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Optimization of Simultaneous Flavanol, Phenolic Acid, and Anthocyanin Extraction from Grapes Using an Experimental Design: Application to the Characterization of Champagne Grape Varieties

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Optimization of polyphenol extraction from grape skin, seed, and pulp was performed on *Vitis vinifera* L. cv. Pinot Noir, by response surface methodology using a Doehlert design. An acidified mixture of acetone/water/methanol was the best solvent for simultaneous extraction of major polyphenol groups from all berry parts, while optimum extraction times and solid-to-liquid ratios varied according to the part. The determined composition from the model agreed with independent experimental results. Analysis of the three Champagne grape varieties showed that proanthocyanidins were the major phenolic compounds in each part (60−93%). The total berry proanthocyanidin content was highest in Pinot Meunier (11 g kg^{-1}) and lowest in Chardonnay (5 g kg^{-1}), but Pinot Meunier pulp contained lower amounts of proanthocyanidins and phenolic acids (210 and 127 mg kg^{-1} berry, respectively) than that of the other two varieties. The berry anthocyanin content was equivalent in both Pinot Noir and Pinot Meunier (632 and 602 mg kg^{-1}, respectively).

KEYWORDS: Champagne grapes; polyphenol; extraction; experimental design; *Vitis vinifera* L.; optimization

INTRODUCTION

Grape polyphenols are characterized by a large range of structures diversely distributed in every part of the berry. Phenolic acids, mainly represented by hydroxycinnamic acids, are localized in both skin and pulp and are considered to be the major constituents of pulp (*1*, *2*). Flavonoids comprise mostly flavanols, under monomeric and polymeric (proanthocyanidins, also called condensed tannins) forms, which have been known to be present in seeds and skins for a long time. Trace amounts of monomers and oligomers have also been reported in pulp (*3*, *4*), but the presence of highly polymerized condensed tannins has only recently been shown in this part (*5*). In red grape varieties, another flavonoid family, the anthocyanins, are responsible for the red color of the skin (*6*).

Champagne wine is made by three major grape varieties used in different proportions, creating the typical organoleptic characters of each Champagne house, namely Chardonnay, a white variety, and Pinot Noir and Pinot Meunier, which are red varieties. The particularity of Champagne winemaking is a soft and progressive pressing so as to limit in particular staining due to extraction of anthocyanins from the skin. Pulp is thus the major part of the berry providing polyphenolic compounds to the juices and wines of Champagne.

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Phenolic acids of pulp and must from Pinot Noir and Chardonnay have already focused interest (*7*, *8*) and, more particularly, in the Champagne context through a study on Pinot Noir and Chardonnay must oxidation (*9*). Trace amounts of flavanol monomers have also been reported in musts, but no data is available on tannins contained in the pulp of Champagne grape varieties. This information may, however, be of interest because the browning susceptibility of white wines has been related to their flavanol content (*10*, *11*).

The aim of the work reported herein was thus to study the phenolic composition of the three grape varieties involved in Champagne winemaking and to determine the distribution of phenolic compounds, namely flavanols, phenolic acids, and anthocyanins, in the pulp, skin, and seeds. This information is essential to confirm or infirm the potential implication of tannins in the browning of Champagne wines.

To achieve this goal, extraction of all three classes of polyphenols had to be optimized for each berry part. Extraction of polyphenols depends on their diffusion into the extraction solvent (*12*), which is determined on the one hand by their structure and on the other hand by their interactions with other fruit components. Thus, literature gathers various extraction conditions (time, solvent) according to the accessibility of polyphenols in the different matrices. Moreover, because of the acidic lability of interflavan linkages within tannins and of the

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oxidizability of polyphenols, a valid extraction method should provide an extraction of the polyphenol pool as complete as possible while limiting their degradation (*13*). Acidified water, hydroalcoholic solutions of methanol (*14*, *15*), methanol (*3*), or acetone (*16*) are the common solvents used for extracting polyphenols from grape berries. In particular, lower molecular weight polyphenols, such as phenolic acids, anthocyanins, and flavanol monomers and oligomers, are well extracted with methanol, while the higher molecular weight flavanols are better extracted with aqueous acetone than with methanol (*17*−*20*).

The adjustment of the extraction conditions is usually performed by optimizing successively the main parameters influencing the efficiency of extraction without taking into account the eventual interactions between them. As an illustration, Nawaz et al. studied individually the influence of liquid-to-solid ratio and the number of extraction stages on polyphenol extraction from grape seed by ethanol/water (50:50) (*21*). They came to the conclusion that the best conditions were 0.2 g mL^{-1} solid-to-liquid ratio and a double-stage extraction. Extraction times varying from 5 min (*20*) to 24 h (*22*) have been used. Short extraction times aim to decrease tannin degradation and long ones to maximize extraction, but the concentration of tannins in the extracts tends to fall rather than rise after 2−5 h (*23*).

In our work, a response surface methodology (RSM) using Doehlert design was applied to optimize the procedure. This approach allows minimizing the number of experiments and evaluating simultaneously the effect of each parameter (extraction duration, solid-to-liquid ratio, percentage of methanol in the extraction solvent) and that of interactions between them on the extraction efficiency. The contribution of each factor is thus determined with better precision than would be obtained from successive optimizations of the various factors.

Such a methodology has been recently used to determine the most influential parameters for simultaneous extraction of phenolic compounds and organic acids from white Vinho Verde grapes (*24*), but no precision was given on the part studied. Moreover, extraction efficiency was evaluated only on four flavonols and a monomeric flavanol (epicatechin), which are known to be minor phenolic compounds of grapes, while phenolic acids, anthocyanins, and tannins were not considered.

