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Analytical conditions for the determination of 23 phenylthiocarbamyl amino acids and ethanolamine in musts and wines by high-performance liquid chromatography

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Abstract

An improved HPLC method, using phenyl isothiocyanate (PITC) as derivatization agent, was developed for the separation and determination of 23 amino acids and triethanolamine in must and wine, without any pretreatment of the samples. The stability of dry and dissolved phenylthiocarbamyl derivatives was studied. The effects of pH, polarity, temperature and the addition of an ion-pairing reagent to facilitate the chromatographic separation were investigated. Four Penedès musts and their wines, from Macabeo and Parellada varieties, were analysed. The free amino acid contents found were compared with published results for the same varieties.

1. Introduction

Nitrogen compounds, especially amino acids, are particularly important in enology with regard to their influence on the organoleptic profile of wines and they can contribute to varietal characterization [1].

High-performance liquid chromatography in the reversed-phase mode (C_{18} column) and detection after derivatization is the technique of choice for the separation and identification of these compounds. Several compounds have been used as derivatization agents, e.g., dansyl chloride [2,3], orthophthaldehyde (OPA) [4,5] and phenyl isothiocyanate (PITC) [6–8]. All of them have advantages and disadvantages. We chose PITC, because it combines with primary and secondary amine groups (proline and hydroxyproline), it has sufficient sensitivity, the reaction times are short and their derivatives are more stable than the others. Moreover, it is a pre-column derivative that does not need any special equipment, so the versatility of the instrumental is not lost.

Other investigators have also used the advantages of phenylthiocarbamyl (PTC) derivatives in application to wines; however, a prior amino acid extraction was applied to the sample [6]. We propose an elution system that allows the separation of 23 PTC-amino acids and ethanolamine (ETA) in musts and wines without any prepurification of the sample.

The mobile phase plays a very important role in the resolution of these compounds with regard to the amino acid structure. The amino acids have a dipolar character and some of them have

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an additional acidic or basic group. The basic amino acids show less resolution owing their interaction with the stationary phase. Hence the selection of the ionic strength, pH and polarity of the mobile phase is important. Buffer solutions with a pH between 5.0 and 6.5 and acetonitrile have been reported as eluents [6–8]. Sodium pentanesulphonate is added to the mobile phase because it forms an ion pair with the amino acids, improving the elution of basic compounds.

A diode-array detector was used because it not only gives results as a fixed-wavelength detector, it also indicates the peak purity and corraborates their identification, comparing the absorbance spectrum of the sample with those of standards. With these data correct amino acid identification and determination are possible; the mobile phase and chromatographic column are used frequently and, as no sample pretreatment was carried out, the peaks of some compounds present in must and wine could overlap with those of the PTC-amino acids.

2. Experimental

Stock standard solutions of amino acids (obtained from Sigma) were prepared by dissolving each acid in 0.1 *M* acetic acid to give a 20 nmol/μl concentration; tyrosine was dissolved in 1 *M* NaOH. From these solutions working standard solutions of 8, 12, 40, 80 and 120 m*M* for each amino acid were prepared; as proline is present at a higher concentration in must and wines, working standard solutions containing 0.4, 0.6, 0.8, 1.6 and 2.4 *M* were prepared. A 400 m*M* solution of norleucine (Nle) in 0.1 *M* acetic acid was used as an internal standard.

The derivatization reagent [PITC–water–ethanol–triethylamine (1:1:7:1)] was prepared daily. PITC was kept in a freezer (−20°C).

The samples were four white Penedès musts from the 1991 harvest, two Macabeos (M1 and M2) and two Parelladas (P1 and P2), and their four wines. The samples were kept at −20°C until analysis; also, 1 g/l of sodium fluoride was added to the must samples to prevent fermentation. The samples were centrifuged at 4000 *g* for 15 min to eliminate large particles before derivatization.

2.1. Derivatization procedure

A 20-μl volume of the standard solutions or 40 or 100 μl of must or wine, with 20 μl of the internal standard (Nle) and 20 μl of a triethylamine (TEA) solution [TEA–water–ethanol (1:2:2)] were added to a 35 × 4 mm I.D. tube. The solution was evaporated to dryness under vacuum and then the procedure described by Bidlingmeyer et al. [8] was followed. The dry residues containing the PTC derivatives were kept in a freezer. The dry residue was dissolved in 100 μl of acetonitrile–water (95:5) and filtered through a 0.45-μm cellulose acetate filter before injection.

