Gel Permeation Chromatography of Anthocyanin Pigments from Rosé Cider and Red Wine

Toshihiko Shoji,* Akio Yanagida, and Tomomasa Kanda

Institute for Production Research and Development, The Nikka Whisky Distilling Company, Limited, 967 Matsuyama, Masuo, Kashiwa, Chiba 277-0033, Japan

Anthocyanin pigments from rosé cider and red wine, which is a sparkling wine made from apples, were separated by gel permeation chromatography (GPC) using a TSK-GEL Toyopearl HW-40 (F) column with a 6:4 mixture of acetone and 8 M urea (pH 2.0) as the eluent. Under this condition, all phenolic compounds containing monomeric anthocyanins (mainly, cyanidin-3-galactoside; Cyn-3-gal), oligomeric and polymeric anthocyanins, chlorogenic acid, catechin, epicatechin, procyanidin B2 (PB2), and procyanidin C1 (PC1) in the apples and rosé cider were found to elute according to molecular weight. Bleaching of the anthocyanin pigments by SO_2 was gradually effective in the fractions separated by GPC according to elution volume. In the case of rosé cider, the levels of Cyn-3-gal decreased markedly during fermentation and then decreased gradually during maturation. We confirmed that anthocyanin polymers are not detectable in apple juice; these polymers are produced during fermentation and maturation as determined by GPC. The polymeric anthocyanins from red wine could be separated by this method, too.

Keywords: *Rosé cider; red wine; anthocyanin pigments; gel permeation chromatography; fermentation; maturation*

INTRODUCTION

The anthocyanin pigments in red wine are known to change from monomeric anthocyanins to polymeric anthocyanins continuously during the wine-making process (maceration, fermentation, maturation, bottling, and aging). This change is attributed to the formation of condensed polymeric anthocyanins resulting from interactions between anthocyanins and other phenolic compounds such as flavonols (e.g., catechin, PB2).

The structures and mechanisms of formation of these polymeric anthocyanins have been proposed in reports concerning the condensation processes which are suggested to occur through direct condensation between anthocyanins and flavonols (Liao et al., 1992; Santos-Buelga et al., 1995), the reactions between them in which acetaldehyde is involved (Bakker et al., 1993; Baranowski et al., 1983; Dallas et al., 1996a,b; Rivas-Gonzalo et al., 1995), and the copigmentation (Figueiredo et al., 1996; Mistry et al., 1991). However, because of the complicated nature of the polymerization reactions in red wine, many studies have been carried out using various model systems to examine the condensation processes (Bakker et al., 1993; Dallas et al., 1996b; Francia-Aricha et al., 1997; Rivas-Gonzalo et al., 1995; Saucier et al., 1997).

For the detection of polymeric anthocyanins in red wine, different types of spectrophotometric methods have been used (Rivas-Gonzalo et al., 1992). One method is based on the increase in color intensity at low pH, and others are based on the bleaching of colors in the presence of SO_2 or H_2O_2. High-performance liquid chromatography (HPLC) has been used for the separation and quantification of individual monomeric and oligomeric anthocyanins from red wine, but the polymerized anthocyanins have not been detected by HPLC. In addition, attempts to isolate the polymeric anthocyanins actually produced in red wine have failed. Previously, we established a GPC method using a Toyopearl HW-40 (F) column, with a mixture of acetone and 8 M urea (pH 2.0) as the eluent, for molecular sieve separation of oligomeric and polymeric procyanidins from apple juice (Yanagida et al., 1999). In the present study, we applied this method for the separation of the anthocyanin pigments from rosé cider and red wine.

The aim of our studies was to reconfirm the changes from monomeric anthocyanins to polymeric anthocyanins that occur in rosé cider and red wine and to demonstrate the applicability of GPC in separation of anthocyanin pigments. We used rosé cider, which is a sparkling wine made from apples, to study the changes in anthocyanin pigments during the vinification process. As the only anthocyanins reported to be present in apples are cyanidin glycosides (Sun and Francis, 1967; Timberlake and Bridle, 1971), it seemed to us that there would be fewer kinds of anthocyanin polymers generated during vinification than in the case of red wine. A spectrophotometric method based on bleaching by SO_2 was used to evaluate the degree of polymerization of the anthocyanin pigments separated by GPC.

