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Proteins in Musts Estimated by Size-Exclusion HPLC

Francesco S. Interesse, Vincenzo Alloggio,
Francesco Lamparelli & Gerolmina D'Avella

Istituto di Industrie Agrarie, Università degli Studi di Bari,
Via Amendola, 165/a, 70126 Bari, Italy

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ABSTRACT

Proteinaceous materials in musts were separated by high performance liquid chromatography (HPLC) on the basis of molecular size, using a TSK G3000 SW column.

The size and the nature of the various proteinaceous fractions were estimated by comparing them with protein standards and proteins isolated from the must by gel-chromatography on a Sephadex G-100 column.

Seven fractions of different molecular weight: (I) > 300 000; (II) 83 000; (III) 18 700; (IV) 15 000; (V) 6600; (VI) 4200; (VII) <255, were obtained.

Among these the most abundant was fraction VI with a relative percentage of 36·4%.

Although this method did not completely separate all the proteinaceous fractions in the must, it showed a significant development in this field, due to its high level of resolution and its speed, when compared with traditional methods.

INTRODUCTION

Musts and wines contain a complex and variable amount of nitrogenous substances, the nature of which has been the object of numerous investigations. The results of such research show the presence not only of nitrogenous substances with a simple, or relatively simple, composition, but also of complex macromolecular structures (Ribéreau-Gayon, 1932; Usseglio-Tomasset & Di Stefano, 1977). A closer study of the latter has led to the ascertainment of the presence of proteins, peptides, pectins, leucoanthocyanins and glucide polymers variously associated with each other. The

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types of association and the ways in which such substances are linked, however, have not yet been clearly defined (Usseglio-Tomasset & Di Stefano, 1977).

The protein–peptide fraction, both in itself and as a sub-component of the macromolecular complexes, is considered the most important nitrogenous component in wines, because it can cause the formation of unwanted hazes or deposits during storage (Ribéreau-Gayon, 1932; Kean & Marsh, 1957; Moretti & Berg, 1965; Ferenczy, 1966; Bayly & Berg, 1967; Somers & Ziemelis, 1973; Usseglio-Tomasset & Di Stefano, 1977).

The influence of such fractions on the clarity and quality of the wine depends, however, on many factors; for example, grape variety, environment where the grapes were grown (Bayly & Berg, 1967), climate, soil, time of harvest (Ferenczy, 1966; Koch & Sajak, 1959), method of winemaking (Ferenczy, 1966), conditions of fermentation (Bayly & Berg, 1967; Ferenczy, 1966), and some other factors (Cordonnier, 1966).

In spite of extensive research, there is still a great deal of uncertainty about the effect of each of these factors on the qualitative and quantitative composition of the fraction under consideration. This is mainly due to the lack of an adequate analytical methodology, to use as a routine analytical tool (Cordonnier, 1966; Ferenczy, 1966; Somers *et al.*, 1973), and to the unclear nature of the peptide fraction (Cordonnier, 1966).

Our work aims at achieving a more detailed characterization of the protein–peptide fraction of the must and the wine, using a new rapid separation method. To this end, we have used a high-performance liquid chromatography technique with a size exclusion column (HPLC–SE), that provides good resolution levels and fast analysis, both when used to replace and to complement traditional methods.

In the present study, we have compared the results obtained by HPLC with those obtained by traditional gel-chromatography on a Sephadex G-100 column used for preparative purposes.

With both techniques the proteinaceous material of the must was separated according to molecular size. The fractions obtained were revealed by absorbance in the UV region of the spectrum, thus allowing the detection of proteins, glycoproteins, pectins, polyphenols, nucleic acids, or other acids. Therefore, the HPLC–SE and gel-permeation chromatogram peaks may be attributed to any of these components or their complexes.