2.2. Chromatographic conditions

A Hewlett-Packard Model 1040 gradient liquid chromatograph with an HP 1050 diode-array UV–visible detector was used. The separation was carried out using a Spherisorb C_{18} ODS-2 column (25 × 4.6 mm I.D.) of 5-μm particle size; the precolumn contained the same stationary phase.

The following solvents were used: (A) an aqueous buffer containing 0.1 *M* sodium acetate, 0.680 ml/l of triethylamine and 0.2 mg/ml of

Table 1
Linear solvent gradient for elution

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	35.0	62.8	2.2
3	35.0	62.0	3.0
15	35.0	62.0	3.0
16	35.0	58.0	7.0
30	35.0	50.0	15.0
45	35.0	42.0	23.0
50	0.0	0.0	100.0
60	0.0	0.0	100.0
65	35.0	62.8	2.2

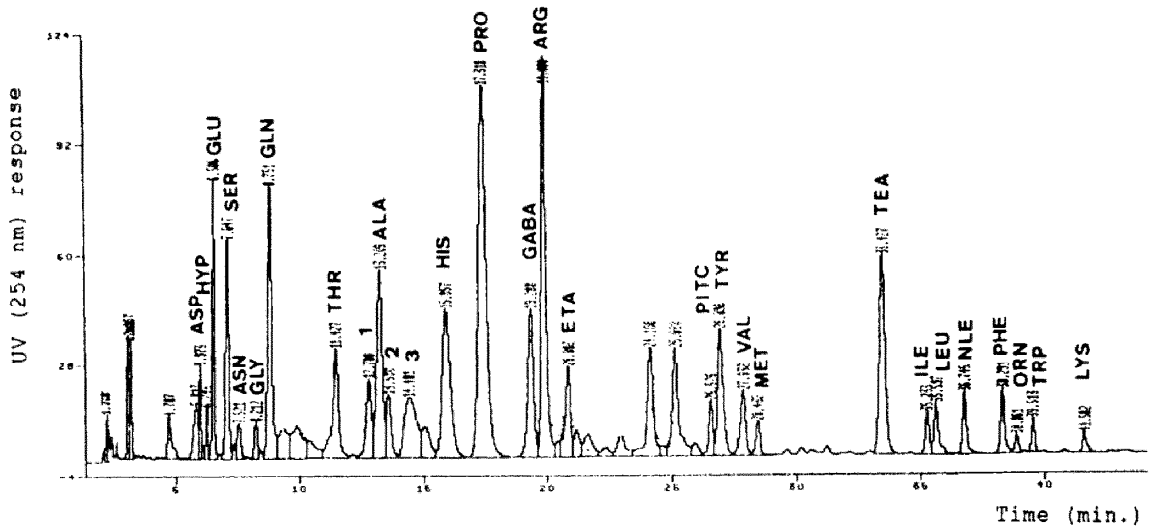

Fig. 1. Chromatogram of Parellada (P1) grape must.

sodium pentanesulphonate, adjusted to pH 5.27 with glacial acetic acid; (B) water; and (C) acetonitrile. The separation was carried out using a linear multi-step solvent gradient, as shown in Table 1. The flow-rate was 1 ml/min, the column temperature was 35°C and the detection wavelength was 254 nm.

3. Results and discussion

Twenty-three amino acids and ethanolamine (ETA) were satisfactorily separated from must and wine samples (Figs. 1 and 2). The effects of ionic strength, pH, mobile phase polarity, ion pair formation and column temperature are

