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Short communication

# Development of direct size-exclusion chromatography separation for the determination of molar mass of native procyanidins in the phenolate form

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**Abstract**

A size-exclusion chromatographic method using an aqueous solvent as eluent was developed for the determination of the molecular mass of procyanidins has been developed. Size-exclusion chromatography is a chromatographic technique used to determine molecular weight distribution of polymers. During the development work, PL$^{TM}$ aquagel-OH columns and several different eluent compositions were investigated. The best separation of procyanidins was obtained with an eluent consisting of water–methanol (50:50, v/v), $NaNO_3$: 0.18 M, pH < p$K_a$ + 2. The results from this aqueous SEC method were compared with results obtained using SEC with organic solvents.


*Keywords:* Size-exclusion chromatography; Procyanidins; PL-aquagel-OH columns

## 1. Introduction

Phenol compounds are found in grapes and wines in the form of non-flavanoids (phenol acids, stilbenes) and flavanoids such as condensed tannins (e.g. proanthocyanidins, PA) and monoglucoside anthocyanidins. They are important in the colouration and organoleptic properties of berries and give specific characteristics to the wine. PAs are mainly present in the seeds and skin of grape, and are polymers of flavan-3-ols units and in the case of grape seeds (procyanidins, PC): catechin and epicatechin (Fig. 1). Grape seed procyanidins have been characterized by many analytical methods [1]. Several chromatographic approaches have been developed in order to obtain molecular mass information concerning proanthocyanidins. Cleavage techniques consist on the conversion of proanthocyanidins into their constitutive subunits via acid catalysis in the presence of excess nucleophile [2]. Subsequent analysis by reversed-phase high-performance liquid chromatography of the subunit products can provide the mean degree of polymerization of procyanidins, the composition of the terminal and extension units, and the mean molecule weight of procyanidins. Also, with the additional knowledge of subunit composition, the number-average molecular mass can be obtained. These methods, however, are unable to provide mass distribution information.

Chromatographic methods exist that analyze intact proanthocyanidins. Normal- and reversed-phase HPLC methods can be used to separate proanthocyanidins. Again, these methods only provide complete molecular mass distribution information. Size-exclusion chromatography (SEC) is the method routinely employed to determine polymer molecular mass [3]. The molecules are separated according to their hydrodynamic volume, which depends on their molar mass, the solvent and the chemical composition of the polymer. By calibrating the column with known standards, the molar mass of an analyte can be calculated. If the analytes do not

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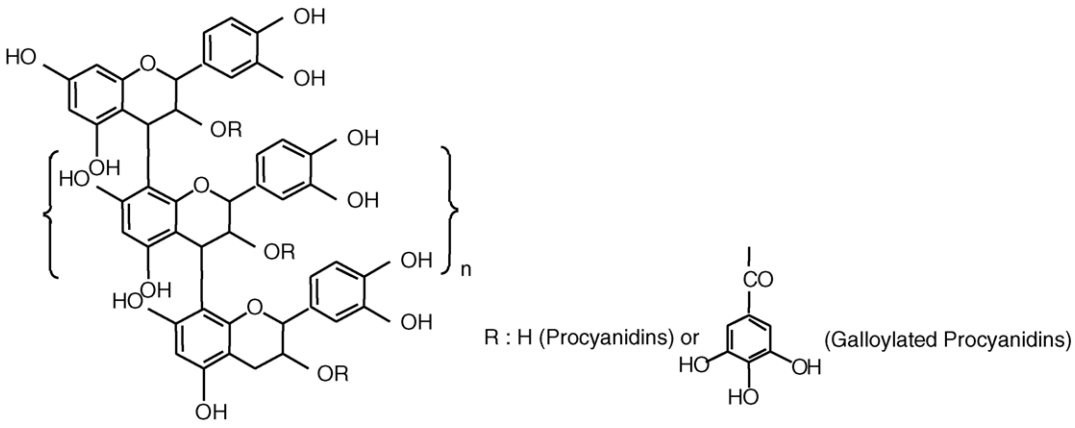


