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Article

Electrospray Ionization Mass Spectrometry Applied to Study the Radical Acetylation of Amino Acids, Peptides and Proteins

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Recentemente nosso grupo demostrou experimentalmente que a hipótese de produção de radical acetil em meio tamponado no sistema diacetil/peroxinitrito era possível. Diacetil é um flavorizante em alimentos, cigarros e bebidas. O peroxinitrito é encontrado na mitocôndria, e em certas condições como um processo de infeção em humanos, a concentração aumenta significativamente. Em sistemas biológicos, radicais podem facilmente modificar a estrutura e atividade de ácidos nucleicos, proteínas e outras biomoléculas, causando significativo estres oxidativo. Baseado em dados de ressonância magnética e espectrometria de massas, este artigo apresenta os produtos que provam a produção do radical acetil e a formação de compostos estáveis devido à ligação covalente entre o acetil e os aminoácidos (produtos acetilados), e os adutos de peptídeos e proteínas. Estes materiais foram separados por eletroforese capilar e identificados por espectrometria de massas. O meio reacional consistiu da mistura de diacetil e peroxynitrito em uma relação de 1:2 e em 20 mmol L^{-1} de fosfato de sódio, no valor de pH 7,2. Estes experimentos também revelam a dupla acetilação da lisina, demonstrando a alta reatividade do composto com biomoléculas contendo grupos de nitrogênio, abundantemente encontradas em sistemas biológicos. A mudança estrutural de uma molécula acetilada é uma fonte de modificações pós-tradução com inúmeras consequências biológicas.

Recently, our group proposed a process that generated acetyl radicals in a reaction medium buffered with a diacetyl/peroxynitrite system. Diacetyl is a flavoring agent in food, cigarettes and drinks. Peroxynitrite is found in mitochondria, and in certain conditions, such as an infection in humans, its concentration is augmented significantly. In biological systems, radical compounds can easily modify the structure and activity of nucleic acids, proteins and other biomolecules, causing significant oxidative stress. Based on paramagnetic resonance and mass spectrometry data, this work discusses products that prove acetyl radicals are produced and are able to form stable covalent bonds with amino acid (acetylated products), peptide and protein adducts. These materials were separated and detected by capillary electrophoresis coupled with tandem mass spectrometry or offline mass spectrometry. The reaction medium contained a 1:2 mixture of diacetyl and peroxynitrite dissolved in 200 mmol L^{-1} of pH 7.2 sodium phosphate buffer. These experiments also reveal the double acetylation of lysine, demonstrating the high reactivity of these compounds when in contact with nitrogen-containing biomolecules readily found in biological systems. These structural changes might be an epigenetic source of post-translational protein modification.

Keywords: acetylation of biomolecules, acetyl radical, peroxynitrite, diacetyl, mass spectrometry, CE/ESI-MS/MS

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Introduction

Mass spectrometry (MS) is an important technique in the post genomic era because it can rapidly and reliably investigate the relationship between the genome and phenotype of cells and organisms.[1] Understanding the gene expression and protein states in living systems requires quantitative *in vitro* and *in vivo* studies of substrates, intermediates and products.[2] The modification of proteins by chemical reactions involving compounds released through distinct metabolic pathways may lead to deleterious results.[3] Therefore, post-translational modification (PTM),[4] which is undoubtedly the most widely studied enzyme-catalyzed modification event in proteins, and other non-enzymatic processes can be investigated using capillary electrophoresis coupled with electrospray ionization mass spectrometry (CE-ESI-MS).[5,6]

ESI-MS is a powerful tool for analyzing the plethora of intermediate and final products in biological samples.[7] Generally, CE-MS is successful platform for revealing the physiological and pathological biomarkers in various body fluids. In addition to defining biomarker analytical data, such as migration time and molecular mass, CE-MS is an indispensable tool for gaining deeper insight into the molecular mechanisms underlying the manifestation of several acquired and inborn errors in metabolism. While CE provides high resolution and fast separation during analysis, MS offers selectivity and sensitivity, making the combined CE-ESI-MS a very effective analytical tool.[8,9] Furthermore, capillary electrophoresis belongs to a category of analytical separation techniques that produces minimal residue.

Lysine acetylation is a reversible post-translational modification involved in various biochemical processes in an organism; an acetyl group is transferred to an internal lysine residue Є-amino group in a protein. The acetylation process in histones has been known for almost 30 years. Enzymes that regulate these acetylation processes in non-histone proteins have recently been discovered to play a crucial role in biological events.[10-12] These enzymes contain flexible and highly basic tails that are targeted by several types of post-translational modification reactions, including methylation, citrullination, phosphorylation,[13] sumoylation, ubiquitination, ADP-ribosylation,[14,15] acetylation and glycation.[16]

The significance of lysine acetylation has been exhaustively studied in the context of nuclear histone modifications. However, the implications of the changes transcend gene regulation.[17] The molecular changes caused by lysine acetylation in an organism may alter the activity of metabolic enzymes and force the cell to adapt to metabolic changes. These alterations may cause unanticipated changes that may alter an individual metabolic profile.[18,19] Recently, Zhang *et al.*[20] demonstrated epigenetic chromatin process for remodeling of histone proteins by acetylation. This process was diagnostic for late-onset cognitive impairment. Histone acetylation affects the regulation of gene transcription. The loss of learning induced by deacetylation in specific histone sites may provide biomarkers for memory loss and Alzheimer's disease.

Nearly a decade ago, Oya *et al.*[21] reported the modification of bovine serum albumin (BSA) by methylglyoxal, resulting in arginine/methylglyoxal adducts and structural changes in BSA. This compound is a well-known α-dicarbonyl catabolite derived from triose phosphates, acetone and aminoacetone that has cyto and genotoxic effects. Diacetyl produces acetyl radicals after treatment with peroxynitrite, which is a powerful nucleophile and oxidant formed by the diffusion-controlled reaction between superoxide radical anions and nitric oxide in biological systems.[22] Diacetyl is a flavoring agent used in food (milk, coffee, tea, beef, butter, yogurt and alcoholic drinks) at concentrations ranging from 0.5 to 28 nmol L^{-1}.[23-26] Diacetyl is a volatile product of citrate metabolism produced by *Lactococcus lactis* ssp. lactis biovar. *diacetylactis* and *Leuconostoc citrovorum*. In the USA, diacetyl-based ingredients are generally considered safe, although their use in food products is limited by good manufacturing practices. Diacetyl has recently been implicated as a causative agent for certain lung diseases among plant workers. However, little information has been published regarding the volatile composition of this ingredient and the levels of diacetyl or other flavoring agents in finished dairy products. A detailed review of the literature on this topic reveals few studies regarding biological samples and their biochemical implications.[27]

