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ORIGINAL PAPER

Determination of the β-Glucosidase Activity in Different Soils by Pre Capillary Enzyme Assay Using Capillary Electrophoresis with Laser-Induced Fluorescence Detection

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Abstract Enzyme activities can provide indication for quantitative changes in soil organic matter (SOM). It is known that the activities of most enzymes increase as native SOM content reflecting larger microbial communities and stabilization of enzymes on humic materials. β-Gucosidase (β-Glu) activities have been frequently used as indicators of changes in quantity and quality of SOM. In this study we propose a simple and very sensitive method, which has lower limit of detection compared with classic spectrophotometric method with the aim of determinate the β-Glu activity in soil samples using Fluorescein mono-β-D-glucopyranoside (FMGlc) as a substrate. The fluorescein released by the enzymatic reaction was quantified by capillary electrophoresis-laser induced fluorescence (CE-LIF) method. The background electrolyte (BGE) consisted in 40 mM phosphate buffer, pH 6. The LOD and LOQ for fluorescein were 1.3 10^{-7} mg mL^{-1} and 6.4 10^{-6} mg mL^{-1}, respectively. This work deals with the minimization of the mixture for the enzymatic reaction and with the optimization conditions of CE separation. To the best of our knowledge, this is the first time that an enzymatic activity was detected in soil using CE-LIF system.

Keywords Capillary electrophoresis · Laser induced fluorescence · β-glucosidase · Enzyme activity · Fluorescein

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Introduction

Intensive cultivation leads a rapid decline in organic matter and in nutrient levels, besides affecting physical properties of soil. Conversely, management practices with organic materials influence the agricultural sustainability by improving the physical, chemical and biological properties of soil. However, a better understanding of the nutrients cycle, and the factors that govern their decomposition in soil is imperative for the implementation of the sustainable practices. Soil microorganisms and enzymes are the primary mediators of soil biological processes, including organic matter degradation, mineralization and nutrient recycling. They play an important role in maintaining soil ecosystem quality and functional diversity [1, 2].

Enzymes activity are important indicators of soil quality because their strong connection with organic matter, physical properties and microbial activity; they respond earlier than other soil properties and they can be relatively simple to test [3]. Some enzyme activities can provide indication for quantitative changes in soil organic matter (SOM). It is known that the activities of most enzymes increase with the native SOM content increases, reflecting larger microbial communities and stabilization of enzymes on humic materials [4, 5]. Enzymes allow microbes to access energy and nutrients present in complex substrates and catalyze decomposition and nutrient mineralization as well as humification processes [6–9].

Structural polysaccharides include cellulose, xylane, chitin and polyphenol, while starch is the fundamental storage polysaccharide in plants. Once being incorporated into soil, these polysaccharides are hydrolyzed to oligosaccharides by polysaccharidases, e.g. xylanase for xylane and hemicellulose, and amylase for starch. They are further degraded to monosaccharides by heterosidases, i.e.

β-glucosidase (β-Glc) for cellobiose, invertase for sucrose, N-acetyl-b-D-glucosaminidase for chitooligosaccharides. Sugars with low molecular-weight are mineralized as energy sources by soil microbes. Accordingly, the activities of enzymes involved in the cycle of organic C are considered to be useful index [10, 11], or representing modification of microbial communities, because this community composition determines the potential for soil enzyme synthesis [12, 13]. Among hydrolytic enzymes, acid phosphatase and β-Glc activities have been frequently used as indicators of changes in quantity and quality of SOM [14].

β-Glucosidase activity has been found to be sensitive to soil management [15–20] and has been proposed as a soil quality indicator [21]. However, it needs to be recognized that the classic enzyme assays are often neither correlate with the microbial activity nor predict the nutrient availability to plants [22, 23]. However, this does not detract from using enzyme assays as an integrative index for detecting changes in soil quality due to soil management. As mentioned above many papers have shown it to be sensitive to various treatments relative to C inputs and disturbance. Research is needed on understanding what mechanisms control the sensitivity of this enzyme to management in order to develop it as a practical tool to guide sustainable soil management.

