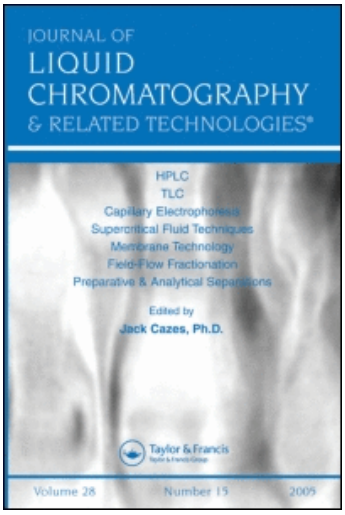

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Dansyl Amino-Acids Behavior on a Radial Pak C_{18} Column. Derivatization of Grape Wine Musts, Wines, and Wine Vinegars

Paloma Martín[a]; Carmen Polo[a]; M. Dolores Cabezudo[a]; Manuel V. Dabrio[b]
[a] Instituto de Fermentaciones Industriales, Madrid, Spain [b] Instituto de Química Orgánica General. (C.S.I.C.), Madrid, Spain

To cite this Article Martín, Paloma , Polo, Carmen , Cabezudo, M. Dolores and Dabrio, Manuel V.(1984) 'Dansyl Amino-Acids Behavior on a Radial Pak C_{18} Column. Derivatization of Grape Wine Musts, Wines, and Wine Vinegars', Journal of Liquid Chromatography & Related Technologies, 7: 3, 539 — 558
To link to this Article: DOI: 10.1080/01483918408073984
URL: http://dx.doi.org/10.1080/01483918408073984

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 539-558 (1984)

DANSYL AMINO ACIDS BEHAVIOR ON A RADIAL PAK C_{18} COLUMN. DERIVATIZATION OF GRAPE WINE MUSTS, WINES AND WINE VINEGARS.

Paloma Martín*, Carmen Polo*, M. Dolores Cabezudo* and Manuel V. Dabrio**

*Instituto de Fermentaciones Industriales, **Instituto de Química Orgánica General. (C.S.I.C.) Juan de la Cierva, 3. Madrid-6. Spain.

ABSTRACT

Investigation was carried out into the retention behaviour of dansyl amino acids with the concentration of the organic modifier, the pH and the ionic strenght of the eluting buffer. The quantitative result precision obtained using gradients of polarity and the stability of dansyl amino acids have been calculated. Finally, the dansylation conditions of grape wine musts, wines and wine vinegars are fixed.

INTRODUCTION

Dansylation has been largely used as a method for determining free amino acids, as well as protein hydrolyzing amino acids and terminal amino acids from proteins and peptides (1-4). Dansyl amino acids (Dns-aa) are highly fluorescent compounds, which can easily be obtained; thus they are very adequate for the analysis of these nitrogenized compounds.

Dns-aa have been separated using electrophoresis (5) and thin layer chromatography (1, 6, 7) but during recent years High Performance Liquid Chromatography (HPLC) has been used to separate and detect these

 0148-3919/84/0703-0539$3.50/0

compounds, with either silica as a stationary phase (8, 9) or phases linked with silica (10, 11, 12).

The reaction of amino acids with dansyl chloride in conventional pH and temperature conditions produces Dns-aa and HCl (I). At the same time a hydrolysis of the reagent is produced and dansic acid is obtained (II). For some amino acids, dansyl chloride excess reacts in turn with the Dns-aa and the reaction (III) takes place.

$$AA\text{-}NH_2 + Dns\text{-}Cl \longrightarrow AA\text{-}NH\text{-}Dns + HCl \quad (I)$$

$$H_2O + Dns\text{-}Cl \longrightarrow DNS\text{-}OH + HCl \quad (II)$$

$$AA\text{-}NH\text{-}Dns + Dns\text{-}Cl \longrightarrow Dns\text{-}NH_2 + \text{other products} \quad (III)$$

Neadle and Pollit (13) show that dansyl amide formation is an unavoidable limitation of the method, the quantity formed depending upon the amino acid concerned and the dansyl chloride excess. Therefore some reaction conditions should be found for each kind of sample, conditions in which the reaction (I) is favoured (which implies a dansyl chloride excess) and in which the reaction (III) is minimized. This excess should not be very high.

In this survey Dns-aa behaviour with regard to the buffer molarity, pH and concentration of the organic modifier has been investigated. To do that a Radial Pak C_{18} column was used.

The variability of the results obtained with this method of analysis and the stability of these derivatives have also been calculated.

In addition, the dansylation conditions of grape wine must, wine and wine vinegar amino acids have been determined so that the reagent quantity should be sufficient but not excessive.

In fact, it is interesting to know the amino acid contents for these kind of samples, because amino acids

are yeast and bacteria nutrients and due to they are flavour precursors as well.

EXPERIMENTAL

Instrumentation

All separations were performed on a Waters Associates instrument with two 6000 A pumps, a 660 solvent flow programmer, radial compression module RCM-100 and a U6K injector. Fluorescence was detected using a fluorometer 420AC with standard flow-cell and standard filters: excitation filter 340 $\pm$ 6 nm; emission filter 425 nm (long pass). Reversed phase column (10 cm x 8 mm ID), Radial Pak C_{18} (10µm). Bondapak C-18/Corasil (37-50 µm) guardcolumn.

Chemicals and Buffers

Methanol was HPLC grade from Scharlau. All buffers were prepared from analytical grade chemicals and Milli-Q (Millipore Corp. Bedford, MA) water. Before use, all buffers were filtered using a Millipore Type HA filter with a pore diameter of 0.45 µm, and degassed.