Otsuka *et al.*[28] developed a method to determine the diacetyl concentration using gas chromatography with electron capture detection. Using this method, diacetyl was detected in different rat tissues (liver, heart, kidney, muscle, brain and liver).[29] The most important finding was that diacetyl influences several aspects of amyloid-β (Aβ) peptide aggregation. The process is one of the two primary pathologies associated with Alzheimer's disease.[23]

In biological systems, nitric oxide reacts with the superoxide anion, producing peroxynitrite ($^{-}$OONO) extremely rapidly (k_2 ca. 7-20 × 10^9 mol^{-1} L s^{-1} at 25-37 °C).[30,31] This rate constant is almost one order of magnitude higher than that of the superoxide dismutase-catalyzed decomposition of the superoxide radical ion. In living organisms, the simultaneous formation of nitric oxide and superoxide radicals is often observed in the

presence of high peroxynitrite concentrations, particularly in endothelial cells and near inflammatory sites.[32] Therefore, the evaluation of peroxynitrite reactivity toward various biomolecules is highly relevant. Peroxynitrous acid (HOONO) has a pK_a of 6.8, meaning that 80% of it is deprotonated in the biological environment.[33] The combined presence of peroxynitrite and exogenous diacetyl or endogenous methylglyoxal in a slightly alkaline physiological cellular medium creates the perfect conditions for producing acetyl radicals that may chemically alter biomolecules.[10,11]

We report a method that uses capillary electrophoresis-electrospray ionization-tandem mass spectrometry (CE-ESI-MS/MS) or only ESI-MS coupled to an ion trap mass analyzer to separate and identify 20 acetylated amino acids, peptides and proteins digested by acetylation generated from a diacetyl/peroxynitrite system. Consequently, concomitantly with the transacetylase-catalyzed reaction, the diacetyl/peroxynitrite system may acetylate proteins (*N*-terminal and chain lysine residues) and nucleobases. We propose that similar analytical conditions might be employed to identify acetylated free amino acids or proteins exposed to diacetyl and peroxynitrate *in vitro*.

Experimental

Reagents and solvents

The chemicals used were analytical grade. Diacetyl, formic acid, phosphoric acid, ammonium formate, sodium tetraborate, sodium nitrite, Chelex-100, 2-methyl-2-nitrosopropane (MNP), amino acids (L-Gly, L-Ala, L-Val, L-Leu, L-Ile, L-Met, L-Phe, L-Tyr, L-Trp, L-Ser, L-Pro, L-Thr, L-Cys, L-Asn, L-Gln, L-Lys, L-His, L-arg, L-Asp and L-Glu) and proteins were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, ammonium hydroxide, acetic acid, hydrochloric acid, sodium carbonate, sodium hydroxide, disodium hydrogen phosphate decahydrate and sodium phosphate monobasic monohydrate were supplied by Merck (Darmstadt, Germany). The peptides and peroxynitrite were synthesized in our laboratory.

Synthesis of peroxynitrite

Peroxynitrite was synthesized from $NaNO_2$ (0.6 mol L^{-1}) and H_2O_2 (0.7 mol L^{-1}) in HCl (0.6 mol L^{-1}) and NaOH (1.5 mol L^{-1}) in a quench-flow reactor.[34] Excess H_2O_2 in the alkali peroxynitrite solution was eliminated with MnO_2. The H_2O_2 and peroxynitrite concentrations were quantified spectrophotometrically at 240 nm (ε = 42 L mol^{-1} cm^{-1}) and 302 nm (ε = 1670 L mol^{-1} cm^{-1}), respectively. The peroxynitrite concentrations obtained using this method ranged from 250 to 350 mmol L^{-1}. The stock solutions of peroxynitrite were stored on ice in the dark. Aliquots were stored at −80 °C. Diacetyl was distilled before use at 88 to 90 °C and 40 rpm. All the solutions were prepared in distilled water prepared in a Millipore Milli-Q system (MA, USA), and the phosphate buffer (200 mmol L^{-1}, pH 7.2) was pretreated with Chelex-100 to eliminate any metal contaminants. Excess hydrogen peroxide in the peroxynitrite-containing alkaline solution (1.0 mol L^{-1} NaOH) was eliminated with MnO_2. Amino acid stock solutions were prepared in deionized water obtained from a Millipore Milli-Q system (MA, USA), and the buffers were pretreated with Chelex 100 to eliminate any free metals in solution. A diacetyl (50 mmol L^{-1}) stock solution was prepared in a 200 mmol L^{-1} phosphate buffer. The radical acetylation reactions involved mixing peroxynitrite (2.0 mmol L^{-1}), diacetyl (2.0 mmol L^{-1}) and amino acid (1.0 mmol L^{-1}) in a phosphate buffer solution (200 mmol L^{-1}, pH 7.4) under constant Vortex agitation for 1 min to maintain the dissolved oxygen concentration at a saturated and non-limiting level. Reaction mixtures with pH values ranging from 7.1 to 7.5 were analyzed.

The reaction between peroxynitrite and α-dicarbonyl substrates, such as diacetyl ($k_2 = 1.4 \times 10^4$ L mol^{-1} s^{-1}, pH 7.2, 25 °C), proceeded faster than reactions with monocarbonyl substrates (e.g., $k_2 < 10^3$ L mol^{-1} s^{-1} for acetaldehyde, propionaldehyde, and pyruvate).[35] Uppu *et al.*[36] reported that low yields of carboxylic acids were formed when aldehydes were exposed to peroxynitrite (ca. 0.2 mol acetate *per* mol peroxynitrite), while Yang *et al.*[35] demonstrated that carboxylic fragments formed in reactions between various ketones and peroxynitrite.

Synthesis and purification of synthetic peptides

A peptide was synthesized with the following sequence: Ac-A-E-F-K-F-A-L-NH_2. This peptide was constructed to contain a lysine residue. The peptide was synthesized according to the following protocol. The peptide was obtained using solid phase synthesis, as described by Hirata *et al.*[37] and reviewed by Korkmaz *et al*.[38] Specifically, Fmoc chemistry (9-fluorenylmethoxycarbonyl) was used to protect the α-amino group, while NovaSyn TGR® resin and HBTU / HOBT were the solid support and coupling reagents, respectively. The Fmoc group was removed using *N*-methyl-20% piperidine in dimethylformamide, and the peptide was cleaved from the resin using trifluoroacetic acid: anisole:ethanedithiol:water (TFA:ANS:EDT:H_2O/85:5:3:7).

The synthesized peptides were characterized and assayed for purity using fast high performance liquid chromatography (fast HPLC) coupled to mass spectrometry (EV LC/MS-2020 - Prominence; Shimadzu Corp., Tokyo, Japan or /MS EV-2020 - Prominence; Shimadzu Corp., Tokyo, Japan), equipped with a LC-10AD pump for the fast mobile phase, a Fast XR-ODS column (5 μm, 4.6 × 75 mm), SPD-10AV UV-Vis detector operating at wavelength of 220 nm, as well as by electrospray ionization mass spectrometry or matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry in a Bruker Daltonics MALDI-TOF mass spectrometer. The matrix used during all the analyses was α-cyano-4-hydroxycinnamic acid.