Capillary electrophoresis (CE) is applicable in the analysis of a wide range of compounds including natural products and plant metabolites [24]. Several analytical methods were used to determinate enzymatic activities in different biological samples [25–27]. Nowadays, the use of capillary electrophoresis (CE) in enzyme assays has advantages over conventional assays in terms of time of analysis, and sample size required. Also the ability to separate and quantify substrates and products, that are very similar in structure, could be easy to study using CE. Another advantage of CE is that organic buffers such as diethanolamine (DEA), which are compatible with bioanalytes, can be used. Moreover, the high resolution, short analysis time, low simple and reagent consumption make CE become a rapidly growing separation technique. It has become one of the most powerful tools for the analysis of a wide variety of species, including inorganic compounds, proteins, organic acids, amino acids, and neurotransmitters [28]. In spite of its powerful separation ability, CE faces the challenge of improving the detection sensitivity required by the small injection volume and as a consequence a small amount of reactive. So electrochemical [29], chemiluminescence (CL) [30] and laser induced fluorescence (LIF) detectors [31] stand out for their perfect sensitivity. Fluorescence spectrometry is one of the main detection methods of CE and is one of the most sensitive detection techniques in CE, which is capable of achieving the concentration detection limits below 10^{-13} M [32, 33]. Due to the advantages of high sensitivity, rapid resolution, high separation efficiency and small sample size, CE-LIF system has been demonstrated to be powerful for the determination of low-concentration of different compounds in several kinds of biological samples.

The aim of this work was to establish a selective, precise and accurate method to determine the β-Glc activity using Fluorescein mono-β-D-glucopyranoside (FMGlc) as a substrate and labeling reagent with CE-LIF in soil. This work deals with the minimization of the mixture for the enzymatic reaction and with the optimization conditions of CE separation. This assay was performed in order to reduce the amount of solvents required and also to diminish the generation of wastes, which is an important requisite in green analytical chemistry [34]. To the best of our knowledge, this is the first time to determine an enzymatic activity in soil using CE-LIF system.

Experimental

Instrumentation

A Beckman P/ACE MDQ instrument (Beckman Instruments, Fullerton, CA, USA) equipped with a LIF detector. The excitation light from an argon ion laser (3 mW) was focused on the capillary window by means of a fiber-optic connection. Excitation was performed at 488 nm and the electropherograms were recorded by monitoring the emission intensity at 520 nm. The data handling system comprising an IBM PC and P/ACE System MDQ Software (ESANCO) was used. The fused silica capillaries were obtained from MicroSolv Technology Corporation and had the following dimensions: 47 cm total length, 40 cm effective length, 75 μm ID, and 375 μm OD. The temperature of the capillary and the samples were maintained at 25 °C.

Reagents and solutions

All reagents used, were of analytical reagent grade. FMGlc (Fig. 1) was purchased from Sigma Chemical Co. (St. Louis, MO) and fluorescein was from Merck (Buenos Aires, Argentina). The disodium hydrogen phosphate dehydrate and sodium dihydrogen phosphate salts were perchance from Merk (Darmstadt, Germany).

All the solutions were degassed by ultrasonication (Testlab, Argentina). The water used in all studies was ultrapure water (18 MΩ cm) obtained from a Barnstead Easy pure RF compact ultrapure water system. Running electrolytes and samples were filtered through a 0.45 μm Titan Syringe filters (Sri Inc., Eaton Town, NJ, USA).

Soil samples

The samples were obtained from the upper horizon (0–15 cm) of four Entisols soils of San Luis and two Mollisols soils of Santa Fé. The samples were from Argentinean soils used for agricultural activities. The coordinates of the obtained samples were soil 1 (33°19′ S, 66°20′ O), soil 2 (33°74′ S, 65°55′ O), soil 3 (32°32′ S, 65°14′ O), soil 4 (34°06′ S, 66°44′ O), soil 5 ((32°53′ S, 60°56′ O), soil 6 (31°37′ S, 61°01′ O).

The moist soil sample was sieved (≤2 mm) after removing the plant material and roots. Soil samples were kept at 4 °C in plastic bags for a few days to stabilize the microbiological activity disturbed during soil sampling, handling. The analyses were achieved within 2 weeks after the sample collection. The physical and chemical characteristics of the soil are given in Table 1.

Enzymatic assay

The development of the β-Glc assay 0.1 g of soil was added in a 1.5 ml ependorf tube with 1 mL of a solution of phosphate buffer 40 mM (pH 6) contain different concentrations of FMGlc for the enzymatic assay. The flask was placed in a shaker at 37 °C. After 1 h, 500 µl of 0.5 M sodium hydroxide was added. Immediately after the flask containing the mixture was swirled for a few seconds, and centrifuged at 3,000 rpm for 5 min. A 500 µl aliquot of the supernatant phase was filtered through 0.45 µm Titan Syringe filters (Sri inc., Eaton Town, NJ. USA) and was transferred into the CE sample vial.