Fig. 1. Structures of procyanidins.

have the same relationship between molar mass and hydrodynamic volume as the standards used, the calculated molar mass will only be relative to the standards. SEC separates the molecules in a polymeric sample according to size, allowing the determination of both number-average molecular mass ($M_n$) and the weight-average molecular mass ($M_w$) and the polydispersity (I) according to the following equations:

$$M_{w,n} = \frac{\sum(N_i M_i^{x+1})}{\sum(N_i M_i^{x})}$$

$$I = \frac{M_w}{M_n}$$

where $N_i$ represents the number of moles of a polymer with $i$ repeating units, $M_i$ is the molecular mass of a polymer with $i$ repeating units, $x = 0$ for $M_n$ and $x = 1$ for $M_w$.

The gross structure of proanthocyanidin polymer is also characterized by its degree of polymerization (DP), i.e. average number of units in the polymer.

As mentioned previously, these two equations give information concerning the molar mass distribution of the polymer, which is an essential parameter for characterizing the polymer.

Several SEC methods have been developed for proanthocyanidin analysis. Early methods consisted on prederivatization of proanthocyanidins prior to analysis in order to eliminate the interactions between phenolic functional groups and SEC packing material [4]. Hence, SEC is usually used with organic eluents, but then peracetylation of the samples is requires.

The purpose of our work was to develop a reliable method for the characterization of procyanidins, regarding their molecular mass, by SEC using aqueous eluents.

The operating conditions for SEC using aqueous eluents are very dependant on the analytes and the column packing material. The operating conditions must be optimised in order to suppress non-size exclusion phenomena resulting from hydrophobic or ionic interactions since the risk of occurrence of non-exclusion behaviour is in this case greater than when using organic eluents. In using aqueous SEC, the risk of non-exclusion behaviour is greater than when using non-aqueous SEC. The non-exclusion behaviour can be controlled by adding salt [5–7].

## 2. Experimental

### 2.1. Samples

The investigated samples are grape seeds from *Vitis vinifera* L. Grapes were collected at commercial maturity. Seeds and skins were separated manually. Isolated seeds were lyophilized, crushed to obtain a fine powder and stored at −18 °C until required.

### 2.2. Chemicals

Poly ethylene oxide (PEO) standards ranging between 106 and 200,000 Da (Polymer Laboratories, Marseille, France) were used to calibrate the SEC columns used. The following reagents were used to prepare the different eluent compositions and for the procyanidin isolation: Ultrapure water, sodium chlorure (Analytical reagent quality, Prolabo, Pessac, France), sodium nitrate (99%, Aldrich, Saint-Quentin, France), sodium phosphate (99%, Aldrich, Saint-Quentin, France), sodium bicarbonate (analytical reagent quality, Aldrich, Saint-Quentin, France), ammonium acetate (98%, Aldrich, Saint-Quentin, France), methanol (analytical reagent quality, Prolabo, Pessac, France), ethanol (analytical reagent quality, Prolabo, Pessac, France), acetic acid (99.8%, Riedel-de-Haën, Saint-Quentin, France), pure chloroform (SDS, Riedel-de-Haën, Saint-Quentin, France).

### 2.3. Procyanidin isolation

Portions (4 g) of seeds were extracted with 20 mL of ethanol–water–acetic acid (50:45:5, v/v/v) under nitrogen atmosphere with mechanical stirring for 12 h. A liquid–liquid

extraction was performed adding 20 mL of chloroform to the extract, in order to eliminate lipids and pigments. Then, the mixture was centrifuged at $2500 \times g$ for 10 min. The lower green phase corresponding to the chloroform phase was discarded and the upper yellow phase corresponding to the hydroalcoholic (HA) phase was recovered. The liquid–liquid extraction with chloroform was repeated three times and all the HA extracts of each sample were mixed, the ethanol of the extract was evaporated (at 35–40 °C under reduce pressure), and the water freeze-dried (lyophilized) and then kept under nitrogen and stored at −18 °C until analysis.

