Large-volume sample stacking-capillary electrophoresis used for the determination of 3-nitrotyrosine in rat urine☆

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Abstract

Large-volume sample stacking using the electroosmotic flow (EOF) pump technique has been investigated for the quantification of 3-nitrotyrosine in urine of diabetic rats. The best separation conditions for these highly complex samples were obtained using capillary electrophoresis (CE) in the reversed polarity mode (i.e., injecting at the cathode and detecting at the anode) using cetyltrimethylammonium bromide (CTAB) in the running buffer. The optimum CE separation conditions were achieved using a phosphate buffer prepared with 0.15 M phosphoric acid and 0.5 mM CTAB adjusted to pH 6.4 with sodium hydroxide. In such CE conditions, the limit of detection (LOD) was 1.77 μM for 3-nitrotyrosine with normal injection mode, meanwhile with the large-volume sample stacking technique a more than 20-fold improvement was observed (i.e., LOD = 0.08 μM was obtained) without noticeable loss of resolution. This value allowed the detection of 3-nitrotyrosine in urine from diabetic rats. To our knowledge, this work is one of the few applications showing the great possibilities of these stacking procedures to analyse biological samples by CE.
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1. Introduction

There are direct and indirect evidences that connect oxidative stress and diabetes [1]. Research has shown that levels of oxidized proteins increase with diabetes [2], while oxidized proteins seem to be selectively targeted for proteolytic breakdown in vivo. This opens the exciting possibility of using oxidized amino acids, such as 3-nitrotyrosine, in urine as a non-invasive marker for oxidative stress. Due to its potential significance several analytical methods have been described for the determination of 3-nitrotyrosine alone or bound to proteins. These methods have to overcome the important limitation that the low concentration in which this analyte is usually found in such complex matrices brings about.

Techniques for the determination of 3-nitrotyrosine in biological samples by HPLC with electrochemical detection were described by Hensley et al. [3], after concluding that UV detection was not sensitive enough for the routine quantification of the compound in vivo. Immunochemical analysis [4] was also studied by these authors and they concluded that this procedure is cumbersome and subject to variability. HPLC with fluorescence detection was employed for 3-nitrotyrosine measurement in human plasma [5]. GC–MS has also been employed but derivatization for the full quantification of 3-nitrotyrosine can lead to the artifactual nitration of tyrosine if performed under acidic conditions [6]. The analysis by GC–MS avoiding artifacts requires a HPLC separation prior to derivatization [7]. An accurate, sensitive and specific assay for 3-nitrotyrosine based on combined liquid chromatography tandem mass spectrometry has been developed [8], but this is not a routine analytical tool yet and the instrumentation is very expensive. In other cases, even derivatization prior to HPLC-tandem MS was employed to enhance sensitivity and selectivity [9].

More simple methods are therefore needed that can be employed in routine analysis of a large number of samples. Capillary electrophoresis (CE) presents important features related to the problem such as: (i) the ability to separate small molecules in complex matrices without sample pre-treatment; (ii) the small sample volume employed, which is of especial interest for some body fluids, mostly working with small experimental animals and (iii) the low consumption of reactives: a few milliliters of aqueous buffers are enough for 1 day. However, CE has a very important drawback, its lack of sensitivity.

Several methods to increase the amount of analyte injected in CE without impairing peak shape or resolution have been employed and several recent reviews [10,11] describe the principles and procedures used in capillary zone electrophoresis for sample stacking.

The objective of the present work is the development of a simple CE method for 3-nitrotyrosine analysis in rat urine to be employed for monitoring oxidative stress in a large number of samples coming from groups of experimental animals. Furthermore, the exploration of stacking principles applied to the biological sample is also carried out.

2. Materials and methods

2.1. Capillary electrophoresis

CZE was performed on a Beckman System P/ACE 5010, Beckman Instrument, Palo Alto, USA) equipped with a UV detector set at 214 nm, an uncoated capillary (Beckman Coulter, Madrid, Spain) with total length, of 57 cm and an internal diameter (i.d.) of 50 μm. The detector window was set at 50.6 cm from the inlet. All the experiments were carried out at 25 °C. Normal sample injections were made by pressure at 0.5 psi from 5 to 30 s, as indicated. The separation potential was −15 kV. Injection was done at the cathode and detection at the anode. For large-volume sample stacking injections were made at high pressure (20 psi) for 12 s.

