The Effects of Ultra-high Pressure Treatment on the Phenolic Composition of Red Wine

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Wine is usually aged in oak barrels. In this study, young red wines were treated with ultra-high pressure (UHP) to stimulate the ageing process. Changes in phenolic acids, flavan-3-ols and proanthocyanidins were determined by reverse-phase high pressure liquid chromatography (RP-HPLC). The concentration of phenolic acids increased, while the levels of flavan-3-ols decreased. The content and structure of proanthocyanidins also changed and the tendency was similar to that of natural ageing.

INTRODUCTION

Ultra-high pressure (UHP), or high hydrostatic pressure (HHP), processing refers to a method in which food is sealed in containers or placed in water or other liquids under pressure (100 MPa) to sterilise and inactivate enzymes or to change the functional properties of a product (Chen, 2005; Zhang *et al*., 2008b). Under ultra-high pressure, the volume of the product is compressed, which results in the deeper penetration of proteins and other macromolecules into the product, resulting in the destruction of its three-dimensional structure. Thus, UHP not only affects cell morphology, but also changes the hydrogen, ionic and hydrophobic bonds in the three-dimensional structure of biological material. This may, for instance, lead to starch and protein denaturation and enzyme inactivation, all of which may have an effect on the texture of the product. Pressure is instantly and evenly applied to the product, regardless of its size, shape and volume (Bian, 2009). Hite and co-workers have shown that the shelf life of milk, fruits, vegetables and other foods could be prolonged by UHP treatment. Proteins coagulate at 500 MPa and form a gel at 700 MPa, which also destroys microorganisms (Bridgman, 1914).

In Japan, UHP technology was applied for the first time in 1986, at the University of Tokyo (Kinugasa *et al*., 1993). In 1991, the first ultra-pressure products (jam) were introduced into the market, followed by fruit juice and other products. A number of studies on UHP have been performed in Germany and the United States, and many different products have been introduced into the market. In China, UHP technology is focused mainly on food sterilisation and macromolecular degeneration. Litchi juice treated with UHP was shown to have a longer shelf life and to retain its nutritional value (Huang *et al*., 2007). The treatment of pineapple juice at 400 MPa for 10 min resulted in improved organoleptic quality (Li *et al*., 2010a). In sauerkraut, the total number of bacteria was significantly reduced when treated at 600 MPa. *Lactobacillus* spp. were killed and the shelf life of sauerkraut was increased (Li, 2010c). The number of microorganisms and the degree of browning in watermelon juice was reduced after UHP treatment, without there being an effect on the flavour profile (Liu, 2010).

Wine is traditionally aged in oak barrels, which is a lengthy process. This process has been simulated by using high-voltage pulsed electric field ageing, electromagnetic field ageing and microwave ageing (Liu *et al*., 2006). Some studies have shown that the treatment of wine with UHP results in physical and chemical changes, including changes observed in the ultraviolet and visible spectrum, with an overall improvement in quality (Li, 2005). The method accelerated wine ageing (Liu *et al*., 2006). Thus far, little research has been conducted on the UHP treatment of wine.

Phenolic compounds play an important role in the quality of red wine, as they contribute to certain sensory characteristics, particularly colour and astringency. Polyphenols in wine are strong antioxidants and have health benefits. During the ageing of wine, phenols play an important role in wine colour and taste. Thus, specific phenols can be used to follow the ageing process in wine (Minussi *et al*., 2003; Revilla & González-SanJosé, 2003; Proestos *et al*., 2005; Monagas & Garmen, 2006). The phenols are divided into non-flavonoids and flavonoids. Non-flavonoids are mainly phenolic acids, mostly derivatives of hydroxybenzoic acid and hydroxycinnamic acid. Flavonoids include flavanols, flavonols, anthocyanins and proanthocyanidins. In this paper, the effects of different UHP treatments on the phenolic compounds in wine have been studied.

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MATERIALS AND METHODS

Equipment and reagents

Liquid chromatography was performed using the WATERS-2695XE HHP-700-6 high hydrostatic PATS device and the WATERS Empower Software chromatography data handling system. Methanol and acetonitrile were of HPLC grade (Fisher), and all the remaining reagents were of analytical grade. Catechin [(+)-catechin, CAT], epicatechin [(-)-epicatechin, EC], epigallocatechin [(-)-epigallocatechin, EGC], epicatechin gallate [(-)-epicatechin gallate, ECG] and epigallocatechin gallate [(-)-epigallocatechin gallate, EGCG] were used as flavan-3-ol standards. Gallic acid, protocatechuic acid, p-hydroxy benzoic acid, syringic acid, chlorogenic acid, caffeic acid, coumaric acid, erucic acid, ferulic acid, vanillic acid and gentisic acid were used as phenolic standards. All standards were from Sigma (Sigma Aldrich 3050, Spruce St. St. Louis, Mo 63103) and of 95% and higher purity.

