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The use of extract-ion chromatograms to quantify the composition of condensed tannin subunits

Short title: A better analysis of tannin subunits by mass spectrometry

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/rcm.8619

ABSTRACT

RATIONALE: The analysis of tannins is currently usually done by liquid chromatography following their chemical depolymerization. One of the main limitations of this method, however, is the difficulty with specifically detecting each constitutive tannin monomer in a complex matrix, as numerous compounds co-elute with the monomers, thereby compromising the analysis.

METHODS: Tannin depolymerization under acidic conditions and in the presence of thioglycolic acid releases the various constitutive monomers, either as terminal or extension units. The tannin subunits were then quantified by extracted-ion chromatogram (EIC) mass spectrometry, which required determination of the ionization efficiency of the monomers. Thus, we used AgBF4-assisted coupling for the hemisynthesis of the non-commercial ECG dimer.

RESULTS: The EIC showed that the derivatives of the extension units were twice as ionized as the terminal unit. Unlike the use of UV chromatograms, this new EIC-based method is more specific and accurate because it is not impacted by the co-elution phenomenon. This result, when added to the linearity obtained on a large range of the calibration curves, allowed for quantification of tannin subunits from EIC in complex mixtures with less pretreatment of the samples.

CONCLUSIONS: As a result of its specificity and sensitivity, this EIC-based method represents a significant step toward improvement of the quantification of the tannin composition of samples. The results of this study should allow the oxidation markers to be quantified more accurately and taken into account, thereby providing a better indication of the actual tannin composition.

Keywords: Tannin, polyphenol, average degree of polymerization aDP, extracted-ion chromatogram EIC, ECG-ECG

INTRODUCTION

Condensed tannins (also called 'proanthocyanidin') are polyphenol biopolymers of flavan-3-ol subunits and they are widely distributed in the plant kingdom. As a first approximation, they can be considered to be a linear[1] or cyclic[2, 3] sequence of monomeric units such as catechin (C), epicatechin (EC), epicatechin gallate (ECG), and epicatechin 3-*O*-gallate (EGC) (Figure 1). The link between the monomeric subunits is called the interflavanic bond, and it is usually a C4-C8 bond (rarely a C4-C6 bond)[4, 5]. Tannins are well known for their antioxidant activity, which is thought to inhibit cellular aging[6]. They are, consequently, highly sensitive to oxidation, which results in their chemical structure becoming more complex.

Due to the high structural diversity of condensed tannins and the relatively low concentration of some of their constituents, analyses of such polymers can be quite challenging, especially for wines, as the chemical structure of tannins has a direct impact on their organoleptic properties such as astringency[7], bitterness[8, 9], color[10, 11], and colloidal stability[12, 13].

The most common methodology to establish the average molecular composition of condensed tannins, and to quantify them, starts with chemical depolymerization. This is carried out in an acidic medium in the presence of a nucleophile. Chemical depolymerization of condensed tannins leads to cleavage of the interflavan bonds, thereby releasing terminal units and extension units that are substituted by the nucleophile at their C4 carbon. These depolymerized products are then typically analyzed by liquid chromatography using UV detection (Figure 1 and Table S1). The most commonly used nucleophiles are benzyl mercaptan, thioglycolic acid, mercaptoethanol, and phloroglucinol[14-22]. The depolymerization method does not provide access to the polydiversity polymeric distribution in tannins, although it is a suitable way to generate ample information in regard to their chemical characterization. The advantage of this method is that it readily allows for determination of both the composition of constitutive flavan-3-ol (terminal and extension) subunits of tannins, in addition to the average degree of polymerization (aDP). However, this method fails to take into account the different transformations that tannins undergo throughout the winemaking process and its storage. These transformations are mainly induced by oxidation.

The oxidation of tannins results in several changes to their chemical structure. It generally results in new covalent bonds that connect at least two flavan-3-ol constitutive subunits. Since these new bonds are not broken under the depolymerization conditions, a set of biaryl-C-C connected dimers, trimers, or higher oligomers of flavan-3-ol derivatives are generated by this reaction[23]. These compounds are thought to be good markers of oxidation[23, 24]. They exhibit a very high level of structural diversity, and each of them is present at a very low concentration. Hence, in chromatogram profiles, the corresponding peaks exhibit very low intensities and poor resolution. They present as a broad "hump", instead of separate peaks. As none of these markers can be detected as a definite peak on UV chromatograms, they cannot be quantified based on this method of detection[23, 25]. They co-elute with non-oxidized depolymerized flavan-3-ol units and other phenolic compounds such as phenolic acids and anthocyanins, and they are not taken into account in the analysis of the tannic fraction.

In light of the limitations of the current analytical procedure for quantification of the composition of condensed tannin subunits of a sample, there is a clear need for a specific analytical procedure that is not compromised by this co-elution phenomenon and the overlapping of peaks.

New and improved approaches using mass spectrometry have been developed in recent years. Mass spectrometry has proven to be a more efficient method than UV detection for the quantification of flavonoids[26-28]. Indeed, mass spectrometry appears to be a more accurate method with greater selectivity and sensitivity than UV detection[26]. However, due to the complexity of natural samples, an LC-separation step is required prior to MS analysis in order to limit the ionization suppression[29, 30] and to increase the quality of the MS analysis[31]. Liquid chromatography coupled to mass spectrometry is a very attractive method for flavonoid analysis of complex samples[32]. A UPLC system is also used nowadays, in part to reduce the time required for the analysis[33-36].

More recently, flavonoids have been quantified by various mass spectrometry methods such as MRM[27] or extract-ion chromatograms (EIC)[26].

Engström *et al.*[34] developed a method to analyze tannins by depolymerization directly in the ion source after separation by liquid chromatography. This innovative method has many advantages because no chemical depolymerization is required and because of the tannin content, the procyanidin/prodelphinidin ratio, and the average degree of polymerization can

be estimated based on mass spectrometry analysis. However, this method still suffers from co-elution of tannins in complex mixtures because a large hump containing a mixture of tannins is observed in the UPLC chromatograms of some sample. The separation of tannins is one of the limiting factors of liquid chromatography. Moreover, a single cone voltage cannot be used for optimal detection of all tannin types and the results can be compromised based on which tannins are co-eluted.

