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The Effect of Methyl Jasmonate-Doped Nano-Particles and Methyl Jasmonate on the Phenolics and Quality in Monastrell Grapes during the Ripening Period

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Abstract: The effect produced by the application of methyl jasmonate (MeJA) in vineyards is clear, but this is a product that, despite its efficiency, is expensive, volatile and difficult to dissolve. Regarding increasing the MeJA use efficiency, new forms of application are proposed in this article, such as the use of calcium phosphate nanoparticles with two different morphologies: amorphous (ACP) and crystalline (Ap). In addition, few of the studies addressed so far have assessed MeJA's effect during the ripening period of the grapes. As a result of this, in this article, we evaluated/studied for first time the effect of the different MeJA formats on the phenolic composition of the grape during the ripening period. The results showed small differences between the two morphologies of the nanoparticles, which promoted a significant a delay in the sugar accumulation and an increase in the different phenolic compounds compared to the control. Such improvements were not as significant as those induced by the conventional MeJA treatment. However, it is remarkable that when the nanoparticles were applied, we used a concentration 10 times lower than when it is used conventionally. Therefore, these findings revealed that both types of calcium phosphate nanoparticles are potential MeJA nanocarriers allowing for the increase in the quality of the grapes at the time of harvest in a more sustainable way, although future studies must be carried out in order to optimise the concentration with which these nanoparticles are doped.

Keywords: grape; elicitors; nanotechnology; sustainability; maturation; amorphous; crystalline

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1. Introduction

Grape ripening starts at veraison and lasts about forty-sixty days until harvest, depending on different factors such as the variety, environment and agricultural practices [1]. Several characteristics of grapes are modified during this period, including their physical and chemical characteristics, such as modifications in size, composition, colour, texture and flavour. In addition, the harvest time directly affects the sensory characteristics of the grape and its wine such as the colour, aroma and taste [2]. Therefore, the quality of the harvested fruits and the obtained wines is strongly related to the characteristics gained by the grapes during maturation.

On the other hand, it is known that phenolic compounds are quality indicators of red wines, because they can affect to its colour and biological properties. Anthocyanins are the main compounds responsible for the colour in grapes and red wines. Flavonols can increase the colour intensity by several times through copigmentation, particularly in younger red wine. Proanthocyanidins play a role in the colour stabilisation processes over time and also in the taste and mouth-feel properties of red wines [3]. The composition and content of polyphenols are different during grape ripening and are directly responsible for

the special characteristics in the different grape varieties and of the resulting wine. The concentration of polyphenols in grape berries depends on the grapevine variety and is influenced by viticultural and environmental factors [4,5], and they are the most common chemical compounds present in grapes.

Climate plays an important role in optimal grape growth and quality wine production [6,7]. The increase in temperature, as a consequence of climate change, has resulted in advanced harvest dates in most of the wine producing countries [8]. In our area, southeast Spain, the climate is very warm and dry. Therefore, the current situation has resulted in an uncoupling of technological and phenolic maturity in red grape cultivars, resulting in unbalanced grape maturity and berry composition [9].

Different alternatives are being adopted in different world regions to mitigate the effects of climate change. This effect is noticeable in crops such as wine grapes (*Vitis vinifera* L.), a very important crop in many places, which covers more than 7.4 million hectares worldwide with high social and economic impact [10]. In recent years, phytochemical contents and other quality parameters have been improved with pre- and post-harvest plant growth regulator treatments [11,12]. Methyl jasmonate (MeJA) is among the most common of such plant growth regulators. It is an organic volatile compound derived from jasmonic acid that is present in several plant tissues. Its biological behaviour in plants is that it triggers the activity of the enzymes responsible for the biosynthesis of polyphenols, such as phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), peroxidases (POD) and other related enzymes [13–16]. Since it has a regulatory role in fruit growth and ripening, it can easily affect the ripening processes of both climacteric and non-climacteric fruits [17,18].

Different authors have studied MeJA's effect on grapes at harvest and its wines by investigating its effect on different families of analytes, such as phenolic and nitrogen compounds [19,20]. Specifically, in wine grapes, several reports have shown that MeJA treatments on vineyards led to increased phenolic content, mainly anthocyanins, flavonols and stilbenes, in grapes and wine, although huge differences between growing seasons and varieties were found [21–24]. Nevertheless, no information is available in these papers regarding the effects of MeJA treatments on the grape ripening process. Only in a recent paper has a 10-day delay on the technological maturity (°Brix and pH), as a consequence of MeJA vineyard treatment, been reported in the wine variety 'Sangiovese' [25]. Gómez-Plaza et al. [26] studied the evolution of phenolic compounds during ripening in grapes treated with MeJA and benzothiadiazole (BTH) in three different varieties (Monastrell, Syrah and Merlot). However, in all the mentioned works, to achieve an increase in the phenolic composition of grapes, it was necessary to use high concentrations of MeJA (10 mM). This concentration is the minimum to use in order to corroborate its effect, since other authors, such as Parra-Torrejón et al. [27], have showed how the application of MeJA in two concentrations (5 mM and 10 mM) on Monastrell grapes in veraison only increased stilbene content when the higher concentration was applied (10 mM).

In addition, a novel nanocomposite (i.e., Nano-MeJA) with amorphous structure (ACP), allowed to reduce the MeJA dosage to 1 mM while maintaining a stilbene content similar to the values obtained for MeJA at 10 mM [27]. Foliar application of Nano-MeJA (1 mM MeJA) on the Monastrell variety during two consecutive seasons also improved the amino acid composition in grapes and wines to a similar extent as the conventional treatment using a 10 times higher MeJA dosage (10 mM) [28]. This considerable MeJA dosage reduction is due to the role of the amorphous calcium phosphate nanoparticles, which provide a sustainable release and protection against thermal degradation of the elicitor, ensuring elicitor activity over longer period of times on the surface of the leaves [25]. These amorphous nanoparticles have also been proposed as potential nanocarriers of nitrogen containing compounds such as urea (U-ACP). Field experiments on Tempranillo grapevines demonstrated that grapes treated with U-ACP contained similar yeast assimilable nitrogen (YAN) and amino acids beneficial to wine aroma and taste as those treated with pure urea using a 15 times higher nitrogen dose [29]. Another study on wheat revealed a N dosage

reduction of 40% compared to conventional urea treatments through foliar application of U-ACP [30].

In this work, we evaluated the effect of pre-harvest methyl jasmonate (MeJA) treatment applied in two different ways (conventionally or as nanoparticles) on fruit quality parameters and phenolic composition of the grape during the period between veraison and harvest. In addition, two types of calcium phosphate nanoparticles with the same composition but different morphology, i.e., nanocrystalline apatite (Ap) and its ephemeral precursor, amorphous calcium phosphate (Nano), were used as nanocarriers of MeJA (Ap-MeJA and Nano-MeJA, respectively) in order to verify if their function was the same or not during the ripening period.