Detection of phenolic acids

Phenolic acids were detected by using a 100RP-18e column (250 mm × 4.0 mm, inner diameter 5 μm) from Merck LiChrospher (Darmstadt, Germany) and a guard column RP-18 (10 mm × 4 mm) from Merck (Darmstadt, Germany). For the first 11 to 15 min the wave length was set at 320 nm, while it was set at 280 nm for the rest of the run. The column temperature was set at 30°C and 10 μL was injected. External standards were used to detect the peak area. Mobile phase A was methanol, acetic acid and water (10:2:88), and mobile phase B was methanol, acetic acid and water (90:2:8). The flow rate was 1 mL/min. The gradient elution was 0 to 25 min with phase B from 0% to 15%; 25 to 45 min with phase B 15% to 50%; and 45 to 53 min with phase B 50% to 0%. Samples were filtered through a 0.45 μm Millipore membrane before injection.

Detection of flavan-3-ols

Detection was with a 100RP-18e column (250 mm × 4.0 mm, inner diameter 5 μm) from Merck LiChrospher (Darmstadt, Germany) and a guard column RP-18 (10 mm × 4 mm) from Merck. The wavelength used was 280 nm, the column temperature 30°C, and the injection volume was 10 μL. External standards were used to detect the peak area. Mobile phase A was water and mobile phase B was 10% glacial acetic acid (90/10). The flow rate was 1 mL/min. The gradient elution was 0 to 20 min with B 7.5% to 65%; 20 to 30 min with B 65% to 80%; 30 to 48 min with B 80% to 90%; 48 to 55 min with B 90%; and 55 to 63 min with B 7.5%. Samples were filtered through a 0.45 μm Millipore membrane before injection.

Detection of proanthocyanidins

The method of Pekić *et al*. (1998) was used for the detection of proanthocyanidins. Solution A was 30% (v/v) H_2SO_4/methanol and solution B was 1% (w/v) vanilla aldehyde/methanol. The solutions were mixed rapidly and used at a ratio of 1:1. A 0.2 mL sample was added to 6.0 mL vanillin and the absorbance was measured at 510 nm after 5 min. A standard curve was prepared with catechin, using the same protocol.

Separation and purification of proanthocyanidins

Wine (10 mL) was injected into the TSK HW-50 (F) column (18 mm × 250 mm) and eluted with 90 mL ethanol/water/TFA (55/45/0.05) and 50 mL acetone/water (60/40, containing 1g/L of vitamin C, Vc). The flow rate was 1.5 mL/min. The samples were freeze-dried and stored at 20 C.

Degradation of proanthocyanidins

The freeze-dried samples were dissolved in 2.5 mL methanol (HPLC). One 1 mL was added to 0.5 mL 0.2 N HCl/MeOH (containing 20 g/L ascorbic acid) and 0.5 mL 100g/L phloroglucinol. This was heated to 50°C for 20 min and then added to 40 mM b-sodium to terminate the reaction. Samples were stored at -20°C.

Chromatography conditions to detect proanthocyanidins degradation

A 100RP-18e column (250 mm × 4.0 mm, inner diameter 5 μm) from Merck LiChrospher (Darmstadt, Germany) and a guard column RP-18 (10 mm × 4 mm) from Merck (Darmstadt, Germany) were used. Detection was at 280 nm, the column temperature was 30°C and the flow rate 1.5 mL/min. The injection volume was 10 μL. A standard curve was prepared with catechin, prepared using the same protocol. Mobile phase A was 1% (v/v) acetic acid and mobile phase B was 80% acetonitrile (1%, v/v, acetic acid). The following gradient was applied: elution 0 to 8 min with 3.8% B; 8 to 28 min with 3.8 to 22.5% B; 28 to 32 min with 22.5 to 50% B; 32 to 50 min with 50 to 100% B; 50 to 52 min with 100% B; and 52 to 60 min with 3.8% B. Samples were filtered through a 0.45 μm Millipore membrane before injection.