### 2.4. Size-exclusion chromatography (SEC) coupled with UV detection

The study of the $M_w$ (weight-average molecular mass) distribution of catechin and seed samples was performed using the native forms.

The analyses were performed using a Thermo Quest™ (Les Ulis, France) instrument equipped with two (300 mm × 7.5 mm, 8 μm) columns, PL aquagel-OH 30™ and PL aquagel-OH 40™, in series, protected with a guard column (50 mm × 7.5 mm, 8 μm) of the same material (Polymer Laboratories, Marseille, France). Detection was made at 280 nm, using an UV detector (SPECTRA SERIES™ UV-150) and a refractive index detector (RI-150). PL Caliber™ (Polymer Laboratories, Marseille, France) software was used for data acquisition.

Approximately 8 mg of the standards and 8 mg of the samples (grape seed isolated procyanidins) were dissolved in 10 mL of eluent. The solutions were filtered through a Millex-LH (Millipore) PTFE filter (0.45 μm), before analysis by SEC. Standards and samples were injected in the same volume, 20 μL. The flow-rate of the mobile phase was 1 mL/min and analysis were performed under isocratic elution mode. Eluents of different compositions were tested with the PL aquagel-OH columns. Eluents consisting of ultrapure water, mixtures of ultrapure water with an organic modifier, and mixtures of salt aqueous solutions with organic modifier were used. The analyses were performed at room temperature. The columns were equilibrated overnight before use. All eluents were degassed before use.

### 2.5. Calibration

The calibration curve (Fig. 2) for $M_w$ determination was constructed using PEO standards dissolved in mobile phase at concentrations of 8 mg/ml.

Among the polymer standards available, PEO were chosen for calibration. They are soluble in water and give a good response with refractive index detector (RI). They were prepared in the same mobile phase solution as the samples for direct comparison. Whether dissolved in mobile phase or in water, PEO eluted with identical retention volumes.

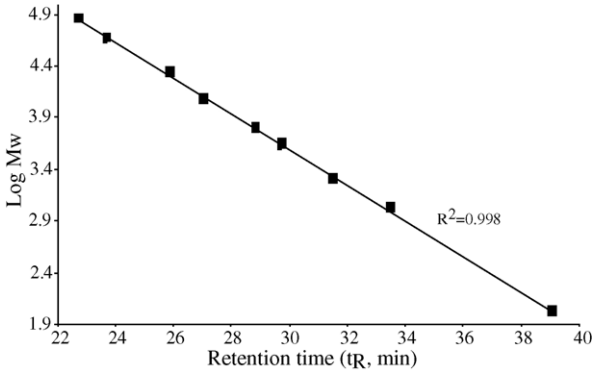


Fig. 2. A calibration curve of PEO standards in the range 106–78,450 g/mol.

### 2.6. Titration

Acidity constants of polyphenols were determined by titrating 50 mL of 5 g/L catechin solution or procyanidin extract with 1 N NaOH, and the ionic strength of the solution was adjusted with 0.5 g $(NH_4)_2SO_4$, using the Mettler Toledo™ DL50 apparatus. The temperature was maintained at room temperature. Experiments were performed in triplicate.

## 3. Results and discussion

The molecular weight distribution (MWD) of PC can be determined by SEC using organic solvents and specific columns, but then it is required to perform a pre-derivatization of the PC. In this paper, an alternative method is presented (without pre-derivatization step). Due to the complex nature of water soluble polymers, it is often necessary to modify the eluent in order to avoid sample–sample and sample-to-column interactions which can result in poor aqueous SEC separations. The good stability of the PL aquagel-OH packing material allows the eluent to be modified to suit the polymer, while retaining high column efficiency.

