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Application of off-line size-exclusion chromatographic fractionation–matrix assisted laser desorption ionization time of flight mass spectrometry for proanthocyanidin characterization

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Abstract

In this work, we wanted to devise a reliable method to characterize polymerized forms of tannins, their structural information and mass distribution. Size-exclusion chromatography (SEC) is a chromatographic technique used to determine molecular mass (weight) distributions of polymers. One important step in the data treatment is the modeling of the calibration curves. Polystyrenes (PS) are standards usually used because no commercial procyanidin (PC) standards are available. An off-line coupling of SEC and MALDI was carried out to measure differences between polystyrenes and procyanidins. Thus, a new calibration curve was established; from 1000 to 8000 Da, there is a good correlation between the MALDI and PS calibration curves, in this field the PS calibration is correct and enables true mass determination. For masses above 8000 Da, PS calibration overestimates the real molecular weight of PC, overestimation of 53%. And for masses below 1000 Da, PS calibration underestimates their real molecular weight (10–15%). This means that to truly characterize PC, calibration based on PC standards is required.

Keywords: Calibration curves; Size-exclusion chromatography; Matrix-assisted laser desorption ionization mass spectrometry; Molecular mass distribution; Procyanidins

1. Introduction

Phenolic compounds are found in grapes and wines in the form of no flavanoids (phenol acids, stilbenes) and flavanoids: condensed tannins (e.g. proanthocyanidins (PA)) and anthocyanidins monoglucosid. They occur in the coloration and gustatory properties of berries and impart specific characteristics to the wine. PA come from solid parties: seeds and skins; they are polymers of flavan-3-ols units; in the case of grape seeds (procyanidins (PC)), there is only one B-ring: catechin and epicatechin. Grape seed procyanidins have been characterized by many analytical methods [1]. Several chromatographic approaches have been developed for obtaining molecular mass information for proanthocyanidins. Cleavage techniques rely on the conversion of proanthocyanidins into their constitutive subunits via acid catalysis in the presence of an excess nucleophile [2]. Subsequent analysis by reversed-phase high-performance liquid chromatography of the subunit products can provide mean degree of polymerization. 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 that are used to analyze intact proanthocyanidins exist. Normale-phase and reversed-phase HPLC methods can be used to separate proanthocyanidins. Again, however, these methods are restricted in their ability to provide complete molecular mass distribution information. Size-exclusion chromatography (SEC) is the method routinely employed to determine polymer molecular mass

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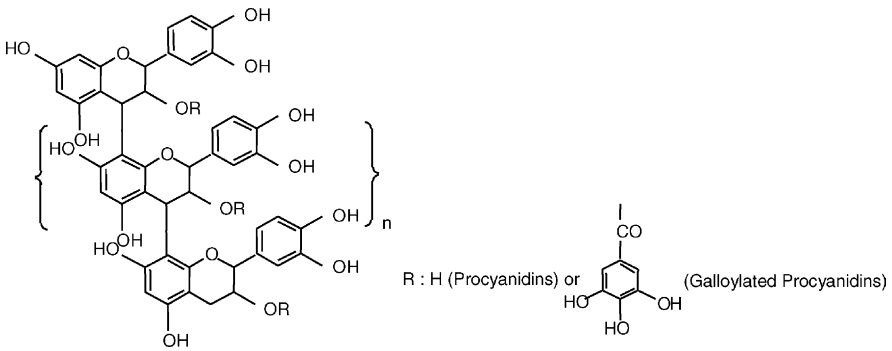
[3]. SEC methods have been developed for proanthocyanidin analysis. Early methods have relied on pre-derivatization of proanthocyanidins prior to analysis to eliminate interaction between phenolic functional groups and SEC packing material [4]. Kennedy and Taylor [5] developed a SEC method for the direct analysis of proanthocyanidins but this method consisted of two PL gel columns utilizing a mobile phase consisting of *N*,*N*-dimethylformamide containing 1% glacial acetic acid, 5% (v/v) water and 0.15 M lithium chloride. This method requires columns packaged in a specific mobile phase and so can limit the different applications in a laboratory.

The purpose of our work is to devise a reliable method to characterize polymerized forms of tannins, their structural information and mass distribution. All these parameters will enable us to establish a comparative map of PA of different origins.