Standard curves of 11 phenolic acids

Standard samples of 10 different phenolic acids were prepared, as shown if Fig. 1. The peak order is gallic acid at 4.904 min; protocatechuic acid at 9.332 min; P-hydroxy benzoic acid at 15.788 min; chlorogenic acid at 20.102 min; vanillic acid at 21.508 min; caffeic acid at 22.656 min; syringic acid at 26.358 min; p-coumaric acid at 33.232 min; ferulic acid at 36.714 min; and erucic acid at 37.792 min. Fig. 2 shows gentisate at 13.743 min.

Standard working curves of five flavan-3-ols

The samples are presented in Fig. 3. The peak order is as follows: (+)-catechin at 22.866 min; (-)-epigallocatechin at 23.823 min; (-)-epigallocatechin gallate at 31.611 min; (-)-epicatechin at 35.925 min; (-)-epicatechin gallate at 50.913 min.

Standard curve of proanthocyanidin degradation

The proanthocyanidin standard curve is shown in Fig. 4. The peak order is as follows: CAT, 16.298 min; EC 21.750 min; ECG 28.152 min. Other degradation products were qualitatively analysed as described by Pengfei (2005).

Preparation of wine samples

The samples were wine from Xinjiang's 2009 Cabernet Sauvignon. The first group of wine was treated at 100 MPa, 200 MPa, 300 MPa, 400 MPa, 500 MPa and 600 MPa for 30 min. The second group of wine was treated for 5 min, 10