MATERIALS AND METHODS

Chemicals. All HPLC-grade solvents, i.e., methanol, acetone, and acetonitrile, as well as formic and hydrochloric acids, were purchased from Merck (Darmstadt, Germany). (+)-Catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin-3-gallate, phloroglucinol, L-ascorbic acid, and methylparaben were purchased from Sigma (Saint Louis, MO). Malvidin-3-*O*-glucoside was purchased from Extrasynthèse. Sodium acetate was purchased from VWR Prolabo (Fontenay sous Bois, France). For the preparation of all solutions, purified deionized water was used (MilliQ purification system, Millipore, France).

Fruit Sampling and Preconditioning. Clusters of grapevine (*Vitis vinifera* L.) varieties Pinot Noir, Pinot Meunier, and Chardonnay were collected at maturity from the same parcel in Champagne during the 2005 grape harvest by the Comité Interprofessionnel des Vins de Champagne. For each variety, a representative set of 50 berries kept with their pedicel were frozen and stored at −80 °C in order to limit oxidation. The frozen berries were then peeled with a scalpel, and the three parts composing the berries, i.e., the skin, the pulp and the seeds, were separately placed in Dewar vases full of liquid nitrogen. The different parts were immediately milled with a liquid nitrogen precooled Dangoumau grinder (Longjumeau, France) and stored at −80 °C until used for analysis.

Extraction of Polyphenols from Skin, Pulp, and Seed. All extractions were performed on a mechanical stirrer at room temperature (22 °C). Methylparaben was used as internal standard and added to the extraction solvents at 200 mg L^{-1} in the cases of skin and seed, and at 100 mg L^{-1} for an extraction from the pulp.

The development of the extraction process was performed on the variety Pinot Noir.

Preliminary experiments consisted of 2 h extractions using water/acetone (40:60, v/v) acidified with trifluoroacetic acid 0.05% as the extraction solvent, in a single-step or multiple-step process. In the case of single-step extraction, the supernatant was removed after a 2 h extraction followed by a centrifugation for 10 min at 10 000*g*. For the multiple-step extraction, a centrifugation for 10 min at 10 000*g* was operated after 30 min of extraction. The supernatant was then removed, and fresh solvent was added on the solid residue. The operation was repeated three times. All preliminary experiments were performed in triplicate, and the standard deviation was calculated from this triplicate.

Further optimization was achieved using a Doehlert experimental design (see below) with mixtures of (1) methanol acidified with trifluoroacetic acid 0.05% and (2) water/acetone (40:60, v/v) acidified with trifluoroacetic acid 0.05% in variable proportions of (1) and (2) (from 0:100 to 30:70, v/v) as the extraction solvent.

For the runs including methanol, the powder was first suspended in a defined volume of methanol and the mixture was stirred during 2 min before adjusting to the final extraction volume with the water/acetone mixture.

In the optimized extraction conditions, each experiment was triplicated. The standard deviation was evaluated from this triplicate.

Polyphenols Analysis. Two aliquots of each extract (200 μL for skin and seed, half of the extraction volume for pulp, so as to get an acceptable signal-to-noise ratio in chromatographic analyses) were taken to dryness with a centrifugal evaporator Genevac (New York, NY). One of the two residues was dissolved in 200 μL of methanol/water (20:80, v/v) containing 1% HCl and analyzed by HPLC for the determination of flavanol monomers, phenolic acids, and anthocyanins. The second one was used for tannin characterization that was achieved by HPLC analysis after phloroglucinolysis following a protocol adapted from earlier studies (*25*, *26*). The solid residue was dissolved in 200 μL of methanol−HCl 0.2 N containing phloroglucinol (50 g L^{-1}) and L-ascorbic acid (10 g L^{-1}). The solution was then heated in a water bath at 50 °C for 20 min, and the phloroglucinolysis reaction was stopped by adding an equal volume of sodium acetate 200 mM. Samples were then analyzed by HPLC.

HPLC analysis was carried out using an Agilent Technologies system (Waldbronn, Germany) equipped with a photodiode array detector and a Shimadzu spectrofluorimeter (Kyoto, Japan). Samples (10 μL injection volume) were injected onto a reversed-phase Atlantis dC18 column (5 μm packing, 250 mm × 4.6 mm) supplied by Waters (Milford, MA) protected by a guard column (20 mm × 4.6 mm) of the same material and maintained at 30 °C. A binary solvent system was used at a 0.25 or 1 mL min^{-1} flow rate with solvent A being water/formic acid (98:2, v/v) and solvent B being acetonitrile/water/formic acid (80:18:2, v/v/v). The elution gradient was as follows: 0−5 min, isocratic 0% B; 5−65 min, linear 0−20% B; 65−70 min, linear 20−100% B; 70−75 min, 100−0% B. Concentrations were calculated using peak areas from calibration curves established using external standards, either commercial ((+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin-3-gallate for flavanol quantification, and malvidin-3-*O*-glucoside for anthocyanin quantification) or purified in the laboratory (phloroglucinol derivatives for flavanol polymers quantification, and caffeoyl tartaric acid for hydroxycinnamic acid quantification). They were determined with the PDA detector at 280, 320, and 520 nm, respectively, for flavanols, phenolic acids, and anthocyanins, or with the spectrofluorimeter at emission 275 nm and detection 322 nm for non-galloylated flavanol units. Eventual differences in the dilution or injection volumes were compensated for by taking into account the peak area of the internal standard (methylparaben). Tannin concentration was calculated as the sum of the concentrations of constitutive units, terminal units being detected as flavanol monomers and extension units as the corresponding phloroglucinol adducts. Their average degree of polymerization (aDP) was calculated from the proportions