In our case, SEC in the organic phase is usually used [4] and requires peracetylation of the samples. Currently, molecular mass values are obtained in polystyrene (PS) equivalents as commercial PC standards are not available. But SEC is not an absolute method, structural and conformational differences between PS and PA must be taken into account. Mass spectrometry (MS) is an effective method for characterizing the molecular mass distribution of oligomeric constituents in phenolic samples. Numerous applications have been utilized: fast-atom bombardment (FAB)–MS [6–8], liquid secondary ion–MS (LSIMS) [9], electrospray ionization (ESI)–MS [10,11] and MALDI-Tof–MS [8,12,13]. A variety of methods have been prepared to fractionate proanthocyanidins. Labarbe et al. [14] and Saucier et al. [15] proposed separation methods based on the relative solubility of proanthocyanidins of different molecular sizes in different solvents and solvent mixtures. Sephadex LH-20 has been one of the more widely used materials for proanthocyanidin chromatography since 1974 [16], both for size fractionation and more frequently for sample cleanup.

In order to evaluate possible differences, an off-line coupling of SEC and MALDI was carried out, MALDI being a well-known technique able to accurately determine the mass of low dispersity samples [17]. In this work, we present the new calibration curve and a comparative study on grape seed samples based on the two different calibrations.



2. Experimental

2.1. Chemicals

The investigated samples are grape seeds from *Vitis vinifera* L. 2,5-Dihydroxybenzoic acid (DHB) and sodium iodide were purchased from Aldrich (Saint-Quentin, France). PS standards were purchased from Polymer Laboratories (Marseille, France). Ethanol (EtOH) (analytical reagent), methanol and acetic anhydride were obtained from Prolabo (Pessac, France); extra-pure, stabilized tetrahydrofuran (THF), pure chloroform (SDS), acetic acid (99.8%) and pyridin were obtained from Riedel-de-Haën (Saint-Quentin, France).

2.2. Procyanidin isolation

Grapes were collected at commercial maturity. Seeds and skins were separated manually. Isolated seeds were lyophilized, reduced to powder and frozen at −18 °C until used.

Portions (4 g) of seeds were extracted in 20 ml of EtOH/acidified water at 10% with acetic acid (1:1 (v/v)) under nitrogen with mechanical stirring for 12 h. A same volume of chloroform was added to eliminate lipids and pigments. The mixture was centrifuged for 10 min. The lower green phase corresponding to the chloroform phase was eliminated and the upper yellow phase corresponding to the hydroalcoholic (HA) part was recovered. Extraction was repeated three times and all the HA extracts of the same sample were collected, evaporated to dryness, lyophilized and frozen at −18 °C until analysis.

2.3. Analysis by size-exclusion chromatography (SEC)

The study of the M_p (peak-average molecular mass) distribution of seed samples was performed using the acetyl derivatives. A 10 mg sample of freeze-dried material was acetylated with 20 ml of pyridine–acetic anhydride (1:1 (v/v)) for 3 days at room temperature. The precipitate obtained by pouring the mixture into cooled water was recovered by centrifugation. This precipitate was then washed three times with distilled water, then methanol and finally chloroform. It was dried, dissolved in 1 ml THF and filtered

using PTFE type, 0.45 μm pore size filters (Millex-LH, Millipore) before analysis by SEC.

SEC analysis was performed using a Thermo Quest™ (Les Ulis, France) instrument equipped with three columns (300 mm × 7.8 mm i.d.): TSK™ Gel G 1000 HXL, TSK™ Gel G 2000 HXL, TSK™ Gel G 2500 HXL, in series, protected with a guard column of the same material (Polymer Labs., Marseille, France). Analysis conditions were: THF as the eluent, flow-rate: 1 ml/min at ambient temperature, injection volume: 20 μl and analysis time: 45 min. Detection was made at 280 nm, by an UV detector (SPECTRA SERIES™ UV-150). PL Caliber™ software was used for data acquisition.

2.4. Procyanidin fractionation

Fractionation of seed procyanidins was performed by SEC using the aforementioned equipment and in the same analysis conditions except for the injection volume, in this case we used a 100 μl loop. Fractions were collected using a FRAC-100 type fraction collector (Amersham pharmacia biotech™, Uppsala, Sweden) connected to an UV detector. To optimize fractionation, collector parameters were adjusted; the principal programming characteristics were: collection mode: 0; fraction size: 5.5 min; peak threshold: 3%; peak fraction size: 0.1 min.