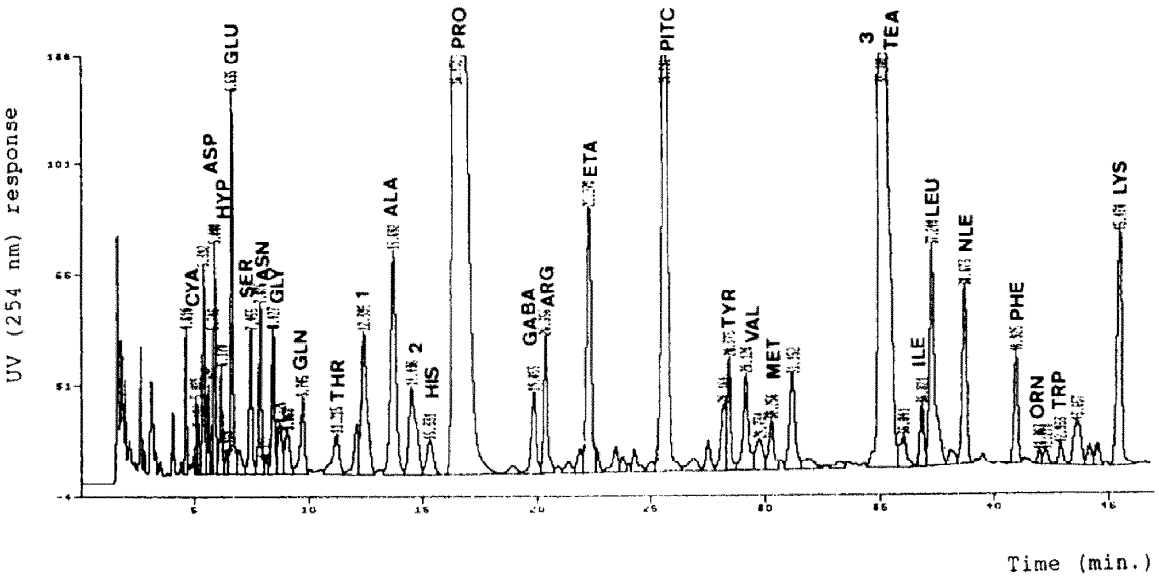

Fig. 2. Chromatogram of Parellada (P1) wine.

different for each amino acid, depending on their structure. An increase in the ionic strength of the mobile phase leads to an increase in the retention times of the amino acids, except for the basic compounds, which appear earlier. An increase in the buffer acidity produces a delay in the retention times. The addition of sodium pentanesulphonate or any other salt that produces an ion pair with the amino acids improves the resolution of the basic amino acids and prolongs their elution times. The effect of the ion pair depends not only on the salt concentration in the mobile phase, but also on the ionic strength, the amino acid polarity and the percentage of acetonitrile in the mobile phase. Almost all the amino acids, with or without basic