The high-throughput analysis of polyphenols using the MRM method quantifies constitutive units of tannins as being equivalent to catechin for flavan-3-ols due to the lack of standard products, as described by Lambert *et al.*[27]. Depending on the various monomers present in tannins, the ionization efficiency can nevertheless differ and skew the result.

The EIC method offers several advantages over the previously used methods. The method developed by Stanoeva *et al.* has shown that EIC had a higher selectivity and sensitivity compared to UV spectrophotometry when it was used for HPLC detection in the analysis of 20 flavonoid monomers[26].

In light of the difficulty analyzing tannins in complex mixtures, the novel method developed in this work was aimed at improving the characterization of tannins in such mixtures. We decided to use UPLC coupled to ion trap MS. Compared to HPLC, the higher column pressure with UPLC increases the working flow and thus improves the sensitivity of the analysis. This method is based on the relative quantification of the different tannin subunits, released after chemical depolymerization, from the EIC. The EIC method offers several advantages. As a result of its high specificity and sensitivity, it provides better detection of tannin subunits. By overcoming the issues related to co-elution, the tannin samples need to undergo less pretreatment. However, development of this method requires determination of the ionization efficiency of the extension subunit derivatives (EC-Nu, C-Nu, EGC-Nu, and ECG-Nu) and of the terminal (EC, C, EGC, and ECG) units. For this reason, proanthocyanidin dimers were depolymerized and investigated by means of their EIC. This method was then applied to wine samples, for which the condensed tannins are composed of catechin, gallocatechin, epicatechin, epigallocatechin, and epicatechin gallate[4, 37-40].

In the present work, this method was applied using non-oxidized units. Unlike oxidation markers, standards tend to be more readily available for non-oxidized units. Hemisynthesis of oxidation markers is required to obtain standards of oxidation markers, but this is an arduous

process. Once the method has been implemented with non-modified subunits, it can then be applied to oxidized units after calibration with the standard obtained by hemisynthesis. The aim of this work was to facilitate tannin characterization by improving the analytical procedure.

EXPERIMENTAL

Reagents and samples

(+)-Catechin (≥ 99%), (-)-Epicatechin (≥ 99%), (-)-Epicatechin gallate (≥ 99%), and procyanidin B2 (≥ 90%) were purchased from Extrasynthese S.A. (Genay, France). Thioglycolic acid (minimum 98%) and $MgSO_4$ (≥ 99.5%) were purchased from Sigma-Aldrich. EtAcO (pure) was purchased from Carlo Erba Reagents S.A.S.(Peypin France). Grape seed polyphenols were purchased from Sucren Groupe UDM (Vallon Pont d'Arc France). A polyphenolic extract was obtained from red wine (a six-year-old wine made by traditional winemaking using the Marselan grape variety, produced by the INRA Pech Rouge experimental unit, Gruissan, France) according to the following process: 1 mL of red wine was added to a reverse-phase C18 cartridge (20 cc, 5 g) after column conditioning (addition of 2 x 5 mL of MeOH then 2 x 5 mL of H_2O/CH_3COOH, 98/2, v/v, this operation was carried out twice). Two 5 mL volumes of H_2O/CH_3COOH, 98/2, v/v were introduced to remove the sugar fraction. The polyphenol extract was eluted with methanol (2 x 5 mL), and the methanol was then removed under vacuum. Fractionation of the polyphenolic extract from the one-year-old red wine was performed using fractogel chromatography[41, 42] (methyl acrylate copolymer solution in aqueous ethanol 20%, HW-50 F) with a flow rate of 5 $mL.min^{-1}$. The fractionation was achieved with EtOH/TFA (99.95/0.05, v/v), H_2O/TFA (99.95/0.05, v/v) gradients. The highest molecular weight phenols were then eluted with a CH_3COCH_3/H_2O/TFA solution (70/29.95/0.05 v/v/v). The gradient conditions were solvent A (H_2O/TFA, 99.95/0.05, v/v); solvent B (Ethanol/TFA, 99.95/0.05, v/v); solvent C (Acetone/H_2O/TFA, 69.97/29.97/0.05, v/v/v); 0-10.2 min isocratic 100% A; 10.2-11 linear 84% A and 16% B; 11-12.2 linear 60% A and 40% B; 12.2-22.2 isocratic 60% A and 40% B; 22.2-24.2 linear 45% A and 55% B; 24.2-34.2 isocratic 45% A and 55% B; 34.2-36.2 linear 100% C; 36.2-46.2 isocratic 100% C. The fraction studied in this work was the one that eluted with H_2O/TFA (99.95/0.05, v/v) 45%; EtOH/TFA (99.95/0.05, v/v) 55%.

Analytical procedures

UPLC-ESI/MS analyses

The analyses were performed by liquid chromatography coupled to a mass spectrometer (UPLC-ESI-MS) controlled by HyStar 3.2 software. The compounds were separated on a UPLC HSS C18 column (1.8 µm; 1.0 x 150 mm) with a flow of 0.10 mL.min^{-1} using an Acquity UPLC liquid chromatography system (Waters, Milford, MA) equipped with a diode array detector. The gradient conditions were solvent A (H_2O/HCOOH, 99/1, v/v); solvent B (CH_3CN/H_2O/HCOOH, 80/19/1, v/v/v); initial 0.1% B; 0-3 min linear 25% B; 3-5 min linear 35% B; 5-7 min isocratic 65% B; 7-8 min linear 99.9% B; 8-9 min isocratic 99.9% B; 9-10 min linear 0.1% B; and 10-11 min isocratic 0.1% B. The MS analyses were performed with an amaZon X ESI Trap mass spectrometer (Bruker Daltonics, Bremen, Germany). In source, the nebulizer pressure was 44 psi, the temperature of the dry gas was set at 200 °C with a flow of 12 L.min^{-1}, and the capillary voltage was set at 5.5 kV. The mass spectra were acquired over a mass range of 70-2000 Th in positive ionization mode. The speed for the mass spectrum acquisition was set at 8.1 Th.min^{-1}.

