

1 **Use of chromatographic and electrophoretic tools for assaying elastase, collagenase, hyaluronidase, and**  
2 **tyrosinase activity**

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16 **List of abbreviations:** **BGE:** background electrolyte; **BTH:** bovine testicular hyaluronidase; **CS:** chondroitin  
17 sulfate; **ECM:** extracellular matrix; **EMMA,** electrophoretically mediated microanalysis; **FAC:** frontal  
18 analysis chromatography; **HA,** hyaluronic acid; **HNE,** human neutrophil elastase; **Hyal,** hyaluronidase; **IB:**  
19 incubation buffer; **LIF,** laser induced fluorescence; **MMP,** matrix metalloproteinases; **MST:** microscale  
20 thermophoresis; **PMMA,** pressure-mediated microanalysis; **SFE:** skin fibroblast elastase; **TDLFP,** transverse  
21 diffusion of laminar flow profiles; **Tyr,** tyrosinase.

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24 **Keywords:** Capillary electrophoresis, enzymatic assays, high performance liquid chromatography, microscale  
25 thermophoresis, kinetic constants

35 **ABSTRACT**

36 Elastase, collagenase, hyaluronidase and tyrosinase, are very interesting enzymes due to their direct  
37 implication in skin aging and as therapeutic hits. Different techniques can be used to study these enzymes and  
38 to evaluate the influence of effectors on their kinetics. Nowadays, analytical techniques have become  
39 frequently used tools for miniaturizing enzyme assays. The main intention of this article is to review  
40 chromatographic and electrophoretic tools that study the four enzymes above mentioned. More specifically,  
41 the use of high-performance liquid chromatography and capillary electrophoresis and their derivative  
42 techniques for monitoring these enzymes will be investigated. The advantages and limitations of these assays  
43 will also be discussed. The original use of microscale thermophoresis and thin layer chromatography in this  
44 domain will also be covered.

## 1. Introduction

The skin is composed of three layers; the epidermis, dermis and the subcutaneous layer [1]. The extracellular matrix (ECM) is the outermost part of the skin and contains essential components for wound healing [2]. The molecules associated with the ECM of each tissue, including collagens, proteoglycans, laminins and fibronectin, and the manner in which they are assembled determine the structure and the organization of the ECM [3]. The degradation of ECM is thus linked to skin aging. Different intrinsic and extrinsic factors can affect the skin and cause its damage [4, 5]. Intrinsic structural changes occur as a natural consequence of aging and are genetically determined while extrinsic factors are, to varying degrees, controllable. The extrinsic agents include exposure to UV sunlight, pollution or nicotine, repetitive muscle movements like squinting or frowning, and miscellaneous lifestyle components such as diet, sleeping position, and overall health [6-8]. Solar radiation can damage human skin such as an acute overexposure that causes clinical sunburn. Chronic exposure can lead to skin changes such as plaque-like thickening, loss of skin tone, deep furrowing and fine wrinkle formation. Collectively, these changes may be termed 'photoaged skin' or 'solar scar'. The solar UV radiation has particularly a negative effect on the health of the skin, since they involve the generation of reactive oxygen (ROS) species and cellular oxidative damage [9]. Figure 1 shows the induction of skin aging caused by environmental factors and more specifically by solar UV. The generated ROS induce angiogenesis, which is the process of generating new blood vessels [10, 11]. However, the blood vessels are also decreased in chronically UV-damaged human skin. This damage is known to result in marked degenerative changes of the upper dermal connective tissue with degradation of elastic fibers, collagen fibers and hyaluronic acid (Figure 2). This is probably due to increased expression of elastase, matrix metalloproteinase (MMP), and hyaluronidase (hyal) produced in keratinocytes, fibroblasts and various inflammatory cells (Figure 1). On the other hand, UV sunlight and ROS activates melanogenesis, which is the process of melanin production and distribution [12-14]. Tyrosinase (tyr), the key protein involved in skin pigmentation due to the presence of melanin pigment, is activated and a hyperpigmentation is produced [12]. Therefore, understanding the evolution of enzymes in the human body is important to understand and to find new therapeutics for a broad range of biological phenomena [15, 16].

Elastase, known for its capacity to cleave proteins, prefers the amino acids valine and alanine (Figure 2.a). Available evidence suggests that there are at least two main types of elastases implicated in the skin degradation, human neutrophil elastase (HNE) and skin fibroblast elastase (SFE). HNE is derived from neutrophil cells and SFE from fibroblasts and these two differ in their substrate specificity [17]. HNE is a 29 kD serine endoprotease of the proteinase S1 family. It exists as a single 238 amino acid-peptide chain with four disulfide bonds and contains two or three N-linked glycans of variable composition that account for its three major isoforms with an isoelectric point of 8.77 - 9.55. Natural substrates include elastin, cartilage proteoglycans, collagen types I, II, III and IV, and fibronectin [18]. SFE has been characterized as a membrane-

80 bound metalloproteinase with little information about its structure, amino acid sequence, and encoding gene.  
81 It acts on oxytalan fibers and elaunin fibers but has limited effects on mature elastic fibers. Several studies  
82 suggests that SFE is identical to neprilysin, (neutral endopeptidase 24.11 or NEP) due to similarities in their  
83 molecular weights and activity profiles [19, 20]. HNE and SFE are also implicated in the metabolism of elastic  
84 fibers in various types of tissues during inflammation or diseases [21, 22].

85 The matrix metalloproteinases (MMPs) are a family of zinc and calcium dependent enzymes which are  
86 important in the resorption of ECM in normal physiological processes and pathological states [7]. These  
87 enzymes are responsible for the degradation of ECM molecules and are also implicated in the tissue  
88 remodeling, which accompanies inflammation, bone resorption, atherosclerosis, and the invasion of tumors  
89 [23]. Most of collagnases belongs to the MMP family. Collagenases are responsible for collagen degradation  
90 by breaking the peptide bonds (Figure 2.b). Collagen is the major insoluble fibrous protein in the ECM and  
91 the connective tissue and its primary function is to maintain skin firmness. Sixteen types of collagen exist and  
92 around 80 – 90 % of the collagen in human body only consists of types I, II, and III. Different types of  
93 collagenases exist like gelatinase B, which cleaves gelatine type I and collagen types IV and V, gelatinase A  
94 which cleaves gelatine type I and collagen types IV, V, VII and X and neutrophil collagenase which cleaves  
95 interstitial collagens with a preference for collagen type I [24].

96 Skin aging is also associated with loss of skin moisture where the most important key molecule involved is  
97 hyaluronic acid (HA). This high molecular weight mucopolysaccharide ( $1.5-1.8 \times 10^6$  Da) was discovered in  
98 1934 by Karl Meyer in the vitreous of bovine eyes [25]. This non-sulphated glycosaminoglycan is composed  
99 of repeating polymeric disaccharides of D-glucuronic acid and N-acetyl-D-glucosamine linked by a  
100 glucuronic  $\beta$  (1→3) bond (Figure 2.c) [26]. Its function is, amongst other things, to bind water and to  
101 lubricate movable parts of the body, such as joints and muscles. Its consistency and tissue-friendliness allow  
102 it therefore to be beneficial in skin-care products as an excellent moisturizer. This acid is naturally synthesized  
103 by a class of integral membrane proteins called hyaluronan synthases, and degraded by a family of enzymes  
104 called hyaluronidases or Hyal [27], which display both hydrolytic and transglycosylation activities. In the  
105 presence of NaCl, at pH 4-5 and at 37°C, Hyal mainly catalyzes a hydrolysis reaction with a preference for  
106 HA over chondroitin sulfate (CS). In the absence of NaCl, at higher pH of 7 and at 37°C or higher, Hyal mainly  
107 catalyzes a transglycosylation reaction and prefers CS over HA [28, 29]. In the human genome, six Hyal  
108 sequences occur with similar catalytic mechanisms. The most recent overview dealing with these enzymes  
109 was prepared in 2008 by Stern et al [30].

110 Tyrosinase is present in melanosome granules present in melanocytes. This multifunctional, glycosylated, and  
111 copper-containing oxidase catalyzes the first of the two steps in mammalian melanogenesis. This is the entire  
112 process leading to the formation of dark macromolecular pigments, like melanin in the skin. Melanogenesis is  
113 initiated with the first step of tyrosine oxidation to dopaquinone catalyzed by Tyr (Figure 2.d). In mammals,

114 there are two types of melanin, the brownish black eumelanin and the reddish yellow pheomelanin. A  
115 suractivity of Tyr leads to overproduction of melanin leading to hyper-pigmentation of the skin. These  
116 phenomena have therefore encouraged researchers to study the activity of Tyr and to seek new potent inhibitors  
117 for use in cosmetics.

118 This review summarizes the different studies made on elastase, collagenase, hyaluronidase and tyrosinase by  
119 chromatographic (Table 1) and electrophoretic tools (Table 2). Analytical assays usually measure the product  
120 formation or the excess of substrate by separating the reaction mixture into its components. High performance  
121 liquid chromatography or HPLC and capillary electrophoresis or CE are the two main analytical techniques  
122 used for monitoring enzyme kinetics. The compatibility of these techniques with various detection modes (UV,  
123 DAD, LIF, C<sup>4</sup>D and MS) makes them interesting for studying all types of molecules with low sample  
124 consumption, speed, automation of analysis and satisfactory sensitivity. The usefulness of HPLC and CE stems  
125 from their power as separation techniques and from their versatility.

## 127 2. Enzyme kinetics

### 128 a) Michaelis-Menten model

129 Enzymes are highly specific since they catalyze particularly a single chemical reaction or a set of closely  
130 related chemical reactions without the alteration of the equilibrium point of the reaction. When the reaction  
131 between an enzyme and its substrate occur, instead of a linear plot of velocity versus the substrate  
132 concentration, the experiment yields a hyperbolic curve. The analysis of the data suggested that the reaction  
133 is first-order at very low concentrations of substrate (S) and with the increase of S concentration ([S]), the  
134 reaction became progressively closer to zero<sup>th</sup>-order. This phenomenon was explained by the fact that the  
135 enzyme and substrate interact to form an intermediate enzyme-substrate complex. The substrate binds to a  
136 very specific region of the enzyme molecule, called the active site that is highly active for specific substrates.  
137 The mechanism of enzyme catalyzed reactions is often studied by measuring rates of the enzyme-catalyzed  
138 reactions at different substrate and enzyme concentrations. The simplest model of an enzymatic reaction is:



140 Where E is the enzyme, S is the substrate, E-S the enzyme substrate complex and P the product of the enzyme-  
141 catalyzed reaction.  $K_m^{app}$  is the apparent Michaelis-Menten constant and  $k_{cat}$  is the catalytic constant or the  
142 turnover number of the forward reaction of E-S forming E + P.

