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Note

Rapid and sensitive method for the determination of proline by reversed-phase high-performance liquid chromatography with automated pre-column fluorescence derivatization

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The analysis of amino acids using high-performance liquid chromatography (HPLC) with a reversed-phase C_{18} column has recently become very popular[1–5]. The preference for this method over other possible approaches[6–10] is undoubtedly due to the fact that the *o*-phthalaldehyde (OPA) derivatives of the amino acids present in a hydrolysed protein or a fruit juice are all easily and totally separable on a reversed-phase column and that these fluorescent derivatives permit the achievement of high sensitivity. In fact, under the appropriate conditions it is possible to determine amino acids in the sub-picomole range[2].

However, there is the great disadvantage that proline does not react directly with OPA and only after it has been oxidized with chlorinated oxidizing agents to a primary amino acid does its give a fluorescent derivative with OPA[11–16].

Recently, some workers[13,14] have proposed the use of 4-chloro-7-nitrobenzofurazan (NBD-Cl) as a reagent for the derivatization of proline and hydroxyproline, and also other secondary amines. The products of the reaction of the two secondary amino acids are easily separated on a C_{18} column, not only from each other but also from 4-methoxy-7-nitrobenzofurazan and 4-hydroxy-7-nitrobenzofurazan, which are likely to be formed during the derivatization reaction. Separation is speeded up and its completeness enhanced by the fact that the reaction between NBD-Cl and secondary amino acids is significantly greater than that between NBD-Cl and primary amino acids. As a result, the latter, even although always present in large amounts in the products subjected to analyses, do not interfere.

All of the derivatization methods mentioned using either chlorinated oxidizing agents or NBD-Cl are, however, manual methods that have the disadvantage of not being perfectly controllable and reproducible and, further, of not permitting automation such as the type developed by Waters Assoc.[15] for the analysis of amino acids with OPA.

The method described in this paper, which can be utilized for the analysis of amino acids in both fruit juices and protein hydrolysates, is entirely automatic.

EXPERIMENTAL

Equipment and column

All the analyses were performed using a liquid chromatograph (Waters Assoc.,

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Milford, MA, U.S.A.) equipped with a Model 6000A pump and an M45 pump, a Wisp 710B automatic injector, an M420 fluorescence detector with λ_{ex} = 450 nm and λ_{em} = 530 nm, an M721 system controller, an M730 data module and an Eldex oven to maintain the reactor and reaction coil at 65°C. The column was a Nova-Pak C_{18}, 5 μm (Waters Assoc.), 150 × 4 mm I.D., made of stainless steel. The reactor was a 25 × 4 mm I.D. stainless-steel pre-column filled with 600-μm glass beads. The reaction coil was a capillary stainless-steel tube (6 m × 0.2 mm I.D.).

The analytical column was heat-regulated by means of a glass jacket in which liquid circulated at 21°C.

Buffers and eluents

For the dissolution or dilution of the samples to be analysed we used a 0.4 *M* sodium borate buffer (pH 10) prepared by dissolving 24.7 g of boric acid (Fluka, Buchs, Switzerland) in 900 ml of water, adjusting it to pH 10 with 50% sodium hydroxide solution and then diluting to 1 l with water.

Eluent A was a phosphate–acetate 5.4 m*M* buffer (pH 6.5), obtained by dissolving in 800 ml of water 0.77 g of anhydrous dibasic sodium phosphate, 0.74 g of sodium acetate trihydrate (RPE) (Carlo Erba, Milan, Italy), 5 ml of tetrahydrofuran (THF) (LiChrosolv) (Merck, Darmstadt, F.R.G.) and 13 ml of methanol (RS-HPLC) (Carlo Erba), adjusting it to pH 6.5 with 50% acetic acid and then diluting to 1 l with water.

Eluent B was prepared by mixing 650 ml of methanol with 350 ml of water.

All the water used was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Prior to use the buffers were filtered through a 0.45-μm Millipore filter.

Materials

All the amino acids and NBD-Cl were purchased from Fluka and casein from BDH (Poole, U.K.). Grapefruit juice was obtained in the laboratory using a lemon-squeezer. The concentrated proline solution was prepared by dissolving 115 mg of proline in 50 ml of water containing 0.2% of sodium azide. Hydroxyproline solution was prepared by dissolving 131 mg of hydroxyproline in 20 ml of water containing 0.2% of sodium azide.

The working standard solution was prepared by placing 0.2 ml of proline solution in a round-bottomed flask, together with 0.2 ml of hydroxyproline solution and deluting to about 20 ml with 0.4 *M* borate buffer (pH 10). The hydroxyproline was the internal standard (I.S.).