2. Materials and Methods

2.1. Materials

Sodium citrate tribasic dihydrate ($Na_3(C_6H_5O_7)\cdot 2H_2O$, ≥99.0% pure), potassium phosphate dibasic anhydrous (K_2HPO_4, ≥99.0% pure), sodium carbonate (Na_2CO_3, ≥99.0% pure), calcium chloride dihydrate ($CaCl_2\cdot 2H_2O$, ≥99.0% pure), methyl jasmonate ($C_{13}H_{20}O_3$, 95%, racemic), phloroglucinol and quercetin were purchased from Sigma-Aldrich (Barcelona, Spain). Methanol was purchased from Carlo Erba (Vall de Reull Cedex, France), acetone was purchased from Honeywell Riedel-de Haën (Charlotte, NC, USA), sodium acetate was purchased from Panreac (Barcelona, Spain) and malvidin 3-*O*-glucoside chloride was purchased from Extrasynthèse (Genay, France). All the solutions were prepared with ultrapure water (0.22 μS, 25 °C, Milli-Q, Millipore, MA, USA).

2.2. Synthesis and Characterisation of MeJA Nanocomposites

MeJA-doped nanoparticles (Nano-MeJA) were synthesised using a simple and eco-friendly protocol, as previously described [27,28]. Firstly, amorphous calcium phosphate nanoparticles (ACP) were precipitated just after mixing an aqueous solution (2 L) containing 0.2 M $CaC_{l2}\cdot 2H_2O$ and 0.2 M $Na_3(C_6H_5O_7)\cdot 2H_2O$ with a phosphate solution of equal volume containing 0.12 M K_2HPO_4 and 0.1 M Na_2CO_3. The mixture was agitated for 5 min and then ACP precipitates were collected and repeatedly washed with ultrapure water by centrifugation (3700 rpm, 15 min, 4 °C). Nano-MeJA was obtained by adding 5 mL of MeJA solution to 40 g of ACP nanoparticles dispersed in 2 L of water. The mixture was kept under magnetic stirring for 24 h and then Nano-MeJA was collected by centrifugation (3700 rpm, 15 min, 4 °C).

Nanocrystalline apatite (Ap) nanoparticles were synthesised by slightly modifying the batch precipitation protocol described above. Two solutions, (A) $CaCl_2\cdot 2H_2O$ (0.1 M) and $Na_3(C_6H_5O_7)\cdot 2H_2O$ (0.4 M) and (B) K_2HPO_4 (0.12M) and Na_2CO_3 (0.1 M), were mixed (1:1 v/v, 4 L total) and kept for 24 h at 60 °C. After that, the precipitates were collected and repeatedly washed with ultrapure water by centrifugation (3700 rpm, 15 min, 4 °C). The Ap nanoparticles were then functionalised with MeJA (Ap-MeJA) through the same protocol previously described for ACP nanoparticles.

The morphology and size of Nano-MeJA and Ap-MeJA were evaluated by transmission electron microscopy (TEM) with a LIBRA 120 PLUS instrument (Carl Zeiss SMT, Centre for Scientific Instrumentation of the University of Granada, CIC-UGR) operating at 120 kV. To this aim, Nano-MeJA and Ap-MeJA nanoparticles were ultrasonically dispersed in ethanol, and some drops of the slurry were deposited on 200 mesh copper grids covered with thin amorphous carbon films. ImageJ software (version 1.48v; NIH, Bethesda, MD, USA) was used to analyse the size of the nanoparticles (n = 40).

2.3. Field Experiments on Vineyards

To evaluate the efficiency of Nano-MeJA and Ap-MeJA in the field, four treatments were carried out on Monastrell (*Vitis vinifera* L.) grapevines from experimental vineyards in Jumilla (Murcia, Spain), which were grafted onto 1103-Paulsen rootstocks and trained in a vertical trellis system. Vine rows were arranged N–NW to S–SE with a between-row

and within-row spacing of 3 × 1.25 m in a completely randomised block design, with 30 vines for each treatment and 10 vines for each replication. The treatments applied were as follows: (1) an aqueous solution of MeJA at a concentration of 10 mM (MeJA), (2) an aqueous suspension of 3.6 g L^{-1} Nano-MeJA (resulting in a total concentration of 1 mM MeJA), (3) an aqueous suspension of 7.8 g L^{-1} Ap-MeJA (resulting in a total concentration of 1 mM MeJA) and (4) an aqueous solution of Tween 80 only (control) used as a wetting agent (0.1 v/v) in all treatments. Foliar applications were made at veraison and one week later and approximately 200 mL per plant was sprayed. This assay was conducted during the 2020 season and climatological data are shown in work by Gil-Muñoz et al. [28].

2.4. Physicochemical Parameters in Grapes

Sampling was carried out every ten days from veraison to harvest and total soluble solids, pH, total acidity [14] and berry weight were evaluated. Total soluble solids were measured with an Abbé-type refractometer (Atago RX-5000, Tokyo, Japan) and pH and total acidity were measured using an automatic titrator (Metrohm, Herisau, Switzerland) with 0.1 N NaOH.

2.5. Phenolic Composition of the Grapes during Ripening

Different polyphenolic compounds were analysed during the ripening period: anthocyanins, flavonols and proanthocyanidins.

2.5.1. HPLC Analysis of Anthocyanins and Flavonols

Twenty grape berries from each of the treatments and their replicas were peeled using a scalpel. The skin obtained was frozen at −20 °C. Subsequently, an extraction was made in methanol (20 mL ethanol/g of skin) for 2 h on a plate at 150 rpm and 25 °C. After that, the methanolic extract was taken and filtered through a 0.45 µm nylon filter (OlimPeak; Teknokroma, Barcelona, Spain). A chromatographic analysis (HPLC) was carried out following the protocol of Gil-Muñoz et al. [31]. Anthocyanins and flavonols were quantified at 520 nm and 360 nm, respectively, using malvidin 3-*O*-glucoside chloride and quercetin as external standards.

2.5.2. HPLC Analysis of Proanthocyanidins

Ten skins were peeled with a scalpel and rinsed with deionised distilled water. Subsequently, an extraction was made using the skins and 10 mL 2:1 acetone/water. The mixture was kept shaking for 24 h in an orbital shaker at 200 rpm in the dark. In order to eliminate acetone, the extract was concentrated in a vacuum concentrator at 35 °C. Finally, the pellet was redissolved in 2 mL methanol in a volumetric flask and the skin's proanthocyanidin were analysed by HPLC using the protocol described by Busse-Valvered et al. [32].