Synthesis of ECG propylsulfanyl derivative (1)

Ninety-nine milliliters of MeOH, 9.61 mL (13.5 mmol, 15 eq) of propan-1-thiol, and 1 mL of HCl 37% w/v were added to 2 g of grape seed polyphenols in a round-bottom flask. The mixture was stirred for 1 hour at 40 °C and 100 mL of water was added. The methanol was removed under vacuum and the aqueous phase was extracted three times with 100 mL of ethyl acetate. The combined organic layers were dried over anhydrous $MgSO_4$ and the EtAcO was removed under vacuum, yielding 2.2 g of a brown solid. Five-hundred milligrams of this crude mixture were purified on a C18 reverse-phase silica column (50 g of C18 silica, 40-60 µm particle size) at a flow rate of 35 mL.min^{-1}. The gradient conditions were solvent C (H_2O); solvent D (CH_3CN); 0-2 min isocratic 0% D; 2-4 min linear 25% D; 4-8 min isocratic 25% D; 8-9 min linear 35% D; 9-15 min isocratic 35% D; 15-19 min linear 100% D; and 19-24 min isocratic 100%. After CH_3CN evaporation and freeze-drying for 24 hours, 46.49 mg (purity ≥ 90%) of a light brown fine powder of ECG propylsulfanyl derivative were recovered. Analysis: UPLC-MS rt=7.4 min; m/z (calculated)=517.11 Th; $[M+H]^+$(found)=517.2 Th.

Synthesis of ECG-ECG dimer (2)

Thirteen milligrams of ECG (29.41 µmol, 1 eq) and 15.18 mg (29.41 µmol, 1 eq) of epicatechin gallate propylsulfanyl derivative were added to a round-bottom flask (**1**). The substrates were dissolved in 433 µL of THF at 0 °C. Then, 14.31 mg of $AgBF_4$ were added

(73.56 µL, 2.5 eq). The reaction mixture was stirred for 10 min at 0 °C, and then 8 mL of water was added. The aqueous phase was extracted three times with 10 mL of EtAcO. The combined organic layers were dried over anhydrous $MgSO_4$ and the EtAcO was removed under vacuum, yielding a pale yellow-orange solid. The entire crude mixture was purified by preparative HPLC. A 940 HPLC preparative LC Varian device controlled by Galaxy software was used. A UV-visible dual channel detector was used and the column was a Microsorb 100-3 µm C18 (100 x 21.4). The pressure was 2,400 psi at the beginning of the run. The gradient conditions were solvent A1 (H_2O/CH_3COOH 99/1 v/v) and B1 (CH_3CN/H_2O/CH_3COOH 80/19/1 v/v/v); initial 20% B1; 0-10 min linear 22% B1; 10-13 min linear 25% B1; 13-15 min linear 80% B1; and 15-17 min linear 20% B1.

The compound was eluted with 22% of B1 from 8.6 min to 9.2 min. Following elution, the solvent was removed under vacuum. The aqueous layer was extracted three times with 50 mL of EtAcO. The combined organic layers containing ECG-ECG were dried under vacuum. The ECG-ECG dimer was dissolved in H_2O and lyophilized, yielding 2.85 mg of a light-brown solid resin of ECG-ECG dimer (purity ≥ 95%; yield=11%). Analysis: UPLC-MS rt=4.4 min; m/z (calculated)=883.16 Th; $[M+H]^+$(found)=883.2 Th.

Chemical depolymerization

Thioglycolysis: A 1 mg. L^{-1} solution of each sample (B2, ECG-ECG, the polyphenolic extract, and the polyphenolic fraction) was prepared in methanol. One-hundred microliters of the solution was introduced into a 250 µL glass insert and 100 µL of thioglycolic acid solution (20 µL of thioglycolic acid, 4,880 µL of methanol, and 100 µL of HCl 37% w/v) was added. After sealing the glass insert with an inert cap, the depolymerization reactions were carried out at 90 °C for 6 min.

For each tannin fraction, the thioglycolysis reactions were performed in triplicate (indices 1, 2, or 3 on Table 1) and, after depolymerization, each sample was injected by UPLC-MS three times (indices a, b, or c on Table 1).

RESULTS AND DISCUSSION

Development of the MS method

The way that laboratories currently characterize the tannin fraction of samples is by determination of the aDP[43-47]. The aDP is defined, after the chemical depolymerization of the tannins, as $\frac{\sum Total\ subunits}{\sum Terminal\ subunits}$, and is determined by integration of the extension and terminal unit areas from the UV chromatogram obtained after HPLC analysis (Figure 1).

The quantification of subunits of tannins has been based on integration of the various peak areas from the UV chromatogram. This UV-based quantification is distorted to a certain degree as a result of many co-elution phenomena due to the presence of other compounds in the samples, as well by oxidation of a portion of the tannins.

This limitation is linked with the use of UV analysis as a quantification method for tannins, and it is possible to overcome this by developing a new method based on EIC generated by mass spectrometry (Figure 2). After chemical depolymerization, the various released subunits are separated by UPLC and analyzed by ESI-MS. In order to be able to transpose the work already done, this current work is based on the same UPLC-MS method used previously to detect and identify oxidation markers [23, 24].

The quantification of the subunits requires evaluation of the ionization efficiency ratio between the terminal units and the extension derivatives generated after the depolymerization. Estimation of the ionization efficiency based on each unit obtained after purification is not sufficiently accurate. Indeed, a large number of errors could arise in the purification and in the preparation of the standards, which could distort the analysis. To be sure of the ionization efficiency ratio, pure tannin dimers, in which the amounts of terminal and extension units are equivalent, were depolymerized.

In this work, the ionization ratios were determined after depolymerization of the commercially available B2 (EC-EC) dimer and the synthesized ECG-ECG dimer (which is not commercially available).