143 The Michaelis-Menten equation presents the reaction velocity versus the substrate concentration. The  
144 Michaelis-Menten kinetics ( $K_m$  and  $V_{max}$ ) can be determined by measuring velocity at a variety of substrate  
145 concentrations. These constants are observed when the entire enzyme present is fully saturated with substrate.

146  $K_m$  is equal to the substrate concentration at which the reaction rate ( $v$ ) is half its maximum value ( $V_{max}$ ). The  
147 equation of the determination of Michaelis-Menten constant is:

$$148 \quad v = \frac{V_{max} [S]}{K_m + [S]} \quad (2)$$

149 The value of  $K_m$  for an enzyme depends on the particular substrate, the pH of the incubation buffer (IB) and  
150 the temperature at which the reaction is carried out [31].  $V_{max}$  is also related to  $k_{cat}$  by the equation:

$$151 \quad k_{cat} = \frac{V_{max}}{[E]_T} \quad (3)$$

152 where  $[E]_T$  corresponds to the total concentration of the enzyme.

### 153 **b) Enzyme effectors**

154 Many factors play an important role in the running of an enzymatic reaction (the ionic strength or salt  
155 concentration of the buffer, the presence of a major cation ( $Na^+$ ,  $K^+$ ) and pH). The optimal pH of an enzymatic  
156 activity depends also on the origin of the enzyme. Therefore, the human dermal fibroblast elastase has an  
157 optimum pH of 8.5 [32], the collagenase a pH of 7.4 [33], the hyaluronidase a pH of 3.7 [34] and the tyrosinase  
158 a pH of 6.4 [35]. The temperature plays also an important role for the activation of an enzymatic reaction [36].  
159 Since the enzymes guide and regulate the metabolism of a cell and an organism, they tend to be controlled by  
160 regulatory molecules called effectors. These molecules can either increase or reduce enzyme's activity.  
161 Molecules that increase the activity are called activators, while those that decrease the activity are called  
162 inhibitors.

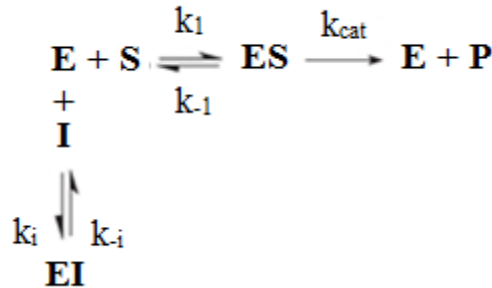
163 In order to determine the efficiency of an inhibitor/activator, a quantification of the effector potency is  
164 required. The two most common parameters for quantifying an effector are  $IC_{50}$  and  $k_i$  (the dissociation  
165 constant) for an inhibitor and  $AC_{50}$  and  $k_A$  (the activation constant) for an activator [37].  $IC_{50}$  is the  
166 concentration of an inhibitor required to reduce the enzyme activity by 50% while  $AC_{50}$  is the concentration  
167 of an activator required to increase the enzyme activity by 50 %. A series of experiments are necessary to  
168 determine  $IC_{50}$  or  $AC_{50}$  value by maintaining a constant concentration of substrate so that the enzyme can react  
169 at an appreciable rate.

170 Activators or inhibitors can be natural, synthetic or semi-synthetic and are classified into two types: non-  
171 specific and specific [38, 39]. A non-specific inhibitor for example induces physical or chemical changes,  
172 which ultimately denatures the protein part of the enzyme and is therefore irreversible (pH, temperature, acids  
173 and bases).

174 The enzyme inhibition or activation can be either reversible or irreversible.

175 An irreversible reaction dissociates very slowly from its target enzyme because it has become tightly bound  
176 to the enzyme, either covalently or noncovalently. The reversible one, in contrast, is characterized by a rapid

dissociation of the enzyme-inhibitor/activator complex. A reversible reaction can be competitive, uncompetitive or non competitive. The competitive effector behaves such as the substrate and binds then to the active site of the enzyme. The substrate is thereby prevented from binding to the same active site. The following scheme represents a competitive inhibition:



where  $K_d = k_{-1}/k_1$  and  $K_i = k_{-i}/k_i$ ; the classic expression is:

$$K_i = \frac{IC_{50}}{(S / K_m + 1)} \quad (4)$$

$$\text{if } \begin{cases} S = K_m \rightarrow K_i = IC_{50} / 2 \\ S \gg K_m \rightarrow K_i \ll IC_{50} \\ S \ll K_m \rightarrow K_i \cong IC_{50} \end{cases}$$

The uncompetitive inhibition occurs when the inhibitor binds to the enzyme-substrate complex and not to the free enzyme. In noncompetitive inhibition, the inhibitor and substrate can bind simultaneously to an enzyme molecule at different binding sites. The noncompetitive inhibition, in contrast with competitive inhibition, cannot be overcome by increasing the substrate concentration. A more complex pattern, called mixed inhibition, is produced when a single inhibitor both hinders the binding of substrate and decreases the turnover number of the enzyme.

Enzymatic reactions can be studied either by continuous assays, that give a continuous reading of activity, or by discontinuous assays, where samples are taken, the reaction is stopped and the concentrations of substrates and/or products are determined. The assay is the act of measuring how fast a given amount of enzyme will convert substrate to product by determining the velocity and requires the determination of the concentration of a product or substrate after a certain incubation time. Different methods can be used depending on the type of reaction catalyzed and the nature of substrate and product.

### 3. Chromatographic and electrophoretic tools for enzyme assays

In HPLC and CE, assays can be performed using two different modes (Figure 3). The first one, called offline mode, consists in mixing the enzyme and its substrate (in the presence or absence of the effector) outside the

201 separation column (or the capillary in CE). The pre-column assay is the most frequently used due to its  
202 simplicity. The enzymatic reaction occurs during a certain time of incubation in a vial containing an incubation  
203 buffer (IB). Then, a few nanolitres (1-10 nL) by CE or microliters (5-20  $\mu$ L) by HPLC of the reaction mixture  
204 are injected in the separation column for analysis and quantification. For the reaction mixture, the enzyme may  
205 also be immobilized on silica particles or magnetic beads. In this case, the injection of the reactants can be  
206 done by continuous flow injection especially in HPLC. In the online mode, the separation column acts as an  
207 enzymatic nano- or micro- reactor and not only as a separation tool. Indeed, the enzymatic reaction takes place  
208 directly inside the column. In the heterogeneous mode, the enzyme (or occasionally the substrate) is  
209 immobilized inside the column. Moreover, multiple enzymes can be co-immobilized onto chromatographic  
210 supports. Reaction time can thus be shortened and fast screening of enzyme effectors can be performed.  
211 Immobilized enzymes generally maintain high catalytic specificity but may lose some of their catalytic  
212 efficiency. Their stability against heat, pH, and organic solvents is commonly increased by immobilization.  
213 However, the main problem in this process is related to the properties of support materials. The ideal support  
214 material should have good biocompatibility, known three-dimensional porous structure and chemical groups  
215 to avoid unspecific interactions with substrates and/or effectors. Many approaches for enzyme immobilization  
216 can be used depending on the properties of the target enzymes like entrapment, cross-linking, adsorption, or a  
217 combination of these methods [40]. In order to maintain the stability of the enzyme, covalent method is usually  
218 used to immobilize enzymes in the separation column [41].

219 Nowadays, capillary electrophoresis (CE) is one of the most promising analytical techniques for the study of  
220 enzymatic reactions [42-44]. Homogeneous assays can be used specifically in CE. In that case, all reactants -  
221 the enzyme, the substrate (and the inhibitor) - are free in solution and are introduced as separate plugs (plug-  
222 plug mode) in the capillary previously filled with the separation buffer also called the background electrolyte  
223 (BGE). A critical step of the successful development of such assays is to achieve an effective mixing of enzyme  
224 and substrate plugs into the capillary. For this, two main mixing modes have been employed based either on  
225 the electrophoretic migration or on the diffusion of the reactants inside the capillary. The first approach was  
226 introduced in 1992 by Bao and Regnier [45]. It is named electrophoretically mediated microanalysis or EMMA  
227 since it uses the different electrophoretic mobilities of the enzyme, substrate(s) and effector(s) for mixing  
228 reactants inside the capillary [44]. General parameters that have to be optimized for EMMA methods include  
229 reactant concentrations, plug lengths and injection sequence. The mixing by longitudinal diffusion of reactants  
230 through the interface between the plugs was named "at-inlet reaction" since the enzyme and the substrate plugs  
231 are placed at the inlet of the capillary. Another method called pressure-mediated microanalysis or PMMA was  
232 introduced in 2012 by our group [46] where mixing was obtained by longitudinal diffusion that is enhanced  
233 by applying a continuous pressure until detection. However, mixing using longitudinal diffusion is inefficient  
234 when relatively long plugs or more than two plugs are to be mixed because longitudinal diffusion is a rather



235 slow process. In order to overcome these limitations, mixing by transverse diffusion of laminar flow profiles  
236 (TDLFP) has been developed by Krylov et al. [47] in 2005 to assess  $\beta$ -galactosidase activity. This method is  
237 efficient for mixing two or more reactants inside a capillary. The injection of the reactants is conducted by  
238 applying relatively high pressure during short time to obtain plugs with a parabolic shape [47, 48] responsible  
239 for the mixing process by transverse diffusion [49]. Krylov et al. [50] also developed a software able to  
240 accurately simulate the spatial overlapping of plugs taking into account injection pressure, plug length, reactant  
241 coefficient of diffusion and concentration, and inner radius and length of the capillary.

242 More specifically, Tables 1 and 2 details the conditions optimized in literature for assaying elastase,  
243 collagenase, hyaluronidase and tyrosinase using chromatographic and electrophoretic approaches. These  
244 studies are detailed in the following sections.

#### 246 **4. Elastase activity monitored by HPLC, CE and microscale thermophoresis (MST)**

247 The activity of elastase has been studied by several methods like photometry [51], nuclear magnetic resonance  
248 (NMR) [52], UV spectrophotometry [53], surface plasmon resonance [54] and also by chromatographic (Table  
249 1) and electrophoretic (Table 2) tools as discussed below .