Dns- amino acid standards and amino acids were obtained from Sigma (St. Louis, Mo. USA) and Dns-Cl from Fluka.

Methods

Dns-derivatization was carried out under conditions similar to those used by Tapuhi et al (14). The reactant solution consisted of Dns-Cl disolved in acetone (1.5 mg/ml, 5.56 mM). Dansyl derivatization of a standard mixture of amino acids was carried out with Dns-Cl 5-10 fold higher in concentration than amino acids and 40 mM lithium carbonate buffer, pH 9.5, during 1 hour in the dark, at room temperature.

RESULTS AND DISCUSSION

Methanol Concentration Influence upon Retention

In order to study the influence of the methanol concentration on retention and therefore the Dns-aa separation, a series of tests has been carried out using respectively 20%, 40% and 60% (w/w) of methanol. Phosphate buffer concentration (0.03 M) and pH 6.9 were constant.

In most cases of separations in HPLC reversed phase, the capacity factor (k') logarithm of solutes decreases linearly when the organic modifier percentage increases in the mobile phase. Karger et al. (15) have verified this linear relationships of log. k' with methanol percentage for two solutes n-hexane and octanol. However we have observed that this not happen in the case of Dns-aa, at least in the conditions we have used (Fig. 1).

This fact was observed by Hearn and Grego (16) for polypeptides and acetonitrile as organic modifier. Bij et al. (17) who observed it for very polar compounds, interpreted this as something due to interaction between the solute and silanol groups which are not blocked during the union between the stationary phase and the support.

As Fig. 1 shows, a total Dns-aa separation in isocratic conditions cannot be carried out as low methanol concentration should be used. This would imply very long time analysis. For instance, using 20% methanol, proline would be eluted after 50 minutes. The remaining amino acids of which polarities are lower than proline (among which there are leucine and isoleucine) would be retained in the column and their elution could even last several days. On the other

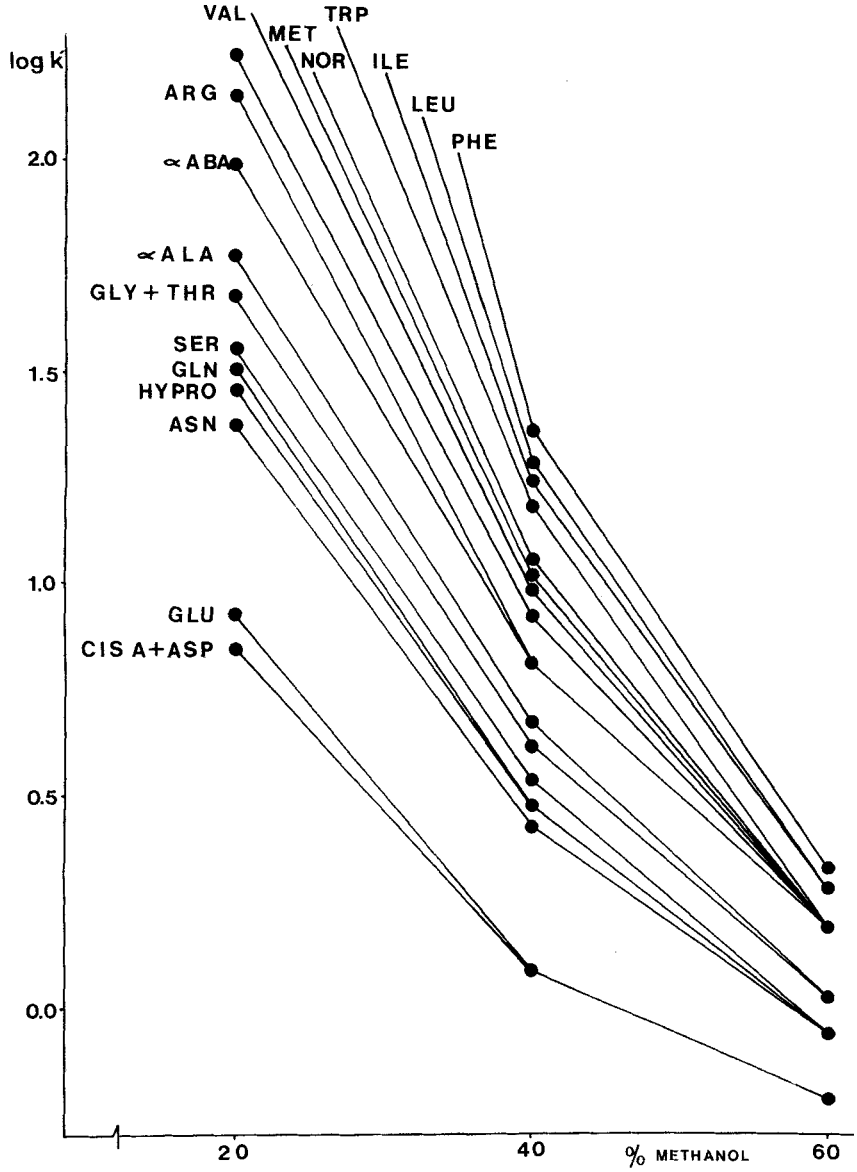

FIGURE 1. Plots of the log k' of dansyl amino acids against concentration of methanol. Isocratic elution, mobile phase: 0.03 M sodium phosphate buffer, pH 6.9.

hand, some amino acids are not resolved at concentration higher than 20%. However, if 40% methanol concentration is used the most of the amino acids can be eluted in a reasonable time and with a fear good separation. For subsequent tests.

Molarity and pH Influence upon Retention

The effect of the ionic eluent concentration is interesting as related to the ionic exclusion phenomenon due to the carboxilate group. An interaction exists between the carboxilate groups of the dansyl aminoacids and the sodium ions presents, the concentration of which increases as the phosphate molarity does.

