



## PROTEASE EXTRACTION FROM SOIL BY SODIUM PYROPHOSPHATE AND CHEMICAL CHARACTERIZATION OF THE EXTRACTS

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**Summary**—Two arable soils and one pasture soil had previously been air-dried for 6 d and stored at room temperature. The enzyme activities remaining after this treatment were constant. The soils were then extracted with 140 mM sodium pyrophosphate at pH 7.1. Amino acid N and total organic C content of soils and soil extracts, together with humic and fulvic acids content of soil extracts were determined. Total organic C was determined in soil residues obtained after extraction. Chemical characterization of the organic matter of soils, soil extracts and soil residues was carried out by pyrolysis–gas chromatography (Py–GC). Protease activity was determined in soil extracts and soil residues by using three different substrates: N-benzoyl-L-argininamide (BAA), specific for trypsin; N-benzyloxy-carbonyl-L-phenylalanyl L-leucine (ZPL), specific for carboxypeptidases, and casein, essentially non-specific. Comparative studies between specific activities referred to organic C in soils, soil extracts and soil residues and their corresponding pyrogram composition, and also between total extracted or residual activity and the humine or unhumified organic matter content of the corresponding soil, allowed us to establish hypotheses about the type of organic matter the enzymes are associated with. From 12% to 21% of the soil organic C (33% to 39% of which were humic acids) and from 3% and 18% of amino acid N were extracted from soil using pyrophosphate. Py–GC analyses showed that pyrophosphate was effective in extracting condensed humic substances and glycoproteins and that the organic matter present in soil extracts was especially rich in intact or partially-decomposed fresh residues of carbohydrate origin and also in certain humus-associated proteins. Extracted BAA-hydrolysing activity accounted for 11% to 36% of the soil activity, depending on soil type. Extracted ZPL- and casein-hydrolysing activities were, with one exception, remarkably high, accounting for about 100% or even more of the soil activity, depending on soil type. According to the results BAA-hydrolysing proteases are probably mostly associated with highly condensed humus, ZPL-hydrolysing proteases with less condensed humic substances and casein-hydrolysing proteases with fresh organic matter. © 1998 Published by Elsevier Science Ltd. All rights reserved

### INTRODUCTION

Active extracellular enzymes in soil may be associated with inorganic and organic colloids (Burns, 1982; Nannipieri *et al.*, 1996). The amount of this extracellular enzyme activity may be indicative not only of the biological capacity of soil for the enzymatic conversion of the substrate, which is independent of the extant microbial activity, but it might also have an important role in the ecology of microorganisms (Burns, 1982). There is the need to study the properties of naturally-occurring enzyme–organic complexes present in soil. These complexes must be extracted in high yields before investigations on their activity and physical and chemical state.

Different hydrolases have been extracted from pasture or forest soils by salt solutions: 0.1 M Tris–borate at pH 8.1, 0.1 M Tris at pH 8.1, 0.1 M Tris–citrate at pH 8.0 and 0.1 M Tris–EDTA at pH 8.0 (Ladd, 1972); 0.2 M phosphate–0.2 M EDTA buffer at pH 8.0 (Batistic *et al.*, 1980); 140 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> at about neutral pH (Nannipieri *et al.*, 1980). Cultivation did not consistently influence the content or composition of soil peptides in molecular weight fractions of extracts obtained from two podzols using a mild extraction procedure (Warman and Isnor, 1991). Extracellular  $\beta$ -glucosidases have been extracted from arable soils either by phosphate–KCl–EDTA buffer at pH 7.0 (Hayano and Katami, 1977) or by 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> at pH 7.0–7.3 (Busto and Pérez-Mateos, 1995). Pyrophosphate was more effective than phosphate in extracting urease and amylase from different podzols (Nannipieri *et al.*, 1975; Shcherbakova *et al.*, 1981).

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It was also demonstrated that 140 mM  $\text{Na}_4\text{P}_2\text{O}_7$  at about neutral pH extracted extracellular urease from a podzol (Nannipieri *et al.*, 1974). Ruggiero and Radogna (1984) reported that sodium pyrophosphate was more efficient in extracting laccase from a forest soil than the phosphate-EDTA.

Mayaudon *et al.* (1975) and Batistic *et al.* (1980) suggested that extracted protease activities occurred partly in soil as a carbohydrate-enzyme complex and partly as a humo-carbohydrate complex. Proteases have been extracted from arable soils by 0.1 M Tris-borate at pH 8.1 (Ladd, 1972), 0.2 M phosphate-0.2 M EDTA buffer at pH 8.0 (Mayaudon *et al.*, 1975) and 0.1 M phosphate at pH 7.0 (Hayano *et al.*, 1987).

Proteases in soil play a role in N mineralization (Ladd and Jackson, 1982), a process regulating the amount of plant available N (Stevenson, 1986). Studies on the composition of protease-organic complexes in arable soils are important to determine the role of these biochemically-active organic fractions in soil fertility. More insights are required to know the chemical composition of organic molecules extracted by  $\text{Na}_4\text{P}_2\text{O}_7$  under neutral conditions (Stevenson, 1982). This chemical characterization may give indications on the chemical structure of matrices on which the extracted enzymes are associated. Proteases have been extracted by 50 mM  $\text{Na}_4\text{P}_2\text{O}_7$  at pH 9 from a municipal solid waste compost (Rad *et al.*, 1995) and also by 140 mM  $\text{Na}_4\text{P}_2\text{O}_7$  at pH 7.1 from forest or permanent grassland soils (Nannipieri *et al.*, 1980, 1982, 1985) but not from arable soils. The presence of humic molecules in pyrophosphate-extracted enzyme complexes from forest soils was demonstrated by pyrolysis-gas chromatography (Py-GC) (Ceccanti *et al.*, 1986).

Our aims were: (1) to determine the efficacy of 140 mM  $\text{Na}_4\text{P}_2\text{O}_7$  at pH 7.1 in extracting different proteases from two arable soils and one pasture soil; (2) to chemically characterize (amino acid N content, fractionation in humic and fulvic acids, analysis by Py-GC) organic matter of soils, soil extracts and soil residues; (3) to compare the data obtained in Py-GC analysis and enzyme activity to find out the type of organic matter the proteases

are associated with. Proteases were differentiated by assaying soil extracts and soil residues with different substrates as reported by Ladd and Butler (1972). To achieve our aims data obtained with soil extracts and residues were compared with those reported by Bonmatí (1989) in the same soils.

