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

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Abstract

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

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

Introduction

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

In the global functional food market and in light of societal developments and climatic change, the demand is mainly for so-called natural, non-ultra-processed products, with low environmental impacts and among these, collagen peptides have become a high priority must for many consumers. According to a new report from Grand View Research, Inc., the global collagen market is expected to reach 5.87 billion Euros by 2025, with a compound annual growth rate (CAGR) of 6.5% over the forecast period. The interest in collagen peptides generated from gelatine lies in their bioavailability and ease of digestion. They have many applications in health care, such as incorporation in designed formulas or as a powder that is to be diluted. Collagen peptides do not contain hormones, chemicals, or antibiotics. Their innocuousness is also one of the arguments for their consumption, whether by people who are ill or people concerned about their health. They are used by athletes for

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joint and tendon health during their daily workouts [2]. The increasing prevalence of several diseases such as obesity and diabetes [3], as well as a host of pathologies associated with metabolic syndrome, has led to a surge in demand for natural food sources containing such bioactive peptides as an early treatment option [4]. Bioactive peptides are used in a wide range of therapeutic areas, from alteration of the immune system to the gastrointestinal system to antihypertensive uses [5]. Indeed, the market for bioactive peptides, worth USD 48.65 billion in 2020, continues to grow and is expected to reach USD 95.71 billion by 2028, at a CAGR of 8.86% between 2021 and 2028 [6].

The novelty of this review lies in its propensity to discuss collagen under integrative aspects, which have often been eluded to date. First of all, via its historical use in ancient Egypt, Chinese traditional medicine, as well as in more contemporary times in Europe, to finally describe the different uses in our current societies. Then, the fundamental aspects of its structure and physicochemical properties, as well as the sources and methods of extraction, are addressed. Additional novelty is added regarding the methods of identifying peptides generated by the hydrolysis of food matrix to understand all the processes involved in the exploitation of such products, which are often hard to identify exhaustively in complex mixtures. Finally, a section on the bioactivities of collagen hydrolysates shows the versatility of their uses in today's and tomorrow's health, with the significant added value of these products, which is often overlooked in contemporary therapeutic arsenals, such as in glucose metabolism management for instance.

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

Historical use of collagen: According to the literature, collagen and especially its hydrolysed form gelatine have been used by humans for thousands of years. In ancient Egypt, gelatine was extracted by boiling animal skins such as cowhides and bones [7]. This method produced glue that was then used for the confection of clothes and supplies [8], but it has also been found in coloured wall paintings dating back 4400 years [9]. In addition to this use, the consumption of gelatine extracted from bone broths was also a common practice in everyday life. The culinary use of gelatine quickly became commonplace in medieval Baghdadi culture, being incorporated into many preparations from the early 10th century [10]. In China, its use as an adhesive agent can be traced back to 1980-1450 BCE [11]. From a medicinal point of view, oriental and particularly traditional Chinese medicine describes the use of gelatine as far back as 771 BC [12]. The use of gelatine in the medical field was for a long time confined to China, Japan, and some East Asian countries.

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

Collagen and the gelatine derived from it are sources of very high added value that can be found in several fields of application. In medical areas, collagen films are used as a drug delivery system for infected corneal tissue [20,21], as well as in tissue engineering for wound healing [22] and cosmetology/dermatology [5,23]. Collagen solutions have significant advantages, notably their low production cost, and the large quantities indirectly produced by the food industry, as well as their bioavailability, biocompatibility, and lack of adverse effects [24].

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

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

The structure of the collagen alpha chain is determined by the amino acid sequence. Out of the 20 proteinogenic amino acids that can be found in collagen, glycine, proline, and hydroxyproline are the most frequent [29]. Indeed, the alpha chain consists of the repeating unit Gly-X-Y, where X is often proline (Pro) and Y is often hydroxyproline (Hyp). The content of proline and hydroxyproline is approximately 30% in mammalian gelatine, 22%–25% in warm water fish gelatine (Tilapia and Nile perch), and 17% in cold water fish gelatine (cod) [30,31]. Comparative studies on the rheological properties of terrestrial mammalian and fish gelatine concluded that the differences observed are due to the content of the specific imino acids, proline and hydroxyproline, which stabilise the conformation when gelatine forms a gel. Thus, a lower content of proline and hydroxyproline results in a less compact gel with a lower melting temperature [32-35]. Therefore, it is important to keep in mind the composition of the collagen molecule and also post-translational modifications (PTMs) such as hydroxylation of prolines, in order to make relevant and comprehensive identifications of the peptides generated following hydrolysis, using bioinformatic tool features accordingly.

Physicochemical properties: Gelatine is a natural polymer similar to its collagen precursor. After collagen separation, gelatine can be extracted by either alkaline hydrolysis or acid hydrolysis. The latter determines the isoelectric point (pI) of the gelatine. With acid hydrolysis, gelatine is classified as type A, with an pI ≈ 5. Extraction with alkaline media yields type B gelatine, with an pI ≈ 9. Gelatine is classified as a physical gel [34]. That is to say, the interactions or bonds between the chains that make up the gel are physical in nature (van der Waals interactions and hydrogen bonding at E ≈ 2 kcal/mole). Some physical gels, such as alginate, are not thermoreversible. However, the binding energy of gelatine is relatively weak and can form thermoreversible gels. The most

