Protein fingerprinting of *Staphylococcus* species by capillary electrophoresis with on-capillary derivatization and laser-induced fluorescence detection.

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Abstract

Capillary electrophoresis (CE) coupled with laser-induced fluorescence detection (LIF) has allowed to obtain protein fingerprints, which have demonstrated to be useful in microorganisms characterization. In this work, protein fingerprints of two species of *Staphylococcus* grown in different culture media and submitted to temperature and nitrosative stress were studied by CE-LIF. After the growth of the bacteria, protein extracts were obtained by cell lysis using sonication. The water soluble fraction of these lysates was derivatized on-capillary with a fluorogenic dye, 3-(2-furoyl)quinoline-2-carboxaldehyde. The fluorescent products were analyzed by CE using phosphate buffer containg submicellar concentrations of sodium pentanesulfate and detected by LIF. Different protein fingerprints were obtained depending on the bacterial specie studied, indicating the usefulness of this method for identification of different species of the same bacterial genus. It was also demonstrated that the CE protein fingerprints were dependent on the culture conditions, such as growth medium, or on stressing conditions, such as heat shock or nitrosative stress.

Keywords: capillary electrophoresis, laser-induced fluorescence detection, protein fingerprinting, *Staphylococcus*, growth conditions, stressing conditions.

1. Introduction

Staphylococcus forms part of the normal microbiota of the humans. However, they have gained notoriety as opportunistic pathogens capable of causing a wide variety of infections in individuals with a weak immune system. In fact, *Staphylococcus* are pathogens responsible for most of the infections caused in hospitals [1]. Besides, these bacteria can be present in food due to an inadequate handling and storage, even causing food poisoning [2].

The diseases caused by *Staphylococcus* range from skin lesions to more serious complications including endocarditis, septic arthritis, and toxic shock syndrome. Moreover, pathological activity of *Staphylococcus* is different among species, for instance *Staphylococcus aureus* is more pathogenic than *Staphylococcus epidermidis* [3]. It is therefore necessary first to distinguish *Staphylococcus* from other type of bacteria and second to have methods that allow identification of different species of *Staphylococcus*. This identification is usually performed by classical microbiological assays, such as microscopic observation and the coagulase test [4]. However, in some cases the correct identification of the species is difficult, leading to false positives [5] which makes necessary the development of alternative methods for identification. The development of polymerase chain reaction (PCR) alternative techniques, based on the nucleic acids analysis, has increased the sensitivity of these assays without the need for bacterial cultures [6].

Bacteria are able to rapidly adapt to changing environments. In fact, bacteria deploy only those factors required for growth and survival within a particular environment and show a number of genetic responses to environmental conditions [7]. In particular, under stress conditions bacterial cells synthesize proteins which are different from those produced under normal conditions. Most of them are highly conserved proteins which appear as response to different stimuli [8]. This is the case of the proteins known as heat shock proteins (hsp) [9] or those known as cold shock proteins (csp) [10]. For instance, Laport *et al.* [9] reported the synthesis of three new proteins with a molecular weight higher than 90 kDa when the growth temperature of a culture of *S. aureus* was increased from 37 to 42 ºC. Also, Richardson *et al.* [11] reported *S. aureus* stress responses to the

nitric oxide produced by mammalian hosts as a defense against the bacteria. The protein maps obtained by two-dimensional gel electrophoresis (2D-PAGE) [12] or by transcriptomic analysis [13] of the nitrosative stress response of *S. aureus* have allowed to demonstrate changes in protein patterns under stressing conditions. However, these techniques are laborious and time-consuming and the protein maps obtained are usually complex.

Capillary electrophoresis (CE) has demonstrated to be a powerful tool, either to separate intact microorganisms [14-16] or to obtain protein fingerprints of different microorganisms [17,18]. When combined with laser-induced fluorescence detection, minute amounts of proteins can be analyzed, even at the level of single cells [19,20]. In addition, the development of on-capillary, covalent [19,20] or non-covalent [21, 22] derivatization methods for CE-LIF allows the reaction time and the consumption of reagents to be reduced, the use of a small sample volume, and automation of the analytical process. In the case of bacteria, their protein fingerprints obtained by CE-LIF have allowed to establish differences between species that can contribute to their identification and classification [23]. Therefore, the identification of bacterial species based on these protein profiles could complement the information obtained by classical microbiological assays. In addition CE could be useful to detect proteins produced by certain bacteria under different medium and growth conditions.

In this paper the feasibility of the CE-LIF method to obtain protein fingerprints that allow differentiating *S. aureus* from *S. epidermidis* is presented. In addition, by using this method, protein profiles of *S. aureus* cultured under different growth and stress conditions (temperature and nitric oxide onset) are obtained.

2. Experimental

2.1. Reagents, standards, and solutions

Borax, potassium cyanide, and potassium chloride were purchased from Sigma Chemical (St. Louis, MO, USA). Disodium hydrogenphosphate dihydrate, sodium hydroxide, sodium

chloride, and sodium dihydrogenphosphate were from Merck (Darmstadt, Germany). 3-(2-Furoyl)quinoline-2-carboxaldehyde (FQ) was from Molecular Probes (Eugene, OR, USA). Methanol (HPLC grade) and tryptic soy broth (TSB) were from Scharlau Chemie (Barcelona, Spain). Nutrient broth (NB) was from Microkit Iberica (Madrid, Spain). Sodium pentanesulfate (SPS) was from Kodak (Rochester, NY, USA). 2-(N,N-Diethylamino)-diazenolate-2-oxide (DEA NONOate) was from Alexis Biochemicals (San Diego, CA, USA). Water from a Milli-Q water system (Millipore, Bedford, MA, USA) was used throughout.

CE running buffer was 50 mM phosphate, 15 mM SPS (pH 7.0). A volume of approximately 20 μL of 1 M NaOH was added to 10 mL of the running buffer to make a pH 11.0 solution which was used to dissolve the sample. Variations in the concentration of the buffer components due to the addition of NaOH were not considered significant as the volume added was very small. A 200 mM KCN stock solution was made in 2.5 mM borax (pH 9.3).

