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HPLC and ninhydrin photometric determination of amino acids in $CaCO_3$ rich lacustrine sediments of the Jura region, France.

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Keywords : amino acids, calcium, sediment, lake, HPLC, ninhydrin.

A study has been carried out on the acid soluble fraction (hydrosysis at pH 1.5, 3N HCl, 20 % KCl) of organic matter (OM) in the highly calcareous lacustrine sediments of the Jura region, France. Amino acids (AA's) were quantified and determined ninhydrin photometry and HPLC respectively. There was a high degree of interference by calcium in both procedures. The mechanism of interference is explained and methodology adopted to overcome it, by removal of calcium, are dealt with. The results of both methods are compared.

Dosage des acides α-aminés dans les sédiments lacustres jurassiens en présence de fortes teneurs en $CaCO_3$. Comparaison de deux méthodes : colorimétrique et chromatographique

Mots clés : acides aminés, calcium, sédiment, lac, CLHP, ninhydrine.

Des études sont réalisées sur des sédiments de nombreux lacs Jurassiens, à matrice calcaire. Elles portent en particulier sur la matière organique. Nous nous sommes particulièrement intéressés à la fraction acidosoluble extraite à pH 1.5 à l'aide de HCl 3N à 20 % KCl et avons cherché à quantifier globalement les acides aminés puis à identifier les plus abondants d'entre eux. Que ce soit pour le dosage global, par colorimétrie ou pour leur identification par CLHP, nous avons rencontré des difficultés dues à une forte teneur en calcium dans nos échantillons. Nous traitons ici du rôle du calcium sur les deux méthodes analytiques, de son élimination et comparons les résultats des deux méthodes.

1. Introduction

This study was carried out on Jura (France) lake sediments listed in table 1 (Verneaux 1987). It concerned the organic matter (OM) and its characterization. To extract the OM the sediment was submitted to various increasing pH hydrolysis solutions (Bruckert 1983). The pH ranged from 1.5 to 12 leading to three kinds of organic matter: acidic organic matter (AOM), which is soluble at low pH (pH < 1.5), basic organic matter (BOM), soluble at intermediate pH's (5 < pH < 12), insoluble matter (IOM), which is not soluble at low or high pH.

AOM was mainly studied and amino acids (AA's) were found in abundance (Decau 1969). The aim was to determine the total amount of AA's and their individual identification. Thus, two methods were used to obtain the relevant information. Firstly a photometric method to obtain the amount of AA's, which is a modification of the procedure described by Pesez (1982) and secondly an HPLC method described by Peter (1982) for the identification.

This paper only deals with the methodological aspect of the determination of AA's in presence of high contents of calcium involving modifications of both analytical methods.

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Table 1. Analyses of the lakes sediments (results expressed as mg/g of dry sediment).
Tableau 1. Analyses des sédiments lacustres (les résultats sont exprimés en mg/g de sédiment sec).

Lake	Depth (m)	C/N	Ca^{2+} mg/g	SiO_2 mg/g	COT mg/g
L'Abbaye	17	9.2	200	212	171
Ilay	25	11.4	254	109	97.9
Saint Point	30	11.0	336	38.2	35.0
Clairvaux	22.5	8.5	302	120	23.6

2. Materials and methods

2.1. Sampling procedure

Sediments cores were taken using a gravity core sample (Rofes 1980, 1981). Surface sediments (0-5 cm) were collected. The first five centimeters correspond to the sedimentation of the past twenty years (Verneaux 1988). On each location, two cores were taken and mixed to obtain a sample of 1 l. The samples were kept at 4°C prior to analysis.

2.2. Processing of sediments

The sediments were dried to a constant weight at 80°C, ground, then sieved at 2 mm and homogenized. Hydrolysis was carried out using 3 N HCl for 24 h so that the pH remained constant at 1.5. Hydrolysis was carried out on 20 g of dry sediment using 60 ml of 3 N HCl with 20 % KCl leading to a 250 ml solution (H solution). The AA's were determined on H solution.