Separation development

Different concentration of BGEs were tested (20–60 mM), but the one producing the best results considering selectivity, reproducibility, baseline and current performance, was sodium phosphate 40 mM. Increases in migration times as well as current were observed when the concentration of buffer increased.

The separations parameters were as follows: the capillary temperature was maintained at 25 °C and the voltage was set at 20 kV. Samples were pressure-injected at the cathodic side at 0.5 psi for 5 s. To assure a good reproducibility, the capillary was rinsed sequentially after all running with sodium hydroxide for 1 min., followed by water for 1 min., and then equilibrated with the running buffer (40 mM phosphate buffer, pH 6) for 1 min.

The new capillary was preconditioned prior to use by rinsing sequentially with 0.1 M NaOH for 15 min, distilled water for 2 min, running buffer for 15 min and, finally, equilibrated at 20 kV with running buffer for 20 min.

β-Glucosidase activity measurements by classical methods

Soil β-Gluc activity was determined using *p*-nitrophenyl-β-D-glucopyranoside (pNPG) as an analogue substrate [35]. Briefly, we put 0.1 g of each soil sample (<2 mm) in a 1.5 ml Ependorf flask, added 1 ml of THAM solution (Tris-hydroxymethyl-aminomethane, with citric, maleic and boric acids), buffer (pH 6.5 for acid phosphatise assay or pH 11 for alkaline phosphatase assay) and 0.25 ml of *p*-NPP solution prepared in the same buffer, and swirled the flask for a few seconds to mix the contents. After stoppering the flask, we placed it in an incubator at 37 °C. After 1 h of incubation, we removed the stopper, added 0.1 ml of 0.5 M $CaCl_2$ and 0.4 ml of 0.5 M NaOH, and swirled the flask for a few seconds to stop the reaction. Then, we filtered (0.45 mm HA nitrocellulose, Millipore) the soil suspension in order to prevent the interference of possible precipitates. The fading of the intensity of yellow colour in the calibration standards, samples and controls was measured with a spectrophotometer at 405 nm against the reagent blank. We calculated the *p*-nitrophenol content by referring to a calibration curve.

Results

Determination of the enzymatic product by CE-LIF

The first step in the development of a separation based enzyme assay for monitoring β-Glc activity was the development of a suitable CE separation method. The choice of buffer for the separation of analytes by CE is very important. Different concentrations of sodium phosphate were studied and we observed optimal results with concentrations of a 40 mM of sodium phosphate. Increases

Table 1 Physical and chemical characteristics of soils

Characteristic	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6
pH	8.1	8.0	8.3	6.9	5.3	6.0
Clay (%)	5.3	6.5	8.2	9.0	29.2	35.0
Sand (%)	80	75	63	64	20	13.4
Organic matter (%)	0.63	0.84	0.97	0.59	1.63	2.07
Total N mg kg^{-1}	842	1070	1208	798	206	317

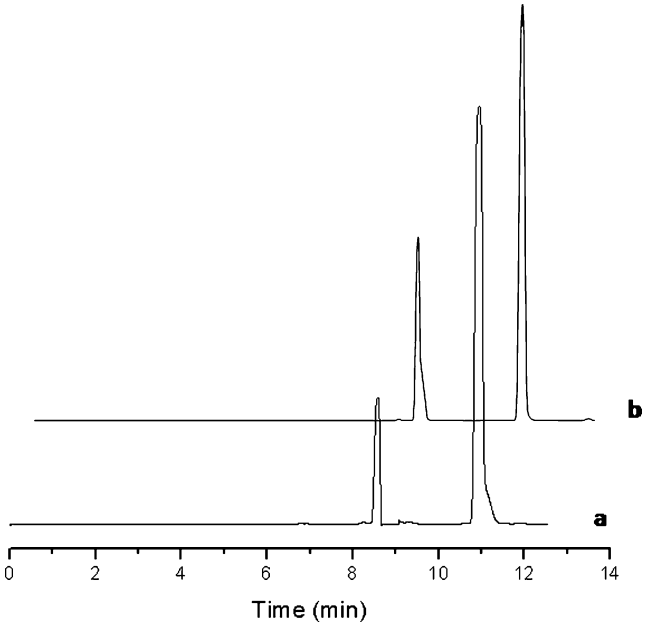

Fig. 1 Electropherogram of the FMGlc and fluorescein for **a** β-glucosidase activity at 30 min with 3 μM FMGlc; **b** β-glucosidase activity at 60 min with 3 μM FMGlc; Conditions: 40 mM phosphate buffer (pH 6); capillary, 47 cm full length, 40 cm effective length, 75 μm ID, 375 μm OD; hydrodynamic injection at 0.5 psi, 5 s; 20 kV constant voltage; Excitation was performed at 488 nm and the electropherograms were recorded by monitoring the emission intensity at 520 nm

in migration times as well as current were observed when the concentration of buffer increased. A high resolution was obtained with increased buffer concentrations. However, we didn't observe appreciable improvements over the separation with concentrations above 40 mM.