Since FQ in solution, even when stored at -20 ºC in darkness, degraded slowly, small aliquots of dried FQ were prepared. To do so, a 50 mM stock solution of FQ was prepared in methanol and 10 μL aliquots of it were placed into 500 μL microcentrifuge tubes. The solvent was removed under vacuum at room temperature using a model RC10-10 centrifugal evaporator (Jouan, Saint-Herblain, France). The dried FQ was stored in darkness at -20 ºC. On the day of the experiments, an aliquot was thawed and dissolved in the running buffer to obtain a 5 mM FQ solution.

The nitric oxide generating compound, DEA NONOate, was dissolved at a concentration of 500 mM in 0.01 M NaOH and aliquots were stored at -80º C. Immediately before adding this NO donor to the cultures, an aliquot was thawed and diluted in the growth medium to obtain a 1.1 mM DEA NONOate concentration.

Safety precautions. Potassium cyanide is highly poisonous and reacts readily with acids to form lethal HCN gas. Stock solutions should be made in a basic buffer. Neutralization of waste containing KCN should be made by addition of 1 % NaOH solution (about 50 μL/g of cyanide)

followed by slow addition of bleach (about 70 μL/g of cyanide). *Staphylococcus* were handled using biosafety level 2 practices.

2.2. Bacterial strains and culture conditions

The strains employed were *S. aureus* CECT 4465 and *S. epidermidis* CECT 231 from the Spanish Type Culture Collection. Bacteria were grown in tubes of 16 mm internal diameter with orbital shaking at 200 rpm for 15-60 h in nutrient broth (NB) or tryptic soy broth (TSB) at 20, 37, or 42 ºC.

2.3. Bacterial extracts

To obtain the bacterial lysates, the culture medium was eliminated by centrifugation at 6000 x g for 10 min at room temperature. The pellet was washed 3 times in saline solution (9‰ w/v NaCl in water), resuspended in Milli-Q water, and then disrupted on ice by ultrasonication (4 x 60 s) (Misonix, Farmingdale, NY, USA) into 15 mL falcon tubes. The crude sonicate was centrifuged at 6000 x g for 20 min at room temperature, and then the supernatant (water soluble fraction) was recovered and stored at -20 ºC. Total protein concentration was estimated by the Bio-Rad (Hercules, CA, USA) protein assay based on the Bradford dye-binding procedure [24].

On the day of the experiment, samples were thawed and mixed with the corresponding volume of the 200 mM KCN stock solution and then diluted with the 50 mM phosphate, 15 mM SPS (pH 11.0) sample solvent, so that a final concentration of 5 mM KCN and 6.4 x 10^{-7} M of total protein in the final mixture were obtained in all the protein extracts studied.

To carry out the nitrosative stress experiments, bacteria inoculated from the exponential phase of a *S. aureus* culture in NB at 37 ºC were grown in two 50 mL erlenmeyer flasks in 10 ml of NB with orbital shaking at 200 rpm. The purpose of this inoculation was to have a longer exponential phase of growth and a more accurate sampling process of the cultures where NO was produced. Both erlenmeyers flasks were sealed with microbiology cotton to avoid the gas escape.

To know the nitrosative stress response of *S. aureus*, the effect on bacterial growth of a synthetic NO donor, DEA NONOate, was studied. In one of the erlenmeyers, the logarithmic phase culture (OD_{600nm}~1) was stressed by the addition of 22 μL of 500 mM DEA NONOate in 0.01 M NaOH. A control culture without NO donor addition was performed in parallel for each treatment. In this case addition of NO donor was substituted by a similar volume of the solvent (0.01 M NaOH). Measurements of the optical density were performed at 600 nm every 30 min in both cultures. Growth curves were completed for nine hours until the optical density had reached a stable value.

2.4. Instrumentation

The laboratory-made CE apparatus with LIF detection employed was described previously [25]. Briefly, high voltage was provided by an RS/EH50R Glassman power supply (High Bridge, NJ, USA) used in the normal configuration (anode connected at the inlet end of the capillary). A Spectra Physics Ar-ion laser (9 mW) (Mountain View, CA, USA) was used for excitation at 488 nm. Fluorescence was collected at right angle to the laser beam with a 40x microscope objective, filtered successively through a 550 nm cut-off filter and an interference filter centered at 590 nm, imaged onto an iris to block stray light, and detected with an R928 Hamamatsu photomultiplier tube (Masse, France) operated at 600 V. The photocurrent was processed by a 7070 Oriel photometer (Stratford, CT, USA) and a 406 System Gold A/D Beckman converter (Fullerton, CA, USA). Data were collected on a 486 computer. A laboratory-made special device was used to heat a small part at the inlet of the capillary (10 cm) where the derivatization reaction took place, using an F3 thermostatic water bath (Haake, Karlsruhe, Germany).

Uncoated capillaries (Composite Metal Services, Worcester, UK) 60 cm length (50 cm to detector) and 50 μm I.D. (375 μm O.D.) were used. New capillaries were first rinsed with 1 M NaOH (100 μL), followed by a rinse with Milli-Q water (100 μL). Between runs, the capillary was sequentially rinsed with Milli-Q water (100 μL), 0.1 M NaOH (20 μL), Milli-Q water (100 μL), and

the separation buffer (50 μL). Rinses were made manually employing a model 1710 glass syringe (Hamilton, Bonaduz, Switzerland).

The amount of NO released from DEA NONOate recoverable as NO_2^- was determined in the bacterial cultures by a diazotization reaction originally described by Griess [26] and the reaction product read at 540 nm in a Molecular Devices (Menlo Park, CA, USA) plate reader.