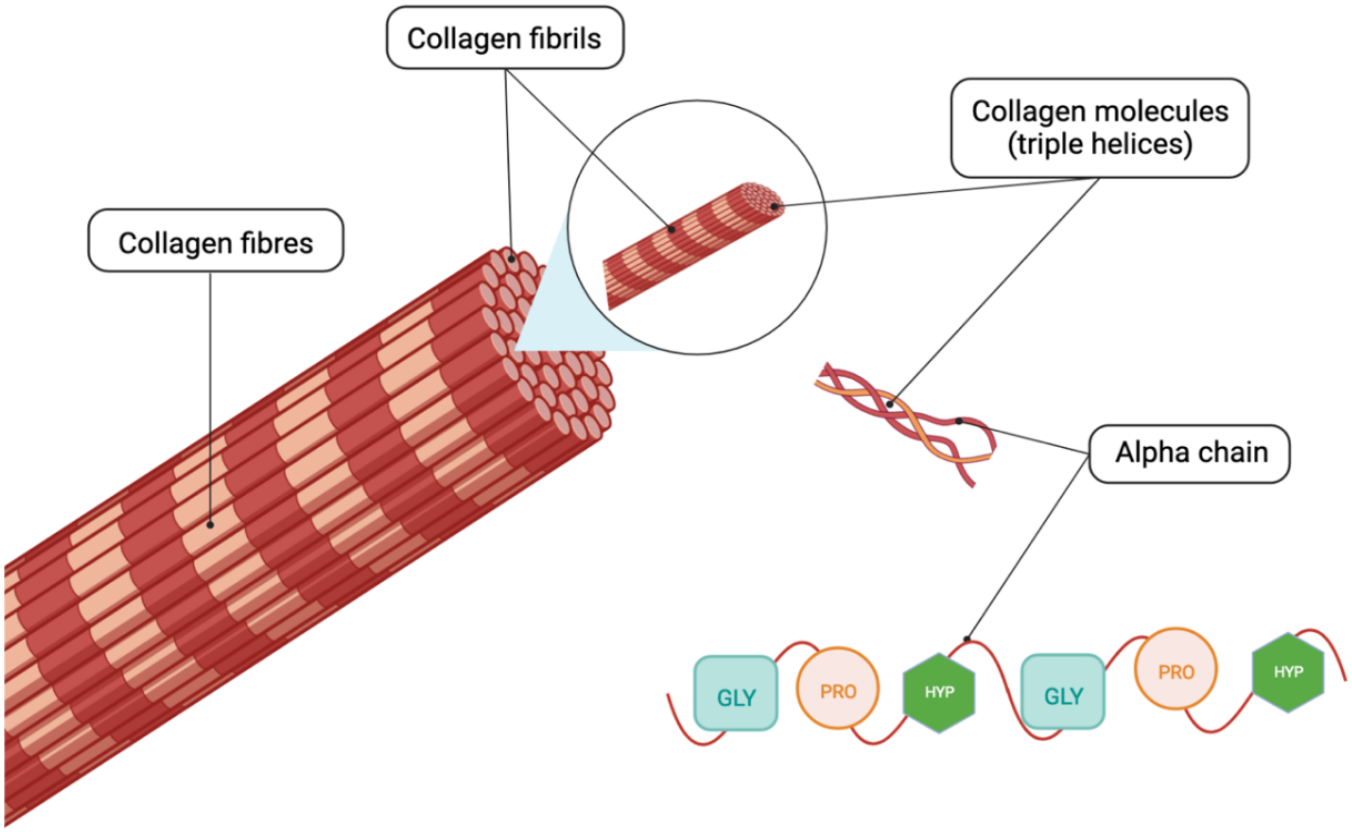

Figure 1: Schematic of the four-level structure of type I collagen fibres.

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

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Table 1: Types, forms, distribution, and functions of collagens [27].

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 Ab toxicity
XVII	Transmembrane	Dermo–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

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important physical properties of gelatine gels are strength and melting point. These are determined by molecular masses and complex interactions that depend on the amino acid composition and the ratio of α/β chains present in gelatine [36]. There is a strong correlation between gelatine gel strength and chain content. Indeed, differential rheological properties observed between mammalian and fish gelatines are mostly due to the content of the amino acids, proline and hydroxyproline, which are responsible for stabilisation of the gel network. Moreover, gelatines with a higher proportion of α-chains have higher gel strengths. On the other hand, a high percentage of peptides with molecular masses higher or lower than the α chain reduces the gel strength [37]. The gel strength of commercial gelatines is expressed in Bloom values, which is the weight in grams required by a specific plunger to push a standard temperature-controlled gel surface to a specific depth under standard conditions [38]. The gel strengths of commercial gelatines range from 100 to 300, but gelatines with Bloom values of 250 to 260 are the most desirable [39].

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

The wide range of Bloom values found in different gelatines is due to differences in the proline and hydroxyproline content of different types of collagens and is also related to the temperature of the animal's habitat. Badii and Howell showed that hydrophobic amino acids (Ala, Val, Leu, Ile, Pro, Phe, and Met) may also contribute to the high Bloom value of Tilapia fish gelatine [45].

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

Nuclear level: At the nuclear level, the genes coding for type 1 collagen, *COL1A1* and *COL1A2*, are transcribed into pre-mRNA and then capped at the 5'-end and polyadenylated at the 3'-end. The mRNAs are translated by ribosomes into collagen precursors, the α1 or α2 procollagens. They possess globular polypeptide domains at their ends, the N-terminal and C-terminal propeptides, 15 kDa on the N-terminal side and 30 kDa on the C-terminal side, respectively, which help maturation of the protein figure 2(1).

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

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

Extracellular matrix*:* Two specific peptidases cleave the N-terminal and C-terminal propeptides, namely procollagen N-proteinase and procollagen C-proteinase, respectively, allowing the formation of mature tropocollagen [52]. After cleavage of the propeptides, tropocollagen bundles aggregate into fibrils near the cell surface. The formation of aldehydes at the level of collagen telopeptides by lysyl oxidase allows the formation of covalent cross-links *via* the substitution of a carbonyl group for the amine group of a lysine residue, which results in spontaneous bridging into several tropocollagen bundles. Minerals, such as hydroxyapatite crystals, are present between the fibrils. The monomers are 300 nm long and there are 40 nm gaps between consecutive monomers,

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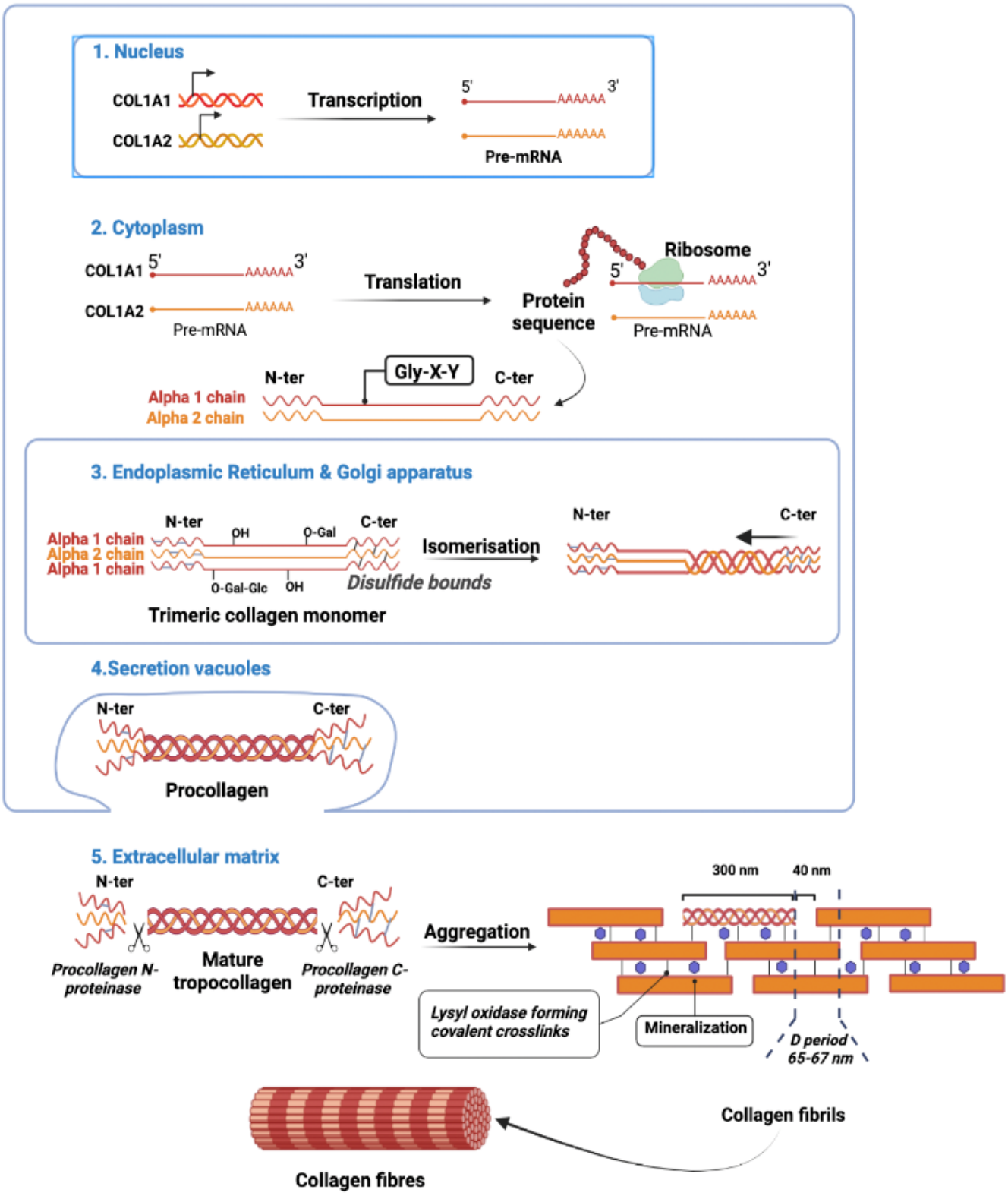

Figure 2: Schematic representation of collagen synthesis.