## MATERIAL AND METHODS

### Site, soils and sampling

Three soils were selected among the most representative soil types of Catalonia and classified according to FAO-UNESCO (1974). Soil 5, a Calcaric Fluvisol, under maize (*Zea mays*)-rye grass (*Lolium perenne*) rotation, was sampled after cropping at Figueres (Alt Emporda); soil 13, a Calcaric Fluvisol, under tomato (*Lycopersicon esculentum*), was sampled after cropping, at Malgrat (Maresme); soil 19, a Dystric Cambisol, under permanent meadow, was sampled in winter at Viella (Vall d'Aran). Soils were collected (0 to 20 cm) from 15-20 soil cores that were pooled together. The bulked samples (1 kg) were air-dried for 6 d, sieved (<2 mm) and stored at room temperature for 3 yr prior to investigation. Physico-chemical characteristics of the three soils are shown in Table 1 (from Bonmatí, 1989). Organic matter had been extracted with 0.1 M sodium pyrophosphate-0.1 M NaOH (1/1) and fractionated into Humic acids, Fulvic acids, humine and unhumified organic matter, by using the method of Duchaufour and Jacquin (Pleven *et al.*, 1967).

### Soil extraction and preparation of humic and fulvic acids

Soils were extracted with 140 mM sodium pyrophosphate at pH 7.1 (soil-solution ratio 1:10) at 37°C for 24 h in a shaking water bath. Before initiating the shaking process  $\text{N}_2$  was passed into the Erlenmeyer flasks containing the mixture and they were hermetically closed immediately afterwards. Centrifugation and bacteriological filtration were carried out as reported by Nannipieri *et al.* (1974). Soil extracts were exhaustively dialysed against

Table 1. Physico-chemical characteristics of soils

Soil	pH <sup>a</sup> (H <sub>2</sub> O)	C <sup>b</sup>	FA <sup>c</sup> (g C kg <sup>-1</sup> dry soil)	HA <sup>d</sup> (g C kg <sup>-1</sup> dry soil)	HUM <sup>e</sup> (g C kg <sup>-1</sup> dry soil)	UOM <sup>f</sup> (g C kg <sup>-1</sup> dry soil)	N <sup>g</sup>	CEC <sup>h</sup>	Clay (%)	Silt (%)	Sand (%)
5	7.5	23.2	1.30	1.87	19.16	0.88	2.74	31.6	25	61	14
13	7.4	13.1	4.99	1.02	6.40	0.71	1.40	30.4	22	26	52
19	5.5	47.6	6.03	7.58	29.95	4.08	5.56	36.5	26	36	38

<sup>a</sup>Soil:water ratio 1/2.5.

<sup>b</sup>Units: g kg<sup>-1</sup> dry soil.

<sup>c</sup>FA = fulvic acids.

<sup>d</sup>HA = humic acids.

<sup>e</sup>HUM = humine.

<sup>f</sup>UOM = unhumified organic matter.

<sup>g</sup>Units: g kg<sup>-1</sup> dry soil.

<sup>h</sup>Cationic exchange capacity (cmol kg<sup>-1</sup> dry soil).

water as reported by Ceccanti *et al.* (1978). Soil extraction was replicated at least three times.

Humic and fulvic acids were prepared from soil extracts as reported from Pujolà *et al.* (1990); 0.5 ml of 2.5 M H<sub>2</sub>SO<sub>4</sub> were added to 5 ml of extract; the mixture was left at 4°C for 24 h and then centrifuged (4000 × *g*, 10 min); the precipitate was washed with acidulated water and centrifuged.

#### *Amino acid N determination*

Amino acid N in soils and soil extracts was determined as described by Stevenson (1982). Soil extracts were previously concentrated by ultrafiltration (Amicon 202 200 cm<sup>3</sup> cell with a PM 10 diafmembrane) up to a total N content of about 0.1 g l<sup>-1</sup>. Three replicates were carried out. In the case of soil extracts each replication concerned a separate extraction from a new sample coming from the same bulked soil sample. The same procedure was followed for the rest of the determinations in soil extracts and soil residues in our work. Thus variability obtained for the results would include not only that of the analytical technique but also that of the extraction procedure.

#### *Organic C determination*

Total C and humic acids C of soil extracts were determined according to Pujolà *et al.* (1990). An excess of dichromate and 5 M H<sub>2</sub>SO<sub>4</sub> were added to the samples and then the organic C was oxidated by heating at 150°C for 15 min; the mixtures were left for 24 h at room temperature and the formed Cr<sup>3+</sup> determined by measuring the absorbance at 590 nm (Nikitin, 1972). Total C of extraction residues was determined by dichromate oxidation (Walkey, 1935). We performed at least three replicates for each sample. Each replication concerned, as reported for amino acid N determination, a separate extraction from the same soil.

#### *Enzyme assays*

Total activities and specific activities referred either to organic C or amino acid N of soil extracts and soil residues were assayed using three different substrates: N-benzoyl-L-argininamide (BAA), N-benzyloxycarbonyl-L-phenylalanyl-L-leucine (ZPL) and casein. N-benzoyl-L-argininamide hydrolysing activity of soil residues was determined as reported by Nannipieri *et al.* (1980); 1 g of residue was reacted for 1 h with 4 ml of 7.5 mM BAA dissolved in 0.1 M pH 7.1 phosphate buffer; ammonium concentration was determined by Orion Ammonium electrode. N-benzyloxycarbonyl-L-phenylalanyl-L-leucine-hydrolysing activity of soil residues was determined as reported by Ladd and Butler (1972); 1 g of residue was reacted for 1 h with 4 ml of 1 mM ZPL dissolved in 0.1 M pH 8.1 Tris-borate buffer; leucine concentration was determined by the ninhydrin reaction. Assays of these two activities

were performed at 40°C and replicated a minimum of three times, each replication concerning a different extract from the same soil. Casein-hydrolysing activity of soil residues was determined as reported by Bonmati *et al.* (1991) after four different incubation periods (1, 1.5, 2 and 3 h); 1 g of residue was reacted at 51°C with 2.5 ml of 1% casein dissolved in 0.1 M pH 8.1 Tris-HCl buffer; the concentration of trichloroacetic acid soluble peptides was colorimetrically determined by Folin reagent. Each assay concerned a sample coming from a different extract of the same soil. With the three assayed activities two types of control were carried out; buffer substituted for the substrate solution in one control and no soil residue was present in the other.

Soil extracts were concentrated by ultrafiltration (as reported for the determination of amino acid N) up to an organic C content of 1 g l<sup>-1</sup> and then assayed for protease activities. The activities in the concentrated extracts (at least three replicates for each sample) were determined as follows:

*BAA hydrolysis* 0.6 ml, 0.1 M pH 7.1 phosphate buffer and 0.2 ml 30 mM BAA were added to 0.4 ml of soil extract. Mixtures were reacted for 1 h at 40°C and then diluted with 15 ml of distilled water. Ammonium concentration was determined by Orion ammonium electrode. In the control, the substrate solution independently incubated for 1 h at 40°C, was added after diluting the mixture with water.