2.5. Procedures

FQ was used as the labeling reagent. This compound generates stable fluorescent isoindol derivatives when reacted with primary amines in the presence of nucleophilic agents, such as KCN [27-29]. The procedure used for on-capillary derivatization was optimized previously employing a mixture of three standard proteins [30]. Briefly, a plug of a mixture of the sample (total protein content in the final protein solution 6.4×10^{-7} M) plus a KCN solution at final concentration 5 mM in the sample solvent was injected into the capillary. Next, a plug of a 5 mM solution of FQ in separation buffer was injected. Both injections were performed by gravity (20 cm height) during 12 s. After each injection of sample plus KCN or of FQ, the inlet end of the capillary was washed by immersion in a vial containing Milli-Q water. Next, vials containing the separation buffer were placed at the inlet and at the outlet ends of the capillary and a voltage of 3 kV was applied for 6 min (mixing step). Then, the power supply was switched off for 0.25 min (reaction step). Reagents mixing and reaction were carried out at 65 ºC. After reaction, separation was performed at 15 kV at room temperature (the temperature of the room was controlled to be 24 ºC). Each experiment was made, at least, in duplicate.

3. Results and discussion

The study was carried out using the water soluble fraction from the lysates of *Staphylococcus* species studied. Among the different intracellular compounds, only compounds with primary amino groups can be derivatized with FQ and consequently detected. Proteins,

peptides and free amino acids are the main compounds that can be labeled with FQ. The relative proportion of these compounds in bacterial cells is very different, proteins being the most abundant (38 % for proteins versus less than 3 % for free amino acids plus nucleotides) [31]. Metabolites containing primary amino groups can also be inside bacterial cells. However, most probably, the number of amino groups is higher in proteins that in these potential metabolites and therefore proteins are derivatized more quickly and easily. In consequence, in this study, the collection of peaks observed in the electrophoretical profiles will be called “protein profile” or “protein pattern” although other components could be present. This assignment has already been used in the literature [19,20,23] when referring to the electrophoretical profiles obtained in conditions similar to the ones presented in this work.

3.1. On-capillary derivatization of proteins with FQ

To carry out the derivatization reaction, KCN, FQ, and the sample containing the proteins have to react inside the capillary. The sample and KCN were mixed before injection into the capillary to simplify the number of steps involved in the analysis. FQ was introduced into the capillary after the injection of KCN plus sample and then a potential of 3 kV was applied to mix the sample and reagents. In these conditions, the FQ plug and the plug containing the negatively charged proteins and the cyanide ion approach each other, at the same time they are swept toward the detector by the electroosmotic flow, generating a region in the sample-FQ interface where these proteins are labeled with high efficiency. In contrast, positively charged proteins are driven away from the reaction region, decreasing the reaction efficiency for these cationic proteins. As separation buffer, a mixture 50 mM phosphate, 15 mM SPS (pH 7.0) was employed following the experiments developed by Zhang *et al.* [23] in order to be able to compare the protein profiles obtained for the *staphylococcal* species with the ones previously published. To assure the labeling of most of the proteins, samples were dissolved in a buffer, with almost the same composition than that of the separation buffer but at pH 11.0. In these conditions, the proteins with a pI lower than the

sample pH are negatively charged. As, in general, proteins have a pI lower than 11, most of the proteins are efficiently derivatized with FQ in the conditions selected. However, it is unlikely that the buffer of the sample influences the separation of the derivatized proteins, because it gets diluted in the background electrolyte soon after the start of the separation, being the pH of the separation buffer the one controlling the charge of the derivatized proteins and therefore their migration. In the separation buffer submicellar concentrations of the surfactant SPS were used. The presence of anionic surfactants at these concentrations avoids the band broadening or multiple peak effect which would be due to the binding of a different number of FQ residues to the protein molecules (multiple labeling of proteins) [23,28].

In the selected conditions, CE protein patterns of cultures of different species of *Staphylococcus* grown in different culture media and submitted to different stress conditions (heat shock or NO onset) were obtained.

3.2. Protein fingerprinting of different species of Staphylococcus

In this study the protein fingerprint of two different species of *Staphylococcus*, *S. aureus* and *S. epidermidis*, were investigated. Each electrophoretical profile consisted of 10-15 peaks that tend to cluster in three different regions (see Figures 1-6). This general aspect of the electropherogram is in accordance with those reported by Zhang *et al.* [23] for *S. aureus* and other *Staphylococcus* species.

The reproducibility of the protein patterns was analyzed for injections of the same culture and for different cultures grown under identical conditions for each bacterial species. Figure 1 shows an example of this study for *S. aureus*. Profiles were very similar, the number and the position of the peaks in the protein patterns were always reproducible, however slight differences in the height of some of the peaks were observed. The same results regarding reproducibility of peaks profile were obtained in the case of the two cultures of *S. epidermidis* grown under the same conditions (results not shown). Therefore, the peak height cannot be considered as a differentiation

factor between protein fingerprints for different species or different conditions. Probably, these differences observed among cultures could be due to variations in bacterial growth and the consequent protein content (compare Fig. 1B with Fig. 1C, 1D, or 1E), even thought the total amounts of protein in the different samples was the same (6.4×10^{-7} M according to the Bradford assay). No peaks were observed in the electropherograms if a blank (culture broth where no microorganisms were grown) was used as a sample (see Fig. 1A).

It is worth mentioning that for pattern reproducibility it is of special importance to work, whenever possible, with the same fused silica capillary for CE-LIF experiments. Protein fingerprints obtained with two different capillaries from the same type are shown in Figure 2, where it can be observed that migration time of the peaks and the peak heights are different from capillary to capillary. It is well known that small modifications on the EOF of the capillary causes slight differences in the migration time of analytes, thus influencing reproducibility. Moreover, when a capillary has been used for a large number of runs (> 20) it is degraded and different migration times are obtained with respect to new capillaries. However the three different regions of the electropherogram in which the peaks seem to cluster can be easily recognized.