In order to prove this point, several tests were carried out with constant methanol (40%) and pH (6.9) values; phosphate concentration being changed: 0.01, 0.03 and 0.05M. These low molarities have been chosen in order to avoid precipitations in the analytical system.

Fig.2 shows the increase in the retention of the most of the Dns-aa used when the phosphate buffer concentration increases. Dns-arginine is an exception, probably due to the existence of a highly polar guanidinic group in its lateral chain. As much as phosphate buffer concentration increases, more sodium ions are present, being predominant the guandidinic group effect.Therefore, the retention decreases because of the polarity increases as it was suggested by Hill et al. (18) on the basis of amino acid ortho--phthaldialdehide/ethanethiol derivatives. The guanidinic group polarity is inversed to the carboxilate one and in certain conditions, these opposed polarities could be compensated up to a certain point.

The pH effect in the separation was studied for a 5.9 - 7.9 interval. Within these limits, the most of

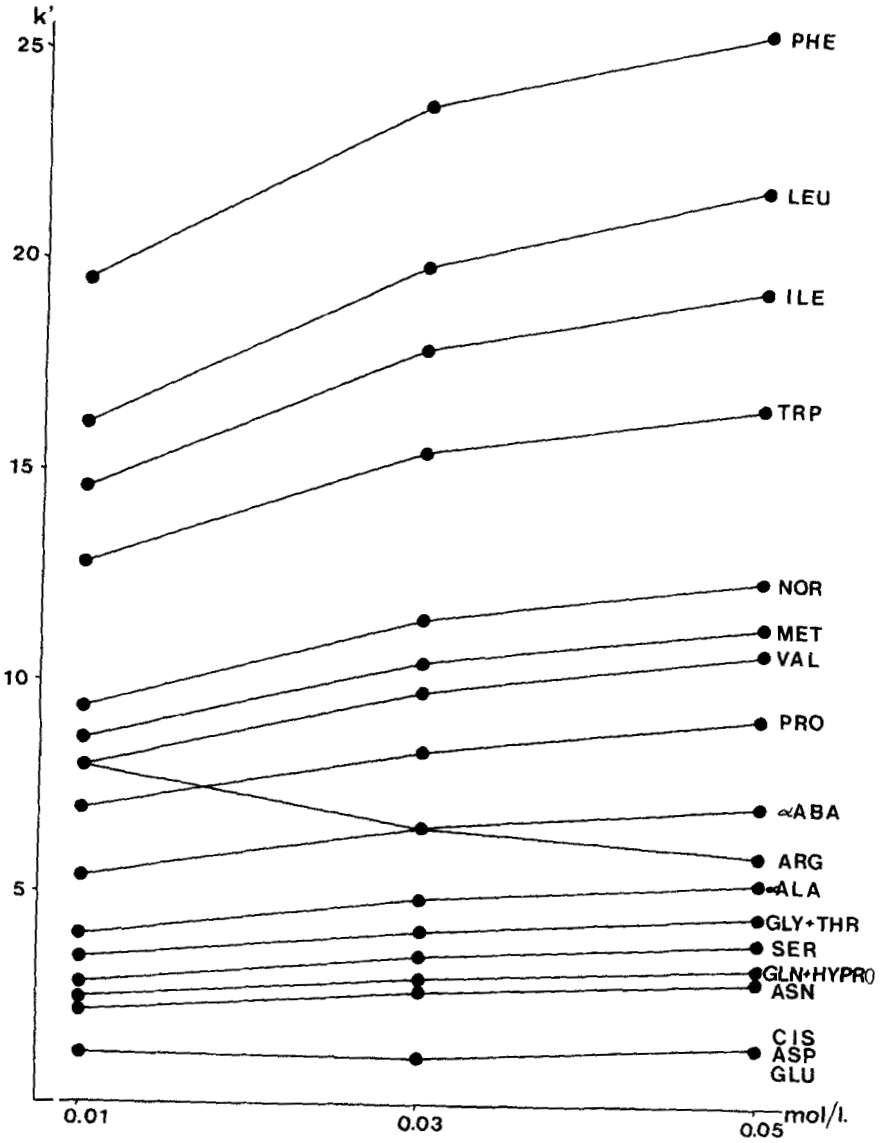

FIGURE 2. Plots of the log k' of dansyl amino acids againts phosphate buffer concentration. I Isocratic elution: methanol - sodium phosphate buffer pH 6.9 (40-60 w/w).

the carboxilic groups of the Dns-aa are ionized. The Dns-arginine presents the nitrogen of the guanidinic group protoned and a zwitterion is formed; this is why its net charge is null in these tests.

Fig. 3 shows a retention increase as pH decreases. The k' increase when pH decreases may be due to an ionic suppression phenomenon. According to Lindroth and Mopper (19), when pH decrease Dns-aa carboxilate protonization is favoured and its hydrophobicity increases; therefore retention is favoured in the apolar stationary phase.

Fig. 4 shows the chromatogram obtained in the conditions of the central point of these tests: Methanol 40%, phosphate buffer 0.03 M, pH 6.9. Although several double peaks appear most of the amino acids are resolved.

Quantitative Dansyl Amino Acids Analysis. Derivatives Stability

A synthetic solution of the dansyl amino acids in which they are in similar proportions to those in musts, wines and vinegars (20 - 22) has been chromatographied.

Owing to the fact that Dns-aa cannot be separated in isocratic conditions, from now on, gradient of polarities described by Martin et al. (23) have been used.

Eluent A: 15% (w/w) methanol/phosphate buffer 0.01 M, pH 6.3.