250 The first study of elastase by HPLC was done in 1989 by Stein et al. [55]. These authors developed a RPLC  
251 method to study the activity of elastase by measuring the quantity of elastin peptides obtained by the  
252 degradation of elastine. A simple offline assay was used to mix the enzyme with its natural substrate, the  
253 elastin. After 6, 24 and 48 h of incubation, the mixture was injected in a C<sub>18</sub> column using a linear gradient  
254 from 0 to 10 % of 1-propanol containing 50 mM of heptafluorobutyric acid (pH 3.0). An online post column  
255 derivatization of the products obtained was then performed by mixing the mobile phase with 0.2 M of borate  
256 buffer (pH 8.0) followed by the addition of fluorescamine. More precisely, the dipeptide Val-Pro released was  
257 marked with the fluorescamine and followed for quantification at excitation and emission wavelengths of 395  
258 and 455 nm, respectively. This method was also shown to be useful in the measurement of the elastolytic  
259 activity of tissues. However, the purification of the reaction mixture and the derivatization step were time- and  
260 reactant- consuming.

261 To facilitate the kinetic study of elastase, Viglio et al. [56] used short sequences of peptides that are  
262 representative of elastin. Three types of elastase were studied, human neutrophil elastase (HNE), *Aspergillus*  
263 *fumigatus* elastase, and porcine pancreatic elastase. Their appropriate substrates are presented in Table 1. An  
264 offline RPLC-based assay was developed based on the quantification of the released product (nitroaniline or  
265 NA) in each case. A C<sub>18</sub> column was used and UV detection at 214 nm was conducted. Good linear regression  
266 was obtained and the K<sub>m</sub> and V<sub>max</sub> values evaluated were in good agreement with those obtained by  
267 conventional spectrometric assay at 410 nm. The aim of this work was also to develop a new approach by CE  
268 for the rapid determination of elastase activity, which could eventually become a promising alternative to the

269 spectrophotometric and HPLC-based techniques. Therefore, micellar electrokinetic chromatography (MEKC)  
270 was used to separate the products of the enzymatic reaction using 35 mM of tetraborate (pH 9.3) with 65 mM  
271 SDS and 15 % MeOH as running buffer. The peaks of the substrate and products were well resolved with a  
272 migration time of 15 min.  $K_m$  of MeO-Suc-Ala-Ala-Ala-Pro-Val-pNA ( $S_0$ ) cleaved by HNE was successfully  
273 determined to be equal to 0.10 mM. These results were successfully comparable to those obtained by  
274 spectrophotometric and RPLC assays [56].

275 Using similar electrophoretic conditions, the same authors [57] analyzed the proteolytic activities of several  
276 elastases having different specificities of bond cleavage and present simultaneously in the incubation mixture.  
277 *Pseudomonas aeruginosa* elastase (PsE) and human leukocyte elastase (HNE) were incubated with Suc-Ala-  
278 Ala-Ala-NA ( $S_0$ ) as substrate. PsE cleaves the substrate after the first alanine, giving then two products Suc-  
279 Ala-OH and Ala-Ala-NA while HNE gives Suc-Ala-Ala-Ala-OH and NA. The identification of all fragments  
280 obtained from the cleavage of the substrate by the two enzymes showed that MEKC was able to detect two  
281 different proteolytic activities simultaneously in a simple and efficient assay.  $K_m$  of PsE and of HNE were  
282 determined to be 0.67 and 4.5 mM, respectively. However, some limitations were present due to the use of  
283 SDS micelles, low detection sensitivity and relatively high reagent consumption (few tens of microliters).

284 Based on this work, our group studied the kinetics of HNE by developing new offline and online CE-based  
285 assays [58]. First of all, the short-end injection at the outlet side of the capillary was used in order to decrease  
286 the analysis time (3 min instead of 15 min) and the incubation buffer (25 mM HEPES and 20 mM NaCl) was  
287 optimized based on the “high salt stacking” method for a better sensitivity [59]. In these conditions,  $K_m$  for  
288 the substrate  $S_0$  was determined to be  $0.17 \pm 0.04$  mM and found to be similar to that determined by Viglio et  
289 al. [56] (Table 2). The consumption of analytes was minimized by using two online approaches based on  
290 EMMA and TDLFP for in-capillary mixing. With EMMA assay, the injection (~18 nL) sequence was  
291 conducted as follows: IB (incubation buffer), HNE,  $S_0$  and IB. A plug of the IB was injected at the beginning  
292 of the injection sequence to prevent the inactivation of the enzyme by the background electrolyte or BGE  
293 (partial filling mode). A negative potential of -5 kV was then applied for 30 s across the capillary to  
294 electrophoretically mix the reactants (Table 2). The incubation time was only of 5 min and a voltage of - 25  
295 kV was applied to separate the substrate from the products. For the determination of  $K_m$  and  $V_{max}$ , TDLFP was  
296 used. The injection sequence, presented in Figure 4.a, was therefore conducted in a sandwich mode to offer a  
297 good mixing of the enzyme with the substrate when the transversal diffusion will occur: IB, HNE,  $S_0$ , HNE  
298 (~7 nL) and then IB (~18 nL). The incubation time was also equal to 5 min and a voltage of -15 kV was applied  
299 for electrophoretic separation. This first IB plug must have a rectangular shape to avoid its transversal  
300 diffusion.  $K_m$  for  $S_0$  was determined to be  $0.13 \pm 0.02$  mM, which corresponds to the value obtained by offline  
301 MEKC assay (Figure 4.b). To increase the sensitivity of the developed assay, the laser induced fluorescence  
302 (LIF) detector was employed instead of UV and three synthetic peptides were tested as new potential substrates

for HNE by evaluating the corresponding catalytic constants; S<sub>1</sub> or 5-FAM-Ala-Ala-Ala-Val-OH, S<sub>2</sub> or 5-FAM-Ala-Ala-Ala-Phe-Tyr-Asp-OH and S<sub>3</sub> or 5-FAM-Arg-Glu-Ala-Val-Val-Tyr-OH. The tested substrates were chosen taking into account their ionization properties (pK<sub>a</sub>) to avoid the use of MEKC mode and the narrow cleavage site specificity of HNE. CZE-LIF used a 50 mM tetraborate buffer at pH 8.75 as BGE. Using TDLFP online assay, HNE was found to cleave S<sub>2</sub> and S<sub>3</sub> but failed with S<sub>1</sub> [60]. This was explained by the fact that HNE does not cleave the substrates containing in their structures the large hydrophobic amino acid Tyr in the position P'1 [61]. The catalytic constants of the two new substrates were successfully determined and the values showed good affinity (K<sub>m</sub> = 0.07 mM) towards HNE compared to the reference substrate S<sub>0</sub> (K<sub>m</sub> = 0.13 mM). The inhibition of HNE by two standard inhibitors from the triterpene family, the ursolic and the oleanolic acids, was assessed [60]. The solubility of the pentacyclic triterpenes was evaluated using two organic solvents, ethanol and DMSO. The effect of these solvent on HNE was monitored using a novel technique named microscale thermophoresis (MST) (Figure 5.a). This technique is based on the unique physical principle of thermophoresis, which is the directed motion of molecules in temperature gradients [62-64]. Under constant buffer conditions, thermophoresis probes the size, charge and hydration shell or conformation (solvation entropy) of the molecules. When ursolic acid was prepared in 5 % DMSO (v/v), a bumpy signal was observed on the thermophoretic curves indicating an aggregation of HNE. When stock solutions of ursolic acid were prepared in EtOH before their dilution in the IB, the aggregation of HNE was not observed as long as EtOH does not exceed 20 % (v/v). In these conditions, the K<sub>i</sub> value for ursolic acid was equal to 2.72 ± 0.66 μM (Figure 5.b). IC<sub>50</sub> values were also determined by online-CE assay using the following injection sequence: IB; HNE; inhibitor; S<sub>3</sub>; inhibitor; HNE and IB (Table 2). The incubation time was 5 min. and the IC<sub>50</sub> values were equal to 5.62 ± 0.10 μM for ursolic acid and 8.21 ± 0.23 μM for oleanolic acid. K<sub>i</sub> values for ursolic and oleanolic acid, were deduced from Cheng-Prusoff equation as 2.81 ± 0.05 μM and 4.11 ± 0.12 μM, respectively. The LOQ of the CE-LIF assay was equal to 3 nM with no pre-concentration step.

To conclude, few studies were done for assaying elastase activity by offline based HPLC-assays. Offline and online based CE-assays were developed for the determination of kinetic constants (K<sub>m</sub>, V<sub>max</sub>, IC<sub>50</sub> and K<sub>i</sub>), and good repeatabilities, resolutions and sensitivities were obtained. In addition, the use of MST for the first time to study elastase-inhibitor interactions demonstrated it as powerful technique providing complementary information to CE and HPLC for monitoring enzyme-inhibitor affinity.

### 5. Collagenase activity monitored by HPLC and CE

HPLC was used for the first time to study vertebrate collagenase by Gray and Sanell in 1982 [65]. A simple offline assay was used to mix the collagenase present in *Rana catesbeiana tadpoles* with a synthetic substrate. A RPLC-UV method was developed to separate and quantify both the disappearance of the substrate 2,4-

337 dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg and the appearance of the products 2,4-dinitrophenyl-Pro-  
338 Gln-Gly and Ile-Ala-Gly-Gln-D-Arg after collagenase cleavage.  $K_m$  and  $V_{max}$  were successfully determined  
339 to be 1.7 mM and 310 nmol.min<sup>-1</sup>.mg<sup>-1</sup>, respectively. Despite the efficiency of the assay, the method did not  
340 specify if substrate hydrolysis comes from the collagenase or other peptidases present in the skin of *Rana*  
341 *catesbeiana tadpoles*.