MATERIALS AND METHODS

Materials. Cyn-3-gal, cyanidin-3-rutinoside (Cyn-3-rut), cyanidin (Cyn), malvidin-3-glucoside (Mal-3-glu), and malvidin-3,5-diglucoside (Mal-3,5-diglu) were obtained from Funakoshi Co., Ltd. (Tokyo, Japan). PB2 (epicatechin-(4β→8)-epicatechin) and PC1 (epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin) were isolated from the apples by using Sephadex LH-20 and preparative ODS columns.

* To whom correspondence should be addressed (e-mail xls05673@niftyserve.ne.jp).

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Red wine made in the region of Bordeaux, France, in the 1993 season was commercially obtained from the winemaker. It was preserved at 18 °C and used as a sample in 1998.

Unless otherwise stated, all other chemicals used were of reagent grade.

Preparation of Rosé Cider. Rosé cider was made from commercial Starking Delicious apples (6.5 kg). At the time of crushing the apples, SO_2 was added to the must at 200 mg/L. The must was brought into contact with apple skins overnight at room temperature to extract anthocyanins from the apple skins. After the apples were pressed, the juice (4.5 L) was clarified and inoculated with a yeast starter culture (*Saccharomyces bayanus*) at 6 °C. After fermentation, the cider was centrifuged and allowed to stand at 4 °C in an atmosphere of CO_2.

Sample Preparation for GPC. To obtain the samples of phenolic compounds containing anthocyanidins to be applied to GPC, rosé cider or red wine was passed through a preparative ODS (FS 1830 FT, Organo Co., Ltd., Tokyo, Japan) packed column (90 × 25 mm i.d.) to absorb these compounds. After the column was washed with the distilled water, the phenolic compounds were eluted with MeOH. The eluate was concentrated by means of a rotary evaporator at 45 °C and lyophilized.

Samples of phenolic compounds were prepared from apple juice (day 0) and from cider during fermentation (day 10) and maturation (days 45, 80, 115, 160, and 190).

GPC Fractionation. The Toyopearl HW-40 (F) (TOSOH Co., Ltd., Tokyo, Japan) (950 × 25 mm i.d.) column was packed at atmospheric pressure. Each sample prepared from rosé cider or red wine was diluted to a concentration of 25 mg in 0.5 mL of eluent and applied to the column. A 6:4 mixture of acetone and 8 M urea (adjusted to pH 2.0 with HCl) was used as the eluent. The flow rate was 1.0 mL/min, and the operation was carried out at room temperature.

After adding 5 N HCl (200 μL) to each fraction (2 mL), the anthocyanins were detected by measuring the absorbance at 520 nm. Phenolic compounds in each GPC fraction were detected by a modified Folin−Ciocalteu method (Slinkard and Singleton, 1977).

The anthocyanins and other phenolic compounds were recovered from each GPC fraction according to the method of Yanagida et al. (1999). The acetone in each GPC fraction was removed by evaporation, and the remaining solution was 10-fold diluted with the distilled water. The anthocyanins and other phenolic compounds were adsorbed to a C_{18} Sep-Pak cartridge (Waters Associates, Tokyo, Japan), and the column was washed with the distilled water to remove the urea. Finally, the absorbed compounds were eluted with MeOH. The eluate was concentrated by means of a rotary evaporator at 45 °C and lyophilized.

HPLC Analysis of Anthocyanin Pigments. Anthocyanins were analyzed by means of an HPLC equipped with a L-6200 intelligent pump (Hitachi Ltd., Tokyo, Japan), a AS-2000 autosampler (Hitachi), and a Inertsil ODS-3 (GL Sciences Inc., Tokyo, Japan) reverse-phase column (150 × 4.6 mm i.d.) at 30 °C. A mixture of 0.01 M KH_2PO_4 solution (adjusted to pH 1.8 with H_3PO_4) and MeOH was used as the mobile phase, and the flow rate was 1.0 mL/min. For the first 10 min, the initial eluent used was 20% MeOH and 80% 0.01 M KH_2PO_4 solution, followed by a linear gradient from 20% to 50% MeOH for 40 min; subsequently the concentration was held at 50% MeOH for 15 min and then returned to the initial conditions (20% MeOH) to reequilibrate for 10 min. Detection was performed using a L-4200 UV−vis detector (Hitachi) at 520 nm.

