

Review

Use of insoluble polyvinylpyrrolidone and isoelectric focusing in the study of humic substances in soils and organic wastes

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ABSTRACT

The use of insoluble polyvinylpyrrolidone and isoelectric focusing in the study of humic substances is reviewed. Chromatography on insoluble polyvinylpyrrolidone is widely used to characterize humic materials extracted from soils, organic fertilizers, soil amendments and organic wastes in order to evaluate their degree of humification. The isoelectric focusing technique is employed mainly to evaluate the stability of the organic matter in organic wastes before their use in agriculture.

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1. INTRODUCTION

The total amount of soil organic matter worldwide has been estimated to be *ca.* $2 \cdot 10^{12}$ – $3 \cdot 10^{12}$ kg [1,2], while the amount of soil humic substances can be estimated as 50% of the total soil organic matter.

In general, organic matter (especially humic sub-

stances) plays an important role in soil and water environments. Hayes *et al.* [3] have summarized the role of organic matter in soils as follows: (1) formation and maintenance of a good soil structure; (2) improvement of water capacity; (3) retention in available form of plant nutrients by cation-exchange processes; (4) slow release of nitrogen, sulphur, phosphorus and some trace elements; (5) transport of metals into plant roots; (6) stimulatory effects on plant growth; (7) immobilization of some anthro-

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With this aim the fulvic fraction is purified from non-humic (NH) substances by using small columns (10 cm) packed with insoluble PVP (3–5 cm³) after acidification of the alkaline extract [17,18,41,42]. The retained fraction on PVP (generally brown) is then re-eluted by adding 5–10 ml of 0.5 M NaOH and collected (FA), while the non-adsorbed fraction (NH, non-humic fraction) is discharged.

Humic substances have been defined as amorphous, polymeric, brown compounds [43,44] and other classes of organic compounds, such as polysaccharides, polypeptides or altered lignins, are not considered to be humic compounds. It has recently been demonstrated [45] that carbohydrates, peptides, amino sugars and amino acids contained in the fulvic fraction separated from three different types of soil are concentrated mainly in the fractions which are not adsorbed on PVP. Indeed, the FA fraction adsorbed on the PVP resin contained a large number of aromatic rings and carboxyl groups, while non-humified (NH) compounds remained in the fraction not adsorbed on the PVP [45].

The International Humic Substances Society (IHSS) has recommended the use of Amberlite XAD-8 resin as the adsorbent to obtain only humic substances in the FA fraction. However, Kuwatsuka *et al.* [46] have recently demonstrated that fractionation using insoluble PVP gives a higher recovery and more distinct fractions of FA than the resin pro-

posed by the IHSS. In fact, after extraction and fractionation of FA from forest soils and from ando soils, the amounts of FA recovered using the Nagoya method [46] were 5 and 40 times higher, respectively, than those found after application of the IHSS method.

Recently, in studies of soil organic matter, some workers (*e.g.*, refs. 17, 18, 47 and 48) have used insoluble PVP to calculate various humification parameters, namely (i) a humification index [17], $HI = NH/(HA + FA)$, *i.e.*, the ratio between non-humified (NH) and humified compounds (HA + FA), (ii) the degree of humification [49], $DH (\%) = [(HA + FA)/TEC] \cdot 100$, *i.e.*, the percentage of humified compounds with respect to total extracted organic carbon (TEC) and (iii) the humification rate [49], $HR (\%) = [(HA + FA)/TOC] \cdot 100$, *i.e.*, the percentage of humified compounds with respect to total organic carbon (TOC) in the sample.

Some of the results obtained after application of PVP in the separation of the fulvic fraction of organic extracts arising from the A horizons of typical Italian soils are reported in Table 1. The calculated humification rate (*HR*) and degree of humification (*DH*) are values typical of these two parameters for soil samples [18]. The role of PVP in the separation of humified (FA) from non-humified (NH) compounds in the fulvic fraction is well indicated by the *DH* values. In fact, the difference

TABLE 1

TOTAL ORGANIC CARBON AND NITROGEN, HUMIFICATION RATE (*HR*) AND DEGREE OF HUMIFICATION (*DH*) OF REPRESENTATIVE SAMPLES OF SOILS

All samples were taken from the A horizon of the soil profile and the data are expressed on the basis of the air-dry mass.