Table 1. Doehlert Experimental Design for Three Variables[a]

no. exp	time (min)		solvent volume (mL)		methanol (%)	
1	120	[1]	5.5	[0]	15	[0]
2	93.75	[0.5]	8.53	[0.866]	15	[0]
3	41.25	[–0.5]	8.53	[0.866]	15	[0]
4	15	[–1]	5.5	[0]	15	[0]
5	41.25	[–0.5]	2.47	[–0.866]	15	[0]
6	93.75	[0.5]	2.47	[–0.866]	15	[0]
7	93.75	[0.5]	6.51	[0.289]	27.24	[0.816]
8	41.25	[–0.5]	6.51	[0.289]	27.24	[0.816]
9	67.5	[0]	3.48	[–0.577]	27.24	[0.816]
10	93.75	[0.5]	4.49	[–0.289]	2.76	[–0.816]
11	41.25	[–0.5]	4.49	[–0.289]	2.76	[–0.816]
12	67.5	[0]	7.52	[0.577]	2.76	[–0.816]
13	67.5	[0]	5.5	[0]	15	[0]
14	67.5	[0]	5.5	[0]	15	[0]
15	67.5	[0]	5.5	[0]	15	[0]

[a] Values between square brackets are the corresponding coordinates in a normalized space.

of the different constitutive monomeric units of tannins by the formula given below.

$$\mathrm{aDP} = \sum\nolimits_{\mathrm{terminal+extension}} / \sum\nolimits_{\mathrm{terminal}}$$

Experimental Design and Statistical Analysis. Optimization of extraction conditions was carried out on Pinot Noir variety using response surface methodology.

A Doehlert experimental design was built for determining the experimental conditions that provide the highest responses. This design was built with three factors at 7, 5, and 3 levels respectively, and was composed of 15 runs, including three replicates at the central point. The three independent factors chosen were extraction time, solvent volume, and rate of methanol (**Table 1**), the weight of sample analyzed being fixed at around 50 mg for the skin and seed, and 100 mg in the case of pulp.

The proposed model to which the experimental data were fitted is a second-order polynomial model. The following equation was used:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2$$

where Y is the amount of extracted compound per g of raw material, a_i are the regression coefficients, and X_1, X_2, and X_3 are the experimental factors. Confidence intervals were evaluated with the $T_{95\%,3}$ as the Student coefficient.

The same Doehlert experimental design was applied to each of the three berry parts.

RESULTS AND DISCUSSION

Effect of the Solid-to-Liquid Ratio. A first study was performed on each part of the Pinot Noir berry in order to determine the most appropriate range of solid-to-liquid ratios to be considered afterward. The weight of solid residue being fixed in the further experimental design, this led to the choice of a range of extraction volumes. Three solid-to-liquid ratios were tested for each part, namely 100 mg in 2 mL, 50 mg in 2 mL, and 50 mg in 4 mL for skin and seed extraction and 200 mg in 2 mL, 100 mg in 2 mL, and 100 mg in 4 mL for pulp extraction, building experimental domains from 12.5 to 50 mg mL^{-1} and from 25 to 100 mg mL^{-1}, respectively.

The influence of solid-to-liquid ratio on the concentration and characteristics of flavanols, anthocyanins, and phenolic acids extracted from the different parts of the Pinot Noir berries is illustrated in **Figure 1**.

The extraction yield of anthocyanins from skins was the same with the three solid-to-liquid ratios tested. Those of flavanols and phenolic acids were significantly lower, with the highest ratio than with the other two, for all parts. Further decrease in the solid-to-liquid ratio induced no significant increase in the extraction yields of hydroxycinnamic acids from pulp nor of flavanols from skin and pulp, indicating that saturation of the solvent occurred only at the highest ratio. Therefore, the extraction conditions selected for further experiments used 25 mg mL^{-1} (for seed and skin) and 50 mg mL^{-1} (for pulp) as the upper limit of the range of solid-to-liquid ratios. In contrast, flavanol extraction yield from seeds and phenolic acid extraction yield from skin significantly increased for each decrease in the solid-to-liquid ratio so that solvent saturation could not be ruled out within the range tested. Consequently, lower ratios were tested in further optimization steps.

Influence of the Number of Extraction Steps. A series of experiments consisting of extractions at constant total volume of extraction and constant extraction time, i.e., a single 2 h extraction step against four successive 30 min extraction steps, was performed in order to determine if increasing the number of extractions improves the extraction efficiency. Indeed, previous studies described experiments based on multistep extractions with a single solvent (*21*, *27*), while others preferred to use a single-step extraction (*28*).

In the multistep extractions performed on each berry part, the major part of phenolic compounds was extracted in the first extraction step, less and less being extracted in successive steps (**Figure 2**). This result was expected, and optimization by the exhaustion method is actually based on this observation (*29*). For seed and pulp, the amounts of flavanols extracted in the single-step and four-step procedures were not significantly different. Extraction of anthocyanins from skins did not depend either on the number of extraction steps. In contrast, extraction of flavanols from skin and of phenolic acids from both skin and pulp was significantly lower with a four-step extraction than with the corresponding single-step extraction (**Figure 2**). Skin tannins, which are located in vacuoles and partly bound to the cell wall and the vacuolar membrane (*30*, *31*), have been shown to be rather easily extracted in winemaking compared to seed tannins (*32*). A possible explanation is that exposure to organic solvents leads to irreversible adsorption of tannins on plant cell walls or other cell components (e.g., proteins, polysaccharides), thus reducing their extractability in subsequent extraction steps. Another possibility is that the rather small solvent volume used in each extraction of the four-step procedure is insufficient to inhibit enzymatic oxidation, resulting in degradation of hydroxycinnamic acids and flavanols.

Simultaneous Optimization of Solvent, Solid-to-Liquid Ratio, and Time of Extraction Using a Doehlert Experimental Design. A Doehlert experimental design was used to evaluate the weight of different factors, namely solvent volume, extraction time, and methanol rate in the solvent and that of interactions between them on the extraction efficiency, in order to determine the optimal extraction conditions.

