# QUALITATIVE COMPOSITION AND EXTRACTABILITY OF GRAPE SKIN TANNINS DURING THE RIPENING PERIOD. ROLE OF THE EXTRACTION SOLVENT

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## Abstract

**Aims** : The changes that skin tannins undergo during ripening, which are crucial for their extractability and transfer to wine, were followed in Monastrell grape skins. To elucidate whether the commonly used analytical method involving extraction with 70 % acetone overestimates the quantity of tannins that could be extracted during the winemaking process, the extraction with aqueous 12.5 % ethanol was also monitored throughout the ripening period.

**Methods and results** : When 70 % acetone was used as extraction solvent, the results showed that skin tannin concentration decreased towards maturity. When tannins were extracted with 12.5 % ethanol, their concentration during the first part of maturation was much higher than that obtained with the acetone extraction. However, both methods gave similar quantitative results for the last sampling dates, although the mean degree of polymerization (mDP) of the extracted tannins was significantly lower using the ethanol method.

**Conclusions** : The acetone method provides, for ripe grapes, an accurate prediction of what may be quantitatively extracted by a fermenting medium, although it overestimates the tannin mDP.

**Significance and impact of the study** : The structural composition of tannins (and not only their concentration) might be essential for defining the sensorial characteristics of the wine and the differences observed in tannin mDP between the acetone and the ethanol method may be of importance when predicting wine organoleptic characteristics.

**Key words** : tannins, proanthocyanidins, grape skin, berry, acetone, ethanol, ripening

## Résumé

**Objectifs** : Les changements que subissent les tannins de la peau au cours de la période de maturation, essentiels pour leur extractibilité et transfert dans le vin, ont été suivis dans la peau de raisin Monastrell. Pour élucider si les résultats obtenus avec la méthode analytique couramment utilisée, impliquant l'extraction avec l'acétone à 70 %, surestime ce qui se passe pendant le processus de macération fermentaire dans le vin, l'extraction avec une solution d'alcool à 12.5 % a également été suivie.

**Méthodes et résultats** : Quand l'acétone à 70 % a été utilisée comme solvant d'extraction, les résultats ont montré que la concentration des tanins de la peau diminuait vers la maturité. Quand les tanins ont été extraits avec la solution d'alcool à 12.5 %, leur concentration au cours de la première partie de la maturation était beaucoup plus élevée qu'avec la méthode de l'acétone. Cependant, les deux méthodes ont donné des résultats quantitatifs similaires pour les derniers échantillons, bien que le degré moyen de polymérisation (mDP) des tanins extraits fût significativement plus faible lorsque l'éthanol a été utilisé.

**Conclusions** : La méthode de l'acétone fournit pour les raisins mûrs une prévision précise de la quantité de tanins pouvant être extraits dans un milieu de fermentation, mais cette méthode surestime la valeur du mDP.

**Signification et importance de l'étude** : La composition structurelle des tanins (et non seulement leur concentration) pourrait être essentiel pour définir les caractéristiques organoleptiques du vin et les différences observées dans le mDP des tanins entre les deux méthodes pourraient être importantes dans la prédiction de ces caractéristiques.

**Mots clés** : tanins, proanthocyanidines, peau de raisin, baie, acétone, éthanol, maturation

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## INTRODUCTION

Grape tannins are important to wine quality. The results of Kassara and Kennedy (2011) pointed to a positive relationship between projected wine price and the extraction of phenolics from skin. Therefore, the measurement of tannins in grape skins before harvest may give an indication of the tannins that could be extracted into wine during winemaking and of the potential quality of the wine.

Several studies have shown that skin tannins start to accumulate at flowering, reach a maximum around veraison and decrease towards maturity (Downey *et al.*, 2003 ; Ortega-Regules *et al.*, 2008 ; Obreque-Slier *et al.*, 2010). This tannin variation during ripening and the different extractability have to be considered when grapes in different maturity stages are used in winemaking (Bordiga *et al.*, 2011).

The most commonly used methods for measuring tannins in winegrapes generally involve direct extraction with 70 % acetone, which has been regarded as the most effective condensed tannin extraction solvent (Kallithraka *et al.*, 1995 ; Harbertson and Downey, 2009 ; Downey and Hanlin, 2010). Although this method may provide insight into the total extractable potential of condensed tannins in grape, previous results have suggested it may overestimate the amount that can be extracted during fermentation in an alcoholic medium (Downey and Hanlin, 2010). Therefore, an extraction method using a model wine may be more appropriate for estimating potential grape tannin extraction.