MATERIALS AND METHODS

Preparation of must

In this study *Vitis vinifera* (cv. Pinot Bianco) grapes were used. The grapes were chilled to 0–4°C, crushed, basket-pressed, centrifuged at

10 000 g min^{-1} for 15 min at 4°C and filtered, first through Whatman No. 1 paper and then through a Millipore S.A. (SMWP) membrane with a pore size of 0·45 μm.

Gel-chromatography: Apparatus and operating conditions

For the preparative separation of must components, 10 ml of the sample were submitted to gel-permeation, carried out on a Sephadex G-100 (Pharmacia Fine Chemicals) column (2 cm inside diameter × 54 cm). The swelling of the gel, packing, equilibration and elution of the column were performed according to the gel manufacturer's instructions.

The eluent was the same as that used in HPLC and was pumped into the column at a rate of 0·55 ml min^{-1}. The void volume, determined with Blue Dextran 2000, was 35 ml.

The material in the column effluents was continuously monitored by absorbance at 280 and 254 nm, using UVICORD III (LKB) connected to a 6520 LKB recorder. Each fraction (total volume 2·75 ml) was recovered by an automatic fraction collector 7000 Ultrorac (LKB).

An excellent repeatability of the elution curve was ensured by using the same gel-bed and flow-rate. After each test, a few centimetres of the first filtering layer were substituted. The fractions corresponding to well-separated peaks were collected. Aliquots (20 μl) of the collected fractions of each peak were submitted to further separation by HPLC–SE. The remaining part was dialyzed with benzoylated tubing (Sigma Chemical Co.), concentrated and then assayed for total protein by the Bio-Rad protein assay and biuret method.

HPLC–SE apparatus and operating conditions

The analysis was performed with a Beckman isocratic system, which included a 112 solvent delivery module, a Model 210 sample injection valve with a loop of 20 μl, and a Model 160 absorbance detector with a zinc lamp for 214 nm.

Samples were separated on a (7·5 ml inside diameter × 60 cm) TSK G3000 SW column (Toyo Soda, Japan), protected by a precolumn (0·46 cm inside diameter × 4·5 cm) containing ultrasphere Si 5 μm, placed between the pump and the injector. A guard column (7·5 mm inside diameter × 7·5 cm) TSK was placed between the injector and the analytical column, connected by capillary tubes of 0·1 mm inside diameter (entire volume, 2 μl), and packed with the same material as the TSK G3000 SW. The column mobile phase, pumped at 0·5 ml min^{-1}, was a buffer Na–phosphate 0·15M (pH 7·0), containing 0·3M NaCl and 0·02% NaN_3. Absorbance of the eluate was monitored at 214 nm.

TABLE 1
Typical Data and Repeatability of the Elution Times of the Standards

Standards	*Molecular weight*	*Composition of standard solution* (*μg/μl*)	*Area* (*Mean value*) (%)	*Capacity factor K*	*Elution time* (*Mean value*) (*min*)	*Standard deviation* (*SD*) (*min*)	*Relative standard deviation* (*RSD*)
Blue Dextran	2 000 000	0·27	4·7	0	20·59	0·01	0·05
Catalase	240 000	0·07	14·5	0·40	28·75	0·01	0·03
Bovine Serum Albumin	67 000	0·10	12·3	0·67	34·38	0·03	0·09
Albumin egg	45 000	0·20	17·8	0·83	37·60	0·03	0·08
Chymotrypsinogen	25 000	0·11	17·1	1·02	41·55	0·05	0·12
Cytochrome C	12 300	0·10	14·6	1·07	42·65	0·05	0·12
Melittin	2 840	0·13	6·0	1·54	52·25	0·07	0·13
DNP–Alanine	255	0·03	13·0	1·82	58·13	0·09	0·15

The analytical conditions for ten chromatographic runs are indicated in Fig. 1.

Since the absorbance of the must fraction with a molecular weight of around 4000 Daltons was much higher than the others, the separation was performed twice, in different conditions: the first monitored to 0·150 absorbance units full scale (AUFS), the second to 0·600 (AUFS). The chromatograms were then superimposed. For the standards, the AUFS was 0·150.