342 In 1988, Biondi et al. [66] studied the activity of bacterial collagenase using a synthetic peptide (Table 1). The  
343 product Gly-L-Pro-D-Arg obtained after collagenase hydrolysis was quantified by RPLC-UV using L-Pro-L-  
344 Phe as an internal standard.  $K_m$  and  $V_{max}$  were estimated to be 7.5 mM and 50.5  $\mu$ moles. min<sup>-1</sup>.mg<sup>-1</sup> with 0.2  
345  $\mu$ g of collagenase at pH 7.0 and 25°C. The reproducibility of the method was checked by analyzing 1  $\mu$ g of  
346 collagenase and the resulting specific activity was 5.23  $\pm$  0.27 U/mg with RSD of 5.2 % (n=5). This assay  
347 demonstrate that HPLC as a suitable technique to study collagenase activities. Using the same technique, the  
348 hydrolysis of dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg by MMPs present in tissues around site of  
349 loose total hip arthroplasty prostheses was studied in 1996 [67]. A RPLC-UV was used for the quantification  
350 of the synthetic substrate. Results demonstrated that tissues extracted from periprosthetic total hip arthroplasty  
351 tissues presented elevated MMP activity (52.9  $\pm$  19.7 %) compared with that in non-inflammatory synovial  
352 tissues from the knee joint (2.7  $\pm$  61.9 %). After incubation with different antibiotics, MMP activity decreased  
353 significantly in the presence of cephalothin at 100 mg.mL<sup>-1</sup> (31.9  $\pm$  8.8 %) and 1000 mg.mL<sup>-1</sup> (13.6  $\pm$  0.1 %)   
354 in a dose-dependent manner. In addition, 500  $\mu$ g of tetracycline inhibited MMP activity by 45.2  $\pm$  8.5 %.  
355 However, doxycycline and gentamicin did not inhibit the MMP activity of the interface tissue extract. These  
356 findings conducted by HPLC show that cephalothin, a  $\beta$ -lactam antibiotic, can directly inhibit MMP activity  
357 in diseased human tissue when it is administered at therapeutically effective concentrations. Later on, Efsen  
358 et al. [68] developed an *in-vitro* offline assay to examine and quantify the proteolytic activity of MMPs in  
359 Crohn's disease fistulas specimens. Since different types of proteases exist in these fistulas, a specific  
360 fluorogenic substrate for MMP was used. Using HPLC with fluorescence detection, it was found that 50 % of  
361 the protease activity was ascribed to MMP activity and especially to MMP-3 and MMP-9. Furthermore,  
362 inhibition tests were done with ethylenediaminetetraacetic acid (EDTA), the synthetic broad-spectrum  
363 inhibitor GM6001, the angiotensin-converting enzyme inhibitor, ramiprilate, tetracycline, and doxycycline.  
364 Results have shown that ramiprilate decreased the total MMP activity level by 42 % and suppressed the  
365 specific MMP-3 activity by 72 %, which is comparable to the effect of GM6001 (87 %). Moreover, MMP-9  
366 activity was completely blunted by ramiprilate. Doxycycline showed no effect on MMP activity, which  
367 corresponds with the findings cited previously [67]. The assay had good sensitivity and efficiency although  
368 the analysis lasted more than 30 min.

369 MMPs were studied by different offline and online CE-based assays as summarized in Table 2. In 1996, a  
370 simple preliminary study was done to separate collagen peptides derived from collagen type I using acid

371 buffers (pH 2.5) [69]. This separation was necessary before conducting any enzymatic reaction of collagenase  
372 by CE. Deyl et al. [70] used a pre-capillary assay where collagenase tissues were incubated with collagen type  
373 I according to the procedure described by Krane et al. [71]. The reaction was stopped by the addition of EDTA  
374 after 12 h. The mixture was injected by overpressure (3.5 kPa, 1s) and MEKC was used with a high  
375 concentration of surfactant. The analysis was carried out either in a 25 mM phosphate buffer (pH 2.5) or in a  
376 25 mM phosphate buffer (pH 4.5) diluted to 0.1%, with respect to SDS. This study shows that when the  
377 separation is done in the straight mode at pH 2.5, the small peptides migrated faster than the larger ones with  
378 a shorter analysis time (roughly by a factor of three) compared to separations of collagen peptides which have  
379 come in contact with SDS during their preparation. When the separation was done at pH 4.5 with SDS, the  
380 larger peptides migrated ahead of the smaller ones. However, since the system must be run in the reversed  
381 polarity mode, the differences between the migration times of individual components were much higher and  
382 allowed an easy accumulation of the individual compounds in the anodic vial.

383 Miksik et al. [72] applied a model protein structure for detecting the nonenzymatic post-translational changes  
384 under various physiological conditions like high fructose diet and hypertriglyceridemic state. A mixture of  
385 collagen types I and III were incubated with bacterial collagenase then injected in CE using sodium phosphate  
386 buffer (pH 2.5). Almost 60 peaks of peptides were detected; however the origin of these peptides was unknown  
387 since no purification step was done. Principle component analysis (PCA) enabled to identify changes in the  
388 peptide profiles, allowing the determination of a section (part) of the electropherogram in which the  
389 physiological state changes occurred and was an excellent method for the analysis of these profiles. This  
390 approach was good to reveal which regime affects the collagen molecules most and can be used as guidance  
391 for targeted pre-separation of the very complex peptide mixture. Tosi et al. [73] used MEKC to study the  
392 activity of collagenase present in four species of *Arthrobotrys*, a genus of mitosporic fungi. Separations were  
393 performed using 50 mM sodium tetraborate, pH 9.3, containing 65 mM SDS and methanol (85/15, v/v). The  
394 calculated  $r^2$  were  $\geq 0.996$  for the different products. Results showed that the total amount of collagenase  
395 produced by the Antarctic strain of *Arthrobotrys tortor* was about threefold higher than that observed for the  
396 other species. In the Antarctic strain, collagenase was shown to be a constitutive enzyme, which means it is  
397 produced in constant amounts regardless to the physiological demand or the concentration of the substrate.

398 In 2004, Sano et al. [74] developed a pre-capillary CE-LIF assay to study the effect of different inhibitors on  
399 three types of MMPs (-1, -8 and -13). These assays included the measurement of the activities of interstitial  
400 collagenase (MMP-1) and neutrophil collagenase (MMP-8) against type I collagen, and collagenase-3 (MMP-  
401 13) against type II collagen by determining the  $\frac{3}{4}$  fragments produced after the cleavage. The mixture was  
402 injected in CE apparatus and the separation was done in 15 min using an electrolyte composed of 4 % of  
403 polyacrylamide and 0.05 % SDS (pH 8.8). The high sensitivity of the assays was achieved by employing  
404 NanoOrange, a non-covalent fluorescent dye for proteins. This method showed good repeatability and

405 sensitivity and was useful to estimate the IC<sub>50</sub> values of three inhibitors: MMI-166, Metisam and BB-2516. It  
406 is therefore useful for proteinase assays since it enables conducting collagenase activity using native substrate  
407 without the need of antibody preparation. To improve the separation of the mixture of collagen peptides after  
408 a bacterial collagenase cleavage, Eckhardt *et al.* [75] added alkylamines, as dynamic coating agents, to the  
409 background phosphate buffer at pH 2.5. Ethylenediamine or 1,7-diaminoheptane used as background  
410 electrolyte modifiers improved the separation and yielded the best resolution. CZE was used in 2004 by Sheu  
411 *et al.* [76] to identify the presence of MMP-9 in human platelets and systematically examine its activity. To  
412 elucidate subcellular localization of MMP-9 in human platelets, intraplatelet MMP-9 was investigated by  
413 immunogold labeling and visualized using electron microscopy. Another way to separate a mixture of peptides  
414 consisted of a partial filling approach with combined gel and non-gel separation mechanisms in capillary  
415 electrophoresis [77]. In this work, a pre-capillary assay was used at high temperature with Pluronic F 127 as  
416 the gel matrix. Pluronic F was inserted as the first plug in the capillary, and the separation was done according  
417 to molecular weight and/or hydrophobicity. This method was applied firstly to the separation of a test  
418 peptide/protein mixture as well as on real samples containing peptides arising from collagen after collagenase  
419 cleavage and albumin after trypsin cleavage.

420 The first online CE-assay of MMPs was done in 2011 by Hai *et al.* [78]. This CE assay based on EMMA with  
421 LIF detection was used for the screening of MMP inhibitors. Two enzymes, MMP-2 and MMP-9, which have  
422 been considered as promising targets for cancer therapy, were selected as model enzymes. The hydrolysis of  
423 a fluorogenic substrate (Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH<sub>2</sub>) catalyzed by MMPs was determined by  
424 measuring the increase in fluorescence. The enzyme was injected first, due to its lowest electrophoretic  
425 mobility. After the application of an electric field, the plugs of enzyme and then of the substrate and inhibitor  
426 mixture interpenetrated due to the differences in their apparent mobilities. The resultant reaction products as  
427 well as the unreacted substrate were electrophoretically transported toward the detector, where they were  
428 individually detected. In this study, the partial filling mode of EMMA was used (Figure 6.a). This establishes  
429 conditions under which inhibitors, regardless of charge, will mix with the enzyme when the mixing potential  
430 is applied. Compared with the normal-end injection at the inlet side of the capillary (Figure 6.b), short-end  
431 injection provided reduction in the analysis time to only 70 s, as well as an increase in peak sensitivity. Offline  
432 assay was also assessed in this study. K<sub>m</sub>, V<sub>max</sub> and IC<sub>50</sub> of standard inhibitor (epigallocatechine gallate or  
433 EGCG) towards MMP-2 and MMP-9 were determined by offline and EMMA assays. To evaluate the  
434 screening potency of the developed assay, six known MMP inhibitors (oleanolic acid, caffeic acid, quercetin,  
435 doxycycline, resveratrol, and glucosamine) were tested on MMP-2 and MMP-9 by determining the inhibition  
436 percentage (I %). The comparison of the values with the offline assays indicated that EMMA with fluorescence  
437 detection is well suited for initial screening of a large number of compounds for MMP inhibition. For more  
438 sensitive and selective detection for MMP-9 study, mass spectrometry (MS) was coupled to CE by the same

group [79]. The advantage of in-capillary assay coupled with MS compared to fluorescence detection was that it allowed label free screening of enzyme inhibitors. Offline and online assays with MS detection were used for MMP-9 inhibitor screening. Different parameters were optimized for the coupling including sheath liquid flow rate, nebulizer pressure, dry gas flow temperature, and ESI voltage by two level half fractional experimental designs. The volatile buffer ammonium acetate (pH 6.8) was selected for both enzyme reaction and electrophoretic separation. Substrate and products were detected in only 5 min. After the optimization of different parameters (sheath liquid nature and flow, nebulizer pressure, drying gas flow, temperature and ESI voltage), the screening of five known inhibitors (GM6001, EGCG, doxycycline, minocycline, and tetracycline) and of four unknown inhibitors (chrysophanol, emodin, dioscin, and tetrahydropalmatine) was assessed. EMMA and PMMA were used for online incubation. The results demonstrated that the compound GM 6001 inhibited most of the activity of MMP-9. The I % of EGCG (~70 %) and doxycycline (~20 %) was consistent with the ones obtained in their previous study [78]. The RSDs on the peak area and the migration time of the product were 3.2 % and 3.9%, respectively for EMMA and 4.8 % and 0.9 %, respectively for PMMA. Good linearity was obtained in the tested range with  $r^2 \geq 0.997$  for online assays. The LOD was equal to 10 nM for both methods.