*From collagen genes in the nucleus (**1**), mRNA translation into ribosomal protein synthesis in the cytoplasm (**2**), post-translational modifications (PTMs) in the rough endoplasmic reticulum (**3**), vacuolar secretion (**4**), and maturation of tropocollagen into fibril formation, leading to collagen fibres in the extracellular matrix (**5**). Illustration created with BioRender.com*

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which results in the characteristic striated appearance of type I collagen fibrils by electron microscopy. Finally, collagen fibrils aggregate into collagen fibres. Altogether, a collagen fibre's molecular mass is 300,000 Da, the length is 280 nm, and the width is 1.4 nm [53].

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

Unlike fish-derived collagen, mammalian collagen generally requires a higher pH to remove non-collagenous proteins [54]. Lipids are also partially eliminated during this process [55]. Nevertheless, to achieve maximal lipid removal, organic solvents, such as chloroform-methanol [56] and hexane-extraction [57], are usually required. The main issue with the use of organic solvents is the potential for adverse effects on public health, the environment, and general safety [58].

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

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

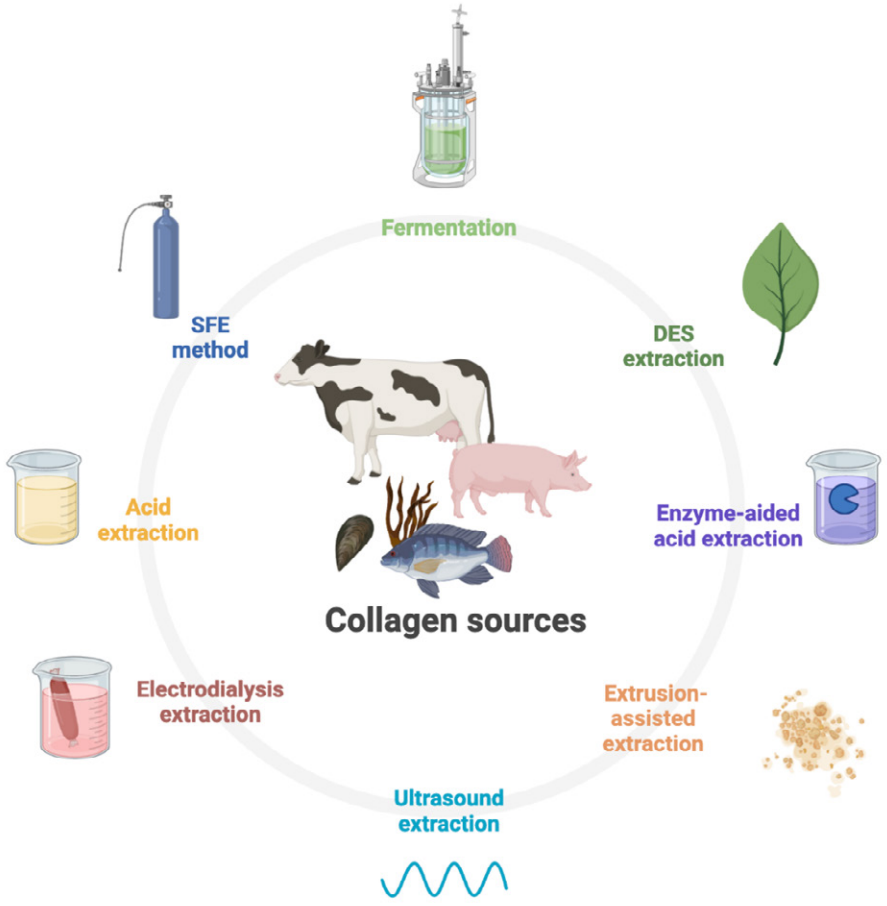

Figure 3: Schematic representation of current collagen extraction methods.

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

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

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

The method using AcOH is regularly used. Indeed, literature data describe the extraction of collagen from several aquatic species, both marine and freshwater: grass carp (*Ctenopharyngodon idella*) skin [26], Alaska pollock skin [59]; swim bladders of yellowfin tuna (*Thunnus albacares*) [63], catla (*Catla catla*) and rohu (*Labeo rohita*) skins [64]; small-spotted catshark skin [65]; Tilapia (*Oreochromis niloticus*) skin and scale [66]; scales of five species from Vietnam and Japan lizard fish (*Saurida spp.*) horse mackerel (*Trachurus japonicus*), grey mullet (*Mugil cephalis*), flying fish (*Cypselurus melanurus*), and yellow black seabream (*Dentex tumifrons*) [67]; three Vietnamese freshwater fish skins [68]; elasmobranch by-products [69]; and jellyfish (*Acromitus hardenbergi*) [70].

Most of these methods use concentrations of AcOH ranging from 0.1 to 1 M. This method is not the only one used since other solutions are also described, such as the use of hydrochloric, citric, and lactic acid [71].

Acid and enzyme-aided acidic collagen extraction: As an example, the extraction of collagen from the swim bladder of Atlantic cod (*Gadus morhua*) has been carried out using two different methods. The first method is with AcOH, and the second involves combining AcOH and pepsin. The authors demonstrated that the combined approach increased the final yield from 5.72% to 11.14% [72]. Other studies of the combined use have demonstrated their effectiveness in the extraction of collagen from Tilapia skins and gills [73] and from Chilean mussel byssus [74].

Various extraction methods: Unusual processes such as fermentation for collagen extraction have been described for Nile Tilapia skins [75], or by electrodialysis for Pufferfish (*Takifugu flavidus*) skins [76]. The latter appears to be rather promising as it allows this extraction to be carried out efficiently and cost-effectively, notably by reducing the amount of water waste by 95% while still maintaining a high extraction yield.

Deep eutectic solvent (DES) extraction is a method involving a two-compound tandem: a hydrogen bond receptor and a hydrogen bond donor. This approach favours the use of non-toxic, abundant, and biodegradable green solvents such as choline chloride, oxalic acid, urea, and ethylene glycol, among others, allowing extraction of high-value-added products from numerous by-products from plants, mammals, and aquatic sources. A study on the extraction of collagen from cod skins using a mixture of oxalic acid and choline chloride resulted in a yield of almost 90% [77], thus demonstrating the benefits of this extraction technique versus conventional ones.

Supercritical fluid extraction (SFE) is based on the use of fluids at pressures and temperatures beyond the critical point, allowing the capacities of a solvent to be modified through physical change. CO_2 is most often used as it is inexpensive, stable, and has high availability, as well as rather simple conditions of use. Studies from the same author provided evidence of the unambiguously better performance of SFE versus acid and pepsin-aided AcOH extraction methods on cod skins [72].

Among the various unconventional methods, several studies have investigated the use of **ultrasound** to improve collagen extractability. Ultrasound induces cavitation in the liquid solvent, creating micro bubbles that in turn cause tissue damage, thereby increasing the tissue/solvent contact area. The higher the frequency, the smaller the bubbles and the more effective they are. This method is suitable not only for marine sources but also for plant and animal sources [78], although the harsh conditions of ultrasound could affect the physicochemical and molecular characteristics of the extracted collagen.