According to the preliminary experiments, the maximum of extraction was reached with a single-step 2 h extraction. The extraction time was varied between 15 min and 2 h as it was expected to influence the amount of extracted phenolics, but longer extraction times may also induce degradation of the extracted compounds. In particular, artifacts arising from anthocyanin reactions with acetone have been reported (*33*). Indeed, the main objective of the study was to determine a suitable compromise to ensure proper extraction while minimizing degradation. A second objective was to decrease the extraction duration so as to make it possible to handle high-

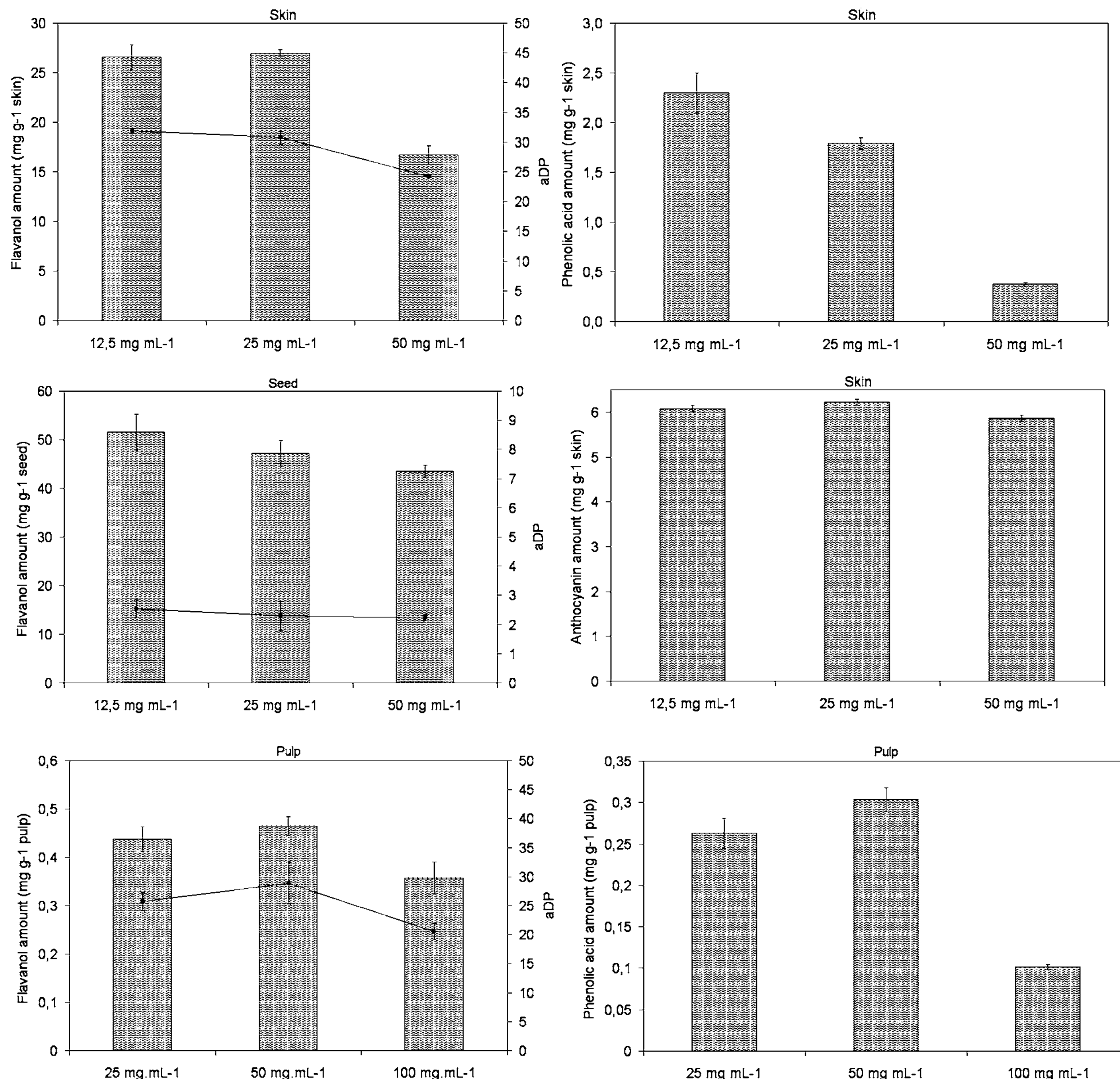

Figure 1. Influence of solid-to-liquid ratio on flavanol, phenolic acid and anthocyanin extraction (histogram: amount in mg g^{-1} part, ■ aDP) from skin, pulp, and seed. Standard deviations are calculated from a triplicate and are visualized by error bars.

throughput experiments. The preliminary results have shown that the solid-to-liquid ratios have to be sufficient to avoid saturation. For modifying the solid-to-liquid ratio, the volume of extraction solvent was varied and the amount of sample was maintained at 50 mg (for skin and seed) or 100 mg (for pulp). However, rather small solvent volumes should be preferred in the development of environment-friendly protocols and to reduce evaporation times while enabling appropriate final concentration factors. The extraction volume was thus varied between 2 and 9 mL.

Moreover, previous studies have shown that extraction of anthocyanins and of lower molecular weight flavanols by acidified methanol and methanol/water mixture is efficient (*34*, *35*), while the use of acetone/water mixture is more adapted for the extraction of tannins with higher aDP (*17*, *36*). Therefore, we evaluated the extraction efficiency with acetone/methanol/water mixtures in different proportions. Such a mixture (acetone/MeOH/water 40:40:20, v/v/v) was chosen by Perret (*37*) for extracting tannins from a grape powder. Furthermore, in our experiments, methanol was first added on the solid residues to reduce PPO activity, which may lead to oxidation of hydroxycinnamic acids and coupled oxidation of flavanols (*38*) and anthocyanins (*39*) by the *o*-quinone derived from caffeoyl tartaric acid. Valero et al. actually showed that primary alcohols partially and temporarily inhibit PPO activity (*40*). The rate of methanol in the extraction solvent was thus studied in a reasonable range, from 0 to 30%.