Once it had been observed that the protein fingerprints for different cultures of the same species were reproducible, the protein patterns obtained for two *staphylococcus* species *S. aureus* and *S. epidermidis*, were compared. As shown in Figures 2B and 2C significant differences were observed between the protein patterns of the two species. In particular in the region 3 of the electropherogram of *S. epidermidis* the two peaks flanking the major one are higher than those observed at the same migration time for *S. aureus*. These differences were observed repeatedly for different cultures of both *Staphylococcus* species grown under the same conditions (results not shown). Thus, the protein profile could allow a clear differentiation between cultures of *S. aureus* and *S. epidermidis*. This is a promising result that could be useful to differentiate these two species in foods or clinical samples as a complementary method to the classical microbiological methods and to the CE methods previously developed [16,32] for the separation of intact microorganisms.

As discussed above, the peaks appearing in the electrophoretical profiles mainly correspond to the major proteins present in the bacteria studied. This is due to the fact that the major proteins are the ones with the highest concentration in the protein extracts and are responsible for producing major CE peaks when derivatizing with FQ. In order to have an idea which proteins were appearing in these protein fingerprints, the profiles were compared to the protein spots obtained by two-dimensional gel electrophoresis (2D-PAGE) of *S. aureus* [13,33,34]. However these 2D-PAGE protein maps are of a different *S. aureus* strain and had been grown in a different medium (no proteomic studies have been found about the CETC strains used in this work). The major proteins found in these maps, and which could also correspond to the major peaks in the electropherograms are of three different types: 1) enzymes of the energetic metabolism, mainly enzymes taking part in glycolitic processes, 2) proteins and factors related with the translation as ribosomal proteins and elongation factors, respectively, and 3) proteins characteristic of stress processes such as Asp23, Dnak or GroEL.

3.3. Protein fingerprinting of cultures of S. aureus grown under different conditions

As mentioned above, bacteria synthesize different proteins depending on the growth conditions. In consequence, differential peaks obtained under different growth conditions could help to identify the proteins necessary for adaptation of bacteria to specific environmental factors.

Firstly, protein patterns of cultures of *S. aureus* grown in two different media, NB and TSB, (at 20 ºC for 24 h) were compared. As it can be seen in Figure 3, the profiles corresponding to both cultures were different, some peaks correspond to over-expressed proteins while others are related to infra-expressed proteins. For instance, the profile corresponding to the culture grown in NB presents three peaks in region 3 that are not observed in the profile of the culture grown in TSB; however, a shift in migration time of the peaks due differences in electroosmotic flow could not be ruled out. In consequence, the method developed allows the differentiation of cultures of *Staphylococcus* grown in different media on the basis of these protein patterns.

Afterwards, to study if the growth phase of the bacteria had any effect over the protein patterns obtained, cultures of *S. aureus* grown for 24, 40, and 60 h in TSB medium at 20 ºC were compared (Figure 4). The profiles obtained were very similar, the region 3 had the highest peak density. However differences were found mainly in the height of the peak in region 1 and in the major peak appearing in region 3. This last peak was higher for the culture grown for 60 h than in that grown for 24 h, and higher in this one than in that grown for 40 h. Also differences were found in the minor peaks in region 3 (note, for instance, the differences in peak height for those at 15.5 min relative to other peaks in region 3). Therefore, it was not possible to establish a direct relationship between the growing time of the cultures and the height of the peaks in the protein profiles.

3.4. Protein fingerprinting of S. aureus grown in stressing conditions: the effect of temperature

It has been reported that the growth temperature is of crucial importance in the growth rate of the microrganisms [2]. To study the effect of the growth temperature on the protein profiles, two cultures of *S. aureus* were grown at 20 and 42 ºC (in TSB medium for 60 and 15 h, respectively). These are suboptimal temperatures for *S. aureus* which has an optimum growth temperature of 37 ºC [9]. In these experiments two different growing times (60 and 15 h) were used in order to obtain approximately the same number of bacterial cells at the end of the same growing phase of the microorganism than that obtained at the optimal growth temperature. In Figures 5A and 5B the protein profiles obtained for cultures of this microorganism grown at these two temperatures are shown. Although, several peaks could differentiate both electrophoretic profiles, only the peaks showing major differences will be considered here. For instance, by comparing Figures 5A and 5B with Figure 5C it can be readily seen that peak 1 is only shown in the profiles of the cultures grown at 42 ºC and peak 5 is the major peak in the cultures at 20 ºC, which is scarcely present when the microorganism is grown at 42ºC . Minor peaks, such as peaks 3, 4, and 10, could in addition serve to differentiate both cultures, but these minor components are more sensitive to variations among

culture, as indicated before. As an example, note the differences among the high of these minor peaks in Figure 5A and in Figure 4C, which correspond to two different cultures of *S. aureus* grown in exactly the same conditions. Temperatures of around 42 ºC are considered extreme for *S. aureus* and it could be hypothesized that these peaks could be related to proteins induced in stress conditions, such as the heat shock proteins Dnak and GroEl [9] or the cold shock protein csp [10], but further studies are necessary to confirm or to reject this hypothesis. This finding indicates the potential of the CE-LIF method to study proteins induced in microorganisms under different thermal conditions.

3.5. Protein fingerprinting of S. aureus grown in stressing conditions: the effect of nitrosative stress

Nitric oxide (NO) is an important antimicrobial agent and it has been shown to be indispensable for the clearance of diverse pathogens including viral, fungal, bacterial and parasitic microorganisms [35]. Several studies have suggested that NO is bacteriostatic and bactericidal for *S. aureus* [35-37], however the adaptative response of these bacteria to nitrosative stress is not clear.