Extrusion-assisted extraction (EAE) increases the yield of collagen extracted from Tilapia scales by two- to three-fold compared with a simple acid extraction, making this method relevant in the context of tissue pre-treatment [79].

After centrifugation, the contaminating particles and the remaining fat are eliminated [80], and hydrolysis can be performed, whether enzymatic, physical, or chemical. The batches are decolourised and deodorised with activated carbon

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and then filtered. The salts are removed from the hydrolysates *via* ion-exchange columns, which are then concentrated by vacuum evaporation [58]. The hydrolysates are sterilised by heat treatment before pulverisation and then packaged [81], as depicted in figure 4.

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

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

Sources of collagen and gelatin: Collagen can be retrieved from skin, bones, and tendons of several sources, such as porcine, bovine, avian, and marine species. Many applications have been described in the literature due to intrinsic features such as biocompatibility and degradability [82]. Bovine collagen is mostly used for extra-oral wounds [83], and marine collagen such as Tilapia skin is used as a xenograft after burn injuries [84]. With its low immunogenicity and reduced chance of rejection, it is a material of choice for several fields of application such as surgery and cosmetology, drug delivery, and food [85,86].

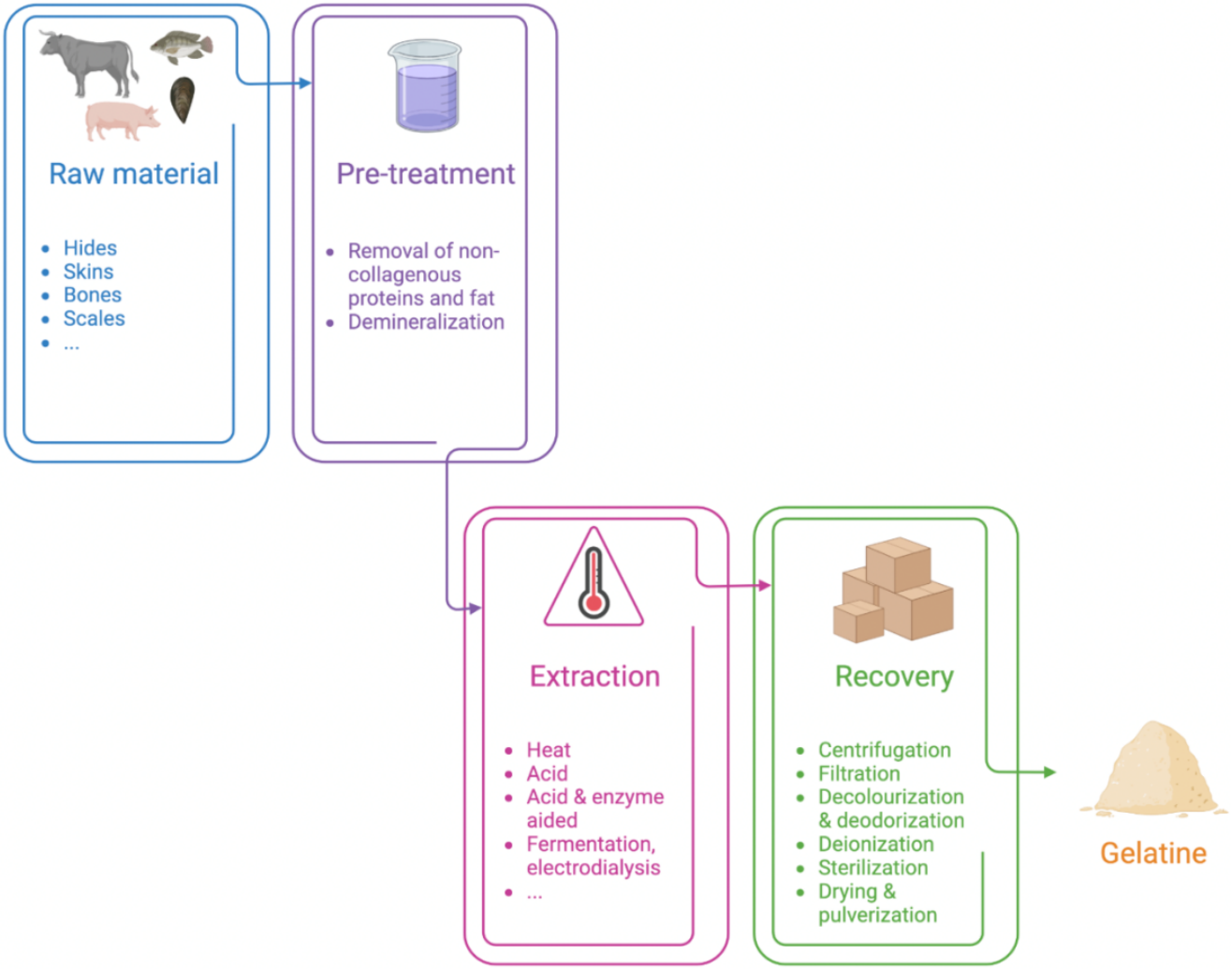

Figure 4: Schematic representation of collagen extraction from raw material to gelatine finished product.

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Due to the variability of collagen sources, the versatility of use is equally broad, like no other available protein. Nevertheless, the use of animal collagen has been severely compromised over the past several decades due to the emergence of severe diseases. Sanitary crises, such as the emergence of bovine spongiform encephalopathy (BSE) in 1985, forced the market to be more cautious in its use [87]. The use of mammalian collagen also increases the risk of immune reaction in 3% of the population, as well as the transmission of zoonoses such as foot and mouth disease and transmissible spongiform encephalopathy [46]. Additionally, in several cultures and religious backgrounds, such as Jews, Muslims, and Hindus, porcine and/or bovine collagen use is prohibited or subjected to special religious requirements [88]. Thus, for the past three decades, marine collagens (in blue) as alternatives have emerged as a solution of choice as the number of scientific researches had increased over time (figure 5).

As the interest in marine collagen sources increased in the past 30 years, two categories emerged: vertebrate and invertebrate marine animals. The latter category is the most widely exploited, such as molluscs comprising cuttlefish and mussels, sponges, echinoderms comprising sea urchins, starfish, jellyfish, and crustaceans such as prawns [89-94]. Regarding vertebrate animals, the added value of such sources lies in the possibility to use by-products derived from industrial processes [95,96] such as scales and skin.

Analytic aspects and identification methods for peptides derived from agro-food

Background

Food scientists have faced new challenges in the past several decades. To this end, they rely on methods developed in medical, pharmacological, and/or biotechnological fields. Thus, advanced analytical methodologies, omics approaches,

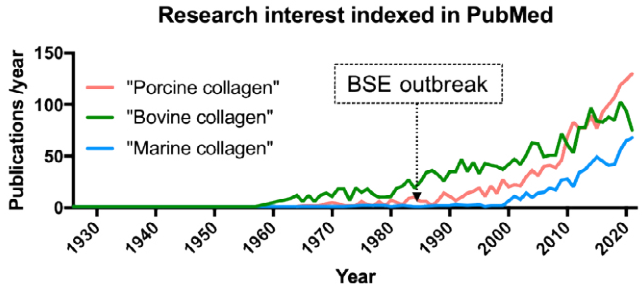

Figure 5: Increase in research interest in porcine, bovine, and marine collagens.