Soil samples	Total organic carbon (%)	Total organic nitrogen (%)	Humification rate, <i>HR</i> (%)	Degree of humification, <i>DH</i> (%)
Typic Xeropsamment	1.72	0.17	21.2	65.7
Typic Xerorthent	2.71	0.13	13.6	63.4
Mollic Xerorthent	6.30	0.34	18.4	67.4
Fluventic Xerochrept	1.91	0.21	17.9	68.2
Typic Haplumbrept	5.13	0.37	20.9	68.7
Typic Pelloxerert 1	3.78	0.25	21.2	80.3
Typic Pelloxerert 2	0.99	0.10	26.3	88.9
Typic Chromoxerert	2.21	0.16	36.5	97.7
Mollic Andept 1	13.1	0.65	27.5	79.1
Mollic Andept 2	12.0	0.90	32.9	78.4

between the *DH* value found and 100 represents the amount (as a percentage) of the NH fraction present in the fulvic fraction of the soil organic extract. Without the use of the PVP resin, these fractions, mainly composed of polysaccharides, amino acids and amino sugars [44], are often erroneously included in the humic fraction. The mean values of the overestimate are around 30%; the overestimate is less only in the case of samples of Vertisols (*e.g.*, Pelloxerert and Chromoxerert). It should be emphasized that the role of PVP in the separation of FA from NH compounds in the fulvic fraction is higher in the soil samples where the organic matter is less humified (*e.g.*, Entisols, Inceptisols, Mollisols), where the amount of NH compounds is generally high (Table 1).

2.2. PVP in studies of organic amendments

The procedure for the extraction and separation of the soil organic matter reported in section 2.1. has also been used to characterize organic amendments, such as peats, lignins and leonardites [18]. Owing to the high organic carbon content of these samples (normally 30–55%) [7], as reported above, the ratio between the volume of the extractant solution and the mass of the sample used is greater than that for soil humic substances (50:1 compared with 10:1,

v/w, respectively). This procedure has recently been introduced by the Italian Ministry of Agriculture and Forestries (MAF) [50] as the official method for determining humic substances in peats, leonardites, suspensions of humic acids and farmyard samples.

Some selected results after fractionation of the organic carbon extracted from samples of peats, lignites and leonardites are reported in Table 2. Organic amendments are generally characterized by a high content of organic carbon and small amounts of organic nitrogen. The percentage of humic substances (HA + FA) with respect to total organic carbon in the sample (TOC) is low for the lignite samples, around 60% for the peat samples (except peat sample 2 with *HR* = 26.7%) and over 80% for the leonardite samples. As expected, the presence of NH substances in organic amendments is generally low, owing to the progressive decrease in easily oxidized compounds (*i.e.*, polysaccharides and amino sugars). Indeed, the *DH* of the organic extracts is very high for all samples and close to 100% for the leonardite samples and highly humified peats [7].

2.3. PVP in studies of organic fertilizers and wastes

In the case of organic fertilizers and wastes the use of insoluble PVP in the separation of humified

TABLE 2

TOTAL ORGANIC CARBON AND NITROGEN, HUMIFICATION RATE (*HR*) AND DEGREE OF HUMIFICATION (*DH*) OF SOME SAMPLES OF ORGANIC AMENDMENTS

The data are expressed on a dry mass basis.