Rosé Cider Color at Acid (RCCA) and Polymeric Pigment Color (PPC). The RCCA and PPC were determined according to the modified method of Somers and Evans (1977). After polyphenol samples for GPC analysis were dissolved in 4.5 g/L malic acid solution (adjusted to pH 3.5) and 5 N HCl was added to them, the RCCA was determined by measuring the absorbance at 520 nm. Similarly, after adding 10% (w/v) bisulfite to polyphenol samples, the PPC was determined by measuring the absorbance at 520 nm.

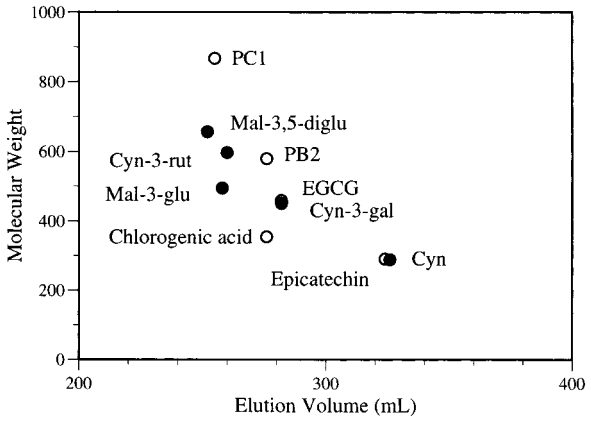

Figure 1. Relationship between elution volume resulting from GPC and molecular weight of phenolic compounds containing anthocyanins: (○) phenolic compounds without anthocyanins; (●) monomeric anthocyanins.

Measurement of Acetaldehyde. The concentration of acetaldehyde in rosé cider was measured by the enzymatic method of McCloskey et al. (1981).

RESULTS AND DISCUSSION

In studying the structures and mechanisms of formation of polymeric anthocyanins in rosé cider and red wine, there have been many difficulties encountered in separation of the polymeric anthocyanins from monomeric anthocyanins and many other phenolic compounds. In general, Toyopearl HW-40 and Sephadex LH-20 columns have been used to separate such phenolic compounds. However, the larger molecular weight compounds such as polymeric anthocyanins and procyanidins cannot be separated by these molecular sieve methods. Previously, we showed that polymeric procyanidins from unripe apples can be separated by GPC using Toyopearl HW-40 with a mixture of acetone and 8 M urea as the eluent (Yanagida et al., 1999). As shown Figure 1, anthocyanins and other phenolic compounds eluted according to molecular weight. There was a good correlation between elution volume and molecular weight. Therefore, we applied this method to separation of anthocyanin pigments from rosé cider and red wine and examined the changes in anthocyanin pigments that occur during the vinification process.

The results of the GPC fractionation of polyphenolic compounds separated from apple juice and rosé cider are shown in Figure 2. The anthocyanins present in fraction 142 were recovered using a C_{18} Sep-Pak cartridge, and it was confirmed by HPLC that fraction 142 mainly contained Cyn-3-gal. Similarly, chlorogenic acid, which is one of the polyphenols present in apples, was found in fraction 150, and catechin and epicatechin were found in fraction 170. We demonstrated that there were no polymeric anthocyanins present, only monomeric anthocyanins were present, in Starking Delicious apple juice. GPC fractionation of phenolic compounds from Orin (Golden Delicious × Indo) apple juice, which is a green apple containing only anthocyanins, indicated that there were no anthocyanin pigments present at all (data not shown). On the other hand, we examined the changes in anthocyanin pigments occurring in rosé cider during fermentation and maturation and found that there were not only monomeric anthocyanins but also polymeric anthocyanins present in rosé cider which had matured for 180 days after fermentation.