Preparation of samples

The reaction mixture consisted of BSA (68.4 μmol L^{-1}), diacetyl (5.0 mmol L^{-1}) and peroxynitrite (1.3 mmol L^{-1}) in 200 mmol L^{-1} pH 7.2 phosphate buffer at room temperature. MALDI studies of the RNase and lysozyme were carried out with the protein alone and protein treated with either peroxynitrite, diacetyl, or both reagents without trypsinization. The reaction mixture was ultrafiltered (Vivaspin MWCO 3000, GE Healthcare Bio-Sciences, Uppsala, Sweden) and lyophilized; subsequently, the product was rehydrated with 50 μL of 0.1% TFA.

Digestion of samples

The protocol for lysozyme digestion was the classical procedure used for proteomic studies.[39] After the reaction and desalinization via ultra-filtration, the volume of the lysozyme and acetylated lysozyme 0.04 mg mL^{-1} solutions was halved. 100 μL corresponded to 80 μg and was diluted in 100 μL methanol *per* 50 mmol L^{-1} NH_4HCO_3 solution at pH 8.0, (60/40 v/v) in a 1.5 ml screw cap tube for 5 min at 95 °C in an Eppendorf Thermomixer (Germany) at 600 rpm. This step broke down the protein 3D structure, facilitating the enzymatic action. The mixture was cooled to room temperature before 100 μL of 10 mmol L^{-1} DTT were added and incubated for 30 min at 37 °C. This reagent reduced the protein disulfide bonds. Afterward, 100 μL of 20 mmol L^{-1} iodoacetic acid were added, and the mixture was incubated at room temperature in the dark to promote the alkylation of the cysteine residues. Trypsin was dissolved in 50 mmol L^{-1} NH_4HCO_3 at pH 8.0 to reach 5 μg mL^{-1}, and 100 μL of this solution were added to the reaction tube; the reaction tube was placed in the thermomixer and vortexed for 20 h at 37 °C in the dark. To end the enzymatic reaction, 56 μL of 0.1% TFA were added.

Purification of acetylated peptide

A homemade zip-tip C18 purification column was assembled in a 200 μL micropipette tip with a small amount of glass fiber inserted into the tip to hold 50 μL 100 mg mL^{-1} C18 reversed-phase resin in isopropyl alcohol.[40] The resin was flushed with 100 μL methanol to activate the solid support and 100 μL 0.05% of TFA for equilibration. A 100 μL sample was added, and 100 μL of 0.05% TFA were eluted. Finally, 100 μL of 80% ACN solution with 0.05% TFA were passed through the column to elute the peptides. The samples were lyophilized and rehydrated using 50 μL of 0.1% TFA.

Electron paramagnetic resonance (EPR) spin trapping spectra with 2-methyl-2-nitrosopropane (MNP) were recorded at room temperature, using a Bruker EMX spectrometer (Bruker AXS Inc., Madison, USA). All spectra were recorded after incubating the reagents for 4 min in 500 mmol L^{-1} phosphate buffer (pH 7.2). At the end of the experiment, the pH was measured to detect any changes caused by adding the alkaline peroxynitrite stock solutions to the buffered reaction mixtures. The following instrumental conditions were utilized: microwave power of 19.91 mW, modulation amplitude of 0.1 mT, time constant of 163,840 ms and receiver gain of 2.5×10^3.

The separation and detection of amino acids in all completed reaction mixtures were performed with a Beckman Coulter MDQ™ capillary system (California, USA) and a Thermo Finnigan model LCQ Ion Max Advanced mass spectrometer equipped with an ESI source and an ion trap analyzer (MA, USA). Electrophoresis was carried out in fused silica capillaries from Polymicro Technologies (AZ, USA) with 50 μm i.d. × 70 cm total length. The electrolyte used during the CE/ESI-MS analyses was 1.0 mol L^{-1} formic acid. Before injection, the capillary was preconditioned by flushing with 1.0 mol L^{-1} ammonium hydroxide for 3 min, followed by water for 2 min, and finally, the electrolyte for 5 min. The samples were injected under 2.0 psi for 10 s. The effective applied electric field was 280 V cm^{-1}, the capillary temperature was maintained at 25 °C, and the sheath flow interface was used. The 50.0:49.5:0.5 water/methanol/acetic acid solution was delivered at 10 μL min^{-1} by a Gilson Model 402 Pump (MI, USA). The MS and MS/MS experiments were performed in positive ion mode at 4.5 kV. This voltage was previously evaluated; although this value may seem high, this was the best value to analyze amino acids because the CE-MS system potential combines that of the ESI and CE systems. The experimental conditions were as follows: number of microscans = 3, microscan time = 200 ms, capillary temperature at 275 ºC, spray voltage of 27 V and tube lens

offset of −8 V. The mass spectrometer was calibrated using 20 μmol L^{-1} L-Lysine directly infused into the CE under 10 psi. The data acquisition was performed in full scan and MS/MS scan modes by examining the most intense peaks; the normalized collision energy and the isolation width were set to 30-35% and 1.0 *m/z*, respectively for these peaks in the MS/MS. The minimum signal intensity threshold was 1×10^3 while the MS/MS data were collected. Nitrogen was used as both the nebulizer and sweep gas and was delivered at flow rates of 15 and 5 L min^{-1}, respectively.

The micro-HPLC-MS/MS (AmaZon Ion Trap (Bruker-Franzen Analytic GmbH) analysis of protein digests was carried out with a Shimadzu micro liquid chromatograph (Kyoto, Japan), and the data were processed using the Mascot 2.3 program peptide sequences. Chromatography was performed using a Shim-pack XR-ODS II column at 0.2 mL min^{-1} with a 5 min gradient time, 70% of solvent A (20/79.9/0.1 acetonitrile/water/acetic acid v/v) and 30% of solvent B (79.9/20/0.1 acetonitrile/water/ acetic acid v/v), 30-50% B in 20 min and 90% of B in 80 min, for a total analysis time of 100 min. The mass spectrometer was optimized for 70-3000 *m/z*, with a 4.5 kV positive polarity, a 300-2800 *m/z* trap scan speed, a 4500 V capillary voltage, 8.0 psi nebulizer gas, 5.0 L min^{-1} dry gas, a 220 °C heater interface temperature, and MS/MS with a 5.0 eV quadrupole ion energy. Smart Defrag was used to obtain satisfactory MS/MS data from the different compounds with varying stabilities. This technique ramps up the fragmentation amplitude from 20% at the start amplitude by 30 to reach 300% of the specified value. A $-CH_3CO^{\bullet}$ adduct was obtained for a MNP solution containing peroxynitrite (2.0 mmol L^{-1}) and diacetyl (2.0 mmol L^{-1}) in phosphate buffer (200 mmol L^{-1}, pH 7.4) (Figure 1).