2.6. Statistical Analysis

Data were presented as means ± SD (standard deviation), calculated from three repeated samples by using Excel 8.0.1 software (Microsoft Excel Software, Redmon, WA, USA). Significant differences among grapes for each variable were assessed by analysis of variance (ANOVA) using Duncan's test to separate means ($p < 0.05$). Finally, a discriminant analysis was also performed to determine whether the groups were sufficiently discriminated based on the original variables available. These analyses were performed using statistical analysis.

3. Results

3.1. Characterisation of Amorphous and Crystalline Nanoparticles

The nanoparticles were synthesised through a simple batch precipitation method previously reported, with reagent concentration, temperature and maturation time being the parameters controlling the formation of ACP or Ap [33,34]. Both nanoparticles are similar in composition (calcium phosphate) but with different structures and morphologies.

Subsequently, the nanoparticles were functionalised with MeJA following a previously reported procedure [31], resulting in the nanocomposites Ap-MeJA and Nano-MeJA. Figure 1 shows TEM micrographs of the two types of nanoparticles used in this work. The TEM micrographs showed that Ap-MeJA are elongated platelet-like nanoparticles with a length of about 22.2 ± 5.3 nm, similar to those found in mineralised tissues [35]. Nano-MeJA nanoparticles showed a rounded morphology with a diameter of 15.4 ± 3.8 nm.

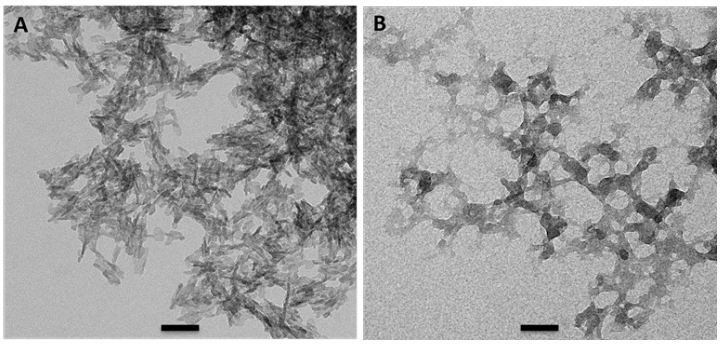

Figure 1. TEM micrographs of Ap-MeJA (**A**) and Nano-MeJA (**B**) nanoparticles. Scale bar = 50 nm.

3.2. Field Experiments on Vineyards

3.2.1. Evolution of Physicochemical Parameters in Grapes

The evolution of the different physicochemical parameters studied (°Brix, total acidity, pH and berry weight) can be seen in Figure 2 (numerical data can be seen in ST1 of the Supplementary Materials). The results showed that physiological changes in the berries are evident after veraison, with a constant increase in sugar concentration, pH and weight of the berries, and a reduction in acidity. Other authors have observed the same results in grapes during grape maturation [36,37].

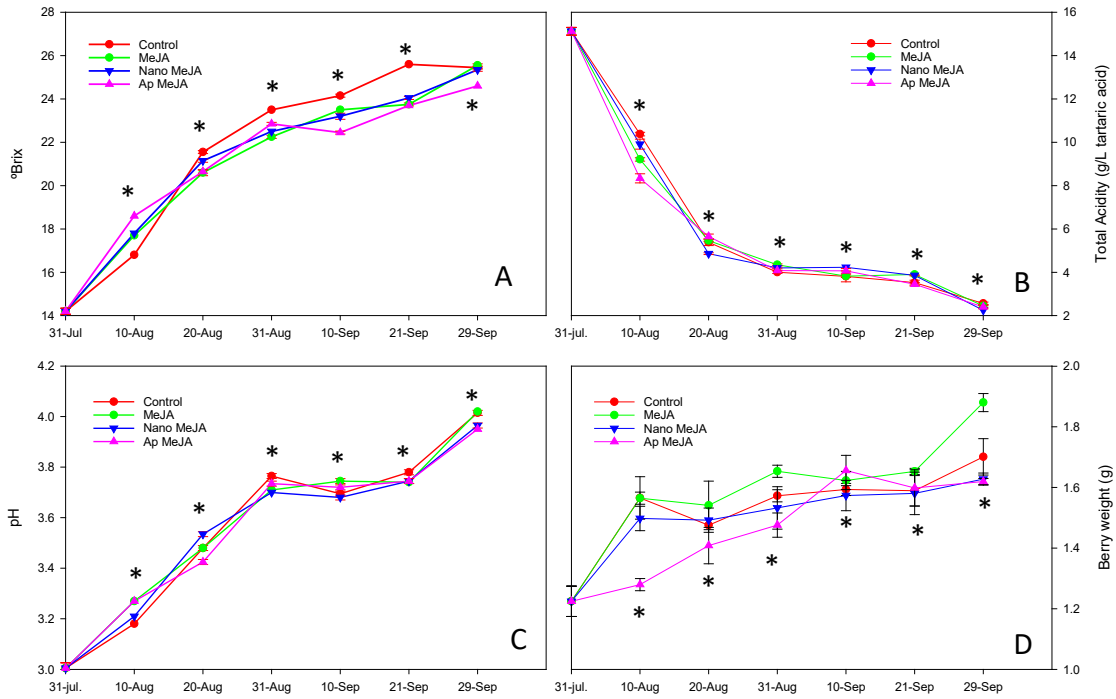

Figure 2. Physicochemical parameters in grapes during ripening period ((**A**) evolution of °Brix; (**B**) evolution of total acidity; (**C**) evolution of pH and (**D**) evolution of berry weight). Abbreviations: MeJA: methyl jasmonate; Nano-MeJA: methyl jasmonate adsorbed on amorphous calcium phosphate; Ap-MeJA: methyl jasmonate adsorbed on nanocrystalline apatite. * Indicates significant differences among treatments according to Duncan's test ($p < 0.05$).