2.5. MALDI analysis

MALDI–MS spectra were acquired using a TofSpec™ MALDI-Tof mass spectrometer from Micromass™ (Manchester, UK). The instrument is equipped with a pulsed N2 laser (337 nm, 4 ns pulse width) and a time-delayed extracted ion source. Spectra were recorded in the positive-ion mode using the reflectron and with an accelerating voltage of 20 kV.

Samples were dissolved in THF at 10 mg/ml. The DHB matrix solution was prepared by dissolving 10 mg in 1 ml of THF. A methanol solution of sodium iodide was prepared at 10 mg/ml. The solutions were combined in a 10:1:1 (v/v/v) matrix to polymer to cationization agent. One to two microliters of the obtained solution were deposited onto the target sample and vacuum-dried.

3. Results and discussion

3.1. SEC and MALDI analysis of an acetylated total extract

A SEC analysis was performed on a sample of grape seeds under the previously described conditions. Fig. 1 represents the obtained chromatogram. The wide profile does not make it possible to determine the classical parameters that characterize polymers: M_p (peak-average molecular mass), M_n (number-average molecular mass), M_w (weight-average molecular mass), I (polydispersity) and DP (degree of polymerization); for this reason, we divided the chromatogram into sections. Calculations of molecular masses for each portion are summed up in Table 1. Thanks to this partitioning, we were able to obtain a true illustration of the profile and will be able in the future to compare different procyanidin samples: study of the variety, origin, vineyard. The final goal is to find a variable permitting to highlight the influence of these parameters.

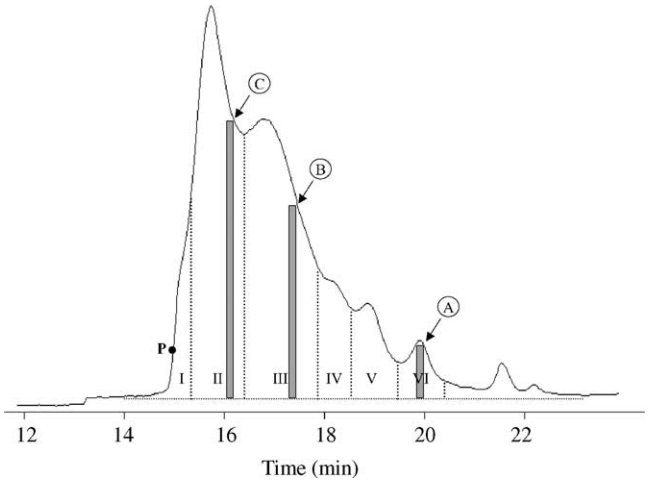


Fig. 1. SEC chromatogram of an acetylated extract of grape seeds and identification of three fractions used as standards. Columns (300 mm × 7.8 mm i.d.): TSK™ Gel G 1000 HXL, TSK™ Gel G 2000 HXL, TSK™ Gel G 2500 HXL, mobile phase THF at 1 ml/min, UV detection at 280 nm.

Narrow PS standards were used to establish the calibration curve. Many works [18] show that calibration curves applied in SEC are generally non linear [19], their shape depending on the columns used. In our study, a third-order polynomial regression best fits the different calibration points (Fig. 2). The corresponding equation is: $y = -0.0084x^3 + 0.4954x^2 - 9.8157x + 68.561$ where y represents $\log(M_p)$ and x retention time. With this calibration, the point named P on the chromatogram corresponds to a mass of 20,000 Da, that means a 40-mer is present in our grape seed extract.

Table 1
Molecular mass data obtained by SEC using polystyrene calibration[a] compared to data obtained with MALDI procyanidins calibration

	Retention time, t_r (min)	M_p		E_{max} (%)[b]
		PS calibration	PC calibration	
I	15.215	15 716	10 287	53
II	15.851	7242	6355	14
III	16.899	2917	3283	−11
IV	18.439	1381	1633	−15
V	19.045	1148	1336	−14
VI	20.121	865	1029	−16

[a] SEC conditions: 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.

[b] E_{max}, maximum error.