In conclusion, different types of MMPs were studied by HPLC and CE-based assays. As for elastase, no immobilization was needed for collagenase study in HPLC.  $K_m$ ,  $V_{max}$  and  $IC_{50}$  of different natural inhibitors were determined using both techniques. Online assays based on EMMA and PMMA were used for inhibitors screening; however TDLFP was not conducted. The use of LIF detection allowed better sensitivity and MS allowed selective detection and provided confirmation for all components within the reaction. Most of the applications mentioned in these studies were for therapeutic fields rather than for cosmetic ones, due to the important role of collagenases in tissue destruction.

## **6. Hyaluronidase activity monitored by HPLC and CE**

The determination of Hyal activity involves the quantification of its ability to degrade HA. The increase in the number of sugar reducing ends was first monitored by simple chemical assays like calorimetry [80]. Benchetri *et al.* [81] used spectrophotometric assays to determine the amount of Hyal by measuring the interaction between undegraded HA and various dyes. Turbidity [82, 83], ELISA-like assay [84], agarose-gel electrophoresis [85], colorimetry [86] and zymography [87] can also be used.

In 1980, Knudsen *et al.* [88] followed by HPLC the products obtained after degradation of HA by bovine testicular hyaluronidase (BTH). This enzyme is commercially available and shows similar activity to human Hyal. An offline assay was used and the incubation was done for 120 h in order to reduce to the maximum the size of HA. After stopping the reaction by heating for 5 min, 20  $\mu$ L of the mixture was injected into a  $C_{18}$

472 column and the products were separated by size exclusion chromatography. HA was added to the mobile phase  
473 to prevent the adsorption of the products onto the column and for a better recovery. The chromatograms  
474 obtained showed the formation of tetra- and hexa- saccharide fractions and a low quantity of disaccharides.  
475 This method was efficient for the characterization of HA compound and of the enzymatic digestion products  
476 within 30 min of analysis time.

477 Later on, an HPLC method was developed to follow the quantity of HA present in synovial fluids [89].  
478 Different incubation temperatures (4, 20 and 35 °C) with Hyal were tested. The temperature of 20 °C  
479 corresponded to the production of the highest quantity of tetra- and hexa- saccharide fractions. A reversed-  
480 phase octadecylsilyl column was used to separate the products of the Hyal reaction. An internal standard, the  
481 benzoic acid, was added to the mixture to improve precision. A good correlation factor ( $r^2 = 0.99$ ) was found  
482 between HPLC and radiometry results. The separation of the main products tetra- and hexa- saccharides and  
483 the internal standard was done in almost 12 min with a good repeatability ( $CV \leq 2.8 \%$ ;  $n=10$ ). This method  
484 was suitable for direct determination of HA in synovial fluids and therefore as a biomarker.

485 Later on, Suzuki *et al.* [90] studied the inhibition of BTH by fully O-sulfated hyaluro-oligosaccharides using  
486 an amide-80 HPLC column and UV detection at 232 nm. The offline digestion was achieved in 48 h by  
487 determining the separation pattern of HAoligos that correspond to 40 % of digestion. CE was also used (Table  
488 2).  $IC_{50}$  values of fully O-sulfated HA oligos were determined by HPLC with the flow injection assay method.  
489 Both CE and HPLC showed that each oligosaccharide ranged 4- to 20- mer was isolated with a purity higher  
490 than 90 %. The inhibitory action for Hyal of the oligosaccharides ranged 16- to 20- mer corresponded to 79 %  
491 through both competitive and noncompetitive effects.

492 In 2015, Kakizaki *et al.* [91] investigated the effects of HA oligosaccharides and chondroitin sulfates (CS) on  
493 BTH hydrolysis and transglycosylation activities. HA oligosaccharides corresponded to di-, tetra- and hexa-  
494 saccharide fractions while CS corresponded to tetra-, hexa-, octa- and even deca- saccharide fractions with a  
495 sulphatation of the position 4 or 6. More precisely, for the hydrolysis reaction, HA was used as a substrate and  
496 BTH was added in the presence or absence of HA and CS oligosaccharides at 37 °C for 1 h in acetate buffer  
497 (pH 5.0). These oligosaccharides were also tested in the presence of HA octasaccharide as a substrate. The  
498 products of this reaction were followed by HPLC with UV (215 nm) and fluorescence detection ( $\lambda_{ex}/\lambda_{em} =$   
499 320/400 nm) using a polyamine II column (more details in Table 1). The chromatograms showed that HA  
500 oligosaccharides had no effect on BTH, while CS oligosaccharides inhibited its activity. In addition, results  
501 revealed that CS hexasaccharides or larger oligosaccharides inhibited BTH hydrolysis and transglycosylation  
502 more efficiently than CS tetrasaccharides. This is due to the increasing sulphate number improving the  
503 electrostatic interactions with the enzyme. Moreover, when the sulphation degree in the same chain is similar,



504 the inhibition of C4S (sulfate chondroitin in position 4) was shown to be greater than that of C6S  
505 oligosaccharide. In this study, the effect of homogeneous and heterogeneous sulphation was not assessed.

506 Aside from its presence in human skin, Hyal can also be found in animal venoms. Its presence increases the  
507 permeability of the tissues and accelerates therefore the spread of the venom. The determination of Hyal  
508 activity in snake and bee venom was done by CE in 1996 by Pattanaargson et al. [92] (Table 2). The method  
509 used was based on the addition of a quantity of HA to an aliquot of crude venom sample and then mixing  
510 during a fixed period of incubation. Hyal activity was measured by following the decrease in HA area due to  
511 its enzymatic hydrolysis. However, due to the complexity of Hyal mode of action, many authors [93-95] have  
512 observed that the degradation by Hyal of the products of HA hydrolysis also happens immediately after mixing  
513 of HA with Hyal. Their migration times may overlap with migration time of HA which makes the approach  
514 developed by Pattanaargson et al. inappropriate for quantitative analyses.

515 In 1997, Park et al. [96] studied the hydrolysis of HA by *Streptomyces hyaluronate lyase*. Since the products  
516 of this reaction are saccharide fractions with a similar charge density (a carboxylate group *per* disaccharide  
517 unit), their electrophoretic separation was difficult. These compounds have few chromophoric groups.  
518 Therefore, fluorescence detection was used to increase sensitivity after labeling with 7-amino 1,3-naphthalene  
519 disulfonic acid (AGA). The CE analysis required the use of a phosphate buffer of pH 2.3. Using this acidic  
520 pH, the AGA sulphonate group ( $pka < 1$ ) which is more acid than the carboxylate group of the saccharides  
521 ( $pka \sim 3.5$ ) becomes the only group responsible for the total charge of the oligosaccharides, ending up with a  
522 constant charge of -2 for each oligosaccharide. In this case, the electrophoretic separation of the HA  
523 oligosaccharides will only depend on their size. The formation of the various products of this enzymatic  
524 reaction was followed by detection of the fluorescence ( $\lambda_{ex}/\lambda_{em} = 250/420$  nm). Figure 7 shows that the  
525 electropherograms present a complicated mixture of oligosaccharides in the early reaction time. When the  
526 reaction is completed, only two major products are detected, the tetra- and hexa- saccharide fractions. This  
527 study demonstrated that Hyal acts on the HA according to an endolytic mechanism. Since only the labeled  
528 products are detected, the selectivity of this method makes possible the kinetic study of Hyal catalyzed  
529 reactions in more complex matrices.

530 HA depolymerization products were investigated by online CE-electrospray ionization-MS (CE/ESI-MS) for  
531 the first time in 2003 by Kuhn et al. [97]. The identification of several HA oligomers up to 16-mers was done  
532 using an ion trap mass spectrometer in less than 12 min. This coupling was shown to be promising for the  
533 qualitative analysis of negatively charged oligosaccharides. For the validation of this assay, extra experiments  
534 were conducted using gel-permeation chromatography (GPC) and viscosimetry [98]. Linear relationships were  
535 found between peak areas of the observed oligosaccharides and reaction times. To monitor the extent of  
536 degradation of HA by Hyal, the viscosity of HA fragments was measured. Indeed, the decrease in viscosity of  
537 the substrate solution corresponds to the number of cleavages of glycosidic bonds *per* time unit.

538 This coupling was later optimized by Grundmann *et al.* [99] using a CE-ESI-time-of-flight-MS (CE-ESI-TOF-  
539 MS) for the analysis of hyaluronan oligomer mixtures. An offline assay was performed with BTH (Table 2).  
540 The analytes showed best response in the negative ionization mode with an acidic sheath liquid (2-  
541 propanol/water/formic acid 50:50:0.2, v/v). Capillaries of 28 cm length and 15  $\mu\text{m}$  i.d. were found to give best  
542 results. Optimal BGE contained 25 mM ammonium acetate (pH 8.5). CE-MS runs were completed in only 65  
543 s which allowed a sample throughput of about 35 samples *per* hour.

544 Matysiak *et al.* [100] recently developed a new method for the determination of Hyal (from bovine testes and  
545 honeybee venom) activity using CZE with UV detection of the reaction products at 220 nm. A simple pre-  
546 capillary assay was used as shown in Table 2. Results showed that after 1.5 h of incubation, the products  
547 formed are tetra-, hexa- and octa- saccharide fractions. After increasing the incubation time to 24 h,  
548 disaccharide fractions appeared and octasaccharide fractions disappeared. A multiple regression analysis  
549 involving the heights of the peaks of the main HA degradation products was also performed. The method was  
550 validated by comparing the results with those obtained using pharmacopeia methods [101, 102].

