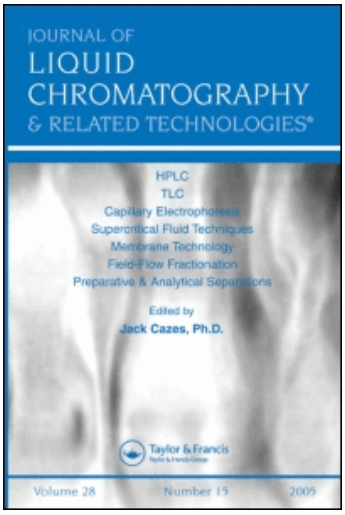

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THE SEPARATION OF 24 OPA-AA OF NATURAL ORIGIN AND QUANTITATIVE ANALYSIS OF TYROSINE BY MEANS OF HPLC

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ABSTRACT

The separation of a mixture of 24 amino-acids (AA) of natural origin is carried out by column HPLC μ Bondapak C18 10 μm, following pre-column derivatization using orthophthalaldehyde (OPA). Because of the large numbers of AA to separate, the use of a linear gradient making use of the diversity of the AA polarities ensures the resolution of the mixture in less than 45 min.

The gradient is established through the progressive addition of acetonitrile to monopotassic phosphate solution (pH 6.80). The thioisoindol derivatives, which are thus formed and wich absorb greatly at 340 nm, are detected with a spectrophotometer. Quantitative analysis of tyrosine is carried out by means of the internal calibration method with two calibration substances : glutathion and 6amino caproic acid.

This analysis, which is directly applicable in industrial surroundings to the separation of primary AA, is both reproducible and sensitive.

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INTRODUCTION

The beginnings of modern HPLC coincide with those of analyses involving AA by Stein and Moore in 1958 : these authors used resin chromatography exchanging ions with post-column derivatization by ninhydrine. This technique, however, requires the preparation of many buffer solutions, an unreasonably long analysis time, and equipment specific to this type of analysis. And yet, the importance of AA has never stopped increasing, and their quantitative analysis are more and more important both from medical, alimentary and pharmaceutical standpoints.

In order to make up for the disadvantages of the first analyses, and fulfill modern requirements, other reagents enabling the carrying out of pre-column derivatization habe been used, such as phenylthio-hydantoine (PTH) (1, 2, 3) dansyl-chloride (4, 5) dimethylazobenzene sulfonyl (DABS) 6, fluorescamine, and orthophthalaldehyde (OPA).

The PTH derivatives obtained by Edman degradation display heating constraints which may lead to partial hydrolysis of amides. Moreover, this methods is time consuming for routine use, and all AA do not react to the same reagent (taurine, β alanine, GABA).

Dansyl chloride is difficult to use as it may bring about secondary reactions causing serious interference when high sensitivity is concerned.

The formation of DABS alco requires heating at a temperature of 70°C, causing the same problems as PTH. Derivatization with fluorescamine is rapid and quantitative, but the derivatives formed are voluminous and are often double. OPA is interesting because the fluophoric derivatives are in aqueous medium, at room temperature, and without purification previous to their injections. In the presence of a reducing agent, 2 mercapto-ethanol (2ME) or ethanethol (Et-SH), OPA reacts specifically and quickly with the amine group of amino acids to form substituted thioisoindols which greatly absorb at 340 nm and are fluorescent. However, like the previously mentioned reagents, they have certain disadvantages : the nature of the formed derivatives, and the

reconstitution of the reagent influence the stability of the OPA-AA. Nonetheless, the technique can be adapted to get around these difficulties.

That is the reason we attempted to develop a system for analyzing AA which could be rapid, effective, sensitive and specific, using basic equipment flexible enough for the methodology to be applicable to a great number of analyses.

EQUIPMENT AND METHOD

Equipment :

The Waters Associates chromatograph (Milford, Mass.) used for the operation is composed of 2 pumps (M6000A and F6000A), an M660 solvent programmer, a U6K injector, and an M440 spectrophotometer with a fixed wave length. Separation takes place using a column (μ Bondapak C18 Waters - 300 x 3.9 mm. i.d. 10 μm), proceded by a precolumn lined with Corasil C18.

