogenic amino acids that can be found in collagen, glycine, proline, and
 152 hydroxyproline are the most frequent [29]. Indeed, the alpha chain consists of the repeating
 153 unit Gly-X-Y, where X is often proline (Pro) and Y is often hydroxyproline (Hyp). The content of
 154 proline and hydroxyproline is approximately 30% in mammalian gelatine, 22%–25% in warm
 155 water fish gelatine (Tilapia and Nile perch), and 17% in cold water fish gelatine (cod) [30], [31].
 156 Comparative studies on the rheological properties of terrestrial mammalian and fish gelatine

157 concluded that the differences observed are due to the content of the specific imino acids,
158 proline and hydroxyproline, which stabilise the conformation when gelatine forms a gel. Thus,
159 a lower content of proline and hydroxyproline results in a less compact gel with a lower melting
160 temperature [32]–[35]. Therefore, it is important to keep in mind the composition of the
161 collagen molecule and also post-translational modifications (PTMs) such as hydroxylation of
162 prolines, in order to make relevant and comprehensive identifications of the peptides
163 generated following hydrolysis, using bioinformatic tool features accordingly.
164

165 **Table 1. Types, forms, distribution, and functions of collagens (Lin et al., 2019)**

Type	Molecular form	Tissue distribution	Function
I	Fibrils	Noncartilaginous connective tissues, e.g., skin, bone, tendon, dentin, ligaments, cornea	Structural component
II	Fibrils	Hyaline cartilage, vitreous body, nucleus pulposus	Structural component
III	Fibrils	Idem as type I	Structural component
IV	Network	Basement membranes	Presynaptic organizer
V	Fibrils	Idem as type I	Structural component
VI	Beaded filaments	Muscle, skin, cartilage, placenta, lungs, vessel wall, vessel	Structural component
VII	Anchoring fibrils	Skin, dermo-epidermal junction, oral mucosa, cervix	Retaining dermal-epidermal adhesion
VIII	Network	Descemet's membrane	Structural and signaling component
IX	FACIT	Idem as type II	Retaining tissue integrity
X	Network	Hypertrophic cartilage, growth plate	Regulating endochondral ossification
XI	Fibrils	Idem as type II	Structural component
XII	FACIT	Tendon, skin, periodontal ligament	Not clear
XIII	Transmembrane	Epidermis, hair follicle, endomysium, intestine, chondrocyte, lung, liver, dermo-epidermal junction	Regulating bone formation
XIV	FACIT	Skin, tendon, vessel wall, placenta, lungs, liver	Maintaining mechanical tissue
XV	Endostatins	Fibroblast, smooth muscle cell, kidney, pancreas	Not clear
XVI	FACIT	Fibroblast, amnion, keratinocyte	Protecting neurons against A β toxicity
XVII	Transmembrane	Derma-epidermal junction	Structural component
XVIII	Endostatins	Lung, liver, vascular, epithelial basement membrane	Associated with eye development and basement membrane integrity
XIX	FACIT	Central neurons, human rhabdomyosarcoma	Associated with hippocampal synapses
XX	FACIT	Corneal epithelium, embryonic skin, sternum cartilage, tendon	Not clear
XXI	FACIT	Heart, placenta, stomach, jejunum, skeletal muscle, kidney, lung, pancreas, lymph node	Not clear
XXII	FACIT	Myotendinous junction, cartilage-synovial fluid, hair follicle-dermis	Retaining tissue junctions
XXIII	Transmembrane	Prostate	Associated with prostate cancer
XXIV	Fibrils	Cornea, bone	Regulating osteoblast differentiation
XXV	Transmembrane	Precursor protein for collagenous Alzheimer amyloid plaque component	Associated with Alzheimer's disease
XXVI	Beaded filaments	Testis and ovary	Associated with generation and modeling of tissues
XXVII	Fibrils	Cartilage, skin, cartilage, cornea, retina, major arteries of the heart	Cartilage calcification
XXVIII	Beaded filaments	Peripheral nerves, skin calvaria,	Not clear
XXIX	Beaded filaments	Skin, lung, small intestine, colon	Associated with atopic dermatitis

166

167

168 2.3 Physicochemical properties

169

170 Gelatine is a natural polymer similar to its collagen precursor. After collagen separation,

171 gelatine can be extracted by either alkaline hydrolysis or acid hydrolysis. The latter determines

172 the isoelectric point (pI) of the gelatine. With acid hydrolysis, gelatine is classified as type A,

173 with an pI \approx 5. Extraction with alkaline media yields type B gelatine, with an pI \approx 9. Gelatine is

174 classified as a physical gel [34]. That is to say, the interactions or bonds between the chains that
175 make up the gel are physical in nature (van der Waals interactions and hydrogen bonding at E
176 ≈ 2 kcal/mole). Some physical gels, such as alginate, are not thermoreversible. However, the
177 binding energy of gelatine is relatively weak and can form thermoreversible gels. The most
178 important physical properties of gelatine gels are strength and melting point. These are
179 determined by molecular masses and complex interactions that depend on the amino acid
180 composition and the ratio of α/β chains present in gelatine [36]. There is a strong correlation
181 between gelatine gel strength and chain content. Indeed, differential rheological properties
182 observed between mammalian and fish gelatines are mostly due to the content of the amino
183 acids, proline and hydroxyproline, which are responsible for stabilisation of the gel network.
184 Moreover, gelatines with a higher proportion of α -chains have higher gel strengths. On the
185 other hand, a high percentage of peptides with molecular masses higher or lower than the α
186 chain reduces the gel strength [37]. The gel strength of commercial gelatines is expressed in
187 Bloom values, which is the weight in grams required by a specific plunger to push a standard
188 temperature-controlled gel surface to a specific depth under standard conditions [38]. The gel
189 strengths of commercial gelatines range from 100 to 300, but gelatines with Bloom values of
190 250 to 260 are the most desirable [39].

191 Fish gelatine typically has a Bloom value ranging from 0 to 270 (tested under standard Bloom
192 test conditions), compared to the high Bloom values of bovine or porcine gelatine of 200–240.
193 Certain gelatines from warm-water fish have been reported to have relatively high Bloom
194 values, as has high-Bloom porcine gelatine [40]. Only gelatine from the skin of warm-water fish
195 such as Tilapia [41]–[43] and grass carp [44] has a very high gel strength.

196 The wide range of Bloom values found in different gelatines is due to differences in the proline
197 and hydroxyproline content of different types of collagens and is also related to the

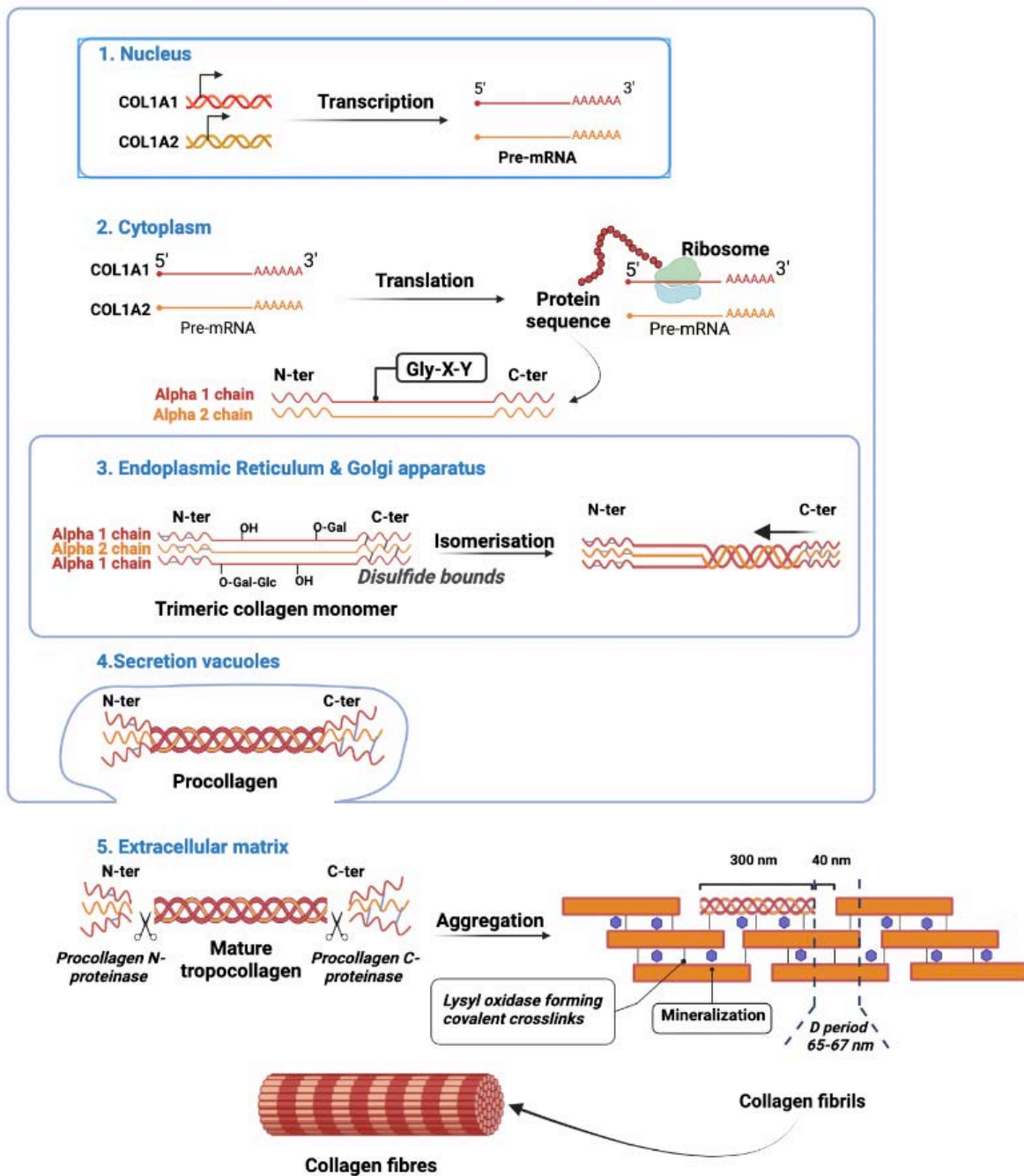
198 temperature of the animal's habitat. Badii and Howell showed that hydrophobic amino acids
199 (Ala, Val, Leu, Ile, Pro, Phe, and Met) may also contribute to the high Bloom value of Tilapia fish
200 gelatine [45].