The reagent solution for derivatization was prepared by dissolving 100 mg of NBF-Cl in 20 ml of methanol. This reagent should be stored in a refrigerator in a brown-glass vessel.

Preparation of analytical solutions

A 0.5-ml volume of fruit juice and 0.2 ml of I.S. were diluted to 20 ml with 0.4 *M* borate buffer (pH 10). The hydrolysate corresponding to 2.5 mg of protein, dried in a rotary evaporator at 50°C so as to eliminate most of hydrochloric acid, to which 0.2 ml of I.S. was added, was reconstituted with 20 ml of 0.4 *M* borate buffer (pH 10).

Chromatographic procedure

Analytical separations were performed as follows. The Wisp apparatus was programmed to charge the sampling coil, without injection, with 5 μl of NBD-Cl reagent solution, 5 μl of the solution to be analysed and a further 5 μl of NBD-Cl reagent solution, in this order. After the last sampling the Wisp injected into the reactor. The following programme was used: initial flow of 0.08 ml/min with 100% of eluent A for 11.5 min; linear flow gradient from 0.08 to 1 ml/min n 0.5 min still with 100% eluent A; linear gradient from to 84% B in 11 min; linear gradient from 84 to 100% B in 1 min.

The column had then to be conditioned for 8 min with eluent A at 1 ml/min prior to the start of the subsequent analysis. During the entire analysis procedure the reactor and the reaction coil were kept at 65°C and the column 21°C.

RESULTS AND DISCUSSION

We chose hydroxyproline as the I.S. because it is a secondary amino acid that is very similar to proline and thus behaves identically during the derivatization reaction. In addition, hydroxyproline is perfectly separated and exits before proline but fairly close to the latter (Fig. 1). The only disadvantage is that it cannot be used as an I.S. when the sample to be analysed contains any significant amount to collagen

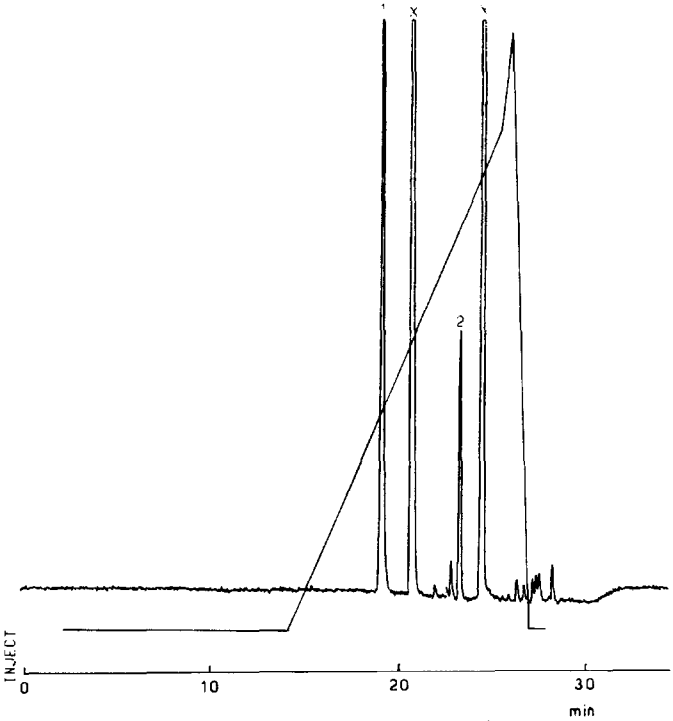

Fig. 1. Chromatogram of a standard solution. Proline, 200 nmole/ml. The thinner line represents the gradient profile. For conditions see text. Chart speed = 0.5 cm/min. Peaks: 1 = hydroxyproline (I.S.); 2 = proline; X = unknown, probably 4-methoxy-7-nitrobenzofurazan and 4-hydroxy-7-nitrobenzofurazan from the derivatization reaction.

because collagen has a high hydroxyproline content. In this instance, the additions method could be usefully employed for the quantitative determination of proline.

The substantial difference in the reaction speeds of secondary and primary amino acids is such that, with an appropriate reaction time and temperature, primary amino acids are not detectably derivatized. Given this, the latter do not interfere and this make it possible to separate the I.S. and proline in a relatively short time.

In the reactor, because it has a larger section than the tube coming from the sampler and also owing to the very low initial flow-rate, there is a very efficient mixing of the NBD-Cl reagent with the sample being analysed. The sample is diluted or reconstituted with 0.4 *M* borate buffer (pH 10) in order to have an optimal pH of 9.50 during the derivatization reaction. The total volume of the reactor and coil is designed to ensure that the sample + NBD-Cl mixture remains, at the flow-rate of 0.08 ml/min, for 9 min at the reaction temperature of 65°C.