551 The kinetic constants of Hyal were determined for the first time by CE-UV and CE-HRMS by our group [103].  
552 A simple pre-capillary CE-UV/HRMS assay was used. Different parameters were studied to have the best  
553 sensitivity and are summarized in Table 2. The standard tetrasaccharide which is the final product of HA  
554 degradation by Hyal, was used for quantification.  $K_m$  and  $V_{max}$  were determined (n=3) using CE/UV at 200  
555 nm as  $0.24 \pm 0.02 \text{ mg}\cdot\text{mL}^{-1}$  and  $150.4 \pm 0.1 \text{ nM}\cdot\text{s}^{-1}$ , respectively (Figure 8.a). They were also successfully  
556 obtained by CE/HRMS (n=3) with  $K_m$  of  $0.49 \pm 0.02 \text{ mg}\cdot\text{mL}^{-1}$  and  $V_{max}$  of  $155.7 \pm 0.2 \text{ nM}\cdot\text{s}^{-1}$  (Figure 8.a).  
557  $IC_{50}$  of a standard natural inhibitor, EGCG, was also determined by CE-UV/HRMS (Figure 8.b). In addition,  
558 the activity of homemade tetrasaccharides of biotinylated chondroitin sulfate CS-A or CS-C (4- or 6- sulphated  
559 in a homogeneous or heterogeneous way) and of two trisaccharides from truncated linkage region of  
560 proteoglycans on the hydrolysis reaction of BTH was evaluated. Results showed that BTH was mostly dose-  
561 dependently inhibited by CS-A tetrasaccharides sulphated in a homogeneous way and that a small modification  
562 of one hydroxyl group changes the influence on BTH activity.

563 Later on, the previously developed CE-UV assay was used to screen the effect of extracts of a brown algae,  
564 *Padina pavonica*, on BTH [104]. The extraction of this alga was optimized using several extraction techniques.  
565 A novel online CE-assay using TDLFP was developed allowing the efficient monitoring of BTH kinetics;  $K_m$   
566 and  $V_{max}$  of  $0.46 \pm 0.04 \text{ mg}\cdot\text{mL}^{-1}$  and  $137.1 \pm 0.3 \text{ nM}\cdot\text{s}^{-1}$  ( $r^2=0.99$ ; n=3), respectively. Figure 9 shows the  
567 electropherograms obtained with pressurized liquid ethyl acetate and water extracts. The water extract of  
568 *Padina pavonica* obtained by pressurized liquid extraction and microwave assisted extraction showed the  
569 highest anti-hyaluronidase activity (Figure 9). Therefore, its  $IC_{50}$  was determined by offline and online assays  
570 to be  $25.2 \pm 0.1 \mu\text{g}\cdot\text{mL}^{-1}$  and  $35.1 \pm 0.1 \mu\text{g}\cdot\text{mL}^{-1}$ , respectively.

571 To summarize, we can notice that different HPLC- and CE- methods were developed to study Hyal  
572 activity on HA. Various detectors were used to improve sensitivity and to identify the products obtained after  
573 HA cleavage. We can notice that BTH was mostly used to represent human Hyal activity. Compared to elastase  
574 and collagenase assays, the effect of a brown algae extracts were tested on hyaluronidase by offline and online  
575 CE-assays. These assays can be used for the screening of natural and synthetic inhibitors of Hyal activity for  
576 cosmetic and therapeutic applications.

### 577 578 **7. Tyrosinase activity monitored by HPLC, HPTLC and CE**

579 Tyr activity and inhibition was studied by many techniques such as UV/Vis spectroscopy [105]. In 1993,  
580 Shosuke Ito [106] developed an HPLC method to study the regulation of melanogenesis by analyzing  
581 quantitatively the contents of eu- and pheo- melanin in tissue samples without any purification procedure.  
582 Pyrrole-2,3,5-tri-carboxylic acid (PTCA) served as a quantitative indicator of eumelanin, whereas  
583 aminohydroxyphenylalanine (AHP) as a specific indicator of pheomelanin by UV absorbance at 254 nm. This  
584 method required 5 mg or less of tissue samples or 10<sup>6</sup> cultured cells for each analysis. The yields of PTCA  
585 and AHP were 2 % and 20 % from eu- and pheo- melanin, respectively. This study indicated that when Tyr  
586 activity is low, dopaquinone, a reactive intermediate in melanogenesis, is converted quantitatively to  
587 glutathionyl-dopa, which gives exclusively pheomelanin. However, when Tyr activity is high, an excess of  
588 dopaquinone is obtained, resulting in the inactivation of the essential enzymes for pheomelanogenesis. This  
589 method is very efficient since it has been successfully applied for the analysis of synthetic melanins,  
590 melanosomes, hair, feathers, melanomas, human epidermis and cultured melanocytes. In 2011, our group [107]  
591 developed a continuous-flow step gradient method for online kinetic tests of immobilized mushroom Tyr using  
592 MS for selective detection of product formation. This approach combined a continuous flow bioreactor and a  
593 frontal analysis chromatography (FAC) of ligand-Tyr binding methodology. FAC is an approach based on a  
594 LC-MS system enabling simultaneous ranking of the ligands/inhibitors according to their affinities to the  
595 biological target (enzyme/receptor). It involves the constant infusion of the studied ligand/inhibitor (or their  
596 mixture) and a non-interacting marker into the bioreactor with the immobilized target. Various flow rates of  
597 the mobile phase were tested, and in the different conditions, the values of  $K_m$  obtained were constant  
598 confirming the robustness of the developed assay. The use of MS allowed a selective monitoring of the  
599 enzymatic reaction products and for better sensitivity (500 pmol/min/column).  $K_i$  of kojic acid, a competitive  
600 inhibitor of Tyr, was determined for free and immobilized enzymes to be  $3.56 \pm 0.44$  mM and  $3.10 \pm 0.05$   
601 mM, respectively. This method was automated. It allowed a simultaneous determination of specific inhibitor-  
602 Tyr interactions in complex mixtures and of  $k_i$  value of a given inhibitor. Later on, Yang *et al.* [108] developed  
603 a new assay based on ultrafiltration HPLC-DAD-MS<sup>n</sup> for the rapid screening and identification of ligands for  
604 Tyr. This system is illustrated in Figure 10. The method was composed of three steps: incubation,

605 ultrafiltration, and characterization. The incubation process was designed to allow the sample solution to come  
606 into contact with the enzyme. Ethyl acetate extracts of mulberry leaves at 5 mg.mL<sup>-1</sup> were incubated with 160  
607  $\mu$ L of Tyr in 100 mM phosphate buffer (pH 6.8) at 25°C. Then, the unbound low-mass molecules were  
608 separated from the ligand–enzyme complexes by ultrafiltration after incubation for 30 min. The ultrafiltrates  
609 were injected directly into the HPLC–DAD–MS<sup>n</sup> system for analysis. Tyr binding activity was determined by  
610 a decrease in peak areas of the detected chemicals corresponding to incubation with active Tyr. For  
611 comparison, control experiments with a denatured enzyme were also carried out which allowed to identify  
612 non-specific interactions. The identities of the “hits” were identified by online HPLC–DAD–MS<sup>n</sup> analysis and  
613 by comparison with reference standards. Twelve compounds found in mulberry leaf extracts were  
614 characterized by HPLC-DAD-MS<sup>n</sup> and two compounds, quercetin-3-O-(6-O-malonyl)- $\beta$ -D-glucopyranoside  
615 and kaempferol-3-O-(6-O-malonyl)- $\beta$ -D-glucopyranoside were identified as new Tyr inhibitors with an IC<sub>50</sub>  
616 of 267.8  $\mu$ M and 103.7  $\mu$ M, respectively. The developed method was efficient for the screening and the  
617 identification of Tyr inhibitors in complex mixtures. The same approach was then used to identify inhibitors  
618 from *Xanthium strumarium* fruit extracts [109]. The false negatives were distinguished by optimizing the  
619 ultrafiltration-HPLC-DAD parameters to reduce the background noise and the false positives were  
620 distinguished by introducing a blocked Tyr in the control for comparison. Three competitive inhibitors 3,5-di-  
621 O-caffeoylquinic acid and 1,5-di-O-caffeoylquinic acid and one mixed-type inhibitor (chlorogenic acid) were  
622 identified in this study with IC<sub>50</sub> of 1.07  $\pm$  0.08 mM, 1.19  $\pm$  0.03 mM and 1.05  $\pm$  0.06 mM, respectively. O-  
623 caffeoylquinic acid and 1,5-di-O-caffeoylquinic acid were successfully distinguished as Tyr inhibitors from  
624 the false negatives and the false positives. Our group [110] studied again Tyr in 2014 using direct surface-  
625 assisted laser desorption/ionization (SALDI) and ESI-qTOF-MS. Mushroom Tyr was covalently coupled to  
626 core-shell-type silica-coated iron oxide magnetic nanoparticles (EMPs) that were applied as non-organic  
627 SALDI matrix suitable for studying low-mass compounds using a classic matrix-assisted laser  
628 desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometer. The enzymatic conversion of glabridin  
629 and 3-(2,4-dihydroxyphenyl)propionic acid (DHPA) were also conducted by ESI-QqTOF-MS. This study  
630 showed that glabridin and DHPA were converted into the corresponding quinones by Tyr only in the presence  
631 of the auxiliary monophenol or o-diphenol substrates (L-Tyr and catechin, respectively) capable of  
632 regenerating the active site of Tyr. A post-TLC technique was developed in 2007 by Wangthong et al. [111]  
633 to make the isolation and purification of mushroom Tyr inhibitors easier and to differentiate between Tyr  
634 inhibitors and other components present in Licorice extract. A conventional TLC was used for a sample  
635 mixture containing octyl methoxy-cinnamate (not a Tyr inhibitor) and kojic acid (a standard Tyr inhibitor) as  
636 shown in Figure 11.a) and for arbutin and glabridin. The procedure consists of dropping the extract onto the  
637 bottom of the stationary phase and then to put the plate into 1:1 (v/v) hexane/ethylacetate as mobile phase.  
638 When the solvent front reached the mark, the plate was taken out and allowed to dry at room temperature.