201

202 2.4 Endogenous production

203

204 Collagen biosynthesis, from gene transcription in the nucleus to the aggregation of collagen
205 heterotrimers into large fibrils, is a complex multistep process [46]. The endogenous
206 production of collagen takes place in multiple successive stages, described hereafter and
207 outlined schematically in **Figure 2**, and is carried out within specialised cells, the fibroblasts.



208

209 *Figure 2. Schematic representation of collagen synthesis.*

210 *From collagen genes in the nucleus (1), mRNA translation into ribosomal protein synthesis in the*

211 *cytoplasm (2), post-translational modifications (PTMs) in the rough endoplasmic reticulum (3),*

212 *vacuolar secretion (4), and maturation of tropocollagen into fibril formation, leading to collagen*

213 *fibrils in the extracellular matrix (5). Illustration created with BioRender.com*

214

215 **2.4.1 Nuclear level**

216

217 At the nuclear level, the genes coding for type 1 collagen, *COL1A1* and *COL1A2*, are transcribed
218 into pre-mRNA and then capped at the 5'-end and polyadenylated at the 3'-end. The mRNAs
219 are translated by ribosomes into collagen precursors, the $\alpha 1$ or $\alpha 2$ procollagens. They possess
220 globular polypeptide domains at their ends, the N-terminal and C-terminal propeptides, 15 kDa
221 on the N-terminal side and 30 kDa on the C-terminal side, respectively, which help maturation
222 of the protein **Figure 2** (1).

223

224 **2.4.2 Endoplasmic reticulum and Golgi apparatus**

225

226 These procollagens are introduced into the lumen of the rough endoplasmic reticulum during
227 their synthesis by the presence of a signal peptide. After removal of the signal peptide by signal
228 peptidase, several PTMs occur, such as the hydroxylation of proline and lysine by prolyl 3-
229 hydroxylase, prolyl 4-hydroxylase, and lysyl hydroxylase, respectively. These three enzymes
230 require cofactors to function: ferrous ions, 2-oxoglutarate, molecular oxygen, as well as
231 ascorbic acid, the latter being of great importance in the synthesis of collagen by acting as an
232 electron-donor that keeps iron in the ferrous state, thereby maintaining the full activity of
233 collagen hydroxylases [47], [48]. The 4-hydroxyprolines form hydrogen bonds between collagen
234 polypeptides and thus play a key role in the thermal stability of the triple helix domain, but also
235 that of the monomer and on a larger scale that of the collagen fibril [28]. Concomitantly the
236 role of 3-hydroxyprolines still needs to be determined, but when prolyl 3-hydroxylase does not
237 catalyse the addition of hydroxyl groups for various reasons (e.g., mutation, inhibition, etc.),
238 their absence causes pathologies such as osteogenesis imperfecta [49]. In addition to their

239 stabilising role, hydroxyprolines also provide attachment sites for carbohydrates. Through
240 hydroxylysyl-galactosyltransferase and galactosylhydroxylysyl-glucosyltransferase enzymes,
241 glucosyl- and galactosyl groups, respectively, can be transferred to the collagen molecules
242 **Figure 2 (3)**.

243 The presence of disulphide bridges also plays a structure-stabilizing role, on the N-terminal
244 propeptide side, the bridges are intracatenal, whereas, on the C-terminal propeptide side, they
245 are formed between the three alpha polypeptide chains. The C-terminal propeptide plays a
246 crucial role in the assembly of the three alpha chains into the trimeric collagen monomers.
247 Indeed, the formation of disulphide bonds between each C-terminal propeptide provides the
248 alignment and initiation of the formation of the triple helix in the direction of the N-terminal
249 extremity **Figure 2 (3)**. This step relies on the activity of peptidyl-prolyl *cis-trans*-isomerase and
250 collagen-specific chaperones (HSP47) [50], [51]. Once procollagen is formed in the Golgi
251 apparatus, procollagen molecules are secreted in the extracellular environment *via* secretory
252 vacuoles **Figure 2 (4)**.

253

254 **2.4.3 Extracellular matrix**

255

256 Two specific peptidases cleave the N-terminal and C-terminal propeptides, namely procollagen
257 N-proteinase and procollagen C-proteinase, respectively, allowing the formation of mature
258 tropocollagen [52]. After cleavage of the propeptides, tropocollagen bundles aggregate into
259 fibrils near the cell surface. The formation of aldehydes at the level of collagen telopeptides by
260 lysyl oxidase allows the formation of covalent cross-links *via* the substitution of a carbonyl
261 group for the amine group of a lysine residue, which results in spontaneous bridging into
262 several tropocollagen bundles. Minerals, such as hydroxyapatite crystals, are present between

263 the fibrils. The monomers are 300 nm long and there are 40 nm gaps between consecutive
264 monomers, which results in the characteristic striated appearance of type I collagen fibrils by
265 electron microscopy. Finally, collagen fibrils aggregate into collagen fibres. Altogether, a
266 collagen fibre's molecular mass is 300,000 Da, the length is 280 nm, and the width is 1.4 nm
267 [53].

268

269 2.5 Collagen hydrolysate obtention processes

270

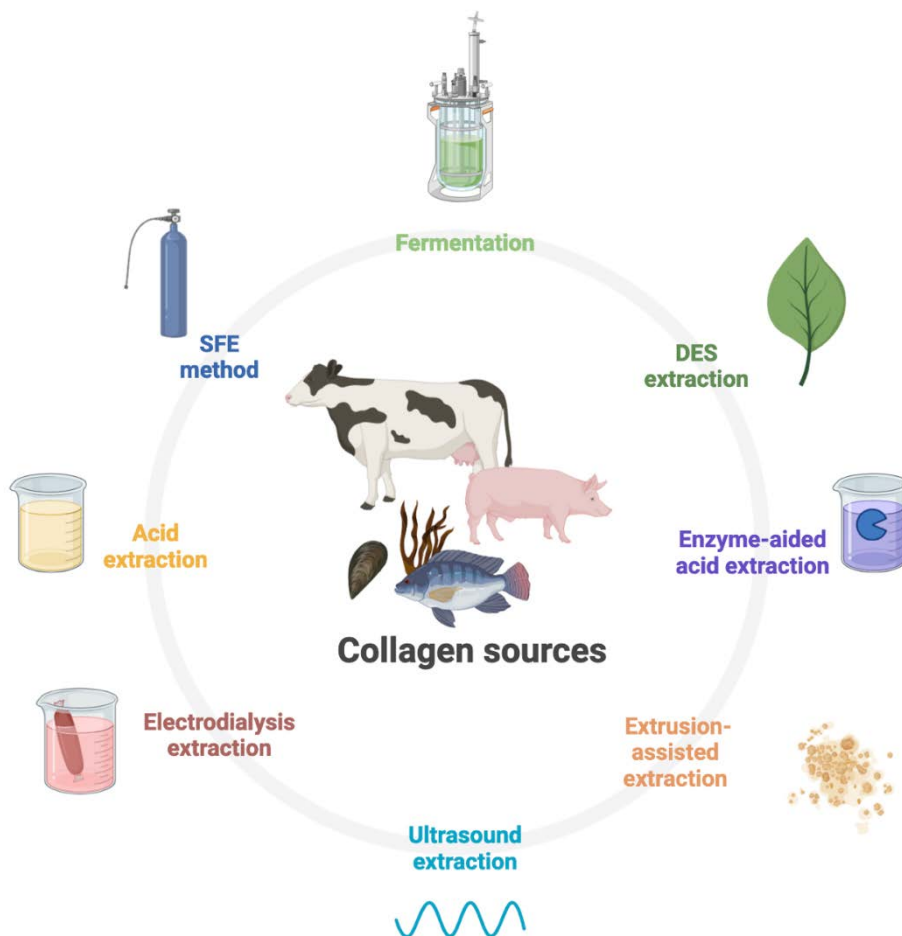
271 The process of obtaining low-molecular-size collagen hydrolysates requires several treatments,
272 as described below. Raw materials such as bones, cartilage, skin and tendons, and scales from
273 different by-product sources (cattle, poultry, and aquaculture industries) are first pre-treated
274 after grinding to remove fat and minerals. The pre-treatment process ensures removal of non-
275 collagenous materials and enrichment of collagen (as gelatine). However, as a result of the
276 presence of cross-linked collagen in connective tissue, the collagen dissolves very slowly,
277 including in boiling water. Furthermore, the triple helix conformation makes collagens very
278 resistant to most proteases [29]. Thus, gentle pre-treatment is often required to disrupt the
279 triple helix structure to extract the collagen (or gelatine).

280 Unlike fish-derived collagen, mammalian collagen generally requires a higher pH to remove
281 non-collagenous proteins [54]. Lipids are also partially eliminated during this process [55].

282 Nevertheless, to achieve maximal lipid removal, organic solvents, such as chloroform-
283 methanol- [56] and hexane-extraction [57], are usually required. The main issue with the use
284 of organic solvents is the potential for adverse effects on public health, the environment, and
285 general safety [58].

286 Acid pre-treatment for demineralisation followed by alkaline neutralisation can provide a
287 neutral extraction medium, resulting in a high yield of gelatine that has desirable gelling
288 properties [59]. An example in the literature has demonstrated the benefits of using phosphoric
289 acid instead of acetic acid (AcOH) to disrupt skin tissues from unicorn leatherjackets (*Aluterus*
290 *monoceros*) [60].

291 Once the pre-treatment stage has been completed, the process continues with extraction of
292 the collagen. There are several different methods, as depicted in **Figure 3**, and the most
293 common ones are those based on the solubilisation of collagen in acidic solutions, with or
294 without the help of enzymes such as pepsin, and alkali solutions [61]. There are also options to
295 optimise this extraction, such as the application of heat treatment or high-pressure [58].