On the one hand, to eliminate ionic interactions, the eluent can be modified by the addition of salt and/or the adjustment of pH. On the other hand, to inhibit hydrophobic interactions, the addition of a weak organic solvent, such as methanol, was enough for water soluble polymers with hydrophobic character. The PL aquagel-columns can be used to analyze a wide range of neutral, polar, anionic and cationic samples.

### 3.1. Optimisation of SEC parameters

In order to obtain a good elution of the procyanidins with the PL aquagel-OH columns, eluents of different compositions were tested. Eluents consisting of ultrapure water, mixtures of ultrapure water with an organic modifier (20 and 50% of methanol), and mixtures of salt aqueous solutions (sodium nitrate, sodium chlorure, sodium phosphate, sodium

bicarbonate, ammonium acetate) with organic modifier (20 and 50% of methanol) were used.

And in order to optimize the method, different concentrations of salt solutions (0.05, 0.10, 0.18 and 0.20 M) were tested and the influence of pH and sample concentration on this elution were studied.

### 3.1.1. Ultrapure water as eluent

When ultrapure water was used as eluent, catechin was adsorbed to the columns. Using ultrapure water as eluent was not successful in eluting catechin from the PL aquagel-OH columns. Therefore, mixtures of water and an organic solvent such as methanol had to be used.

### 3.1.2. Ultrapure water with an organic modifier as eluent

Ultrapure water with methanol as organic modifier was chosen as the eluent. Several assays were performed with different percentages of methanol: 20 and 50%. The optimum percentage of methanol was determined. Using only 20% methanol, part of the catechin samples was adsorbed to the columns. Since the response intensity was low, only a small amount of catechin was desorbed. As the percentage of methanol increased, the amount absorbed decreased. With 50% of methanol, desorbtion of catechin samples improved. So, the higher the percentage of methanol was, the better the desorbtion was. The best result was obtained with 50% of methanol; this percentage being the maximum quantity of methanol in the eluent recommended by the column manufacture. Therefore, initially water–methanol (50:50, v/v) was chosen.

### 3.1.3. Salt solutions with an organic modifier as eluent

These columns were tested with different eluents consisting of mixtures of salt solutions and methanol in the proportion 50:50 (v/v). Different salts (five different salts: sodium nitrate ($NaNO_3$), sodium chlorure (NaCl), sodium phosphate ($NaH_2PO_4$), sodium bicarbonate ($NaHCO_3$), and ammonium acetate ($NH_4C_2H_3O_2$)) and different salt concentrations (0.05, 0.10, 0.18 and 0.20 M) were tested. Salt solutions allowed us to modify ionic interactions and to suppress them. We noticed that the nature and the concentration of a salt are both very important parameters. They do not have the same effect on sample elution. Fig. 3 shows the evolution of catechin desorbtion from the column according to the nature of the salts. Elution volumes increase markedly if salt is present, indicating a decrease in the hydrodynamic volume of the polymer when salt is added. Assays were performed in the middle of the basic range so the choice of the nature of salts was very important. Salts must not react with hydroxide ions present. Therefore, we could not use salts which could give protons, such as ammonium acetate. Following our different assays, sodium nitrate ($NaNO_3$) was found to be the most appropriate salt under our analysis conditions and was the selected salt, since it does not react with the eluent and desorbs catechin efficiently. The sodium nitrate solution ensured that the polymers were eluated without interfering with chromatographic mechanisms, i.e. adsorption. Special care must be taken to wash out the salt solution from the chromatographic system unit after sample analysis to avoid precipitation and blockage within the hydraulic pathway.