Eluent B: 35% (w/w) methanol/phosphate buffer 0.01 M, pH 6.3.

Linear gradient from 0 to 100 B in 30 minutes.

Flow: 2 ml/min.

Fig. 5 shows a standard solution chromatogram obtained in these conditions.

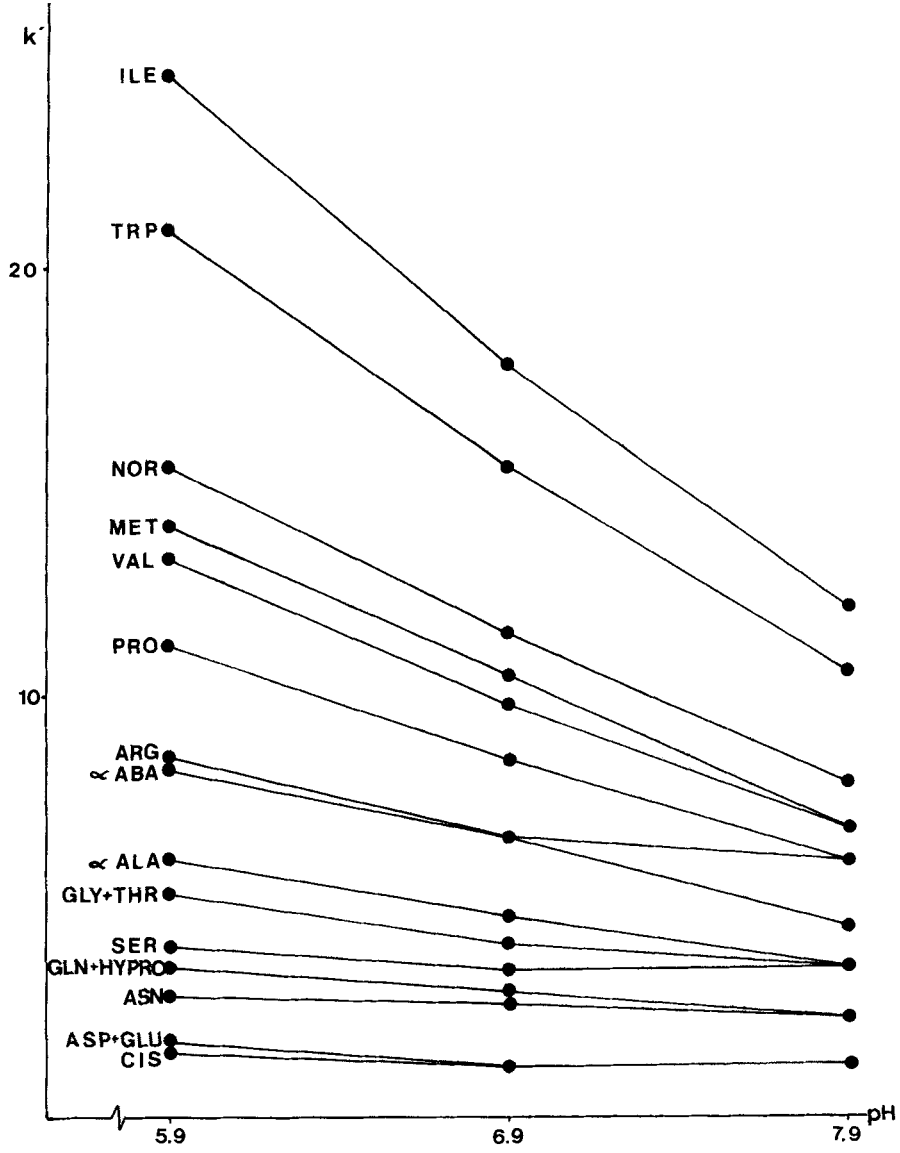

FIGURE 3. Plots of the log k' of dansyl amino acids againts pH. Isocratic elution: methanol - 0.03 M sodium phosphate buffer.

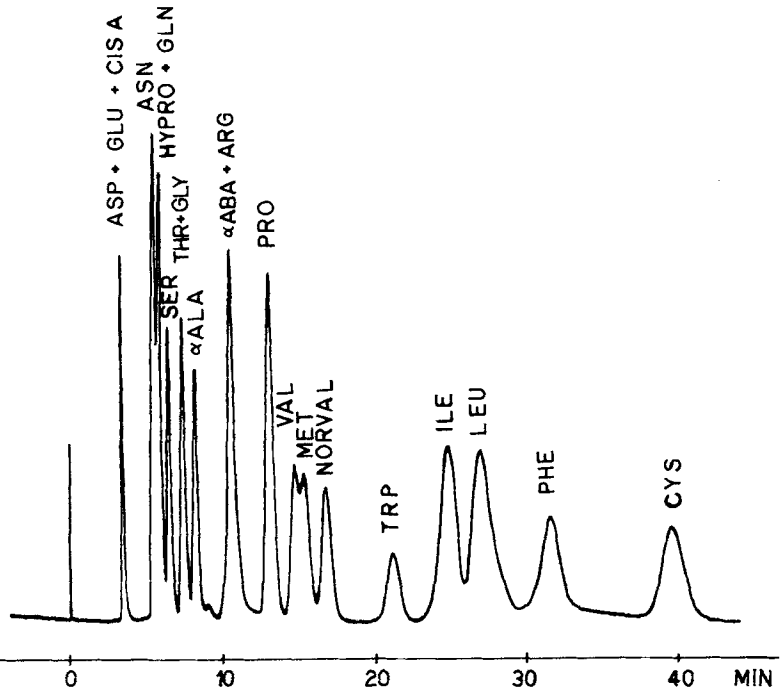

FIGURE 4. Chromatogram of a synthetic solution of Dns-amino acids. Isocratic elution, mobile phase: methanol-0.03 M sodium phosphate buffer pH 6.9 (40-60 w/w).