Electronic integration was performed with a Shimadzu Chromatopac CR2A.

The total elution time in our operating conditions was around 70 min.

Standard for the HPLC–SE system

The standards used for calibrating the TSK G3000 SW column were: DNP-Alanine (molecular weight 255), Melittin (molecular weight 2840), Cytochrome C (molecular weight 12 300), Chymotrypsinogen (molecular weight 25 000), Albumin egg (molecular weight 45 000), Bovine Serum Albumin (molecular weight 67 000), Catalase (Bovine) (molecular weight 240 000) purchased from Serva Feinbiochemia, and Blue Dextran 2000 purchased from Sigma Chemical Co.

To determine the revelation limit of each standard, 20 μl of solutions, containing different amounts of the standard dissolved in a buffer (the same as for the elution), were preliminarily chromatographed.

To determine the resolution limit, chromatographic runs were carried out on 20 μl of a solution, containing all the standards indicated, at the concentrations shown in Table 1.

Determination of protein

The protein concentration of the must and of the fractions derived from the gel-permeation was determined by the biuret method, according to the procedure of Amerine & Ough (1973). To confirm the results, Bradford's (1976) dye-binding method was also used, as indicated in the Bio-Rad protein assay (Bio-Rad Laboratories, München, West Germany). In both cases, Bovine Serum Albumin was used as a standard.

The determination of total nitrogen, by micro-Kjeldahl, was also carried out on the must, which was obtained as previously indicated.

RESULTS AND DISCUSSION

HPLC separation of standards

First of all, we tried to create optimal analytical conditions. For this purpose, we used the standards listed in the 'Materials and Methods'