*ZPL hydrolysis* 0.6 ml, 0.1 M pH 8.1 Tris-borate buffer and 0.2 ml 4 mM ZPL were added to 0.4 ml of soil extract. Mixtures were reacted for 1 h at 40°C and then diluted with 6 ml of 2% ninhydrin reactive. Leucine concentration in mixtures was determined by the ninhydrin reaction. In the control, substrate solution independently incubated for 1 h at 40°C, was added after diluting the mixture with ninhydrin.

*Casein hydrolysis* Soil extract (2 ml) was added to 2.5 ml of 1% casein dissolved in 0.1 M pH 8.1 Tris-HCl buffer and reacted for 1, 1.5, 2 and 3 h respectively at 51°C. At the end of the reaction, 1 ml of 17.5% trichloroacetic acid was added to precipitate proteins and concentration of soluble peptides was colorimetrically determined by Folin's reagent. In the controls, substrate solutions independently incubated at 51°C for the same time as reaction mixtures, were added after the trichloroacetic acid solution. Activity was determined by calculating the slope of the straight regression line absorbance vs time, which was considered not to cross the origin of coordinates (Ladd and Butler, 1972).

Replications of activities of extracts concerned, as in their corresponding extraction residues, a different extract from the same soil.

For all the activities that were determined (both in soil extracts and in soil residues), there was a linear relationship between the amount of formed pro-

duct and reaction time throughout the assay period; BAA-hydrolysing activity was expressed in  $\mu\text{mol N-NH}_4^+ \text{ g}^{-1} \text{ dry soil h}^{-1}$ , ZPL-hydrolysing activity in  $\mu\text{mol leucine g}^{-1} \text{ dry soil h}^{-1}$  and casein-hydrolysing activity in  $\mu\text{mol tyrosine g}^{-1} \text{ dry soil h}^{-1}$ .

*Organic matter characterization by pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS)*

Soils, soil extracts and soil residues (two replicates for each sample) were analysed by Py-GC-MS. Composite samples of soil extracts and soil residues were especially prepared for this technique by pooling together samples of at least three extractions of the corresponding soil.

Py-GC was performed as reported by Alcañiz *et al.* (1987a), with some modifications. Composite samples of soils and soil residues were finely pulverized and amounts of these samples equivalent to 400  $\mu\text{g}$  of organic matter were introduced in pyrolysis quartz tubes as a water suspension and dried with a rotatory procedure as reported by Gassiot-Matas *et al.* (1982). Composite samples of soil extracts were concentrated by ultrafiltration (as reported for the determination of amino acid N) up to an organic C content of 6  $\text{g l}^{-1}$  and amounts of these samples equivalent to 400  $\mu\text{g}$  of organic matter were introduced in quartz tubes and dried as described before.

A CDS Pyroprobe 190 heated-filament with a platinum coil probe was used. Pyrolysis was carried out at 700°C for 10 s, heating rate 10°C  $\text{ms}^{-1}$ . The probe was coupled directly to a Hewlett-Packard HP-5995 A gas-chromatograph with a flame ionization detector. Separations were performed on a bonded phase capillary column (Supelcowax, Carbowax 20 M, Supelco, 25 m, 320  $\mu\text{m}$ ) with a temperature program of 3 min at 60°C up to 240°C at 6°C per min. Injection was carried out using the splitless mode. Carrier gas was He at 1.5  $\text{ml min}^{-1}$ . Mass spectra were obtained in a HP5995A quadrupole mass spectrometer. The corresponding identifications were performed by Py-GC-MS on a representative sample of each type, and extended to the others by coincidence on retention times.

To perform semi-quantitative comparisons between organic matter composition of samples, this procedure was followed.

— Among the 30 peaks which were identified by mass spectrometry, 18 were selected according to their frequency of occurrence and major relative area throughout the pyrograms, as well as taking into account, after consulting published data, the information they supplied on the samples organic matter composition. The mean peak areas in the duplicated pyrograms were calculated and successively normalized as a percentage of the sum of the 18 areas; then the logarithm of each normalized area was calculated. The ulterior statistic process,

according to the SPSS program (SPSS Inc., 1986), was performed by using the latter values.

— Correlation coefficients (SPSS-X 2.1-PEARSON COR) between pyrograms were calculated; then Multidimensional Scaling (MDS), based on the Euclidean Distance (SPSS-X 2.1-ALSCAL), was carried out according to Cuadras (1981). The data obtained were used to assess differences between pyrograms. This type of statistical treatment had been used before by Bonmati *et al.* (1991) and proved to be a useful tool to study the overall differences among the samples investigated.

— Correlations between the area of peaks and Principal Components Analysis (PCA) based on the obtained correlation values were carried out (SPSS-X 2.1-FACTOR); interpretation was in accord with the conceptual approach of Gabriel (1971); the PCA treatment has been frequently utilized for interpretation of Py-MS data (Halma *et al.*, 1984; Alcañiz *et al.*, 1987b, 1994). Multivariate Regression, using the stepwise variable selection procedure, of every principal component and each peak (SPSS-X 2.1-REGRESSION) was calculated according to Cuadras (1981), so as to select the peaks being most responsible for the differences among pyrograms; Beck *et al.* (1997) used this analytical system to find the most responsible factors for the variability in the results obtained when using different methods to measure soil microbial biomass. The two mentioned successive statistical analyses (PCA and Multivariate Regression) allowed us to reduce from 18 to 12 the number of Py-GC peaks being most responsible for the pyrograms differences.

— The aforementioned pyrolysis products were distributed into different families, according to the organic matter pool which they represented, by using the data obtained above as well as published data. This distribution led to an interpretation of the differences among organic composition of tested samples.

*Comparative studies between the enzyme activity and the organic matter composition of samples*

Specific BAA-, ZPL- and casein-hydrolysing activities of soil extracts and soil residues were obtained. These activities were compared with those reported by Bonmati (1989) of their corresponding soil. Soil, soil extract and soil residue specific activities, referred to organic C, of each sample were also compared with their corresponding pyrogram composition. Finally total activities of the samples were obtained from among them and with the humine and unhumified organic matter content of their corresponding soil. These procedures allowed us to confirm previously reported hypotheses, or to establish new ones, on the composition of the protease-organic complexes.