Hemisynthesis of ECG-ECG dimer

The synthesis of proanthocyanidin dimers has already been described in the literature by Steynberg *et al.*[48]. The optimized protocol is based on reacting 4β-benzenesulfanyl epicatechin and catechin in the presence of $AgBF_4$ (2.5 equiv.) for 1 h at 0 °C to obtain proanthocyanidin B1 with a yield of 38%. We altered this reaction scheme by using ECG propylsulfanyl derivative (**1**) and epicatechin gallate to generate ECG-ECG dimers (**2**) with a yield of 11% (Figure 3). Propanethiol was used instead of benzenethiol due mainly to the polarity of the former, thereby enabling good purification and isolation.

Analytical study of dimers by mass spectrometry

The calibration curves for each monomer showed that catechin and epicatechin had a similar degree of ionization, but this differed for EGC and ECG. Indeed, the EGC unit was 1.14

times less ionized than catechin and epicatechin and the ECG was 1.35 times more ionized than C and EC.

The UV chromatogram at 280 nm generated before and after the chemical depolymerization (by means of thioglycolysis) of the ECG-ECG dimer showed that the area of the dimer was equal to the sum of areas of the extension and the terminal units released after the depolymerization. Furthermore, the area for the terminal units was equal to that of the extension units. The ratio between the terminal and the extension units in the UV profile was, therefore, equal to one. The sulfanyl 2-methyl acetate group was not expected to change the absorbance coefficient at 280 nm of the extension unit derivative. Therefore, the same area should be obtained for the terminal and extension units. However, the EIC indicated that the area of the extension units was two-fold greater than that of the terminal units (Figure 4 and Table S2). This was also observed in the case of B2. We can, therefore, assume that the extension units EC and ECG were ionized twice as much as the terminal units. As C and EC are two epimeric forms of one monomer, and as EGC is structurally closer to EC and C than to ECG, it can be expected that their respective extension units should exhibit similar ionization properties. Thus, we can make the assumption that the extension units of C and ECG were twice as ionized as the terminal as EC and ECG. The UPLC-MS analysis allows comparison of the ionization factor of the extension and terminal units by integration of the EIC. Moreover, the ratio of ionization of each monomer (C, EC, EGC, and ECG) must be taken into account in the non-oxidized subunits estimation.

Using the EIC method to assay the non-oxidized subunits of wine tannins

Two polyphenol samples that had been extracted from red wine were studied: the entire polyphenol extract and a polyphenolic fraction, eluted in the first part of the fractionation of the polyphenol extract. These samples were chosen because it is difficult to determine their tannin composition by the routine UV method. Indeed, the polyphenolic extract is composed of various classes of phenolic compounds (anthocyanins, phenolics acids, flavonols, etc.)[11] that co-elute under UPLC separation conditions. To limit this co-elution phenomenon, it is necessary to separate the tannins before the UV analysis takes place. However, the first fractions are still difficult to analyze after fractionation because they often contain low monomers (Figure 5a). The current UV analysis procedure is mainly only suitable for fractions with high molecular weight entities (i.e., tannin fractions eluted at the end of a CH_3COCH_3/H_2O/TFA solution gradient, Figure 5b) and not for the entire tannin fraction. In

addition, as a result of their reactivity, tannins undergo structural modifications, while a portion of them can also be lost by adsorption on the stationary phases[49]. Thus, limiting pretreatments appears to be essential in order to be able to determine tannin compositions with a greater degree of accuracy.

The quantities of total non-oxidized subunits estimated by UV chromatogram integration exceeded those obtained from EIC 18-fold and more than 200-fold for the polyphenolic extract and the polyphenolic fraction, respectively (Table 1). This highlights the need to replace UV detection with EIC when analyzing complex samples. Indeed, this overestimation by UV chromatogram analysis is mainly due to the substantial co-elution phenomenon that stems from the complexity of the samples. We have observed numerous co-elutions with anthocyanins on UV chromatograms: malvidin-3-*O*-glucoside co-eluting with epicatechin, malvidin-3-*O*-(6-*O*-acetyl-glucoside) co-eluting with the extension units of EGC (EGC-Nu), and malvidin-3-*O*-(6-*O*-coumaroyl-glucoside) co-eluting with extension units of EC (EC-Nu) (Supplementary data, Figure S2). For the samples analyzed in this work, the co-elution of malvidin-3-*O*-(6-*O*-acetyl-glucoside) with the extension units of EGC (EGC-Nu) profoundly skewed the estimation. Depending on the extent of these co-elutions, they tend to result in varying degrees of over or underestimation of the amounts of non-oxidized tannin subunits (co-elutions with terminal or extension units, respectively). In addition to the co-elutions with anthocyanins, we also observed co-elutions with oxidation markers, which are sometimes not insignificant depending on the oxidation state of the wine product. For example, the mass spectrum obtained at the retention time of EGC-Nu revealed co-elution with malvidin-3-*O*-(6-*O*-acetyl-glucoside) as well as with numerous compounds (Supplementary data Figure S3). The analyses of these samples by the conventional HPLC system also revealed the limitations of UV chromatograms for non-oxidized subunits of tannins. Indeed, with HPLC, although anthocyanins did not co-elute with the EC-Nu subunits, other co-elutions could also distort the results. The non-oxidized subunit content was also overestimated with HPLC analysis of total polyphenolic extract. For the polyphenolic fraction, no subunits were detected by the current UPLC method, while the EIC method allowed their detection.

These results show that, depending on the sample, the levels of non-oxidized subunits of tannins from UV chromatograms can be significant. The EIC method allows the problems associated with co-elution to be circumvented, thereby allowing more accurate determination of the quantity of non-oxidized subunits of tannins.