This article focuses on the study of the qualitative and quantitative changes of Monastrell skin tannins as maturation progresses and on elucidating whether the results obtained using 70 % acetone as extraction solvent overestimate the quantity of tannins that could be extracted from skins during winemaking.

## MATERIALS AND METHODS

### 1. Grape sampling and preparation for analysis

*Vitis vinifera* L. cv. Monastrell grape samples were obtained from a commercial vineyard in Jumilla (Spain). Grape samples were obtained at different stages of ripeness from preveraison (green) to commercial harvest for the 2010 growing season. To obtain a representative vineyard sample, a 1000-berry sample was collected from three rows distributed within the vineyard block and then pooled and transported to the laboratory where samples were kept at –20ºC until use.

### 2. Tannin extraction with 70 % acetone

All the experiments were done in triplicate. The skins of 10 berries were separated from the mesocarp and rinsed with distilled deionized water. Whole skins were extracted in covered Erlenmeyer flasks with 10 mL of 70 % acetone in water at room temperature for 24 h on an orbital shaker at 200 rpm. To minimize tannin oxidation, solutions were purged with nitrogen and extraction was carried out in the dark. Following extraction, the extract was concentrated under reduced pressure at 35ºC to remove acetone and then lyophilised to a dry powder. This powder was redissolved in 2 mL methanol in a volumetric flask.

### 3. Extraction and quantification of skin tannins using a model solution of 12.5 % ethanol

The skins of 10 berries were manually separated from seeds and pulp and rinsed with distilled deionized water. Whole skins were extracted in flasks with 15 mL of a hydroalcoholic solution containing 12.5 % ethanol and 2 g/L potassium hydrogen tartrate (pH 3.6) at room temperature for 72 h on an orbital shaker at 200 rpm. To minimize oxidation, the solutions were purged with nitrogen and the extraction was carried out in the dark. Following extraction, the extract was concentrated under reduced pressure at 35ºC and then lyophilised to a dry powder. This powder was redissolved in methanol in a volumetric flask and analysed for tannins.

### 4. Skin tannin analysis

Skin tannins were determined according to the method described by Kennedy and Jones (2001) with some modifications, as follows. A solution of 0.2 N HCl in methanol, containing 100 g/L phloroglucinol and 20 g/L ascorbic acid was prepared (phloroglucinolysis reagent). 100 µL methanolic extract was reacted with 100 µL phloroglucinolysis reagent (1:1) in a water bath for 20 minutes at 50ºC and then combined with 2 volumes of 200 mM aqueous sodium acetate to stop the reaction.

HPLC analysis followed the conditions described by Busse-Valverde *et al.* (2010). Proanthocyanidin cleavage products were estimated using their response factors relative to (+)-catechin, which was used as the quantitative standard. These analyses

allowed the total tannin content, the apparent mean degree of polymerization (mDP) and the percentage of each constitutive unit to be determined. The mDP was calculated as the sum of all subunits (flavan-3-ol monomer and phloroglucinol adducts, in moles) divided by the sum of all flavan-3-ol monomers (in moles).

### 5. Statistical data treatment

Significant differences between wines and for each variable were assessed by analysis of variance (ANOVA). LSD test was used to separate the means ($p<0.05$) when the ANOVA test was significant. This analysis was conducted using Statgraphics 5.0 Plus.

## RESULTS AND DISCUSSION

Figure 1 compares the evolution of the tannin content observed when the extraction was carried out with 70 % acetone for 24 hours or 12.5 % ethanol for 72 hours. When 70 % acetone was the extracting solvent, the concentration of tannins was maximum on the first sampling date (before veraison, which occurred on August 7th), and then decreased towards maturity. Other authors have also stated that the bulk of tannin synthesis occurred immediately after fruit set and finished several weeks before veraison while a second phase of tannin accumulation occurred just prior to veraison, when the maximum levels of tannins were found (Downey *et al.*, 2003). From veraison, Bogs *et al.* (2005) found that the expression genes most related to tannin synthesis could no longer be detected. This could partly explain why tannins do not accumulate during ripening, but it does not explain the observed decrease. Other authors have attributed the postveraison decrease in tannin levels to a less efficient extraction process that occurs as grapes ripen or to degradation processes. Hence, it has been suggested that extractability decreases following the conjugation of tannins with other cellular components (Downey *et al.*, 2003), the simultaneous polymerization of tannins near cell walls (Gagne *et al.*, 2006) or the oxidative cross-linking of polymeric tannins (Kennedy *et al.*, 2000 ; Downey *et al.*, 2003 ; Cadot *et al.*, 2006). Gagne *et al.* (2006) observed that skin tannins can be found either inside skin cells or attached to skin cell walls and they reported the decrease in tannin content to be greater in the internal part of skin cells than in the cell walls, where they remain nearly constant from the early stages of development to maturity. The changes that tannins undergo during fruit ripening are crucial for their extractability and transfer from the solid parts into wine.