Table 2. Organic carbon, humic acids (HA), fulvic acids (FA) and amino acid N contents of samples

	Sample	g C kg <sup>-1</sup> dry residue (soil residues) or g kg <sup>-1</sup> dry soil (soil extracts)			mg amino acid N kg <sup>-1</sup> dry soil		
		replicate	mean	conf. interval <i>P</i> < 0.05	replicate	mean	conf. interval <i>P</i> < 0.05
5	soil						
	residue	3	13.06	2.16	3	1600	180
	extract	3	2.87	0.59	3	106	38
	HA in extract	3	1.12	0.21			
13	FA in extract		1.75	0.84			
	soil				3	1100	180
	residue	3	9.22	2.19			
	extract	4	1.80	0.52	3	36	7
19	HA in extract	3	0.59	0.21			
	FA in extract		1.21	0.27			
	soil				3	1950	90
	residue	3	29.3	2.19			
	extract	8	10.20	0.98	3	345	107
	HA in extract	4	3.40	0.51			
	FA in extract		6.80	0.94			

## RESULTS AND DISCUSSION

The soils had an organic C content ranging from 13 to 48 g kg<sup>-1</sup> and had been air-dried for 6 d before their storage at room temperature for 3 yr. The stability of the protease activities remaining after this treatment had been demonstrated by Bonmatí (1989).

### Extraction yields of organic C and amino acid N

Pyrophosphate was more effective in extracting organic C than amino acid N from soil (Table 2) thus confirming results reported by Stevenson (1982). As can be calculated from Tables 1 and 2, extraction yields of organic C for soils 5, 13 and 19 were 12%, 14% and 21%, respectively, whereas those for amino acid N were 7%, 3% and 18% of the respective total soil contents, which means that an important portion of the soil proteins and peptides are associated with pyrophosphate non-extractable organic matter. About 11% of organic C was extracted by phosphate-EDTA buffer from a fresh pasture soil (Batistic *et al.*, 1980). Percentages of organic C and organic N extracted by 140 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> at pH 7.1 from two forest and one permanent grassland soil ranged from 11% to 43% and from 19% to 38% respectively (Nannipieri *et al.*, 1980).

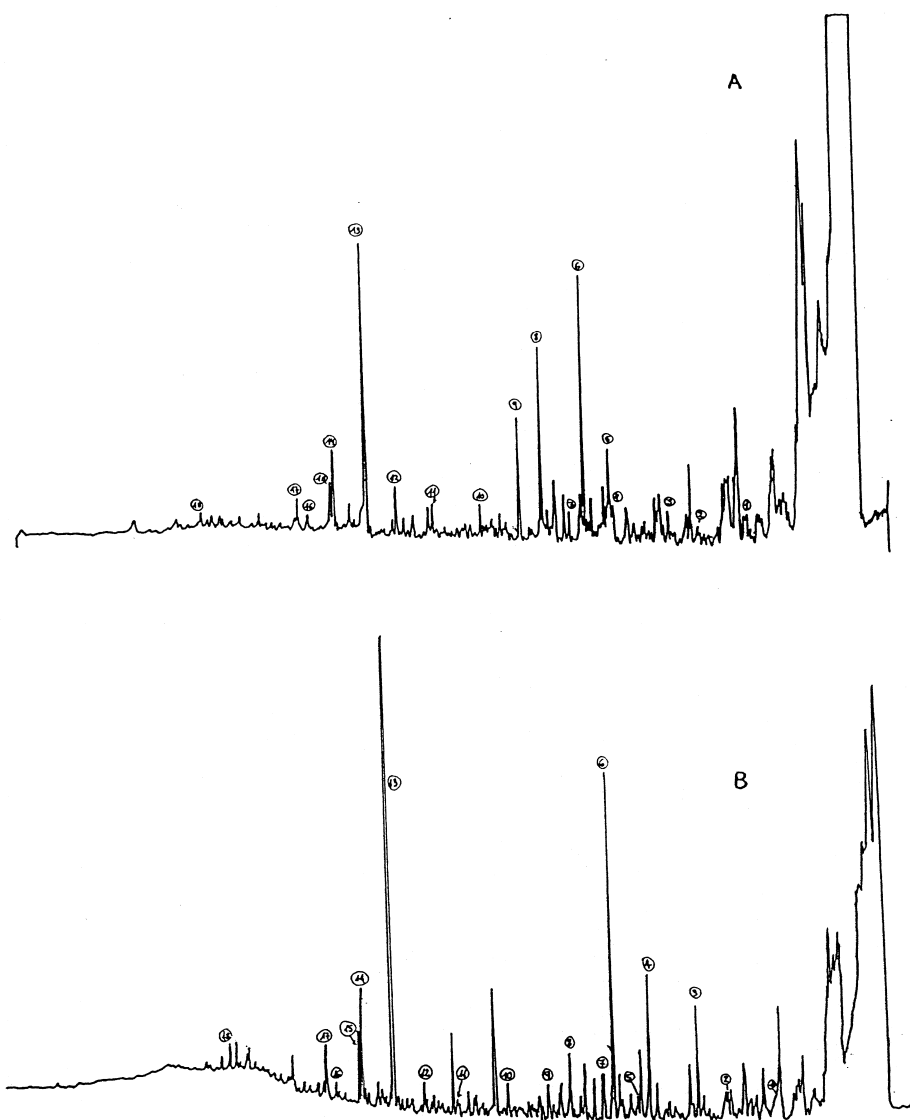
As can be verified by comparing the data in Tables 1 and 2, the extracted humic acids ranged from 45% to 60% of their amounts in soil. Sodium pyrophosphate being a mild extractant, is less efficient than the mixture of Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>-NaOH for extracting humic acids from soil (Giesecking, 1975). The percentage of extracted organic C content present as humic acids ranged from 33% to 39% (data were calculated from Table 2).

### Pyrogram characteristics and overall differences among soil and soil extract organic matter (MDS analysis)

As an example, Fig. 1 shows the pyrograms for soil 5 and its corresponding extract identifying the 18 chosen pyrolysis fragments and listing their corresponding retention times. The normalized peak areas in the whole of pyrograms are reported in Table 3.

Pyridine, pyrrole, 2-methyl-pyrrole, benzonitrile, *p*-toluenitrile and indole mainly proceed from the pyrolysis of polypeptides but partly also from the non-hydrolyzable nitrogen (Alcañiz *et al.*, 1987a). Styrene and *o*-xylene were obtained by the pyrolysis of proteins as well as from carbohydrates and from the aromatic fraction of humus, and they clearly increase during humification (Alcañiz *et al.*, 1987a). Acetic acid, furfural, acetophenone and acetamide derive from the thermal degradation of carbohydrates (Saiz-Jiménez *et al.*, 1979; Irwin, 1982; Ceccanti *et al.*, 1986; Alcañiz *et al.*, 1987a). Guaiacol, ethylphenol and vinyl-guaiacol are characteristic products of the pyrolysis of lignins, which tend to decrease during humification (Bracewell *et al.*, 1980b; Alcañiz *et al.*, 1987a). Phenol and cresols have a less specific origin, being produced from humus, proteins and the pyrolytic cyclization of carbohydrates (Alcañiz *et al.*, 1987a). The origin of cyclopentenone will be commented upon later.