To investigate if the exposure of the microorganism to NO influences the electrophoretic fingerprint, we have studied in the optimum growth temperature of the bacteria (37 ºC), which had been grown for 18 h, the effect of the presence of a NO donor (DEA NONOate) in the culture media of *S. aureus.* NONOates are synthetic adducts of NO that release NO at predictable rates [38]. DEA NONOate is a rapid-releasing compound known to liberate NO with a half-life of 2 min at pH 7.2. By using the Griess assay, it was determined in this experiment that the NO_2^- concentration produced was 1.1 mM in 30 min that decreased slowly until a concentration 0.9 mM after 4 h.

As shown in Figure 6, for cultures grown in the presence of the donor, the heigh of the peaks marked as 1 and 2 in the electropherogram have increased, however peaks in region 2 of the electropherogram has decreased, or even some peaks are not observed. Therefore, the method described allows one to differentiate the profiles of NO-exposed and not exposed *S. aureus.*

Transcriptomic analysis of the nitrosative stress response of *S. aureus* has identified both up-regulated and down-regulated genes (66 and 18 genes, respectively) in response to NO donors [13]. Some of the down-regulated genes could correspond to peaks of proteins under-represented in the treated cultures, such as soluble enzymes involved in redox homeostasis or amino acid metabolism. It is possible to speculate, according to other authors' studies, that proteins such as the regulatory system SrrAB and the protein Hmp, among others [39,40], and the regulatory systems PerR, Fur and Zur [12] are implicated in the adaptative response of the microorganism in environments containing NO.

These results corroborate the capability of the CE-LIF method presented in this paper to differentiate proteins patterns of *S. aureus* submitted to stressing conditions.

4. Concluding remarks

The usefulness of a capillary electrophoresis method with on-capillary derivatization and laser-induced fluorescence detection to obtain protein fingerprints of different species of *Staphylococcus* under different conditions has been demonstrated. The analysis (derivatization plus separation) requires less than 25 min. The protein patterns obtained for cultures of *S. aureus* and *S. epidermidis* are species-specific and they could be useful to differentiate microorganisms of different species belonging to the same genus. However, the dependence observed for the protein profiles obtained upon the culture conditions (medium) should be taken into account for identification purposes. Finally, the CE-LIF method described seems to offer good potential for studying proteins induced under stressing conditions such as heat shock or cold shock and nitric oxidative onset.

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Figure legends

Fig 1. Capillary electrophoresis patterns of a blank assay (A), patterns of three consecutive injections of the same culture of *S. aureus* (B, C, and D) and two cultures of *S. aureus* grown in the same conditions (D and E). Growth conditions: temperature 37 ºC, medium NB, and time 18 h. CE-LIF conditions: running buffer 50 mM phosphate-15 mM SPS (pH 7.0), uncoated capillary (L = 60 cm, l = 50 cm, 50 μm I.D.), injection by gravity (h = 20 cm, 12 s) of a mixture of the sample extract with a 5 mM KCN solution followed by an injection of 5 mM FQ in running buffer, mixing 6 min at 3 kV, reaction 15 s at 0 kV, mixing and reaction temperature 65 ºC, separation performed at 15 kV and 24 ºC.

Fig. 2. Capillary electrophoresis patterns of proteins for two injections of a culture of *S. aureus* using two different capillaries (A in a new capillary, B in an old capillary) and a culture of *S. aureus* (B) and *S. epidermidis* (C). Other conditions as in Fig. 1.

Fig. 3. Capillary electrophoresis patterns of proteins for cultures of *S. aureus* grown in (A) NB medium and (B) TSB medium. Other growth conditions: temperature 20 ºC, time 24 h. Rest of the conditions as in Fig. 1.

Fig. 4. Capillary electrophoresis patterns of proteins for cultures of *S. aureus* grown for (A) 24 h; (B) 40 h; and (C) 60 h. Other growth conditions: temperature 20 ºC, medium TSB. Rest of the conditions as in Fig. 1.

Fig. 5. Capillary electrophoresis patterns of proteins for (A) a culture of *S. aureus* grown at 20 ºC for 60 h, (B) a culture of *S. aureus* grown at 42 ºC for 15 h and (C) a mixture 1:1 of the protein extracts of both cultures shown in (A) and in (B). Medium: TSB. Other conditions as in Fig. 1.

Fig. 6. Capillary electrophoresis patterns of proteins for (A) a culture of *S. aureus* grown in NB and (B) *S. aureus* grown in NB supplemented with 1.1 mM DEA NONOate. Other conditions as in Fig. 1.