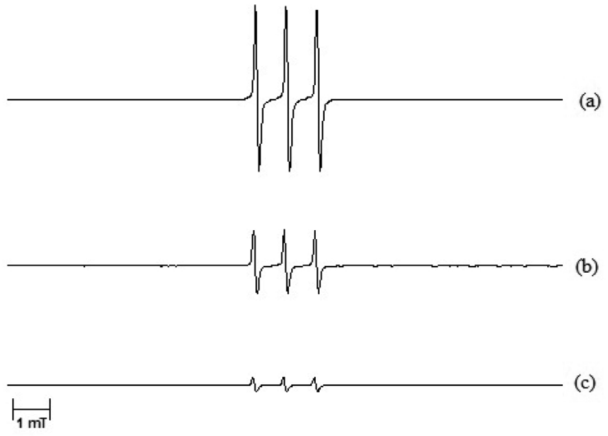

Figure 1. Effect of histidine on the amplitude of the EPR signal for the putative MNP-$CH_3CO^{\bullet}$ adduct. The peroxynitrite/diacetyl reaction was performed at room temperature in 200 mmol L^{-1} aerated phosphate buffer (pH 7.2) after a 4 min incubation. The reaction mixture contained diacetyl (5.0 mmol L^{-1}), peroxynitrite (2.0 mmol L^{-1}), MNP (2.0 mmol L^{-1}) and varying concentrations of histidine: (a) 0.0 mmol L^{-1}, (b) 0.5 mmol L^{-1} and (c) 1.0 mmol L^{-1}.

Results and Discussion

The hypothesis that the acetyl radical produced by adding peroxynitrite to diacetyl is consumed by amino acids was first investigated using EPR spin-trapping studies with MNP (20 mmol L^{-1}) and L-histidine (0.50 and 1.0 mmol L^{-1}).[10,11] A three-line EPR spectrum with $a_H = a_N = 0.83$ mT characteristic of the MNP was collected.

The radical adduct signal decreased as the concentrations of L-histidine increased, indicating that the acetyl radical was consumed by the L-histidine. Figures 2a, 2b and 2c depict the CE-ESI-MS traces obtained after adding free L-lysine to the diacetyl/peroxynitrite solutions that produced the acetylated amino acid. Peak 1 was assigned to the free L-lysine (peak 1, 147 *m/z*), while the other two peaks (2 and 3) were the *N*-α- and *N*-ε-acetyl-L-lysine isomers (189 *m/z*), demonstrating that both lysine nitrogen atoms underwent acetylation. These data, as well as those obtained with other polar and nonpolar amino acids, confirmed that the chemical yield of acetylation was less than 30% of the peroxynitrite concentration. This value was a rough estimation based on the ratio of the CE peak areas for the acetylated and free amino acids (2.0 mmol L^{-1}) in the consumed reaction mixture. This calculation also accounts for the fact that 70% of added peroxynitrite (2.0 mmol L^{-1}) isomerizes, forming the nitrate ion.[41] Figures 2b and 3c reveal the two different radical fragmentation pathways for the molecular ions of both isomers generated from the acetylation of lysine. These data support our hypothesis that dual acetylation is a competitive process, while partial acetylation occurs at each lysine nitrogen atom. Full scan mass spectra of the ion precursor (189 *m/z*) were obtained during the experiments.

Figure 3 depicts the CE-ESI-MS traces for eight representative acetylated amino acids, 1: acetyl-L-Ser (148 *m/z*); 2: acetyl-L-Tyr (224 *m/z*); 3: acetyl-L-Phe (208 *m/z*); 4: acetyl-L-His (198 *m/z*); 5: acetyl-L-Lys (189 *m/z*); 6: acetyl-L-Met (192 *m/z*); 7: acetyl-L-Arg (217 *m/z*); and 8: acetyl-L-Thr (162 *m/z*). All twenty amino acids were acetylated.

The synthetic peptide was dissolved in acetic acid/H_2O/methanol (50/49/1 v/v) to produce the spectra without acetylation, as illustrated in Figure 4a. When this peptide was subjected to the same reaction conditions as the amino acid acetylation, lysine was also acetylated in these chain peptides, as displayed in Figure 4b. However, the spectral data presented in Figure 4c validates our hypothesis because a protein in a physiological buffer