296

297 *Figure 3. Schematic representation of current collagen extraction methods.*

298 *Collagen extraction methods are varied and are tailored to meet specific demands. These*
299 *methods are used on many sources of bovine, porcine, and marine origin. Among these*
300 *extraction methods are those using conventional methods via acid extraction and enzyme-aided*
301 *acid extraction and also non-typical methods such as fermentation, deep eutectic solvent (DES),*
302 *ultrasound, electrodialysis, and supercritical fluid (SFE) extraction. Illustration created with*
303 *BioRender.com*

304

305 **2.5.1 Acid extraction methods**

306

307 Acid treatment is used to extract type 1 collagen from porcine tissues and fish skins. The
308 different concentrations of acid tend to result in thorough extraction and change of the
309 physicochemical parameters of the matrices by decreasing their final pH, modifying the
310 electrostatic interactions and thus affecting their structures. This also has an impact on the
311 solubility of the final product [26].

312 The literature describes several protocols for the extraction of marine and fish
313 collagen/gelatine, which generally involves use of acid solutions. Indeed, the H⁺ ions liberated
314 by the acids allow water molecules to be attracted to the collagen fibres, which are thus
315 trapped between the charged polar groups by hydrogen bonds or by the uncharged polar
316 groups and the negatively charged atoms by electrostatic forces. The collagen fibre thereby
317 becomes saturated with water and swells, ultimately causing it to dissolve [62].

318 The method using AcOH is regularly used. Indeed, literature data describe the extraction of
319 collagen from several aquatic species, both marine and freshwater: grass carp
320 (*Ctenopharyngodon idella*) skin [26]; Alaska pollock skin [63]; swim bladders of yellowfin tuna

321 (*Thunnus albacares*) [64], catla (*Catla catla*) and rohu (*Labeo rohita*) skins [65]; small-spotted
322 catshark skin [66]; Tilapia (*Oreochromis niloticus*) skin and scale [67]; scales of five species from
323 Vietnam and Japan lizard fish (*Saurida spp.*) horse mackerel (*Trachurus japonicus*), grey mullet
324 (*Mugil cephalis*), flying fish (*Cypselurus melanurus*), and yellow black seabream (*Dentex*
325 *tumifrons*) [68]; three Vietnamese freshwater fish skins [69]; elasmobranch by-products [70];
326 and jellyfish (*Acromitus hardenbergi*) [71].

327 Most of these methods use concentrations of AcOH ranging from 0.1 to 1 M. This method is
328 not the only one used since other solutions are also described, such as the use of hydrochloric,
329 citric, and lactic acid [72].

330

331 **2.5.2 Acid and enzyme-aided acidic collagen extraction**

332

333 As an example, the extraction of collagen from the swim bladder of Atlantic cod (*Gadus*
334 *morhua*) has been carried out using two different methods. The first method is with AcOH, and
335 the second involves combining AcOH and pepsin. The authors demonstrated that the combined
336 approach increased the final yield from 5.72% to 11.14% [73]. Other studies of the combined
337 use have demonstrated their effectiveness in the extraction of collagen from Tilapia skins and
338 gills [74] and from Chilean mussel byssus [75].

339

340 **2.5.3 Various extraction methods**

341

342 Unusual processes such as **fermentation** for collagen extraction have been described for Nile
343 Tilapia skins [76], or by **electrodialysis** for Pufferfish (*Takifugu flavidus*) skins [77]. The latter
344 appears to be rather promising as it allows this extraction to be carried out efficiently and cost-

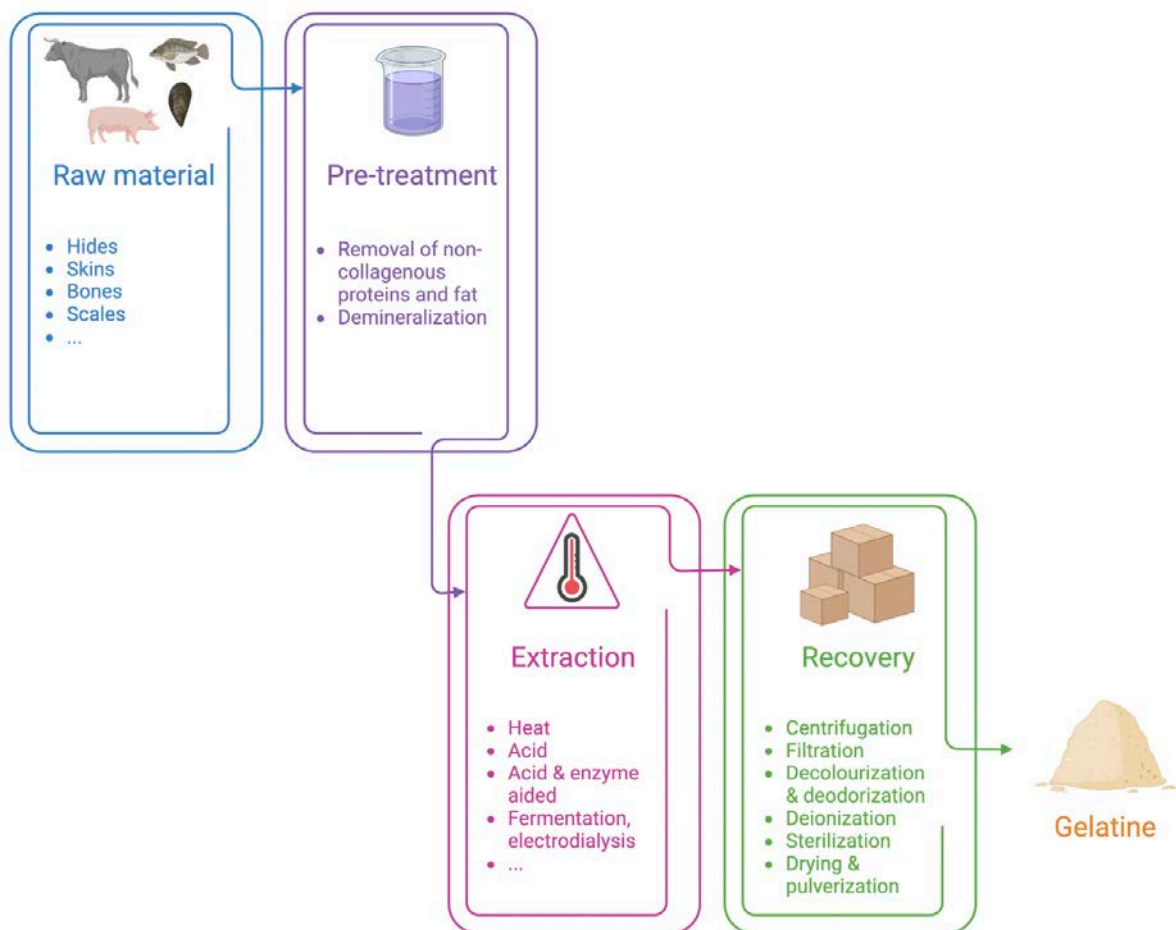
345 effectively, notably by reducing the amount of water waste by 95% while still maintaining a high
346 extraction yield.

347 **Deep eutectic solvent** (DES) extraction is a method involving a two-compound tandem: a
348 hydrogen bond receptor and a hydrogen bond donor. This approach favours the use of non-
349 toxic, abundant, and biodegradable green solvents such as choline chloride, oxalic acid, urea,
350 and ethylene glycol, among others, allowing extraction of high-value-added products from
351 numerous by-products from plants, mammals, and aquatic sources. A study on the extraction
352 of collagen from cod skins using a mixture of oxalic acid and choline chloride resulted in a yield
353 of almost 90% [78], thus demonstrating the benefits of this extraction technique versus
354 conventional ones.

355 **Supercritical fluid extraction** (SFE) is based on the use of fluids at pressures and temperatures
356 beyond the critical point, allowing the capacities of a solvent to be modified through physical
357 change. CO₂ is most often used as it is inexpensive, stable, and has high availability, as well as
358 rather simple conditions of use. Studies from the same author provided evidence of the
359 unambiguously better performance of SFE versus acid and pepsin-aided AcOH extraction
360 methods on cod skins [73].

361 Among the various unconventional methods, several studies have investigated the use of
362 **ultrasound** to improve collagen extractability. Ultrasound induces cavitation in the liquid
363 solvent, creating micro bubbles that in turn cause tissue damage, thereby increasing the
364 tissue/solvent contact area. The higher the frequency, the smaller the bubbles and the more
365 effective they are. This method is suitable not only for marine sources but also for plant and
366 animal sources [79], although the harsh conditions of ultrasound could affect the
367 physicochemical and molecular characteristics of the extracted collagen.

368 **Extrusion-assisted extraction (EAE)** increases the yield of collagen extracted from Tilapia scales
369 by two- to three-fold compared with a simple acid extraction, making this method relevant in
370 the context of tissue pre-treatment [80].
371 After centrifugation, the contaminating particles and the remaining fat are eliminated [81], and
372 hydrolysis can be performed, whether enzymatic, physical, or chemical. The batches are
373 decolourised and deodorised with activated carbon and then filtered. The salts are removed
374 from the hydrolysates *via* ion-exchange columns, which are then concentrated by vacuum
375 evaporation [58]. The hydrolysates are sterilised by heat treatment before pulverisation and
376 then packaged [82], as depicted in **Figure 4**.



377

378 *Figure 4. Schematic representation of collagen extraction from raw material to gelatine finished*
379 *product.*

380 *The first step consists of washing the tissues, and the second step is a pre-treatment to remove*
381 *non-collagenous proteins and fat as well as minerals. The collagens can then be extracted by*
382 *several processes according to the manufacturer such as heat, acid, or enzyme-aided acidic*
383 *methods, or the less conventional methods discussed above. The extracted gelatine is recovered*
384 *after centrifugation and filtration, as well as decolourisation and deodorisation. Batches are*
385 *deionized and sterilized to avoid contaminations before being dried and pulverized.*

386

387 All of these extraction methods are carried out with the aim of reducing or even eliminating
388 the release of solvents that are harmful to the environment and, via the "One Health" principle,
389 preserving human and animal health at the same time. These innovative methods also reduce
390 the energy costs associated with extraction by reducing the heating time of the matrices and
391 the addition of costly enzymes and by implementing methods such as sonication, which provide
392 a more efficient way of obtaining high-quality peptides of interest at a lower energy cost. These
393 aspects are all the more important as the current challenges linked to the energy crisis do not
394 allow these issues to be ignored at any scale of society.