Articles indexed in PubMed (https://pubmed.ncbi.nlm.nih.gov) with the keywords "porcine collagen", "bovine collagen", and "marine collagen" published between 1926 and 2021 (last accessed on August 2022) and the time when the BSE outbreak occurred, inducing an increase of research interest for marine collagen in the late 1990s.

and bioinformatics, often combined with *in vitro*, *in vivo,* and/or clinical trials, are applied to investigate topics in food science and nutrition.

Within this framework, several research projects are directed toward the study of peptides, which are significant and multifaceted components of the human diet. They can be present naturally in food, but they can also be formed from protein precursors during physiological processes, for example, gastrointestinal digestion (GID) or food processing such as fermentation or enzymatic hydrolysis [97]. Chemical hydrolysis is more commonly used in the industry nowadays, but biological processes using enzymatic hydrolysis are more promising when products with high nutritional value and improved functionality are required. In addition, enzymatic hydrolysis generates less waste and is less costly and time-consuming [61].

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

In response to advances in omics technologies, research in the field of food science has developed the concept of "foodomics" approaches to traceability, authenticity [104], well-being, health, and food safety, which are synonymous with consumer confidence in the agro-food sector [105]. Foodomics is defined as a discipline that studies the fields of food and nutrition through the application of omics technologies to, for example, characterize and demonstrate the beneficial effects of innovative food products on human health [106,107]. One of the main challenges is, therefore, to improve our currently limited understanding of the interaction of food components with genes, lipids, proteins, sugars, metabolites, etc. and the consequences of these interactions.

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This knowledge will undoubtedly in the future allow a rational design of strategies to achieve a beneficial impact on, for example, human health [108], but also to ensure food safety by determining/measuring harmful components or organisms that may be present in food at very low concentrations and much more.

Food peptidomics

The term peptidomics was introduced at the beginning of this century to represent the various peptide analysis technologies for the complete identification of endogenous peptides in a biological sample [109] and was subsequently rapidly applied in the clinical field and food industry. Clinical peptidomics thus relates to the analysis of peptides from a cell, organ, or organism, whereas food peptidomics relates to the analysis of the entire pool of peptides present in food items or generated during processing, storage, or GID of foods [110].

Thus, different workflows have proven useful to help identify, characterise, and quantify peptides in a particular approach. Among these, peptidomics has demonstrated that allergenicity due to the presence of certain food protein allergens (e.g., wheat gluten, β-lactoglobulin, milk casein, collagen [111] can be greatly reduced after protein cleavage into peptides. Liang and colleagues showed by *in vitro* ELISA that the immunoreactivity due to cow milk allergenicity could be significantly reduced by a two-hour enzymatic incubation [112]. These findings have many applications, such as food formulations containing hydrolysed proteins for feeding infants and children with food allergies or intolerances. In the same context, peptidomics has also been applied to monitor the extent of proteolysis to ensure that allergenic epitopes are destroyed [112].

Furthermore, the quality, safety, and authenticity of foods can also be studied using peptidomics [113]. As the sequence of proteins in original and altered products differs to a certain extent, the disparities are, therefore, reflected through peptidomics tools to identify peptides that can serve as biomarkers [114]. For example, most commercial gelatines are manufactured from porcine or bovine skin and/or bones. However, in some cases, such as outbreaks of bovine spongiform encephalopathy, it may be necessary to differentiate between bovine and porcine gelatine for safety considerations. Another aspect is that some religions ban the consumption or use of pork derivatives. Therefore, it is necessary to develop biomarker-based analytical methods to trace the species origin of gelatine [115,116].

Peptidomics is also an asset for studying the sensory aspects of hydrolysates. Indeed, work on predicting the bitterness of milk hydrolysates using peptidomics made it possible to identify the peptides responsible for bitterness, combined with a targeted bioinformatics approach and consumer sensory tests [117]. However, sensory study combined to peptidomics dedicated to collagen peptide study are not numerous [118]. On the other hand, studies have highlighted that such peptides from food-derived proteins may, beyond their nutritional role, confer additional health benefits [119,120].

In this respect, food peptidomics has identified bioactive peptides found in different types of foods such as milk [121], soy [122], and egg white [123]. They can also be derived from agro-food by-products, which refer to the parts discarded after processing for direct consumption, such as fish by-products [124] containing collagen for instance in tilapia and halibut skin gelatines [125], Atlantic salmon skin collagen [126] and boarfish [127], meat by-products [128], and plant by-products (pulp, kernels, skins, etc.) [129]. Once peptide bioactivity has been identified, the potential activities in the organism, can be assessed after GID. However, many studies take the reverse approach, which is all the more interesting, as the whole hydrolysate is tested. Indeed, a protein matrix digested with *in vitro* simulated GID is tested for various bioactivities (e.g., inhibition of DPP-IV, ACE, anti-microbial activity, etc.) and then the potential bioactive peptides are identified by the use of methods that allow their identification, such as peptidomics. Thus, bioactive peptide sequences have been identified from bovine haemoglobin after GID [130] or from Tilapia by-product [131], halibut [125], Alaska pollock [132], and even Blue whiting [133,134].

Understanding and identification of food peptide components of the peptidome have become crucial for the study of food allergenicity, biomarker discovery, combating fraud, identification of bioactive peptides, study of the digestome, as well as valorisation of agro-food by-products [110].

Current strategies for collagen-derived peptide characterisation

From an analytical point of view, after the cleavage of the proteins, the samples are usually centrifuged to remove insoluble material. The peptide-enriched mixture is then subjected to separation and/or fractionation processes for characterisation. Note that the prerequisites for gas chromatography (GC) separation of molecules are volatility of the analytes and their thermal stability. As peptides are not naturally volatile enough and, therefore, require chemical derivatisation to vaporise, they are not suitable for GC separation [135,136]. For this separation purpose, liquid chromatography (LC) has been widely used. However, capillary electrophoresis (CE) and non-capillary electrophoresis (peptide isoelectric focusing) have also been applied, albeit to a lesser extent due to the accumulation of peptides on the walls of the CE capillary as well as due to the potential precipitation of analytes for non-capillary techniques, which leads to a deterioration in analytical performance (loss of efficiency and non-repeatability) [137].

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

One-dimensional chromatographic separation methods

Steric-exclusion chromatography (SEC) allows separation of peptides according to their hydrodynamic volume, which takes into account their shape and size. Although the molecules migrate at the same speed, peptides with a low hydrodynamic radius have access to a larger part of the total pore volume of the stationary phase than solutes with a high hydrodynamic radius, which will, therefore, be eluted first. The choice of the column is crucial, as the porosity and volume of the particles determine the separation capacity of this technique using an isocratic elution mode. Dextran gel (Sephadex®), polyacrylamide gel (Bio-Gel®), or polystyrene gel (Styragel®) resins can be used. The mobile phase must be capable of solubilising the sample. It should also be noted that the sample should not be too concentrated, as the gel matrix pores may become saturated [138]. Therefore SEC is currently used to characterise the peptide molecular mass distribution of collagen hydrolysates [139,140] but also the lot-to-lot reproducibility of collagen hydrolysates [141] as well as the purification of collagen peptides according to their average steric hindrance [142,143].

Ion-exchange chromatography (IEX) separates peptides according to their overall charge. The separation is based on a reversible interaction between the charged peptides and a resin of opposite charge. Elution of the molecules is then carried out using a pH or salt concentration gradient (NaCl or KCl). The choice of the type, composition, and pH of the mobile phase, which is usually a buffer, is important, as it affects the elution of the peptides and the shape of the peaks. The use of cationic or anionic columns is described in the literature [144,145]. The choice of strategy depends on the stability of the peptides at a given pH [146]. In overall, IEX is currently used to purify collagen peptides from various origins and test different bioactivities [147-150].