Peaks are registered on an Omniscribe B5000 recorder.

Mobile phases :

One of the pumps is fed with pure acetonitrile (CH_3CN, Uvasol Merck), known as solvent B. The other is fed with a monopotassic phosphate solution KH_2PO_4 0.02 M pH 6.8 (solvent A) made by dissolving 0.68 g of monopotassic phosphate (Prolabo) in one liter of distilled water containing 2 % tetrahydrofuran (THF, Uvasol Merck), and a sufficient quantity of NaOH 0.1 N to obtain a pH of 6.80.

This aqueous solution is filtered using Millipore filters 0.45 μm before use, and can be kept for several days at a temperature of + 4°C.

Reagent used for derivatization :

54 mg of OPA (Sigma) are dissolved in 1 ml of methanol (Merck analysis quality). After total solubilisation, 9 ml of borate buffer solution 0.4 M pH 10 are added, as well as 100 µl of 2 ME (Riedel de Haen). The reagent stored at a temperature of +4°C and protected from light, is stable for about ten days as long as 20 µl of 2ME are added every 3 or 4 days. This should be prepared at lest 24 hours before use.

AA standard solutions :

10 mg of each of the 24 AA listed in table 1 are dissolved in 10 ml of hot solvent A ; the solution may be stored at a temperature of +4°C for about two weeks.
In order to quantify the tyrosine, internal standard solutions are prepared : 200 mg of glutathion (Merck) and 6 amino caproic acid (ε Ahx, Fluka) in 10 ml of solvent A constitute the stocksolution. The working solution is a dilution to the 1/16th containing 1.25 g of standard solution per liter of solvent A.
The range of standardisation of the tyrosine is obtained by successive dilution of a stocksolution containing 1 g of tyrosine per liter of solvent A :
The calibration points are in the range : 0,5 - 0.25 - 0.125 - 0.0625 g/1.

Products to be analysed :

The product under study is an autolysate of natural origin.
The distilled water and the solvent A are filtered. Any precipitates which might be found in certain solutions are retained inside the precolumn.

Chromatographic conditions :

The linear gradient is programmed from 5 to 50 % of CH_3CN in 45 min. The other parameters which are experimentally fixed are : the flow rate 1.4 ml/mn, the detection wave-length, 340 nm, the processure of the pumps, 2.500 psi, the sensitivity, 0.2 AUFS, the speed of the recorder, 0.5 cm/mn, and the ambiant temperature. The return to the initial conditions consists of programming the reverse gradient for 10 min. and allowing the column to stabilize for about 15 min. It is essential after work to thoroughly wash the column with distilled water for a period of 30 min. at a flow rate of 2 ml/min. before keeping it for storage.

Protocol for derivatization :

The internal standard working solution is mixed volume for volume with either the standard AA solution, or the tyrosine solution, or the product being analysed.
Then, 40 µl of this mixture are derivati ed using 200 ul of reagent containing the OPA-2ME for exactly 2 min. before an injection of 10 ul.
(AUFS = Absorbance Unit Full Scale).

RESULTS

Figure 1 shows the separation of the standard 24 AA and the 2 internal standards in a solution versus a derivatized blank containing neither AA nor internal standards. The evolution of the percentage in solvent B is shown in dotted lines.
Under these conditions, the complete elution of the mixture is performed within 36 min. Return to the base line is effective for nearly all the peaks.
Assimilation of a peak to an AA is carried out by the relative retention time of this AA injected alone, with the same in a