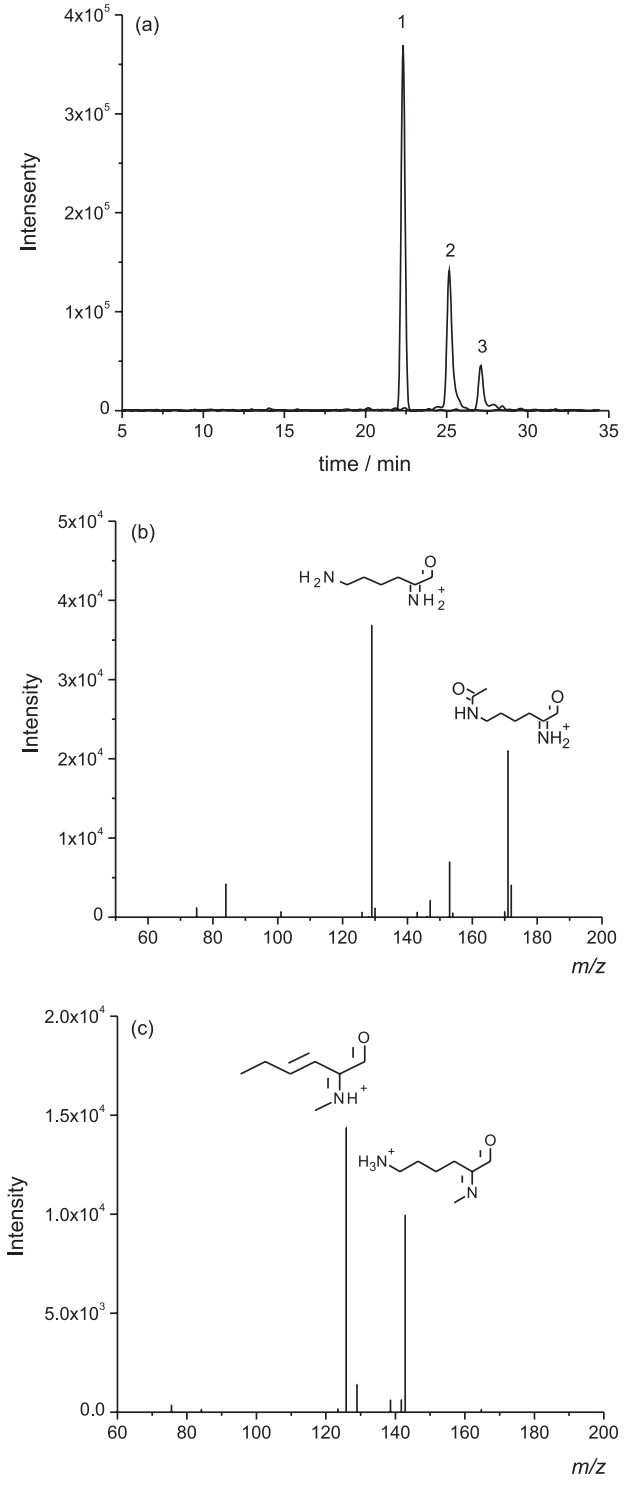

Figure 2. (a) CE-MS base peak electropherogram of radical acetylation detected by CE-ESI-MS/MS, 1: L-lysine (147 *m/z*), 2: *N*-α-acetyl-L-lysine and 3: *N*-ε-acetyl-L-lysine (both at 189 *m/z*); (b) and (c) MS/MS of 189 *m/z* ions. The CE-MS conditions for the free and acetylated amino acid mixtures were as follows: 1 mol L^{-1} acid formic electrolytes with 20% methanol (pH 2.0); uncoated fused silica capillary, 50 μm i.d. × 75 cm; injection under 2 psi of pressure for 10 s; applied voltage 20 kV; temperature of 25 ºC. ESI conditions: positive ion mode; spray voltage of 4.5 kV; sheath liquid methanol/water/acetic acid (50/49.5/0.5 v/v) at 5.0 μL min^{-1}; drying gas flow (N_2) of 3 L min^{-1}; and drying temperature of 275 ºC.

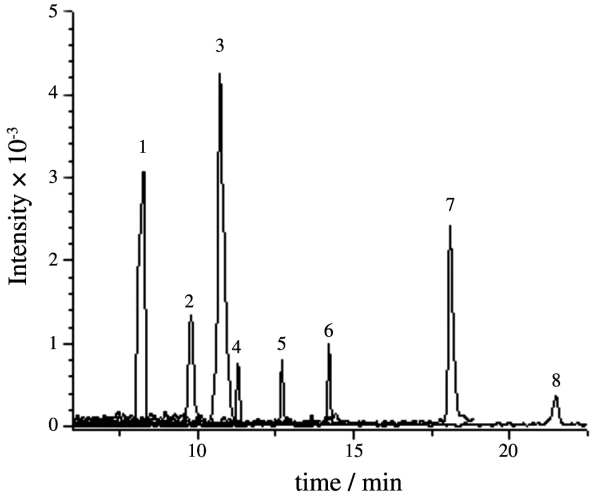

Figure 3. Eight independent traces representative of the CE-ESI-MS acetylation experiments, 1: Acetyl-L-Ser (148 *m/z*), 2: acetyl-L-Tyr (224 *m/z*), 3: acetyl-L-Phe (208 *m/z*), 4: acetyl-L-His (198 *m/z*), 5: acetyl-L-Lys (189 *m/z*); 6: acetyl-L-Met (192 *m/z*), 7: acetyl-L-Arg (217 *m/z*) and 8: acetyl-L-Thr (162 *m/z*). CE-MS conditions for the free and acetylated amino acids mixture containing 1 mmol L^{-1} L-lysine. BGE of 1,0 mol L^{-1} formic acid electrolyte (pH 1.8); uncoated fused silica capillary 50 μm i.d. × 70 cm; 10 s injection under 2 psi of pressure; applied voltage 25 kV; and capillary temperature of 25 ºC. ESI conditions: positive ion mode; spray voltage 4.5 kV; sheath liquid methanol/water/acetic acid (50/49.5/0.5 v/v) at 5.0 μL min^{-1}; drying gas flow (N_2) of 3 L min^{-1}; and drying temperature of 275 ºC.

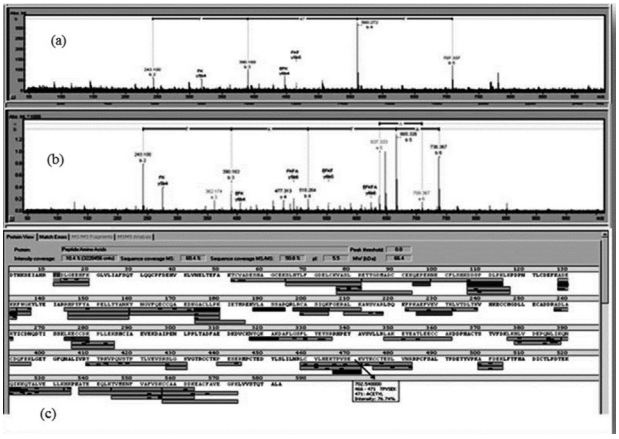

Figure 4. (a) and (b) were radically acetylated peptides and control (c): Tryptic digest of the radically acetylated BSA were obtained in the micro-HPLC-MS/MS coupled with a micro-LC; the acquired data were processed using peptide sequences from the Mascot 2.3 database.

solution (the same condition as that of the amino acids) exhibited an acetylation spectrum for the inner portion of the protein (BSA); this fragment was revealed by a tryptic digest after radical acetylation. The intensity of this fragment (acetylated peptide) was visualized using the MS/MS BioTools data (Bruker Daltonics, USA). This phenomenon was not observed for protein digested without undergoing acetylation beforehand in a reaction medium containing peroxynitrite or/and diacetyl.

Conclusion

Our results support the hypothesis that, unlike inflammatory sites, post-translational protein modification by radical acetylation did not only occur at the protein *N*-terminus of cells under nitrosative and carbonyl stress. In fact, double acetylation may also occur at the *N*-terminus of lysine residues.

Furthermore, CE-MS/MS is an alternative tool for investigating and quantifying the radical acetylation events in regulatory proteins, such as PTM, and small molecules. There is an urgent need for tools that can identify the type and location of protein modifications, as well as determine the extent of structural changes that might be related to a gain or loss of biological function. In this work we demonstrate that the radical acetylation of proteins might contribute to enzymatic acetylation.[42] In addition, acetylated amino acid residues in proteins might be new biomarkers for cell damage.

CE-MS was a demonstrably powerful tool for analyzing small organic molecules (< 500 Da), such as amino acids, their derivatives, and many other catabolites. This technique features highly efficient separation and resolution, as well as relatively easy method development, small sample volume, rapid analysis and low solvent consumption. Recently, a dramatic increase in the number of publications describing and quantifying the type and location of chemical lesions in proteins, DNA and other biomolecules has occurred. We demonstrated that using mass spectrometry in combination with capillary electrophoresis enabled us to examine enzymatic and radical acetylation reactions of protein amino acid residues. CE-ESI-MS also simplifies the process for distinguishing between the *N*-terminal and inner acetylation of L-lysine residues implicated during post-translational cellular events.