The electrophoretic buffer was prepared with 0.15 M phosphoric acid plus 0.5 mM of CTAB adjusted to pH 6.4 with 0.1 M NaOH. The buffer was shaken and filtered (using 0.4 μm filters) prior to its use in CE. Capillary was flushed between runs with NaOH, H₂O and the background electrolyte for 3 min each. Buffer vials (5 mL) used for separation were replaced after each injection.

2.2. Reagents

3-Nitro-L-tyrosine was from Sigma (St. Louis, MO, USA), 3-chloro-L-tyrosine was from Aldrich (St. Louis, MO, USA), L-tyrosine was from Merck (Darmstadt, Germany). Phosphoric acid 85% was from Merck, sodium hydroxide from Panreac (Madrid, Spain). All the buffer solutions were prepared with purified water by a Milli-Q-System (Millipore, Bedford, MA, USA).

2.3. Animals and sample preparation

Urine was obtained from Sprague–Dawley male rats of 450 g weight bred in our animal quarters. Streptozotocin (50 mg/kg) was used to promote diabetes in one group of animals and the effect was monitored by glucose measurement. Urine was collected after 24 h with rats housed into metabolic cages with water and standard commercial diet ad libitum.
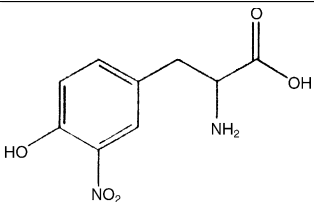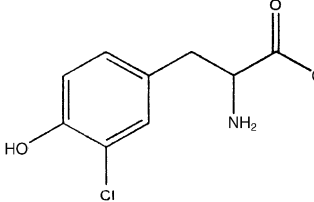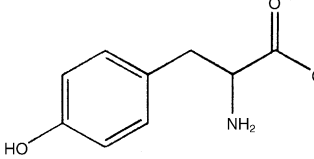
Urine samples and standards were diluted 1:1 (v/v) with acetonirile and centrifuged at 4000 rpm for 10 min at room temperature. The supernatant was directly injected in the CE instrument.

3. Results and discussion

The first step consisted in the development of the CE conditions using standards to obtain an adequate separation of the compounds involved. Tyrosine, 3-nitrotyrosine and 3-chlorotyrosine were employed, the last one as a possible internal standard. As the three compounds present both an amino and a carboxylic moieties, as can be seen in Table 1, normal polarity in acidic conditions, as well as reversed polarity in more basic conditions were tested. The best resolution for standards was obtained in reversed polarity and, afterwards, conditions were optimised in parallel for standards and spiked samples. Phosphate and borate buffers with concentrations from 100 to 200 mM plus 0.5 mM CTAB used to decrease the electroosmotic flow (EOF), pH values from 6 to 9, separation voltages from −10 to −25 kV and different detection wavelengths were tested. Optimum conditions in terms of peak efficiency and resolution were obtained using a buffer composed of 0.15 M phosphoric acid, 0.5 mM of CTAB at pH 6.4, as can be seen in Fig. 1 where a good separation of these three compounds is shown. At this condition CTAB provides a negative EOF of 2×10^{-4} cm^2/(V s). Using this buffer, LODs were calculated after consecutive dilutions of the standards for a signal/noise ratio (S/N) equal to 3. Results from this experiment can be also observed in Fig. 1, obtaining LODs values equal to 1.8 μM for nitrotyrosine, 1.8 μM for chlorotyrosine and 2.2 for tyrosine. As it was expected, due to the low levels of analyte, when non-spiked samples were analysed, 3-nitrotyrosine was not observed. Injection time was increased from 5 to 30 s, but too long injection times produced some band-broadening and the corresponding loss of resolution without noticeable increase of peak heights at injection times higher than 20–25 s, as can be observed in Fig. 2.