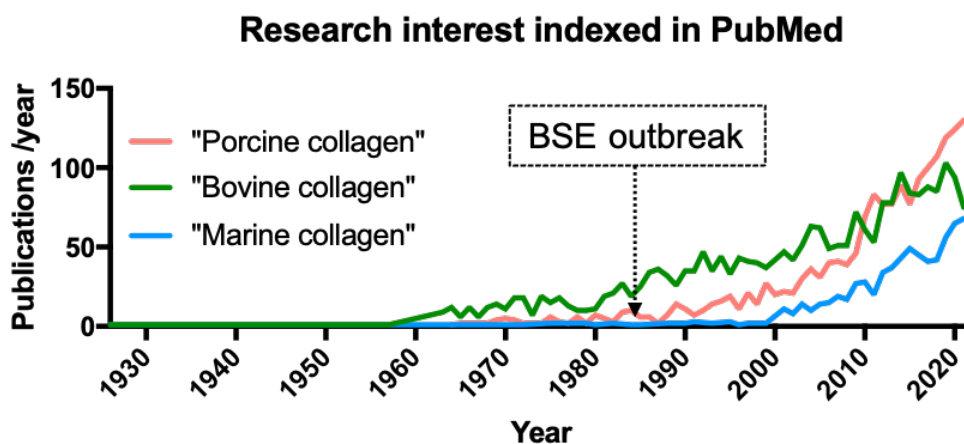
395

396 **2.5.4 Sources of collagen and gelatine**

397

398 Collagen can be retrieved from skin, bones, and tendons of several sources, such as porcine,
399 bovine, avian, and marine species. Many applications have been described in the literature due
400 to intrinsic features such as biocompatibility and degradability [83]. Bovine collagen is mostly
401 used for extra-oral wounds [84], and marine collagen such as Tilapia skin is used as a xenograft

402 after burn injuries [85]. With its low immunogenicity and reduced chance of rejection, it is a
403 material of choice for several fields of application such as surgery and cosmetology, drug
404 delivery, and food [86], [87].
405 Due to the variability of collagen sources, the versatility of use is equally broad, like no other
406 available protein. Nevertheless, the use of animal collagen has been severely compromised
407 over the past several decades due to the emergence of severe diseases. Sanitary crises, such
408 as the emergence of bovine spongiform encephalopathy (BSE) in 1985, forced the market to
409 be more cautious in its use [88]. The use of mammalian collagen also increases the risk of
410 immune reaction in 3% of the population, as well as the transmission of zoonoses such as foot
411 and mouth disease and transmissible spongiform encephalopathy [46]. Additionally, in several
412 cultures and religious backgrounds, such as Jews, Muslims, and Hindus, porcine and/or bovine
413 collagen use is prohibited or subjected to special religious requirements [89]. Thus, for the past
414 three decades, marine collagens (in blue) as alternatives have emerged as a solution of choice
415 as the number of scientific researches had increased over time (Figure 5).



416
417 *Figure 5. Increase in research interest in porcine, bovine, and marine collagens.*

418 *Articles indexed in PubMed (<https://pubmed.ncbi.nlm.nih.gov>) with the keywords "porcine*
419 *collagen", "bovine collagen", and "marine collagen" published between 1926 and 2021 (last*

420 accessed on August 2022) and the time when the BSE outbreak occurred, inducing an increase
421 of research interest for marine collagen in the late 1990s.

422

423 As the interest in marine collagen sources increased in the past 30 years, two categories
424 emerged: vertebrate and invertebrate marine animals. The latter category is the most widely
425 exploited, such as molluscs comprising cuttlefish and mussels, sponges, echinoderms
426 comprising sea urchins, starfish, jellyfish, and crustaceans such as prawns [90]–[95]. Regarding
427 vertebrate animals, the added value of such sources lies in the possibility to use by-products
428 derived from industrial processes [96], [97] such as scales and skin.

429

430 **3 Analytic aspects and identification methods for peptides derived** 431 **from agro-food**

432

433 **3.1 Background**

434

435 Food scientists have faced new challenges in the past several decades. To this end, they rely on
436 methods developed in medical, pharmacological, and/or biotechnological fields. Thus,
437 advanced analytical methodologies, omics approaches, and bioinformatics, often combined
438 with *in vitro*, *in vivo*, and/or clinical trials, are applied to investigate topics in food science and
439 nutrition.

440 Within this framework, several research projects are directed toward the study of peptides,
441 which are significant and multifaceted components of the human diet. They can be present
442 naturally in food, but they can also be formed from protein precursors during physiological

443 processes, for example, gastrointestinal digestion (GID) or food processing such as
444 fermentation or enzymatic hydrolysis [98]. Chemical hydrolysis is more commonly used in the
445 industry nowadays, but biological processes using enzymatic hydrolysis are more promising
446 when products with high nutritional value and improved functionality are required. In addition,
447 enzymatic hydrolysis generates less waste and is less costly and time-consuming [61].

448 Peptidomics applied to food is hence emerging as an important area of food science. These
449 technologies include the use of peptide separation techniques coupled with mass spectrometry
450 and bioinformatics for sequencing, identification, and quantification of peptides as well as the
451 PTMs present on them [99]. The term "Omics" derives from the word "ome" from the Greek
452 terminology -ωμια ("-oma"), which denotes a totality. As a result, when the totality of objects in
453 a given biological system (such as genes, proteins, peptides, or metabolites) is combined with
454 the suffix "ome", the result is genome, transcriptome, proteome, peptidome, and metabolome,
455 respectively [100]. In 1996, Marc Wilkins first defined the proteome as the set of proteins
456 encoded by a genome at a given time and in a given environment [101]. The addition of the
457 suffix "omics" (genomics, transcriptomics, proteomics, peptidomics, metabolomics, etc.) then
458 defines the analytical technologies that make it possible to explore the totality of these
459 different biological molecules [102]. From then on, the combination of these approaches
460 constitutes a multi-omics approach [103], also called integrative omics or pan-omics, which
461 allows integration of data from various omics platforms to highlight the interrelationships of
462 the biomolecules involved and their functions [104].

463 In response to advances in omics technologies, research in the field of food science has
464 developed the concept of "foodomics" approaches to traceability, authenticity [105], well-
465 being, health, and food safety, which are synonymous with consumer confidence in the agro-
466 food sector [106]. Foodomics is defined as a discipline that studies the fields of food and

467 nutrition through the application of omics technologies to, for example, characterize and
468 demonstrate the beneficial effects of innovative food products on human health [107], [108].
469 One of the main challenges is, therefore, to improve our currently limited understanding of the
470 interaction of food components with genes, lipids, proteins, sugars, metabolites, etc. and the
471 consequences of these interactions. This knowledge will undoubtedly in the future allow a
472 rational design of strategies to achieve a beneficial impact on, for example, human health [109],
473 but also to ensure food safety by determining/measuring harmful components or organisms
474 that may be present in food at very low concentrations and much more.

475

476 3.2 Food peptidomics

477

478 The term peptidomics was introduced at the beginning of this century to represent the various
479 peptide analysis technologies for the complete identification of endogenous peptides in a
480 biological sample [110] and was subsequently rapidly applied in the clinical field and food
481 industry. Clinical peptidomics thus relates to the analysis of peptides from a cell, organ, or
482 organism, whereas food peptidomics relates to the analysis of the entire pool of peptides
483 present in food items or generated during processing, storage, or GID of foods [111].

484 Thus, different workflows have proven useful to help identify, characterise, and quantify
485 peptides in a particular approach. Among these, peptidomics has demonstrated that
486 allergenicity due to the presence of certain food protein allergens (e.g., wheat gluten, β -
487 lactoglobulin, milk casein, collagen [112]).can be greatly reduced after protein cleavage into
488 peptides. Liang and colleagues showed by *in vitro* ELISA that the immunoreactivity due to cow
489 milk allergenicity could be significantly reduced by a two-hour enzymatic incubation [113].
490 These findings have many applications, such as food formulations containing hydrolysed

491 proteins for feeding infants and children with food allergies or intolerances. In the same
492 context, peptidomics has also been applied to monitor the extent of proteolysis to ensure that
493 allergenic epitopes are destroyed [113].

494 Furthermore, the quality, safety, and authenticity of foods can also be studied using
495 peptidomics [114]. As the sequence of proteins in original and altered products differs to a
496 certain extent, the disparities are, therefore, reflected through peptidomics tools to identify
497 peptides that can serve as biomarkers [115]. For example, most commercial gelatines are
498 manufactured from porcine or bovine skin and/or bones. However, in some cases, such as
499 outbreaks of bovine spongiform encephalopathy, it may be necessary to differentiate between
500 bovine and porcine gelatine for safety considerations. Another aspect is that some religions ban
501 the consumption or use of pork derivatives. Therefore, it is necessary to develop biomarker-
502 based analytical methods to trace the species origin of gelatine [116], [117].

503 Peptidomics is also an asset for studying the sensory aspects of hydrolysates. Indeed, work on
504 predicting the bitterness of milk hydrolysates using peptidomics made it possible to identify the
505 peptides responsible for bitterness, combined with a targeted bioinformatics approach and
506 consumer sensory tests [118]. However, sensory study combined to peptidomics dedicated to
507 collagen peptide study are not numerous [119].

508 On the other hand, studies have highlighted that such peptides from food-derived proteins
509 may, beyond their nutritional role, confer additional health benefits [120], [121].