The evolution of Monastrell skin tannins was also monitored using 12.5 % ethanol as extraction solvent and a 72-hour extraction time. The results were surprising (Figure 1). At the first sampling date, the levels of tannins extracted with the ethanolic solution were almost three times higher than those extracted with acetone, decreasing significantly after this date. From August 29th (22ºBrix) to the harvest time (25ºBrix), the same range of values was observed for both methods. We

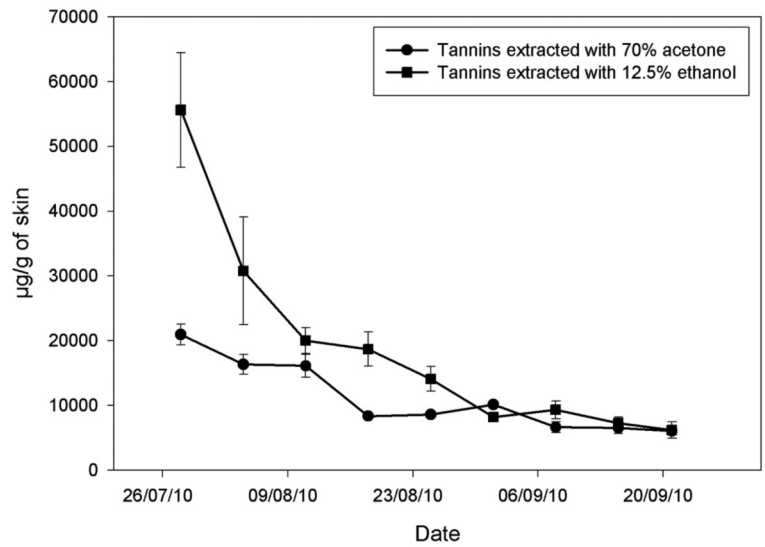


**Figure 1 - Comparison of the evolution of skin tannin concentration during the ripening of Monastrell grapes when determined by the extraction method using 70 % acetone for 24 hours or 12.5 % ethanol for 72 hours.**

are aware that the use of frozen grapes to conduct this experiment may overestimate the level of tannin extraction with both solvents, since ice crystals break the cell structures, thereby facilitating the extraction of those components located inside the cells, as is the case for tannins. However, since the experiments with both solvents have been done with the same grapes, the comparison of the results is valid.

What explains these differences between the two extraction methods as maturation progresses? The answer is probably a combination of two factors: firstly, the different extraction time (72 hours with the ethanol method vs 24 hours with acetone) and secondly, the skin status and tannin interactions with the cell walls. A kinetic study by Geny *et al.* (2003) indicated that tannins are rapidly extracted from the vacuole although complete extraction of the cell wall-associated fraction takes 14 times longer. In unripened grapes, the longer extraction time that the ethanol method entailed could explain the greater quantities of extracted tannins. As maturation progresses, tannins are transferred from vacuoles to cell walls, and the changes in the cell wall structure during berry ripening may reduce tannin extractability as the gel network changes and encapsulates tannins within its structure (Le Bourvellec *et al.*, 2004; Le Bourvellec *et al.*, 2007; Downey and Hanlin, 2010). These compounds seem to be more difficult to extract, even when long extraction times are used, as with the ethanol method. The acetone method implies a shorter extraction time, however, this may be compensated by the greater capacity of acetone to disrupt hydrogen bonding and release cell wall covalently associated tannins.