Table 1 sums up the results obtained from eight replicated analysis of the standard solution. Variation coefficients are from 0.9% for threonine + glycine to 4.5% for aspartic acid. It is observed that the highest values (4.5, 4.1 and 4.0) correspond to the amino acids in lower proportions (aspartic acid, serine and valine). In addition, the valine and the aspartic acid are peaks which are not chromatographically well resolved.

De Jong et al. (24) do not observe loss in the Dns- amino acid when the samples are analyzed within a reasonable time (1 day) after Dns-derivatization while remaining somewhat protected from direct light. We have

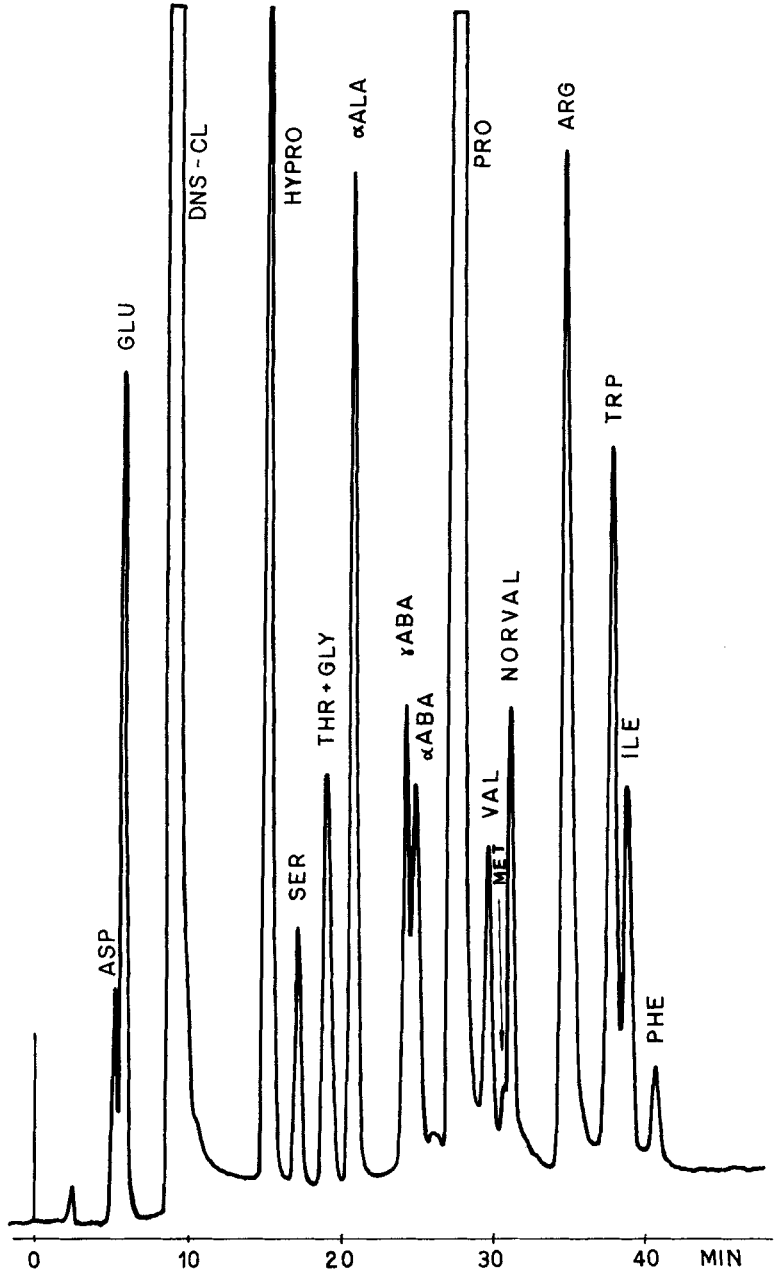

FIGURE 5. Chromatogram of a synthetic solution of Dns-amino acids. Amino acids concentration as stated in Table 1. Gradient elution, mobile phase: A = Methanol-0.01 M sodium phosphate buffer pH 6.3 (15-85 w/w); B = methanol- 0.01 M sodium phosphate buffer pH 6.3 (35-65 w/w), 30 min., flow rate 2 ml/min.

TABLE 1

Variability of the Data Obtained from 8 Replicates of a Test Solution. (Chromatographic Conditions as in Fig.5)

Amino acid	ng	$\overline{Ai/As}$	$s_{Ai/As}$	v
Aspartic acid	400	0.460	0.021	4.5
Glutamic acid	950	0.948	0.019	2.0
Hydroxyproline	300	0.336	0.008	2.4
Serine	174	0.243	0.010	4.1
Threonine+Glycine	301	0.756	0.007	0.9
alfa alanine	592	1.809	0.035	1.9
gamma aminobutyric acid	252	0.959	0.014	1.5
alfa aminobutyric acid	274	0.777	0.014	1.8
Valine	93	0.224	0.009	4.0
Norvaline (IS)	960	1	-	-
Arginine	1210	2.403	0.027	1.1
Tryptophane	400	0.425	0.010	2.4
Isoleucine	50	0.130	0.004	3.2
Phenylalanine	199	0.459	0.013	2.8

	$\overline{As}$	s_{AS}	v
Norvaline	72729380	2849162	3.9

$\overline{Ai/As}$ = mean of peak area / internal standard peak area distribution.

$s_{Ai/As}$ = standard deviation of the Ai/As distribution.

v = coefficient of variation.