Solvent volumes, extraction time, and methanol rate were assigned 7, 5, and 3 levels, respectively. The models built from all the runs of the experimental design representing the amounts of extracted flavanols, anthocyanins, and phenolic acids from each berry part as a function of extraction time, solvent volume, and methanol rate are summarized in **Table 2**.

First, the quadratic terms being negative, maximum extraction of tannins, anthocyanins, and phenolic acids can be found in the experimental domain considered. For each model, only terms higher than the coefficient of variance are significant.

Flavanol extraction from skin was primarily affected by the solvent volume and, to a lesser extent, by the methanol content in opposite ways, while impact of the extraction time was much weaker in the time range considered (**Table 2** and **Figure 3**). More precisely, the response increases as the total volume

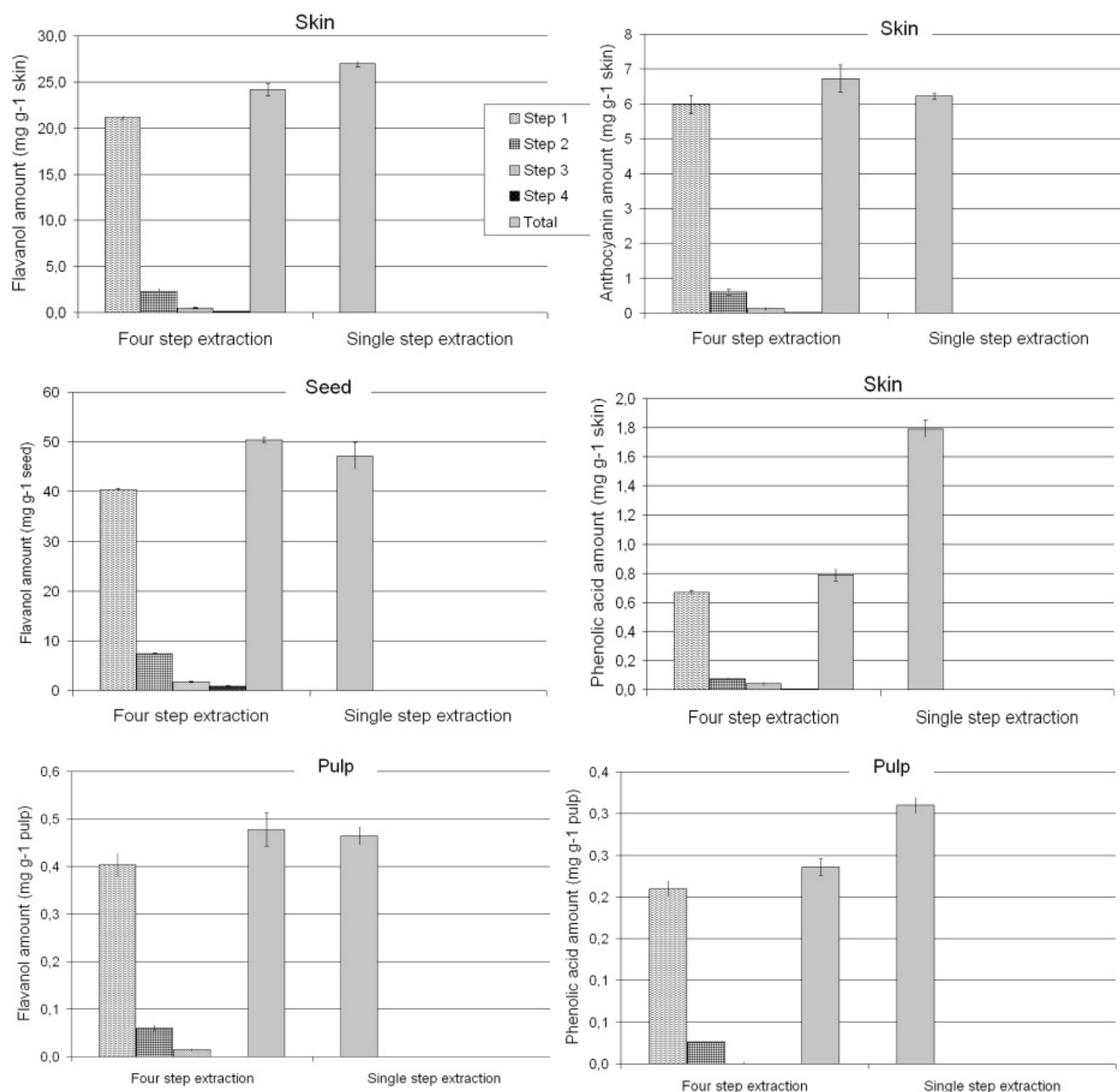

Figure 2. Flavanol, anthocyanin and phenolic acid amounts obtained by four-step and single extractions from skin, seed, and pulp. In the four-step process, individual steps (four histograms on the left) as well as calculated total extraction (histogram on the right) were considered. Standard deviations are calculated from a triplicate and are visualized by error bars.

increases and as the extraction time or the rate of methanol decreases. Regarding the value higher than the coefficient of variance, their extraction from seeds was significantly affected by all three parameters, positively by time and volume and negatively by methanol rate. None of the main effects was significant in the case of flavanol extraction from pulp. The negative effect of methanol is in agreement with earlier work showing that higher molecular weight tannins are better extracted with aqueous acetone than with methanol (*17*−*20*). The amount of extracted anthocyanins is lower with longer extraction times and with higher methanol rate but is increased when larger solvent volumes are used. Phenolic acid extraction from skin or pulp only depends on the solvent volume.

Interactions between extraction time and total volume are significant only in the case of flavanol extraction from seeds and anthocyanin and phenolic acid extraction from skin. Their negative values indicate that higher extraction rates are obtained either with longer times and smaller volumes or with larger volumes and shorter times. Significant and positive interactions between the extraction time and the rate of methanol are observed for all compounds and all parts except phenolic acids from pulp. This indicates that extraction is largely favored in two cases: a long extraction time in presence of high methanol rate and a short extraction time with low methanol rate. Nevertheless, this has to be modulated by the influence of the mains effects and the last interaction term (volume (V) × %MeOH) that is significant for anthocyanins and phenolic acids from skins (positively) and for flavanol from seed (negatively).