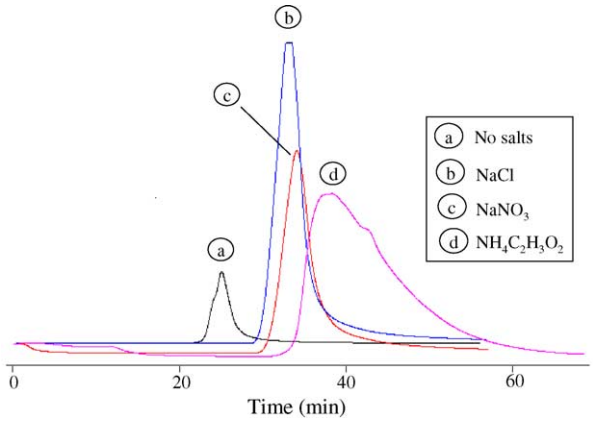


Fig. 3. Superimposed SEC chromatograms of catechin—influence of the nature of salt solutions PL aquagel-OH 30$^{TM}$, PL aquagel-OH 40$^{TM}$ columns; mobile phase water/methanol (50:50, v/v) at 1 ml/min; pH 11.2; UV detection at 280 nm.

### 3.1.4. Concentration of salt solutions

Concentration of salt solutions is an important parameter. A too high concentration could cause plugs (in the tubes and rheodyne injector). Modifying salt solution concentration leads to a change in catechin elution time. Different $NaNO_3$ concentrations were tested: 0.05, 0.10, 0.18 and 0.20 M. Fig. 4 shows the evolution of catechin desorbtion with respect to

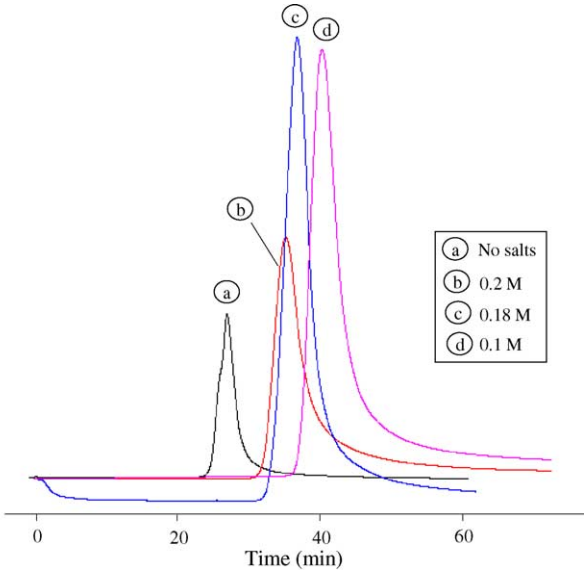


Fig. 4. Superimposed SEC chromatograms of catechin—influence of salt concentration. PL aquagel-OH 30$^{TM}$, PL aquagel-OH 40$^{TM}$ columns; mobile phase water/methanol (50:50, v/v) at 1 ml/min; pH 11.2; salt: $NaNO_3$; UV detection at 280 nm.

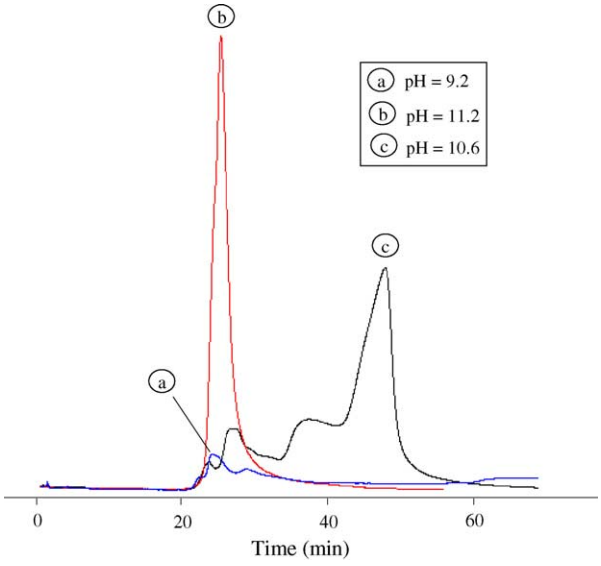


Fig. 5. Superimposed SEC chromatograms of catechin—influence of pH. PL aquagel-OH 30$^{TM}$, PL aquagel-OH 40$^{TM}$ columns; mobile phase water/methanol (50:50, v/v) at 1 ml/min; [$NaNO_3$]: 0.18 M; UV detection at 280 nm.

concentration. When salt concentration was high, we noticed a shift of the catechin peak to a higher mass. Considering the elution time, the calibration area and the form of the peak, the $NaNO_3$ concentration chosen was 0.18 M.