2.3. Analytical methods

— The photometric ninhydrin method

The action of ninhydrin on AA's, using a reducing agent ($SnCl_2$) at pH 5 led to a colored complex (Moore 1954): Ruhemann's crimson, detected at 572 nm by spectrophotometry (Fig. 1). Reagents used were: (a) a buffer solution (pH 5), (b) a 1% ninhydrin solution, (c) a solution at 2.5% $SnCl_2$ in glycerol, (d) HPLC grade water. All reagents were purchased from Merck, Darmstadt (Germany). To an aliquot of 1 ml of AA's solution, 500 µl of (b) and 500 µl of (c) were added in a test tube. The capped tubes were shaken briefly (< 10 s) and heated for 15 min (accurately timed) in a covered boiling water bath. The tubes (kept out of direct light) were cooled to 0°C for 2 min, then 2 ml of (d) were added. The colored complex was then detected at 572 nm using a CARY spectrophotometer (Varian, Les Ulis, France).

— The HPLC method

Free amino acids were determined as their o-phthaldehyde (OPA) derivatives by reversed phase (RP) HPLC (Peter 1982) as shown in figure 2. Derivatisation was carried out in 2 ml pre-baked glass vials by mixing 200 µl of aqueous solution with 200 µl of OPA reagent, prepared by dissolving 40 mg of OPA in 1.0 ml MeOH, at pH 9.5, 150 µl BRIJ (30 % in H_2O) and 50 µl mercaptoethanol. The content was mixed for 30 s. OPA derivatives were separated on a LiChroCART® (125 mm x 4 mm) RP column. A RP-18 4mm cartridge guard column was used. All reagents and columns were purchased from Merck, Darmstadt (Germany). Derivatives were eluted from the column using a gradient program and a ternary solvent system at a flow rate of 1 ml/min (model 9010 solvent system delivery from Varian, Les Ulis, France). Solvent (A) was MeOH, solvent (B) a buffer solution (pH 9.5) and solvent (C) tetrahydofuran (THF). Table 2 shows the gradient of elution used. The OPA derivatives were detected using a UV detector at 336 nm (model 9050 variable wavelength UV-VIS detector from Varian, Les Ulis, France). The data were collected and analyzed using the LC Star® software (developed by Varian, Les Ulis, France) with a compatible IBM computer.

3. Results

3.1. Processing using the photometric ninhydrin method

The reliability of the method was tested on standard solutions of glycine (Fig. 3). However, when the method was applied to the H solution, there was no coloration, even on spiked solutions. Usually coloration arises during the heating step of the procedure at 100°C. A component from the matrix prevented the coloration from appearing. Unless the buffer solution (a) was sufficient to stabilize the pH to 5, suitable for the formation of the Ruhemann's crimson, the reaction