With respect to the °Brix parameter, the evolution of the accumulation of sugars in the berries was faster in the control grapes than in the rest of the grapes treated with MeJA in its different forms. The sugar contents of the control samples, MeJA and Nano-MeJA, at different ripening stages ranged between 14.40 and 26.10, 25.56 and 25.35 °Brix, respectively, whereas in the case of Ap-MeJA it ranged between 14.40 and 24.80 °Brix. Thus, we were able to observe how from sampling 2, the °Brix was always higher in the control samples. On the other hand, it can be seen that up to the third sampling, the grapes treated with conventional MeJA are the ones that accumulate sugar more slowly, however from the fourth sampling, the grapes treated with nanoparticles accumulate sugar more slowly, highlighting the Ap-MeJA treatment. Only at harvest time were sugar concentrations equalised between control grapes and MeJA- and Nano-MeJA-treated grapes, and only Ap-MeJA-treated grapes were harvested with a lower °Brix. Therefore, it could be said that, in general, this treatment delayed the accumulation of sugars in the berries during the ripening period. Some studies showed a delay in the ripening process of grapes after applying MeJA, such as those by Paladines-Quezada et al. [38] or Gil-Muñoz et al. [24], where MeJA or Nano-MeJA were applied on Monastrell grapes, or in work by Portu et al. [23], where this elicitor was applied in Graciano grapes. On the contrary, other authors have found an advance in the maturation of Tempranillo grapes [39]. It should be noted that a delay in the ripening process could be interesting in warm areas such as southeast Spain, where there is a decoupling between technological and phenolic maturity due to the consequences of climate change.

As is normal, in Figure 2B, we can observe how acidity level decreased during the ripening period in the same way for control and treated grapes, finding no differences between Nano-MeJA and Ap-MeJA at the different ripening stages. The acidity values ranged between 15.13 and 2.45 and 2.25 and 2.40 g/L tartaric acid for Nano-MeJA and Ap-MeJA, respectively. We could observe how from the third sampling, the control grapes showed a more accelerated decrease in acidity compared to the treated grapes (see Table S1 of the Supplementary Materials). It can also be observed how in the fourth and fifth samplings, a somewhat less marked decrease was observed in the Nano-MeJA treatment (see Table S1 of the Supplementary Materials), although at harvest time all berries had very similar values. In contrast, other studies have shown an increase in total acidity at harvest time in Monastrell grapes [28,40] or Tempranillo grapes when MeJA was applied [23].

As can be seen in Figure 2C, the evolution of pH in the samples during the ripening period increased until 31st August, but then for three weeks it remained stable and only at harvest time was an increase observed again in all samples. Regarding treatments, small but significant differences could be observed; the pH value ranged between 3.01 and 3.96 for control samples, 3.01 and 4.02 for MeJA samples, 3.01 and 3.96 for Nano-MeJA and 3.01 and 3.95 for Ap-MeJA (see Table S1). In general, treated and untreated grapes were harvested with a high pH, because the climatology suffered in this part of Spain. High pH grape juices may generate technical problems with difficult solutions during alcoholic fermentation [41].

Regarding berry weight, the results are shown in Figure 2D. As can be seen, a great variability was found between control and treated grapes during the ripening period. In general, an increase in weight was observed throughout the ripening period up to the moment of harvest for all treated and untreated grapes. The highest value reached at the time of harvest was for the treatment with MeJA, obtaining a value of 1.88 g, followed by the control grapes at 1.70 g. The lowest values were obtained for the two nanoparticle treatments (1.63 g and 1.62 g for Nano-MeJA and Ap-MeJA, respectively) with a slight decrease in the Nano-MeJA in the fifth sampling. The grapes treated with Ap-MeJA showed the lowest berry weight values in the first weeks of ripening and then reached values similar to other treatments. Some authors explain the decrease in berry weight during ripening in terms of water deficit [42,43], although in this case, all the vines of our experiment were in the same plot and under the same conditions.

3.2.2. Evolution of the Phenolic Composition in Grapes during Ripening Period

Each group of polyphenol families is directly responsible for the important characteristics of specific grape varieties and their products [44]. The polyphenol compositions at different harvest dates were determined by HPLC-DAD analysis and the results are shown graphically for the main polyphenol families in Figures 3–5.

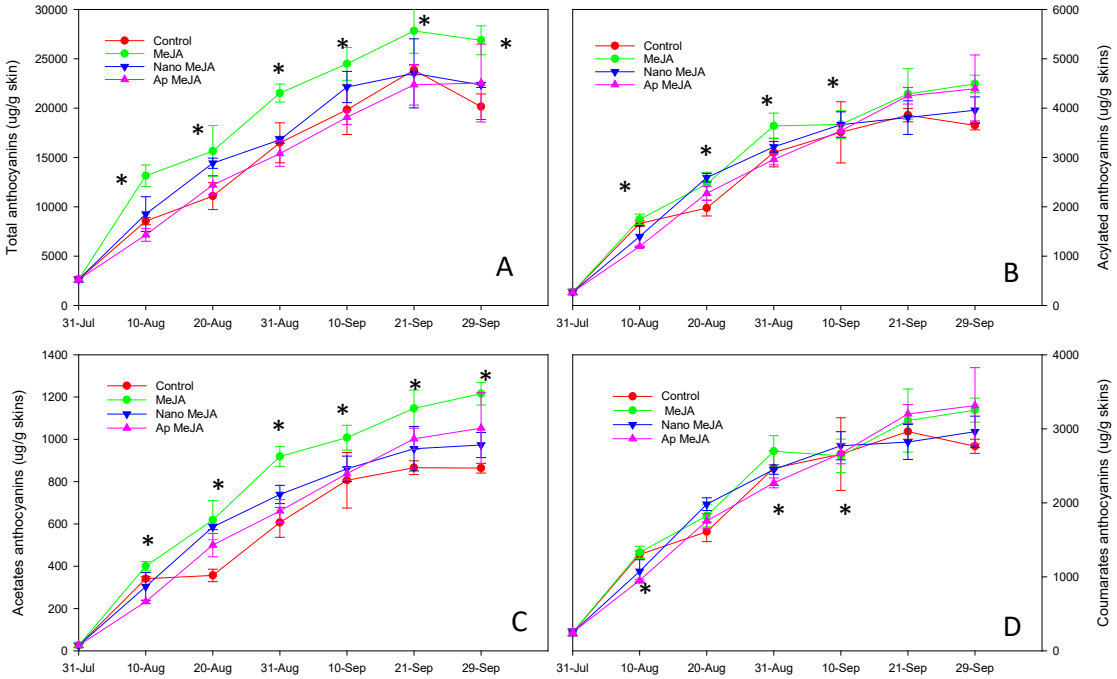

Figure 3. Anthocyanins in grapes during the ripening period. ((**A**) total anthocyanins; (**B**) acylated anthocyanins; (**C**) acetates anthocyanins; (**D**) coumarates anthocyanins.) Abbreviations: MeJA: methyl jasmonate; Nano-MeJA: methyl jasmonate adsorbed on amorphous calcium phosphate; Ap-MeJA: methyl jasmonate adsorbed on crystalline apatite nanoparticles. * Indicates significant differences among treatments according to Duncan's test ($p < 0.05$).