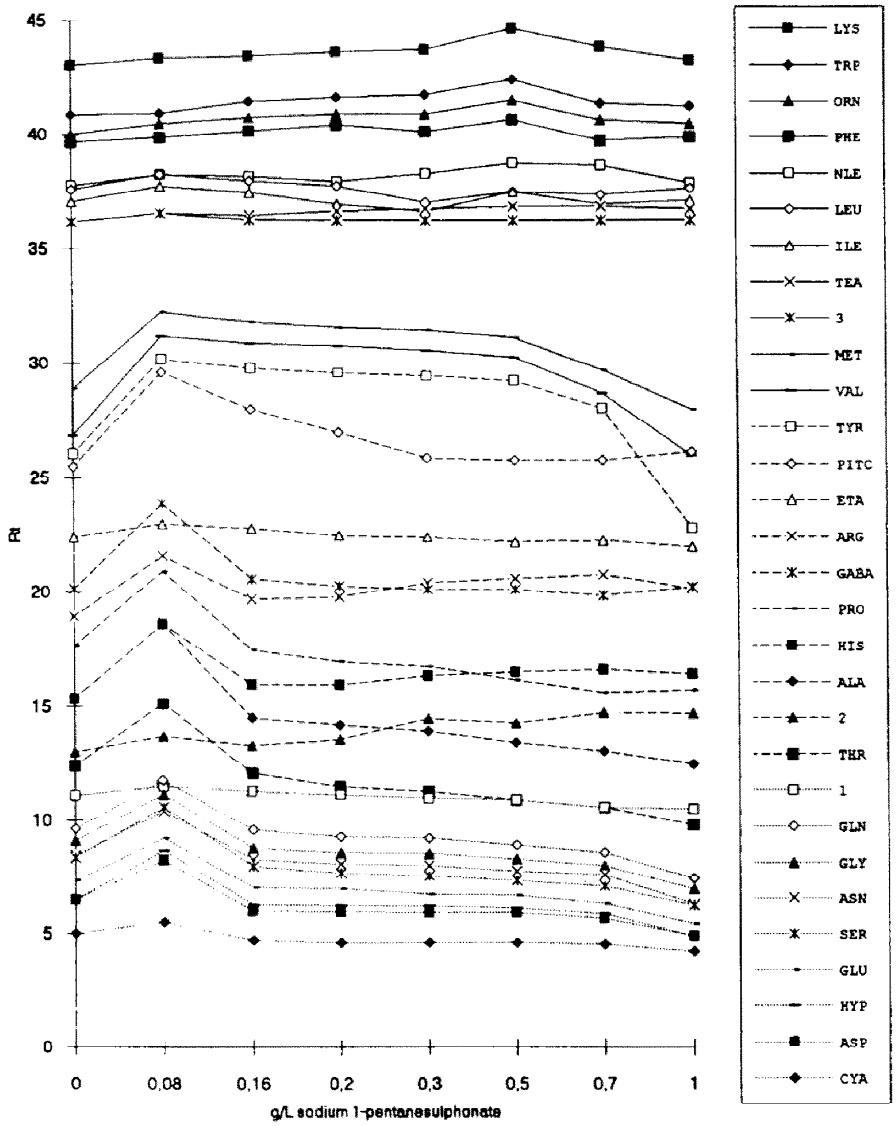

Fig. 3. Changes in retention time in HPLC of wine sample with variation in sodium 1-pentanesulphonate concentration in the mobile phase.

character, show increased retention times on addition of 0.08 g/l of sodium pentanesulphonate to the mobile phase (Fig. 3); however, adding more salt decreases their retention times. This effect was observed previously by Zoest et al. [9], who pointed out that the relationship between the salt concentration and the retention time of the basic compounds follows a parabolic curve. Exceptions are the basic amino acids (His, Arg and Lys) and ETA, which continue to show increasing retention times.

The addition of sodium pentanesulphonate allowed the separations of His–Ala, Asp–Hyp, Gly–Asn and Arg–Pro (Fig. 4). With the salt addition, Arg moves towards GABA, separating from Pro that is present at high concentration compared with the Arg present in must and wines, so Arg can be overlapped by Pro. A better separation of all the amino acids was achieved when 0.2 g/l was added.

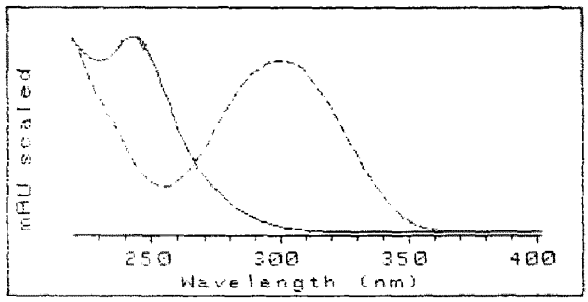

Fig. 5. Comparison of two spectra from a wine chromatogram. Solid line, Pro; dashed line, peak 1 (see Fig. 2).

Changes in the ionic strength, pH and sodium pentanesulphonate affect the first part of the chromatogram (Figs. 1 and 2), but the amino