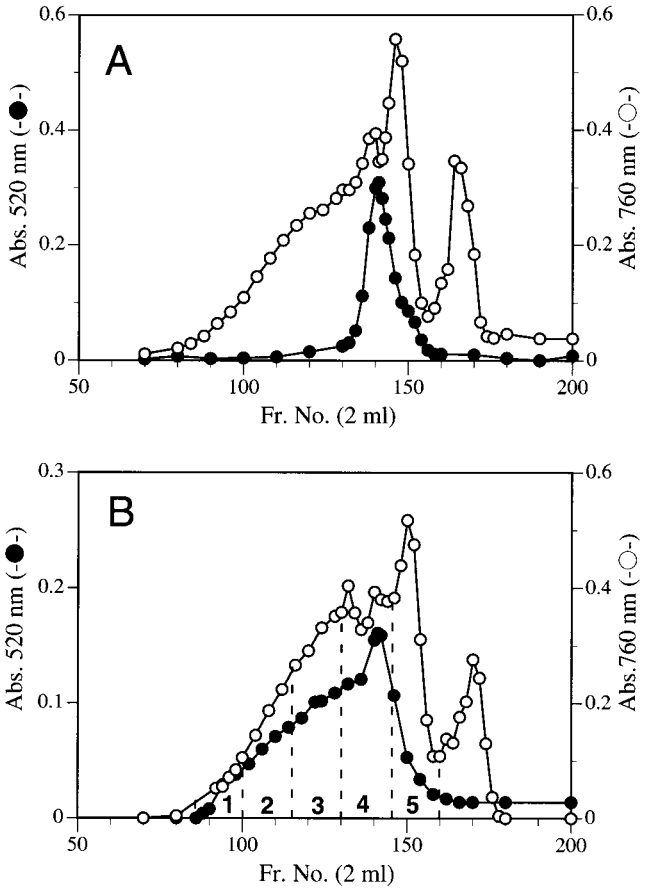

Figure 2. GPC profiles of polyphenolic compounds separated from apple juice (A) and rosé cider (B): (●) Abs. 520 nm (corresponds to the contents of total anthocyanins in each fraction); (○) Abs. 760 nm (correspond to the contents of total polyphenols in each fraction). GPC fractions obtained from rosé cider were numbered from 1 to 5. fr. 1 = fractions 85−100; fr. 2 = fractions 101−115; fr. 3 = fractions 116−130; fr. 4 = fractions 131−145; fr. 5 = fractions 146−160.

In contrast, apples and ciders are known to contain oligomeric and polymeric procyanidins (condensed tannins) (Ohnishi-Kameyama et al., 1997; Guyot et al., 1997; Lea et al., 1978). The high molecular weight fraction obtained from apple juice by GPC may contain oligomeric and polymeric procyanidins.

As shown in Figure 2B, the polyphenolic compounds in rosé cider were separated in GPC fractions 1−5 and were recovered using a C_{18} Sep-Pak cartridge. The results of bleaching by SO_2 are shown in Figure 3. The early fractions (fractions 1, 2, and 3) obtained by GPC showed weaker effects of bleaching by SO_2 than the later fractions (fractions 4 and 5). This finding demonstrated that the early fractions contained polymeric anthocyanins with molecular weights larger than that of Cyn-3-gal (fraction 142) and showed that the polyphenolic compounds from rosé cider were separated according to molecular weight by GPC. The rosé cider was shown to contain condensed polymeric anthocyanins, as found in red wine. The results of HPLC analysis are shown in Figure 4. The component with the retention time of 27.5 min was Cyn-3-gal. Fractions 1, 2, and 3 among the GPC were detected as a bulging baseline which was attributable to polymeric anthocyanins. In fractions 4 and 5, which contained low molecular weight anthocyanins, Cyn-3-gal and peaks corresponding to unidentified compounds were detected. These peaks may be due to