The effect of the pH was investigated within the range of 5.00–8.00 at a fixed buffer concentration, adjusted by 0.1M NaOH and 0.1M HCl. It was found that the resolution decreased as the pH increased, while the time analysis decreased. The separation was achieved at pH 6.

Figure 2 shows the electropherograms of fluorescein and FMGlc standards using the optimized experimental conditions. The migration time of fluorescein and FMGlc were found at 8.65 min and 10.96 min, respectively.

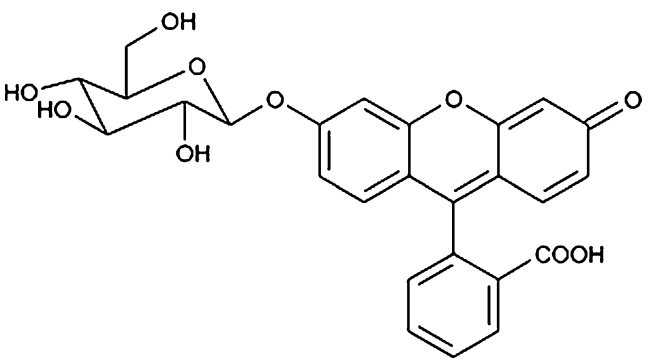

Fig. 2 Structure of Fluorescein mono-β-D-glucopyranoside (FMGlc)

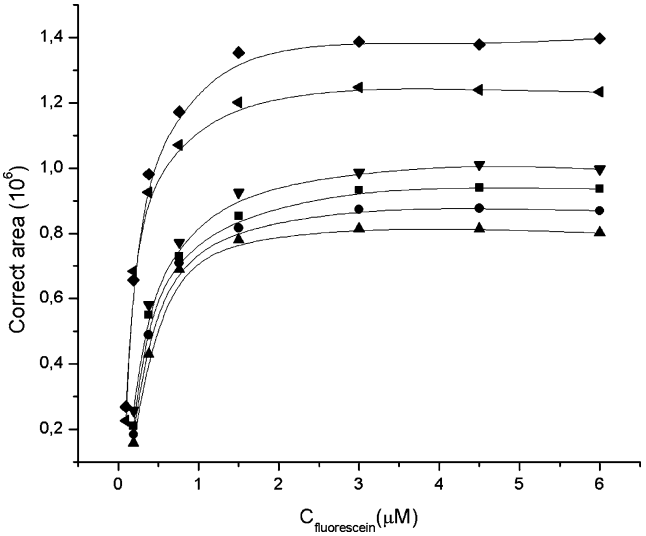

Fig. 3 Enzymatic activities in relation to the increase of the substrate concentration (FMGlc). (n=6 for each analysis). The β-Gluc activity in phosphate buffer (pH 6). Soil 1 (■); Soil 2 (●); Soil 3 (▲); Soil 4 (▼); Soil 5 (◄); Soil 6 (♦)

Identification of the fluorescein and FMGlc were performed by comparison of the migration times obtained in actual samples with those of the standard solutions. Furthermore, spiking experiments (standard addition method) were performed to confirm the peak identity. The repeatability of the separation system was evaluated by replicated analysis of solution (2 10^{-5} mg mL^{-1}) of fluorescein; the relative standard deviations of migration time were 0.98% (n=6, intra-day) and 2.75% (n=15, inter-day).