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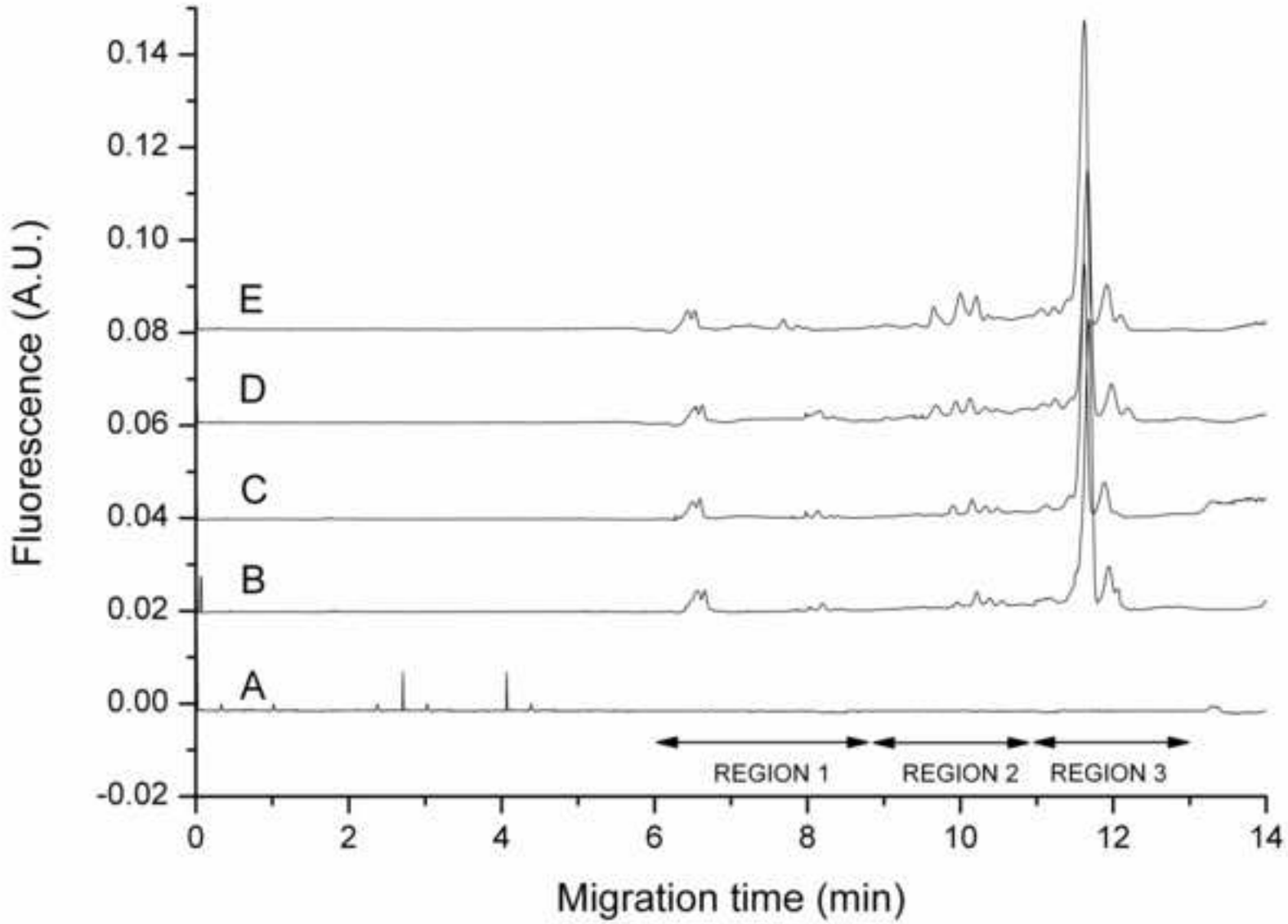

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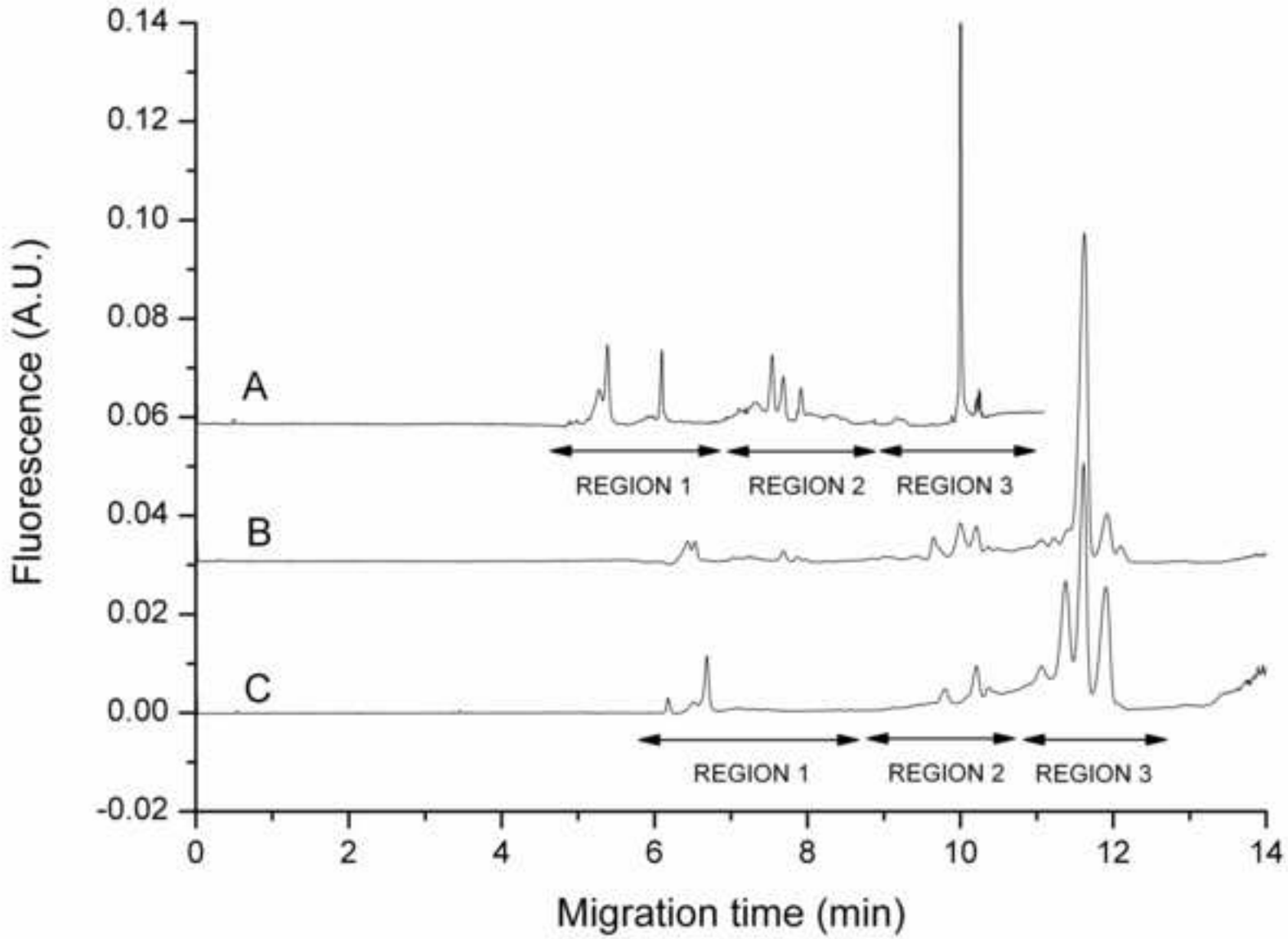