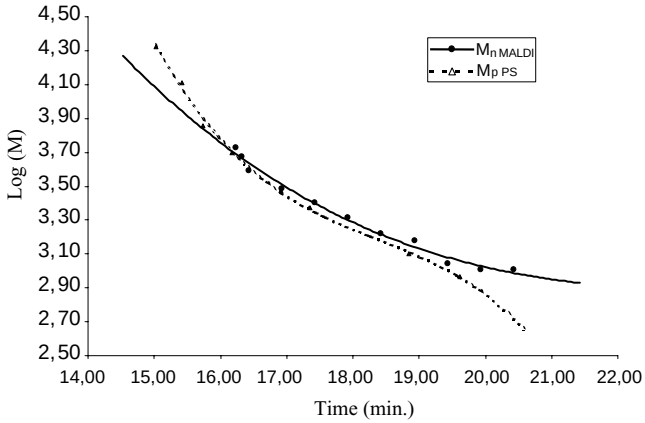


Fig. 2. Comparison of the calibration curves obtained with polystyrene and procyanidin standards.

To complete the study of this extract, we performed a MALDI analysis on the same acetylated sample in the conditions described in the experimental part. In previous works [12,20,21] only the native procyanidins were analyzed and this is the first time, to our knowledge, that a MALDI spectrum of acetylated procyanidin is presented (Fig. 3). The spectrum shows repeating units of 498 Da, corresponding to acetylated monomeric units. The major peaks are sodium adducts (MNa^+), another series of ions 16 Da higher is also observed which is potassium adducts (MK^+). For each oligomer, substructures with mass increment of 236 Da appear (Fig. 4), indicating the presence of galloyl groups. According to this technique, the maximum degree of polymerization observed is $n = 7$, so no correlation exists between SEC and MALDI analysis.

On the one hand, this natural product is highly polydisperse and one of the well-known limitations [22,23] of MALDI analysis is the misestimating of molecular masses for polydisperse polymers (high masses are discriminated against low masses). To overcome this problem, one solution is to fractionate by Size-Exclusion Chromatography into very narrow fractions. Then MALDI analysis can be performed on each fraction and correct molecular masses will be obtained for each fraction.

On the other hand, SEC calibration is obtained with PS standards as no commercial procyanidin standards are available. But at present, we do not know whether PS and PC have the same behavior in these SEC conditions. The values reported in Table 1 are PS equivalents and it is impossible to say if they are true, underestimated or overestimated. The aim of the following study is to answer to this question and obtain a real calibration curve with procyanidin standards.

3.2. Analysis of an acetylated total extract by combination of SEC (off line) and MALDI

A narrow fractionation was performed, represented in Fig. 1, and we indicate the corresponding mass spectrum at three retention times (Fig. 5). Under these fractionation conditions, the polydispersity of each fraction is now a narrow fractionation to allow correct determination of the