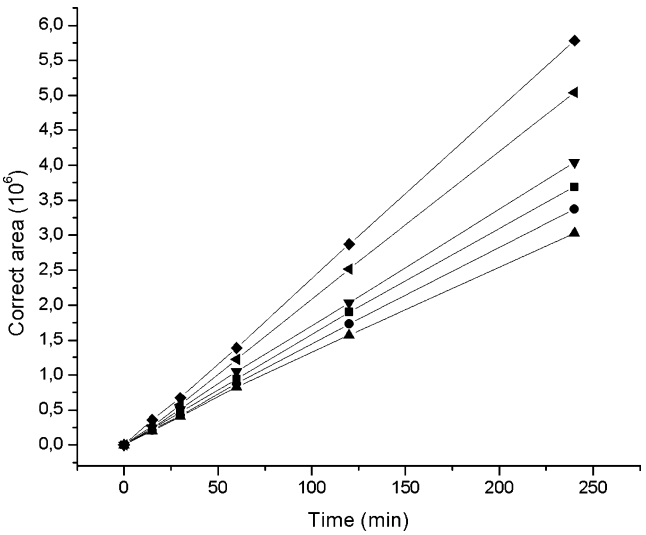

Fig. 4 Effect of time of incubation concerning with the release of fluorescein in assay of soil β-glucosidase activities by described method. Soil 1 (■); Soil 2 (●); Soil 3 (▲); Soil 4 (▼); Soil 5 (◄); Soil 6 (♦)

Table 2 Results obtained from the comparison between different amounts of soil using the proposed method. Conditions: 3 μM of FMGlc, incubation of 1 h at 37 °C

Soil nº	β-Glucosidase	
	0.1[a] ± SD	1[a] ± SD
1	0.110[b]±0.003	0.108[b]±0.008
2	0.131[b]±0.002	0.129[b]±0.007
3	0.134[b]±0.003	0.136[b]±0.006
4	0.115[b]±0.0027	0.116[b]±0.006
5	0.174[b]±0.0035	0.172[b]±0.009
6	0.196[b]±0.0041	0.193[b]±0.008

[a] Amount of soil in gram

[b] μmol of fluorescein g^{-1} soil h^{-1}

Study of the enzymatic process

Reactions catalyzed by enzymes have long been used for analytical purposes in the determination of different analytes such as substrates, inhibitors, and also the activity of the enzymes [36].

Temperature, pH, and substrate concentration influence the enzymatic reactions rates [37]. In order to study the activity of β-Glc, the substrate must be present in excess amount. Thus, the reaction rate must be independent of the substrate concentration. For this reason, the first step in this study was the determination of the concentration at which the enzyme activity was not modified due to the substrate concentration. Thus, increasing concentration of FMGlc was studied in the range of 0.1-6 μM. Figure 3 shows the curves of the enzymatic activity of the different soils in response to the increasing substrate concentrations. As a result, we decided to perform the following studies with 3 μM of substrate concentration.

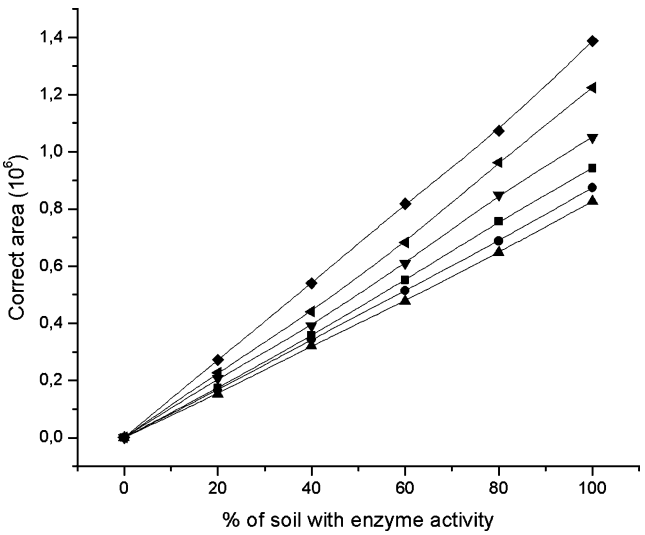

Fig. 5 Method of the standard addition for the enzymes. Soil 1 (■); Soil 2 (●); Soil 3 (▲); Soil 4 (▼); Soil 5 (◄); Soil 6 (♦)

Table 3 The values for the activity of β-Glucosidase in soil

Soil nº	[a]β-Glucosidase	
	Mean ± SD	CV%
1	0.111 ± 0.003	2.7
2	0.132 ± 0.002	1.5
3	0.134±0.003	2.2
4	0.115±0.0027	2.3
5	0.174±0.0035	2.0
6	0.196±0.0041	2.1

[a] μmol fluorescein g^{-1} soil h^{-1}

Afterwards, the reaction time was set up. With this purpose six reaction mixture were incubated at different times: 15–240 min. Figure 4 shows the linearity relationship between time of incubation and the amount of fluorescein released for the six studied soils. Thus, we could demonstrate that this method is not affected by microbial growth or assimilation of enzymatic reaction products by soil micro-organisms [38–40].