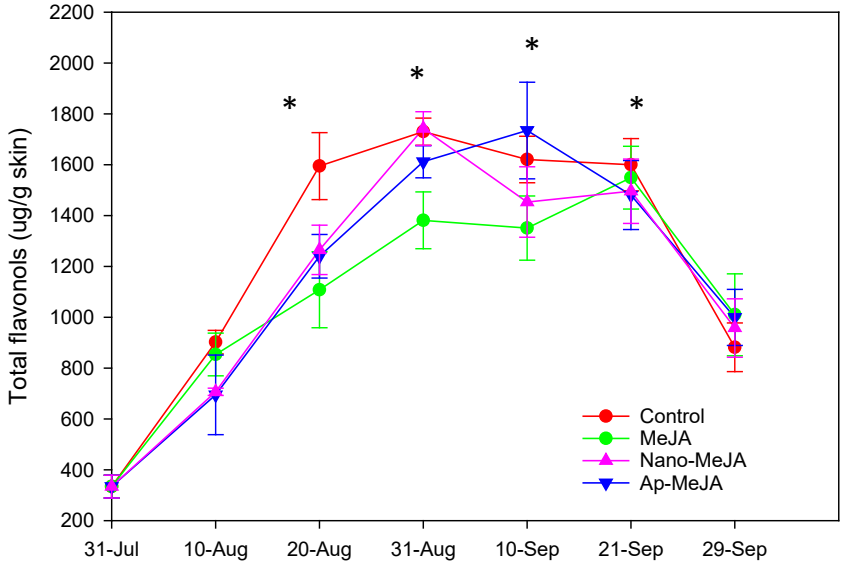

Figure 4. Total flavonols in grapes during the ripening period. Abbreviations: MeJA: methyl jasmonate; Nano-MeJA: methyl jasmonate adsorbed on amorphous calcium phosphate; Ap-MeJA: methyl jasmonate supported on crsiallimum calcium phosphate. * Indicates significant differences among treatments according to Duncan's test ($p < 0.05$).

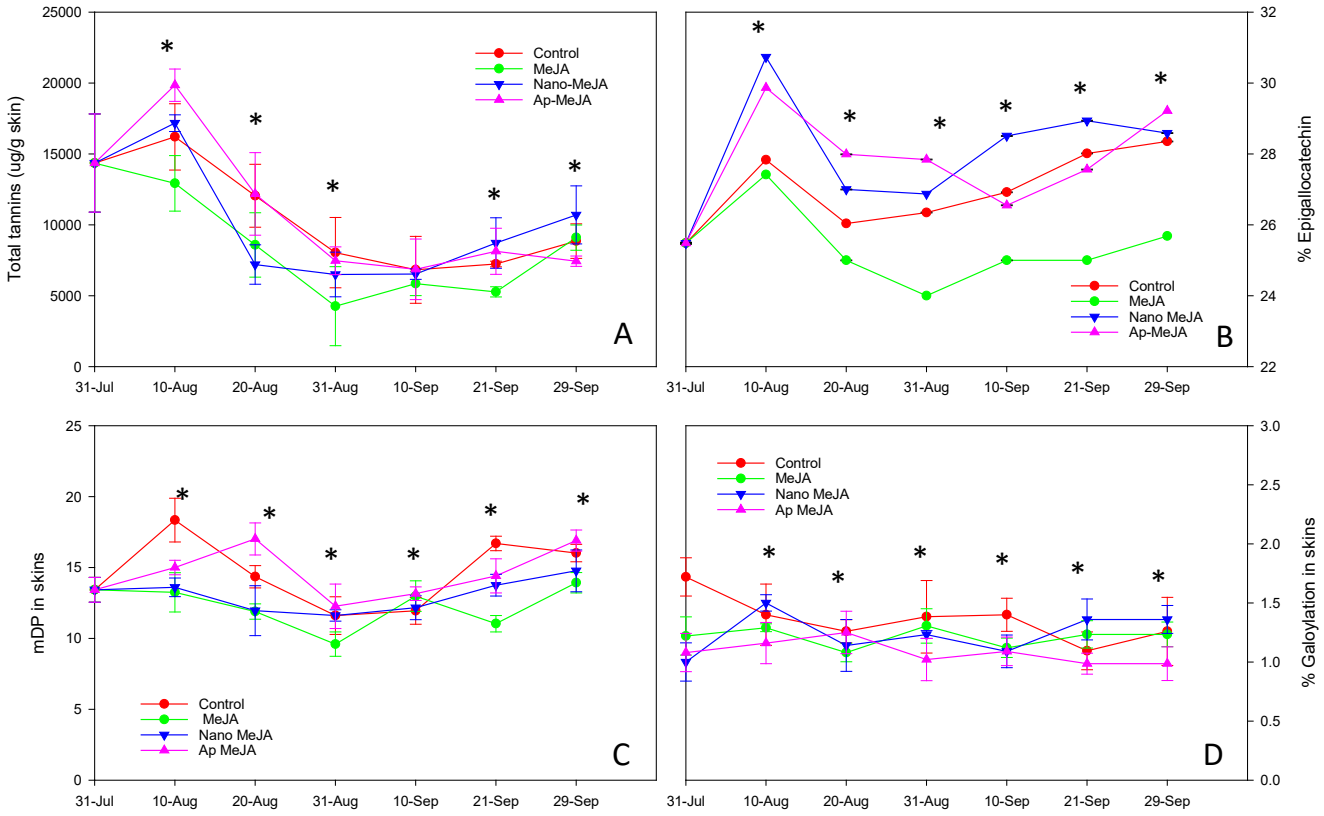

Figure 5. (**A**) Total skin proanthocyanidins in grapes during ripening period. (**B**) % epigallocatechin during ripening period. (**C**) mDP in skins during ripening period. (**D**) % galloylation in skins during ripening period. Abbreviations: MeJA: methyl jasmonate; Nano-MeJA: methyl jasmonate adsorbed on amorphous calcium phosphate; Ap-MeJA: methyl jasmonate adsorbed on crystalline apatite nanoparticles. * Indicates significant differences among treatments according to Duncan's test ($p < 0.05$).

Evolution of Anthocyanins during Ripening Period

The results for the evolution of total anthocyanins in Monastrell grapes in skin extracts of treated and untreated grapes are shown in Figure 3 (numerical data can be seen in ST2 of the Supplementary Materials). The accumulation of anthocyanins starts at veraison and reaches a maximum around harvest time [45], and they are the main pigments responsible for the colour of the grapes and wines.