Samples	Total organic carbon (%)	Total organic nitrogen (%)	Humification rate, <i>HR</i> (%)	Degree of humification, <i>DH</i> (%)
Peat 1 (Czechoslovakia)	50.7	2.38	60.2	89.2
Peat 2 (Russian Federation)	58.4	1.04	26.7	72.2
Peat 3 (Ireland)	57.8	1.27	60.5	90.4
Peat 4 (Ireland)	55.7	1.69	59.2	94.8
Peat 5 (Italy)	33.7	2.11	64.0	96.5
Peat 6 (Italy)	46.2	2.53	53.4	91.3
Peat 7 (Norway)	52.6	3.23	59.4	89.0
Peat 8 (Scotland)	52.0	1.40	52.3	87.5
Lignite 1 (Italy)	51.2	0.79	6.8	77.4
Lignite 2 (Italy)	52.3	0.74	11.6	88.9
Leonardite 1 (USA)	40.5	0.52	84.4	99.7
Leonardite 2 (USA)	41.3	0.55	82.7	94.9

compounds from the organic extract presents many problems [49,51]. According to the definition of humic substances given by Hayes and Swift [43] and Aiken *et al.* [44], without a proper period of maturation organic fertilizers and organic wastes do not contain humic materials. However, during the application of the procedure for the extraction and separation of the organic matter many interferences have been found [18]. In fact, after acidification some of the organic compounds extracted precipitate, or others in the fulvic fraction are adsorbed on PVP (humic-like substances). Ciavatta *et al.* [49] experimented with the use of an acid or an alkaline hydrolysis of the organic extract in order to reduce these interferences, but the results were unsatisfactory. More recently, the same group [51] studied the possibility of using a series of aspecific enzymes added sequentially to the extract to reduce the interferences arising from the humic-like substances. The results obtained were, in most instances, satisfactory and the interferences were reduced or completely eliminated [51,52]. In the determination of the humification parameters the chromatography on PVP of the hydrolysed organic extract only permits the separation of phenolic substances and the reduction of non-humic organic carbon [51,52].

For wastes containing large amounts of organic carbon (*e.g.*, slurries, compost produced from municipal solid wastes and sewage sludges) it is very important to assess the stability of the organic carbon before its addition to the soil. Indeed, the addition of easily oxidized organic compounds to soil, such as those generally present in large amounts in organic wastes, can produce an anoxic environment, phytotoxic substances, leaching to deep waters and damage to crops. For these reasons various humification parameters have been used to characterize, or better to evaluate, the degree of stabilization of the organic matter from a series of materials, including animal manures after digestion by earthworms [21] and raw composts [20,22,24,25,53].

Reported in Fig. 2, for example, is the trend of the *DH* observed during stabilization of the organic matter extracted from samples of compost from municipal solid wastes. Of the two humification parameters, *HR* and *DH*, the latter appears to be the most useful to follow the stabilization processes in organic materials. The values of the *DH* during the period of stabilization are characterized by a sharp