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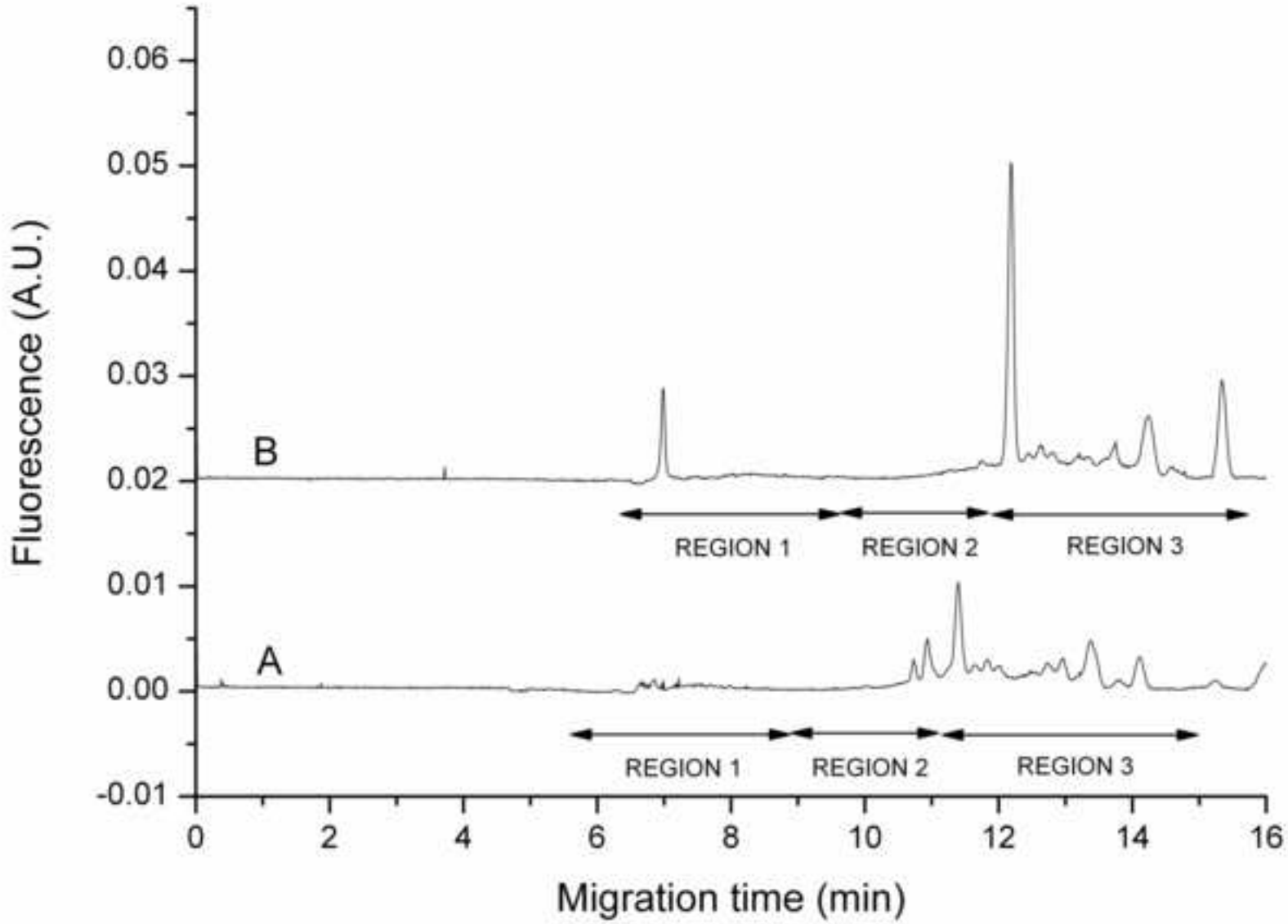