Regarding total anthocyanins (Figure 3A), the treated grapes obtained the highest concentrations of these compounds at harvest, increasing by 33% when MeJA was applied in a conventional way, and 11% and 12% when applied as Nano-MeJA or as Ap-MeJA, respectively. In this regard, it is worth noting that when we applied Nano-MeJA or Ap-MeJA, the concentration of MeJA used was 10 times lower than when applied in a conventional way, and yet we still managed to increase the concentration of anthocyanin at harvest. These results can be explained by the fact that during maturation, MeJA increases the level of secondary metabolites in grapes, specifically by triggering the biosynthesis of anthocyanins [44]. Several authors have found how MeJA treatments increased anthocyanin levels at harvest for different grape varieties, such as Monastrell, by 25% [24], 29% [46] or 44% (for Garnacha). [47]. Likewise, a study during ripening by Gómez-Plaza et al. [26] revealed how Monastrell grapes treated with MeJA and benzothiadiazol (BTH) showed higher anthocyanin levels than control grapes at the fourth sampling date during ripening, and these differences were maintained until harvest.

As we can see, the MeJA and Nano-MeJA treatments obtained higher anthocyanin concentrations from the beginning of the maturation period until harvest. However, at the end of the maturation period the grapes treated with Ap-MeJA also had increased

anthocyanin concentration compared to the control, although during all the previous samplings they obtained the lowest concentration of anthocyanin. The delayed impact of Ap-MeJA treatment on anthocyanin concentrations and berry weight (Figure 2D) could be explained by the lower % of MeJA content in Ap nanoparticles (Ap-MeJA, 2.8 wt.%) compared to ACP nanoparticles (Nano-MeJA, 6.2 wt.%) [48] and thus the need for a higher Ap-MeJA nanoparticle uptake, at least twice that of Nano-MeJA, to provide similar MeJA content to the Nano-MeJA treatment. In fact, Ap-MeJA treatments required 7.8 g of nanoparticles per litre for the application of 1 mM of MeJA, while Nano-MeJA treatment involved 3.6 g of nanoparticles per litre for the same MeJA concentration.

Regarding acylated anthocyanins (Figure 3B), as occurred with total anthocyanins, these compounds increased during the ripening period until harvest time. Acylation is one of the most common modifications of plant phenolics, including anthocyanins, resulting in a significantly large structural diversity of anthocyanins from the addition of aromatic and/or aliphatic constituents linked to the C6″ positions of the glucosyl groups [49]. The amount of acylated anthocyanins is largely influenced by the grape variety and may be absent from some varieties such as Pinot Noir [3]. Regarding treatments, all treatments applied improved the acylated anthocyanin concentrations. It was observed that conventional MeJA treatment had the greatest influence on acylated anthocyanins, promoting an increase of 33% compared to control grapes at harvest time, as occurred with total anthocyanins. In the cases of nanoparticulate treatments, the increases were 22% for Nano-MeJA and 23% for Ap-MeJA. In another study, Portu et al. [23] showed how MeJA enhanced all forms of acylated anthocyanins in Tempranillo grapes in 2016, although they did not find the same results in previous seasons. In addition, Portu et al. [50] also showed how MeJA increased the acylated forms in Tempranillo grapes, although these differences were not found when MeJA was applied together with phenyl alanine.

During the evolution observed in the ripening period, in general, all treatments increased the concentration of acylated anthocyanins in this period when compared to the control grapes. The high concentration reached in the fourth sampling by the grapes treated with MeJA is noteworthy. In the fifth sampling, all samples appeared to be equal, and it is from this moment until harvest when the grapes treated with MeJA and those treated with Nano-MeJA obtained the highest concentrations of acylated anthocyanins, although these differences were not statistical significant.

Regarding acetates (Figure 3C), again, all treatments applied boosted acetate anthocyanins, with MeJA applied in the conventional way increasing it by the most (41%) compared to control grapes. In the case of nanoparticles, the increases were 13% in Nano-MeJA and 22% in Ap-MeJA. The possible explanation for the different increases produced using nanoparticles could be due to their different structure and morphology; the first is spherical and the second is elongated, as it has been specified in Section 3.1 of this work. As can be seen in Figure 3C, the increase in these compounds in MeJA-treated grapes was shown from the beginning of the ripening period until harvest, obtaining the highest concentration. Regarding the rest of the treatments, this increase was evident from the third sample until harvest when compared to control grapes.

Finally, coumarates (Figure 3D) were also increased using the different MeJA treatments, in this case the highest increase was caused by Nano-MeJA (20%), followed by MeJA applied in a conventional way (17%) and then Ap-MeJA (7%) at harvest time. For these compounds, again the difference in the structure and morphology of the nanoparticles influenced the obtained results. During the ripening period a variability in the results was observed for both the control grapes and the applied treatments.

In summary, in all cases, acylated, acetate and coumarate anthocyanins increased with MeJA treatments. It is true that the highest increase occurred when MeJA was applied in a conventional way (10 mM), but in the other two cases, the nanoparticles were applied in a ten times lower concentration (1 mM). Although many efforts are still required to elucidate the mechanism, some authors have proposed that NPs induce reactive oxygen species (ROS) and secondary signalling messengers that lead to transcriptional regulation

in the plant secondary metabolism [51]. Several studies reported that NP application has resulted in an accumulation of ROS in the plant cell, which triggers oxidative stress and thus subsequent direct and indirect changes in primary and secondary metabolites [51]. Therefore, the application of exogenous phytohormones, such as methyl jasmonate conventionally or as nanoparticles, can greatly modify the expression of genes involved in anthocyanin biosynthesis, as well as the production and accumulation of anthocyanins in grape berries [52].

Evolution of Flavonols during Ripening Period

Flavonols are yellow pigments that contribute directly to the colour of white wines and indirectly to the colour in red wines through the co-pigmentation process [53].

Figure 4 shows the results of total grape flavonols during the ripening period for treated and untreated grapes (numerical data can be seen in ST3 of the Supplementary Materials). In general, control grapes had the highest flavonol content during the ripening period compared to the treated grapes, except for in the fifth sampling and at harvest time, with the Nano-MeJA-treated grapes obtaining the highest concentration at these two measurement points.

However, the evolution during ripening was different in the control grapes and in the treatments, with the control grapes exhibiting an increase in their flavonol concentration until the sixth sampling and then a decrease in their flavonol content until harvest. Obreque-Slier et al. [54] reported a similar decrease in the amount of flavonols for Cabernet Sauvignon and Carmenere varieties during the ripening period. On the contrary, some authors have reported an increase in the flavonol content in grapes during the ripening period [55,56].

Regarding treatments, grapes treated with MeJA exhibited an increase in their concentration until the sixth sampling, as did the control grapes, but in the case of Ap-MeJA, the concentration only increased until the fourth sampling, and in Nano-MeJA until the fifth sampling. In all cases afterwards, there was a decrease until the time of harvest. In contrast, Gomez-Plaza et al. [26] showed how MeJA treatment only increased the concentration of flavonols in Monastrell grapes during the first few days of the ripening period, whereas in the last three weeks before harvest, the flavonol concentration of MeJA-treated did not differ from that of control grapes.