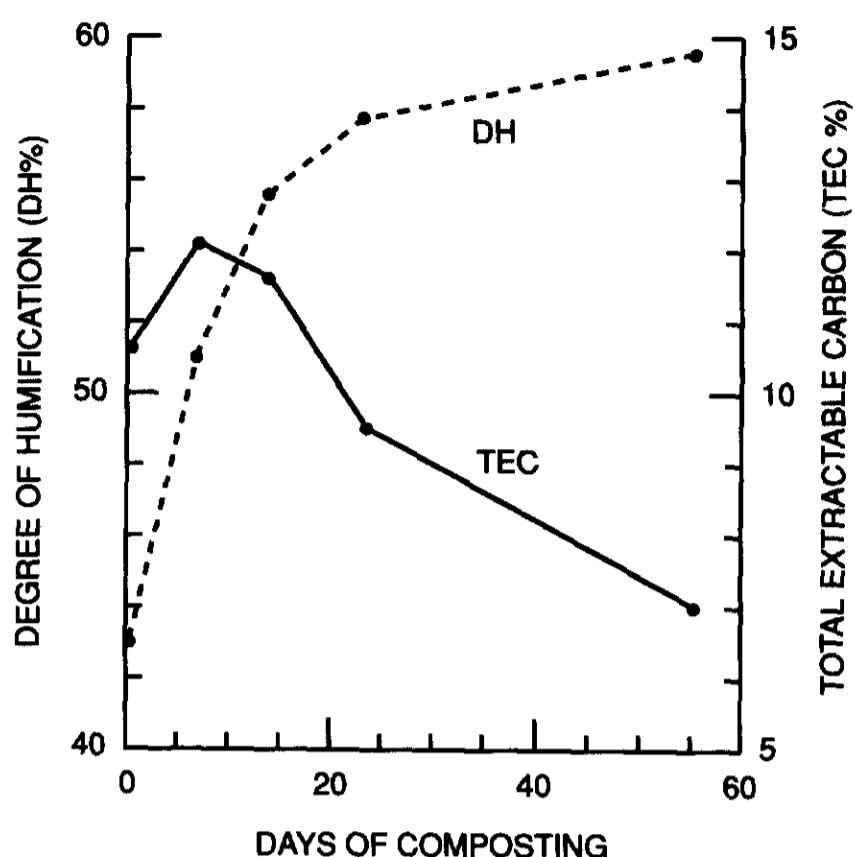


Fig. 2. Trend of both the degree of humification (*DH*) and total extracted organic carbon (*TEC*) during the stabilization process of the organic matter from a pile of compost with municipal solid wastes. From ref. 25.

increase, which is then followed by an asymptotic trend. In other words, the evolution of organic matter during maturation of an organic waste is characterized by a continuous increase in humified or humic-like substances in the alkali-soluble fraction, so that the *DH* effectively represents the development of the process. Sometimes, but not always, the *HR* also has a similar trend to that of the *DH* [8]. Especially with liquid or semi-liquid wastes, the entire mass of organic matter is simultaneously involved in the stabilization process and only the *HR* can accurately describe the process [8,22]. When the processes are effective for a small proportion of the material and involve progressively only further limited parts, while the bulk remains unaltered, the *DH* can describe the process better than the *HR* [7].

In contrast, as shown in Fig. 2, in all instances the total extracted organic carbon (*TEC*) is not a suitable parameter for following the stabilization processes, because its trend is irregular and characterized by a decrease during the formation of humic and humic-like substances.

3. ISOELECTRIC FOCUSING (IEF)

In general, the electrophoretic separation of a charged compound is based on its mobility in an electric field [54–56]. One of the electrophoretic techniques, isoelectric focusing (IEF), is an electrophoresis carried out on a medium with a preformed pH gradient. The principle of this technique has been described well by Righetti and Drysdale [56]: “a stable pH gradient increasing progressively from anode to cathode is established by electrolysis of carrier ampholytes (CAs) in a suitable anticonvective medium”. In this system a charged molecule migrates and reaches a zone where its net electric charge is zero. The final result is that all the molecules of an unknown mixture are fractionated in the pH gradient on the basis of their different isoelectric points (*pI*).

The IEF technique was first reported in 1912 [57] when a mixture of amino acids from hydrolysed vegetable proteins was fractionated in a three-chambered electrolysis cell. One of the most important problems in this and other pioneering studies [58,59] was the absence of an uniform and stable pH gradient, mainly owing to the lack of a suitable CA.

Later, as reported by Righetti and Drysdale [56], the theoretical basis for IEF was developed and can be summarized as follows: the importance of using electrolytes with a high buffering capacity and of stabilizing the pH gradient against convective mixing [60,61] was pointed out; and the law of the monotony of the pH was introduced and the idea of developing a natural pH gradient by electrolysis of amphoteric molecules [62–65] was advanced. Ideally, the ampholytes should have good conductivity, good buffering capacity and good solubility at their isoelectric point and also be easily distinguishable and separable from proteins. In 1969 [66], practical means of synthesizing CAs with many of the properties described by Svensson [62–64] and Rilbe [65] were achieved.

3.1. IEF of soil humic substances

The first electrophoretic separations of humic substances were carried out nearly 70 years ago [67] in free solutions. Then several media, such as a filter-paper matrix [68,69] or cellulose powder [70], were used. The first electrophoresis of humic substances

carried out on polyacrylamide (PAA) gel was reported by Stepanov and Pakhonov in 1969 [71]. At present, PAA gel is the most widely used medium in electrically driven separations of humic substances.