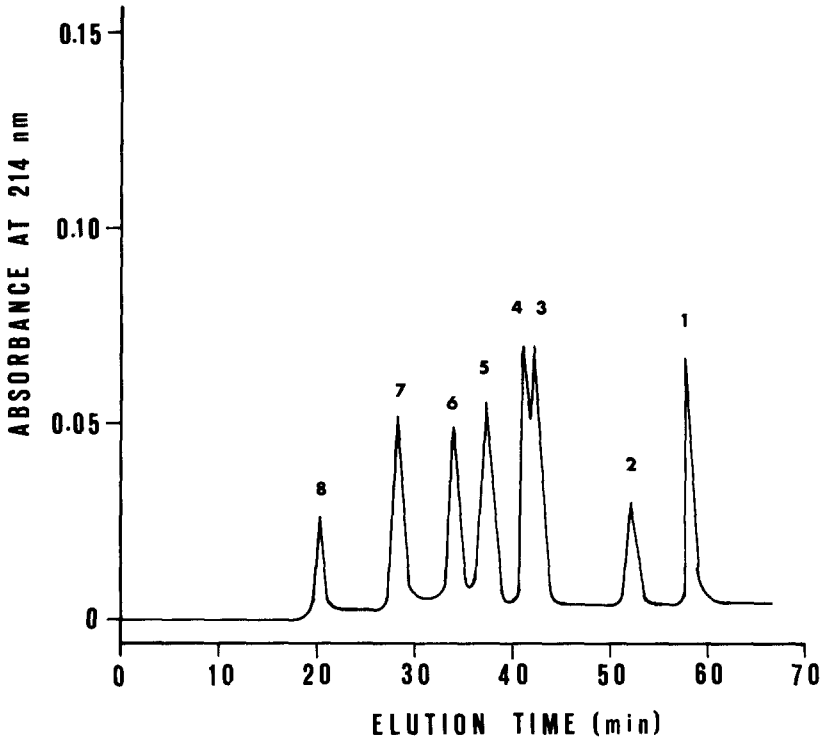

Fig. 1. High-performance liquid chromatography separation of standards. *Standards used*: 1, DNP–Alanine (molecular weight 255); 2, Melittin (molecular weight 2840); 3, Cytochrome C (molecular weight 12 300); 4, Chymotrypsinogen (molecular weight 25 000); 5, Albumin egg (molecular weight 45 000); 6, Bovine Serum Albumin (molecular weight 67 000); 7, Catalase (bovine) (molecular weight 240 000); 8, Blue Dextran 2000 (molecular weight 2 000 000). *Conditions*: Column-TSK G3000 SW (60 cm); Eluent—0·15M Na_2HPO_4/NaH_2PO_4, 0·3M NaCl, 0·02% NaN_3, pH 7·0; Flow rate—0·5 ml min^{-1}; Chart speed—12 cm h^{-1}; Injection volume—20 μl; Detection—214 nm, 0·15 AUFS.

section, with which we defined the resolution and revelation limits, as well as the optimization flow.

The preliminary passage into the column of each standard, at different concentrations, allowed us to exclude, in the operative conditions chosen, both the effects of saturation in the column and poor spectrophotometric revealability.

Figure 1 shows that the protein standards and Blue Dextran were well resolved by HPLC–SE.

The relationship between elution time and log (molecular weight) for the standards listed in Table 1 is shown in Fig. 2. The useful working range for our column is for protein with a molecular weight between 15 000 and 150 000.

Figure 3 shows the results of flow optimization experiments with three

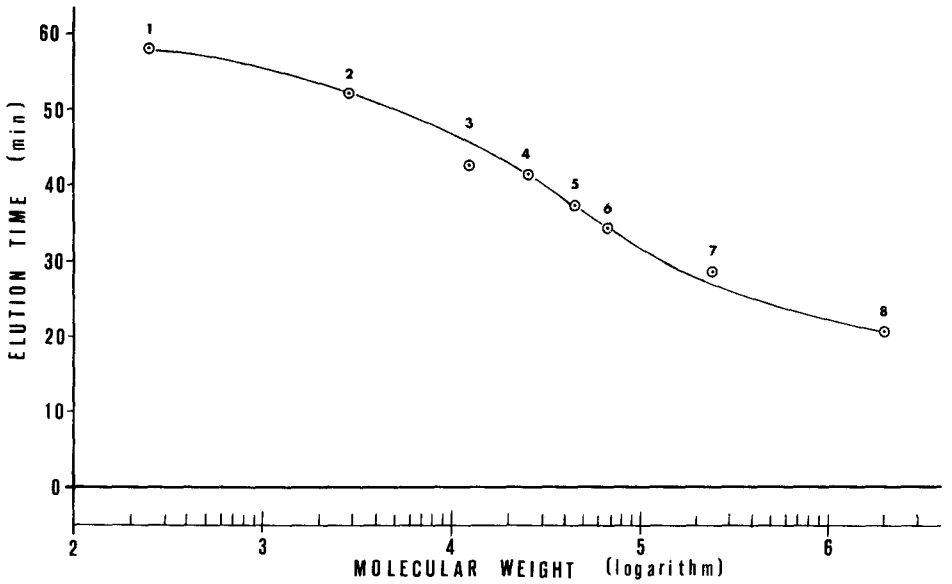

Fig. 2. Correlation between elution time and logarithm of the molecular weight. Standards and analytical conditions are the same as in Fig. 1.