Although ion trap mass spectrometry is not the most suitable system for quantification, calibration curves were generated from the UV chromatograms and from the EIC for each monomer (EC, EGC, ECG) (Supplementary data, Figure S4 and Table S3). The calibration curves from the EIC were linear in the concentration ranges expected in wine samples. Thus, one can envisage using EIC for quantification within a certain concentration range. However, it should be pointed out that the calibration curves were generated for pure monomers. The presence of several co-eluted molecules could impact their ionization in the source of the mass spectrometer, thereby distorting the EIC and their quantification. In the present analytical conditions, EC (which is the terminal unit of B2) was co-eluted with malvidin-3-*O*-glucoside. To test the robustness of this EIC method, various ratios (4/1, 2/1, 1/1, 1/2, and 1/4) of a mixture of B2 and malvidin-3-*O*-glucoside within a concentration range from 7.5 μmol. L^{-1} to 120 μmol. L^{-1} were chemically depolymerized prior to mass spectrometry analysis. This study showed that the co-elution of malvidin-3-*O*-glucoside with EC did not alter the EIC response when the concentration of the malvidin-3-*O*-glucoside did not exceed 60 μmol. L^{-1}, irrespective of the B2/malvidin-3-*O*-glucoside ratio. The concentration of malvidin-3-*O*-glucoside of the samples analyzed in this study was significantly lower than this value (< 7.5 μmol. L^{-1}). Dilution will be required for samples with high concentrations (> 60 μmol. L^{-1}) of anthocyanins, malvidin-3-*O*-glucoside, malvidin-3-*O*-(6-*O*-acetyl-glucoside), and/or malvidin-3-*O*-(6-*O*-coumaroyl-glucoside), in order to accurately determine the amount/concentration of tannin subunits.

Currently, the use of aDP is not suitable for determination of tannin compositions because the oxidized subunits are not taken in account and it is based only on the non-oxidized subunits present in the tannin chains (Supplementary data Figure S1). The tannin fraction can be altered to a certain extent by oxidation. The value of aDP from such fractions does not reflect the actual composition of the entire tannin extract and should not be used for analysis of products containing modified tannins.

To be as exhaustive as possible, the percentage of oxidized tannin should be taken in to account for tannin characterization. For example, more than two-thirds of tannin polymeric chains are modified in the polyphenolic fraction from red wine studied in this work. This can be estimated from the yield of depolymerization. The tools and methods used to date do not allow this modified part to be taken into account in the aDP estimation due to the high level of complexity of the mixture of products. However, establishing the proportion of modified

tannin and accurate quantitation of the non-oxidized subunits of tannins should provide a more accurate assessment of tannin compositions.

CONCLUSIONS

Quantification of tannin subunits is difficult due to the limitations of tannin analytical procedures based on UV chromatography. In this work, we developed a new analytical assay based on EIC. As a result of the specificity and the sensitivity of the EIC, the quantity of tannin subunits could be accurately determined in chemically complex samples.

The depolymerization of flavan-3-ol dimers (B2 and the synthesized ECG-ECG) revealed that sulfanyl-2-methyl acetate derivatives (extension units) were twice as ionized compared to the terminal units. This result allowed for better determination of the tannin levels in wine extract, which was greatly overrated using UV detection. Moreover, the use of EIC allows characterization of complex samples, which have been difficult to study to date, and a reduction in pretreatments. In a given concentration range, tannin quantification may also be feasible.

This method, by using EIC, is a first step toward improvement of the quantification of the tannin composition of samples. These outcomes show that EIC-MS analysis is more amenable to quantification than UV chromatograms. As a result, this study will allow, in the future, oxidation markers to be accurately quantified and taken into account so as to obtain a more representative indication of the actual tannin composition.

ACKNOWLEDGMENTS

We thank Frédéric Véran for helping us with the tannin analyses by HPLC after chemical depolymerization, Jean-Paul Mazauric for his generous assistance with the preparative HPLC, and Chahinez Aouf for proofreading this manuscript.

FUNDING SOURCES

Masters funding was supported by the CASDAR project.

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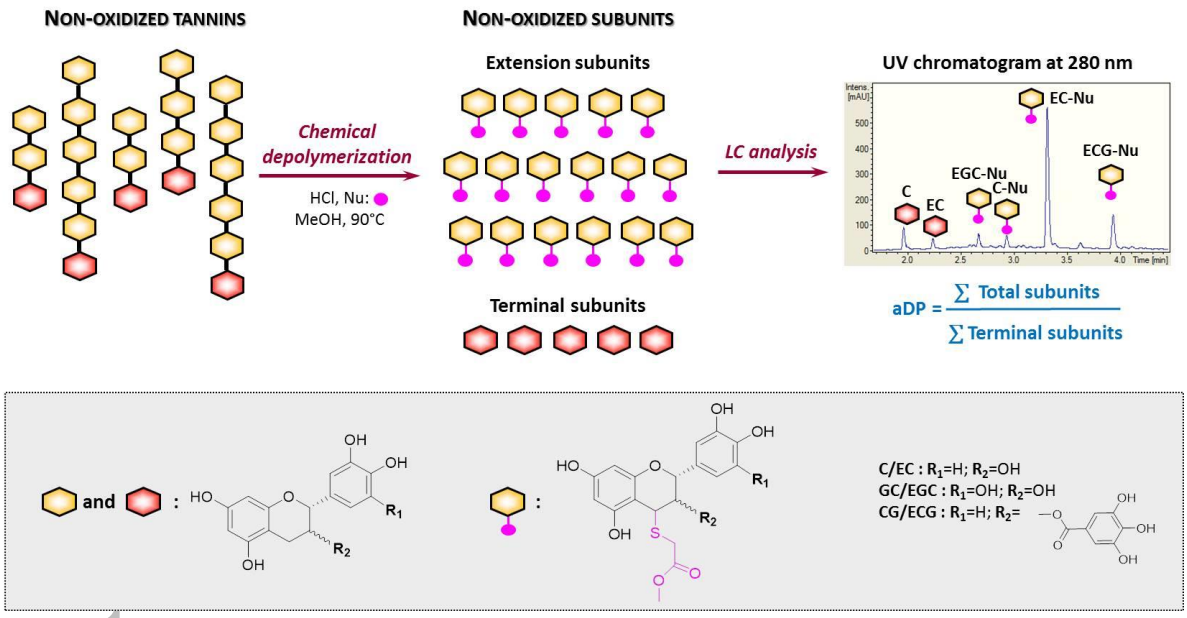

Figure 1: Chemical depolymerization of condensed tannins.