639 Separated spots on the stationary phase were visualized and photographed under UV light. The entire plate  
640 was then sprayed with Tyr (0.02 mL/cm<sup>2</sup>) and L-tyrosine (0.02 mL/cm<sup>2</sup>) already prepared in 20 mM phosphate  
641 buffer (pH 6.8) to determine the presence of Tyr inhibitors in each spot. The different standard inhibitors gave  
642 a white spot whereas the spot obtained by octyl methoxy-cinnamate appeared as a dark background which  
643 validated the developed method. For Licorice extract, conventional two-dimensional TLC (2D-TLC) was used  
644 prior to the post chromatographic Tyr inhibitor detection (Figure 11.b). The first-dimensional TLC was carried  
645 out using 60:40 (v/v) methanol:dichloromethane as mobile phase and the second-dimensional separation was  
646 performed with 40:60 (v/v) hexane:ethyl acetate as a mobile phase. After complete TLC separation, one TLC  
647 plate was developed using the iodine absorption technique (used as a blank) while the other was sprayed with  
648 the Tyr and L-tyrosine. The TLC developed by Tyr/L-tyrosine spraying showed two active spots among six  
649 spots detected by iodine. This result confirmed the inhibitory effect of licorice extract on Tyr and the presence  
650 of at least two Tyr inhibitors with different polarities in the extract. This extract was then fractionated using a  
651 silica gel column eluted with 40:60 (v/v) hexane:ethyl acetate and the active fraction represented glabridin.  
652 These results show that the use of TLC provides valuable information for further isolation and purification of  
653 Tyr inhibitors from sample mixtures.

654 Later on, Momtaz et al. [112] studied the effect of three extracts (acetone, methanol and dichloromethane) of  
655 Sideroxylon inerme on Tyr and on melanocyte cells using the same previous procedure [111]. Results showed  
656 that methanol and acetone extracts presented an inhibition of 70%. However, no significant reduction was  
657 obtained with the dichloromethane extract at any of the tested concentrations. The methanolic extract was then  
658 fractionated with hexane, butanol and ethyl acetate. The ethyl acetate fraction exhibited the highest anti-tyr  
659 activity and the highest inhibition of melanin production in melanocyte cells (40 % at 25 and 50 µg/mL).  
660 EGCG and procyanidin were isolated from this fraction and their IC<sub>50</sub> values were 30 µg/mL and 200 µg/mL,  
661 respectively. The referenced kojic acid and arbutin demonstrated IC<sub>50</sub> values of 1.1 µg/mL and 149 µg/mL.  
662 The effect of the methanolic extract, EGCG, arbutin and kojic acid on cell viability and melanin production in  
663 B16F10 cells was also studied. Sideroxylon inerme extract showed a significant inhibition (37 %) of melanin  
664 production at 6.2 µg/mL while 80 % of cells were viable, thus indicating low levels of cytotoxicity. EGCG did  
665 not show any significant inhibition of melanogenesis at the different tested concentrations. Kojic acid (positive  
666 control) showed no significant toxicity to B16-F10 cells at the highest tested concentration and exhibited  
667 similar reduction in melanin content at 3.1 and 25 µg/mL (60 %). Compared with the positive controls (arbutin  
668 and kojic acid) and to procyanidin, EGCG exhibited more anti-Tyr activity than arbutin but less than kojic  
669 acid. These findings were very important for the identification of skin-depigmenting new agents. Kamagaju et  
670 al. [113] then evaluated *in-vitro* the ability of Rwandese medicinal plants and modulated Tyr activity.  
671 Therefore, five herbs (*Brillantaisia cicatricosa* Lindau, *Chenopodium ugandae*, *Dolichopentas longiflora*  
672 *Oliv.*, *Protea madiensis* Oliv. and *Sesamum angolense* Welw) were selected. Twenty-seven extracts, obtained

673 by treating the herbs with solvents of increasing polarities, were investigated for their effects on cell viability,  
674 on Tyr inhibition and on the measurement of melanogenesis by human melanoma cells. Results demonstrated  
675 that the tested plant extracts were not cytotoxic on human melanoma cell lines, except for *Dolichopentas*  
676 *longiflora*. All the extracts inhibited melanogenesis in melanoma cells. *Protea madiensis* extracts strongly  
677 inhibited melanogenesis and one of the *Dolichopentas longiflora* leaves extracts was found to increase  
678 melanogenesis. Crude cellular extracts obtained from normal melanocytes were used to confirm these results.  
679 The data obtained with the methanol extract of *Protea madiensis* were comparable when assaying on  
680 mushroom Tyr and on melanocyte proteins, while other extracts displayed discording data. This was due to  
681 the differences between human and mushroom Tyr, interference with melanocyte enzymes at later steps than  
682 Tyr or the simultaneous presence of compounds with conflicting activities in a given extract. This interesting  
683 study encourages authors to study the effect of extracts on the mushroom tyrosinase but also on the human  
684 one. In 2015, Taibon et al. [114] developed a simple and rapid HPTLC method to screen plant extracts for the  
685 presence of Tyr-inhibiting substances. Three different mobile phases were used for the separation of polar,  
686 nonpolar and very polar compounds. Several known Tyr inhibitors such as kojic acid, isoferulic acid, benzoic  
687 acid, resveratrol and tropolone were first tested as positive controls. The plates were first sprayed with  
688 *Levodopa* then with Tyr. The inhibitors should present white spots with white light in remission from the plate  
689 as well as with white light transmitted through the plate. However, some extracts included spots with different  
690 behaviors that may lead to false-positive results. This problem was eliminated by adding Triton X-100 to the  
691 *Levodopa* solution. The screening of thirteen extracts on one single HPTLC plate was possible in almost 90  
692 min. The developed method was reproducible and suitable for the screening of plant extracts for Tyr without  
693 causing false-positive results. Additional information such as number or nature of active constituents was  
694 possible due to this technique after direct coupling with MS.

695 Chaita et al. [115] developed an integrated HPTLC-based methodology for the rapid tracing of the bioactive  
696 compounds from *Morus alba* extracts during bioassay-guided processes, using multivariate statistics. Two  
697 methodologies were therefore developed for the processing of the chromatograms; the assignment and the  
698 binning methodology. The first one consisted on the evaluation of the areas under curve of the analyzed peaks  
699 and the second one on applying the data binning technique. The multiple linear regression was chosen in order  
700 to have no correlations between the data and requiring more observations than variables. The conducted Tyr  
701 inhibition assays showed that oxyresveratrol, dihydroxyresveratrol, transdihydromorin and 2,4,3'-  
702 trihydroxydihydrostilbene possess good inhibitory effect, with IC<sub>50</sub> values 1.7 μM, 0.3 μM, 8.0 μM and 0.8  
703 μM, respectively. Chemometric tools were shown in this study to support the development of a HPTLC-based  
704 methodology for the tracing of Tyr inhibitors in *Morus alba* extract and to afford essential key elements for  
705 application in high-throughput screening procedures for drug discovery purposes.

706 More recently, Boka *et al.* [116] aimed to combine high-throughput screening techniques (NMR, HPLC-based  
707 activity profiling and HPTLC-bioautography) in order to establish an integrated high-throughput multivariate  
708 statistics platform for the identification of natural products from *Paeonia mascula*, a greek plant. The extract  
709 of this plant was fractionated by fast centrifugal partition chromatography and the obtained fractions were then  
710 assayed for mushroom Tyr after analysis by HPTLC and NMR. HPTLC-bioautography permits the direct  
711 detection of the active compounds in the fractions, which leads to the target isolation. The use of NMR with  
712 multivariate statistics makes a direct structure elucidation possible. However, NMR is less sensitive compared  
713 to HPTLC and there is a possibility of false detection in case of similar variation of two or more compounds.  
714 Therefore, the combination of HPTLC and NMR employed with multivariate analysis, complemented each  
715 other providing a high-throughput platform for the discovery of natural products with skin whitening activity.  
716 In this study, eight active compounds were identified: 1,2,3,4,6-pentagalloyl glucopyranosid, gallic acid, gallic  
717 acid methyl ester and a mixture of meta and para digallic acid, quercetin-3-O- $\beta$ -glucoside, kaempferol-3-O- $\beta$ -  
718 glucoside-7-O-rhamnoside, 4'-methoxy quercetin 3-O- $\beta$ -glucoside and kaempferol 3-O- $\beta$ -glucoside.  
719 CE was used in 1997 by Robinson and Smyth [117] to probe the biosynthesis of melanin for a better  
720 understanding of the processes involved in the oxidation of L-tyrosine and L-dopa. A simple pre-capillary  
721 assay was used where the intermediate products formed during L-tyrosine and L-dopa oxidation using Tyr  
722 were followed with DAD detection. In this work, an optimization of buffer preparations was done to maintain  
723 optimum working conditions of Tyr and 25 mM of tetraborate (pH 9.0) used as BGE facilitated rapid analyses.  
724 The CE-DAD technique was shown in this study to be useful for the analysis of intermediate compounds  
725 formed and for studies where sample volumes are limited.  
726 The first online CE assay of Tyr was made in 2012 by Zhang *et al.* [118]. An EMMA-based CE assay was  
727 developed to screen Tyr inhibitors from traditional Chinese medicines. This method consisted of injecting a  
728 plug of the enzyme, followed by a plug of the substrate with or without the inhibitor. The activity of Tyr was  
729 detected by following the increase or decrease of quinine peak areas at 214 nm. The effect of the phosphate  
730 buffer concentration and of the incubation time was studied to optimize EMMA conditions. Best results were  
731 obtained with water as IB and 2 min of incubation time.  $K_m$  was determined to be 0.0532 mM and  $K_i$  of kojic  
732 acid was equal to 26.7  $\mu$ M, which corresponds well to those previously obtained [107]. This method simplified  
733 the screening procedure by directly assaying the reaction product and minimized the false-positive effect.  
734 To facilitate the study of Tyr, a chiral ligand exchange-CE (CLE-CE) was developed by Suna *et al.* [119]. This  
735 system employs Zn (II)-L-Alanine complex as a chiral ligand for quantitative analysis of dansylated L-amino  
736 acids. It investigates the activity of Tyr through monitoring the L-tyrosine concentration. Before conducting  
737 the reaction, the separation conditions were optimized with 100 mM boric acid, 5 mM ammonium acetate, 4  
738 mM Zn (II), 8 mM L-Ala and 4 mM  $\beta$ -CD at pH 8.2. Under these conditions and using pre-capillary approach,  
739  $K_m$  and  $V_{max}$  were determined to be 374  $\mu$ M and 172  $\mu$ M.min<sup>-1</sup>, respectively. This method was also used to

740 study the effect of kojic acid and of soy sauce. For the screening of another family of Tyr inhibitors, the  
741 chalcones, the authors optimized CLE-CE method by replacing the chiral ligand by L-leucine [120]. Despite  
742 the good separation of dansylated-D-L-amino acids, the migration time was above 50 min.