Reverse-phase (RP) chromatography is based on the use of an apolar stationary phase that interacts with hydrophobic analytes through a polar mobile phase generally composed of water/acetonitrile or water/methanol. Thus, the elution of peptides is based on their hydrophobicity. The most commonly used RP columns include silicas grafted with linear chains of 8 or 18 carbon atoms (C8 and C18). RP-chromatography is used to purify one or several peptide fractions or just one peptide [151-153]. However, the greatest advantage of this method is its compatibility with the electrospray ionisation (ESI) source in mass spectrometry (MS) for collagen peptide identification [154-156]. To achieve increased chromatographic resolution and mass detection sensitivity, nanoLC separations have been reported [157,158]. The reduced internal diameter of the column (< 100 µm) as well as the reduced mobile phase flow rate (a few $nL.min^{-1}$) lead to increased chromatographic resolution and analyte concentration, which improve detection and mass fragmentation [159-161].

Multidimensional chromatographic separation methods: To improve the analysis of complex food peptidomes, most studies perform separations by orthogonal or multidimensional chromatographic systems. However, these separations present many challenges such as the need for automated or semi-automated systems, the need for specific interfaces, and the compatibility of different mobile phases from one technique to another [162]. SEC and IEX are often applied as a first step for fractionation prior to subsequent peptide characterisation by RP-HPLC since they can be directly combined with MS. Studies adopting this strategy have successfully identified functional peptides produced from different sources; for example, from loach fish protein hydrolysate [163] or carp muscle hydrolysate. Sample prefractionation using two-dimensional offline LC (IEX followed by SEC) was used to test the bioactivities of the fractions. Subsequently, high-resolution RP-HPLC-MS/MS combined with bioinformatics tools identified the peptide sequence Pro-Ser-Tyr-Val in loach protein hydrolysate and Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val in carp muscle hydrolysate as antioxidant sequences [164].

Multi-hydrolysis strategy: In complex matrices of proteins, such as the milk micellar caseins, the identification of particular proteins and their PTMs requires successive and complementary identification methods. Indeed, by combining a four-phase approach, combining a partial-, double-enzymatic dephosphorylation and endoGluC hydrolysis, the authors were able to increase the number of identified peptides to 90% but also increase the identification of PTMs (especially phosphorylation), making this protocol a rapid and reliable method [165].

Analytical techniques for peptide identification

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

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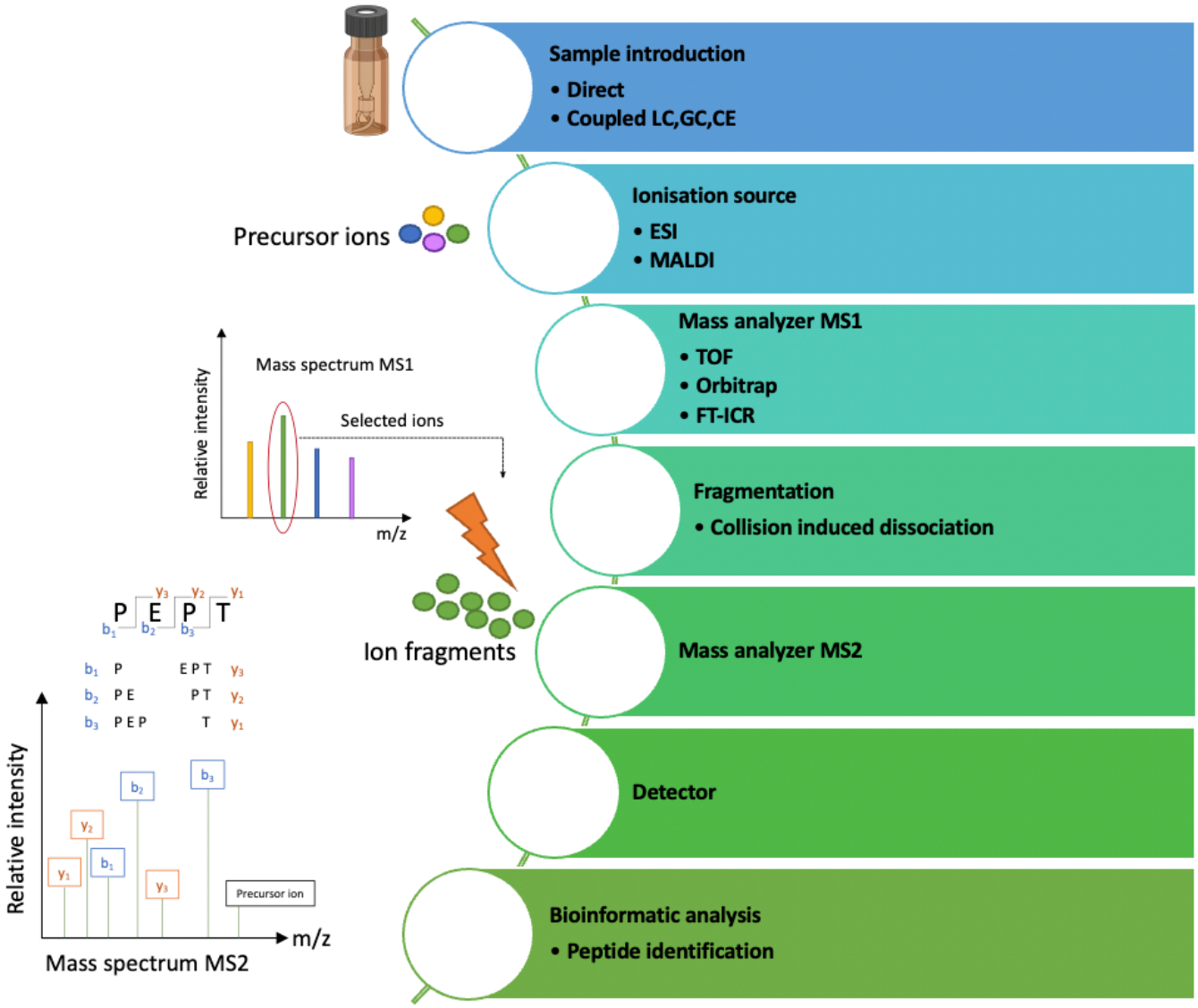

Figure 6: Schematic representation of peptide identification in MS/MS by collision-induced dissociation (CID) fragmentation.

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

Molecules are ionised in the mass spectrometer ionisation source and then a beam of precursor ions is selected in a first mass analyser (MS1) and focused in the mass spectrometer collision region where it is then fragmented by high or low energy input, which can be done in many ways, such as collision-induced dissociation (CID). The kinetic energy is then transformed in part into vibrational energy which leads to the fragmentation of the peptides. The fragments produced are then sorted by a second analyser (MS2) in order to acquire an MS/MS mass spectrum of the fragment ions (the abscissa is a mass/charge ratio scale (m/z) and the ordinate the relative intensity of the ions). The sequencing of the peptides is finally determined on the basis of their fragmentation profiles mainly through the series of b- and y- ions generated. These b- and y- ions correspond to the location of the positive charge with respect to the N-terminus and C-terminus of the peptide, respectively. Finally, the difference in mass between consecutive ions of the same series allows determination of the identity and the sequence of amino acids.

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

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good mass measurement accuracy, and unambiguous determination of peptide charge states.