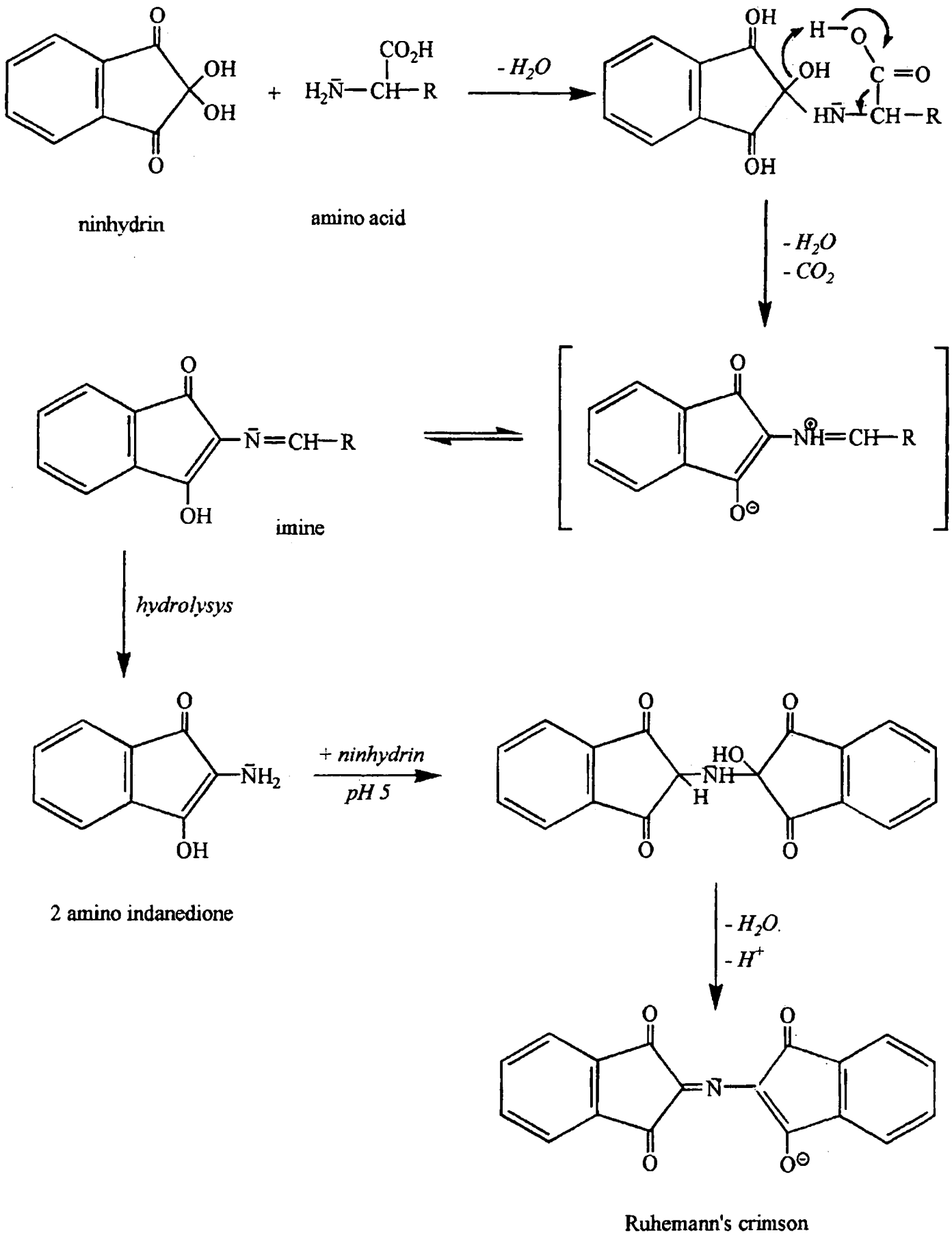

Fig. 1. Mechanism of the reaction between AA's and ninhydrin (Moore 1954).
Fig. 1. Mécanisme réactionnel intervenant entre les acides aminés et la ninhydrine.

