CHARACTERIZATION OF ELECTROCHEMICAL PROPERTIES IN FRACTIONS OF DIFFERENT MOLECULAR SIZE TAKEN FROM NATURAL SOIL ORGANIC MATTER OF ARGENTINE HUMID PAMPA

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Abstract

Soil organic matter (SOM) is mainly formed by humic substances (HSs), which exhibit a highly complex structure. In order to establish causality relationships in redox interactions between HSs-microorganisms or HSs-soil abiotic factors, the characterization of HSs electroactive behavior needs to be researched in depth.

To study the electrochemical characteristics of the molecular assemblies found in humic complexes, the components of HSs must be isolated and identified avoiding the use of drastic methods of separation and characterization. For these investigations a mild extractant, as ethylenediaminetetracetic acid (EDTA), is preferred, even it is less effective than alkali hydroxides in removing SOM. The objectives of this work were to fractionate the HSs compounds extracted with EDTA pH=7 with a molecular sieve (Sephadex G25) and to determine the significance and location in the electrochemical series of the electroactive pairs that make up the most important HSs fractions of these extracts. Eluates of the centrifuged Mollisol soil – EDTA pH=7 extracts were subjected to spectrophotometric scanning (220 and 415 nm) and the amount of electroactive compounds was determined in the fractions with higher absorbance (called *f I* and *f II*) through Differential Pulse Polarography (DPP) within a +300 to –900 mV range of potentials. Results showed that *f I* (excluded fraction which correspond to a molecular weight range assigned of humic acids) had a total concentration of electroactive compounds 10% lower than *f II* (that showed a molecular weight range assigned of fulvic acid). However, *f I* had a higher amount of electroactive compounds concentration at lower potentials (-200 to -900 mV) and a lower concentration of electroactive compounds at higher potentials (-200 to +300 mV) than *f II*. The results also showed that the value of *reducing status* in *f II* was lower (about 23%) than *f I*.

Our results raise the possibility that in front of biotic or abiotic redox effectors may exists electron exchanges between fractions of the HSs in accordance with the general concept that the energetically most favorable available electron acceptors are utilized first. The knowledge of the basic electrochemical nature of the HSs fractions of the studied Mollisol soil needs to be considered in future studies of the impact of many

technological practices in our region, for instance, soil inoculation with microorganisms, soil fertilization practices or soil amendments with organic or inorganic compounds.

Keywords: natural soils, humic substances, electrochemistry, polarography.

Resumen

La materia orgánica del suelo (MOS) está principalmente constituida por sustancias húmicas (SHs), las cuales poseen una estructura sumamente compleja. Para establecer relaciones de causalidad en las interacciones redox entre SHs – microorganismos o SHs – factores abióticos, la caracterización del comportamiento electroactivo de las SHs debe investigarse en profundidad. Para estudiar las características electroquímicas de los ensamblajes moleculares que conforman los complejos húmicos, los componentes de las SHs deben ser aislados e identificados evitando el uso de métodos agresivos de separación y caracterización. Para estas investigaciones un extractante suave, como el ácido etilendiaminotetra acético (EDTA), es óptimo, aunque es menos efectivo que los hidróxidos alcalinos para remover MOS. Los objetivos de este trabajo fueron: fraccionar los compuestos de las SHs extraídos con EDTA pH=7 con tamizado molecular (Sephadex G25), y determinar la significación y ubicación en la serie electroquímica de los pares electroactivos que componen las fracciones más importantes de estos extractos. Eluidos de los sobrenadantes de extractos centrifugados de un suelo Molisol con EDTA pH=7, fueron sometidos a barridos espectrofotométricos (220 y 415 nm) y en las fracciones que presentaron mayor absorbancia (denominadas *f I* y *f II*) se determinó la cantidad de compuestos electroactivos mediante Polarografía de Pulsos Diferenciales (DPP) en un rango de potenciales comprendido entre +300 y –900 mV.

Los resultados mostraron que *f I* (que es la fracción excluida, la cual corresponde a pesos moleculares que se ubican en el rango de los asignados a los ácidos húmicos) presentó una concentración de compuestos electroactivos 10% menor que *f II* (que presentó un rango de pesos moleculares dentro de los asignados a los ácidos fúlvicos).