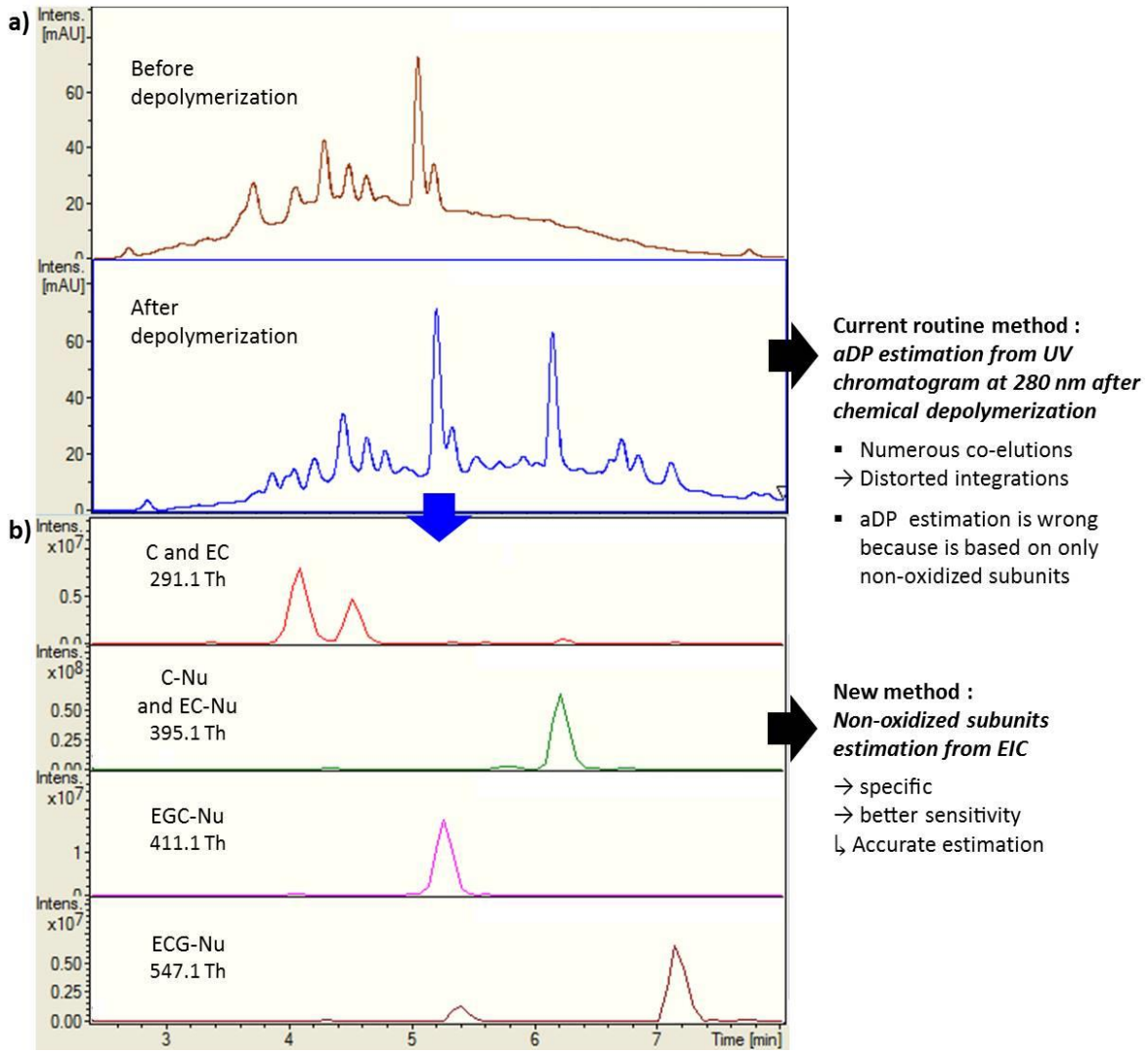

Figure 2: a) UV chromatogram at 280 nm of the polyphenol extract from a red wine sample generated before and after chemical depolymerization; b) EIC from mass spectrometry analysis of each constitutive tannin monomer.