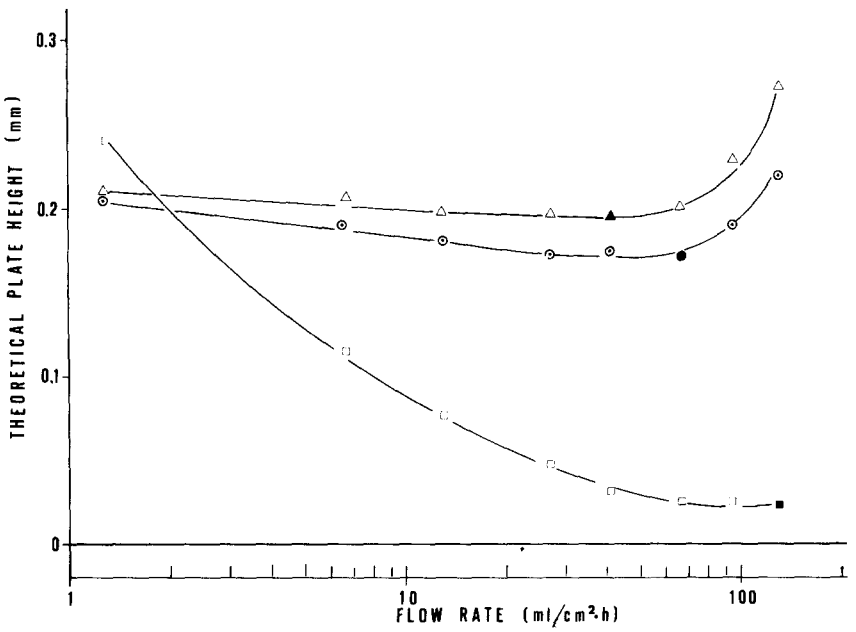

Fig. 3. Correlation between separation efficiency (expressed as height equivalent to a theoretical plate) and linear flow-rate, for standards of different molecular weight; -□-□-□- DNP-Alanine (molecular weight 255); -⊙-⊙-⊙- BSA (molecular weight 67000); -△-△-△- Catalase (molecular weight 240000). The analytical parameters different from the flow are shown in Fig. 1.

standards of different molecular weight (DNP–Alanine, BSA and Catalase, at the same concentrations as in Table 1). The correlation between the flow-rate and the separation efficiency led us to consider $0{\cdot}5\,ml\,min^{-1}$ the optimum flow-rate.

For Chymotrypsinogen and Cytochrome C it was impossible, even varying the flow-rate and the relative concentrations, to obtain a higher resolution value (R) than the experimental one. Since the experimental value of relative retention (α) for the two components was 1·05 (Table 1), we would have had to have a column with a number of effective plates (N_{eff}), higher than that of our column, in order to obtain an R value which was at least equal to 1. This statement derives from the relation connecting those quantities to each other: $N_{eff} = (4R\alpha/\alpha - 1)^2$ (Engelhardt, 1979).

The elution times of the standards, obtained from ten chromatographic runs in the same experimental conditions, are subject to only small fluctuations. In Table 1, besides the composition of the standard mixture, the capacity factor (K), the molecular weights, the elution times for each component and the percentual values of the areas are shown. For each component, the standard deviation (SD) and relative standard deviation (RSD) values are also given. The repeatability obtained is good, since, for the ten samplings carried out, the elution times do not differ by more than 0·1 min.

Separation of proteinaceous material in the must

The 'Pinot Bianco' must, used in this experiment, was submitted to exclusion chromatography separation, both by HPLC and by gel-chromatography on Sephadex G-100 (Figs 4 and 5, respectively).

The latter, more traditional, method was used both for preparative purposes and to compare the elution profile of the proteinaceous fraction of the must with that obtained by HPLC. The first profile was obtained by monitoring continuously at 280 and 254 nm, the second at 214 nm, where the peptide bond absorbs highly.

The gel-permeation profiles of 10 ml of must, loaded on Sephadex G-100, are shown in Fig. 5. The elution patterns at 280 and 254 nm, qualitatively superimposable, revealed the presence of two peaks for each profile.

The fractions corresponding to the two peaks were collected separately. The first (A) was eluted in the void volume (molecular weight $>150\,000$), the second (B) with a K_{av} value (partition coefficient) of 0·90.

The protein contents of the two peaks (A and B) and of the must, which were determined by the two methods, are shown in Table 2, as well as the value of total nitrogen of the must.