Sin embargo, *f I* respecto a *f II*, presentó una concentración mayor de compuestos electroactivos con bajos potenciales (-200 a -900 mV) y una concentración menor con potenciales más elevados (-200 a +300 mV). Los resultados también mostraron que el valor del *status reductor* en *f II* fue menor (alrededor del 23%) que *f I*.

Nuestros resultados plantean la posibilidad de que frente a efectores redox bióticos o abióticos puedan existir intercambios de electrones entre fracciones de SHs de acuerdo con el concepto general de que los aceptores de electrones disponibles energéticamente más favorables son los primeros utilizados. El conocimiento sobre la naturaleza electroquímica básica de fracciones de las SHs en el suelo Molisol estudiado, debe ser tenido en cuenta en estudios futuros sobre el impacto de muchas prácticas tecnológicas en nuestra región, por ejemplo, la inoculación de suelo con microorganismos, las prácticas de fertilización del suelo o el aporte de enmiendas orgánicas o inorgánicas.

Palabras clave: suelos naturales, sustancias húmicas, eletroquímica, polarografía.

Introduction

Soil organic matter (SOM) contributes significantly to the planet's great activated carbon reserve. SOM is mainly formed by humic substances (HSs), which have a highly complex structure. HSs are formed by humic acids, fulvic acids and humin [1]. Traditionally, HSs were considered macromolecular polymers in which

humic precursors (lignine units) were linked by covalent bonds. According to recent studies, HSs appear rather as supramolecular assemblies or associations of heterogeneous and relatively small molecules linked by weak bonds, thus apparently constituting structures of great molecular size [2].

In the past, studies of the chemical characterization of HSs favored methods based generally on chemical fractionation and the degradation of their constituent compounds by means of highly aggressive reagents working under extreme pH conditions. Under these conditions, the products identified often differ significantly from the components provided by parental molecules [3, 4]. The fact that HSs are currently considered complex mixtures of small or midsize molecules further complicates the characterization problem given that the study of mixtures necessarily involves the isolating and identifying of their constituents. Under these conditions, the use of drastic treatments must be thoroughly revised.

On the other hand, redox activity is one of the most important and least known properties of natural organic matter [5, 6]. However, it is well known that fenolic compounds –prominent constituents of humic structures– are active redox effectors in natural environments [7, 8].

Several authors have proven that certain microorganisms are capable of using HSs as electron acceptors in organic compound and hydrogen anaerobic oxidation [9-11]. This electronic transport produces energy and enables microorganism growth. On the other hand, the possibility of microorganisms using reduced HSs as eletron donors for the reduction of electron acceptors with higher positive redox potentials has also been demonstrated [12]. The fact that microorganisms can reduce or oxidize HSs is highly important in the general oxidation-reduction mechanisms driven by microorganisms (by means of which, for instance, they can influence the speciation of inorganic compounds used as fertilizers or in bioremediation processes) [13].

In order to establish causality relations in HSs - microorganisms or HSs - abiotic factors bilateral redox interactions, the characterization of HSs electroactive behavior needs to be researched in depth to a level which may enable us to determine the participation of various molecular components in the humic complex electroactive functioning.

It has been recently proven that the additional use of a “soft” extraction method with ethylenediaminetetracetic acid (EDTA) at pH=7 together with Differential Pulse Polarography (DPP) may be simple and ease operation that can be used to improve the knowledge of the electroactive fractions in SOM, in SOM extracts or in sediments [14].

This combination of techniques allows us to study the changes produced in the redox capacity of the extracted fractions of natural organic matter through the intervention of abiotic factors (incorporation of reduced carbonaceous substances, p_{O2} variation) as well as of biotic factors (soil inoculation with a redox effector microorganism) [14-16].

The objectives of this work were to fractionate the HSs compounds extracted with EDTA pH=7 with a molecular sieve (Sephadex G25) and to determine the significance and location in the electrochemical series of the electroactive pairs that make up the most important HSs fractions of these extract.