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

MALDI-TOF instruments equipped with an ion selector are able to provide fragment ions produced from precursor ions that spontaneously decay in flight by post-source decay (PSD) [168]. The analysis of these so-called metastable ions leads to the determination of the peptide sequence. However, the long spectrum acquisition time and the low resolution of the ion selectors represent major drawbacks of PSD. This is problematic when analysing complex mixtures such as peptidomes of food matrices. For these reasons, the deployment of MALDI-TOF-TOF techniques improves this PSD process by ion fragmentation activation [168]. Fragment ions of given peptides are thus generated by high-energy CID in a cell collision before they enter the reflectron. These ions, therefore, penetrate the reflectron substantially and allow complete fragment ion spectra to be acquired in a single acquisition at a fixed reflectron voltage [169]. For example, peptidome characterisation applying this technique was done on collagen peptides [170,171] and a Tilapia by-product [172]. For this latter, the results revealed the identification of a total of 799 peptides. Due to the complexity of the sample, a prefractionation method by RP-HPLC or SEC is required [173]. As the samples have to be crystallised in the matrix, the chromatographic system cannot be coupled directly to the mass spectrometer. The fractions are, therefore, collected and co-crystallized with the matrix to the MALDI target. The MALDI-TOF-TOF-MS analysis is then performed offline.

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

Coupled with HR analysers such as Q-TOF or FT-MS (LTQ-Orbitrap and FT-ICR), RP-HPLC-ESI-MS/MS provides a technique capable of screening almost the entire peptide pool. Its high sensitivity and ability to detect even trace amounts of peptides in complex matrices provide the most promising approach to analysing the food peptidome [149,176,177]. For example, a study characterising the peptidome of sheep milk led to the identification of 257 peptides through RP-HPLC-ESI-Q-TOF [178], and another study on donkey milk led to the identification of 1330 peptides by nano HPLC-ESI-LTQ-Orbitrap [179], where nano HPLC corresponds to the miniaturisation of the HPLC method to increase the resolving power of the chromatographic device. For these different types of instruments, the low-energy CID activation method was applied to obtain typical MS/MS mass spectra from each selected peptide ion.

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

informatics data analysis: The analytical data is further processed and exploited through bioinformatics software owing to a large data set of up to thousands of ion spectra produced from a single sample. These software packages use mathematical algorithms and statistical tools to archive, retrieve, and analyse HPLC-MS/MS data [183]. Routine software for peptide identification includes Mascot, Sequest, Progenesis QI for proteomics, PEAKS® Studio, FlexAnalysis, X!Tandem, OMSSA, and MaxQuant [184]. Peptide identification can be performed according to two principles: (i) database searching, which compares the m/z ratio list from experimentally acquired MS/MS spectra with theoretical lists of predicted peptide fragments for each peptide contained in the database, the best known of which are UniProtKB and PepBank; and (ii) *de novo* sequencing, which presents itself as an alternative, given that peptide sequences may be absent from the databases and due to certain limitations of peptidomics. This allows determination of the peptide sequence directly from the m/z ratio of the fragmentation spectra. For this purpose, tools can be used to perform sequencing for each MS/MS spectrum such as PEAKS® Studio, PepNovo, MS-Blast, and MassHunter [118,185-187].

Advantages and challenges of peptidomics approaches

Peptidomics has revealed its advantages in the food industry, which produces both food items and by-products, both of which are rich in peptide precursor proteins. On the one hand, in the context of direct feeding of products, allergenicity is reduced and the improvement of functional properties such as solubility, improved digestibility, or intestinal absorption can contribute to solving certain problems such as poor absorption of proteins in the body [188]. On the other hand, the valorisation of food by-products responds both to the concern of recovering and using noble

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proteins and also to better valorisation of their impact on the environment [17,189,190].

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

Despite the progress made in peptidomics, certain limitations exist, such as the analysis of small peptides (di-, tri-, tetra-, or penta-peptides) because their identification requires removal of a certain number of technological constraints [192]. Indeed, they are often poorly separated by RP-HPLC and are difficult to identify by MS/MS due to the low number of fragments obtained by CID. As a result, interpretations of MS/MS spectra can be ambiguous as each spectrum often corresponds to two or more possible peptide sequences owing to a lack of informative ions. In recent work, improvement of their identification through a dimethyl labelling strategy was presented, and 843 small peptides were identified [193]. This chemical derivatisation improves the signals of a1 ions (ions resulting from loss of neutral -CO of b1 ion) in MS/MS analysis and contributes to the unambiguous identification of N-terminal residues. Nevertheless, the *de novo* matching scores remain low owing to the lack of informative fragment ions, which reduces the number of matched fragments. Further study to improve the identification and modification of sequencing algorithms is, therefore, still required to improve the accuracy of automated data analysis.

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

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

The progress of several projects and studies on the proteome in medicine has given rise to clinical peptidomics approaches. By analogy, peptidomics has been rapidly applied by food and nutrient science researchers in the more general framework of "foodomics". Thus, food peptidomics makes a crucial contribution to safety, authenticity, sensoriality, nutritional understanding, healthy food design, and by-product valorisation.

This approach nowadays represents a robust technique to identify the food peptidome through liquid chromatographic separation techniques (SEC, IEX, and RP-HPLC) and high-resolution tandem mass spectrometry combined with bioinformatics. In addition, the coupling of different chromatographic techniques upstream of the analysis provides better separation power and resolution, which result in more complete identification of the peptides present in complex mixtures. However, further research is needed for the near-complete identification of the agro-food peptidome by filling the main gaps related to the identification of size-reduced and modified peptides. The near-complete identification of agro-food proteomes will require the development of even more powerful bioinformatics tools.

Fields of application of collagen and its derivatives

Antioxidant and antimicrobial properties

Antioxidants are molecules with the ability to metabolise free radicals and, therefore, neutralise the deleterious effects of oxidative stress at the cellular as well as tissue levels [197]. The antioxidant activity of gelatine extracted from fish by-products has been the subject of several studies. Indeed, peptides were identified from different species and exerted antioxidant activities, such as PYSFK, GFGPEL, and VGGRP from grass carp [198], DPALATEPDMPF, EGL, YGDEY, and LSGYGP from Nile Tilapia (*O. niloticus)* [199,201], PFGPD, PYGAKG, and YGPM from Spanish mackerel (*Scomberomorous niphonius)* [201], GSGGL, GPGGFI, and FIGP from Bluefin leatherjacket (*Navodon septentrionalis)* [202], and AVGAT from Thornback ray [133]. In a general manner, the particularly low-molecular-mass (MM) peptides (< 3 kDa) exhibited higher radical scavenging properties [203], and it was shown that Tilapia skin collagen peptides (MM < 3 kDa) had protective effects against the D-galactose-induced liver and kidney damage in mice by reducing oxidative stress, for instance [204].

As for antimicrobial activity, bioactive peptides from yellowfin tuna (*Thunnus albacares*) skin collagen have shown efficacy notably with minimum effective concentration (MEC) values of 1.2, 6.5, 17, 8, 3, and 3.2 $\mu g.mL^{-1}$ against *B. subtilis, M. luteus, S. iniae, A. hydrophila, E. coli*, and *V. parahaemolytics* strains, respectively [205]; skipjack tuna (*Katsuwonus pelamis*), with MEC values of 3, 26, 4.8, 25, 2.7, 9, and 16 $\mu g.mL^{-1}$ against *B. subtilis, M. luteus, S. iniae, A. hydrophila, E. coli, V. parahaemolytics, and C. albicans* strains, respectively [206]; and yellow catfish (*Pelteobagrus fulvidraco*), with minimum inhibitory concentration (MIC)

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values of 2, 4, 16, and 64 µg.mL^{-1} against *B. subtilis, S. aureus, E.coli, and C. albicans,* respectively [207].