Under these conditions, proline and hydroxyproline are derivatized to the extent of 95%[13] whereas the other amino acids are only negligibly derivatized, as shown in Fig. 2.

Full automation of the sample/reagent mixing operation and of the derivatization reaction ensures a high response repeatability. In the absence of an automatic sampler, the sample and reagent can be simultaneously placed in a manual sampler, using a 25-μl microsyringe. In this instance, the syringe must take in 5μl of NBD-Cl, 5 μl of sample and 5 μl of NBD-Cl, in this order.

Using the method described, we analysed a casein hydrolysate (Fig. 3) and a

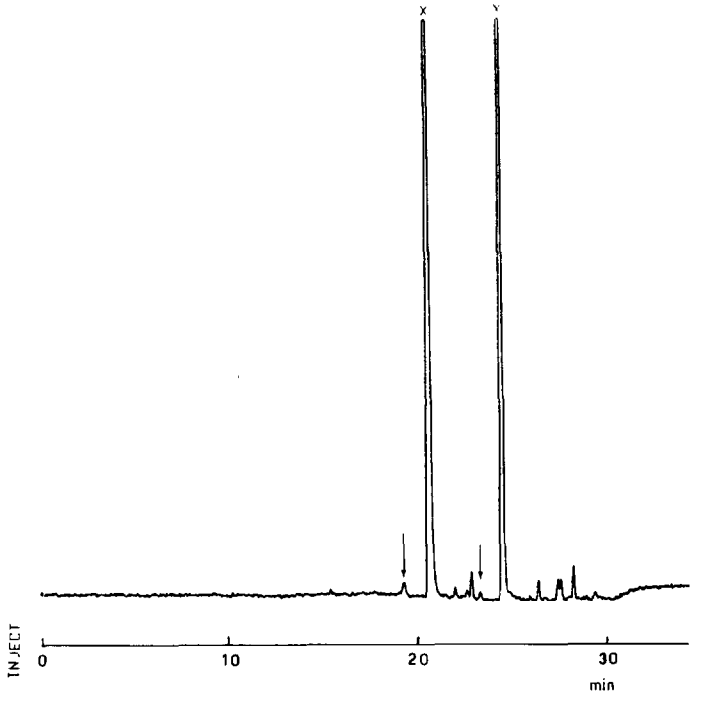

Fig. 2. Chromatogram of a primary amino acid solution. the arrows indicate hydroxyproline and proline positions. Conditions, peak identification and chart speed as in Fig. 1.

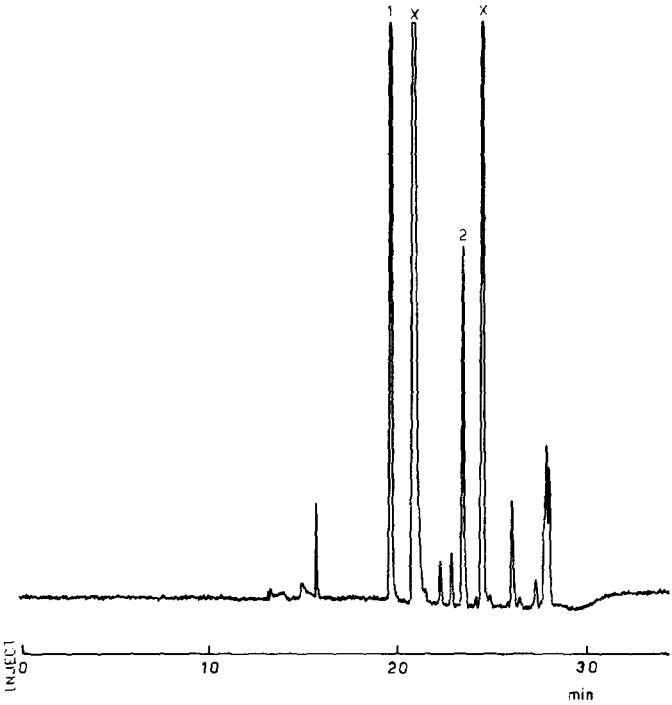

Fig. 3. Chromatogram of a casein hydrolysate. Conditions, peak identification and chart speed as in Fig. 1.