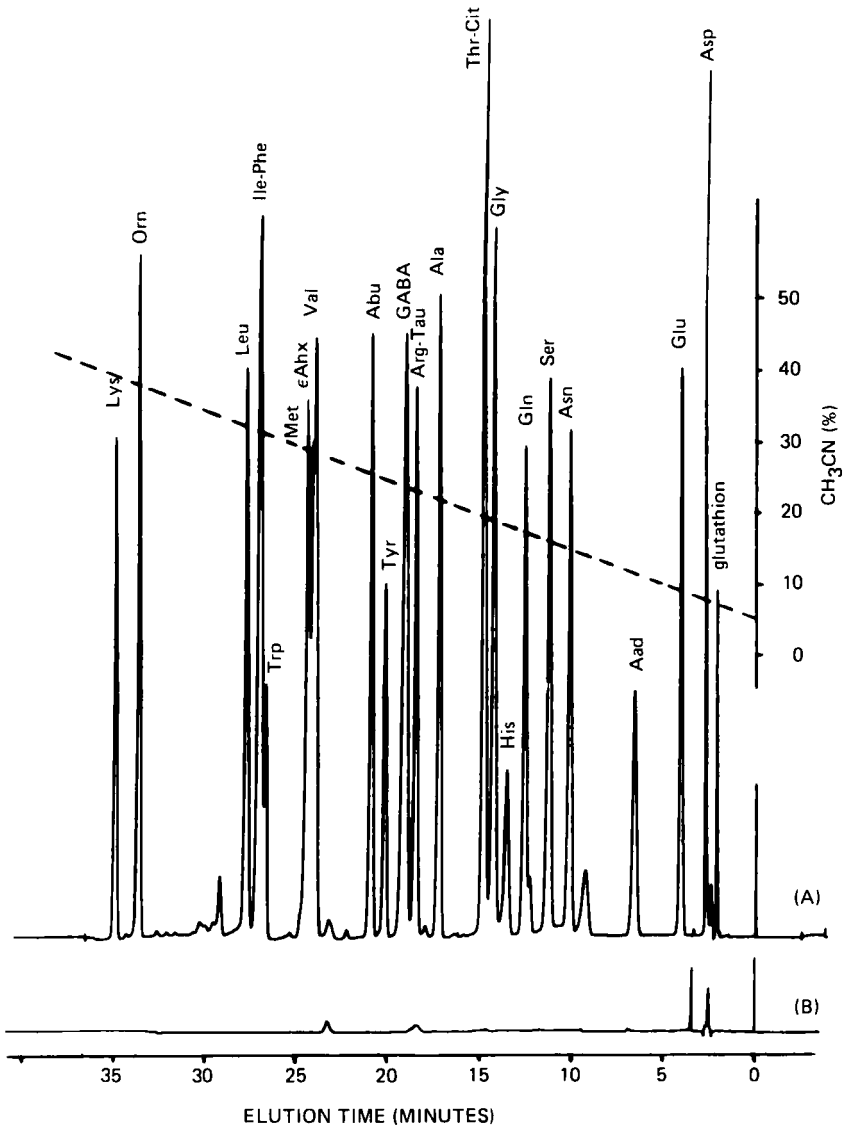

Fig. 1 : Recordings of a standard mixture of 24AA and 2 internal standards (A) versus a derivatizd blank (B), on a µBondapak C18 column using a 45 mn linear gradient at a flow rate of 1,4 ml/mn. The gradient was generated from KH_2PO_4 0,02 M pH 6.80 (95 %) to CH_3CN (50 %).

standard solution. Table 1 shows the retention time of 24AA with reference to their order of elution.

The peaks of the 2 internal standards fit in perfectly with the chromatogram for the 24AA ; moreover, the peaks of 6 amino caproic acid (ε Ahx) and of tyrosine are close to each other.

The derivatization of these standards proves necessary upon detection and therefore makes it possible to check the validity of the reagent used. However, the technique has several disadvantages: 3 pairs of AA could not be totally separated (Thr - Cit, Arg-Tau, Ile-Phe) and a certain amount of interference was shown by the injection of isolated products.

The chromatographic spectra of the His displays a double peak which as yet remains unexplained. The OPA-2ME interfers more or less seriously at the beginning of the resolution : the longer it is kept, the greater the interference.

Under the experimental conditions chosen, the glutathion, the first internal standard eluated, appears with 2 peaks in the chromatogram respectively at 10 and 13 min.