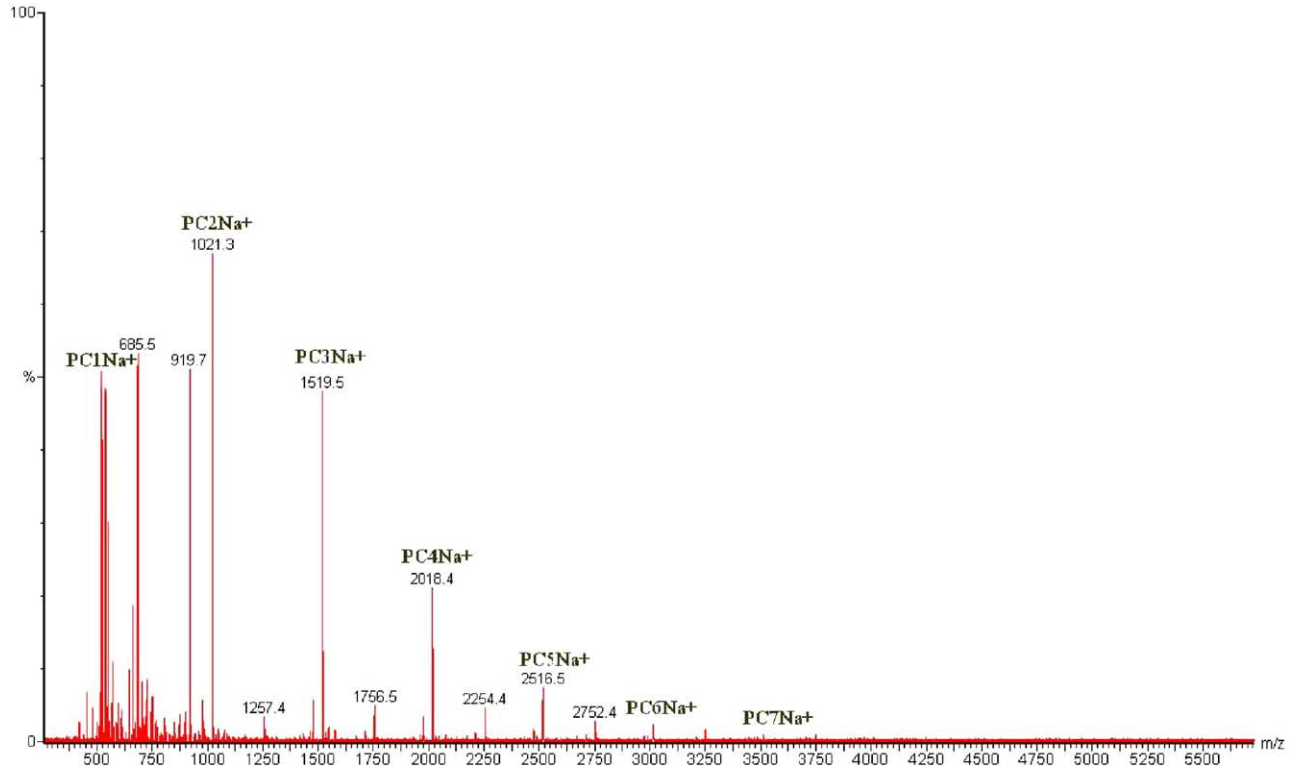


Fig. 3. MALDI spectrum of an acetylated total extract of grape seeds. TofSpec^{TM} MALDI-Tof with a pulsed N2 laser, reflectron mode, matrix: DHB.