Examination of the response surface curves (**Figure 3**) enables selection of optimal conditions for extraction of the different phenolics from each part and confirms that maximum extraction is reached in the range of conditions considered. Thus, the maximum amount of extracted flavanols was obtained with intermediate solvent volumes, extraction times, and methanol rates. This indicates that a minimum volume is requested to avoid a saturation phenomenon limiting the amount of tannins extracted. Moreover, competition occurred between extraction and degradation, degradation overcoming extraction when the extraction time increased. When a higher rate of methanol was used, the amount of flavanol extracted was slightly lower and the aDP of tannins lower, confirming that this solvent fails to extract higher molecular weight flavanols.

Table 2. Regression Coefficients of the Equations Representing the Extraction of Flavanols, Anthocyanins, and Phenolic Acids from Skin, Seed, and Pulp[a]

	skin			pulp		seed
	flavanol	anthocyanin	phenolic acid	flavanol	phenolic acid	flavanol
main effects						
T (a_1)	**–0.53**	**–0.25**	–0.04	–0.01	–0.01	**3.42**
V (a_2)	**3.86**	**0.06**	**0.08**	0.01	**–0.04**	**2.81**
%MeOH (a_3)	**–1.44**	**–0.10**	–0.05	0.04	0.01	**–1.88**
interactions						
$T \times V$ (a_{12})	0. 3	**–0.09**	**–0. 07**	0	–0.01	**–4.1**
$T \times$ %MeOH (a_{13})	**3.04**	**0.46**	**0.46**	**0.09**	–0.01	**3.96**
$V \times$ %MeOH (a_{23})	0.01	**0.26**	**0.27**	0.01	0.02	**–1.16**
quadratic terms						
T^2 (a_{11})	**–2.84**	**–0.44**	**–0.38**	**–0.05**	0.00	**–3.61**
V^2 (a_{22})	**–3.95**	–0.01	**–0.27**	**–0.09**	–0.02	**–7.74**
%MeOH^2 (a_{33})	**–1.58**	**–0.23**	**–0.37**	**–0.06**	**–0.05**	**–3.3**
coefficient variance	0.51	0.02	0.06	0.04	0.02	0.2

[a] The corresponding terms in the model equations are given between brackets. *T*: Extraction time. *V*: Solvent volume. % MeOH: Methanol rate (%). Significant terms are in bold.

Maximum extraction of phenolic acids from skin was obtained using the same conditions. The areas corresponding to the maximum extraction of anthocyanins from skins and phenolic acids from pulp are wider, meaning that the three factors are less influential than for extraction of flavanols. However, the sets of optimized conditions determined on the basis of flavanol extraction for each part appear like good compromises for other phenolic classes. Maximum extraction is thus obtained by using acetone/water/methanol (51:34:15, v/v/v) acidified with 0.05% TFA as the solvent and extraction for 67 min with 7 mL (for skin) or 5 mL (for pulp) or for 90 min with 5.5 mL (for seed).

In these optimized conditions, complementary experiments were carried out by adding sodium metabisulfite in the extraction solvent ([SO_2] = 77 mg L^{-1}) as already used by Cortell et al. (*41*). This molecule is actually known as an inhibitor of PPO activity. The results obtained were not significantly different whatever the polyphenol group considered (results not shown), allowing us to conclude that methanol efficiently inhibited PPO activity.

Validation of the Models. Triplicates of phenolic extraction were carried out on each part of the Pinot Noir berries using the optimized conditions. Results are reported in **Table 3** and compared to the values obtained from the modeling. For all three parts, the phenolic amounts estimated from the model equations are in good agreement with the experimental values, none of the differences between the two values being significant.

Distribution and Characterization of Flavanols, Phenolic Acids, and Anthocyanins in the Different Parts of Pinot Noir, Pinot Meunier, and Chardonnay Grape Berries. The amounts of flavanols, anthocyanins, and phenolic acids in Pinot Meunier and Chardonnay grape varieties were then determined using the same extraction protocol and compared to those of Pinot Noir (**Table 4**).

In all three varieties, 90% of the berry polyphenol content is represented by flavanols, which are the only polyphenols found in seeds but also the major phenolic constituents of the other parts, accounting for 78−93% of skin phenolics and around 60% of pulp phenolics. Pinot Meunier contains larger amounts of flavanols and, consequently, of polyphenols than Pinot Noir, which is richer than Chardonnay. However, it contains lower amounts of phenolic acids and anthocyanins than Pinot Noir, while Chardonnay shows no anthocyanins and intermediate contents of phenolic acids. The majority of flavanols are contained in seeds regardless of the variety, while phenolic acids are distributed almost evenly between skins and pulps. Concentrations of the latter are much higher in the skin than in the pulp for the three varieties considered, but the specific rate (contribution of each part to the berry weight) leads skin and pulp to supply these compounds in similar quantities.

The highest content of seed flavanols is found in Pinot Meunier, with 6400 mg kg^{-1} berry, followed by Pinot Noir (4840 mg kg^{-1} berry), while Chardonnay contains the lowest amount, with 2300 mg kg^{-1} berry. Part of these flavanols actually corresponds to flavanol monomers that represent 17%, 37%, and 11% of the total flavanol amount, respectively, in Pinot Meunier, Pinot Noir, and Chardonnay. The amount of flavanol monomers in Pinot Noir seeds (9.7×10^{-5}g g^{-1} seed) is congruent with other studies evaluating this same parameter in Pinot Noir at 7.7×10^{-5}g g^{-1} seed (*41*). On the contrary, the level of flavanol monomers is 2-fold higher than values found by Cortell et al. (*41*). Chardonnay tannins (calculated after subtracting the concentration of monomers from that of terminal units) are characterized by a slightly higher aDP of 4.2 against 2.9 and 3.7, respectively, for Pinot Noir and Pinot Meunier. These low values compared to those of the literature (*41*) can be explained by the removal of oligomers (dimers, trimers) in the extraction or fractionation procedures used in earlier works (*42*−*44*). The absence of epigallocatechin units and the galloylation rates varying from 9.5% for the Pinot Noir to 13.2% for the Pinot Meunier are consistent with those determined in previous studies (10.6% for the Pinot Noir) (*41*) or other varieties (*5*).