### 3.1.5. Influence of pH

Different pHs were tested: neutral pH, pH range 10–11, pH range 11–12 (Fig. 5) (the pH value was adjusted with a pH-meter and a sodium hydroxide solution 1 N):

- At neutral pH, elution times were much too long, greater than 120 min, i.e. greater than the total permeation time. These observations suggested that ionic or hydrophobic interactions or hydrogen interactions exist between sample and the column packing material.
- pH range 10–11: for catechin analysis, several peaks were eluted (probably due to the fact that the purity of the commercial catechin is 99.9%). In this range of pH, only part of the catechin molecules is in the phenolate form, therefore two peaks appeared in the chromatogram.
- pH range 11–12: Only one sharp peak appeared in the chromatogram. Hence, interactions were suppressed in this pH range. For very basic pH such as pH 11.2, retention time was 20 min. Hence, we have modified considerably the elution time of catechin, respect to the previous pHs assayed.

### 3.1.6. Influence of sample concentration

In this SEC method, we noted that the sample concentration is of minor importance. Assays were performed at different concentrations: 0.6, 0.8 and 1.0 g/L. On the SEC column, the sample is separated according to the size of the polymer molecules and the relative concentration of the individual molecules making up the polymer sample is used to calculate the molar mass. Column overloading may give band broadening which could affect the determination. We must only respect the recommendations of the constructors (of these columns); the concentrations must not be superior to 10 mg/mL. And they recommend 8 mg/L with these specific molecules (such as procyanidins). We must only respect the recommendations of the constructors (of these columns); the concentrations must not be superior to 10 mg/mL. And they recommend 8 mg/L with these specific molecules (such as procyanidins).

### 3.1.7. Linearity

Nine PEO standards were used for calibration. $M_w$ values of PEO standards are 78,450, 43,520, 22,800, 11,840, 6450, 4120, 1900, 1080 and 106 g/mol. The calibration curve is

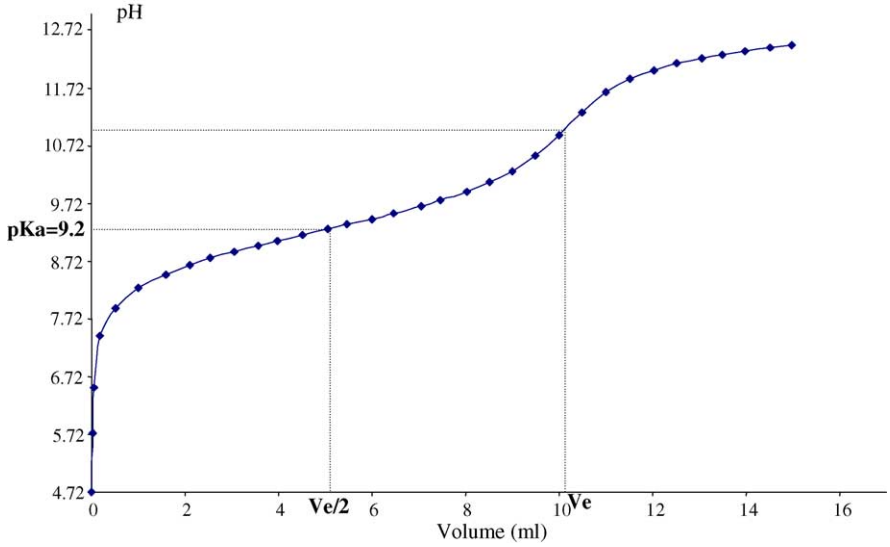


Fig. 6. A titration curve of catechin.