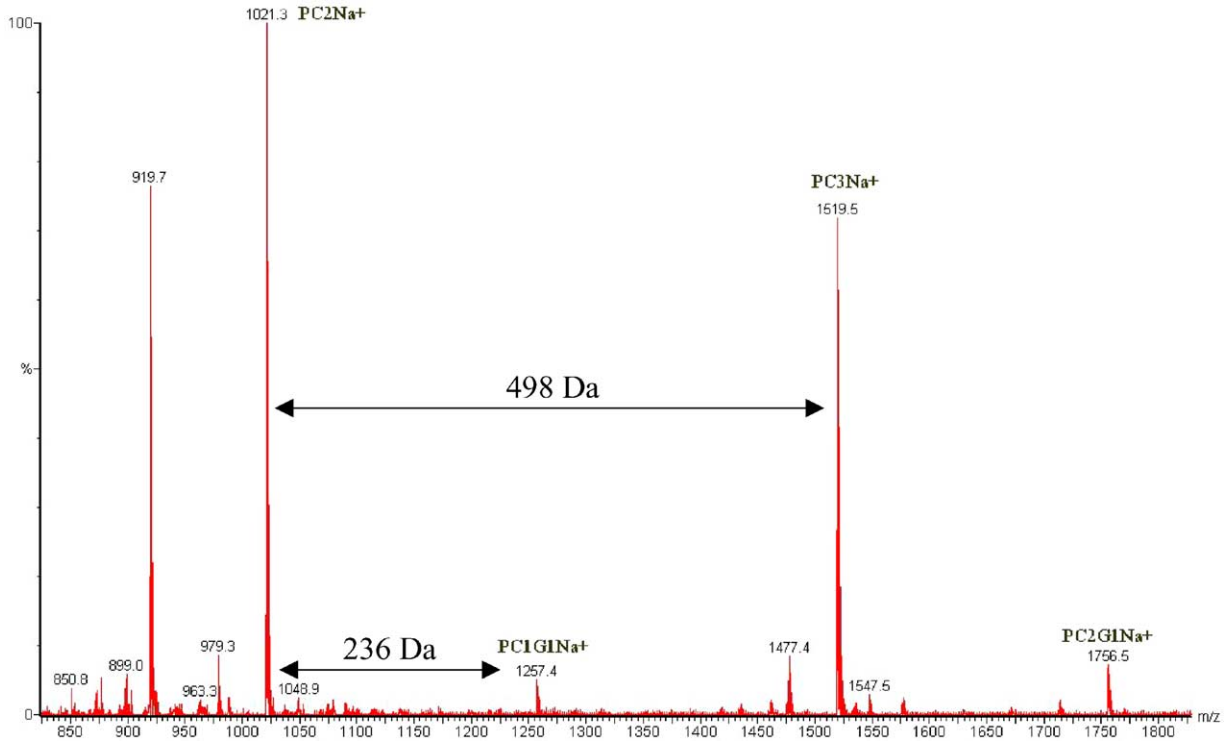


Fig. 4. Part of the MALDI spectrum of an acetylated total extract of grape seeds.

average molecular weight (M_n) by MALDI technique. Owing to the fractionation, we remove the problem of high-mass discrimination.

The number-average molecular mass: M_n, the weight-average molecular mass: M_w values and polydispersity: I are defined by 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 molecules (corresponding to the relative peak intensity) with a molecular mass M_i at each data slice i, $x = 0$ for M_n and $x = 1$ for M_w.

Table 2 reports these characteristics for each fraction. Polydispersity values showed that such fractionation permitted to obtain narrow fractions. Data summed up in Table 2 are used to construct a calibration curve but the MALDI technique restricts us to mass determination below 6000 Da in the particular case of procyanidin studies, hence the necessity to extrapolate. As described in the first part, the best regression model found for PS calibrants was a third-order polynomial regression. The same model was then used until M_n 20,000 Da, which is the upper exclusion limit of our columns. The corresponding equation obtained is $y = -0.0012x^3 + 0.092x^2 - 2.3x + 22$ where y represents $\log(M_n)$ and x retention time, the relationship between the elution time of the fraction and its molecular mass is correct with an R^2 value of 0.989.

Fig. 2 shows the overlay of the two calibration curves. By comparing the two curves, three distinct areas can be identified. From retention time $t_1 = 15.7$ min (corresponding to a mass of 1000 Da) to $t_2 = 19.2$ min (corresponding to a mass of 8000 Da), there is a good correlation between the MALDI and PS calibration curves, in this field the PS calibration is correct and enables true mass determination despite a different structural behavior between PS

Table 2
Average-molecular mass data and polydispersity for each fraction (obtained by SEC) by MALDI analysis

	Retention time (min)[a]									
	16.23	16.33	16.43	16.93	17.43	17.93	18.43	18.93	19.43	19.93
M_n[b]	5301	4736	3930	3049	2512	2064	1660	1511	1098	1022
M_w	5325	4749	3971	3090	2559	2091	1706	1527	1160	1062
I	1.00	1.00	1.01	1.01	1.02	1.01	1.03	1.01	1.06	1.04

[a] Retention time is the calculated average time of the corresponding fraction.
[b] MALDI conditions: TofSpec^{TM} MALDI-Tof with a pulsed N2 laser, reflectron mode, matrix: DHB.