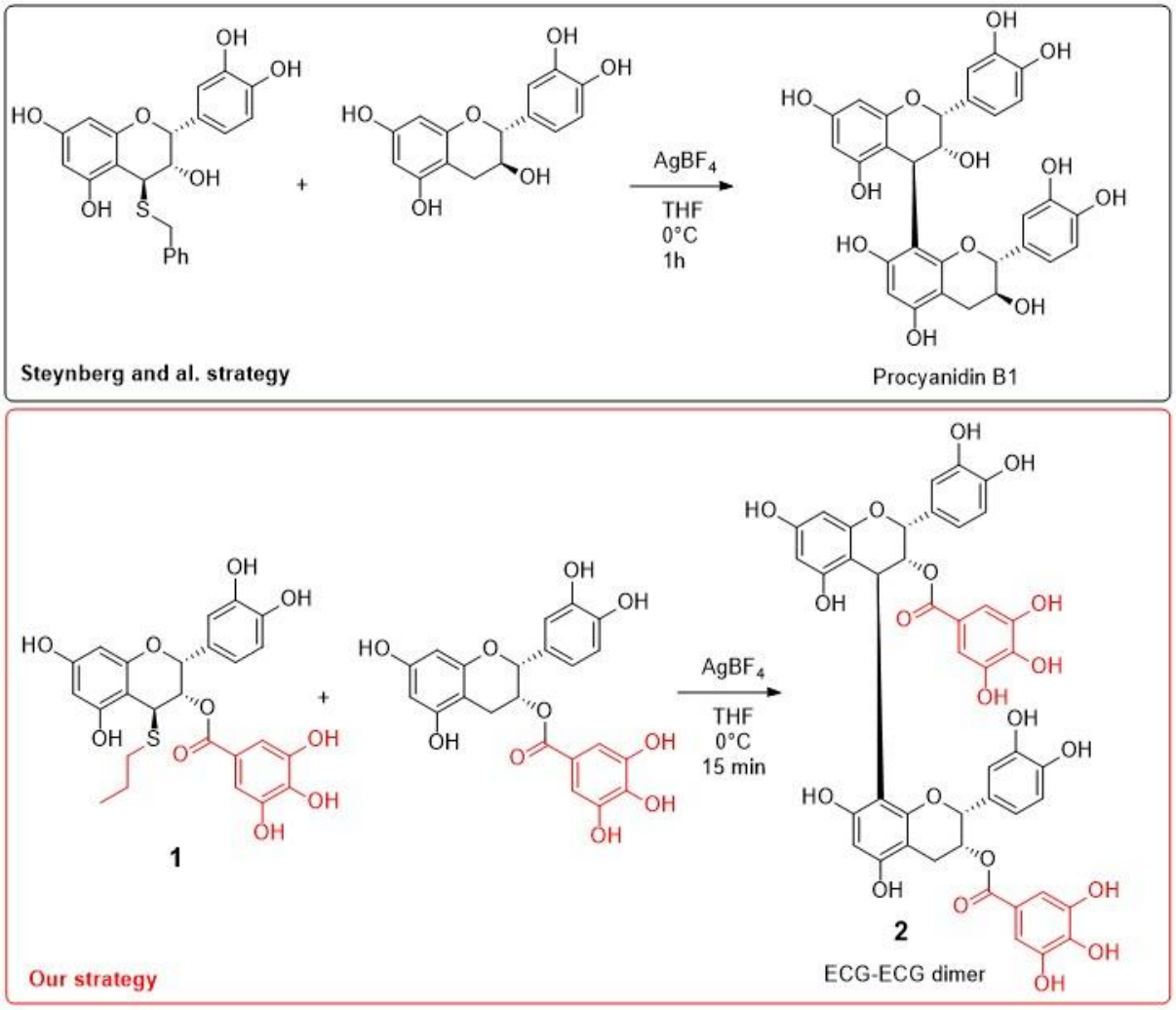

Figure 3: Hemisynthesis of the ECG-ECG dimer.

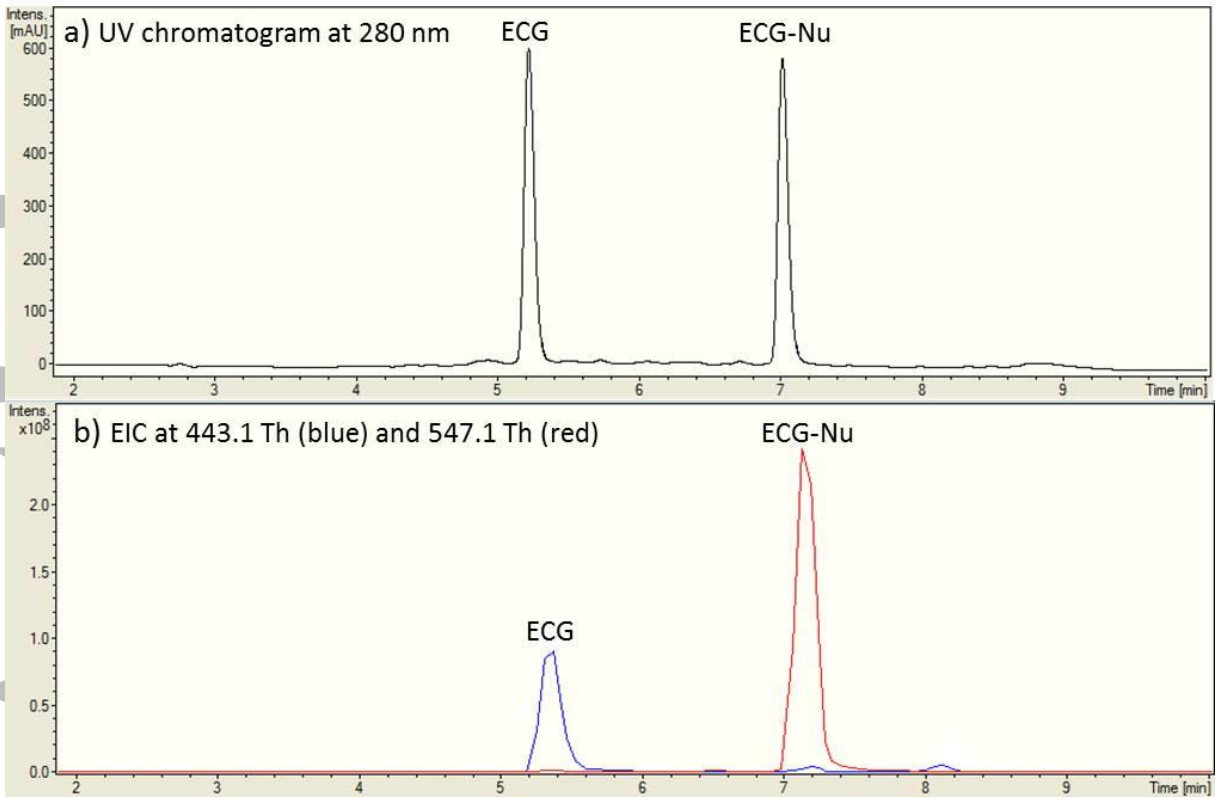

Figure 4: UV chromatogram at 280 nm and extracted-ion chromatogram of ECG-ECG after chemical depolymerization.