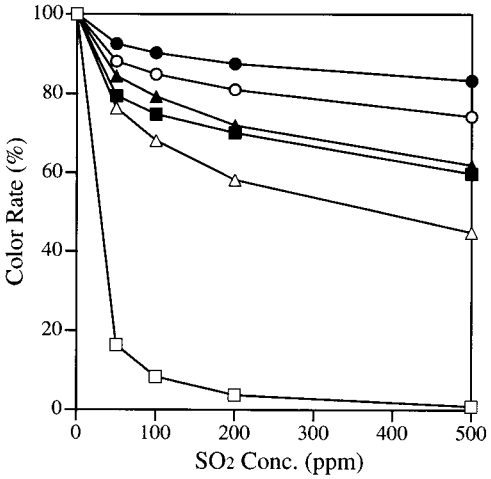

Figure 3. Effect of bleaching by SO_2 on anthocyanins in GPC fractions obtained from rosé cider. Each lyophilized GPC fraction (1−5) corresponding to that in Figure 2B was dissolved in 4.5 g/L malic acid solution (adjusted to pH 3.5). After adding 10% bisulfite to each sample, the absorbance at 520 nm was measured. (●) fr. 1; (○) fr. 2; (▲) fr. 3; (△) fr. 4; (■) fr. 5; (□) Cyn-3-gal.

oligomeric anthocyanins newly produced during the vinification process.

The changes in Cyn-3-gal concentration and the absorbance at 520 nm of GPC fractions (fractions 100 and 120) in apple juice or the must of rosé cider during fermentation and maturation are shown in Figure 5. The apple juice employed for production of rosé cider was colorless or slightly orange in color before fermentation. After 10 days of fermentation, the color of the cider reached a maximum, as indicated by the absorbance at 520 nm, and decreased slowly thereafter through the vinification process. However, the Cyn-3-gal concentration in the must of cider during fermentation decreased rapidly for up to 10 days. The Cyn-3-gal may be oxidized by polyphenol oxidase or it may combine with proteins produced by the yeasts and then precipitate. Anthocyanins in apple juice combined with SO_2 and became colorless. In the course of fermentation, the yeast cells produce carbonyl compounds (e.g., acetaldehyde, pyruvic acid, α-ketoglutamic acid) which combine with SO_2 more strongly than anthocyanins. Acetaldehyde is one of the most important compounds involved in the condensation between anthocyanins and flavonols during maturation and aging of wine or cider. As a result, the must develops color and become bluish pink.

We studied the formation of polymeric anthocyanins in the course of fermentation by GPC. There was little difference in the chromatographic profiles of anthocyanin pigments between day 10 and day 45 of the fermentation period. Somers and Evans (1977) showed that anthocyanin pigments undergo marked changes soon after the start of fermentation. The differences in anthocyanin pigments during fermentation between rosé cider and red wine may be attributed to the pH of the must, the fermentation temperature, and the yeast employed.

After 10 days of fermentation, as the rosé cider matured at 4 °C under an atmosphere of CO_2, the Cyn-3-gal concentration decreased gradually. However, as seen in the GPC profiles, in the fractions (e.g., fractions 100 and 120) which contained components with molecular weights larger than that of Cyn-3-gal (fraction 142),