The first application of the IEF technique in studies of humic substances appeared in 1972 [72], when the fractionation of humic substances from freshwater was described. Later, IEF was used to characterize humic substances from soils, fertilizers, soil amendments and organic wastes.

One of the most important objectives of researchers who use IEF to characterize soil humic substances is to fractionate the compounds of the humic extract and then to conduct further studies on the single fractions. This second objective is not so easy to achieve and, consequently, the use of IEF has often been limited to the characterization of humic substances only by the evaluation of the pattern.

The first applications of IEF to soil humic substances were published in the 1970s. In early work [73], this technique was applied to soil humic substances without performing the pH gradient, but by just mixing the CAs with the sample to be characterized. Later it was found [74], that the use of IEF produced a greater number of electrophoretic and isotachophoretic bands.

It has been reported [75] that the CAs interfered with the humic substances during the IEF fractionation. It was pointed out that urea is apparently unable to break up these interactions and that its presence causes an increase in the intensity in the bands focused in the most acidic region of the pH gradient and a shift in the *pI* of the bands focused at the higher *pI* values. These data, however, in the opinion of De Nobili [76], were not sufficient to demonstrate the presence of interactions because similar results had been obtained previously during the fractionation of humic substances with electrophoresis when CAs were not present.

Another fact that is a cause for discussion about the reliability of the IEF technique for the characterization of humic substances is that, with the exception of the bands focused in the acidic region of the pH gradient, the IEF profile of a single band after refocusing has been found to be characterized by a more or less complex pattern [75]. However, more recently, it was found that the refocusing of a single band obtained in the presence or absence of urea was resolved in its original pattern [77].

In another study [76], humic substances were fractionated with electrophoresis at pH 6.5 into two groups which were subjected to IEF. The group of substances that had migrated towards the anode focused in the pH gradient region below pH 6.5, while the other group focused in the remaining pH gradient region.

As suggested by Duxbury [78] on the basis of experimental data, the formation and stability of complexes between humic compounds and CAs should be pH dependent and it could be possible that the alkaline re-dissolution of a humic–ampholyte complex would create a variety of new complexes that, after refocusing, could resolve in a new IEF profile. Duxbury [78] also reported that the resolution of the bands of the humic substances also depends on the prefocusing time and it was concluded that the technique used was not IEF. In a previous paper published in the 1988 [76], De Nobili used the term electrofocusing (EF) instead of IEF and also more recently the EF of soil humic substances has been reported [48].

Another problem is that commercial CAs are chemically different because their methods of synthesis are protected by patents.

It is not difficult to imagine that the characterization of humic substances with IEF could be influenced by the CA used. Recently, it has been shown [79] that the IEF separation of soil humic compounds using different CAs leads to the formation of different IEF profiles.

Ceccanti *et al.* [80] characterized a soil organic extract and its two fractions previously obtained by ultrafiltration. They found that the higher molecular mass fractions focused in the higher pH gradient region, whereas the fractions with lower molecular mass focused in the lower pH gradient region. Successively, these results were substantially confirmed by combining gel electrophoresis with IEF [81].

More recently [77], using IEF to characterize the different molecular sizes of humic substances in the presence or absence of 8 M urea, the relationship found between molecular mass and the position of resolution of a band in the pH gradient was confirmed but, in addition, it was noticed that the shift in pH due to the presence of urea had a greater influence in the fractions with $M_r > 10^5$. These and other results have demonstrated that most humic sub-

stances are fractionated on the basis of their pI or, at least, by charge neutralization. The lower molecular mass fraction appears to be constituted mainly of polyphenolic compounds with less polymerization than in the other fractions [82].