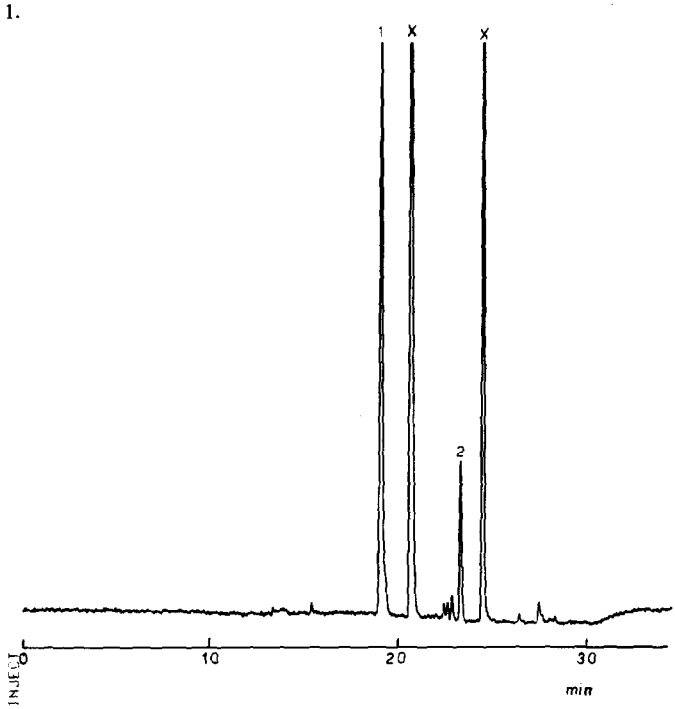

Fig. 4. Chromatogram of grapefruit juice. Conditions, peak identification and chart speed as in Fig. 1.

TABLE I

CONTENT OF PROLINE IN GRAPEFRUIT JUICE AND IN HYDROLYSED CASEIN DETERMINED BY ION-EXCHANGE CHROMATOGRAPHY AND BY THE PROPOSED METHOD

Method	*Casein (g per 100 g)**	*Grapefruit juice (mg per 100 ml)***
Present method	12.22 ± 0.57	58.3 ± 1.8
Ion-exchange chromatography	12.15 ± 0.75	55.4 ± 3.19

* Mean of three determinations, ± S.D.
** Mean of fifteen determinations, ± S.D.

laboratory-prepared grapefruit juice (Fig. 4) for their contents. The results obtained, as can be seen in Table I, are comparable to those obtained by analysing the same products with a traditional method of separation using a cation-exchange resin column and colorimetric detection after ninhydrin reaction.

Recovery and accuracy

The recoveries of proline added in two different amounts (46 and 92 mg per 100 ml) to two aliquots of grapefruit juice and analysed ten times each were 101.2 and 96.5% respectively, with a mean of 98.8%, as can be seen in Table II. This result indicates that the determination of proline with the present method is accurate.

TABLE II

RECOVERY OF PROLINE ADDED TO GRAPEFRUIT JUICE

Sample No.	*Proline present in 0.5 ml of grapefruit juice (mg)*	*Proline added (mg)*	*Proline found (mg)*
1	0.291	0.23	0.522
2			0.543
3			0.511
4			0.490
5			0.495
6			0.550
7			0.534
8			0.548
9			0.599
10			0.519
Mean			0.527 ± 0.024
11		0.46	0.748
12			0.760
13			0.661
14			0.710
15			0.703
16			0.717
17			0.735
18			0.706
19			0.722
20			0.789
Mean			0.725 ± 0.035

Precision, linearity and detection limit

The repeatability of the method was checked by assaying grapefruit juice fifteen times. A mean value of 58.3 mg per 100 ml was found with a standard deviation (S.D.) of 1.8 mg per 100 ml and a coefficient of variation of 3.08%. The detection limit, expressed as the minimum amount of proline present in the analysed solution corresponding to three times the signal-to-noise ratio, was found to be 20 nmole/ml. The linearity of the proline derivative response was found to be excellent in the 100–1000 nmole/ml range.

CONCLUSION

The method described makes it possible to determine the proline content of protein hydrolysate and fruit juices using a completely automatic system of pre-column derivatization and HPLC separation with a reversed-phase C_{18} column. It is therefore a useful complement to the analysis of amino acids with pre-column derivatization using OPA, with which it is not possible to determine proline content. This is particularly important with fruit juices because, in order to judge their authenticity, which is based among other things on total amino acid content, their proline content cannot be ignored as it accounts for almost one third of the total.

In comparison with traditional methods, HPLC analysis of amino acids on a reversed-phase C_{18} column with pre-column derivatization has the undoubted advantage of being faster and much more sensitive, making it possible to analyse much smaller amounts of sample.

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