By comparing the profile in Fig. 5 with the data shown in Table 2, it can

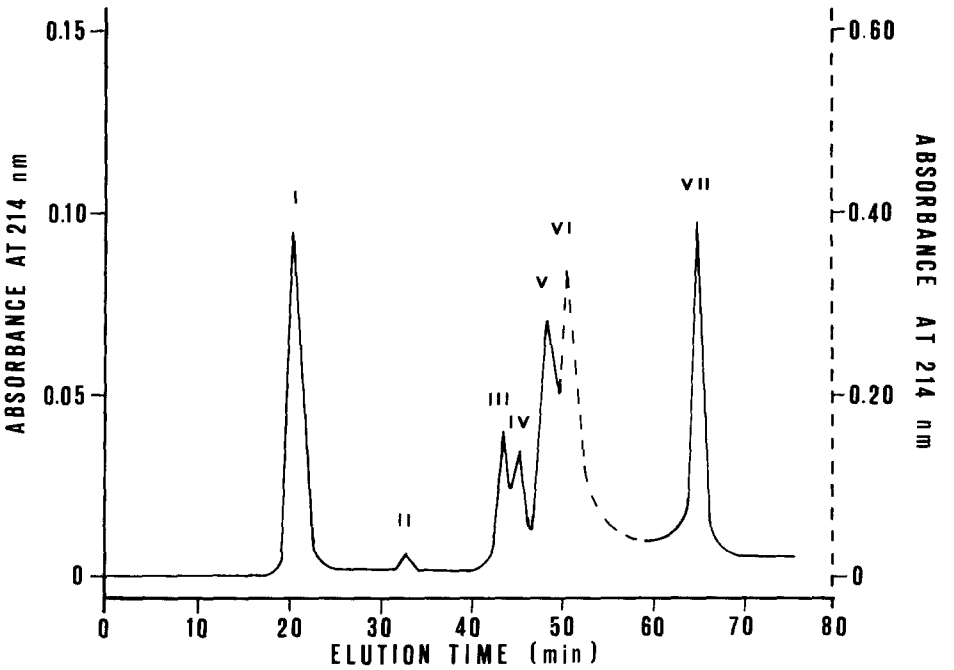

Fig. 4. High performance liquid chromatography profile of 'Pinot Bianco' must. ——— 0·15 AUFS; - - - - 0·60 AUFS. The other analytical parameters are shown in Fig. 1.

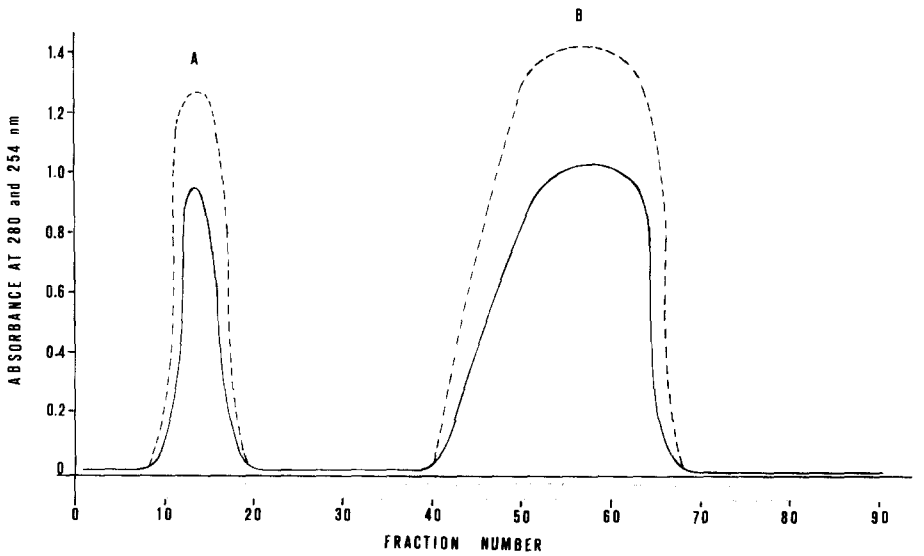

Fig. 5. Gel-chromatography of 'Pinot Bianco' must on a column of Sephadex G-100. ——— absorbance at 280 nm; - - - - absorbance at 254 nm; Column: 540 × 20 mm; Flow-rate: 0·55 ml min^{-1}; Volume of each fraction: 2·75 ml.