It is worth noting that although all treatments increased the concentration of flavonols at harvest, MeJA was the treatment which increased it the most (14%), followed by Nano-MeJA (13%) and then Ap-MeJA (9%). Again, a possible explanation for the difference found in the grapes treated with the two types of nanoparticles could be the difference in the morphology between the amorphous and the crystalline nanoparticles, which could modify the interaction with the plant. Other authors such as Ruiz-Garcia et al. [40] found similar results, in which MeJA increased the flavonol content in Monastrell grapes at harvest. In contrast, authors such as Portu et al. [21] did not observe this increase in Tempranillo grapes.

Evolution of Proanthocyanidins during Ripening Period

Proanthocyanidins or condensed tannins accumulate mainly in berry skins and seeds before veraison. Different authors have found that the greatest synthesis of proanthocyanidins takes place after the fruit sec, which occurs a few weeks before veraison, which is when the highest concentration of these compounds is reached [42]. From this moment on, the skin proanthocyanidins decrease slightly or remain stable [42]. In addition, these compounds play an important role in the final red wine quality as they are responsible for different properties such as bitterness, astringency, structure or colour.

In this paper, we will only show the relationship of the results with the skin parameters because it is known that these are considered of higher quality than those from the seeds. Regarding total proanthocyanidins in skins (Figure 5A, numerical data can be seen in ST4 of the Supplementary Materials), in general, a decrease in their total concentration

was observed until the fifth sampling. From this moment until harvest, this decrease was more attenuated.

Regarding treatments, different behaviours were observed throughout their evolution during maturation. In this case, the grapes treated with MeJA obtained the lowest concentrations of tannins in all the samplings, except at the time of harvest, when the values were equal to those obtained in the control grapes. In contrast, Gomez-Plaza et al. [26] found that MeJA-treated grapes had a higher total concentration of proanthocyanidins in the skin than control grapes throughout the ripening period. In addition, Gil-Muñoz et al. [24] showed an increase in skin proanthocyanidin content in MeJA-treated Monastrell grapes during two consecutive seasons, but in Tempranillo grapes this treatment only increased the tannin concentration during the second season. Therefore, it is evident that factors such as season and variety will also influence the obtained results.

Regarding the treatments with nanoparticles, the grapes treated with Nano-MeJA showed lower concentrations of tannins than the control grapes until the fifth sampling. From this moment until the harvest, these grapes obtained the highest concentrations of tannins. Finally, the grapes treated with Ap-MeJA, in general, obtained similar values to those obtained by the control grapes, except in the sixth sampling, which surpassed the control concentration, and at the time of harvest, were they obtained the lowest concentrations of tannins. As in the case with anthocyanins and flavonols, it seems that the different morphology and structure between ACP and Ap nanoparticles induced the grapes treated with Ap-MeJA to obtain the lowest concentrations of proanthocyanidins in the skin at the end of the ripening process. This effect could be associated with the low % MeJA content of Ap-MeJA and the need for higher Ap-MeJA nanoparticle uptake by the plant, as previously discussed. Other authors have addressed the use of chitosan as a conventional dissolution or as nanoparticles in varieties such as Sousao, and showed a decrease in the concentration of tannins in the skin when both were applied [57].

Other parameters such as mDP (mean degree polymerisation), % epigallocatechin and % galloylation were also measured. Regarding mDP (Figure 5C), this parameter was always higher in the skins than in the seeds and is related to the number of monomeric units that are formed by proanthocyanidins, which in the case of skins can be up to 30 [58]. We were able to observe a great variability in the values obtained from the control and treated grapes during the ripening period. Despite that, it is remarkable that the highest values obtained for this parameter were in the second and sixth sampling for the control grapes and the highest value reached by Ap-MeJA was in the third sampling. On the contrary, treated and untreated grapes equalled their values at harvest time, although Ap-MeJA treated grapes slightly increased their value compared to control grapes. In contrast, authors such as Ruiz-García et al. [40] have found that the mDP values were higher in Monastrell grapes treated with MeJA during 2009–2010 vintages, although the differences were not significant in 2009. In addition, Gil-Muñoz et al. [24] showed that there were higher levels of mDP in control grapes than in MeJ-treated grapes.

Regarding the % galloylation (Figure 5D), this parameter is higher in seeds than in skins and the evolution in treated and untreated grapes during the ripening period was constant although there were some differences. These values ranged between 1 and 1.5% for all treatments, but we can observe from the first sampling to the sampling before harvest that the control grapes obtained the highest values. Otherwise, during the three last samplings, the lowest percentages were obtained by Ap-MeJA treatment. At the time of harvest, the values were similar for the control grapes (1.26%), for the grapes treated with MeJA (1.24%) and for the grapes treated with Nano-MeJA (1.35%), although the lowest values were obtained for the grapes treated with Ap-MeJA (0.98%). On the contrary, other authors such as Gil-Muñoz et al. [28] did not find differences regarding % of galloylation in MeJA-treated and control grapes in the Monastrell variety.

Finally, the % epigallocatechin (Figure 5B) was always lower in grapes treated with MeJA. These results could be explained due to the climatological conditions. Authors such as Gil-Muñoz et al. [19] also showed a decrease in this parameter in Monastrell and

Tempranillo grapes during two consecutive seasons. For the treatments with nanoparticles, when Nano-MeJA was applied, from the third sampling and until the sampling just before the harvest, the percentage increased, whereas when Ap-MeJA was applied, we could observe an increase in this parameter during the first sampling and at the time of harvest, when it obtained the highest percentage (29.2%). This compound is only present in skin and is related to preferable tannins, which is why it is considered softer and of higher quality. On the other hand, the presence of the trihydroxylated flavan-3-ol subunit, epigallocatechin, may reduce the coarse perception of astringency [59] and studies on quality grading of young red wines have indicated that the greater the proportions of skin-derived tannin subunits in the wine, the higher the perception of quality.