Anti-inflammatory properties

Collagen peptides have also been shown to be effective in preventing and reducing inflammation. Collagen peptides extracted from sea cucumber (*Acaudina molpadioides*) were administered by gavage to male mice for 21 days, followed by intraperitoneal injection of 10% CCL_4 (nephrotoxic molecule) to establish an acute model of kidney injury. The results highlighted the protective effects of collagen peptides that significantly decreased the levels of interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF-α [208].

This anti-inflammatory effect has been applied directly in the context of titanium implants. Indeed, when the latter are inserted in the body, a pro-inflammatory reaction takes place, which is necessary for the osseointegration process. However, if this inflammation is excessive and persists over time and becomes chronic, implant failure occurs. Thus, it is important to be able to control this inflammation [209] and the 'use' of collagen peptides can be advantageous. Indeed, a collagen-coated titanium alloy enriched with phenolic compounds has been shown to reduce inflammatory parameters by decreasing the gene expression of IL-6 and TNF-α [210].

Collagen peptides can be used in ways other than as biomaterials. In a study analysing the oral intake of collagen peptides from monkfish (*Lophius litulon*) skin, researchers showed an improvement in renal inflammatory status induced by a high-fat diet in mice, in particular by regulation of nuclear factor erythroid 2-related factor (Nrf2) and nucleotide-binding and oligomerisation domain-like receptors protein 3 (NLRP3) signalling pathways, which are implicated in regulation of the responses to oxidative stress and initiation of the inflammatory cascade [211,212]. Indeed, compared with the control group, the collagen peptide-fed group had decreased levels of IL-1β, IL-6, and TNF-α in kidney homogenate [213].

Antihypertensive activities

Of the numerous pathologies associated with obesity and T2D, as part of metabolic syndrome, hypertension is a growing public health problem worldwide [214]. People suffering from hypertension have systolic blood pressure values ≥ 140 mm Hg and/or diastolic blood pressure values ≥ 90 mm Hg [215]. This condition is partially due to chronic activation of the renin-angiotensin-aldosterone system (RAAS). The presence of excess angiotensin II and aldosterone in the circulation promotes a proinflammatory, fibrotic, and hypertrophic status, particularly in the heart and kidneys [216], making it paramount to address this condition.

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

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

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

Moreover, according to Wu et al., the optimal amino acid residues identified *in silico* for potent ACE inhibition are peptides starting with Tyr or Cys in the first position at the C-terminus; Trp, Met, and His in the second position; Leu, Ile, Val, and Met in the third position, and Trp in the fourth position [227]. Peptides with hydrophobic and aromatic residues at the N- and C-terminal ends generally have higher antihypertensive activities [228].

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

The majority of antihypertensive peptides reported from marine proteins hydrolysates have ACE IC_{50} values ranging from 0.3 to 1 500 µM [231]. Crustacean peptides (shrimp, krill) exhibit IC_{50} values ranging between 0.9-24.1 µM [232,233], between 1.2-51 µM for mollusc peptides

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[234,235], and finally between 2.4-23.4 µM for coelenterates and echinoderms peptides [236,237]. It should be kept in mind that pharmacological ACE inhibitors are 1 000 times more efficient than antihypertensive peptides, but without harmful side effects. Numerous studies have also focused on the ACE-inhibitory properties of peptides derived from gelatine extracted from various fish species. These peptides were generated using various commercial enzymes. Indeed, peptides derived from gelatine extracted from fish skins have proven to be very good candidates, e.g., following the use of commercial Alcalase, the peptide Gly-Pro-Leu (GPL) from Alaska pollock had an IC_{50} value of 2.6 µM, and Nile Tilapia hydrolysate had an IC_{50} value of 62.2 µM [238,239]. For gelatine extracted from the scales of triplecross lizardfish (*Synodus macrops*) and milkfish (*Chanos chanos*), the peptides exhibited IC_{50} values of 420 µM and 472 µM, respectively [240,241]. For collagen extracted from *Theragra chalcogramma* skins, the isolated peptides GPL and Gly-Pro-Met (GPM) exhibited IC_{50} values of 2.6 and 17.1 µM, respectively [238]. Previous work also demonstrated that a tripeptide Leu-Gly-Trp (LGW) was able to reduce blood pressure in SHRs after oral administration [242]. Altogether these results demonstrate the efficiency of food-derived peptides and more specifically gelatine- and collagen-derived peptides in the management of hypertension.

Glucose metabolism regulation

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

Hormone secretion modulation

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

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

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

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

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

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

Inhibition of DPP-IV activity

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

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

Concomitantly, a study assessing oral intake of Tilapia (*Oreochromis niloticus*) collagen hydrolysate has led to establishing, this time in normal mice, the inhibitory activity for DPP-IV, with an IC_{50} value of 0.77 $mg.mL^{-1}$ [254]. Theysgeur *et al*. also studied the effect of Tilapia hydrolysates containing collagen and tested their ability to inhibit DPP-IV both *in vitro* and *in situ*. They were able to identify four sequences of bioactive peptides after SGID and *in vitro* intestinal barrier passage [132].

In addition to marine sources, a hydrolysate of camel gelatine skin has shown beneficial effects on DPP-IV inhibition as well [255]. Recently, Jin and colleagues [127] used Atlantic salmon skin, several peptidases, and fractionation methods of hydrolysates to evaluate DPP-IV inhibition activity, and they identified novel DPP-IV inhibitory peptides from the generated subfractions; the highest IC_{50} value was 0.79 ± 0.13 $mg.mL^{-1}$.

The literature shows, for instance, that after SGID of Alaska pollock skin collagen, the DPP-IV IC_{50} value was 1.39 ± 0.08 $mg.mL^{-1}$ [133]. Investigation of DPP-IV inhibition of barbel skin gelatine-derived peptides [256] led, after performing hydrolysis using Esperase®, the lowest IC_{50} value of 2.21 $mg.mL^{-1}$.

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

Conclusion

The body of literature presented in this review has shed light on the use of gelatine throughout history, its physicochemical and structural properties, and its endogenous production. The various contemporary methods for its extraction as well as the different sources from which it can be extracted are also discussed. Nevertheless, certain technological barriers do not yet allow for exhaustive identification of their bioactive peptide compositions, but thanks to the optimisation of computing tools and a better understanding of preparation methods, this should no longer be a limiting factor in this regard. In the contemporary worldwide trend, the sustainable use of agro-food waste and/or by-products to produce value-added products for potential applications in the cosmetics, pharmaceutical, or food industry can offer considerable additional income opportunities for the dependent industry. In addition, valorisation of agro-food wastes and by-products can guarantee regional food safety and thus ensure sustainable food production at a time when societal and indeed economic pressures can put food-dependent countries or regions at a harmful disadvantage. Until now, most agro-food by-products have been used as a source of fuel, animal feed, or organic fertiliser. Presently, with the availability of modern technologies, new concepts have been established that enable efficient use of by-products from the agro-food sector to produce value-added products and also to accurately identify the high-interest entities, while limiting waste generation and, therefore, the carbon footprint.

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

Author contributions

Mouna Ambli, Benoit Cudennec, Barbara Deracinois and Christophe Flahaut: Conceptualization, visualization and writing the original draft. Benoit Cudennec and Christophe Flahaut: Review and editing the manuscript. Benoit Cudennec, Christophe Flahaut and Rozenn Ravallec: Supervision, funding acquisition and project administration. All authors read and approved the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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