In agreement with other results, humic compounds with a lower electrophoretic mobility focus at higher pH values whereas the humic compounds with a higher electrophoretic mobility focus at lower pH values [83]. A further evaluation by use of infrared spectrometry and pyrolysis–gas chromatography–mass spectrometry has shown a lower content of carboxyl groups and an higher content of ketonic and quinonic carboxyl groups in the fractions with lower electrophoretic mobility. The fraction with lower electrophoretic mobility was more complex and contained a higher content of polysaccharides and also proteins or peptide residues. It was concluded that humic substances with different origins, but which focused in the same pH interval, show evident structural similarities [83].

The application of IEF in the characterization of humic substances extracted from different types of soils showed that different IEF profiles were obtained. This evidence suggested that the systematic application of IEF in the study of soil humic substances could lead to great improvements in the soil taxonomy [84].

The application of IEF in the study of organic matter extracted from soils that had been fertilized differently for 22 years with different types and rates of organic materials showed that the IEF profile obtained depended on the type of organic material, whereas mineral fertilization did not affect the IEF profiles of the native soil humic substances [48].

The esterase activity of a humic extract in the single focused bands has been measured and it was found that the activity was present in each band of the fractionated soil humic extracts [85]. However, in a more recent study [86], it was found that protease activity was present only in some bands and that this activity was higher in the band focused at pH 4.44.

3.2. IEF of organic wastes

The main objective of workers who apply IEF in studies of organic wastes is to define an analytical method that can be useful in order to characterize