The Multidimensional Scaling gave information about the composition of the organic matter extracted by pyrophosphate. The graphical distribution of pyrograms in two dimensions (Fig. 2) shows that the composition of each soil extract is very different from that of the relative soil; the most important differences (Table 3) can be resumed as follows: contributions by benzonitrile,



Ref. n.	Pyrolysis product	Retention time (minutes)
1	Pyridine + o-xylene	6.20
2	Styrene	8.17
3	Cyclopentenone	9.55
4	Acetic acid	11.40
5	Furfural	11.90
6	Pyrrrole	12.91
7	2-Methyl-pirrole	13.77
8	Benzonitrile	14.79
9	Acetophenone	15.73
10	Acetamide	18.14
11	Guaiacol	19.60
12	p-Toluenitrile	20.98
13	Phenol	22.28
14	p-Cresol	23.62
15	m-Cresol	23.74
16	Ethylphenol	24.73
17	Vynil-guaiacol	25.14
18	Indole	29.24

Fig. 1. Pyrograms of soil 5 (A) and its corresponding extract (B), identifying the 18 more relevant pyrolysis fragments and listing their corresponding retention times

Table 3. Normalized areas of the 18 more relevant pyrolysis fragments of soils, soil extracts and soil residues (see Fig. 1 for the peak identification)

Peaks	1	2	3	4	5	6	7	8	9
Soil 5	3.65	2.62	1.16	0.34	9.29	18.82	2.72	11.24	9.16
Residue	2.40	0.24	0.74	0.52	9.76	24.28	3.61	7.99	4.53
Extract	0.18	0.60	0.36	13.26	0.54	18.86	2.39	3.07	2.35
Soil 13	1.63	1.44	0.61	0.24	6.23	13.88	0.64	42.29	11.40
Residue	0.35	0.89	0.52	0.35	17.87	24.19	0.66	27.17	8.65
Extract	7.35	0.12	7.36	16.67	5.15	16.70	2.91	2.83	0.67
Soil 19	12.35	7.21	5.74	8.32	4.86	11.72	2.22	6.58	2.79
Residue	1.40	1.64	1.14	6.20	11.32	16.62	3.43	4.00	2.76
Extract	0.09	4.99	0.61	14.13	3.24	16.01	2.32	3.20	1.96
Peaks	10	11	12	13	14	15	16	17	18
Soil 5	1.60	2.94	3.20	19.78	5.57	2.67	1.56	2.75	0.93
Residue	2.53	2.82	5.20	19.29	5.86	2.04	1.96	3.62	2.59
Extract	0.29	0.18	1.18	38.79	8.02	3.77	0.62	3.80	1.72
Soil 13	0.49	1.12	1.88	15.89	1.13	0.40	0.24	0.24	0.24
Residue	1.00	1.13	1.71	12.57	0.66	0.35	1.05	0.50	0.35
Extract	3.00	0.18	2.78	20.80	5.74	3.66	1.20	0.12	2.73
Soil 19	3.84	1.80	2.46	18.37	4.77	2.41	1.38	2.07	1.12
Residue	3.04	2.74	3.80	24.70	6.41	3.20	1.96	3.80	1.83
Extract	10.21	1.44	1.07	27.44	5.43	3.10	1.07	2.84	0.85

styrene and acetophenone were higher in soil pyrograms, while those of acetic acid, derived from intact or partially-decomposed fresh carbohydrates (Alcañiz *et al.*, 1987a), and phenol were higher in the pyrograms of soil extracts. Thus a selective extractive action of pyrophosphate occurred. Carbohydrates, present in a high content in soil extracts, play a role in stabilizing humus-enzyme complexes (Mayaudon *et al.*, 1975).

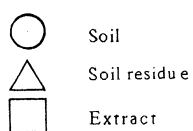
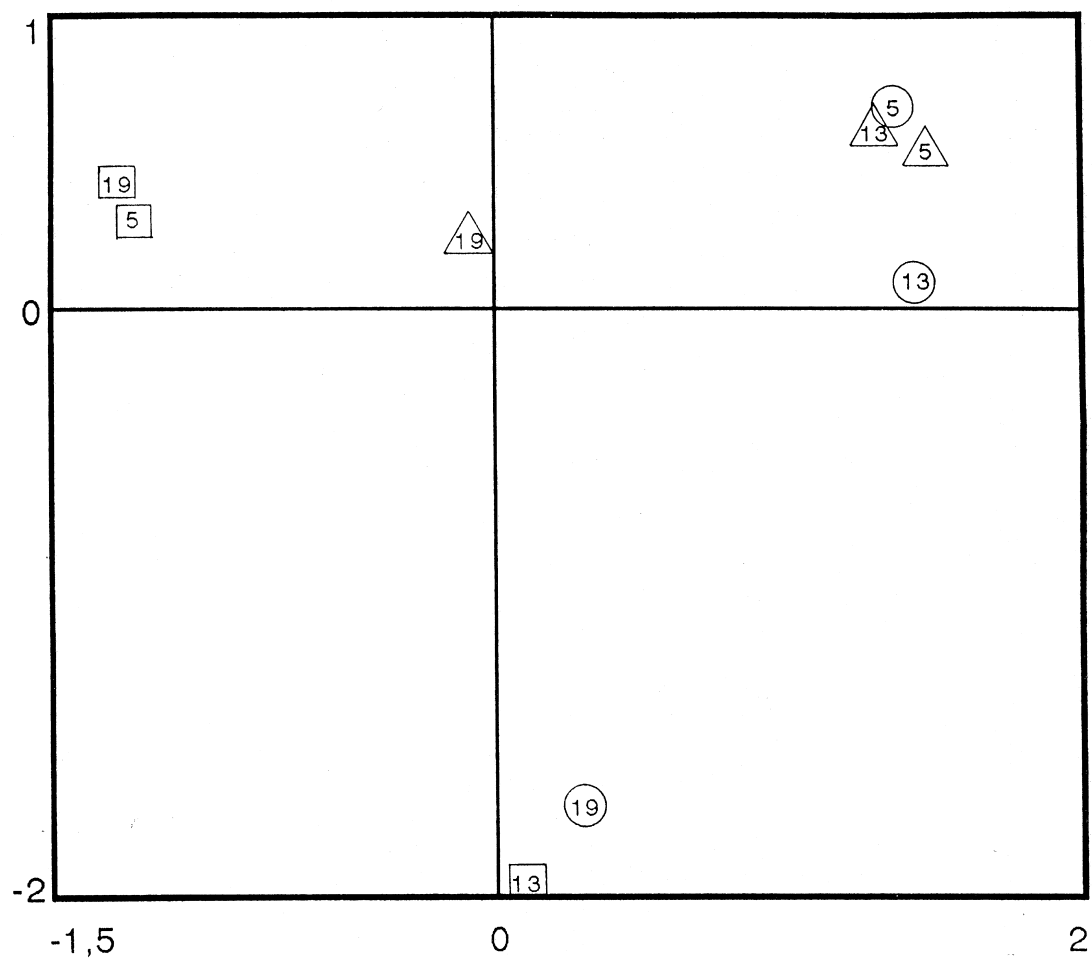
*Selection of the most responsible peaks for the differences between pyrograms (PCA followed by Multivariate Regression analyses)*