The buffer conditions and polarity allowed the use of large-volume sample stacking using the EOF pump [13–17]. A large volume of sample (500 nL versus 5 nL in normal injection) was injected by pressure and under negative voltage. In such conditions, the EOF moves toward the negative electrode in the zone where the sample has been injected and CTAB has been removed. Once the aqueous sample plug has

Table 1
Molecular structures and pK_a values for 3-nitrotyrosine, 3-chlorotyrosine and tyrosine

3-Nitrotyrosine ($C_9H_{10}N_2O_5$)		pK_{a1} = 2.20, pK_{a2} = 7.20, pK_{a3} = 9.11	M_w = 226.19
3-Chlorotyrosine ($C_9H_{10}NO_3Cl$)		pK_{a1} = 2.02, pK_{a2} = 8.09, pK_{a3} = 9.34	M_w = 215.64
Tyrosine ($C_9H_{11}NO_3$)		pK_{a1} = 2.20, pK_{a2} = 9.11, pK_{a3} = 10.07	M_w = 181.19

been removed, negatively charged analytes stacks in a sharp zone and migrate towards the positive electrode due to its own electrophoretic mobilities and because the background electrolyte entering the capillary from the anodic vial deactivates silanol groups and EOF gradually decreases [10]. Obviously, under these conditions the run time is much longer than in normal injection to permit such process. However, it

has to be taken into account that the whole process can be done automatically (i.e., unattended).

Fig. 3 shows that the resolution is still adequate for separation of standards after using the large-volume sample stacking. Interestingly, under these conditions the LODs obtained (for a S/N = 3) were 0.09 μM for 3-nitrotyrosine, 0.11 μM for 3-chlorotyrosine and 0.11 μM for tyrosine. As can be de-
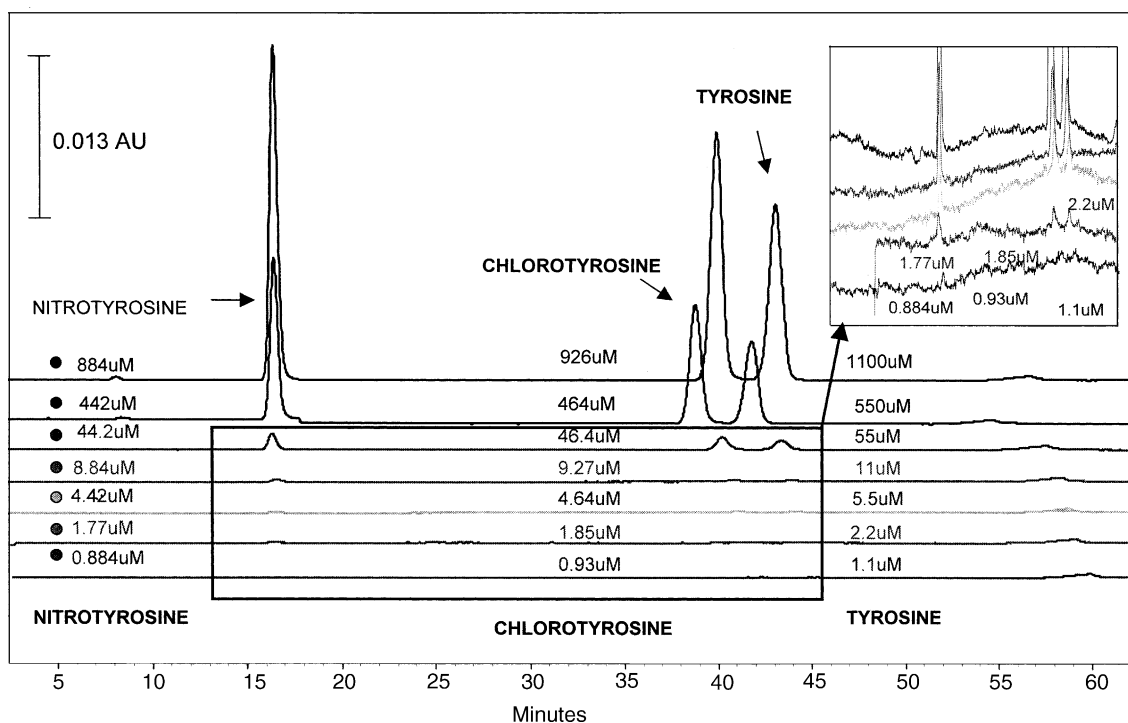