The chromatogram of the complex product to be separated is represented by figure 2 with that of the excipient used for manufacturing it (figure 3). This excipient naturally contains AA, but in relatively small quantities ; the same goes for the tyrosine for which a quantitative analysis is to be carried out. Furthermore, the sample gives only small amounts of interference. Thr-Gt are better separated than in the standard mixture, but an unidentified peak appears between Tau-Arg.

The qualitative aspect of the analysis is studied thanks to the results of the systematic injections of the standard mixture and of the samples over a period of one month ; the relative retention time (T'r) of a few AA in comparison with 6 amino acids are included in table 2 . These results are easily reproductible and the typical standard variations are all less than 0.015.

Moreover, the values of these parameters characteristic of liquid chromatography are all within the theoretical limits required to be able to state that the separation has taken place correctly

TABLE 1

Classification Of The 24AA According To Their Order Of Elution

Nr	NAME OF THE AA	SYMBOL	ORIGIN	Tr(cm)
1	aspartic acid	Asp	Prolabo	1.55
2	glutamic acid	Glu	Cooper	2.55
3	amino adipic acid	Aad	Merck	4.1
4	asparagine	Asn	Prolabo	5.6
5	serine	Ser	Sigma	6.15
6	glutamine	Gln	Sigma	6.8
7	histidine	His	Merck	7.25
8	glycine	Gly	Prolabo	7.6
9	threonine	thr	Merck	7.9
10	citrulline	cit	Merck	7.9
11	β alanine	Ala	Merck	9.2
12	arginine	Arg	Merck	9.8
13	taurine	Tau	Merck	9.8
14	γ amino butyric acid	GABA	Merck	10.15
15	tyrosine	Tyr	Carlo Erba	10.6
16	aminobutiric acid	Abu	Merck	10.15
17	valine	Val	Merck	12.5
18	methionine	Met	Sigma	12.65
19	tryptophanne	Trp	Fluka	13.9
20	isoleucine	Ile	Kochlight	14.05
21	phenylalanine	Phe	Prolabo	14.05
22	leucine	Leu	Merck	14.4
23	ornithine	Orn	Prolabo	17.2
24	lysine	Lys	Fluka	17.95