TABLE 2
Protein Content of the Must and the Two Peaks (A and B), and Total N of the Must

Samples	*N Total* (*mg litre*$^{-1}$)	*Protein* (*mg litre*$^{-1}$)	
		Biuret	*Bio-Rad*
Must 'Pinot Bianco'	401	550	535
Peak A G-100	—	152	159
Peak B G-100	—	321	317

be seen that about 86% of the protein, which was initially present in the must, is recuperated after being passed on Sephadex, and that the first peak eluted represents about 32% of the eluted protein. The area of this peak, both at 280 and 254 nm, corresponds to a relative value of 22%.

The identical tendency of the curves and the coincidence of the maximum absorbances at 280 and 254 nm for both peaks, indicate either the existence of molecular complexes of a certain size, made up of proteins, peptides, phenols and nucleic acids, or the presence of different substances, which, by chance, have the same molecular size.

In addition, by examining the absorbance values of each fraction at the two wavelengths, it can be noted that their ratio, 254 nm/280 nm, is never less than 1·3. This indicates the presence, in the eluted material, of a nucleotidic component (Schleif & Wensink, 1981) as well as the proteinic one. Moreover, the former, present in non-negligible amounts as has already been stated by other researchers (Bourdet & Herard, 1958; Tercelj, 1965; Somers & Ziemelis, 1972; Feuillat & Morfaux, 1976), can also contribute, together with other substances, such as phenolic ones, to the total absorption at 280 nm (Somers & Ziemelis, 1972).

The examination using HPLC included both the analysis of the must and of the two peaks obtained by gel-permeation. In each HPLC run 20 μl of sample were used.

The repeatability, for these analyses, at the two different values of AUFS, tested five times, was good and comparable with the analysis of the standards reported in Table 1.

The elution profile of the must is shown in Fig. 4 where seven fractions can be seen that differ in molecular size.

The mean elution times, together with the capacity factor (K), the estimated molecular weights and the relative percentages of the areas of each peak, are reported in Table 3.

Further separation by HPLC of the two peaks (A and B) that were preparatively isolated by gel-permeation, gave two profiles. These, when superimposed, reproduced excellently the profile obtained for the entire

TABLE 3
Typical Data of Each Fraction of 'Pinot Bianco' Must, Subjected to HPLC

Number fractions	*Elution time (Mean value) (min)*	*Area (Mean value) (%)*	*Molecular weight*	*Capacity factor K*
I	20·59	19·9	>300 000	0
II	33·11	0·4	83 000	0·61
III	43·90	6·3	18 700	1·13
IV	45·42	3·7	15 000	1·21
V	48·88	17·4	6 600	1·37
VI	50·80	36·4	4 200	1·47
VII	65·56	15·9	<255	2·18

Analytical conditions, of five chromatographic runs, as in Fig. 4.

must, already shown in Fig. 4. For this reason, these two profiles have not been shown in this work. In fact, we discovered that peak A, when chromatographed in HPLC, showed only one high molecular weight fraction in the void volume, which was comparable with the first fraction (I) of the must, directly subjected to the same test, shown in Fig. 4.

Peak B, on the other hand, gave rise to six different fractions, with elution times equal to those of fractions II to VII of the must sample separated by HPLC (Table 3). The values of the relative percentages of the corresponding areas (0·6, 8·2, 5·0, 22·1, 44·8, 19·3) are also comparable with those of fractions II–VII in Table 3. In any case, there is no connection between these values and the amounts of protein–peptide, present in the different peaks. In fact, many other substances can affect the elution profile and hence the values of the relative percentages of the areas.