Fig. 1. Limits of detection for 3-nitrotyrosine, chlorotyrosine and tyrosine for a 5 s injection at 0.5 psi. Separation buffer: 0.15 M phosphoric acid, 0.5 mM of CTAB at pH 6.4.
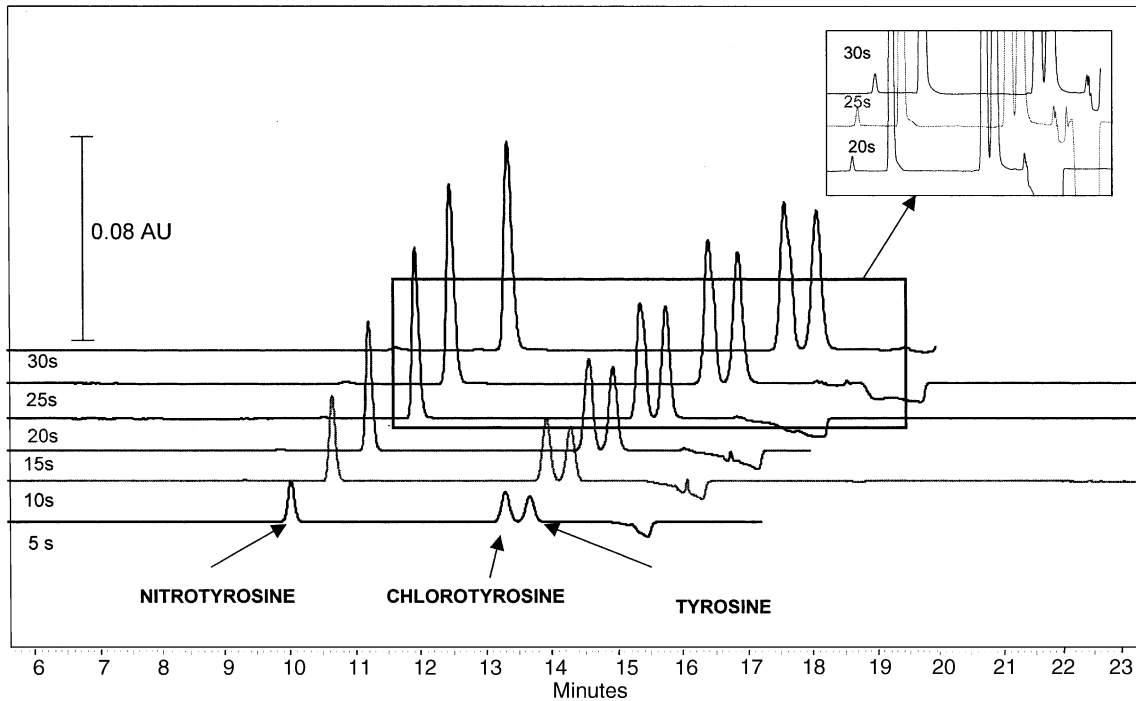
Fig. 2. Increasing injection time from 5 s to 30 s at 0.5 psi. Separation buffer: 0.15 M phosphoric acid, 0.5 mM of CTAB at pH 6.4.