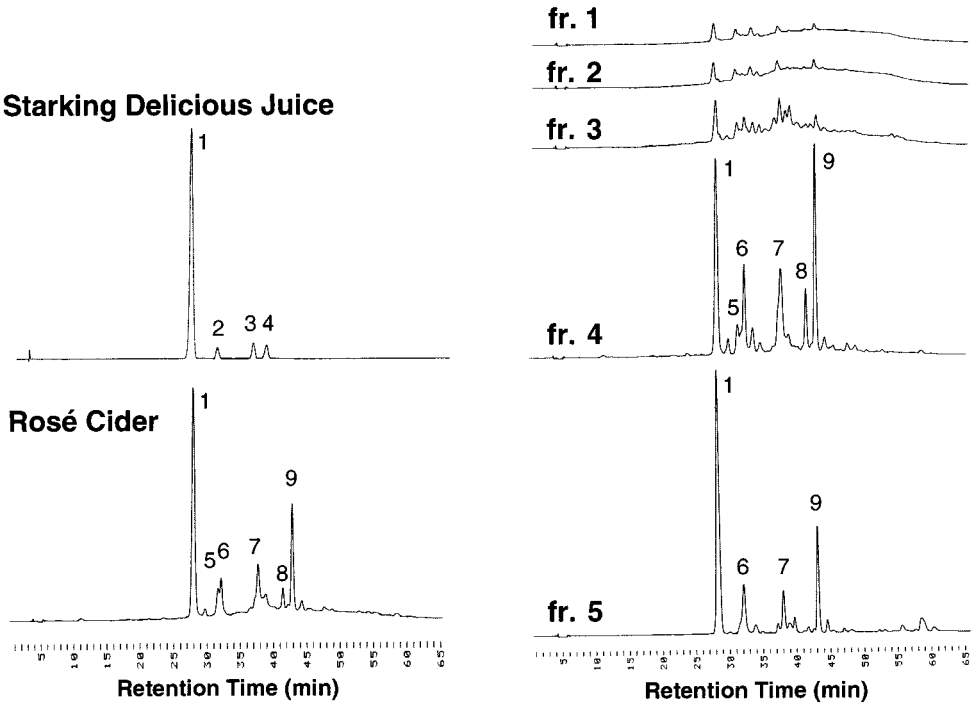

Figure 4. HPLC profiles of Starking Delicious juice, rosé cider, and GPC fractions from rosé cider recorded at 520 nm. Each lyophilized sample was dissolved in 10% EtOH (20 mg/mL). Peak 1 with the retention time of 27.5 min is Cyn-3-gal, which is the main anthocyanin in apple, and peaks 5−9 are the unidentified anthocyanins, which may be newly produced during the vinification process.

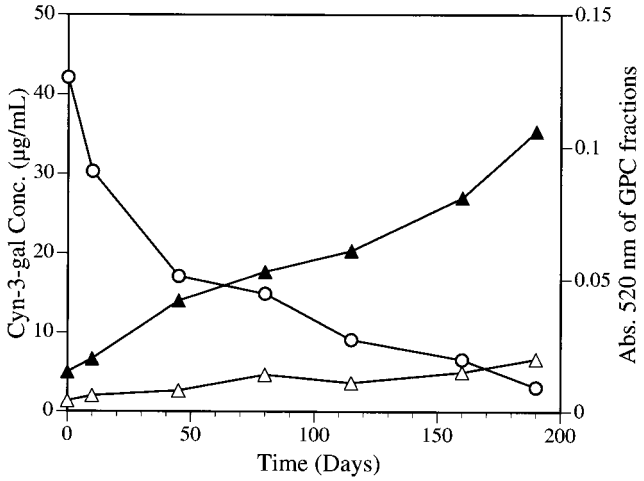

Figure 5. Changes in the Cyn-3-gal concentration of apple juice, or the must of rosé cider, and the absorbance at 520 nm of GPC fractions (fractions 100 and 120) during the vinification process. The Cyn-3-gal of rosé cider during the vinification process was analyzed by HPLC: (○) Cyn-3-gal; (△) fraction 100; (▲) fraction 120.

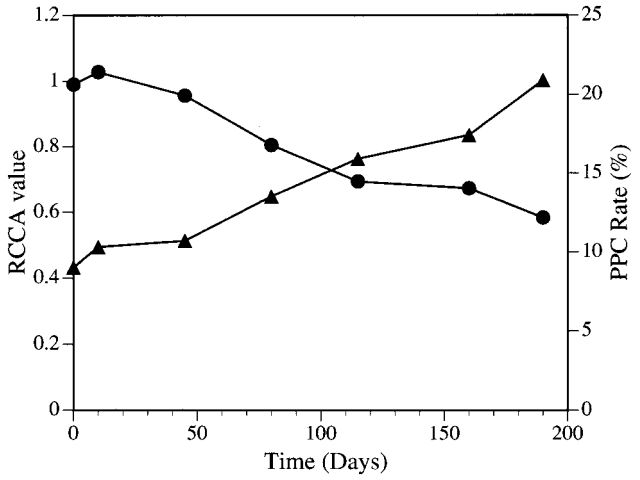

Figure 6. Changes in the RCCA and the PPC rate of rosé cider during the vinification process. RCCA = rosé cider color at acid; PPC = polymeric pigment color. The RCCA and PPC were determined by measuring the absorbance at 520 nm. The PPC rate was PPC/RCCA × 100: (●) RCCA value; (▲) PPC rate.