With the aim of quantifying the fluoresein freed, a calibration curve was obtained by plotting the corrected peak area (Y), versus the corresponding concentrations of the fluoresein (C; μM). By linear regression analysis the following equation was obtained: Peak Area=99229+5.7 $10^7 \times C_{fluorescein}$; R^2=0,999. The sensitivity data calculated as LOD and LOQ were 1.3 10^{-8} mg mL^{-1} and 6.4 10^{-7} mg mL^{-1} (CV=2.7%), respectively.

The enzymatic activity values for both enzymes were obtained using the equation:

$$EA = \frac{2(A - a)}{b\,T\,W}$$

Table 4 Comparison of the result obtained for Soil 1 between proposed and classic method (n=6)

Active soil (%)[a]	β-Glu	
	Proposed method[b]	Classical method[c]
0.1	1.68 10^{-4}	–
1	1.8 10^{-3}	–
10	0.016	–
50	0.058	0.045
100	0.111	0.083

[a] all the assays were performed with 0.1 g of soil

[b] μmol fluorescein g^{-1} soil h^{-1}

[c] μmol p-nitrophenol g^{-1} soil h^{-1}

EA: enzymatic activity (μmol of free fluorescein)/(soil g x digestion time(h)), A: corrected peak area, a: interception fluorescein calibration curve, b: slope fluorescein calibration curve, T: incubation time (h), W: dry soil mass (g).

In order to improve the quantification of β-Glc activity assays, the amount of soil (0.1 g) used in this study was minimized compared with the amount of soil usually used in other spectrophotometric systems (1 g) (Table 2). The assay was performed with the same six soils samples (n=6) used in this study. The results obtained were reasonably close, indicating a good correspondence between the values in both amounts of tried soils (Table 2). The possibility to work with less amount of soil is a very important advantage due to the fact that the reduction of waste generation, which is a basic requisite in green analytical chemistry [25].

As a certified value for β-Glc activity applied to determine the quality of soil, in which fluorescein is the substrate does not exist, and the most used method for determine the activity of this enzyme use *p*-nitrophenyl-β-D-glucopyranoside as substrate, the method of standard addition could be considered to evaluate the accuracy of the proposed method. The development of this assay consisted in warm up a portion of the soil at 200 °C during 24 h. Thought this procedure a soil without enzyme activity or microorganism able to synthesize the enzyme was obtained. Afterwards, increasing amount of non-heated soil with normal enzyme activity was added (0–100%). To the different mixtures of soil we perform the enzymatic determination based on our protocol (described above). The Fig. 5 shows the results obtained for this assay.

The values of the activities of β-Glc in semiarid soil are showed in Table 3. The high precision of the proposed method is probably due to the fact that the used technique, for the determination of the freed fluorescein, was quantitative and the entire assay procedure was simple and readily standardized.

With the purpose of determinate the sensitive of our method we developed an assay in which we compared the proposed method with the classical method proposed by Eivazi and Tabatabai [35]. Table 4 shows the results of this comparison. The percentages in the table correspond to the amount of active soil. The rest of soil added to complete the 0.1 g of soil was heated at 200 °C during a day. This process produces a total destruction of the biological reactions.

Conclusions

The results of the present study clearly demonstrate the potentiality and versatility of CE-LIF method, which could be applied to the routine monitoring of soil enzymes activity. The proposed CE-LIF method appears to be suitable for the rapid and sensitive determination of enzymatic activity in soil samples. The optimum separation was obtained using 40 mM phosphate buffer (pH 6). The detection limit and the linear range of detection for fluorescein were 1.3 10^{-7} mg mL^{-1} and 6.4 10^{-6}–0.01 mg mL^{-1}, respectively. These results demonstrate the simplicity, rapidity, low cost, high sensitivity and good specificityof this method, also this approach displays great potential for the trace determination of β-glucosidase activity soils with low amount of organic matter. Moreover, the results of the present study clearly demonstrate the potentiality and versatility of CE-LIF method, which could be applied to the routine monitoring of soil enzymes activity. The most important advantage of this method is that allows the use of labeled natural substrate in the enzymatic assay.

The use of nano-procedures like capillary electrophoresis has contributed to achieving greener analytical methods not only by automatization and miniaturization, but also by replacing toxic reagents by non-contaminating reagents. In this sense, CE has the advantages of minimizing both reagent consumption and waste generation.

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