duced by comparing these LOD values with those obtained above under no-stacking conditions, the use of large-volume sample stacking brings about an up to 20-fold improvement in the detection of these compounds. These values demon-strate the great possibilities of this stacking procedure to improve the sensitivity in CE separations. Limits of quan-titation (LOQ) were calculated under these conditions fol-lowing EURACHEM recommendations [12] by testing the
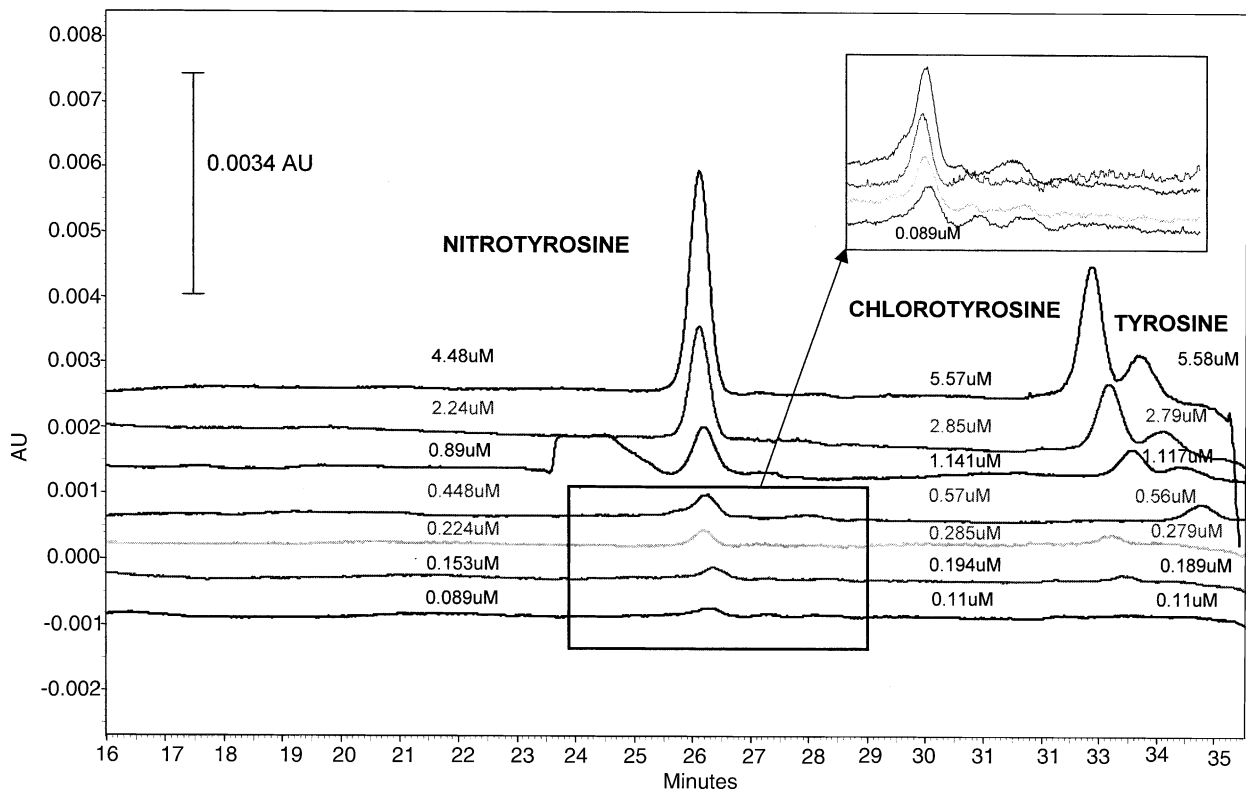

Fig. 3. Limits of detection for 3-nitrotyrosine, at 20 psi for 12 s. Separation buffer: 0.15 M phosphoric acid, 0.5 mM of CTAB at pH 6.4.
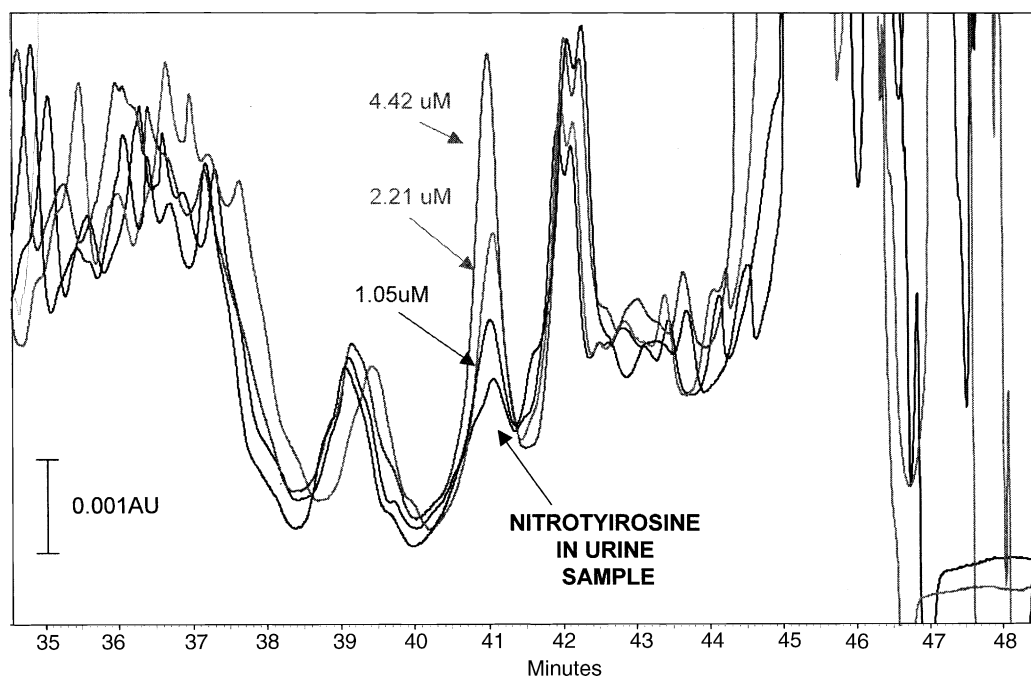
Fig. 4. Nitrotyrosine peak in urine of diabetic rat after LVSEP and the sample spiked with three levels of analyte from 1.05 to 4.42 μM. For conditions see the text.