Materials and Methods

Soil

Soil aggregates were obtained from 5-20 cm layer of a Vertic Mollisol located in Larguía, Provincia de Santa Fe, Argentina (“Humid Pampa” region). The concentration of total organic matter was determined by the dichromate – acid method [17] and total

N were measured using a Tecator Automatic Kjeldahl system. Soil pH was determined at a 1:1 (w/v) soil-to-solution ratio with distilled water. The main soil characteristics were as follows: total organic matter, 37 g kg^{-1}; total N, 2.3 g kg^{-1}; pH 6.1; CEC 21.7 $cmol_c$ kg^{-1}. Nitrate content was usually lower than 10 g kg^{-1}. Soil sample was sieved to a particle size between 2000 and 4000 μm (sieve Number 5 and 10 – IRAM). The mean diameter of the soil aggregates was 2350±14 μm.

Fractionation and Separation of HSs

A 0.05-M EDTA pH=7 solution was used as a dispersing and complexing agent for organic matter, in order to free HSs from associated metallic electroactive compounds [14, 18]. The following additional treatments were carried out: EDTA pH=7 extract from whole soil (A); EDTA pH=7 extract from calcined soil (B); aqueous extract from whole soil (C); aqueous extract from calcined soil (D); control sample without soil (EDTA pH=7 solutions) (EDTA). In the case of extracts with soil, 0.4 g from soil aggregates and 3.5 ml from the EDTA pH=7 solution (treatments A and B) or 3.5 ml of deoxygenated distilled water (treatments C and D) were homogenized for 1 hr in a vertical shaker, and centrifuged at 3,000 *g* for 10 minutes. 0.2 ml of supernatant was eluted on a Sephadex G25 column (1x50 cm) (separation range: 1,000 to 5,000 Da) (Pharmacia®) kept under constant temperature conditions (20ºC). Gel calibration was carried out using Blue Dextran (M.W: 2.10^6 Da; CAS N° 115-39-9) and Bromophenol Blue (M.W: 669.97 Da; CAS N° 87915-38-6), according to Cooper [19]. In the case of control samples without soil, 0.2 ml EDTA pH=7 solution was eluted. Elutions were carried out with distilled water [20] at a rate of 16.8 ml/hour. Fractionations were made in triplicate.

Eluates (2.8 ml) were collected in 24 tubes by means of a fraction collector (Roucaire, Retriever II) and underwent spectrophotometric scanning (220 to 415 nm) (Hitachi, U - 1500) to determine the wavelength corresponding to maximum absorbance. Eluates were subsequently grouped into final volumes of 5.6 ml to determine electrical conductivity (Antares III, Instrumental Parsec SA).

Determination of electroactive compounds

The quantity of electroactive compounds was measured in the fractions obtained from EDTA pH=7 extract from whole soil, which showed significant optical activity after separation with Sephadex G25 gel. 200 μl of each fraction was diluted to 5 ml with deoxygenated distilled water and introduced into the polarographic cell under O_2-free N_2 atmosphere. Differential Pulse Polarography technique (DPP) was used to perform the polarographic analysis. The fractions underwent polarographic scanning in a POL 150 polarographic analyzer (Radiometer Analytical). The polarographic equipment was fitted with a microcell (5-10 ml capacity), a mercury-drop electrode (HMDE-EK 290), a Pt electrode (XM100) and a calomel electrode (SCE-XR 150). All potentials were measured against the SCE. The working conditions of the polarographic equipment were as follows: scanning time: 0.2 s, amplitude of scanning: 5mV; amplitude of pulse (*E*): 25 mV; pulse length: 20 ns. The maximum and minimum current range was 1 mA and 100 nA, respectively. The scanned potential range was +300 to –900 mV. The polarographic study was made in triplicate on samples obtained in two fractionations. Each polarographic scanning was repeated five times on the same sample [21], these values being averaged using TraceMaster 5 software (Radiometer Analytical).

Reducing status calculation

The *reducing status* is the summation of the products of the reduction potential (intensity factor) (E; Volts) and amount of electrochemical compounds (capacity factor) of the linked redox pairs present in a given system [22]. Reducing capacity would be estimated by determining the concentration of the reduced species. Since the quotient nA/mV obtained by TraceMaster 5 software (Radiometer Analytical) is proportional to the concentration, the reducing status could be represented by:

$$\text{Reducing status} = \sum_{i=1}^{n\,(pair)} E_i\,(mV) \times (\,nA/mV)_i$$

where, E_i is the potential found for each redox pair detected when differential pulse polarograms were performed between +300 to –900 mV.

Statistical Analyses

The Student *t* test [23] was used to determine the significance of differences among means.