510 In this respect, food peptidomics has identified bioactive peptides found in different types of
511 foods such as milk [122], soy [123], and egg white [124]. They can also be derived from agro-
512 food by-products, which refer to the parts discarded after processing for direct consumption,
513 such as fish by-products (skin, bones, heads, viscera, muscles, etc.) [125] containing collagen
514 for instance in tilapia and halibut skin gelatines [126], Atlantic salmon skin collagen [127] and

515 boarfish [128], meat by-products (bones, blood, feathers, etc.) [129], and plant by-products
516 (pulp, kernels, skins, etc.) [130]. Once peptide bioactivity has been identified, the potential
517 activities in the organism, can be assessed after GID. However, many studies take the reverse
518 approach, which is all the more interesting, as the whole hydrolysate is tested. Indeed, a protein
519 matrix digested with *in vitro* simulated GID is tested for various bioactivities (e.g., inhibition of
520 DPP-IV, ACE, anti-microbial activity, etc.) and then the potential bioactive peptides are
521 identified by the use of methods that allow their identification, such as peptidomics. Thus,
522 bioactive peptide sequences have been identified from bovine haemoglobin after GID [131] or
523 from Tilapia by-product [132], halibut [126], Alaska pollock [133], and even Blue whiting [134],
524 [135].

525 Understanding and identification of food peptide components of the peptidome have become
526 crucial for the study of food allergenicity, biomarker discovery, combating fraud, identification
527 of bioactive peptides, study of the digestome, as well as valorisation of agro-food by-products
528 [111].

529

530 3.3 Current strategies for collagen-derived peptide characterisation

531

532 From an analytical point of view, after the cleavage of the proteins, the samples are usually
533 centrifuged to remove insoluble material. The peptide-enriched mixture is then subjected to
534 separation and/or fractionation processes for characterisation. Note that the prerequisites for
535 gas chromatography (GC) separation of molecules are volatility of the analytes and their
536 thermal stability. As peptides are not naturally volatile enough and, therefore, require chemical
537 derivatisation to vaporise, they are not suitable for GC separation [136], [137]. For this
538 separation purpose, liquid chromatography (LC) has been widely used. However, capillary

539 electrophoresis (CE) and non-capillary electrophoresis (peptide isoelectric focusing) have also
540 been applied, albeit to a lesser extent due to the accumulation of peptides on the walls of the
541 CE capillary as well as due to the potential precipitation of analytes for non-capillary techniques,
542 which leads to a deterioration in analytical performance (loss of efficiency and non-
543 repeatability) [138].

544 Depending on their physicochemical properties, a distinction is made among the liquid
545 chromatography methods between (i) steric (or size)-exclusion chromatography (SEC); (ii) ion-
546 exchange chromatography (IEX), and (iii) reverse-phase partition chromatography (RP). In this
547 context, separation can be carried out at low pressure (< 6 bar) with constant flow and applied
548 mainly for preparative fractionation techniques or at high pressure (HPLC) ranging from 100 to
549 300 bar used particularly for analytical purposes in order to obtain better separation efficiency
550 and resolution.

551

552 **3.3.1 One-dimensional chromatographic separation methods**

553

554 **Steric-exclusion chromatography** (SEC) allows separation of peptides according to their
555 hydrodynamic volume, which takes into account their shape and size. Although the molecules
556 migrate at the same speed, peptides with a low hydrodynamic radius have access to a larger
557 part of the total pore volume of the stationary phase than solutes with a high hydrodynamic
558 radius, which will, therefore, be eluted first. The choice of the column is crucial, as the porosity
559 and volume of the particles determine the separation capacity of this technique using an
560 isocratic elution mode. Dextran gel (Sephadex®), polyacrylamide gel (Bio-Gel®), or polystyrene
561 gel (Styragel®) resins can be used. The mobile phase must be capable of solubilising the sample.
562 It should also be noted that the sample should not be too concentrated, as the gel matrix pores

563 may become saturated [139]. Therefore SEC is currently used to characterise the peptide
564 molecular mass distribution of collagen hydrolysates [140], [141] but also the lot-to-lot
565 reproducibility of collagen hydrolysates [142] as well as the purification of collagen peptides
566 according to their average steric hindrance [143], [144].

567 **Ion-exchange chromatography** (IEX) separates peptides according to their overall charge. The
568 separation is based on a reversible interaction between the charged peptides and a resin of
569 opposite charge. Elution of the molecules is then carried out using a pH or salt concentration
570 gradient (NaCl or KCl). The choice of the type, composition, and pH of the mobile phase, which
571 is usually a buffer, is important, as it affects the elution of the peptides and the shape of the
572 peaks. The use of cationic or anionic columns is described in the literature [145], [146]. The
573 choice of strategy depends on the stability of the peptides at a given pH [147]. In overall, IEX is
574 currently used to purify collagen peptides from various origins and test different bioactivities
575 [148]–[151].

576 **Reverse-phase** (RP) **chromatography** is based on the use of an apolar stationary phase that
577 interacts with hydrophobic analytes through a polar mobile phase generally composed of
578 water/acetonitrile or water/methanol. Thus, the elution of peptides is based on their
579 hydrophobicity. The most commonly used RP columns include silicas grafted with linear chains
580 of 8 or 18 carbon atoms (C8 and C18). RP-chromatography is used to purify one or several
581 peptide fractions or just one peptide [152]–[154]. However, the greatest advantage of this
582 method is its compatibility with the electrospray ionisation (ESI) source in mass spectrometry
583 (MS) for collagen peptide identification [155]–[157]. To achieve increased chromatographic
584 resolution and mass detection sensitivity, nanoLC separations have been reported [158], [159].
585 The reduced internal diameter of the column (< 100 µm) as well as the reduced mobile phase

586 flow rate (a few nL.min⁻¹) lead to increased chromatographic resolution and analyte
587 concentration, which improve detection and mass fragmentation [160]–[162].

588

589 **3.3.2 Multidimensional chromatographic separation methods**

590

591 To improve the analysis of complex food peptidomes, most studies perform separations by
592 orthogonal or multidimensional chromatographic systems. However, these separations
593 present many challenges such as the need for automated or semi-automated systems, the need
594 for specific interfaces, and the compatibility of different mobile phases from one technique to
595 another [163]. SEC and IEX are often applied as a first step for fractionation prior to subsequent
596 peptide characterisation by RP-HPLC since they can be directly combined with MS. Studies
597 adopting this strategy have successfully identified functional peptides produced from different
598 sources; for example, from loach fish protein hydrolysate [164] or carp muscle hydrolysate.
599 Sample prefractionation using two-dimensional offline LC (IEX followed by SEC) was used to
600 test the bioactivities of the fractions. Subsequently, high-resolution RP-HPLC-MS/MS combined
601 with bioinformatics tools identified the peptide sequence Pro-Ser-Tyr-Val in loach protein
602 hydrolysate and Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val in carp muscle hydrolysate as antioxidant
603 sequences [165].

604

605 **3.3.3 Multi-hydrolysis strategy**

606

607 In complex matrices of proteins, such as the milk micellar caseins, the identification of particular
608 proteins and their PTMs requires successive and complementary identification methods.
609 Indeed, by combining a four-phase approach, combining a partial-, double-enzymatic

610 dephosphorylation and endoGluC hydrolysis, the authors were able to increase the number of
611 identified peptides to 90% but also increase the identification of PTMs (especially
612 phosphorylation), making this protocol a rapid and reliable method [166].

613

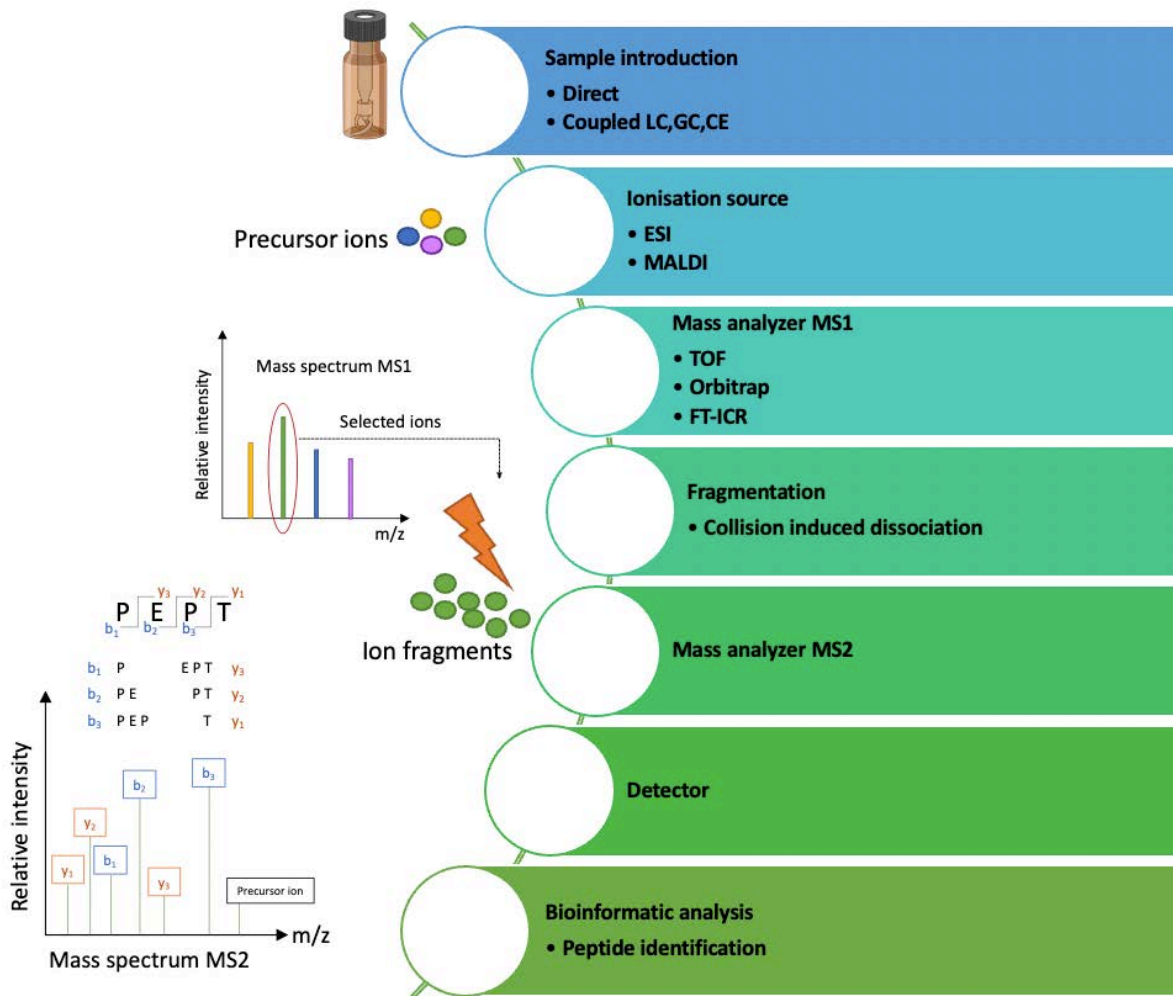
614 **3.4 Analytical techniques for peptide identification**

615

616 **3.4.1 Tandem mass spectrometry**

617

618 After chromatographic fractionation, the detection of peptides depends on their
619 physicochemical properties. Among these, their ultraviolet (UV) absorbance capacity is mainly
620 used through UV-visible-Diode Array Detectors (DAD). However, this mode of detection does
621 not allow the identification of peptides. On the other hand, MS/MS is the ideal detector for the
622 identification of molecules, including peptides (**Figure 6**).