743 In 2013, a heterogeneous assay based on the immobilization of Tyr was developed by Jiang *et al.* [121] (Figure  
744 12). Tyr was immobilized on the surface of fused-silica-capillary by ionic binding with the cationic  
745 polyelectrolyte hexadimethrien bromide (HDB). The HDB solution (0.1 %) was first injected into the capillary  
746 and kept for 5 min to create a positively charged coating on the capillary wall. This injection was followed by  
747 a plug of the enzyme (50 mbar for 10 s) at a concentration of 0.5 mg.mL<sup>-1</sup>. Another plug of HDB solution (0.1  
748 %) was then injected to cover the immobilized enzyme. For activity and inhibition assays, the capillary was  
749 first filled with a BGE of 20 mM phosphate and 0.01 % HDB at pH 7.4, followed by a plug of L-tyrosine with  
750 or without the inhibitor at 50 mbar for 10 s. An illustration of this layer-by-layer assembly is shown in Figure  
751 12. Enzyme activities were quantified by following the decrease or the increase of peak areas of DOPA in the  
752 presence or absence of inhibitors. The  $K_m$  value was determined to be 0.053 mM and the  $K_i$  of kojic acid was  
753 equal to 24.54  $\mu$ M, which is comparable to the work of Zhang *et al.* [118] using EMMA. The inhibitory ratios  
754 of enzymatic activity of traditional Chinese medicine were established. *Fructus crataegi* and *Radix angelicae*  
755 *pubescentis* inhibited Tyr by 62 % and 65 %, respectively. These values were also comparable with those  
756 obtained by Zhang *et al.* [118].

757 A new method based on Tyr immobilization was developed recently by Cheng *et al.* [122]. This reliable online  
758 immobilization enzyme microreactor (IMER) was constructed at the outlet of the capillary by using  
759 glutaraldehyde as cross-linker. The short-end injection was used. The substrate and product were separated  
760 within only 2 min. Immobilization prolonged the storage of the enzyme where immobilized Tyr remained 80  
761 % active for 30 days at 4°C. Fifteen natural compounds from traditional Chinese medicine were screened using  
762 this method and quercetin and kaempferol were shown to have good inhibitory potency, of  $34.7 \pm 3.1$  % and  
763  $24.2 \pm 4.3$  %, respectively. In addition, molecular docking was carried out to demonstrate the inhibitory  
764 potential of quercetin, kaempferol, bakuchiol and bavachinin.

765 From these studies, it can be concluded that immobilization assays were more often used for assaying Tyr  
766 activity in the absence or in the presence of different inhibitors. Several on-line assays were conducted. For  
767 online CE-assays, EMMA was the only method used.  $K_m$ ,  $V_{max}$  and  $IC_{50}$  of different natural molecules were  
768 also determined. It should also be noted that the study on real cells was also presented in the cited studies  
769 which is of great importance in many applications.

## 771 **8. Limitations and future development**

772 As shown in this review, there has been a reasonable amount of papers dealing with the use of chromatographic  
773 and electrophoretic tools for studying elastase, collagenase, hyaluronidase, and tyrosinase kinetics. Continuous



774 innovation in micro- and nano- fluidic research and promising multiplexing of microchip formats revealed  
775 significant potentials in coupling high-throughput to miniaturization of biochemical assays for broader usage  
776 and developments in the cosmetic and the therapeutic fields. For this, further miniaturization by using  
777 microchips hyphenated to mass spectrometry can be very interesting. Moreover, for more accuracy it will be  
778 essential to use these techniques to evaluate enzymatic activity directly in whole cells (as noticed by Kamagaju  
779 et al. [124]). Our group has already showed that cell membranes can be disrupted in-capillary by  
780 electroporation and that the released enzymes can be subsequently studied and quantified in the same capillary  
781 [44] by monitoring the formed products.

## 782

### 783 **9. Conclusion**

784 The examples referenced above confirm that HPLC, HPTLC, CE as well as the more recent MST were  
785 efficiently used for studying elastase, collagenase, hyaluronidase and tyrosinase. Assays were conducted for  
786 the screening of natural or synthetic inhibitors of these enzymes and for applications on cells such as  
787 keratinocytes, fibroblasts and melanocytes. Offline mode in CE and HPLC was the most frequently used since  
788 it is easy to optimize and to perform. The speed of analysis and minimal sample consumption are the main  
789 advantages of these analytical tools. The versatile use of different types of detectors with no limits imposed  
790 by radiolabeling pushed these techniques further and further beyond their limits, encouraging their use by  
791 scientists all over the world for enzymatic assays.

### 792

### 793

### 794 **Acknowledgments**

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797

798 *The authors declare that there are no conflicts of interest.*

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091 **List of figures:**

092 **Figure 1:** Schematic representation of skin aging.

093 **Figure 2:** Schematic illustration of a) elastase-polypeptide reaction; b) collagenase-collagen reaction; c)  
094 hyaluronidase-hyaluronic acid reaction and d) tyrosinase-tyrosine reaction.

095 **Figure 3:** Capillary electrophoresis and liquid chromatography based enzymatic assays.

096 **Figure 4: a)** Kinetic assay of HNE by CE-UV adapted from [58]. TDLFP mode: injection (0.3 psi) in five  
097 steps: 1. IB (6 s); 2. 0.6  $\mu$ M HNE (2 s); 3. MeO-Suc-Ala-Ala-Ala-Pro-Val-pNA ( $S_0$ ; 2 s); 4. 0.6  $\mu$ M HNE (2 s);  
098 5. IB (5 s);  $t_{\text{wait}}$ : 5 min. Conditions: BGE: 35 mM tetraborate, 65 mM SDS and 15% MeOH (pH 9.3), IB: 25  
099 mM HEPES, 20 mM NaCl (pH 7.5); Rinse between analyses: 1 min NaOH (0.1 M), 0.5 min water and 3 min  
100 BGE. Bare silica capillary: 60 cm x 10.2 cm x 50  $\mu$ m i.d.; UV at 380 nm.

101 **b)** Michaelis–Menten curve of  $S_0$  hydrolyzed by HNE. Pre-capillary mode: reaction mixture: HNE 0.9  $\mu$ M  
102 and  $S_0$ : 0.03–0.7 mM.

103  
104 **Figure 5: a)** Schematic illustration of microscale thermophoresis or MST principle adapted from [60]. The  
105 normalized fluorescence in the heated spot is plotted against time. The IR laser ( $1480 \pm 10$  nm) is switched on  
106 at 5 s to induce thermophoresis. The fluorescence decreases as the temperature increases inside the capillary  
107 and HNE-NT647 moves away from the heated spot due to thermophoresis. The steady state occurs during 20 s  
108 before returning to initial state when the laser is turned off at 35 s. **b)** Determination of the dissociation constant  
109  $K_i$  of the ursolic acid inhibitor for HNE using 30% LED and 20% MST power.

110 **Figure 6: MMP-2 online hydrolysis by EMMA. a)** Schematic illustration of EMMA with short-end injection  
111 adapted from [78]. BGE and IB: 50 mM Tris buffer (pH 7.5); Bare silica capillary: 60 cm x 16.5 cm x 75  $\mu$ m  
112 i.d.; **b)** Electropherograms of MMP-2 online hydrolysis with short-end and normal-end injection.

113  
114 **Figure 7:** Electropherograms of AGA labeled oligosaccharide mixtures with reaction completion (%) prepared  
115 by controlling the depolymerization of hyaluronic acid with hyaluronidase adapted from [96]. Peaks number 4  
116 and 6 correspond to tetrasaccharide and hexasaccharide fractions.

117 Conditions: BGE: 20 mM phosphoric acid, 1 M dibasic sodium phosphate (pH 2.3); IB: 20 mM sodium acetate  
118 -acetic acid, 0.15 M NaCl (pH 6.0); bare-fused silica capillary: 70 cm x 50  $\mu$ m i.d.; LIF detection:  $\lambda_{\text{ex}}/\lambda_{\text{em}} =$   
119 250/420 nm.



121 **Figure 8: a)** Kinetic study of HA degradation by hyaluronidase using CE/UV and CE/HRMS; **b)** Dose-  
122 response curves obtained for hyaluronidase inhibition with EGCG using CE/UV and CE/HRMS ( $r^2 \geq 0.94$ )  
123 adapted from [103].

124 Incubation time: 60 min at 37°C. IB: 2 mM sodium acetate (pH 4.0). BGE: 10 mM ammonium acetate (pH  
125 9.0). Injection at the cathodic side: 0.5 psi for 5 s (18 nL). Electrophoretic separation: - 15 kV at 25°C. Bare-  
126 silica capillary: 85 cm x 75  $\mu\text{m}$  i.d.; sheath liquid: MeOH-water (80/20; v/v) with 0.02% formic acid, 10  
127  $\mu\text{L}\cdot\text{min}^{-1}$ ; dry gas flow, 6  $\text{L}\cdot\text{min}^{-1}$ ; nebulizer pressure: 1.1 bar; ESI voltage: - 4 kV.

128  
129 **Figure 9:** Electropherograms obtained for the inhibition of HA hydrolysis by hyaluronidase with different  
130 concentrations of pressurized liquid extraction ethyl acetate (a) and water (b) extracts adapted from [104].  
131 Conditions in **Table 2**.

132  
133 **Figure 10:** Scheme of ultrafiltration HPLC–DAD–MS assay for screening for tyrosinase inhibitors adapted  
134 from [108].

135  
136 **Figure 11: a)** TLC results of about 0.5  $\mu\text{g}$  of the 1:1 (w/w) mixture between kojic acid and octyl methoxy-  
137 cinnamate adapted from [111]. Mobile phase: 1:1 (v/v) hexane:ethyl acetate. Picture taken under UV light (a)  
138 shows kojic acid at the retention value of 0.08 and octyl methoxy-cinnamate at 0.86 and b) after spraying with  
139 tyrosinase/L-tyrosine. **b)** 2D-TLC separation of 3  $\mu\text{g}$  of the chloroform Licorice extract. Mobile phase for the  
140 first dimension: 60:40 (v/v) methanol:dichlorometahne and 40:60 (v/v) hexane:ethyl acetate as a second  
141 dimension mobile phase.

142  
143 **Figure 12:** Schematic representation of the immobilized capillary tyrosinase reactor prepared by the LBL  
144 assembly adapted from [121].