the anthocyanin pigments grew bigger and bigger during the maturation period and were larger than those found in apple juice or must (Figure 5). Similarly, the PPC rates for rosé cider increased during the course of maturation (Figure 6). These results indicate that polymeric anthocyanins were mainly formed in rosé cider during the maturation process. As polymeric anthocyanins increased during vinification, the acetaldehyde concentration in the rosé cider decreased slightly (data not shown). Many studies have been carried out using various model systems to examine the condensation processes, showing acetaldehyde is involved in the reactions between anthocyanins and flavonols (Bakker et al., 1993; Dallas et al., 1996b; Francia-Aricha et al., 1997; Rivas-Gonzalo et al., 1995). Anthocyanins and flavonols in the rosé cider have been shown to condense with acetaldehyde produced by the yeast.

Similarly, we employed GPC to estimate the amounts of anthocyanin pigments from red wine. Red wine made in the region of Bordeaux, France, in the 1993 season was used. The results are shown in Figure 7. Mal-3-glu, which is a major anthocyanin present in grape, was found in fraction 134 by HPLC. We confirmed by a spectrophotometric method based on bleaching by SO_2 that the red wine contained not only polymeric anthocyanins but also polymeric polyphenols (Somers and Evans, 1977; Nagel and Wulf, 1979; Bailey et al., 1994; Prieur et al., 1994; Souquet et al., 1996; Gao et al., 1997). Red wine is known to have food functionality such as antioxidant activity which limits the oxidation of low-

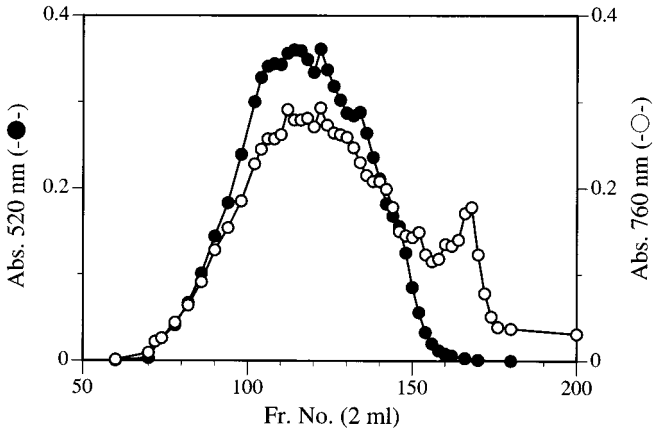

Figure 7. GPC profile of polyphenolic compounds separated from red wine, made in the region of Bordeaux, France, in the 1993 season: (●) abs. 520 nm (correspond to the contents of total anthocyanins in each fraction); (○) abs. 760 nm (correspond to the contents of total polyphenols in each fraction).

density lipoproteins (Frankel et al., 1995; Vinson et al., 1995), and this has attracted a great deal of public attention. New methods for separation of polymeric anthocyanins may contribute to studies which further our understanding of the functionality of red wine.

Molecular sieve separation of anthocyanin pigments produced in rosé cider and red wine during fermentation and maturation was achieved by GPC. We can estimate the degree of polymerization of the anthocyanin pigments in rosé cider and red wine by GPC using a Toyopearl HW-40 column.

ABBREVIATIONS USED

Cyn-3-gal, cyanidin-3-galactoside; GPC, gel permeation chromatography; HPLC, high-performance liquid chromatography; PB2, procyanidin B2; PC1, procyanidin C1; RCCA, rosé cider color at acid; PPC, polymeric pigments color.

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