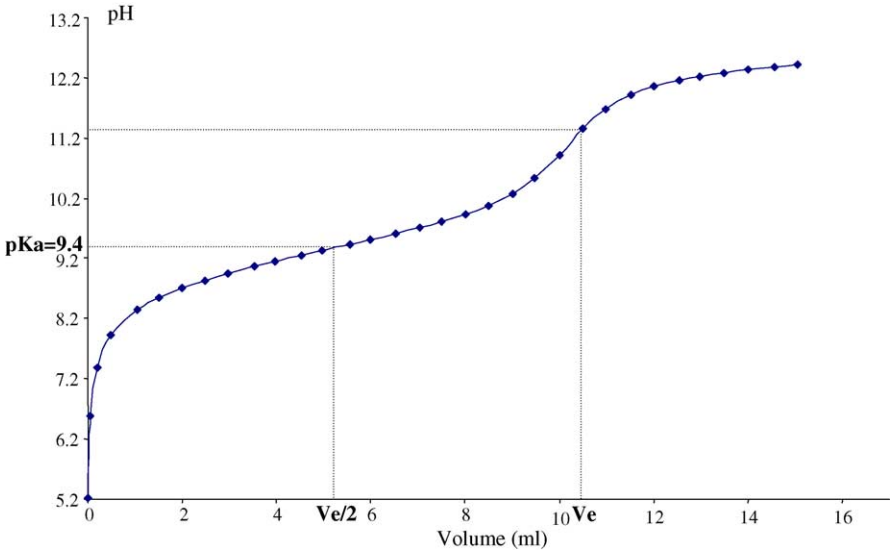


Fig. 7. A titration curve of procyanidins.

shown in Fig. 2 and the corresponding data in Table 1 . The resulting polynomial equation for $M_w$ as a function of retention time ($t_R$) using a (1) was used for all $M_w$ determinations:

$$\log M_w = 8.7615 - 0.1725 t_R \quad (1)$$

with a correlation coefficient ($r$) of 0.998.

### 3.2. Titration

Catechin was titrated (Fig. 6) in order to determinate its p$K_a$ because all previous observations showed that catechin is quantitatively eluted when it is in the phenolate form. The p$K_a$ value, determined from the titration curve (and corresponding to pH value at Ve/2), was 9.2. When we studied the influence of pH, we noticed that when $10 < pH < 11$, only a part of catechin molecules is in the phenolate form. So, it was necessary to set the pH value according to the equation: $pH = pK_a + 2$.

The optimised elution conditions for the determination of catechin by SEC with two PL aquagel-OH columns connected in series are water–methanol (50:50, v/v) containing $NaNO_3$: 0.18 M, being the pH adjusted at 11.2.

Table 1
Data and statistics of the calibration curve

| | $t_R$[a] ± S.D.[b] |
|---|---|
| POE1 ($M_w$: 78,450) | 22.64 ± 0.02 |
| POE2 ($M_w$: 43,520) | 23.66 ± 0.03 |
| POE3 ($M_w$: 22,800) | 25.86 ± 0.03 |
| POE4 ($M_w$: 11,840) | 27.01 ± 0.04 |
| POE5 ($M_w$: 6450) | 28.80 ± 0.02 |
| POE6 ($M_w$: 4120) | 29.66 ± 0.03 |
| POE7 ($M_w$: 1900) | 31.44 ± 0.02 |
| POE8 ($M_w$: 1080) | 33.49 ± 0.03 |
| POE9 ($M_w$: 106) | 30.13 ± 0.02 |

[a] Retention time (min).
[b] Standard deviation.