Multivariable Discriminant Analysis

Discriminant analysis was used to check whether we could classify our samples according to the applied treatments with the measured variables. Three discriminant functions with a p-value lower than 0.05 were calculated and therefore were statistically significant with a confidence level of 95%. These three discriminant functions allowed us to correctly classify 100% of the samples according to the treatment applied (Figure 6). The relative percentage for function 1 was 84.1% and 15.2% for function 2. As can be seen, the separation of the three applied treatments (MeJA, Nano-MeJA and Ap-MeJA) regarding the control samples was good (Figure 6). The MeJA treatment was in the left part of the graph and was the treatment furthest from the control, indicating the greatest differences with respect to the control. The treatments carried out with the nanoparticles (Nano-MeJA and Ap-MeJA) were in the middle of the graph at the top, thus resulting in intermediate values between those found in the control and the treatment with MeJA. In the graph, it was not possible to distinguish between Nano-MeJA and Ap-MeJA, although both treatments could be distinguished from the control, indicating that although we found some differences in the results in the paper, these differences were not so evident so as to distinguish between both morphologies. As can be observed, the Ap-MeJA treatment is a little closer to the control and may suggest less differences with respect to it. In addition, Nano-MeJA is closer to MeJA. These results are logical, since when we applied these treatments, we were using a ten times lower concentration of MeJA than in the conventional form.

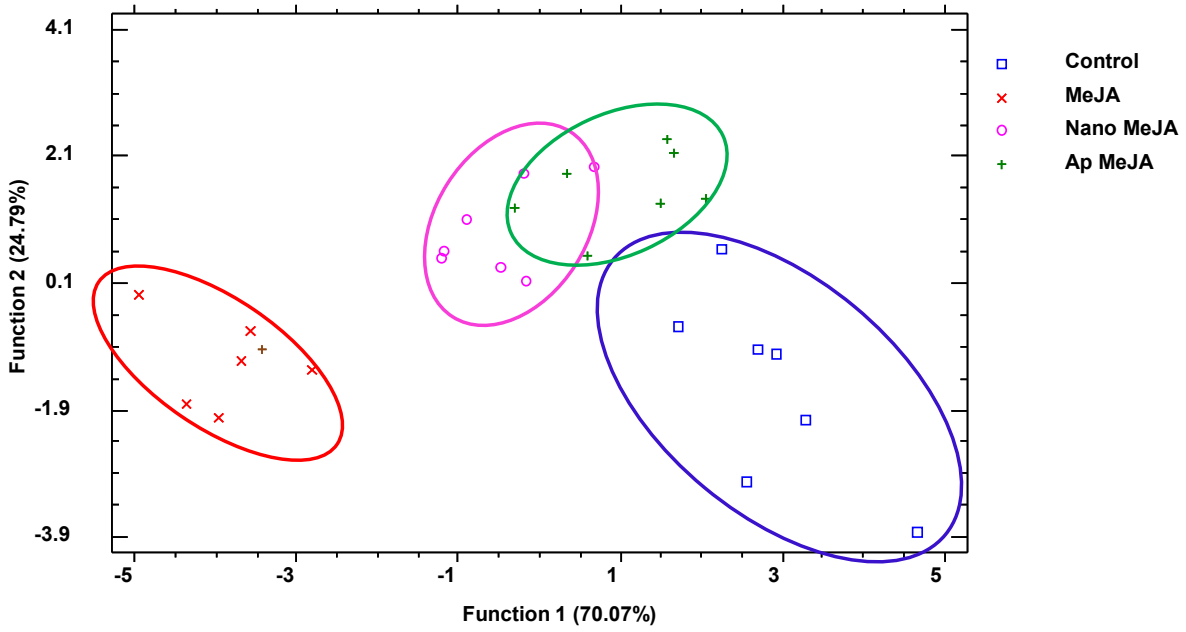

Figure 6. Representation of the first two discriminant functions separating the samples according to the treatments applied in vineyards.

The standardised coefficients of the functions (Table 1) were used to discriminate between the different treatments. From the different magnitude of these coefficients, it can be determined how the independent variables are used to discriminate between groups. The variables with the highest discriminatory power for function 1 were °Brix, total acidity, total anthocyanins and coumarates anthocyanins, and for function 2, they were total anthocyanins, acetates anthocyanins, total acidity and pH.

Table 1. Standardised coefficients of the discriminant functions.

	Function 1	Function 2
°Brix	8.32	−1.36
Total acidity (mg/L)	6.52	−5.09
pH	−1.81	−3.80
Total anthocyanins (μg/g skins)	−7.04	−7.42
Acetylated anthocyanins (μg/g skins)	−2.35	7.35
Coumarated anthocyanins (μg/g skins)	8.81	−0.78
Total tannins (μg/g skins)	−0.06	−1.14
%Epigallocatechin	−0.31	1.54
mDP	0.70	−0.64
% galloylation	−0.16	0.06
Total flavonols (μg/g skins)	0.60	−0.75

4. Conclusions

The effect of treatment with methyl jasmonate (MeJA) applied at veraison in a conventional way or in combination with two different calcium phosphate nanoparticles (Ap and ACP) on the ripening process and fruit quality parameters at harvest were evaluated for the first time in the Monastrell variety.

Regarding the physicochemical parameters of the treatments, the results have shown that MeJA treatments are able to delay the ripening process, although the lowest sugar content was reached in Ap-MeJA-treated grapes. It should be noted that this fact could be interesting in warm areas such as southeast of Spain, in order to decrease the decoupling between technological and phenolic maturity. Regarding berry weight, grapes treated with nanoparticles showed the lowest values compared to control samples, although no differences were found between the two nanoparticle treatments (Nano-MeJA and Ap-MeJA).

Regarding phenolic composition, anthocyanin content increased with all treatments, although MeJA applied in the conventional form was the treatment that increased the concentration of total, acylated and acetate anthocyanins in the grapes by the highest percentage. Flavonol content also increased with the treatments, although the lowest concentration was found in grapes treated with Ap-MeJA. Finally, the highest concentration of tannins was observed in grapes treated with Nano-MeJA.

Therefore, although MeJA applied in the conventional way increased the majority of the parameters measured, the use of nanoparticles is a potential strategy to increase the quality of grapes while reducing MeJA dosage towards a more sustainable agriculture. On the other hand, a priori, both morphologies could be used, but we must account for the fact that the crystalline form will need a higher quantity of nanoparticles to achieve the same percentage of MeJA as when using amorphous nanoparticles.

Finally, more studies must be carried out in the future to optimise the concentration of MeJ nanoparticles in order to get the same results as when MeJA is applied in a conventional way.

5. Patents

Calcium phosphate nanoparticles loaded with jasmonate to induce efficient plant defence responses. Patent PCT. Number: PCT/EP21382662.1. 21 July 2021.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app13031906/s1, Table S1: Physicochemical parameters (°Brix, total acidity, pH and berry weight) in grapes during the ripening period; Table S2: Total anthocyanins, acylated anthocyanins, acetate anthocyanins and coumarate anthocyanins in grapes during the ripening period (expressed as μg/g skin).; Table S3: Total flavonols in grapes during the ripening period (expressed as μg/g skin); Table S4: Total skin proanthocyanidins (expressed as μg/g skin), % epigallocatechin, mDP and % galloylation in skins during ripening period.

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