Supplementary Information

The supplementary data contain the extracted ion electropherogram (BPE) for the free amino acids and acetylate products are available free of charge at http://jbcs.sbq.org.br as PDF file. The amino acids and acetylated products were as follows: A = L-arginine and L-acetyl-arginine; B = L-phenylalanine and L-acetyl-phenylalanine; C = L-methionine and L-acetyl-methionine; D = L-acetyl-tyrosine and L-acetyl-tyrosine; E = L-serine and L-acetyl-serine; F = L-histidine and L-acetyl-histidine; G = L-threonine and L-acetyl-threonine.

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Supplementary Information

Electrospray Ionization Mass Spectrometry Applied to Study the Radical Acetylation of Amino Acids, Peptides and Proteins

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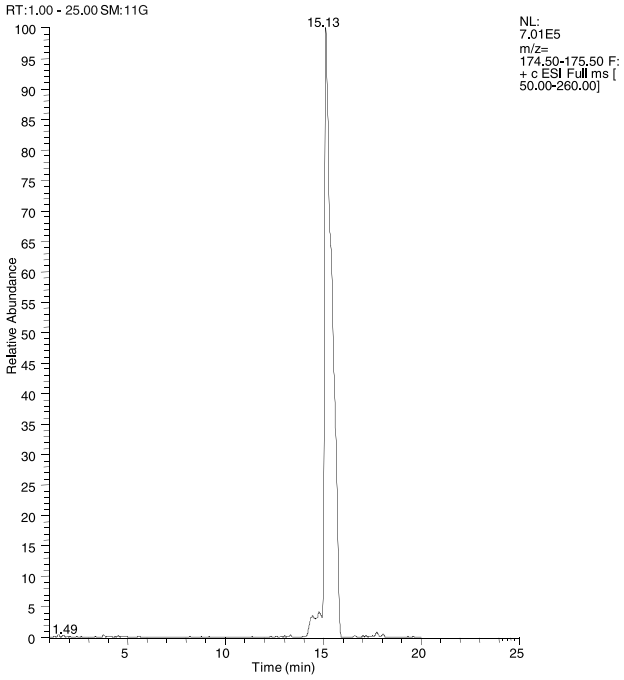

Figure S1. BPE obtained with CE-MS of L-arginine.

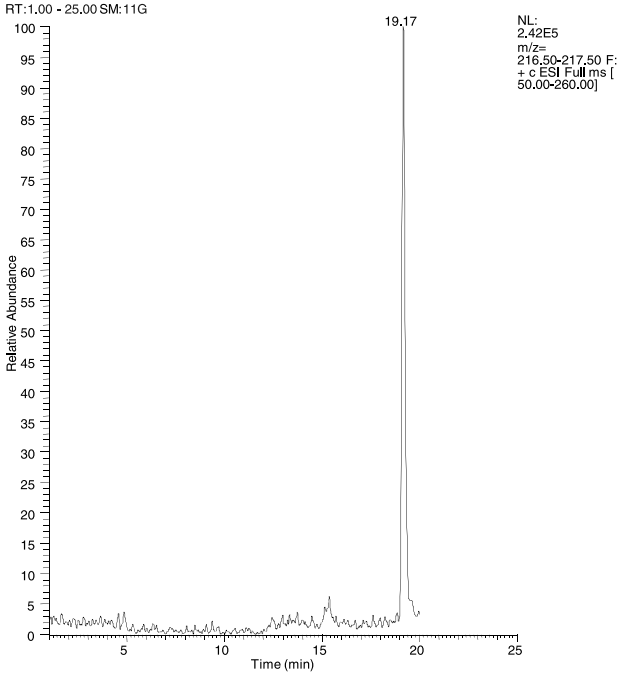

Figure S2. BPE obtained with CE-MS of L-acetyl-arginine.

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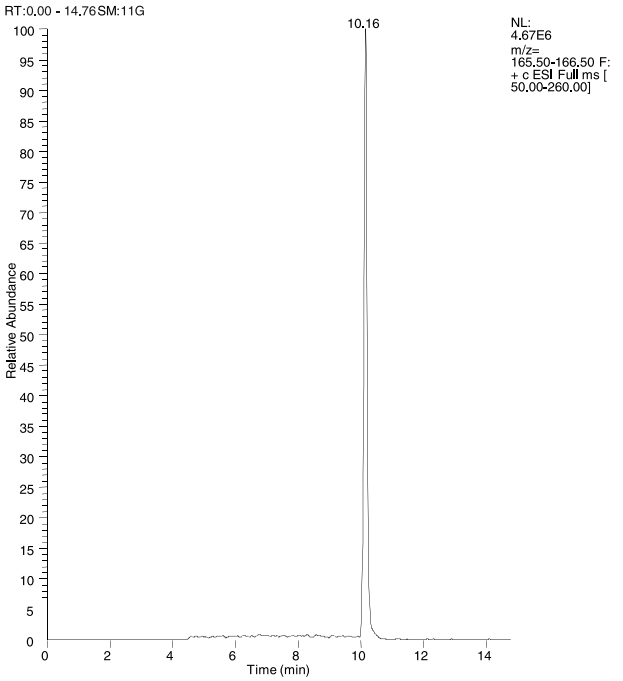

Figure S3. BPE obtained with CE-MS of L-phenylalanine.

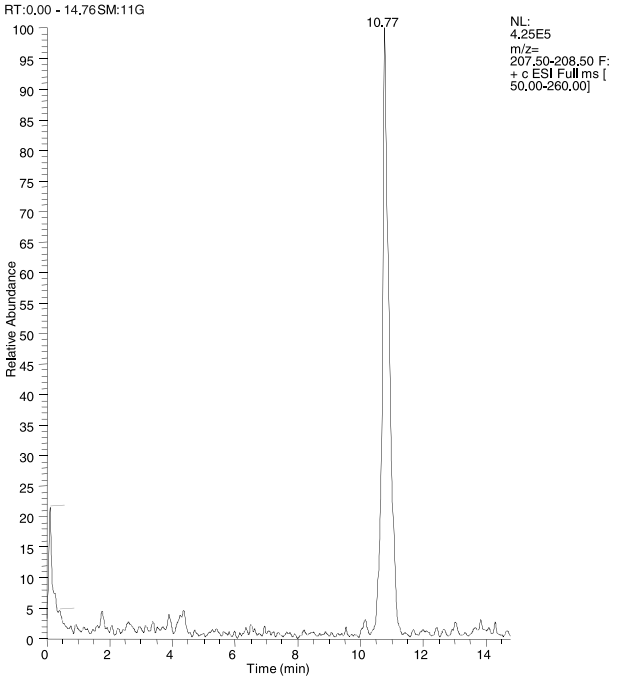

Figure S4. BPE obtained with CE-MS ofL-acetyl-phenylalanine.