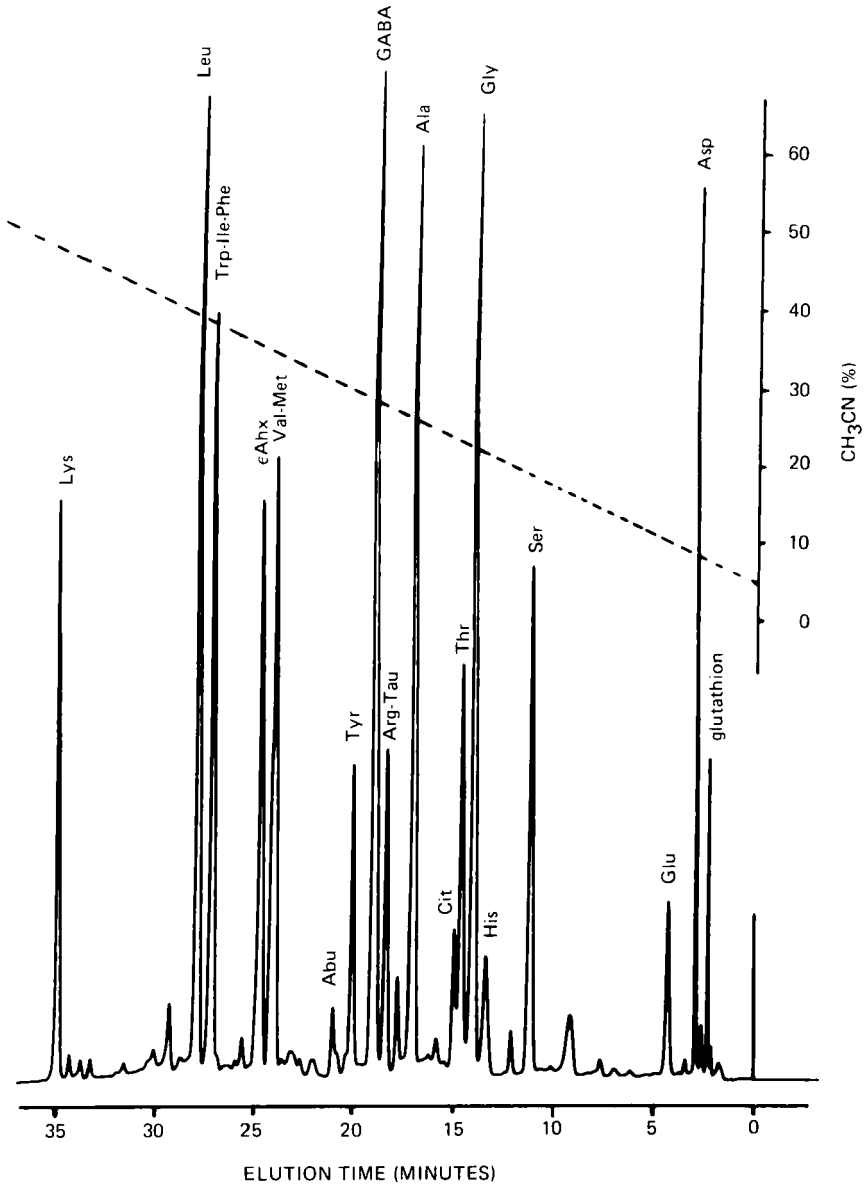

Fig. 2 : Separation of complex mixture of a internal AA hydrolysate
Same conditions as in fig. 1.

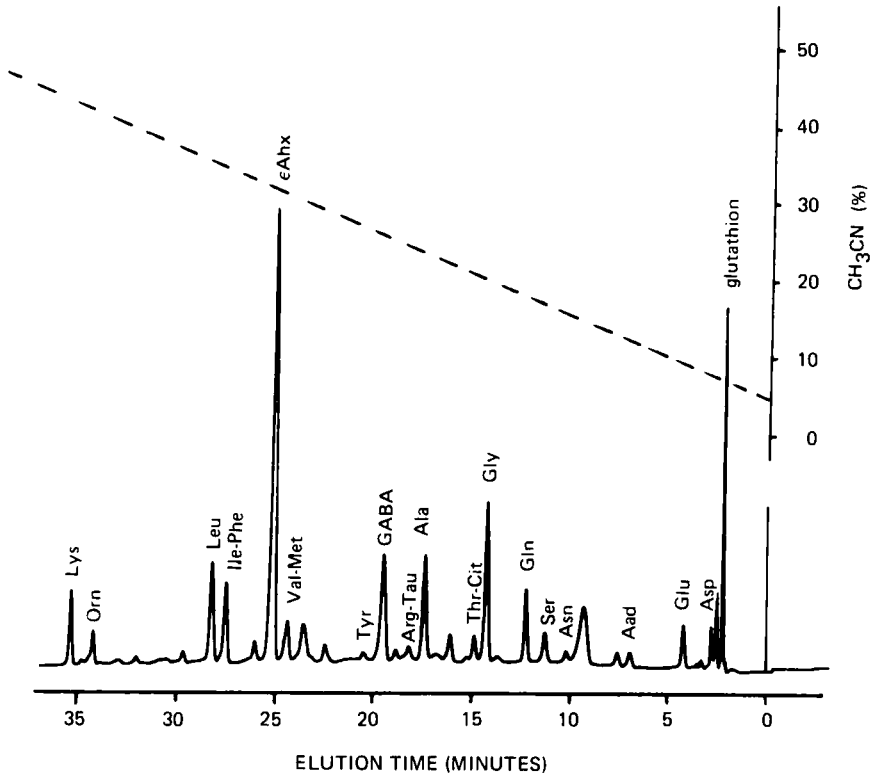

Fig. 3 : Elution profile of the vehicle used to manufacture the AA hydrolysate.