The skin flavanol content is evaluated at 3000, 2400, and 2100 mg kg^{-1} berry fresh weight for Pinot Noir, Pinot Meunier, and Chardonnay, respectively, but as the proportion of skin also varies according to the grape variety (**Table 4**), Pinot Meunier contains the largest concentration of flavanols in skins, followed by Pinot Noir, and finally, Chardonnay. Moreover, skin flavanols of the two red varieties can be differentiated from those of the white variety by a higher mean degree of polymerization (aDP = 38 and aDP = 36 for Pinot Noir and Pinot Meunier compared to aDP = 23 for the Chardonnay) and a higher proportion of epigallocatechin units. The amounts and aDP are consistent with the results obtained in earlier studies on Chardonnay and Pinot Noir harvested in different vineyards (*41*, *45*). The aDP values for Pinot Noir and Pinot Meunier are slightly higher than those obtained for other red varieties such as Mourvedre or Grenache

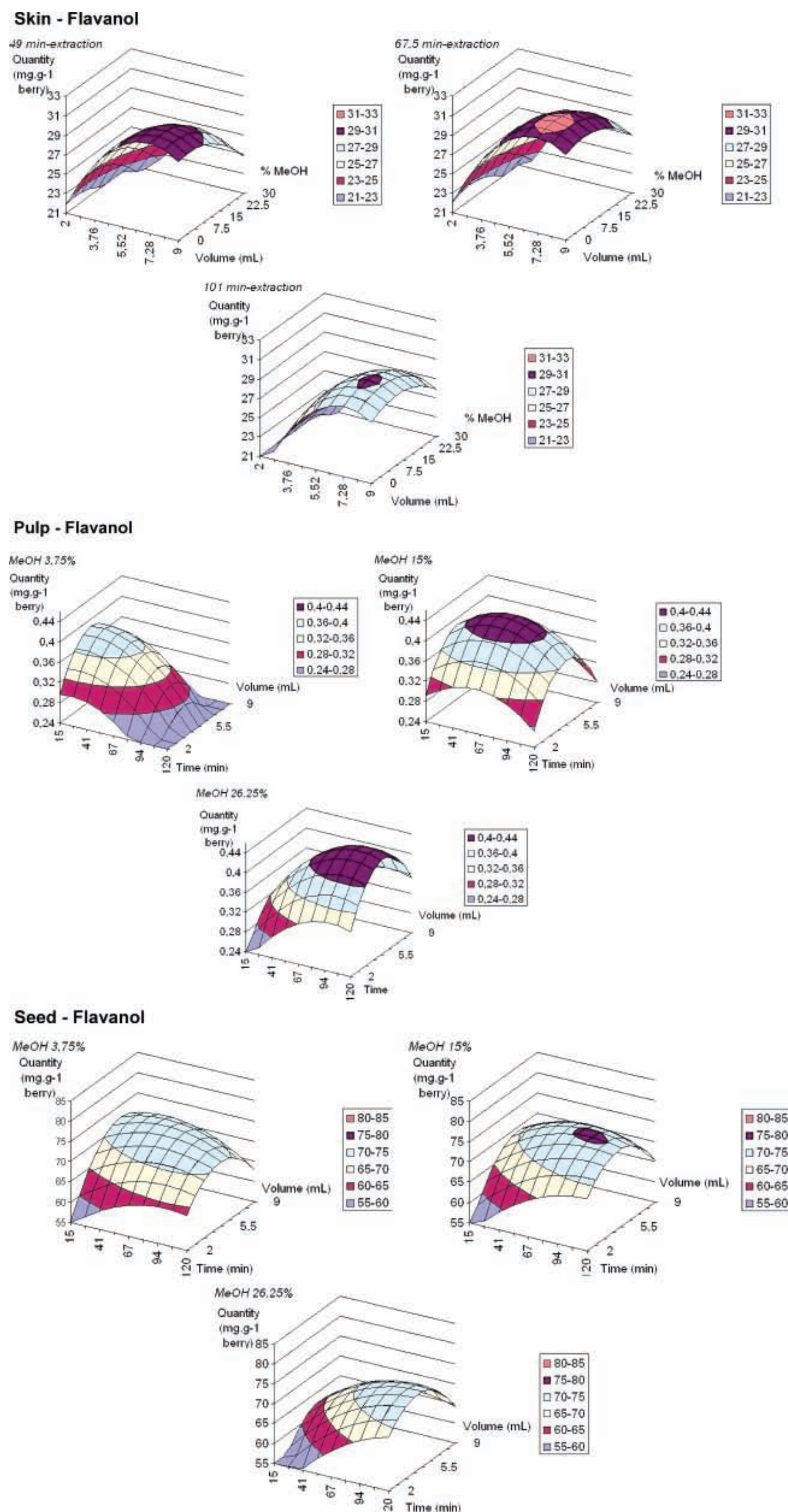

Figure 3. Response surface curves at low, medium, and high levels of time or methanol rate (factor with the lowest impact according to the part) for the extraction of skin, pulp, and seed flavanols.

(aDP = 26) (*5*). Besides, skin tannins of Champagne varieties have a lower galloylation rate (from 1.1 to 1.8%) compared to varieties such as Grenache or Muscat Frontignan, and a proportion of epigallocatechin units (from 9.6 to 14.5%) similar to that usually found in skin tannins (*5*). The galloylation rate is similar to that found for Pinot Noir in an earlier study (*41*).