repeatability of successive injections of standard solutions at the low concentration levels. Based on the R.S.D. obtained, a 10% was considered acceptable for the analytes at the LOQ. Obtained LOQ values were 0.44 μmol/L for 3-nitrotyrosine, 0.46 μmol/L for 3-chlorotyrosine and 0.55 μmol/L for tyrosine.

Once the procedure was optimized using standard solutions, urine samples were analysed. Fig. 4 shows the electropherogram of a urine obtained from a diabetic rat. Samples were mixed with acetonitrile for removing proteins and, simultaneously, lowering sample matrix conductivity to obtain a better stacking effect. Diabetes-induced increased oxida-
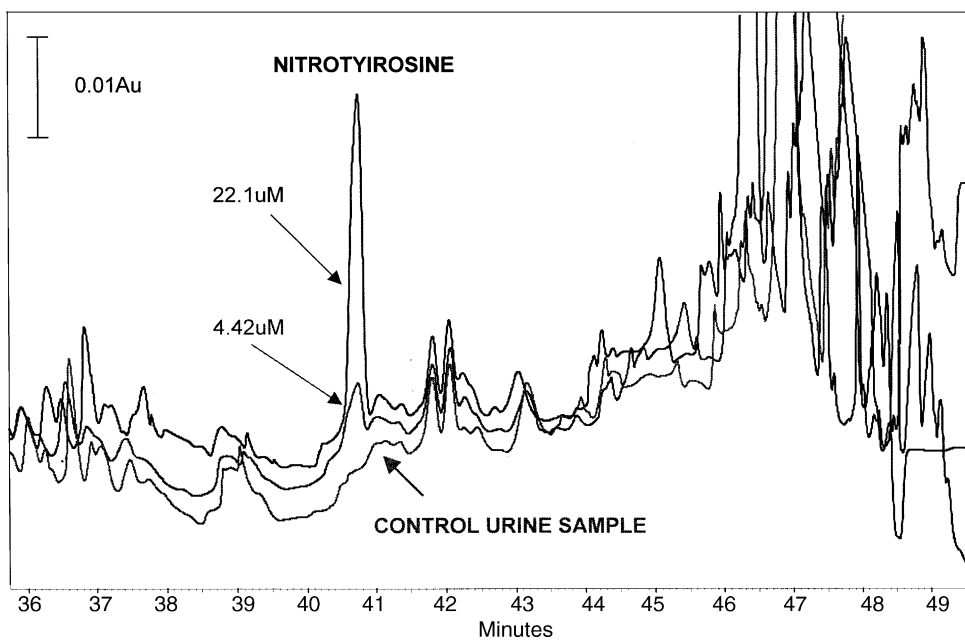

Fig. 5. Urine of control rat after LVSEP and the sample spiked with two levels of 3-nitrotyrosine. For conditions see the text.