Results and Discussion

EDTA Extract Fractionation

Figure 1 shows the results of extracts fractionation with Sephadex G25.

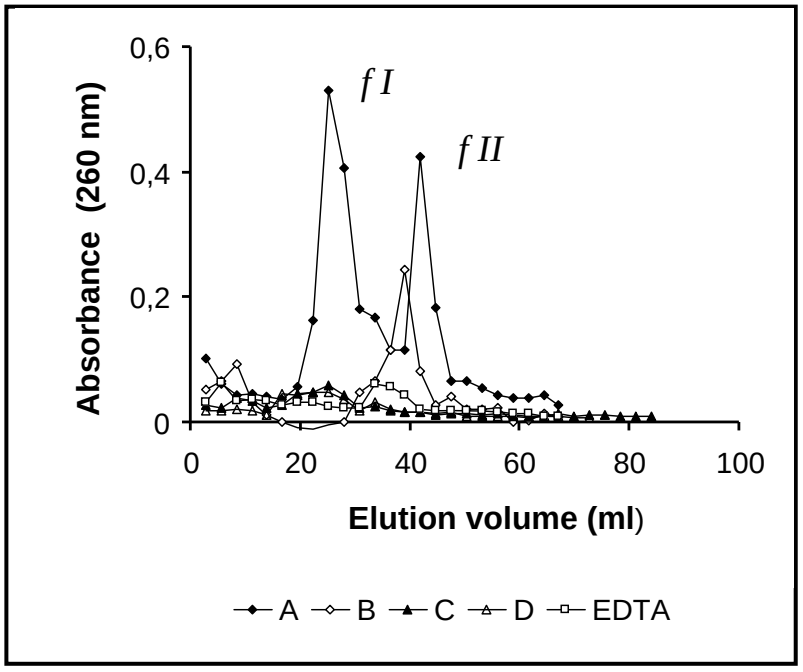

Figure 1 Fractionation of HSs extracts in Sephadex G25. *f I* and *f II* correspond to both fractions obtained with EDTA pH=7 extract from whole Vertic Mollisol extract (treatment A). Extracts were eluted with deoxygenated distilled H_2O, absorbances corresponding to 260 nm. (A) EDTA pH=7 extract from whole soil; (B) EDTA pH=7 extract from calcined soil; (C) aqueous extract from whole soil; (D) aqueous extract from calcined soil; (EDTA) control sample without soil (EDTA pH=7 solution). For further information see *Materials and Methods.*

In EDTA extract from whole soil (A), two greater fractions (maximum absorption at 260 nm) are clearly observed. Upon considering elution volumes in relation to the behavior of the calibration patterns used [19, 20], fraction *I* (*f I*) corresponds to the excluded fraction (size > 5,000 Da) whereas fraction *II* (*f II*) corresponds to a molecular size of about 1,000 Da.

These results may suggest that *f I* (excluded fraction) corresponds to a molecular weight range of the molecular assemblies that constitute humic acids (the average range in the literature is of the order of 50,000 to 100,000 Da) and *f II*, to fulvic acids (the accepted average range of molecular weight of fulvic acid is of the order of 500 to 2000 Da) [1, 24].

The behavior of EDTA extract from calcined soil (B) showed that whereas *f I* was mostly constituted by organic compounds, *f II* could be considered to be constituted by organic compound strongly bonded to inorganic compounds. Fractions obtained from aqueous extracts (C and D) showed low absorbance values at 260 nm, suggesting that little amount of humic substance can be extracted from soil clay humic complexes using this extraction method. Nevertheless, as suggested by some authors, even if the cromatrografic gels have a low tendency to bond irreversibly with biological compounds [25], the possibility of this bonding taking place cannot be dismissed. Taking this into account, humic substances that may have been present in the aqueous extract could have been retained by the gel [24].

The behavior of control extract (EDTA) indicates that EDTA pH=7 did not interfere significantly in fractionation with Sephadex G25.

Figure 2 indicates that the fractions corresponding to the smallest molecular size showed significant conductivity values compared with the excluded fraction (*f I*).