623

624 *Figure 6. Schematic representation of peptide identification in MS/MS by collision-induced*
 625 *dissociation (CID) fragmentation.*

626 *The peptides are first positively ionised in the source and then a precursor ion beam is selected*
 627 *in the first MS1 analyser. This ion beam is then activated and fragmented in the CID collision cell*
 628 *in order to be analysed in the MS2 mass analyser to acquire the MS/MS spectrum. The peptide*
 629 *sequence is finally mainly reconstructed from the series of b- and y-fragment ions obtained.*

630

631 Molecules are ionised in the mass spectrometer ionisation source and then a beam of precursor
 632 ions is selected in a first mass analyser (MS1) and focused in the mass spectrometer collision
 633 region where it is then fragmented by high or low energy input, which can be done in many
 634 ways, such as collision-induced dissociation (CID). The kinetic energy is then transformed in part

635 into vibrational energy which leads to the fragmentation of the peptides. The fragments
636 produced are then sorted by a second analyser (MS₂) in order to acquire an MS/MS mass
637 spectrum of the fragment ions (the abscissa is a mass/charge ratio scale (m/z) and the ordinate
638 the relative intensity of the ions). The sequencing of the peptides is finally determined on the
639 basis of their fragmentation profiles mainly through the series of b- and γ - ions generated.
640 These b- and γ - ions correspond to the location of the positive charge with respect to the N-
641 terminus and C-terminus of the peptide, respectively. Finally, the difference in mass between
642 consecutive ions of the same series allows determination of the identity and the sequence of
643 amino acids.

644 Among the various ionisation methods, the most widely used are: matrix-assisted laser
645 desorption ionisation (MALDI) and ESI, which are soft ionisation techniques that generate little
646 or no fragmentation in the source. Subsequently, high-resolution (HR) mass analysers such as
647 time-of-flight (TOF), quadrupole-TOF (Q-TOF), orbitrap, and Fourier transform-ion cyclotron
648 resonance (FT-ICR) are the analysers of choice. The high resolution provides greater
649 measurement accuracy, good mass measurement accuracy, and unambiguous determination
650 of peptide charge states.

651 MALDI involves the ionisation of analyte molecules assisted by a chemical matrix that can be
652 activated by laser energy absorption [167]. The activated molecules of the matrix (derivatives
653 of cinnamic or benzoic acid) and the analyte are vaporised, ionised, and released into the
654 vacuum of the source. It is often combined with a TOF analyser, which measures the mass of
655 intact peptides.

656 MALDI-TOF instruments equipped with an ion selector are able to provide fragment ions
657 produced from precursor ions that spontaneously decay in flight by post-source decay (PSD)
658 [168]. The analysis of these so-called metastable ions leads to the determination of the peptide

659 sequence. However, the long spectrum acquisition time and the low resolution of the ion
660 selectors represent major drawbacks of PSD. This is problematic when analysing complex
661 mixtures such as peptidomes of food matrices. For these reasons, the deployment of MALDI-
662 TOF-TOF techniques improves this PSD process by ion fragmentation activation [168]. Fragment
663 ions of given peptides are thus generated by high-energy CID in a cell collision before they enter
664 the reflectron. These ions, therefore, penetrate the reflectron substantially and allow complete
665 fragment ion spectra to be acquired in a single acquisition at a fixed reflectron voltage [169].
666 For example, peptidome characterisation applying this technique was done on collagen
667 peptides [170], [171] and a Tilapia by-product [172]. For this latter, the results revealed the
668 identification of a total of 799 peptides. Due to the complexity of the sample, a prefractionation
669 method by RP-HPLC or SEC is required [173]. As the samples have to be crystallised in the
670 matrix, the chromatographic system cannot be coupled directly to the mass spectrometer. The
671 fractions are, therefore, collected and co-crystallized with the matrix to the MALDI target. The
672 MALDI-TOF-TOF-MS analysis is then performed offline.

673 ESI-MS/MS is a more common source-ionisation-mass analyser combination for peptide
674 identification in MS/MS. It consists of generating multi-charged ions by applying a high voltage
675 at the end of the capillary to produce an aerosol from a conductive analyte solution and
676 generate molecular ions in the sample for MS and MS/MS analysis [174]. Its direct association
677 with RP-HPLC to analyse complex samples offers advantages in terms of time, selectivity, and
678 high sensitivity and makes this technique the most commonly used compared to MALDI-TOF-
679 MS/MS, especially for the collagen peptides which are composed of redundant amino acid
680 sequences [152], [175]

681 Coupled with HR analysers such as Q-TOF or FT-MS (LTQ-Orbitrap and FT-ICR), RP-HPLC-ESI-
682 MS/MS provides a technique capable of screening almost the entire peptide pool. Its high

683 sensitivity and ability to detect even trace amounts of peptides in complex matrices provide
684 the most promising approach to analysing the food peptidome [149], [176], [177]. For example,
685 a study characterising the peptidome of sheep milk led to the identification of 257 peptides
686 through RP-HPLC-ESI-Q-TOF [178], and another study on donkey milk led to the identification
687 of 1330 peptides by nano HPLC-ESI-LTQ-Orbitrap [179], where nano HPLC corresponds to the
688 miniaturisation of the HPLC method to increase the resolving power of the chromatographic
689 device. For these different types of instruments, the low-energy CID activation method was
690 applied to obtain typical MS/MS mass spectra from each selected peptide ion.

691 After the investigations, the results are provided reliably, however, there is an urgent need to
692 develop appropriate search databases and algorithms to exhaustively identify the food
693 peptidome since each peptidomics approach is complementary to another [180] because of
694 the complexity of agro-food peptide mixtures [181], [182].

695

696 **3.4.2 Bioinformatics data analysis**

697

698 The analytical data is further processed and exploited through bioinformatics software owing
699 to a large data set of up to thousands of ion spectra produced from a single sample. These
700 software packages use mathematical algorithms and statistical tools to archive, retrieve, and
701 analyse HPLC-MS/MS data [183]. Routine software for peptide identification includes Mascot,
702 Sequest, Progenesis QI for proteomics, PEAKS[®] Studio, FlexAnalysis, X!Tandem, OMSSA, and
703 MaxQuant [184]. Peptide identification can be performed according to two principles: (i)
704 database searching, which compares the m/z ratio list from experimentally acquired MS/MS
705 spectra with theoretical lists of predicted peptide fragments for each peptide contained in the
706 database, the best known of which are UniProtKB and PepBank; and (ii) *de novo* sequencing,

707 which presents itself as an alternative, given that peptide sequences may be absent from the
708 databases and due to certain limitations of peptidomics. This allows determination of the
709 peptide sequence directly from the m/z ratio of the fragmentation spectra. For this purpose,
710 tools can be used to perform sequencing for each MS/MS spectrum such as PEAKS® Studio,
711 PepNovo, MS-Blast, and MassHunter [118], [185]–[187].

712

713 **3.5 Advantages and challenges of peptidomics approaches**

714

715 Peptidomics has revealed its advantages in the food industry, which produces both food items
716 and by-products, both of which are rich in peptide precursor proteins. On the one hand, in the
717 context of direct feeding of products, allergenicity is reduced and the improvement of
718 functional properties such as solubility, improved digestibility, or intestinal absorption can
719 contribute to solving certain problems such as poor absorption of proteins in the body [188].
720 On the other hand, the valorisation of food by-products responds both to the concern of
721 recovering and using noble proteins and also to better valorisation of their impact on the
722 environment [17], [189], [190].

723 However, for collagen and its derived peptides, certain factors limit this hydrolysis process,
724 notably the presence of cross-links and hydroxyproline as well as numerous PTMs [191].

725 Despite the progress made in peptidomics, certain limitations exist, such as the analysis of small
726 peptides (di-, tri-, tetra-, or penta-peptides) because their identification requires removal of a
727 certain number of technological constraints [192]. Indeed, they are often poorly separated by
728 RP-HPLC and are difficult to identify by MS/MS due to the low number of fragments obtained
729 by CID. As a result, interpretations of MS/MS spectra can be ambiguous as each spectrum often
730 corresponds to two or more possible peptide sequences owing to a lack of informative ions. In

731 recent work, improvement of their identification through a dimethyl labelling strategy was
732 presented, and 843 small peptides were identified [193]. This chemical derivatisation improves
733 the signals of a1 ions (ions resulting from loss of neutral -CO of b1 ion) in MS/MS analysis and
734 contributes to the unambiguous identification of N-terminal residues. Nevertheless, the *de*
735 *novo* matching scores remain low owing to the lack of informative fragment ions, which reduces
736 the number of matched fragments. Further study to improve the identification and
737 modification of sequencing algorithms is, therefore, still required to improve the accuracy of
738 automated data analysis.