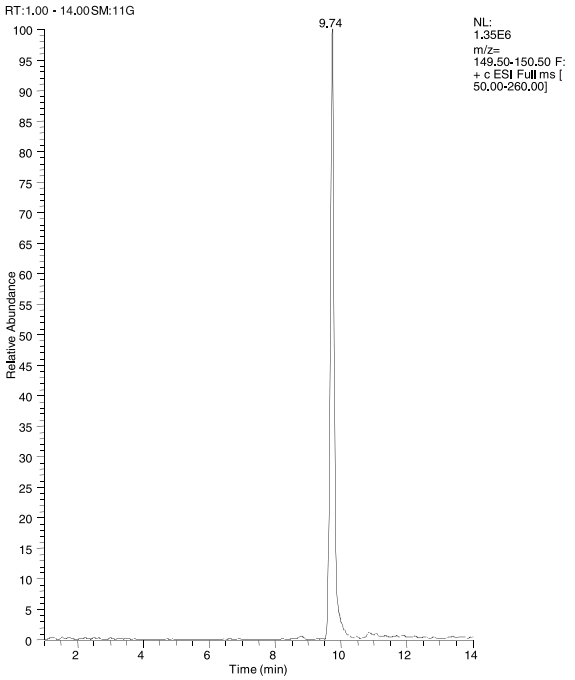

Figure S5. BPE obtained with CE-MS of L-methionine.

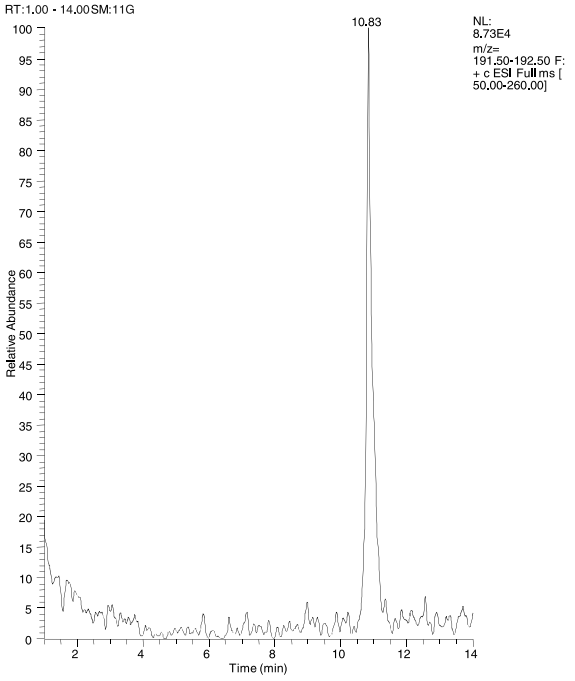

Figure S6. BPE obtained with CE-MS of L-acetyl-methionine.

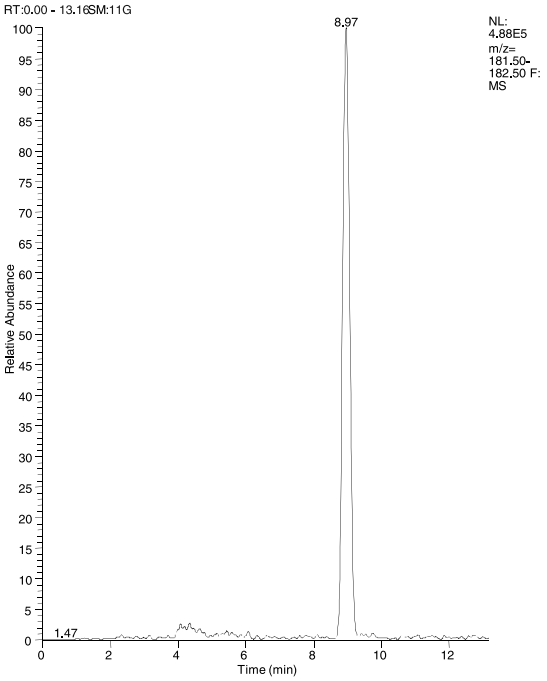

Figure S7. BPE obtained with CE-MS of L-tyrosine.

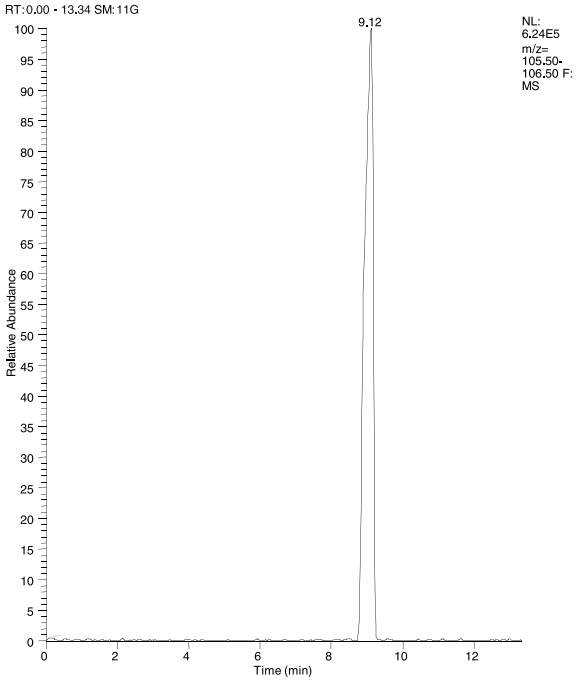

Figure S9. BPE obtained with CE-MS of L-serine.

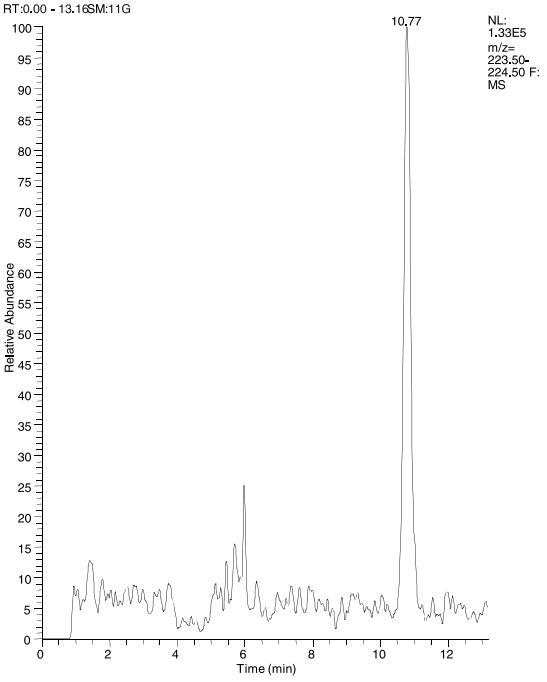

Figure S8. BPE obtained with CE-MS of L-acetyl-tyrosine.