Our previous work on the effect of these two solvents on the extraction of seed tannins (Bautista-Ortín *et al.*, 2012) also showed that at the first sampling dates, the extraction with 12.5 % ethanol gave a much higher tannin concentration (eight times higher) than that obtained using 70 % acetone. However, and contrary to the results reported here, at the last few sampling dates this was reversed and at harvest, the concentration of seed tannins was four times higher (0.43 mg/berry vs 1.80 mg/berry) when the acetone method was used. The localization of tannins in the seed may explain this difference. They are observed in three seed tissues: the epidermis, a large part of the outer integument, and the inner layer of the inner integument (Cadot *et al.*, 2006). It has been described that there is a hardening of the medium integument and intensive lignifications as maturation progresses, which makes it waterproof (Cadot *et al.*, 2006). This would prevent the phenolic compounds of the inner integument from being extracted during ethanolic maceration, whereas this extraction could be possible using 70 % acetone as the extracting solvent.

The mDP obtained with the acetone method (Figure 2) did not vary much during ripening. Bindon and Kennedy (2011) also described a similar evolution for Cabernet Sauvignon grapes, although they observed a higher mDP. In contrast, Bordiga *et al.* (2011) observed a significant increase in mDP values during the maturation of

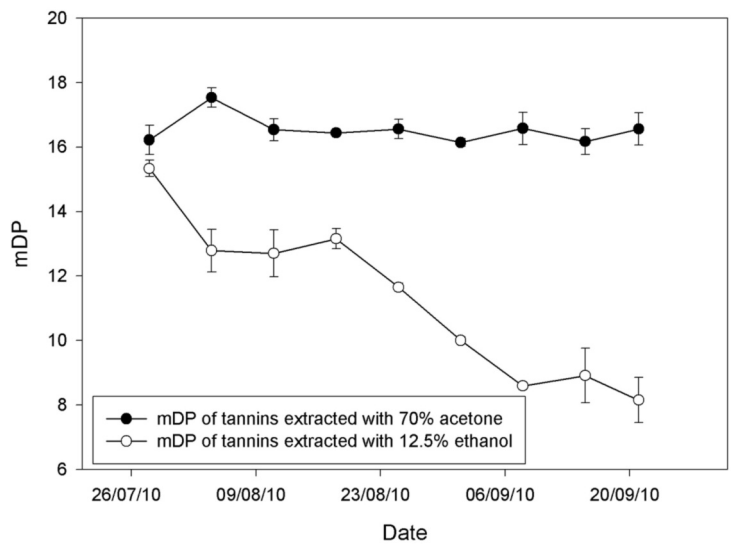


**Figure 2 - Comparison of the evolution of the mean degree of polymerization (mDP) of skin tannins during the ripening of Monastrell grapes when determined by the extraction method using 70 % acetone for 24 hours or 12.5 % ethanol for 72 hours.**

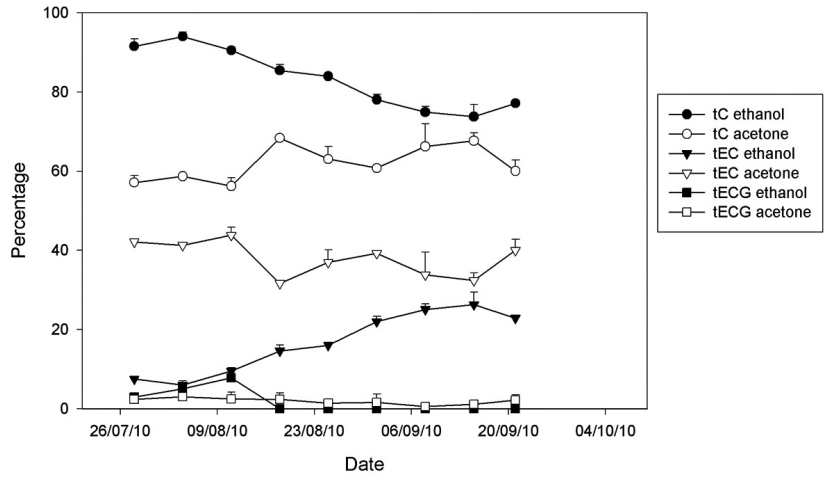


**Figure 3 - Comparison of the evolution of the terminal subunit composition (expressed as percentage) of Monastrell skin tannins during ripening when extracted with 12.5 % ethanol and 70 % acetone (tC : terminal (+)-catechin, tEC : terminal (-)-epicatechin, tECG : terminal (-)-epicatechin-O-gallate).**