By examining all the data at our disposal and opportunely integrating the results shown in Fig. 4 with the data reported in Table 3, it can be deduced that the first fraction eluted in the void volume (10·25 ml) is a molecular complex of large dimensions (molecular weight >300 000), in which the protein–peptide fraction is certainly present. The relative percentage of this fraction (19·9%) is comparable with that of peak A obtained by gel-permeation (22%).

A protein–peptide component is present in each, or in some, of the five fractions (II–VI Fig. 4), alone or combined in various ways with other components.

By comparing the elution time values of these peaks with those of the standards used (Fig. 1), it can be deduced that the five fractions obtained show molecular weights between 83 000 and 4000 (Table 3). However, it should be noted that the values obtained are approximate. This is due, apart from the impossibility of comparing accurately the standards and the matrix used, to the fact that nucleotidic material is certainly present in

peaks A and B. This material, as is well known, has a filamentary structure, and therefore the elution times are different from those of protein of the same molecular weight.

Fraction VII, which is reported in Table 3 with an elution time value of 65·56 min and whose area is about 16% of the total area, gives a value of K ($K = 2{\cdot}18$), which is higher than that of the standard DNP–Alanine ($K = 1{\cdot}82$) shown in Table 1. This indicates that mechanisms, other than exclusion, contribute to the retention. In fact, it is worth remembering that, in general, adsorptive or ion-exchange processes may be associated with exclusion. However, we think that the amino acid component is prevalent in the latter peak.

The examination of the protein fraction of musts and wines, already carried out by many other researchers, using more traditional analytical methods, has led to conclusions which are sometimes different (Bayly & Berg, 1967; Usseglio-Tomasset & Di Stefano, 1977), but which are, however, indicative of the complexity and extent of the problems. Therefore, we thought it essential, before carrying out further tests, to concentrate on a satisfactory separation of the proteinaceous material, in order to understand its nature and molecular size.

Some researchers have already studied this subject (Somers & Ziemelis, 1973; Usseglio-Tomasset & Di Stefano, 1977). For example, Usseglio-Tomasset & Di Stefano (1977), when analyzing freshly pressed musts, found a complex with a very high molecular weight, consisting of glucides, proteins, leucoanthocyanins and pectins.

However, the different analytical method and grapes used by us does not allow a close comparison with these results. We can only observe an analogy between Usseglio-Tomasset's macromolecular complex and our first fraction, besides some further information already pointed out in this work.

On the other hand, the use of the HPLC technique, applied in this case as a complement to the gel-permeation, gave us more specific detailed information on the proteinaceous material of the must. The presence in the must of seven fractions of different molecular weights, resolved quickly and using only the principle of exclusion, provides a decidedly more complex picture than that furnished by more traditional techniques.

These results, which show an important development in the field, were obtained by an analytical use of the method. However, the use of the isocratic technique for large-scale preparative applications, and of the gradient elution system, to favour adequate resolution and recovery of small peptides in the 1000–10 000 molecular weight range, can promote a better understanding of the meaning and function of each nitrogenous component.

CONCLUSIONS

The use of the HPLC–SE technique allowed the separation of the proteinaceous material of the must, according to the size of the components, in the molecular weight range from 250 to more than 300 000.

Analytical separations can be achieved in little more than 1 h, and this time can be reduced.

The method was reliable. The repeatability, in fact, in the operative conditions chosen for the standards and the sample, was good, since it never exceeded an SD value of 0·1 min.

The sample can be applied directly to the column without any pretreatment.

In order to obtain a more detailed knowledge and a better understanding of all the proteinaceous fractions in the must, we believe in developing and applying other methods to use as a complement to this technique.

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