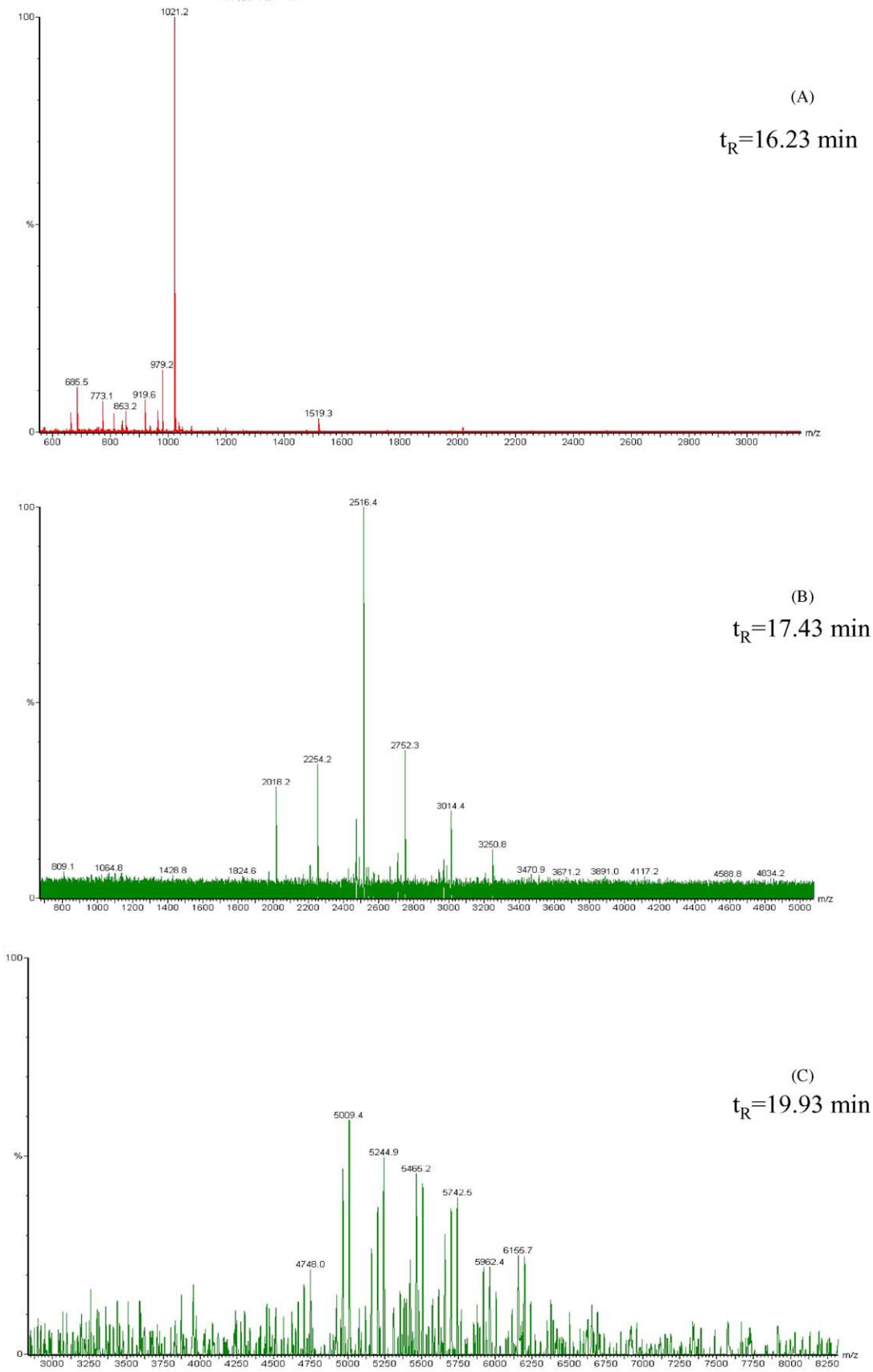


Fig. 5. MALDI spectra of three fractions obtained by SEC fractionation.

and PC. At retention times greater than t_2, there is a small variation, PS calibration underestimates the real molecular weight of procyanidins. Lastly, at retention times less than t_1, we again noted a deviation but unlike field $t > t_2$, PS calibration overestimates the real molecular weight of procyanidins.

The new calibration was applied to the six parts defined in Fig. 1, results are listed in Table 1. With this new calibration, point *P* corresponds now to a mass of 11,889 Da, inducing the presence of a 24-mer in our extract unlike the 40-mer found with PS calibration. We noted that in the case of grape seed studies, the major error is the determination of the molecular weight of part I: we have an overestimation of 53%. This means that to truly characterize PC, calibration based on PC standards is required.

Actually, it is not yet possible to compare these results to those obtained by classical methods like acid-catalysis because fractions collected by SEC technique, in these conditions, are peracetylated and are not under native form.

4. Conclusions

In summary, we developed a method permitting to obtain a real image of PC, we pointed out the weakness of SEC studies based on PS calibration. This study enables us to estimate how high is the misestimating of PC molecular masses with PS standards and to evaluate in which case we have an under or over-estimation. To complete this study, it is necessary to optimize MALDI conditions (sample preparation and ionization) in order to characterize high masse fractions (masses above 6000 Da). Besides this work can only be used in the case of PC characterization from seed grapes, indeed it is well known that seed and skin grapes have a different behavior. So, the same study has to be performed in the case of skin grapes.

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