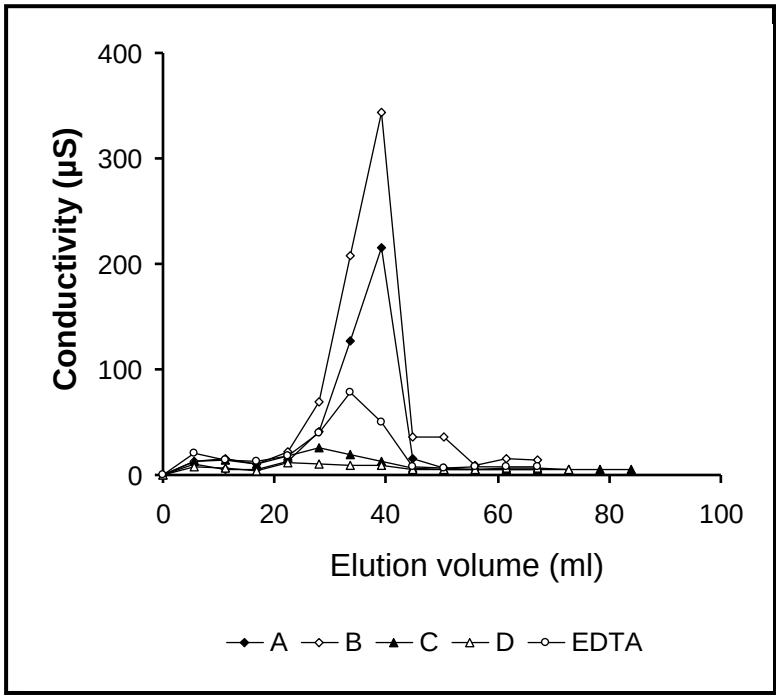

Figure 2. Conductivity values corresponding to the eluates obtained after fractionation in Sephadex G25 for the various HSs extracts. (A) EDTA pH=7 extract from whole soil; (B) EDTA pH=7 extract from calcined soil; (C) aqueous extract from whole soil; (D) aqueous extract from calcined soil; (EDTA) control sample without soil (EDTA pH=7 solution). For further information see *Materials and Methods.*

The fact that the EDTA extract from calcined soil (B) showed high absorbance values suggest that the HSs contained in this fraction would be blocking a sizeable part of the inorganic compounds. On the other hand, the fact that aqueous extracts (C and D) hardly showed any conductivity suggest that, as mentioned before in respect to absorbance results, no significant amount of constituents of HSs of the studied soil are solubilized in an aqueous medium. The figure 2 also indicates that EDTA pH=7 extracting solution helped to increase the conductivity values observed in *f II*.

Distribution of Electroactive Compounds in HS Fractions

Figure 3 shows differential pulse polarograms and the amounts of electroactive compounds found within the range of +300 to –900 mV, obtained with the eluates from *f I* and *f II*, corresponding to the HS fractions showing higher absorbance values at 260 nm (figure 1). The polarograms are well defined and clearly show the different composition between *f I* and *f II* as regards the type of electroactive compounds. The table insert in figure 3 shows the amplitude of the derivative curve (expressed in current units/ potential units; nA/mV) for the peaks identified in both curves in figure 3. These values, which are directly related to electroactive compound concentration, show that *f I* exhibit a total concentration of electroactive compounds 10% lower than *f II* ($p<0.05$).

Peack		E found (mV)	Amount of electroactive compounds (nA/mV)
f I	1	-825	0.396
	2	-785	0.219
	3	-745	0.174
	4	-700	0.266
	5	-335	0.222
	6	-135	0.041
	7	-45	0.113
	8	55	0.026
	9	155	0.247
f II	**1**	-560	0.050
	2	-375	0.499
	3	-190	0.340
	4	75	0.944
	5	150	0.082