Skins of Pinot Noir contain larger amounts of phenolic acids and anthocyanins than Pinot Meunier, the concentration of phenolic acids in Chardonnay being intermediate. However, due to different thickness, skins of the three grape varieties supply phenolic acids in equivalent amounts and the red ones the same amounts of anthocyanins, i.e., around 200 and 600 mg kg^{-1}

Table 3. Amounts of Phenolics Extracted with the Optimized Set of Conditions Compared to the Values Obtained from the Modeling (Results Expressed in mg g^{-1} Fresh Weight Part)

	skin			pulp		seed
	flavanol	anthocyanin	phenolic acid	flavanol	phenolic acid	flavanol
models	31 ± 2	6.55 ± 0.01	2.16 ± 0.05	0.42 ± 0.08	0.33 ± 0.02	75 ± 1
experimental	30 ± 1	6.3 ± 0.2	2.1 ± 0.2	0.45 ± 0.03	0.34 ± 0.01	75.1 ± 0.5

Table 4. Distribution and Characteristics of Polyphenols in the Three Major Champagne Grape Varieties[a]

	Pinot Noir			Pinot Meunier			Chardonnay		
	skin	pulp	seed	skin	pulp	seed	skin	pulp	seed
flavanols (mg g^{-1} f.w. part)	30 ± 1	0.45 ± 0.03	75.1 ± 0.5	24.0 ± 0.7	0.26 ± 0.06	101 ± 1	21 ± 1	0.36 ± 0.03	57.6 ± 0.6
phenolic acids (mg g^{-1} f.w. part)	2.1 ± 0.2	0.34 ± 0.01		1.14 ± 0.05	0.17 ± 0.01		1.65 ± 0.04	0.21 ± 0.03	
anthocyanins (mg g^{-1} f.w. part)	6.3 ± 0.2	ϵ		3.3 ± 0.3	ϵ				
specific rate (%)	10	81.7	6.4	18.2	74.4	6.4	11.4	83.6	4.0
flavanols (mg kg^{-1} berry)	3000 ± 100	370 ± 20	4840 ± 30	4400 ± 100	210 ± 40	6400 ± 100	2400 ± 100	320 ± 30	2300 ± 30
aDP	39 ± 2	21 ± 1	2.9 ± 0.7	37 ± 2	18 ± 2	3.65 ± 0.07	23 ± 1	18.3 ± 0.7	4.24 ± 0.01
% galloylation	1.13 ± 0.08	5.2 ± 0.1	9.5 ± 0.1	1.46 ± 0.08	4.3 ± 0.4	13.2 ± 0.1	1.8 ± 0.1	3.2 ± 0.5	12.7 ± 0.1
% egc	14.5 ± 0.6	1.7 ± 0.2	0	13.2 ± 0.5	1.9 ± 0.2	0	9.6 ± 0.3	4.3 ± 0.8	0
flavanol monomers (mg kg^{-1} berry)	ϵ	ϵ	1800	ϵ	ϵ	1100	ϵ	ϵ	250
phenolic acids (mg kg^{-1} berry)	211 ± 20	276 ± 6		208 ± 9	127 ± 15		190 ± 4	174 ± 2	
anthocyanins (mg kg^{-1} berry)	632 ± 20	ϵ		602 ± 20	ϵ				

[a] Amounts are expressed in both mg g^{-1} fresh weight part and mg kg^{-1} berry, taking into account the specific rate of each part. The standard deviation is estimated from a triplicate analysis. f.w.: Fresh weight. ϵ: Not quantifiable.

berry f.w. for phenolic acids and anthocyanins, respectively. It is worth noting that Pinot Noir berries of our study are richer in anthocyanins than Pinot Noir berries from other vineyards (*41*).

Characteristics of pulp tannins of the three grape varieties are globally similar even if slight differences can be pointed out. The average degrees of polymerization are equivalent, with values varying from 18 for Pinot Meunier to 21 for Pinot Noir. These values are lower than the aDP evaluated in the corresponding skins but remain high compared to those of seed tannins. Similar results were obtained for Maccabeo white grape with aDP of tannins evaluated at 51 and 32 for skin and pulp, respectively (*5*). The proportions of galloylated and epigallocatechin units are intermediate between those found in skin and seed tannins. The main difference between the three varieties lies in the rate of epigallocatechin units (1.7 and 1.9 for the red cultivars vs 4.3% for the white one). The amounts of tannins in the pulp are quite different, varying from 26 mg kg^{-1} for Pinot Meunier to 45 mg kg^{-1} pulp for Pinot Noir. In spite of the high specific rate of pulp, its contribution to the berry tannin content is rather low (4, 2, and 6% for Pinot Noir, Pinot Meunier, and Chardonnay, respectively). These contributions are much lower than those found in the six varieties studied by Souquet, which vary between 8 and 15% (*5*). The lower amount and lower aDP estimated for pulp tannins compared to the values obtained for skin tannins might suggest that the presence of tannins in the pulp is only an artifact of handling and results from tannin diffusion from the skin toward the pulp. But previous studies have shown that few cells containing tannins are present in the skin layer close to the pulp (*31*). Moreover, the particular composition of pulp tannins (higher galloylation rate and lower proportion of epigallocatechin units than in skin tannins) allows us to rule out this hypothesis because galloylation decreases the solubility of tannins while hydroxylation increases it. Finally, Pinot Noir is the variety containing the highest content of phenolic acids in the pulp, with 276 mg kg^{-1} f.w. berry against 174 mg kg^{-1} f.w. berry for Chardonnay and 127 mg kg^{-1} f.w. berry for Pinot Meunier. The flavanol to phenolic acid ratio in Pinot Noir pulp is 1.3, while Chardonnay and Pinot Meunier pulp contain much more flavanols than phenolic acids (ratios 1.8 and 1.7, respectively). This variability may lead to different oxidation susceptibilities of the three Champagne varieties.

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