739 The analysis of modified peptides is equally challenging. Information on the localisation of some
740 PTMs can be provided by CID, but some PTMs, such as N-glycosylation, and proline
741 hydroxylation, for instance, are rapidly lost during the fragmentation mechanism involved in
742 CID. As an alternative, electron-capture dissociation (ECD) and its variant, electron transfer
743 dissociation (ETD), are better suited to solve this problem [194].

744 On the other hand, the identification of these modified peptides and their PTMs is limited by
745 the phenomenon of ionisation suppression occurring in ion sources. A well-known example
746 concerns the detection of phosphorylated peptides, which is impaired by the presence of non-
747 phosphorylated peptides, whose positive ionisation dominates over phosphopeptides, with the
748 negative charge of the phosphate group resulting in a lower ionisation efficiency [195].
749 Therefore, the scientific community have developed various analytical approaches specially
750 dedicated to detect, identify and quantify the phosphopeptides [196].

751 The progress of several projects and studies on the proteome in medicine has given rise to
752 clinical peptidomics approaches. By analogy, peptidomics has been rapidly applied by food and
753 nutrient science researchers in the more general framework of "foodomics". Thus, food

754 peptidomics makes a crucial contribution to safety, authenticity, sensoriality, nutritional
755 understanding, healthy food design, and by-product valorisation.
756 This approach nowadays represents a robust technique to identify the food peptidome through
757 liquid chromatographic separation techniques (SEC, IEX, and RP-HPLC) and high-resolution
758 tandem mass spectrometry combined with bioinformatics. In addition, the coupling of different
759 chromatographic techniques upstream of the analysis provides better separation power and
760 resolution, which result in more complete identification of the peptides present in complex
761 mixtures. However, further research is needed for the near-complete identification of the agro-
762 food peptidome by filling the main gaps related to the identification of size-reduced and
763 modified peptides. The near-complete identification of agro-food proteomes will require the
764 development of even more powerful bioinformatics tools.

765

766 **4 Fields of application of collagen and its derivatives**

767

768 **4.1 Antioxidant and antimicrobial properties**

769

770 Antioxidants are molecules with the ability to metabolise free radicals and, therefore, neutralise
771 the deleterious effects of oxidative stress at the cellular as well as tissue levels [197]. The
772 antioxidant activity of gelatine extracted from fish by-products has been the subject of several
773 studies. Indeed, peptides were identified from different species and exerted antioxidant
774 activities, such as PYSFK, GFGPEL, and VGGRP from grass carp (*Ctenopharyngodon idella*) [198],
775 DPALATEPDMPF, EGL, YGDEY, and LSGYGP from Nile Tilapia (*O. niloticus*) [199]–[201], PFGPD,
776 PYGAKG, and YGPM from Spanish mackerel (*Scomberomorous niphonius*) [201], GSGGL,

777 GPGGFI, and FIGP from Bluefin leatherjacket (*Navodon septentrionalis*) [202], and AVGAT from
778 Thornback ray [133]. In a general manner, the particularly low-molecular-mass (MM) peptides
779 (< 3 kDa) exhibited higher radical scavenging properties [203], and it was shown that Tilapia
780 skin collagen peptides (MM < 3 kDa) had protective effects against the D-galactose-induced
781 liver and kidney damage in mice by reducing oxidative stress, for instance [204].
782 As for antimicrobial activity, bioactive peptides from yellowfin tuna (*Thunnus albacares*) skin
783 collagen have shown efficacy notably with minimum effective concentration (MEC) values of
784 1.2, 6.5, 17, 8, 3, and 3.2 $\mu\text{g}\cdot\text{mL}^{-1}$ against *B. subtilis*, *M. luteus*, *S. iniae*, *A. hydrophila*, *E. coli*, and
785 *V. parahaemolyticus* strains, respectively [205]; skipjack tuna (*Katsuwonus pelamis*), with MEC
786 values of 3, 26, 4.8, 25, 2.7, 9, and 16 $\mu\text{g}\cdot\text{mL}^{-1}$ against *B. subtilis*, *M. luteus*, *S. iniae*, *A. hydrophila*,
787 *E. coli*, *V. parahaemolyticus*, and *C. albicans* strains, respectively [206]; and yellow catfish
788 (*Pelteobagrus fulvidraco*), with minimum inhibitory concentration (MIC) values of 2, 4, 16, and
789 64 $\mu\text{g}\cdot\text{mL}^{-1}$ against *B. subtilis*, *S. aureus*, *E. coli*, and *C. albicans*, respectively [207].

790

791 4.2 Anti-inflammatory properties

792

793 Collagen peptides have also been shown to be effective in preventing and reducing
794 inflammation. Collagen peptides extracted from sea cucumber (*Acaudina molpadioides*) were
795 administered by gavage to male mice for 21 days, followed by intraperitoneal injection of 10%
796 CCL₄ (nephrotoxic molecule) to establish an acute model of kidney injury. The results
797 highlighted the protective effects of collagen peptides that significantly decreased the levels of
798 interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF- α) [208].

799 This anti-inflammatory effect has been applied directly in the context of titanium implants.
800 Indeed, when the latter are inserted in the body, a pro-inflammatory reaction takes place,
801 which is necessary for the osseointegration process. However, if this inflammation is excessive
802 and persists over time and becomes chronic, implant failure occurs. Thus, it is important to be
803 able to control this inflammation [209] and the 'use' of collagen peptides can be advantageous.
804 Indeed, a collagen-coated titanium alloy enriched with phenolic compounds has been shown
805 to reduce inflammatory parameters by decreasing the gene expression of IL-6 and TNF- α [210].
806 Collagen peptides can be used in ways other than as biomaterials. In a study analysing the oral
807 intake of collagen peptides from monkfish (*Lophius litulon*) skin, researchers showed an
808 improvement in renal inflammatory status induced by a high-fat diet in mice, in particular by
809 regulation of nuclear factor erythroid 2-related factor (Nrf2) and nucleotide-binding and
810 oligomerisation domain-like receptors protein 3 (NLRP3) signalling pathways, which are
811 implicated in regulation of the responses to oxidative stress and initiation of the inflammatory
812 cascade [211], [212]. Indeed, compared with the control group, the collagen peptide-fed group
813 had decreased levels of IL-1 β , IL-6, and TNF- α in kidney homogenate [213].

814

815 **4.3 Antihypertensive activities**

816

817 Of the numerous pathologies associated with obesity and T2D, as part of metabolic syndrome,
818 hypertension is a growing public health problem worldwide [214]. People suffering from
819 hypertension have systolic blood pressure values ≥ 140 mm Hg and/or diastolic blood pressure
820 values ≥ 90 mm Hg [215]. This condition is partially due to chronic activation of the renin-
821 angiotensin-aldosterone system (RAAS). The presence of excess angiotensin II and aldosterone

822 in the circulation promotes a proinflammatory, fibrotic, and hypertrophic status, particularly in
823 the heart and kidneys [216], making it paramount to address this condition.

824 Current pharmacological therapies use angiotensin-converting enzyme (ACE) inhibitors and
825 angiotensin receptor blockers. ACE inhibitors are used as a first-line treatment even before
826 treatment with angiotensin receptor blockers. Studies are also looking at the regulation of ACE
827 expression directly [217]. Among the multitude of therapies, bioactive peptides are used as a
828 preventive treatment.

829 Indeed, more and more studies have highlighted the potential of bioactive peptides and in
830 particular, their ACE-inhibiting properties. The antihypertensive effects of protein hydrolysates
831 and their peptides can be determined by measuring ACE inhibitory activity *in vitro* or by
832 monitoring blood pressure in spontaneously hypertensive rats (SHRs) *in vivo* [218], [219]. Many
833 protein hydrolysates and peptides of marine origin have been studied using the ACE inhibitory
834 activity test and in SHRs [219]–[222].

835 ACE inhibitory peptides have molecular masses ranging from 300 to 3000 Da, with a peptide
836 length of 2 to 13 amino acids [223], [224]. *In silico* modelling has revealed optimal motifs for
837 inhibition of the enzyme by candidate peptides. Since ACE is a carboxypeptidase, the C-terminal
838 part of these peptides has been found to be crucial, particularly the C1 amino acid. Quantitative
839 structure-activity relationship (QSAR) studies have shown that the presence of hydrophobic
840 aliphatic amino acids (Ala, Trp, Pro, Phe, Gly, Cys, Leu, and Ile) [225] are good predictors of the
841 peptide effectiveness in inhibiting ACE. It should also be noted that the nature of amino acids
842 in the C2 and C4 positions is also important for ACE inhibition potential [226], [227].

843 Moreover, according to Wu et al., the optimal amino acid residues identified *in silico* for potent
844 ACE inhibition are peptides starting with Tyr or Cys in the first position at the C-terminus; Trp,
845 Met, and His in the second position; Leu, Ile, Val, and Met in the third position, and Trp in the

846 fourth position [227]. Peptides with hydrophobic and aromatic residues at the N- and C-
847 terminal ends generally have higher antihypertensive activities [228].

848 Currently, pharmacological ACE inhibitors comprise enalapril, fosinopril, and captopril, with
849 favourable half maximal inhibitory concentration (IC_{50}) values such as 6 nM for the captopril
850 [229]. Despite the very high inhibitory potential of this captopril, many side effects are
851 associated with its use, including hypotension, cough, and hyperkalaemia, which are the most
852 frequently reported adverse events for the entire drug class [230]. Therefore, alternative
853 preventive measures such as reliance on functional food solutions derived from the agri-food
854 industry, considered safe and affordable to produce, are envisaged.

855 The majority of antihypertensive peptides reported from marine proteins hydrolysates have
856 ACE IC_{50} values ranging from 0.3 to 1 500 μ M [231]. Crustacean peptides (shrimp, krill) exhibit
857 IC_{50} values ranging between 0.9–24.1 μ M [232], [233], between 1.2–51 μ M for mollusc
858 peptides [234], [235], and finally between 2.4–23.4 μ M for coelenterates and echinoderms
859 peptides [236], [237]. It should be kept in mind that pharmacological ACE inhibitors are 1 000
860 times more efficient than antihypertensive peptides, but without harmful side effects.