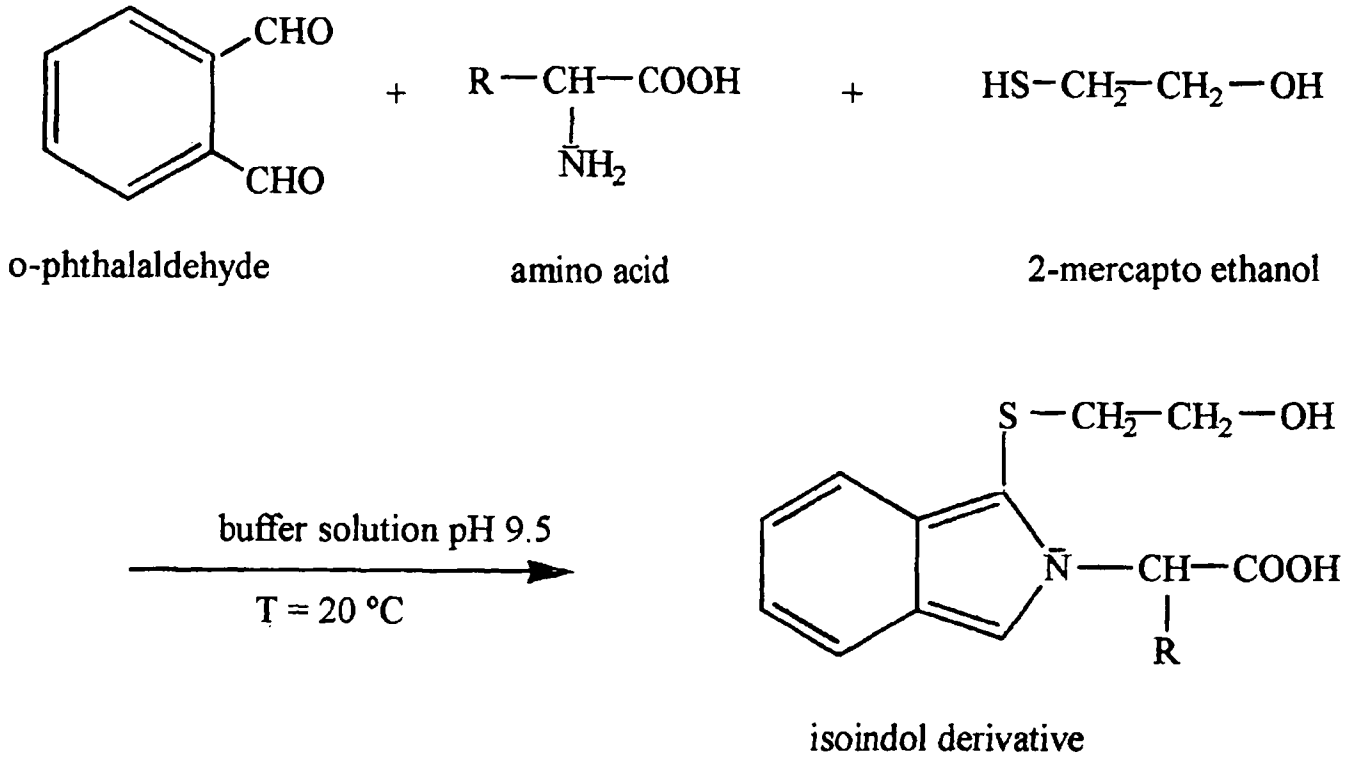

Fig. 2. Mechanism of the reaction between AA's and OPA (Pesez 1982).
Fig. 2. Mécanisme réactionnel intervenant entre les acides aminés et l'OPA.

Table 2. Elution gradient for the HPLC method.
Tableau 2. Gradient d'élution utilisé en CLHP.

time (min)	MeOH %	Buffer pH 9.5 %	THF %
0	20	77	3
5	20	77	3
10	30	67	3
15	40	57	3
20	50	47	3
30	80	17	3

did not occur when reagents (b), (c) and (d) were added. H solution contained K^+, Cl^- from the hydrolysis solution and Ca^{2+} from the dissolution of the $CaCO_3$ abundant in Jura sediments as shown in table 1. The influence of each ion on the titration of the AA's was determined.

3.1.1. Influence of K^+ and Cl^- ions

Spiked solutions of AA's were prepared so that their concentration in K^+ and Cl^- were similar to those found in H solutions. The spiking level was easily recovered. There was an excellent correlation between the results and the spiking level.