$\overline{As}$ = mean of the internal standard peak areas (counts).

s_{As} = standard deviation of the As.

observed that Dns-aa are still stable for at least seven days if the are kept at -4ºC., as may be deduced from Table 2, where the dispersion of the results is within the values obtained for the variability coefficients of the analysis method, as shown in Table 1

TABLE 2

Dansyl Amino Acids Stability in Course of Time

Amino Acid	$\bar{X}$ (ng)	s	v
Aspartic acid	454	11	2.4
Glutamic acid	430	8	1.9
Serine	483	17	3.5
Threonine+Glycine	568	5	0.9
alfa Alanine	345	6	1.7
Proline	365	13	3.6
Valine	420	12	2.9
Methionine	640	34	5.3
Arginine	628	5	0.8
Phenylalanine	740	11	1.5

$\bar{X}$ = mean of the values obtained from the chromatograms of a standard solution stored up 7 days. Each x_i is the mean of two replicates.

s = standard deviation.

v = coefficients of variation.

Must, Wines and Wine Vinegars Dansylation.

For a fixed quantity of sample, dansyl chloride amount has been progressively increased until has been checked that it did not imply an increase in the area of the corresponding peaks. Results show that the amount of dansyl chloride required is at least eight times higher than the amino nitrogen content of the sample. Considering that there is no reliable analytical method for determining amino nitrogen in musts, wines and vinegars (in the usual methods, proline nitrogen is not evaluated), an approach has been taken into account.

There is a quite satisfactory correlation between total nitrogen and amino nitrogen in must as well as in wine samples. These correlation exists for many grape varieties and it is independent of the maturity grade of the grapes (25). Thus, it is possible to estimate the

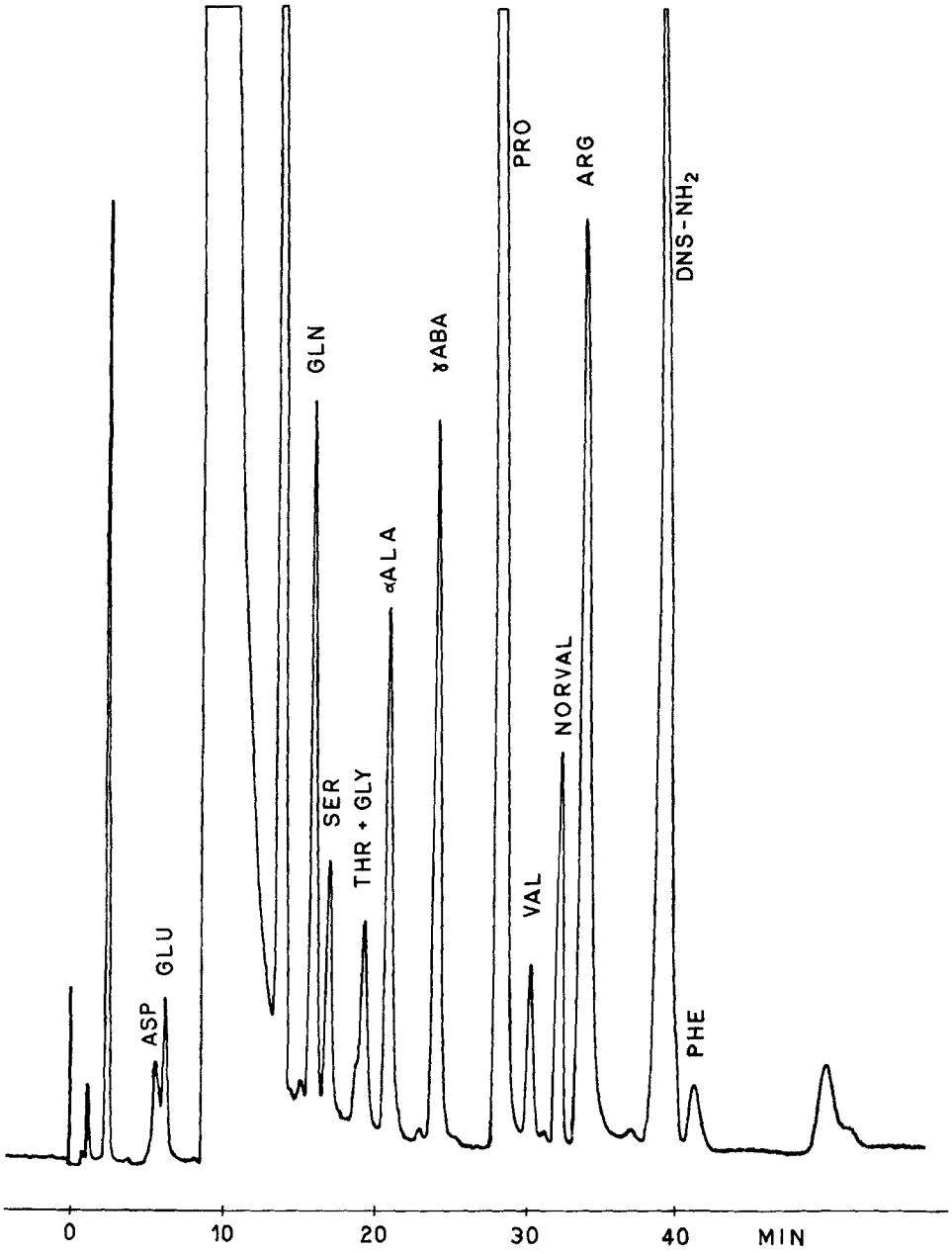

FIGURE 6. Grape must chromatogram after Dns-derivatization. Separation procedure as stated in Fig. 5.