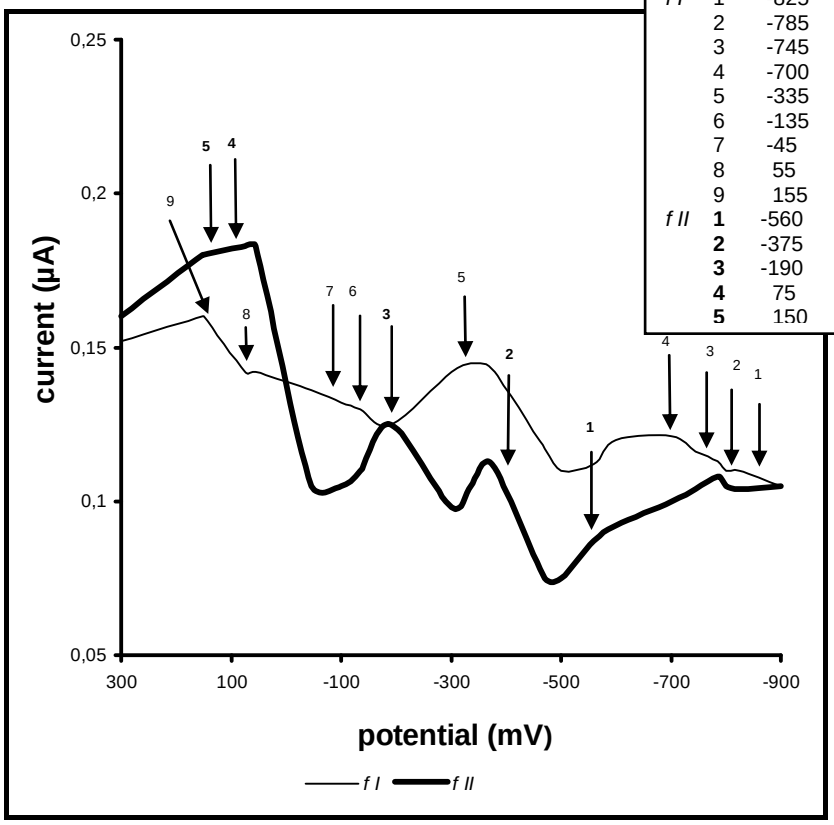

Figure 3. Plot of DPP current (µA) versus potential (mV) for *f I* and *f II* fractions obtained with EDTA pH=7 extract from whole Vertic Mollisol extract, which exhibited maximum absorbance at 260 nm after fractionation with Sephadex G25. The arrows shows the typical point (peak potential) observed in each polarograms. Each typical point was identified with a number of corresponding fractions. The table inserts in figure shows the amount of electroactive compound corresponding to the peak displayed on the curve. For other explanations, see *Materials and Methods*.

However, their distribution in the range of the potentials studied (+300 to –900 mV) was different. *f I* exhibited high electroactive compound concentrations at low potential values (-200 to –900) and low concentrations at higher potentials within the range studied (-200 to +300 mV) in relation to *f II*. However, this polarographic study did not explore more positive potential ranges (higher than +300 mV) because the mercury working electrode has limitations in the zone of potentials higher than +400 mV (against the SCE) [21, 26]. Consequently, the results obtained do not rule out the existence either in *f I* or *f II* of electroactive substances with reduction potentials higher than the ones explored in this polarographic study.

With regard to the nature of electroactive compounds, if one considers that the 0.05 M EDTA solution forms chelates with most of the inorganic soil components [18, 27], we may conclude that the peaks observed corresponded mainly to organic components [13].

In our case, DPP by itself cannot provide specific information on the nature of the redox pairs. It can, however, provide information concerning their total concentration and their place in the redox potential scale. Figure 4 shows the cumulative amount of elecroactive compounds founded in *f I* and *f II* from EDTA whole Vertic Mollisol extract. These curves clearly show that in *f I* and *f II* the concentration of molecules that may act as electroactive species were located in different place on the redox potential scale (Y-axis). The reducing status of these HSs fractions were influenced by the potential of these linked redox couples. Because the concentration of electroactive compounds with lower potentials (the pairs that had a high tendency to donate electrons in the studied systems) is much higher in *f I*, this fraction had a significant reducing status compared with *f II*. This is seen when applying the definition of reducing status (see *Materials and Methods*):