3.1.2. Influence of Ca^{2+}

Spiked solutions of AA's were prepared at different calcium levels. The amount of calcium ranged between 4 and 20 g/l with respect to the level found in H solutions. Figure 4 shows how calcium ion concentration influences the response of the photometric device for a

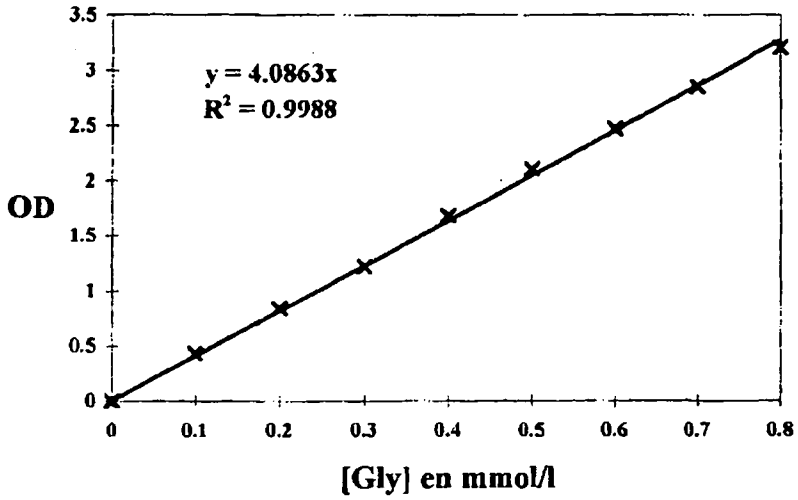

Fig. 3. Standard calibration curve for the ninhydrin photometric method (OD v. [Gly]).

Fig. 3. Courbe de calibration pour la glycine en utilisant la méthode de photométrique à la ninhydrine (DO v. [Gly]).

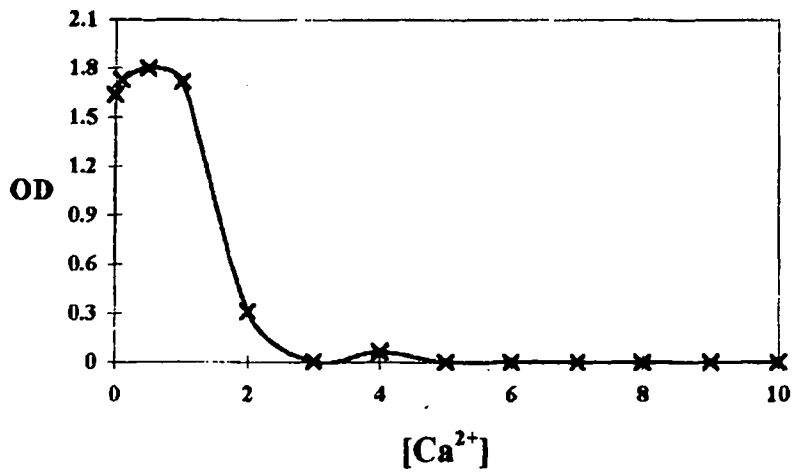

Fig. 4. Calcium interference using the ninhydrin photometric method (OD v. [Ca^{2+}]).

Fig. 4. Interférence du calcium sur la méthode photométrique à la ninhydrine (DO v. [Ca^{2+}]).

known amount of AA's. At low concentration (0< Ca^{2+} <1 g/l) AA's were easily recovered, whereas at intermediate concentration (1< Ca^{2+} < 3 g/l), the optical density (OD) quickly decreased to become nearly zero for a concentration of 3 g/l. Therefore, a high concentration of calcium ions prevented coloration; an explanation could be the formation of a divalent salt at pH 5. AA's were found in form I (Fig. 5) (Siegel 1971), so the presence of calcium would lead to a chelate following the reaction in figure 6 (Evans 1979). This chelate is a barrier to the first step of the reaction between ninhydrin and AA's (Fig. 1), the functional groups involved in the reaction were inhibited by calcium, thus the reaction did not occur. Numerous ways were investigated to remove the calcium.