### 3.3. Applications

The method established above was applied to the characterization of proanthocyanidins of grape seeds. We first determined the p$K_a$s of the extracted procyanidin solutions and then adapted the pH of the eluent according to the equation: $pH = pK_a + 2$. The corresponding titration curve is presented in Fig. 7 and the p$K_a$ value obtained was 9.4 (corresponding to the pH value at Ve/2). Hence, the pH was adjusted to 11.4 for the SEC analysis.

Fig. 8 shows the distribution obtained at pH 11.2 and 11.4. We noticed that at pH 11.2, several peaks are eluted since $pH < pK_a + 2$ and so only part of the sample is in the phenolate form. Whereas at pH 11.4 ($pH = pK_a + 2$), most of the procyanidin molecules are in the phenolate form, so, the distribution of masses of this sample is homogeneous and is eluted in one peak, and inside the calibration range.

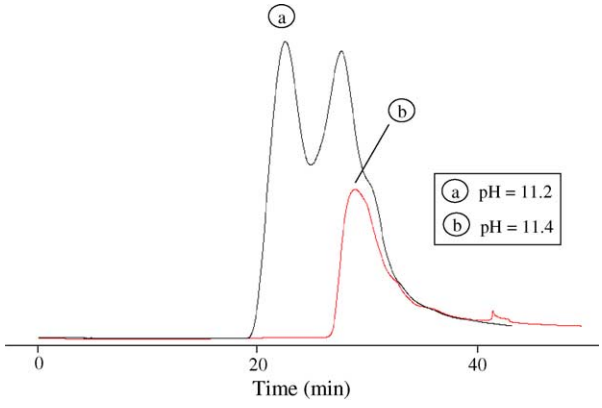


Fig. 8. Superimposed SEC chromatograms of procyanidins–influence of pH. PL aquagel-OH 30$^{TM}$, PL aquagel-OH 40$^{TM}$ columns; mobile phase water/methanol (50:50, v/v) at 1 mL/min; [$NaNO_3$]: 0.18 M; UV detection at 280 nm.

Table 2
Characteristics of grape seed procyanidin obtained with this new SEC method[a] compared to data obtained with a previous method[b]

| | Proposed SEC method[a] | Conventional SEC method[b] [3–4] |
|---|---|---|
| $M_n$[c] | 1763 | 3246 |
| $M_w$[d] | 4556 | 9833 |
| $M_p$[e] | 6215 | 11623 |
| $I$[f] | 2.6 | 3.0 |
| DP[g] | 21 | 23 |

[a] SEC conditions: columns (300 mm × 7.8 mm i.d.): PL aquagel-OH 30™ and PL aquagel-OH 40™, mobile phase water/methanol (50:50, v/v), $NaNO_3$: 0.18 M, pH 11.4 at 1 mL/min, UV detection at 280 nm.
[b] SEC conditions of the acetylated fraction: columns (300 mm × 7.8 mm i.d.): TSK™ Gel G 1000 HXL, TSK™ Gel G 2000 HXL, TSK™ Gel G 1000 HXL, mobile phase THF at 1 mL/min, UV detection at 280 nm.
[c] Number-average momecular mass.
[d] Weight-average molecular mass.
[e] Peak-average molecular mass.
[f] Polydispersity.
[g] Mean degree of polymerization.

The results of the distribution of masses of procyanidins of the seed grape were summarized in Table 2. In the case of previous SEC method [3–4], the values of $M_n$ and $M_w$ were higher because the samples were peracetylated before analysis. Each hydroxyl group was replaced by methoxyl group. The mean degree of polymerization obtained with each method was sensibly equal, 21 by the proposed method and 23 by the previous method. The values of the polydispersity were also very similar whatever the method used. This proposed SEC method (aqueous) gave good results, and not different with those obtained with the classical method. One of the advantages of this method is that we avoid the peracetylation step required by SEC usually used with organic solvents such as THF. It permits to obtain the real profile of native samples. It is less time consuming, the use of organic solvents is reduced.

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