The most important correlations between logarithms of pyrogram peak areas are given in Table 4. There were significant ( $P < 0.001$  or  $P < 0.01$ ) and positive correlations among phenol, *m*-cresol and *p*-cresol; most of these phenolic derivatives were also positively correlated with some nitrogenated products, especially 2-methylpyrrole and indole, and with acetic acid, which derives from the thermal degradation of carbohydrates. A highly significant ( $P < 0.001$ ) positive correlation was observed between acetophenone, also deriving from carbohydrates, and a nitrogenated product, benzonitrile. Cyclopentenone, deriving from both plant and microbial aliphatic polycarboxylic acids (Alcañiz *et al.*, 1987a) and appearing in the pyrograms of humified soils (Bracewell *et al.*, 1980a), was very significantly and positively correlated with pyridine plus *o*-xylene. Acetic acid was very significantly ( $P < 0.001$ ) and negatively correlated with benzonitrile and with acetophenone; the same type of correlation was observed between benzonitrile and *m*-cresol or *p*-cresol.

Table 5 shows the matrix for the five top factors in the principal components analysis explaining 91.5% of the total variation among logarithms of pyrogram peak areas. The contributions to the total variation of factors 1 to 5 (data not shown) were 39.0%, 24.7%, 11.9%, 10.0% and 5.9% respectively. Twelve peaks were the most representative of the overall variability among pyrograms when the multivariate regression of each of the five first factors and every peak was considered. The peaks were benzonitrile (B), furfural (F), guaiacol (G), *p*-toluenitrile (T), acetamide (A), vinyl-guaiacol (V), *p*-cresol (C), styrene (S), cyclopentenone (CY), pyrrole (PY), pyridine + *o*-xylene (P + X) and ethylphenol (E). The mathematical expressions defining the relationship between the selected peak areas and each five factors (*f*), as well as their corresponding  $r^2$  and  $P$  values, were the following (each letter means logarithm of the corresponding peak area):

$$\begin{aligned}
 f_1 &= -6.28 B - 1.54 F + 0.81 G + 16.57 T & r^2 &= 0.9992, P < 10^{-5} \\
 f_2 &= 3.16 T + 1.24 A - 2.94 & r^2 &= 0.7514, P = 2 \times 10^{-3} \\
 f_3 &= 2.33 V - 1.97 C + 4.62 & r^2 &= 0.8686, P = 10^{-4} \\
 f_4 &= 0.86 S + 0.58 A + 0.21 CY + 0.25 & r^2 &= 0.9976, P < 10^{-5} \\
 f_5 &= F - 1.32 PY + 0.78 & & \\
 f_5 &= -0.61 A + 0.56 (P + X) + 0.54 & r^2 &= 0.9541, P = 10^{-4} \\
 &= V - 0.55 E + 0.85 & &
 \end{aligned}$$

By considering the correlation analysis and results reported by Irwin (1982), Ceccanti *et al.* (1986) and Alcañiz *et al.* (1987a,b) we have classified the 12 peaks according to the type of organic matter which they represent. Furfural, guaiacol, acetamide, vinyl-guaiacol and ethylphenol are supposed to derive from fresh organic matter while pyridine + *o*-xylene, cyclopentenone and styrene are the products mainly representing the pyrolysis of the humified substances. Carbohydrate derived pyrolysis products are represented by furfural and acetamide while pyridine + *o*-xylene, *p*-toluenitrile



STIMULUS NUMBER	STIMULUS NAME	DIMENSION	
		1	2
1	log soil 5	1.3655	0.6808
2	log resid 5	1.4660	0.5413
3	log extr 5	-1.2163	0.3085
4	log soil 13	1.4268	0.1020
5	log resid 13	1.3033	0.5960
6	log extr 13	0.1089	-2.0322
7	log soil 19	0.2939	-1.7011
8	log resid 19	-0.0694	0.2320
9	log extr 19	-1.2704	0.4295

Fig. 2. Graphical representation of the sample pyrograms in two dimensions according to Multidimensional Scaling



Table 4. The most important correlations among the logarithms of the 18 more relevant pyrolysis fragment areas of soils, soil extracts and soil residues, ordered according to their significance level

Positive correlations	
$P < 0.001$	pyridine + <i>o</i> -xylene with cyclopentenone benzotrile with acetophenone 2-methyl-pyrrole with <i>p</i> -cresol, <i>m</i> -cresol and indole
$P < 0.01$	<i>p</i> -cresol with <i>m</i> -cresol furfural with guaiacol <i>m</i> -cresol with acetic acid and phenol pyridine + <i>o</i> -xylene with <i>p</i> -toluenitrile phenol with <i>p</i> -cresol indole with <i>p</i> -cresol and <i>m</i> -cresol
Negative correlations	
$P < 0.001$	acetic acid with benzotrile and acetophenone benzotrile with <i>m</i> -cresol and <i>p</i> -cresol
$P < 0.005$	benzotrile with 2-methyl-pyrrole and phenol
$P < 0.01$	acetophenone with <i>m</i> -cresol

trile, pyrrole and *p*-cresol are derived from protein. Benzotrile, highly positively correlated with acetophenone, has been chosen to represent the pool of glycoproteins.

#### Differences in the organic composition of samples

According to the above classification five different organic matter pools were obtained by adding the normalized areas of each peak in the pool considered; Table 6 gives the corresponding values obtained in soils, soil extracts and soil residues. By comparing these values and the normalized areas of the individual peaks constituting the pools (Table 3) it is confirmed that pyrophosphate selectively extracts humic molecules from soil. Indeed pyrograms of soil residues have, compared with those of their relative soil, a lower proportion of humified organic matter derivatives (pyridine + *o*-xylene, styrene and cyclopentenone) and a higher proportion of compounds deriving from fresh organic matter, particularly those (furfural, vinyl-guaiacol

and ethylphenol) deriving from ligno-cellulosic complexes (Alcañiz *et al.*, 1987a).