TABLE 2

Comparison Of Relative Tr of a Few AA

AA	STANDARD T'r	SAMPLE T'r	STANDARD Tr	STANDARD VARIATION
Gluthation	0.088	0.085	0.089	0.003
GABA	0.780	0.774	0.779	0.007
Tyr	0.829	0.822	0.824	0.011
Abu	0.852	0.851	0.857	0.013
Met	0.846	0.975	0.975	0.009

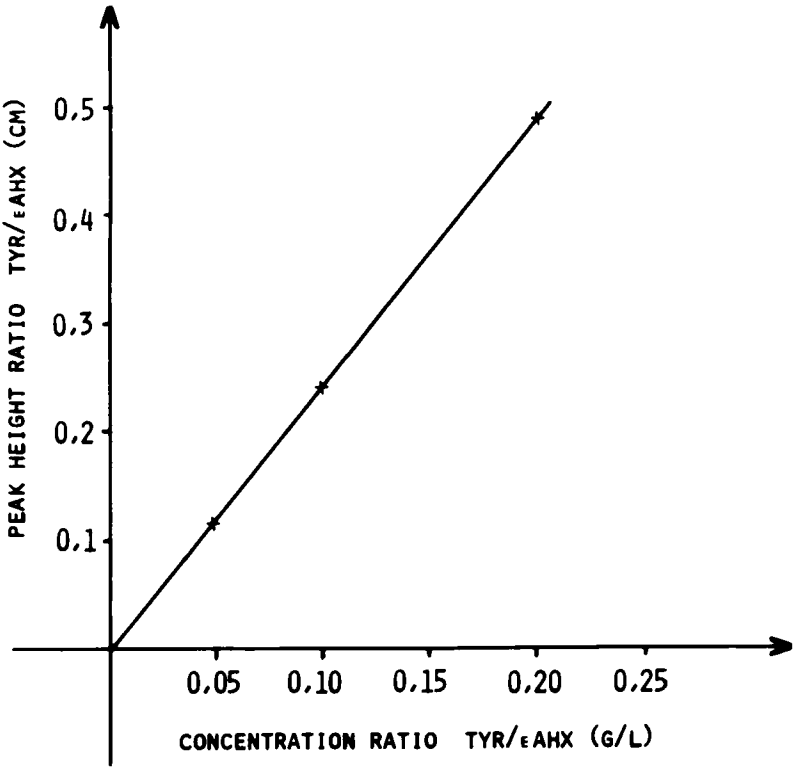

Fig. 4 : Linear relationship between peak height and concentration ratio of TYR/ϵAhx.

From a quantitative standpoint, the concentrations of tyrosine are calculated by the methodof internal standard with a standardisation curve proposed by Yost et al (7).

It consists of analysing a series of standard solutions containing an identical amount of internal standard mixture (1.25 g/l of Ahx) and various amounts of Tyr (0.5 - 0.25 - 0.125 - 0.0625 g/l).

The standardisation curve thus obtained (fig. 4) is linear up to 0.25 g of Tyr/l. The detection limit is about 50 mg.

The concentration of Tyr in the various samples analysed are included in fig. 5 ; this shows great variations in Tyr content depending on the manufacturing batch. The main reason for this is the natural origin of the autolysate.