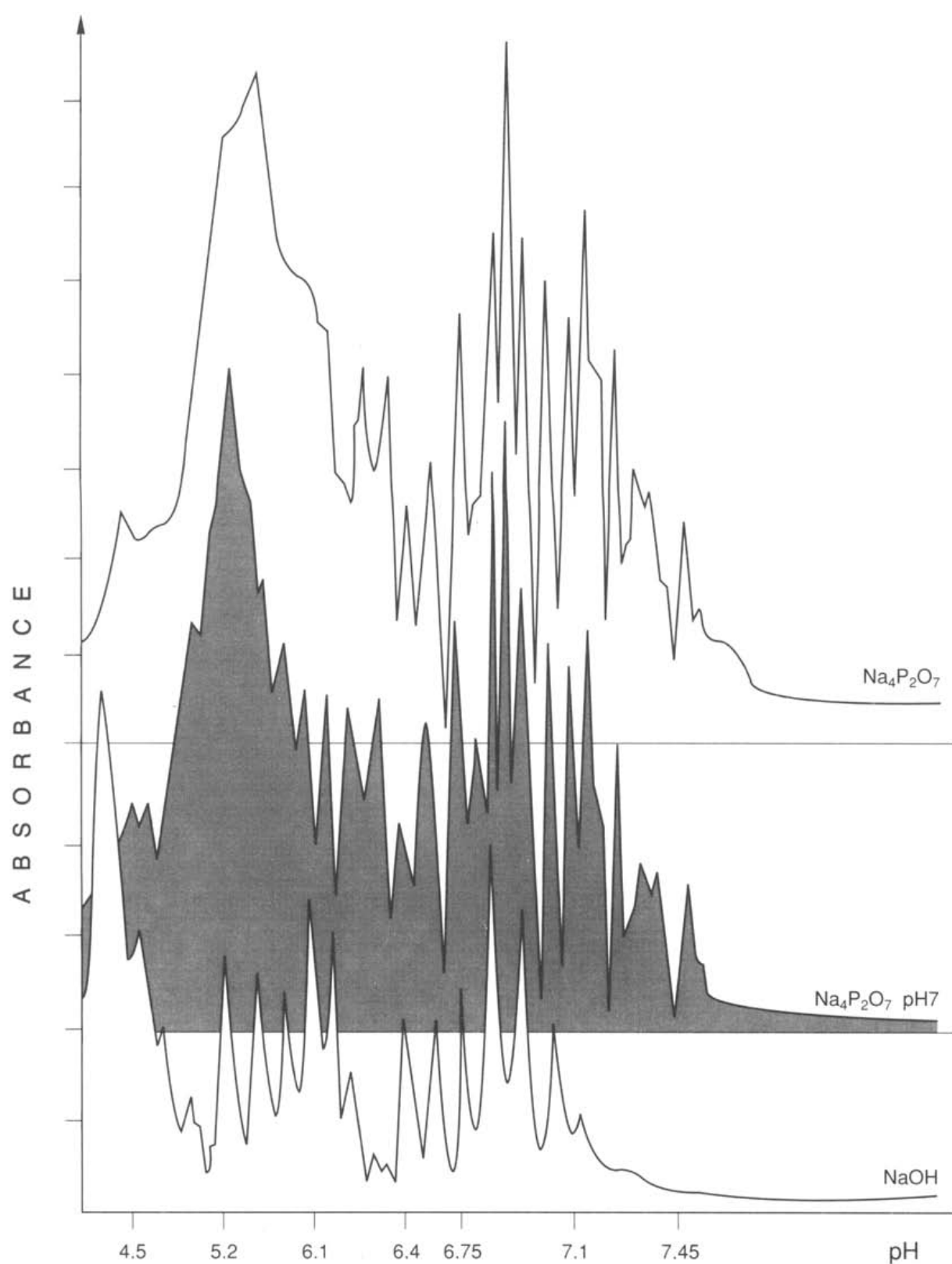


Fig. 3. IEF profiles of a digested sewage sludge extracted with 0.5 M NaOH, 0.1 M $\text{Na}_4\text{P}_2\text{O}_7$ adjusted to pH 7 with H_3PO_4 and 0.1 M $\text{Na}_4\text{P}_2\text{O}_7$. The carrier ampholyte used was Biolyte 3-10 (Bio-Rad). Each scale mark on the ordinate corresponds to 0.1 unit of absorbance.

the organic matter of a waste and accurately evaluate its stability before its use in agriculture. Obviously, this objective is of considerable interest for both agricultural and environmental purposes. Evaluation of the IEF profiles in this case is very different from the case of the soil humic substances because here the main factor is the stabilization of a raw organic material and not the characterization of humic substances. It is not difficult to understand why for this application the problems of the interac-

tions of the organic compounds with the CAs appear to be of secondary importance. The most important question is whether or not an IEF profile of an organic waste can give enough information about its stabilization so that its proper use in agriculture can be assured.

The IEF profiles of raw and mature poultry manure, farmyard manure, worm compost and compost from municipal waste treatment plants have been evaluated [87]. The IEF profiles of the

first three materials were characterized by considerable heterogeneity of the bands in the pH gradient region from 4.5 to 6.5, whereas the composts from municipal waste treatment plants showed an IEF profile with a simpler pattern resolved below pH 5.5. The oldest sample, however, displayed fewer bands in the acidic region.

Comparison of the IEF profiles of differently aged sewage sludges showed the presence of resolved bands in the pH gradient region below pH 5 for the raw samples, whereas the *ca.* 6-month-old samples also focused in a less acidic region (up to pH 6.5) [53]. Similar results were also obtained in another study in which the organic matter was extracted using several extractant solutions [88]. Fig. 3 shows an example of the IEF profiles of a digested sewage sludge extracted using three different extractant solutions.

The evolution of the organic matter of pig slurries has been followed both in summer and in winter [89]. The IEF profiles of the raw samples were poor in bands in the pH gradient region above 5 whereas the IEF profile of the slurry, matured in the summer season, was rich in bands also in the pH gradient region from 5 to 7. The IEF profile of the slurry matured in the winter season was much less complex than that of the summer-matured slurry. These results have also been confirmed in a study of the evolution of organic matter during the stabilization of composts from municipal solid wastes [25].

The most important aims of the use of IEF are (i) to find the relationships between the IEF profiles of organic wastes and their degree of stabilization and (ii) to find the relationships between IEF profiles and the presence of phytotoxic compounds.

Studies to optimize the IEF technique should also provide for comparisons of the IEF profiles obtained using different CAs in order to choose the best CA for this type of work.

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