reducing status *f I* = -859 nA and reducing status *f II* = -192 nA

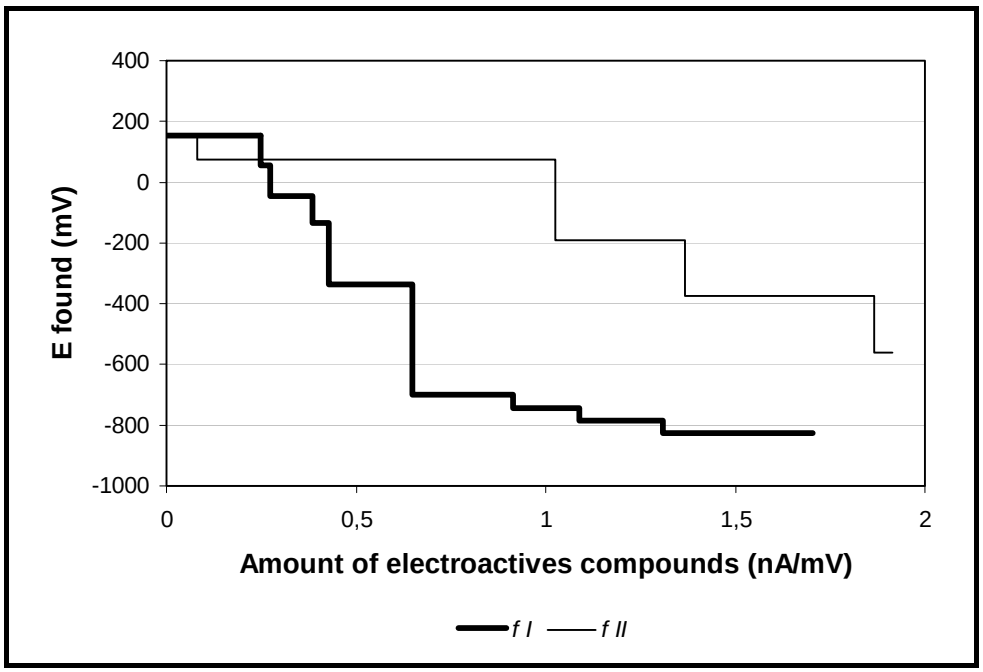

Figure 4. Cumulative curves of the amount of electroactive species founded in *f I* and *f II* fractions (4a and 4b respectively) obtained with EDTA pH=7 extract from whole Vertic Mollisol extract. The plateau's lengths correspond to the oxidizing capacity of each redox pair (for more details see text).

As seen in this calculation, the overall reducing status in *f I* result principally from the contribution of the pairs that correspond with peaks 1 – 4 (table insert in figure 3). This suggest that the exact nature of this HSs redox components should be characterize and their electrochemical behavior need to study in depth in order to determine if they may be a key factor in the electrons transfers processes that involves HSs at the soil aggregate level, phenomena that was well descript in the literature [9 – 13].

Although the nature of the redox compounds involves in the calculated values is not know, the obtained results contribute to the general knowledge of HSs fractions in the studies soil and also informer about the theoretically electron transfers possibilities, inner and outer, of these molecular assemblies.

These results also shows that this theoretically calculation give a numerical indicator of the reducing status in a soil systems that may be employed as a first step into a new area of quantitative biology [22], for instance, to study the inoculation of the redox effector bacteria upon soil redox status at soil aggregate scale [15].

Conclusions

The technique of HSs extraction with ethylenediaminetetraacetic acid (EDTA) with pH= 7 and the subsequent extract fractionation with a molecular sieve (Sephadex G25) enabled the separation of two important HSs fractions. The extractant did not interfere with fractionation according to molecular size and contributed weakly to eluate conductivity.

The polarographic study demonstrated that the two HSs fractions obtained with this extraction method did differ in the amount of total electroactive compounds, (*f I* showed 10% lower than *f II*) and also did differ in their quality. The fraction containing greater molecular size compounds (*f I*, fraction excluded with Sephadex G25) had mainly electroactive pairs with low reduction potentials (lower than –200 mV), whereas the fraction of smaller molecular size (*f II*) had mainly electroactive pairs with potentials between -200 to +300 mV. The reducing status (the summation of the product of redox intensity and redox capacity factors) in *f II* was lower than *f I* fraction.

The fact that in the composition of HSs easy extractable fractions the amount and the place in the electrochemical series of their electroactive components are different has an important implication in microbial ecology. The electronic exchanges between these fractions in accordance with the general concept that the energetically most favorable available electron acceptors are utilized first [10] may contribute to explain, for instance, the metabolic pathway used by microorganisms to act over the HSs and by this way to obtain an adaptive advantage to colonize the soil habitat. On the other hand, these results may be considered in the studies to improve the knowledge of the mechanisms involved in several technological practices in our region, as soil fertilization or soil amendments with organic or inorganic compounds.

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