3.1.3. Removal of the calcium

Three ways of removal were investigated. Firstly a complexation was made with EDTA, a strong chelating agent (Siegel 1971), unless the complex was stable during the heating step, it decomposed releasing Ca^{2+} in solution. Then NaOH was added to the H solution to form $Ca(OH)_2$ and to remove Ca^{2+}. Precipitation occurred followed by centrifugation (5000 rev./min for 20 min), but it was difficult to obtain a pH of 5 suitable for the first step of the reaction. Finally, it was decided to remove Ca^{2+} using $Na_2C_2O_4$. The calcium was precipitate as CaC_2O_4. The precipitate was eliminated by centrifugation. The precipitate was washed to remove any adsorbed AA. The washing solution was analysed showing no adsorption.

Hence, these results led to the modification of the sediment processing step, involving the addition of $Na_2C_2O_4$ after the hydrolysis, followed by centrifugation (5000 rev./min for 20 min). The analysis was carried out on the resulting solution.

3.2. Processing using the HPLC method

The reliability of the method was tested on standard solutions containing a mixture of AA's showing its sensitivity and repeatability (Fig. 7). However, when applied to the H solution, i.e. without treatment using $Na_2C_2O_4$, the top chromatogram in figure 8 was obtained. Assuming calcium prevented the reaction from occurring, the H solution was treated with $Na_2C_2O_4$ to obtain the bottom chromatogram in figure 7 giving the identification of each AA (Table 3).

4. Discussion

A comparison of both methods shows a good correlation of the results (r = 0.9860) if expressed as µmole/g of dry sediment (Fig. 9). The results were expressed as µmole/g of dry sediment because the photometric method was not selective and gave the global

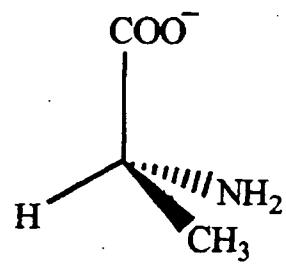

Fig. 5. Structure of the AA at pH 5.

Fig. 5. Structure des acides aminés à pH 5.

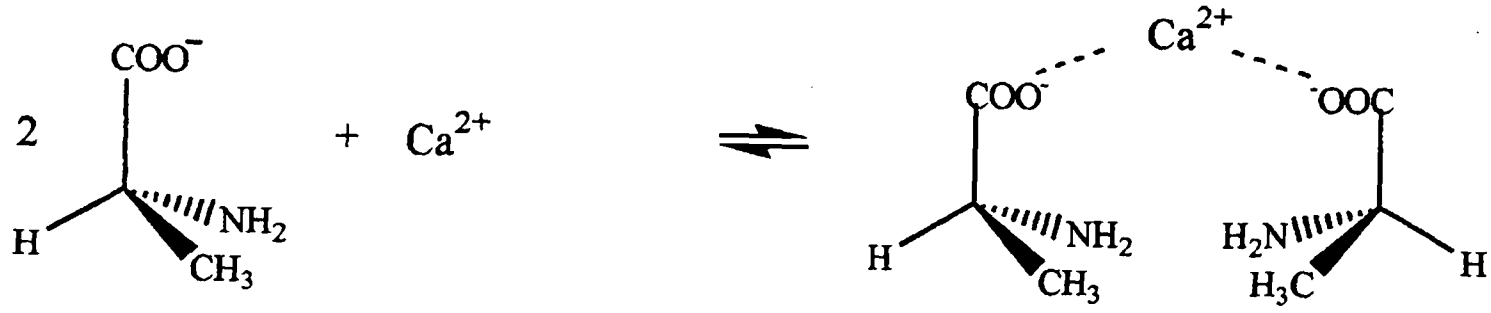

Fig. 6. Possible reaction between calcium and AA.
Fig. 6. Réaction probable intervenant entre les acides aminés et le calcium.