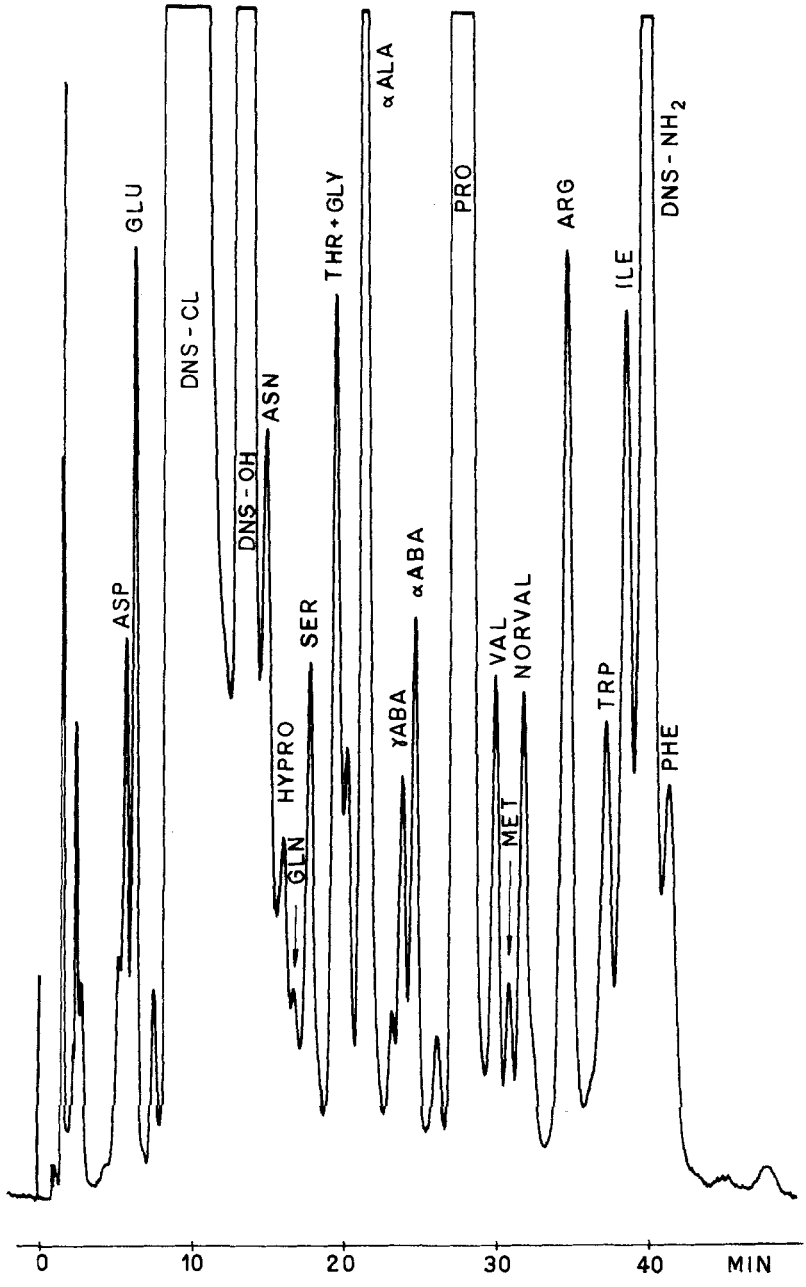

FIGURE 7. Wine chromatogram after Dns-derivatization. Separation as stated in Fig. 5.

amino nitrogen amount of unknown samples on the basis of such a correlation.

The optimun dansylation conditions deduced from our own experience are summed up as follows.

For must samples: The must is raised to a pH of around 9 and a aliquot containing approximately 25 µg total nitrogen is taken. 1 ml dansyl chloride 5.56 mM (1.5 mg in 10 ml acetone without water) and 1 ml lithium carbonate buffer 40 mM, pH 9.5 are added. The reaction is allowed to take place for 1 hour in the dark at room temperature and the internal standard (25 µ litres Dns-norvaline 0.1 mg/ml) is added. It is concentred to dryness at room temperature and is dissolved again in 0.3 ml methanol. It is filtered through a Millipore Type FH Filter with a pore diameter of 0.50 µm before injection.

For wine and vinegars samples: It is raised to a pH of around 9, an aliquot containing approximately 60 µg tctal nitrogen is taken and 2.7 ml dansyl chloride and 2.7 ml buffer are added. It is continued as in the case of musts.

The injected quantities correspond to approximately 4 µg total nitrogen for musts and 10 µg total nitrogen for wines and vinegars (fig. 6 and 7).

CONCLUSIONS

The decrease of Dns-amino acids k' log with the increase of the solvent polarity is not linear, as is the case of most solutes and solvents. When buffer molarity increases, Dns-amino acids retention increases except for arginine, wich does not behave in the same way as the remaining Dns-amino acids. On the other hand, pH increase within a 5.9 - 7.9 interval implies a higher Dns-amino acid retention.

The quantitative results obtained and the Dns-amino acids stability are satisfactory.

Dansyl derivatives of the amino acids from musts, wines and vinegars are suitable ones for the analysis of these samples.

The amount of dansyl chloride required is at least eight times higher than the amino nitrogen content of the sample. In practice, 1 ml dansyl chloride 5.56 mM should be added to the must sample amount equivalent to 25 µg total nitrogen. To carry out wine and vinegar analysis, the use of a higher initial sample amount is suggested.

AKNOWLEDGEMENT

This research was supported (in part) by a grant from the Spanish Vinegar Industries Research Association. We are grateful to Waters Associates by his technical support.