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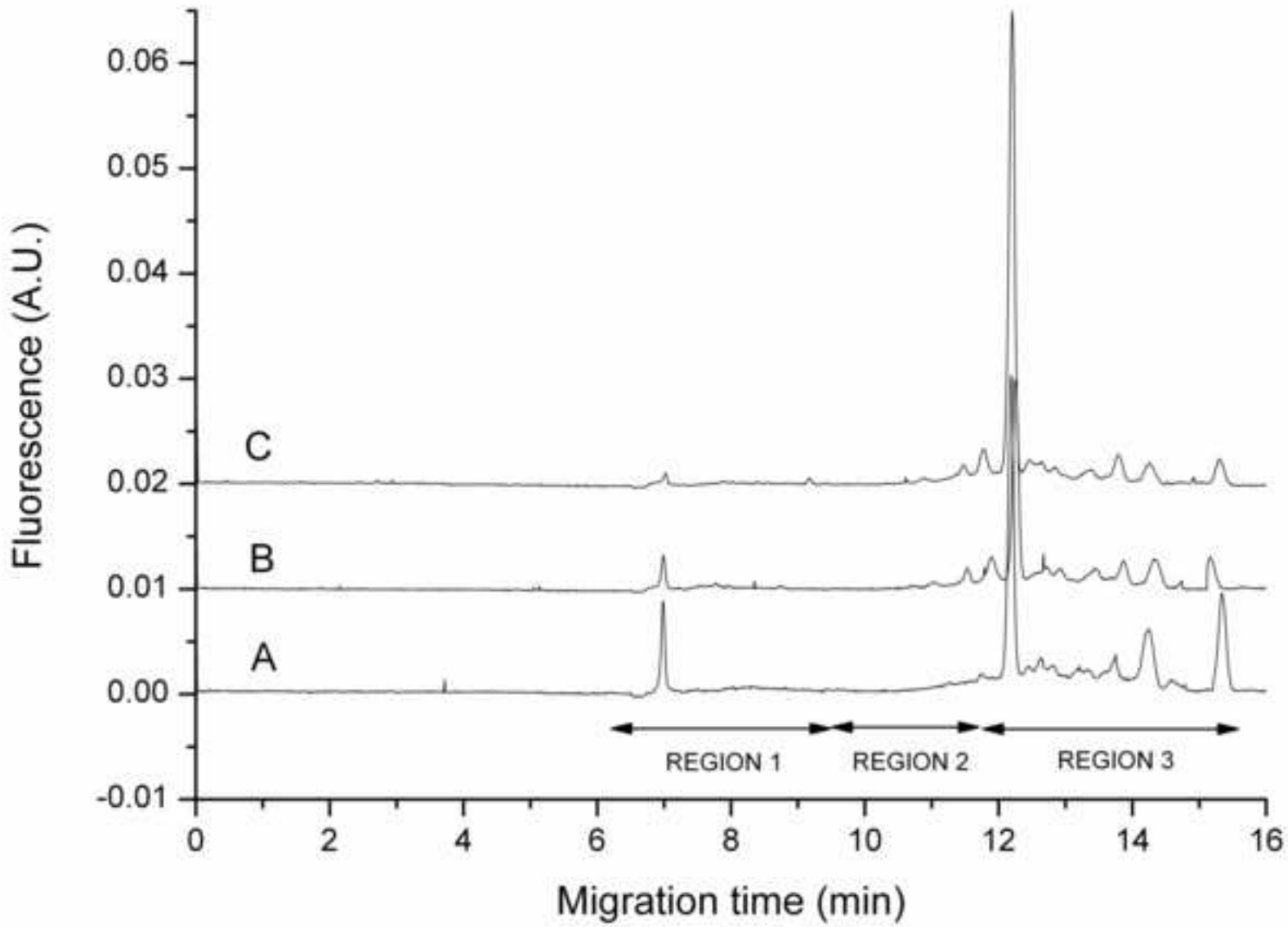

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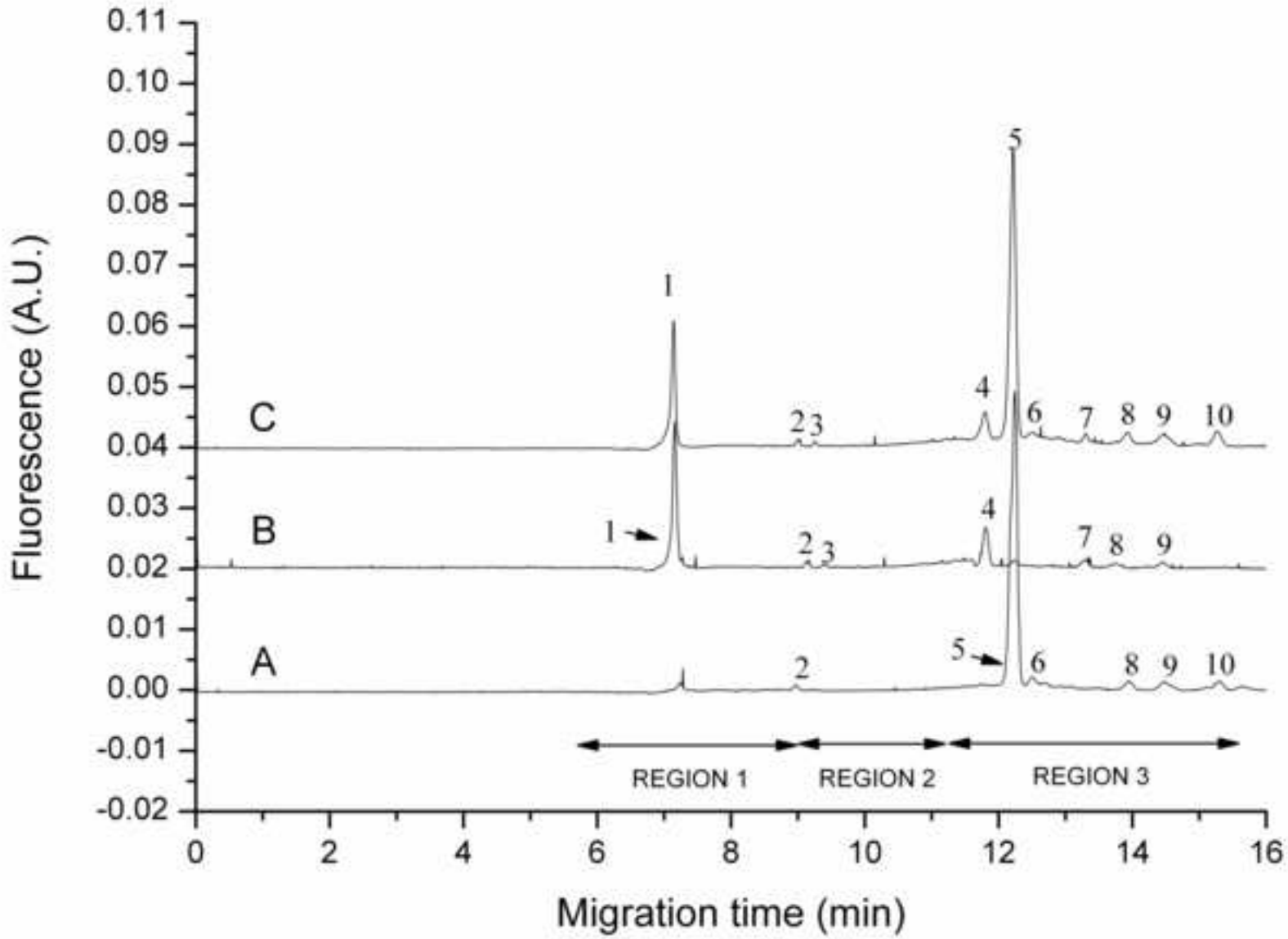

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