tive stress is postulated to play a significant role in nitrotyrosine levels, because superoxides and nitrous oxide can react and form peroxynitrite, a highly reactive intermediate, that can modify tyrosine in proteins to form nitrotyrosine [1]. A very complex electropherogram was obtained by direct injection of the urine samples. Therefore, the nitrotyrosine peak had to be identified by migration time and by spiking, as can be observed in Fig. 4. 3-Chlorotyrosine and tyrosine could not be quantified in real samples because there are several compounds co-eluting in that area, precluding the use of the internal standard procedure. Therefore, nitrotyrosine quantification was performed using both external standard and standard addition procedures. For the external standard quantification method, a calibration line was obtained with standard five levels of standard ranging from 0.88 μM to 4.4 μM (linear regression: peak area $= 2270 + 18\,801 \times$ [nitrotyrosine]; $r = 0.9997$). For the standard addition procedure, urine alone and urine spiked with increasing concentrations of the standard from 0.55 μM to 4.4 μM were processed (linear regression: area $= 14404 + 16924$ [nitrotyrosine]; $r = 0.995$; $n = 5$). The result obtained with the external standard method was 1.90 μmol/L of nitrotyrosine in sample (0.95 μmol/L in the vial) and with standard addition it was 1.72 μmol/L of nitrotyrosine in sample (0.86 μmol/L in the vial). The difference between them was 9.4% which is into the 10% R.S.D. considered acceptable for this level of analyte in the sample and therefore both methods of quantification could be employed. Nevertheless, standard addition permits simultaneously to identify and quantificate the peak solute in a very complex profile. Values to be compared have not been found in the literature for this particular sample, which is an additional indication of the novelty of our approach. One of the main risks of this type of protocols is the possibility of wrong results due to interferences from other unexpected compounds in the real samples. In order to discard this negative effect, urine from control rats was injected under the same conditions and the nytrotyrosine peak was not detected, as can be seen in Fig. 5, and due to this result, the basal level for this compound could not be established. In any case, it has to be kept in mind that the CE method presented in this work would allow to follow in a fast, clean and sensitive way any decrease or disappearance of nitrotyrosine peak in urine samples from rats. This variation can be related to an improvement in the oxidative stress status of these animals during any in vivo assay [2].

4. Conclusions

A capillary electrophoresis method has been developed that permits 3-nitrotyrosine measurement in urine of diabetic rats without any other sample pre-treatment more than dilution with acetonitrile and centrifugation. It employs the large-volume sample stacking using the EOF pump to increase sensitivity. The method allows the detection of 3-nitrotyrosine in urine of a diabetic rat, but not in the corresponding control. To our knowledge, this work is one of the few applications showing the great possibilities of stacking procedures to analyse biological samples by CE.

References

[1] P. Rosen, P.P. Nawroth, G. King, W. Moller, H.J. Tritschler, L. Packer, Diabetes Metab. Res. Rev. 17 (2001) 189.
[2] A. Ceriello, F. Mercuri, L. Quagliaro, R. Assaloni, E. Motz, L. Tonutti, C. Taboga, Diabetologia 44 (2001) 834.
[3] K. Hensley, K.S. Williamson, R.A. Floyd, Free Radic. Biol. Med. 28 (2000) 520.
[4] L. Viera, Y.Z. Ye, A.G. Estevez, J.S. Beckman, Methods Enzymol. 301 (1999) 373.
[5] Y.W.K. Kamisaki, K. Nakamoto, Y. Kishimoto, M. Kitano, T. Itoh, J. Chromatogr. B 685 (1996) 343.
[6] M.T. Frost, B. Halliwell, K.P. Moore, Biochem. J. 345 (2000) 453.
[7] E. Schwedhelm, D. Tsikas, F.-M. Gutzki, J.C. Frolich, Anal. Biochem. 276 (1999) 195.
[8] D. Yi, B.A. Ingelse, M.W. Duncan, G.A. Smythe, J. Am. Soc. Mass Spectrom. 11 (2000) 578.
[9] T. Delatour, J. Richoz, P. Vouros, R.J. Turesky, J. Chromatogr. B 779 (2002) 189.
[10] M. Urbanek, L. Krivankova, P. Bocek, Electrophoresis 24 (2003) 466.
[11] R.-L. Chien, Electrophoresis 24 (2003) 486.
[12] EURACHEM, A Laboratory Guide to Method Validation and Related Topics, 1998.
[13] A. Maciá, F. Borrull, C. Agular, M. Calull, Electrophoresis 24 (2002) 2779.
[14] D.S. Burgi, Anal. Chem. 64 (1993) 3726.
[15] M. Albert, L. Debusschere, C. Demesmay, J.L. Rocca, J. Chromatogr. A 757 (1997) 291.
[16] N. Baryla, C. Lucy, Electrophoresis 22 (2001) 52.
[17] B. Kim, D.S. Chung, Electrophoresis 23 (2002) 49.