REFERENCES

1. Gross, C. and Labousse, B., Study of the dansylation reaction of amino acids peptides and proteins, Eur. J. Biochem., 7, 463, 1969.

2. Seiler, N., Methods of Biochemical Analysis, Vol. 18, D. Glick, ed., John Wiley & Sons, New York, 1970, p. 259.

3. Zanetta, J.P., Vincendon, G., Mandel, P., and Gombos, G., The utilisation of 1-dimethylaminonaphtalene 5-sulphonyl chloride for quantitative determination of free amino acids and partial analysis of primary structure of proteins, J. Chromatogr., 51, 441, 1970.

4. Weiner, S. and Tishbee, A., Separation of Dns-amino acids using reversed-phase high-performance liquid chromatography: a sensitive method for determining N-termini of peptides and proteins, J. Chromatogr. 213, 501, 1981.

5. Gray, W.R., Methods in Enzymology Vol. 11, C.H.W. Hirs and S.N. Timasheff, eds., Academic Press, New York and London 1967, p. 139.

6. Spivak, V.A., Scherbukhin, V.V., Orlov, V.M. and Varshavsky, JA.M., Quantitative ultramicroanalysis of amino acids in the form of their Dns-derivatives. II. On the use of the dansylation reaction for quantitative estimation of amino acids, Anal. Biochem., 39, 271, 1971.

7. Biou,D., Queyrel, M., Visseaux, M.N., Collignon, I. and Pays, M., Separation and identification of dansylated human serum and urinary amino acids by two-dimensional thin-layer chromatography, Aplication to aminoacidpathies, J. Chromatogr., 226, 477, 1981.

8. Bayer, E., Grom, E., Kalteneggar, B. and Uhmann, R., Separation of amino acids by high performance liquid chromatography, Anal. Chem., 48, 1106, 1976.

9. Hsu, K. and Currie, B.L., High performance liquid chromatography of Dns-amino acids and application to peptide hydrolysates, J. Chromatogr., 166, 555, 1978.

10. Wilkinson, J.M., The separation of dansyl amino acids by reversed phase high performance liquid chromatography, J. Chromatogr., Sci., 16, 547, 1978.

11. Hogan, D.L., Kraemer, K.L. and Isenberg, J.I., The use of high-performance liquid chromatography for quantitation of plasma amino acids in man, Anal. Biochem., 127, 17, 1982.

12. Wiedmeier, V.T., Porterfield, S.P. and Hendrich, C.E., Quantitation of Dns-amino acids from body tissues and fluids using high-performance liquid chromatography, J.Chromatogr., 231, 410, 1982.

13. Neadle, D.J. and Pollit, R.J., The formation of 1-dimethylaminonaphtalene-5-sulphonamide during the preparation of 1-dimethylamino naphtalene-5-sulphonyl amino acids, Biochem., J., 97, 607, 1965.

14. Tapuhi, Y., Schimidt, D.E., Lindner, W. and Karger, B.L., Dansilation of amino acids for high performance liquid chromatography analysis, Anal. Biochem., 115, 123, 1981.

15. Karger, B.L., Gant, J.R., Hartkoff, A. and Weiner, P.H., Hidrophobic effects in reversed-phase liquid chromatography, J. Chromatogr., 128, 65, 1976.

16. Hearn, M.T.W. and Grego, B., High-performance liquid chromatography of amino acids, peptides and

proteins. XL. Further studies on the role of the organic modifier in the revesed-phase high-performance liquid chromatography of polypeptides. Implications for gradient optimisation, J. Chromatogr., 255, 125, 1983.

17. Bij, K.E., Horvath, Cs., Melander, W.R. and Nahum, A., Surface silanols in silica-bonded hydrocarbonaceaus stationary phases. II Irregular retention behaviour and effect of silanol masking. J. Chromatogr., 203, 65, 1981.

18. Hill, D.W., Walter, F.H., Wilson, T.D. and Stuart, J.D., High performance liquid chromatographic determination of amino acids in the picomole range, Anal. Chem., 51, 1338, 1979.

19. Lindroth, P. and Mopper, K., High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with o.phthaldialdehyde, Anal Chem. 51, 1667, 1979.

20. Polo, C. and Llaguno, C., Evolution des acides aminés libres dans le moût de raisin sous l'action des levures de fleur. I.- Etúde qualitative et quantitative des acides aminés libres des moûts de raisin, Conn. Vigne Vin, 8, 81, 1974.

21. Polo, C. and Llaguno, C., Evolution des acides aminés libres au cours de la fermentation alcoolique des jus de raisin par les levures de fleur. II.- Composition des vins et des cellules de levures, Conn. Vigne et Vin, 8, 321, 1974.

22. Polo, C., Suárez, M.A. and Llaguno, C., Aportación al estudio de los vinagres españoles. I.- Contenido en aminoácidos libres y nitrógeno total, Rev. Agroquim. Tecnol. Aliment., 16, 257, 1976.

23. Martin, P., Suárez, A., Polo, C., Cabezudo, D. and Dabrio, M.V., Análisis de 19 aminoácidos por HPLC, Anal. Bromatol., 32, 289, 1980.

24. De Jong, C., Hudghes, G.J., van Wieringen, E. and Wison, K.J., Amino acid analysis by high-performance liquid chromatography. An evaluation of the usefulness of pre-column Dns derivatization, J. Chromatogr. 241, 345, 1982.

25. Polo, C., Herraiz, M. and Cabezudo, M.D., A study on nitrogen fertilization and fruit maturity as an

approach for obtaining the analytical profiles of wines and wine grapes. 3th. International Flavour Conference. Corfu. Greece. July 27 - 30, 1983.