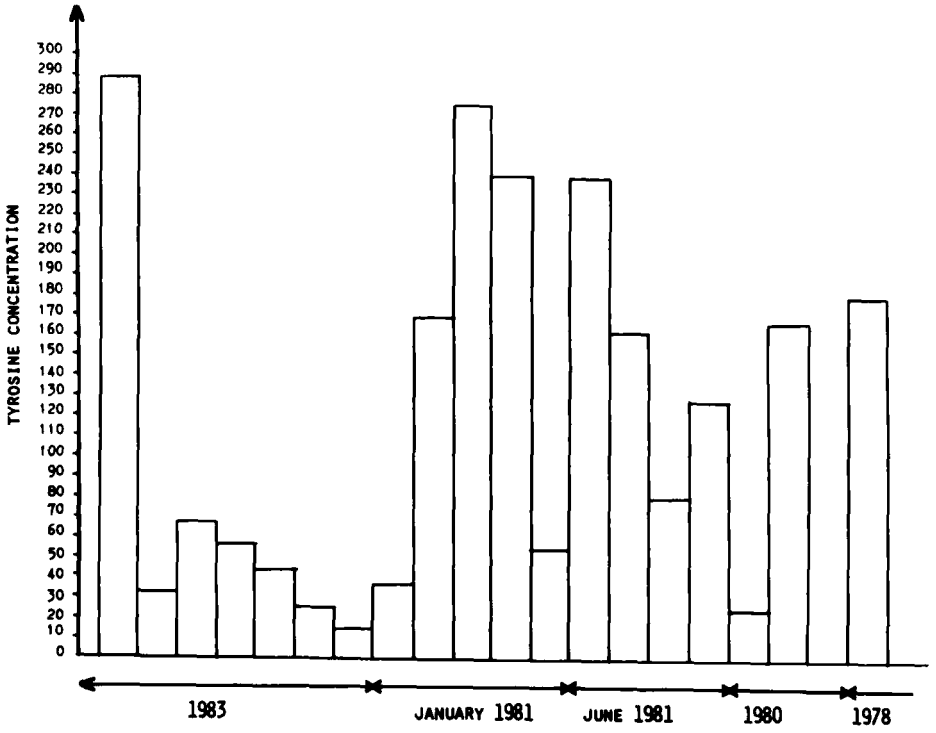

Fig. 5 : Diagram of the concentration of Tyrosine (g/l) between different batches of natural samples.

TABLE 3

Comparaison of the Values of the Parameters Characteristic of the Qualitative Analysis

	GABA	Tyr	Abu	Met	Ahx
Rt	9.66	10.22	10.60	12.14	12.73
Capacity factor $1 < k < 15$	7.86	8.38	8.72	10.14	10.68
Selectivity $d > 1$	1.07	1.04		1.05	
resolution $Rs > 0.8$	2.80	1.90		2.95	

DISCUSSION

Liquid chromatography is a recent technique and all its capabilities are not yet used because they are so varied. It is because of this diversity that complex resolutions such as those presented in this paper are possible. The optimisation of a large number of technical and analytical parameters, work towards the separation of the AA mixture.

Constitution of the buffer solution : effect of the ionic medium

Lindroth and Mopper (8) demonstrated that at constant pH and concentration, the nature of the buffer has an influence on the k'capacity factors of all AA' except Arg, Orn, and Lys ; k' increases and separation consequently improves by replacing water by a borate, phosphate or citrate buffer.
Under the selected chromatographic conditions replacing the phosphate ions with acetate ions in the elution solvent caused a clear loss of resolution of the eluated AA.
Likewise, variations in ionic concentration modify chromatographic resolutions (9) ; the more the concentration of the buffer increases, the better the separation is. However, a gradient of acetonitrile and of KH_2PO_4 0.2 M clogs up the column due to progressive precipitation of the buffer. A 1/10th dilution of the solution gives satisfactory results.
And finally, the pH of the medium, is very important, particularly in the separation of the amphoteric components such as the AA. The evolution of the buffer pH from 6.80 to 7.20 causes an important decrease in the retention of the last eluated AA, thus corroborating the results obtained by Lindroth and Mopper (8).
Precise adjustment of the pH in the separation protocol described is very important for the reproductibility of the analysis.

Effect due to the nature and the concentration of organic modifier

The addition of THF in the mobile phase is extremly important in the separation of all AA, including that of Thr-Gly (10). If the

quantity is right, this product does not affect the quality of the plottings obtained (9).
Wassner and Li (11) prefer to add 2 propanol, but they observe an increase in the pressure of the pumps.