861 Numerous studies have also focused on the ACE-inhibitory properties of peptides derived from
862 gelatine extracted from various fish species. These peptides were generated using various
863 commercial enzymes. Indeed, peptides derived from gelatine extracted from fish skins have
864 proven to be very good candidates, e.g., following the use of commercial Alcalase, the peptide
865 Gly-Pro-Leu (GPL) from Alaska pollock had an IC_{50} value of 2.6 μ M, and Nile Tilapia hydrolysate
866 had an IC_{50} value of 62.2 μ M [238], [239]. For gelatine extracted from the scales of triplecross
867 lizardfish (*Synodus macrops*) and milkfish (*Chanos chanos*), the peptides exhibited IC_{50} values
868 of 420 μ M and 472 μ M, respectively [240], [241]. For collagen extracted from *Theragra*
869 *chalcogramma* skins, the isolated peptides GPL and Gly-Pro-Met (GPM) exhibited IC_{50} values of

870 2.6 and 17.1 μM , respectively [238]. Previous work also demonstrated that a tripeptide Leu-
871 Gly-Trp (LGW) was able to reduce blood pressure in SHR after oral administration [242].
872 Altogether these results demonstrate the efficiency of food-derived peptides and more
873 specifically gelatine- and collagen-derived peptides in the management of hypertension.

874

875 **4.4 Glucose metabolism regulation**

876

877 Several benefits of collagen hydrolysates are slowly emerging, demonstrating their positive
878 effects in preventing and treating T2D, although the illustrations of their beneficial effects in
879 the current literature are not as abundant as those for ACE inhibition. Multiple studies have
880 highlighted the beneficial effects of protein-rich diets from animal and plant sources in the
881 management of T2D [243]. As depicted below, recent studies have investigated the potential
882 of gelatine and gelatine hydrolysates to prevent obesity and diabetes.

883

884 **4.4.1 Hormone secretion modulation**

885

886 In streptozotocin (STZ)-induced diabetic rats, oral intake of fish (*Salmo salar*) skin gelatine
887 hydrolysate (FSGH) resulted in a significant increase in plasma GLP-1 levels relative to diabetic
888 control. Furthermore, the total GLP-1 levels of the FSGH-treated diabetic rats were at the same
889 level as that of the group of normal rats [244]. Closer inspection of the clinical studies revealed
890 that collagen peptides have positive effects after ingestion. Indeed, the incretin-stimulated
891 concentrations of GLP-1 and glucose-dependent insulinotropic peptide (GIP) were increased in
892 a double-blind randomised clinical study in subjects with T2D after 12 weeks of fish collagen
893 peptide ingestion [245]. This study showed an improvement in the biomarkers for unfavourable

894 prognosis of the disease, such as a decrease in fasting blood glycemia, insulin resistance, and
895 glycated haemoglobin compared with the control group (resistant dextrin).

896 Another study, also involving the ingestion of hydrolysed gelatine (the origin of which was not
897 specified) in healthy obese patients, showed an increase in circulating GLP-1 30 min after
898 ingestion, with a peak at 120 min [246].

899 In another study, fish gelatine was administered by gavage to Wistar rats and was also tested
900 after simulated GID in a cell model using Caco-2/TC7 co-culture. The results of this study
901 demonstrated the efficacy of fish gelatine hydrolysates in decreasing intestinal glucose
902 absorption *in vitro* and *ex vivo* concomitantly with improvement of glucose tolerance in Wistar
903 rats [247].

904 A study of a Tilapia by-product hydrolysate after simulated GID demonstrated its effects in
905 stimulating the secretion of the gut hormones, cholecystikinin (CCK) and GLP-1 as well, using
906 the STC-1 enteroendocrine cell model [132]. Blue whiting hydrolysate containing collagen has
907 also been shown to decrease insulin secretion in addition to increasing GLP-1 secretion along
908 with proglucagon production [135], [248].

909 Sasaoka *et al.* demonstrated that sturgeon collagen peptides (SCPs) improved glucose
910 tolerance in a normal mouse model [249]. Following this work, a new study on the mechanisms
911 that ensure these phenomena allowed the authors to hypothesise that SCPs decrease the
912 transport rate of glucose from the stomach to the duodenum, resulting in a delay in its
913 absorption. Thus, SCPs inhibit DPP-IV and maintain a high concentration of plasma GLP-1, which
914 in turn stimulates insulin secretion [250]. Salmon by-products such as skin and trimmings have
915 shown beneficial effects in improving insulin secretion as well as stimulating GLP-1 secretion in
916 BRIN-BD11 and GLUTag cell models [251].

917 Hydrolysed proteins from chicken feet, which are rich in cartilage and, therefore, collagen,
918 showed an improvement in plasma glucose levels and an increase in GLP-1 secretion in glucose-
919 intolerant rats [252]. Still using a chicken source, a study was conducted on the by-products
920 obtained after mechanical deboning, and the peptides isolated from them proved effective in
921 increasing glucose uptake *ex vivo* [253].

922

923 **4.4.2 Inhibition of DPP-IV activity**

924

925 Often going hand in hand with studies on the stimulation of the secretion of intestinal
926 hormones, the study of the inhibition of DPP-IV by protein hydrolysates, and hence those
927 derived from collagen, are unavoidable, all the more so as the methods of evaluation are often
928 not very restrictive and quick to set up *in vitro* or *ex vivo* or even to some extent on serum.

929 The previous study also showed that in STZ-induced diabetic rats, oral intake of fish (*Salmo*
930 *salar*) skin gelatine hydrolysate (FSGH) inhibited plasmatic DPP-IV activity, demonstrating the
931 antihyperglycemic power of a salmon collagen hydrolysate [244].

932 Concomitantly, a study assessing oral intake of Tilapia (*Oreochromis niloticus*) collagen
933 hydrolysate has led to establishing, this time in normal mice, the inhibitory activity for DPP-IV,
934 with an IC₅₀ value of 0.77 mg.mL⁻¹ [254]. Theysgeur *et al.* also studied the effect of Tilapia
935 hydrolysates containing collagen and tested their ability to inhibit DPP-IV both *in vitro* and *in*
936 *situ*. They were able to identify four sequences of bioactive peptides after SGID and *in vitro*
937 intestinal barrier passage [132].

938 In addition to marine sources, a hydrolysate of camel gelatine skin has shown beneficial effects
939 on DPP-IV inhibition as well [255]. Recently, Jin and colleagues [127] used Atlantic salmon skin,
940 several peptidases, and fractionation methods of hydrolysates to evaluate DPP-IV inhibition

941 activity, and they identified novel DPP-IV inhibitory peptides from the generated subfractions;
942 the highest IC₅₀ value was 0.79 ± 0.13 mg.mL⁻¹.

943 The literature shows, for instance, that after SGID of Alaska pollock skin collagen, the DPP-IV
944 IC₅₀ value was 1.39 ± 0.08 mg.mL⁻¹ [133]. Investigation of DPP-IV inhibition of barbel skin
945 gelatine-derived peptides [256] led, after performing hydrolysis using Esperase®, the lowest
946 IC₅₀ value of 2.21 mg.mL⁻¹.

947 Overall, the enzymatic hydrolysates of fish by-products appear to have very promising DPP-IV
948 inhibitor activities compared with other protein hydrolysate-derived peptides such as Japanese
949 rice bran, milk protein, and Gouda cheese [257]–[259].

950

951 5 Conclusion

952

953 The body of literature presented in this review has shed light on the use of gelatine throughout
954 history, its physicochemical and structural properties, and its endogenous production. The
955 various contemporary methods for its extraction as well as the different sources from which it
956 can be extracted are also discussed. Nevertheless, certain technological barriers do not yet
957 allow for exhaustive identification of their bioactive peptide compositions, but thanks to the
958 optimisation of computing tools and a better understanding of preparation methods, this
959 should no longer be a limiting factor in this regard. In the contemporary worldwide trend, the
960 sustainable use of agro-food waste and/or by-products to produce value-added products for
961 potential applications in the cosmetics, pharmaceutical, or food industry can offer considerable
962 additional income opportunities for the dependent industry. In addition, valorisation of agro-
963 food wastes and by-products can guarantee regional food safety and thus ensure sustainable

964 food production at a time when societal and indeed economic pressures can put food-
965 dependent countries or regions at a harmful disadvantage. Until now, most agro-food by-
966 products have been used as a source of fuel, animal feed, or organic fertiliser. Presently, with
967 the availability of modern technologies, new concepts have been established that enable
968 efficient use of by-products from the agro-food sector to produce value-added products and
969 also to accurately identify the high-interest entities, while limiting waste generation and,
970 therefore, the carbon footprint.

971 The growing interest in these sources stems in particular from the versatility of these
972 applications both structurally and in terms of bioactivities. Indeed, for the latter (the theme of
973 our research group), as a result of their antioxidant, anti-inflammatory, and anti-microbial
974 properties, as well as their role in the regulation of glucose metabolism, they are options of
975 choice in the prevention and potential treatment of numerous pathologies, making it possible
976 to imagine natural, inexpensive, effective, and most importantly custom-made therapeutic
977 options in the future. Thus, in order to evaluate their beneficial effects on the prevention and
978 management of chronic diseases such as diabetes and obesity, it is necessary to set up robust
979 and well-set-out clinical trial protocols on humans in order to determine the physiological
980 effects of these by-products with their multiple virtues and thus to allow a large section of
981 people to benefit from them on a broader scale.

982

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984 Mouna Ambli, Benoit Cudennec, Barbara Deracinois and Christophe Flahaut:
985 Conceptualization, visualization and writing the original draft. Benoit Cudennec and Christophe
986 Flahaut: Review and editing the manuscript. Benoit Cudennec, Christophe Flahaut and Rozenn

987 Ravallec: Supervision, funding acquisition and project administration. All authors read and
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996

997 **Conflict of interest:**

998 The authors declare that they have no conflict of interest.

999 **6 References**

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