Pyrophosphate is also effective in extracting glycoproteins because the benzotrile area is less in soil residues than in the unextracted soil. Some of these extracted glycoproteins may occur as enzyme complexes, in which the carbohydrate portion lacks enzyme properties but protects the catalytically-active components (Mayaudon, 1986).

Soil extracts were richer in pyrrole and *p*-cresol (both mainly derived from proteins) when their pyrograms were compared with those of the respective soil.

#### Enzyme activities of soil extracts and soil residues and their relationship with the organic matter composition

Total activities and specific activities referred either to organic C or amino acid N of soil extracts and soil residues are shown in Tables 7–9.

Recoveries of total activity in the extracts were almost 100% or higher for ZPL-hydrolysing activity in soils 13 and 19 (Table 8) and for casein-hydrolysing activity in the three soils (Table 9). In addition casein-hydrolysing activity of soil 5 residue was markedly higher than the respective activity of the unextracted soil (Table 9). Recoveries higher than 100% were found for casein-hydrolysing activities extracted by 140 mM  $\text{Na}_4\text{P}_2\text{O}_7$  at pH 7.1 from a soil sampled under beech-coppice (Nannipieri *et al.*, 1980) and for ZPL-hydrolysing activities extracted by 0.1 M Tris–borate at pH 8.1 from a rendzina-pasture soil (Ladd, 1972). Recoveries were over 100% when the latter activities extracted from the coarser fraction (1.0–2.0 mm) were compared with the unfractionated Chernozem-pasture soil (Ladd, 1972).

Regardless of the substrate used, the specific activities of soil extracts referred to amino acid N were higher than in soil, thus apparently indicating

Table 5. Matrix of the five top factors explaining 91.5% of the total variation among logarithms of the 18 most relevant pyrolysis fragments of soils, soil extracts and soil residues (Principal Components Analysis)

log of peak <sup>a</sup> areas	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
log 1	-0.25966	0.81073	-0.31184	0.12747	0.32717
log 2	0.13880	-0.48437	0.40708	0.66041	0.17406
log 3	-0.13587	0.76398	-0.47583	0.16906	0.17879
log 4	0.87133	-0.24135	-0.28320	0.17826	-0.08616
log 5	-0.58992	0.54630	0.03095	0.13097	-0.14731
log 6	-0.10068	-0.07203	0.37902	-0.81930	-0.27330
log 7	0.79967	0.53854	0.17489	-0.07328	0.10010
log 8	-0.98037	0.00212	0.09247	-0.01711	0.11774
log 9	-0.82931	-0.02253	0.46601	-0.03122	0.25146
log 10	0.26261	0.49303	0.08767	0.54378	-0.57787
log 11	-0.43453	0.56004	0.56517	0.32628	0.06378
log 12	-0.14670	0.82980	0.16570	-0.27823	0.23035
log 13	0.78271	-0.42973	0.04197	-0.07665	0.26021
log 14	0.92190	0.21159	0.11326	-0.02231	0.25795
log 15	0.95575	0.19204	0.02792	0.04340	0.12598
log 16	0.38451	0.61429	0.38151	-0.02641	-0.37437
log 17	0.52204	-0.03359	0.80329	0.05639	0.16193
log 18	0.66311	0.65494	-0.06755	-0.30500	0.00072

<sup>a</sup>See Fig. 1 for the peak identification.

Table 6. Values of five different organic matter pools for soils, soil extracts and soil residues, obtained in each sample by adding, within their corresponding pool, the areas of the most responsible peaks for the pyrograms differences

Pools Py-GC products within the pool <sup>a</sup>	Fresh organic matter 5, 10, 11, 16, 17	Humified organic matter 1, 2, 3	Carbohydrates 5, 10	Proteins 1, 6, 12, 14	Glycoproteins 8
Soil 5	18.14	7.43	10.89	31.24	11.24
Residue	20.69	3.38	12.29	37.74	7.99
Extract	5.43	1.14	0.83	28.24	3.07
Soil 13	8.32	3.68	4.72	18.52	42.29
Residue	21.55	1.76	18.87	26.91	27.17
Extract	9.65	14.83	8.15	32.57	2.83
Soil 19	13.95	25.3	8.7	31.30	6.58
Residue	22.86	4.18	14.36	28.23	4.0
Extract	18.80	5.69	13.45	22.60	3.20

<sup>a</sup> See Fig. 1 for the Py-GC product peak identification.

a marked purification effect of the pyrophosphate extraction. Conformational changes in enzyme chains, including unmasking of the active size of these proteases, separation of inhibitors from enzymes or selective solubilization of proteases may occur during the extraction. Different humic substances have an inhibitory action on protease activities (Ladd and Butler, 1975).

Yields of total extracted BAA-hydrolysing activities were always low, ranging from 11% to 36% (Table 7). Ladd (1972) obtained a low extraction yield for BAA-hydrolysing activity using 0.1 M Tris-borate, pH 8.1, as a soil extractant. Low yields were also found in the extraction of these activities by pyrophosphate from a Mollisol and an Histosol but not in an Alfisol (Nannipieri *et al.*, 1980). In addition Ladd and Paul (1973) found that BAA-hydrolytic activity was relatively stable in soil. Since humine is not extracted by pyrophosphate (Stevenson, 1982) it is reasonable to hypothesize that most of BAA-hydrolysing activity is associated with humine. Indeed, a significant and positive ( $P < 0.001$ ) correlation was found between this enzyme activity and the humine content of soil (Bonmati, 1989). On the other hand BAA-hydrolysing activities of soil residues and also their specific activities referred to the organic C content were lower than those of the corresponding soil (Table 7). If BAA-hydrolysing activity was mostly associated

with humine, the low activity found in the soil residues could be explained by the mechanical disintegration of sodium pyrophosphate over the humine-enzyme complexes during the extraction (Ceccanti *et al.*, 1982, 1986). This action would separate some of these complexes bound through electrostatic bonds, rendering the enzymes unprotected and hence susceptible to proteolysis.

Casein- and ZPL-hydrolysing specific activities of soil extracts and soil residues were, with the exception of the ZPL-hydrolysing activity of the soil 19 residue, higher than those of the corresponding unextracted soils (Tables 9 and 8, respectively). The most marked enhancements were for the ZPL-hydrolysing activity of soil 13 extract and for the casein-hydrolysing activity of soil 5 residue.