- Constitution of the Organic Phase :

Compared to methanol, acetonitrile is more expensive, but it has many advantages which motivated our choice for these experiments :

a) its polarity is lower than that of methanol, making possible to decrease the time needed for analysis and consequently the amount of product consumed, since the less polar AA are eluated with a slightly polar mobile phase.

b) its viscosity is weaker ; pressure reached is therefore less high and efficacy is better.
Furthermore, the viscosity of the water-acetonitrile mixture is less than that of the water-methanol mixture, even for large concentrations of water (12) ; consequently it does not cause great variations in pressure.

c) the water-methanol solvent has a positive mixing-heat and tends to outgas, a disadvantage which the water-acetonitrile medium does not have.
Going from methanol to acetonitrile made it possible, to improve the separation of Phe - Ile (15) or Gly-Thr (14).

Equipment

Most publications talk about the fluorimetric detection of OPA-AA. The results show that it is just as satisfactory to use a spectrophotometer, a less specific apparatus for liquid chromatography, provided one does not have to detect very small amounts of AA (about few FEMTOMOLES, about 10^{-15} moles).
As far as the column is concerned, recent technical progress can certainly improve many separations. Jones and Gilligan demonstrated

that shorter columns decrease the analysis time. Likewise, finer filling particles have a similar effect.
However, the C18 μBondapak 10 μm columns are still widely used. Replacing them during the operation did not modify the chromatograms (16).
Miniaturisation would also appear to be a way of the future (17) as it reduces the size of the columns, the flow-rate and the volume of injection.

Chromatographic conditions

Many experiments have been carried out using complex gradients requiring sophisticated and expensive control apparatuses.
These experiments prove that it is possible, with a lower budget and a simplified protocol, to separate complex mixtures.

Parameters for derivatization

These essentially involve the reagent for OPA which has many advantages : simple to use, active in aqueous medium, and specific for AA. It can, however, only react on free amine groups. That is the reason why Pro and hydroxy Pro are not detected. Ishida et al (18) suggest treating the sample with an oxydant, sodium hypochlorite, before its derivatization, thus breaking up the cyclic structure and liberating a primary amine sensitive to treatment with OPA.
Cysteine (Cys) also poses a problem because, in spite of having chemical structure corresponding to the requirements of the derivatization reagent, it is only slightly detected, if not at all (19). At the moment, no explanation has been proposed, but Larsen and West suggest transforming Cys into its carboxymethyl derivative.
These particular cases prove that no universal reagent exists at the moment ; all of them pose problems involving interferences or contaminations (15).

The reconstitution of OPA solutions

The protocol described makes use of F. Martin's technique (20), one of the simplest, which reduces the manipulations proceeding the injection to an absolute minimum.
Derivatization using OPA-2ME is preferable to OPA-EtSH only in so far as the EtSH gives off the particularly unpleasant odor of thiols.
Both products are identically efficacious but the substitution of one for the other modifies the resolution of the AA.

Storage of reagents

Opinions as to this subject vary greatly. There is no disadvantage to use the reagent over a prolonged period (about two weeks), provided a certain number of conditions are respected :
storage in toned glass bottles at a temperature of +4°C and addition of 20 ul of 2ME every three days. Any failure of the reagents would be noticed immediately with the resolution of the internal standards.
Nevertheless, one needs to wait for 24 hours after the reconstitution of the reagent before using it, as great differences show up in the chromatograms (10).

The duration of the reaction between OPA and AA solutions

As Lindroth and Mopper observed for the fluorescence of OPA-AA, the absorbtion of certain AA decreases when the reaction between OPA and AA is too long. Following several trials, a timed period of 2 minutes seems to be ideal for an optimal reaction. This parameter does not appear as an obstacle to an automatic technique (21-22).

CONCLUSION

The quality of this type of analysis is ensured particularly by the strict observance of operating conditions : stabilisation of the equipment, storage of the reagents, etc.
The protocol used makes possible to begin the analysis rapidly : no extraction previous to the injection, few manipulations, reagents stable for several days.
It is flexible in so far as it may be adapted to several types of natural mixtures (tinctures prepared from Graminaceae, placental extract in cosmetic products, etc...).
It is original because it offers spectrophotometric detection of 24AA in a relatively short period of time.
And finally, it can be directly applied in routine control procedures when carrying out analyses on an industrial level.

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