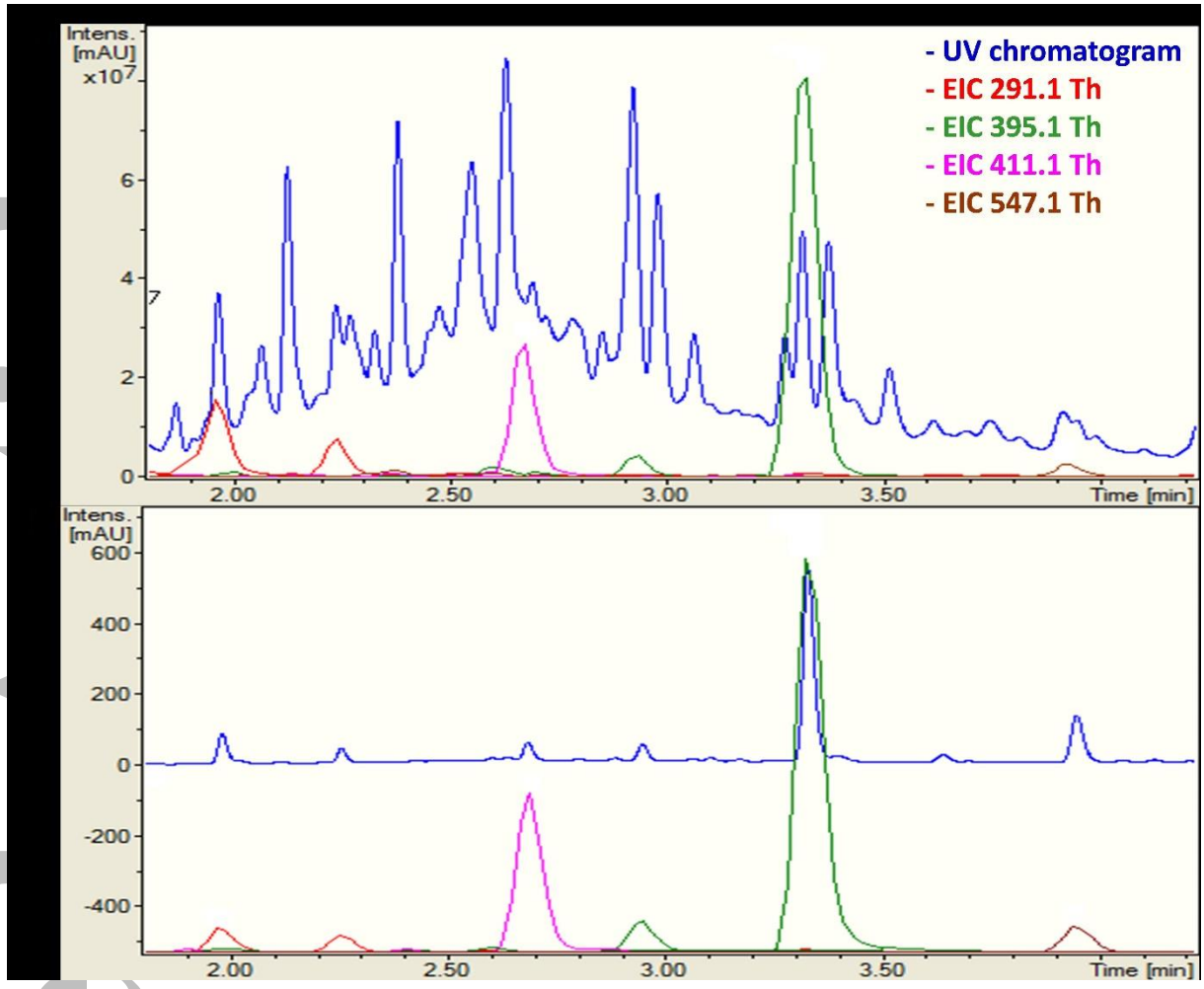

Figure 5: Comparison of UV chromatograms at 280 nm and EIC of two polyphenolic fractions of red wine: (a) the fraction eluted in the first part of the fractionation (b) the fraction eluted in the last part of the fractionation.

			Quantity of non-oxidized tannin subunits (mg/mL)										
		Measurem	EC	C	EGC	ECG	EC-Nu	C-Nu	EGC-Nu	ECG-Nu	Total extension	Total terminal	Total
Polyphenolic extract from red wine	UV	1a	37	11	0	0	61	3	1598	1	48	1663	1711
		1b	39	11	0	0	60	3	1611	1	51	1676	1726
		1c	39	11	0	0	60	3	1603	1	50	1668	1718
		2a	39	11	0	0	60	3	1607	1	51	1671	1722
		2b	39	12	0	0	60	3	1616	1	51	1680	1731
		2c	39	11	0	0	60	3	1600	1	50	1664	1714
		3a	40	12	0	0	61	3	1618	1	51	1684	1735
		3b	40	12	0	0	61	3	1629	1	51	1695	1746
		3c	41	12	0	0	62	3	1639	1	53	1705	1758
	ElC	1a	6	15	0	0	52	1	23	3	22	79	101
		1b	8	16	1	0	50	1	23	3	25	77	102
		1c	7	13	2	0	52	2	19	3	22	75	98
		2a	6	15	3	0	52	2	19	3	25	76	101
		2b	8	15	4	0	56	2	21	3	26	82	108
		2c	6	12	5	0	45	1	20	3	23	69	92
		3a	7	15	6	0	56	1	20	3	28	81	109
		3b	7	16	7	0	55	2	22	3	30	81	111
		3c	7	16	8	0	59	1	22	3	32	85	117
Polyphenolic fraction from red wine	UV	1a	100	0	0	0	31	0	6702	2	100	6735	6835
		1b	100	0	0	0	30	0	6685	2	100	6717	6816
		1c	101	0	0	0	30	0	6653	2	101	6685	6786
		2a	100	0	0	0	31	0	6655	2	100	6688	6788
		2b	101	0	0	0	31	0	6652	2	101	6684	6785
		2c	98	0	0	0	30	0	6654	2	98	6686	6785

	3a	106	0	0	0	31	0	6718	2	106	6750	6855
	3b	103	0	0	0	31	0	6696	2	103	6729	6832
	3c	103	0	0	0	31	0	6661	2	103	6694	6797
	1a	0	0	13	1	6	1	10	20	30	20	30
	1b	0	0	13	1	6	1	10	20	30	20	30
	1c	0	0	13	1	6	1	10	20	30	20	30
	2a	0	0	13	1	6	1	10	20	30	20	30
EIC	2b	0	0	13	1	6	1	10	20	30	20	30
	2c	0	0	13	1	6	1	10	20	30	20	30
	3a	0	0	13	1	6	1	10	20	30	20	30
	3b	0	0	13	1	6	1	10	20	30	20	30
	3c	0	0	13	1	6	1	10	20	30	20	30

Table 1: quantity of non-oxidized subunits estimated from UV chromatograms or EIC.