The pyrogram of soil 13 extract had a higher proportion of pyridine + *o*-xylene and cyclopentenone than the pyrogram of the respective soil, whereas pyrograms of the other two soil extracts showed lower proportion of these compounds than the respective soil pyrograms (Table 3). Indeed soil 13 extract contained more humified organic matter and proteins than the respective soil; the reverse was true for the other two soil extracts (Table 6). The comparison of the same peaks in the pyrograms (Table 3) may also explain why ZPL-hydrolysing specific activity was low in soil 19 residue, in whose pyrogram the proportions of pyridine + *o*-xylene

Table 7. BAA-hydrolysing activities of soils (S) soil extracts (E) and soil residues (RES)

Sample	<i>n</i>	Units <sup>a</sup>		Recovery <sup>b</sup> (%)	Units g <sup>-1</sup> organic C	Units μg <sup>-1</sup> amino acid N
		$\bar{x}$ <sup>c</sup>	conf. interval of $\bar{x}$ ( $P < 0.05$ )		$\bar{x}$	$\bar{x}$
S5 <sup>d</sup>	3	2.153	0.371		93	135
E5	3	0.237	0.071	11	83	2240
RES5	3	0.209	0.063	10	16	ND
S13 <sup>d</sup>	3	0.966	0.193		73	880
E13	4	0.113	0.029	12	63	3140
RES13	5	0.452	0.113	47	49	ND
S19 <sup>d</sup>	3	2.988	0.745		63	1530
E19	4	1.080	0.352	36	106	3130
RES19	6	0.821	0.258	27	28	ND

<sup>a</sup>BAA-hydrolysing activity ( $\mu\text{mol N-NH}_4^+$  g<sup>-1</sup> dry soil h<sup>-1</sup>).

<sup>b</sup>Recovery: percent activity calculated with respect to that of the corresponding soil.

<sup>c</sup> $\bar{x}$  = mean.

<sup>d</sup>Data obtained from Bonmati (1989).

Table 8. ZPL-hydrolysing activities of soils (S) soil extracts (E) and soil residues (RES)

Sample	<i>n</i>	Units <sup>a</sup>		Recovery <sup>b</sup> (%)	Units g <sup>-1</sup> organic C	Units μg <sup>-1</sup> amino acid N
		<i>x</i> <sup>c</sup>	conf. interval of <i>x</i> ( <i>P</i> < 0.05)		<i>x</i>	<i>x</i>
S5 <sup>d</sup>	4	0.860	0.159		37	540
E5	4	0.333	0.095	39	116	2140
RES5	5	0.726	0.174	84	55	ND
S13 <sup>d</sup>	5	0.485	0.111		37	440
E13	3	2.363	1.017	487	1313	65 600
RES13	5	0.472	0.130	97	51	ND
S19 <sup>d</sup>	6	2.336	0.260		49	1200
E19	4	2.110	0.719	90	207	6120
RES19	6	0.657	0.274	28	22	ND

<sup>a</sup>ZPL-hydrolysing activity (μmol leucine g<sup>-1</sup> dry soil h<sup>-1</sup>).

<sup>b</sup>Recovery: percent activity calculated with respect to that of the corresponding soil.

<sup>c</sup>*x* = mean.

<sup>d</sup>Data obtained from Bonmatí (1989).

and cyclopentenone were 89% and 80% respectively, less than in the corresponding soil, whereas in the soil and soil residue pyrograms of samples 5 and 13 these ratios were only 1.5 and 4.5 and 1.6 and 1.2, respectively. It can be concluded that ZPL-hydrolysing activity is probably associated with the humified material represented by pyridine + *o*-xylene and cyclopentenone in the pyrograms.

In the case of soil 5 residue (Table 3) the peak of styrene in the pyrogram of this sample was 9% of its corresponding unextracted soil, whereas in the soil and soil residue pyrograms of samples 13 and 19 these same ratios were 60% and 25%, respectively. This much lower styrene content mainly explains the lower humified organic matter content of the soil residue when compared with the relative unextracted soil in sample 5 (Table 6). Probably the humified organic matter represented by styrene as pyrolytic product has a negative influence on the casein-hydrolysing activity and this may explain the high activity of the soil 5 residue. Bonding of the protease to humus might protect the enzyme protein against proteolysis but might also render it inaccessible and thus inactive towards substrates of high molecular weight such as casein (Rowell *et al.*, 1973). This hypothesis was also supported by the fact that, as commented before, soil residues (with higher specific caseinolytic activity than their rela-

tive soils) have a lower proportion of humified organic matter and a higher proportion of fresh organic matter derivatives than their corresponding soils. Casein-hydrolysing activity was found significantly and positively correlated (*P* < 0.001) with the unhumified organic matter content of soils (Bonmatí, 1989).

In conclusion our results prove that pyrophosphate is an efficient extractant of protease-humic complexes, including those containing glycoproteins, from arable soils and that the extracted organic matter is especially rich in intact or partially-decomposed carbohydrates. Comparison of data obtained in Py-GC analyses and in protease activities confirmed that BAA-hydrolysing proteases are generally associated with highly condensed humic matter, while ZPL-hydrolysing proteases are probably associated with less-condensed humic material. Casein-hydrolysing proteases are generally associated with non-humified organic matter. Further studies are in progress to characterize the organic-proteases complexes extracted from arable and pasture soils. The studies include molecular weight fractionation of extracts and characterization of the relative fractions by the same techniques used in the present work and also by isoelectric focusing. The derivative fractions will be also characterized for

Table 9. Casein-hydrolysing activities of soils (S) soil extracts (E) and soil residues (RES)

Sample	<i>n</i>	Units <sup>a</sup>		Recovery <sup>b</sup> (%)	Units g <sup>-1</sup> organic C	Units μg <sup>-1</sup> amino acid N
		<i>x</i> <sup>c</sup>	conf. interval of <i>x</i> ( <i>P</i> < 0.05)		<i>x</i>	<i>x</i>
S5 <sup>3</sup>	7	0.199	0.022		9	124
E5	4	0.248	0.072	125	86	2330
RES5	4	1.929	0.279	969	145	ND
S13 <sup>3</sup>	6	0.163	0.035		12	150
E13	4	0.164	0.038	101	91	4470
RES13	4	0.163	0.082	100	18	ND
S19 <sup>3</sup>	7	0.605	0.030		13	310
E19	4	0.543	0.142	90	53	1570
RES19	4	0.531	0.163	88	18	ND

<sup>a</sup>Casein-hydrolysing activity (μmol tyrosine g<sup>-1</sup> dry soil h<sup>-1</sup>).

<sup>b</sup>Recovery: percent activity calculated with respect to that of the corresponding soil.

<sup>c</sup>*x* = mean.

<sup>d</sup>Data obtained from Bonmatí (1989).

their thermal stability and optimal pH and temperature values.

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