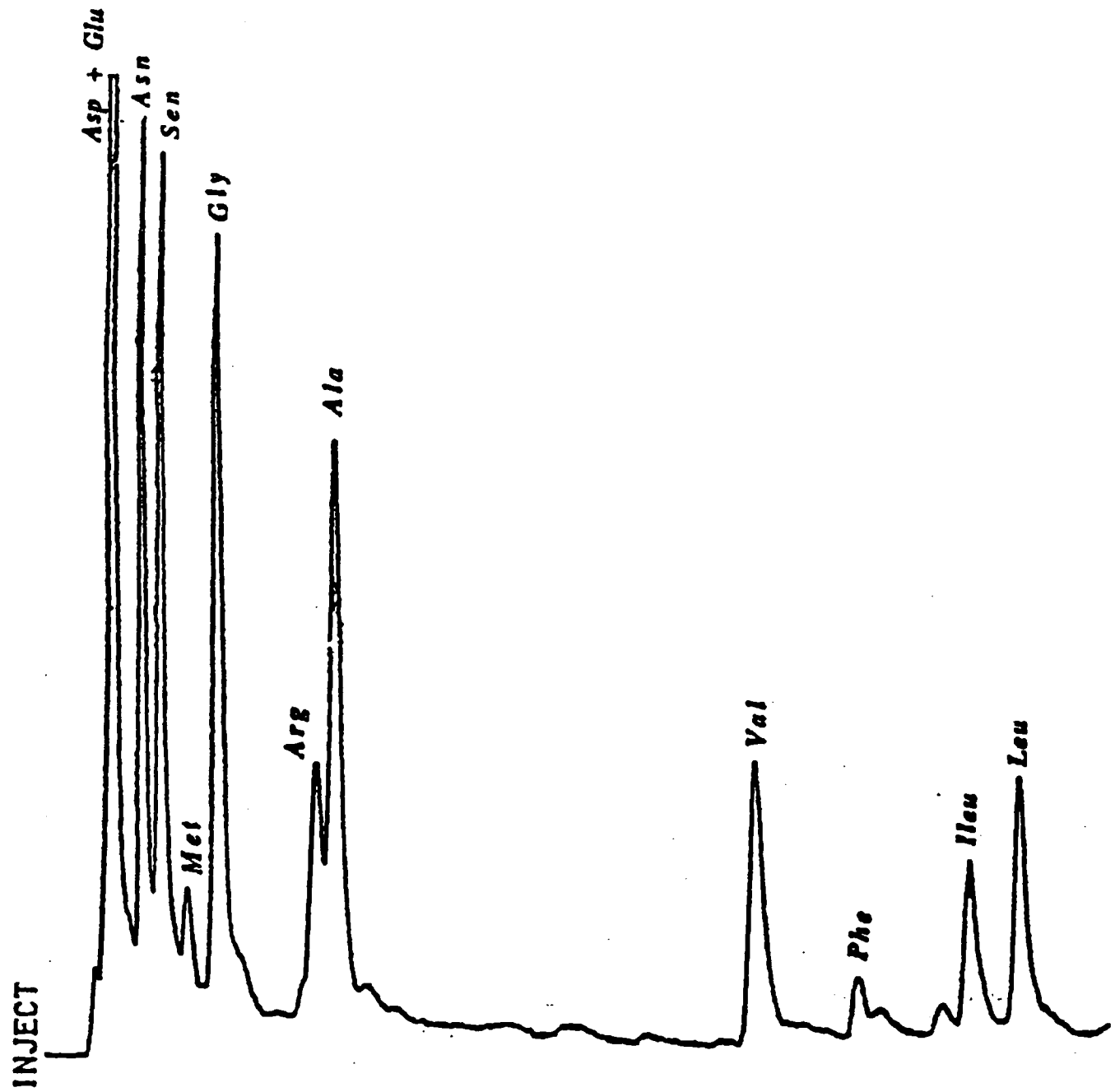

Fig. 7. Chromatogram of a standard solution of 12 AA's.
Fig. 7. Chromatogramme d'une solution étalon de 12 acides aminés.

Table 3. Amino acids found in µmol/g of dry sediment by HPLC (Z_{max} = maximum depth of the lake, 1/2 Z_{max} means half of Z_{max}). n.d. means not detected.