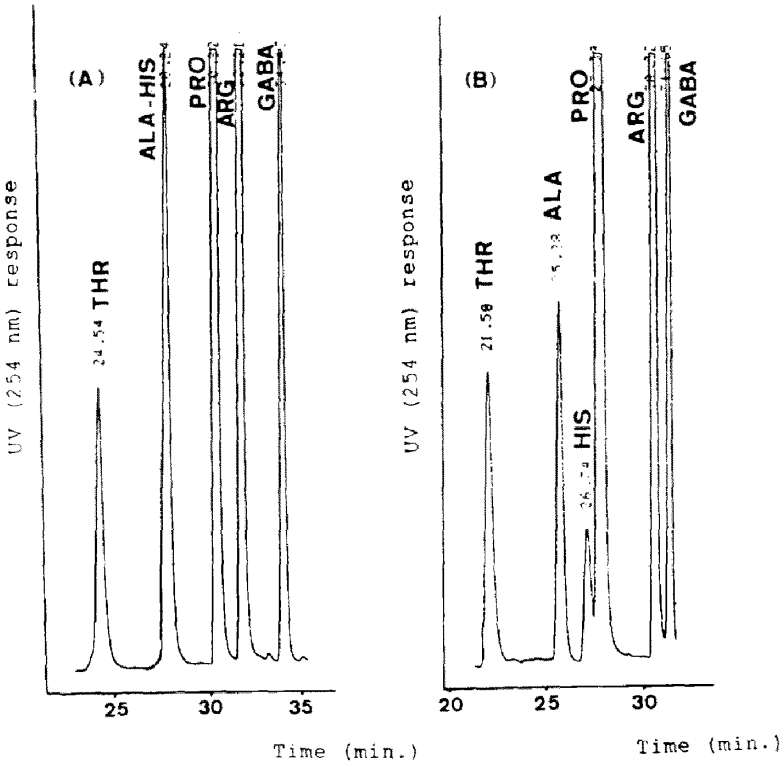

Fig. 4. HPLC of standards (Thr, Ala, His, Pro, Arg and GABA) eluted with mobile phases (A) without sodium 1-pentanesulphonate and (B) containing 0.1 mg/ml of sodium 1-pentanesulphonate.

acids that elute after 30 min are not affected by these parameters and their retention times depend primarily on the polarity of the mobile phase.

Temperature has the same effect on all the amino acids. At higher temperatures the chromatograms are shorter and show better resolution, but the lifetime of the chromatographic column decreases.

Figs. 1 and 2 show the must and wine chromatograms. Very good resolution (separation) was observed not only for the identified peaks, but also for other compounds that elute in the same chromatogram.

The amino acid spectra obtained with the diode-array detector indicate that the purity of each of these peaks is >99% and the correspondence between the standard spectra and the measured peaks is also 99%. The spectra of the other peaks (1, 2 and 3) in Figs. 1 and 2 indicate that they are not PTC derivatives. In Fig. 5, the spectra of a PTC-amino acid (Pro) with an unidentified peak are compared (peak 1).

3.1. Method validation

Different parameters were considered to establish the reliability of the method (derivatization and chromatographic separation) (Table 2).

The variation in the retention time, studied with six aliquots of the same sample (Parellada wine), was <3%. The precision of amino acid determination was lower than 7%, except for

Table 2
Repeatability of retention time (t_R), precision, recovery, linearity, limit of detection (L.D.) and limit of quantification (L.Q.) for amino acids and ethanolamine

Compound	Repeatability, t_R (%)	Precision (%)	Recovery (%) ($n = 4$)	Linearity (r)	L.D. (nmol)	L.Q. (nmol)
Cya	3.00	9.85	100.1 ± 7.5	0.9999	0.020	0.052
Asp	1.83	6.87	100.8 ± 2.7	0.9989	0.025	0.063
Hyp	1.72	2.06	100.7 ± 1.8	0.9995	0.015	0.039
Glu	2.71	5.98	103.1 ± 4.4	0.9991	0.018	0.046
Ser	1.16	4.23	99.4 ± 1.8	0.9992	0.016	0.046
Asn	1.89	4.63	94.0 ± 2.8	0.9992	0.016	0.041
Gly	1.78	5.69	102.7 ± 10.4	0.9997	0.016	0.040
Gln	1.52	3.66	100.6 ± 4.8	0.9997	0.016	0.041
Thr	1.07	5.75	97.9 ± 7.9	0.9989	0.017	0.042
Ala	1.05	3.18	102.9 ± 8.9	0.9999	0.015	0.039
His	0.96	6.15	95.4 ± 2.8	0.9999	0.018	0.045
Pro[a]	1.24	2.16	99.9 ± 0.8	0.9990	0.013	0.033
GABA	0.88	2.46	98.2 ± 4.8	0.9995	0.021	0.054
Arg	0.94	6.94	102.5 ± 3.5	0.9997	0.017	0.044
ETA[b]	0.84	3.22	107.1 ± 1.8	0.9998	0.017	0.044
Tyr	0.77	4.09	96.5 ± 3.3	0.9996	0.017	0.044
Val	0.45	2.79	100.6 ± 4.3	0.9994	0.021	0.053
Met	0.49	9.34	103.3 ± 5.1	0.9999	0.020	0.052
Ile	0.21	4.08	102.3 ± 2.9	0.9998	0.021	0.053
Leu	0.11	5.82	99.6 ± 3.4	0.9999	0.017	0.043
Phe	0.15	4.79	95.0 ± 4.7	0.9999	0.018	0.047
Orn	0.18	3.56	93.7 ± 3.2	0.9997	0.011	0.027
Trp	0.31	6.97	95.2 ± 5.2	0.9995	0.014	0.035
Lys	0.51	6.71	95.6 ± 5.1	0.9992	0.012	0.031

[a] $n = 3$.
[b] Ethanolamine.