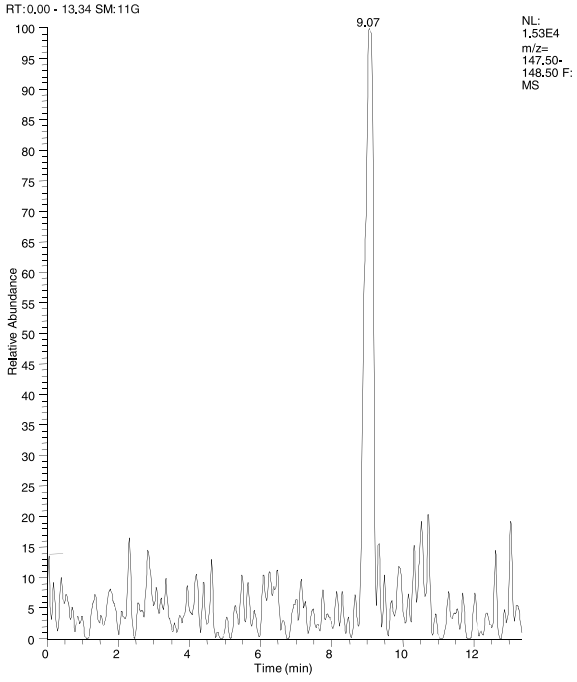

Figure S10. BPE obtained with CE-MS of L-acetyl-serine.

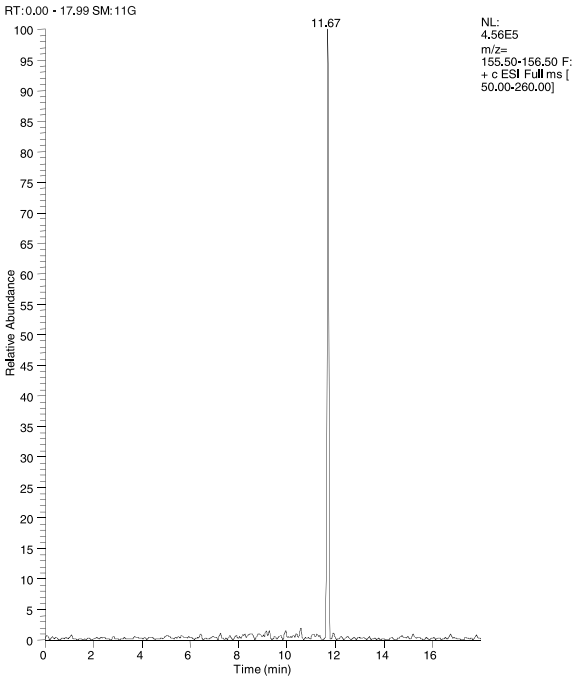

Figure S11. BPE obtained with CE-MS of L-histidine.

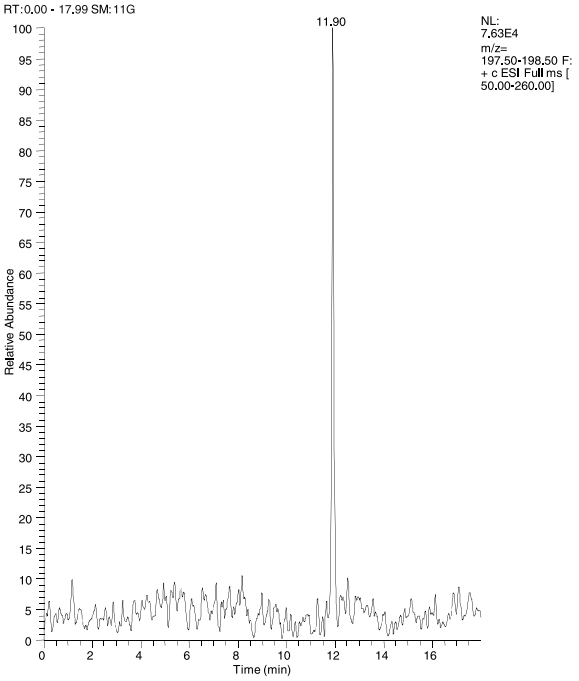

Figure S12. BPE obtained with CE-MS ofL-acetyl-histidine.

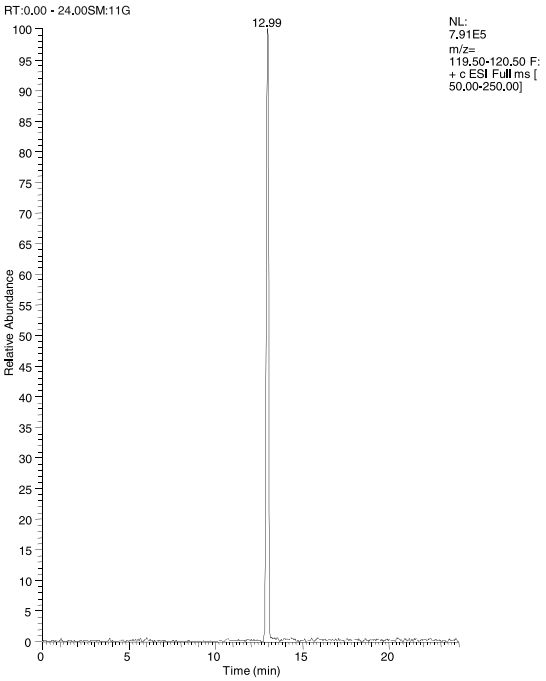

Figure S13. BPE obtained with CE-MS of L-threonine.

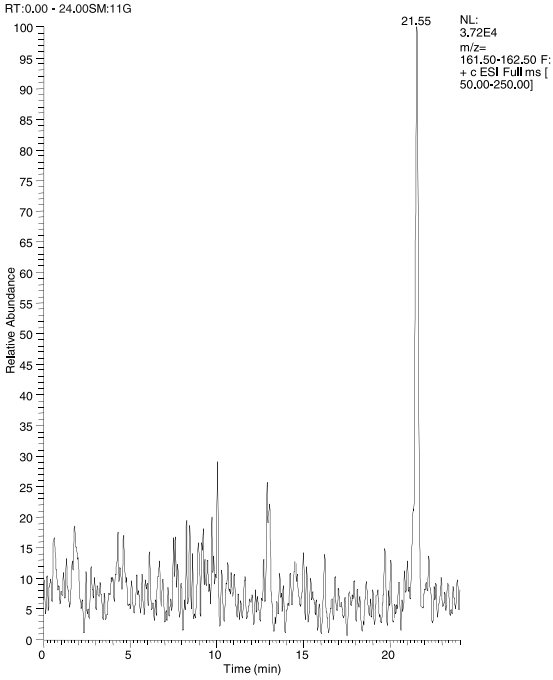

Figure S14. BPE obtained with CE-MS of L-acetyl-threonine.