Tableau 3. Acides aminés détectés par CLHP en µmol/g de sédiment sec (Z_{max} = profondeur maximale du lac, 1/2 Z_{max} à la moitié de cette profondeur). n.d. signifie non détecté.

	L'Abbaye		Ilay		Saint Point		Clairvaux	
amino acid	Zmax	½ Zmax	Zmax	½ Zmax	Zmax	½ Zmax	Zmax	½ Zmax
Asp + Glu	1.8	1.2	0.2	0.4	1.2	0.5	0.5	0.3
Asn	0.5	0.5	n.d.	0.1	n.d.	0.1	0.2	0.1
Ser	0.8	0.8	0.25	0.1	0.1	0.7	0.3	0.3
Gly	4	4	0.2	0.5	2.9	0.8	2.7	0.8
Ala	1.1	0.9	n.d.	0.1	n.d.	0.3	0.7	0.4
Val	0.2	0.7	0.25	n.d.	n.d.	0.2	0.5	0.3
total	8.4	8.1	0.9	1.2	4.2	2.6	4.9	2.2

Fig. 8. Chromatograms of H solution after derivation with OPA (a) without treatment , (b) treated with $Na_2C_2O_4$.

Fig. 8. Chromatogrammes de la solution H aprËs derivation avec de l'OPA (a) sans traitement, (b) avec traitement au $Na_2C_2O_4$.

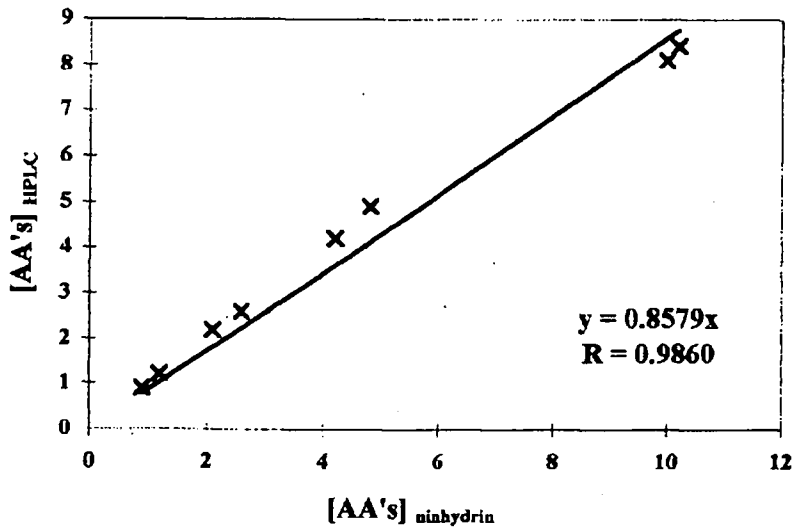

Fig. 9. Comparison of both methods ([AA's from HPLC] v. [AA's using photometry]).

Fig. 9. Comparaison des résultats obtenus par les méthodes (en abscisse les résultats obtenus avec la ninhydrine, en ordonnée les résultats obtenus par CLHP).

amount of AA's reacting with ninhydrin, whereas the HPLC method provided an individual identification. HPLC analysis of Jura sediments showed that the dominant AA's were aspartic and glutamic acids, serine, glycine, alanine and valine. Decau (1969), Goh (1979) and Stanley (1987) found similar results. However, there was no relevant data on the carbonate content of these sediments providing an opportunity to establish a comparison between the results. The AA content was a little less than those reported for other lake sediments (e.g. 145 µmol/g for Lake Haruna: Yamamoto 1992) and ranged from 1 to 10 µmol/g. This may be due to the carbonated matrix involving a basic pH giving adsorption of acidic AA's and none or little adsorption of basic AA's. Mitterer (1968) found a similar amount in natural oolites which consisted mainly of carbonate calcium.

Having accurate analytical methods of determination of AA's in Jura lake sediments, rich in calcium carbonate, many samples have been analysed in order to better understand the transformations of the lacustrin uptake. These results will be discussed in a forthcoming article.

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