Met and Cya, for which it was 9.34% and 9.85%, respectively, because they are present at lower concentrations than the other amino acids in the sample studied.

The determinations of amino acids and ETA were carried out with three calibration graphs for each. The correlation coefficient (r) was >0.9990, except for Asp and Thr ($r = 0.9989$) (see Linearity column in Table 2).

The recovery was studied by adding different concentrations (10, 20, 40 and 80 mM) of each of the amino acids and ETA, except for Pro (400, 600 and 800 mM), to the same sample. The recoveries obtained (see Table 2) for each amino acid and ETA were >90% and independent of the concentration added.

The limit of detection (L.D.) was calculated according to the IUPAC equation [10]:

$$\text{L.D.} = X + tS_{n-1}$$

where X = mean noise, t = Student's t for a probability of 99.99% and S = standard deviation. X was determined by running nine blanks using the maximum sensitivity allowed by the integration system.

The limit of quantification (L.Q.) was calculated according to the equation of the American Chemical Society [11]:

$$\text{L.Q.} = X + 10S_{n-1}$$

where X is the same as for the limit of detection L.D.

The L.D. for the amino acids ranges between 0.011 nmol for Orn and 0.025 nmol for Asp and the L.Q. between 0.027 nmol for Orn and 0.063 nmol for Asp. Hence the method is sufficiently

Table 3
Stability of dry and solution PTC-amino acids (mg of amino acid/l of wine)

Compound	Solution					Dry				
	Time (min)					Time (weeks)				
	0	15	45	60	90	0	1	2	3	4
Cya	10.2	9.9	9.5	10.1	10.2	10.2	12.0	10.7	10.9	11.6
Asp	17.5	17.8	17.4	17.3	17.2	17.5	17.2	17.7	17.0	17.1
Hyp	14.3	14.2	14.7	14.0	13.9	14.3	14.0	13.8	14.7	14.6
Glu	20.1	19.7	20.4	19.8	20.0	20.1	20.5	18.0	20.5	20.5
Ser	12.3	11.9	12.5	12.4	12.0	12.3	12.9	12.3	13.1	12.9
Asn	10.1	9.9	10.0	9.8	10.2	10.1	10.3	10.8	10.2	10.9
Gly	12.5	12.4	12.7	12.3	12.8	12.5	13.1	12.9	13.9	13.0
Gln	11.1	11.0	11.4	11.2	10.5	11.1	11.4	10.2	10.8	7.5
Thr	8.4	8.2	8.0	7.9	8.3	8.4	7.9	8.2	8.7	8.4
Ala	12.1	12.5	11.9	12.3	12.5	12.1	12.1	13.3	13.5	12.6
His	9.1	8.9	9.0	9.1	8.9	9.1	9.0	9.1	9.6	9.0
Pro	287.3	280.1	279.1	290.4	285.4	287.3	302.2	310.6	313.4	298.3
Arg	10.2	10.3	10.6	9.9	10.3	10.2	11.2	10.0	11.7	10.8
GABA	11.8	11.5	11.4	11.9	11.7	11.8	11.6	11.7	11.0	11.6
ETA	21.6	21.5	21.8	21.4	21.5	21.6	20.5	22.3	22.4	22.0
Tyr	12.5	12.4	12.6	12.4	12.0	12.5	13.9	13.6	12.9	13.8
Val	7.5	7.2	7.3	7.9	7.3	7.5	7.9	8.0	8.9	7.6
Met	8.4	8.2	8.4	8.1	8.2	8.4	8.1	8.6	8.4	8.5
Ile	6.9	6.3	6.8	6.4	6.2	6.9	6.7	7.1	7.3	6.5
Leu	12.0	12.4	11.8	11.9	12.0	12.0	13.0	12.0	12.5	12.4
Phe	9.5	9.2	9.1	9.6	9.3	9.5	9.7	9.1	10.1	9.9
Orn	5.4	5.2	5.3	5.9	5.4	5.4	5.6	5.2	5.8	5.6
Trp	18.4	18.2	18.1	18.5	17.2	18.4	17.8	19.3	20.3	15.0
Lys	2.8	2.6	2.9	2.8	2.6	2.8	3.1	2.7	2.7	2.6