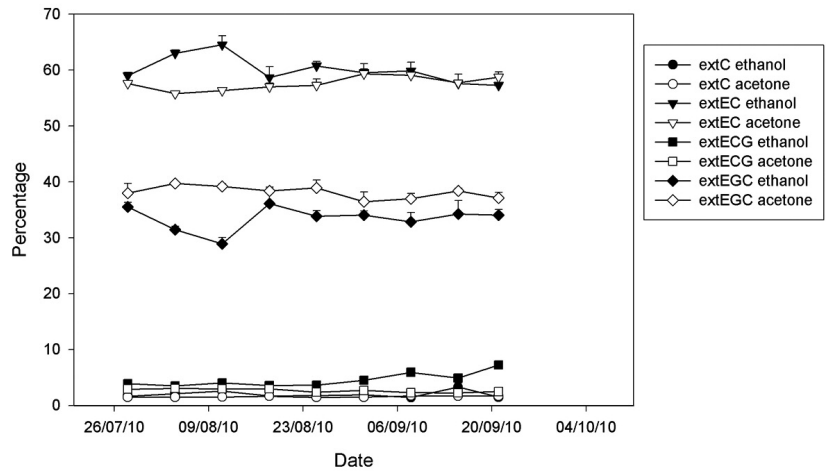


**Figure 4 - Comparison of the evolution of the extension subunit composition (expressed as percentage) of Monastrell skin tannins during ripening when extracted with 12.5 % ethanol and 70 % acetone (extC : extension (+)-catechin, extEC : extension (-)-epicatechin, extECG : extension (-)-epicatechin-O-gallate, extEGC : extension (-)-epigallocatechin).**

Cabernet Sauvignon grapes, with a small reduction at the last sampling date. In our study, the mDP of the extracted tannins was similar for both methods on the first sampling date. After that, it was higher when tannins were extracted with 70 % acetone. Harbertson and Downey (2009) and Downey and Hanlin (2010) compared the extraction of tannins with acetone and ethanol and found that acetone extracted tannins with higher molecular weights. Higher molecular weight tannins are mainly associated with cell walls and, as stated before, acetone disrupts hydrogen bonding to a greater extend than ethanol. Mattivi *et al.* (2009) also found that the estimated mDP of grape tannins extracted from skins into a wine-like solution was lower than that reported for tannins extracted with other solvents. They also stated that the differences were to be expected since large tannins are unlikely to be extracted to any great extent in a wine-like solution.

Also, we monitored the changes in the composition of the skin tannins during ripening with both extraction methods (Figures 3 and 4). According to several studies, the qualitative composition of tannins is also important for the sensory

characteristics of red wines, such as bitterness and astringency (Gawel, 1998). It has been shown, for example, that astringency depends on the structural characteristics of tannins such as mDP and the percentage of galloylation (Vidal *et al.*, 2003). Moreover, Chira *et al.* (2009) found that the correlation between mDP and astringency could be modulated by the presence of (-)-epigallocatechin and Fernández *et al.* (2007) also reported that the presence of (-)-epigallocatechin units in the tannins in Carmenere grapes lowered the "coarse" perception. The knowledge of the structural composition of proanthocyanidins might well be considered essential for defining the sensorial characteristics of the subsequent wine and therefore the knowledge of the evolution of the qualitative composition of tannins during ripening and the effect of solvent on this composition is of importance.

The results with the 70 % acetone extraction method showed that the qualitative composition of Monastrell skin tannins only changed slightly during maturation. (+)-Catechin was the most abundant terminal unit, followed by (-)-epicatechin, and low quantities of (-)-epicatechin-O-gallate were observed (Figure 3). This later terminal subunit was not affected by the extraction method used. However, when 12.5 % ethanol was used, the percentage of (+)-catechin was always higher, especially at the beginning of the maturation, decreasing with time and reaching values more similar to those observed when acetone was used. These results were very similar to those observed by Mattivi *et al.* (2009), with a high percentage of (+)-catechin in the terminal subunits. (-)-Epicatechin behaved just the opposite.

Considering the extension subunits (Figure 4), (+)-catechin and (-)-epicatechin-O-gallate were present at very low percentage, (-)-epicatechin being the most abundant subunit, followed by (-)-epigallocatechin. The extension subunit composition did not change during maturation and only small differences due to solvent were observed.

In conclusion, and contrary to what has been observed in seeds (Bautista-Ortín *et al.*, 2012), the acetone method provides a very accurate prediction of what may be qualitatively and quantitatively extracted by a fermenting medium, especially when the evaluation is made at the end of ripening. However, a fermenting solution will extract tannins with lower mDP than those observed using acetone and this fact should be considered, since tannin mDP may influence wine organoleptic characteristics.

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