sensitive for the determination of the amino acids in the samples studied.

3.2. Stability of PTC derivatives

The stability was studied for dry samples (kept at −20°C) and for samples in solution (kept in a refrigerator at 4°C) (Table 3).

Dry derivatives

The PTC-amino acids were injected every week during 1 month. The variations are lower than those calculated for the method accuracy. However, after the third week Gln and Trp began to decline in concentration. Hence dry derivatives can be kept at 20°C for 3 weeks.

In solution

The same sample was injected at 15, 45, 60 and 90 min, after being dissolved. The amino acids were stable in solution for 1 h, then they became degraded. For this reason, the solution must be injected within 1 h after preparation.

3.3. Sample results

Table 4 gives the results obtained for the amino acids in the four musts and wines are shown. The amino acid concentrations in the wines are very similar to those observed by other workers for other Penedès wines of the same varieties, considering that they are from different harvests and the methods used to analyse them

Table 4
Results for must and wine samples

Compound	Must[a] (mg/l)				Wine[a] (mg/l)			
	M1	M2	P1	P2	M1	M2	P1	P2
Cya	N.D.[b]	N.D.	N.D.	N.D.	1.0	0.1	0.1	0.1
ASp	40.4	6.8	12.4	6.6	6.2	2.8	9.3	5.5
Hyp	1.6	0.7	12.4	6.6	2.1	2.3	3.2	2.9
Glu	51.1	13.2	11.2	11.2	10.2	5.6	13.3	10.1
Ser	28.8	11.8	21.0	16.3	2.1	1.3	2.5	1.6
Asn	8.8	4.7	3.9	2.4	2.5	1.5	8.3	1.0
Gly	3.3	2.9	2.7	2.0	1.4	2.4	2.2	2.5
Gln	73.4	29.9	39.9	37.0	3.8	1.3	6.1	12.3
Thr	27.6	11.9	28.1	18.3	2.8	0.8	1.3	1.2
Ala	39.6	29.4	27.3	27.3	8.3	5.3	9.5	15.8
His	12.0	8.5	8.8	12.3	1.6	1.3	1.8	2.0
Pro	125.1	92.7	103.7	57.5	167.5	162.6	354.0	251.6
Arg	249.6	81.3	241.3	172.4	6.1	4.4	7.3	6.5
GABA	25.3	27.9	16.4	21.2	3.9	2.8	5.0	6.6
ETA	13.4	7.0	14.2	10.5	35.8	33.2	82.0	70.4
Tyr	20.9	8.8	21.8	18.2	3.4	3.1	5.0	3.7
Val	8.6	5.3	8.9	7.1	4.2	2.3	3.1	2.1
Met	2.8	1.2	2.8	1.2	2.4	1.3	2.7	1.8
Ile	6.5	3.5	7.1	5.7	5.1	2.8	4.5	2.0
Leu	15.0	5.1	11.9	11.0	8.2	7.5	10.3	3.9
Phe	15.0	7.5	19.6	15.7	4.4	2.9	5.8	6.4
Orn	5.3	2.1	3.3	5.9	1.9	1.3	6.3	6.1
Trp	8.9	4.7	17.4	12.9	2.5	3.3	2.9	1.8
Lys	5.0	2.2	3.9	6.5	6.7	7.3	6.8	4.3

[a] M1 and M2 = Macabeo variety; P1 and P2 = Parellada variety.
[b] N.D. = not detected.

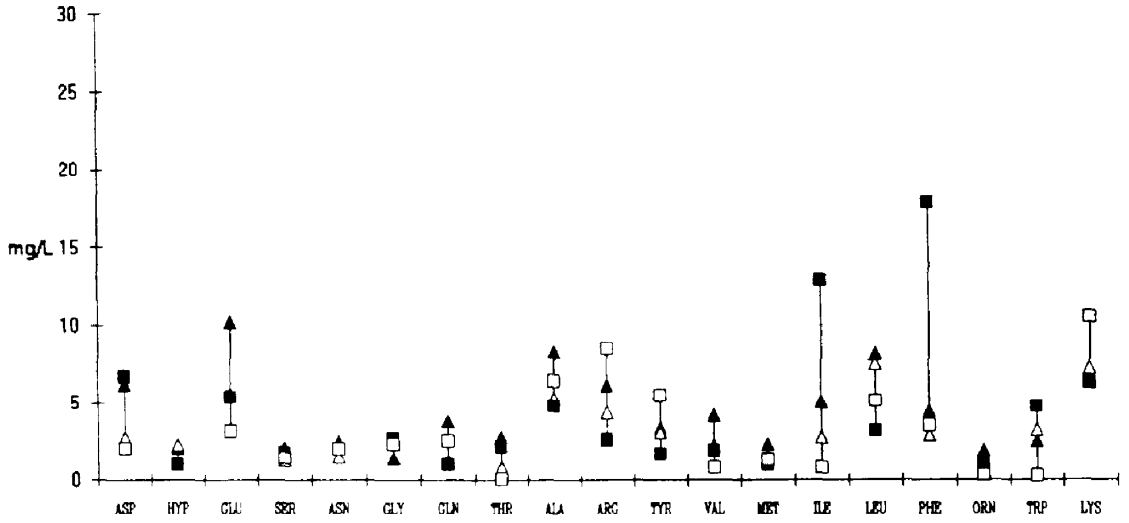

Fig. 6. Amino acid concentrations in Macabeo wine. ▲ = M1; △ = M2; ■ = MARCE (12); □ = CASTRO (4).

are different (Figs. 6 and 7). In one, the analysis was carried out following Sep-Pak pretreatment of the sample using PITC precolumn derivatization [12] and in the other the derivatization was carried out postcolumn with OPA [4].

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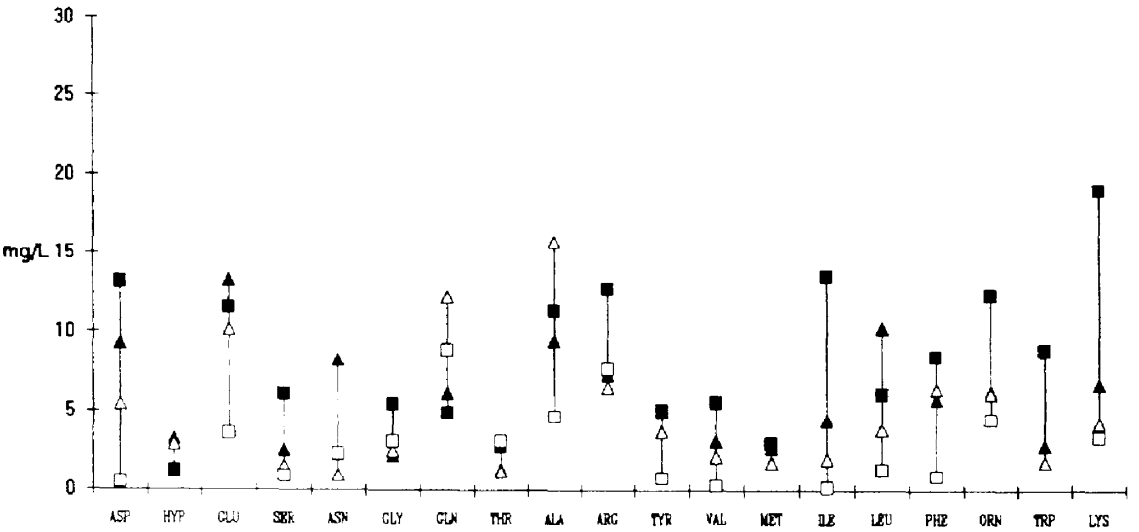

Fig. 7. Amino acid concentrations in Parellada wine. ▲ = P1; △ = P2; ■ = MARCE (12); □ = CASTRO (4).

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