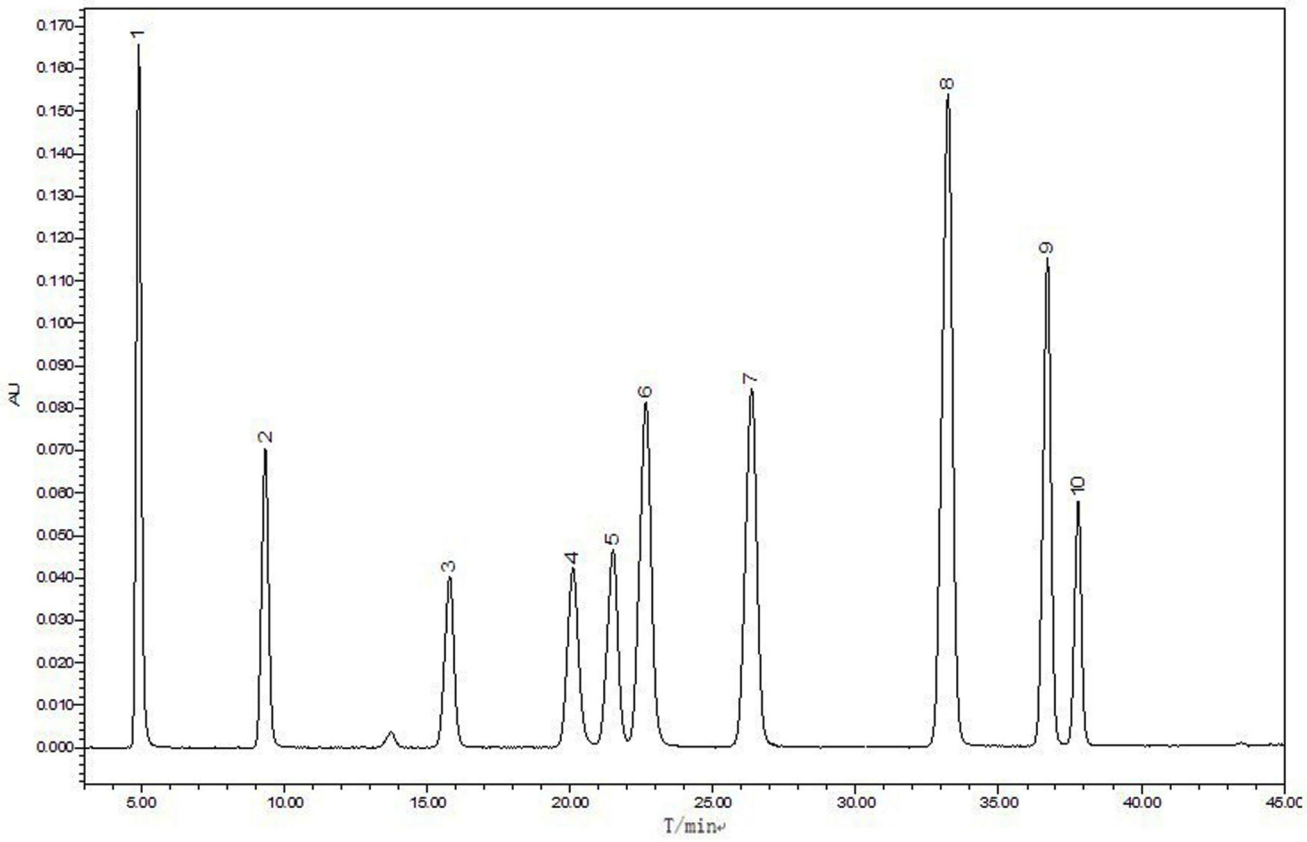

FIGURE 1
Chromatography of 10 phenolic acid standards

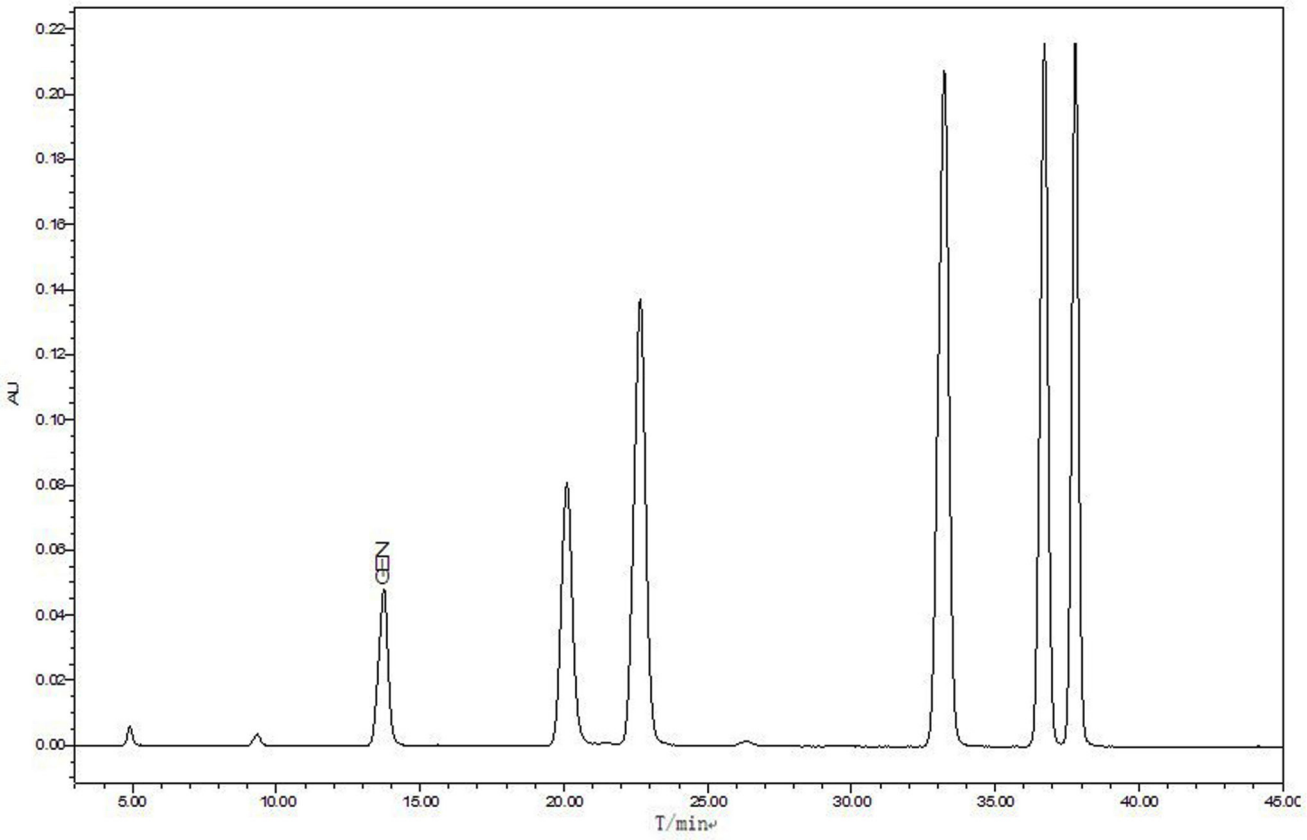

FIGURE 2
Chromatography of gentisic acid standards

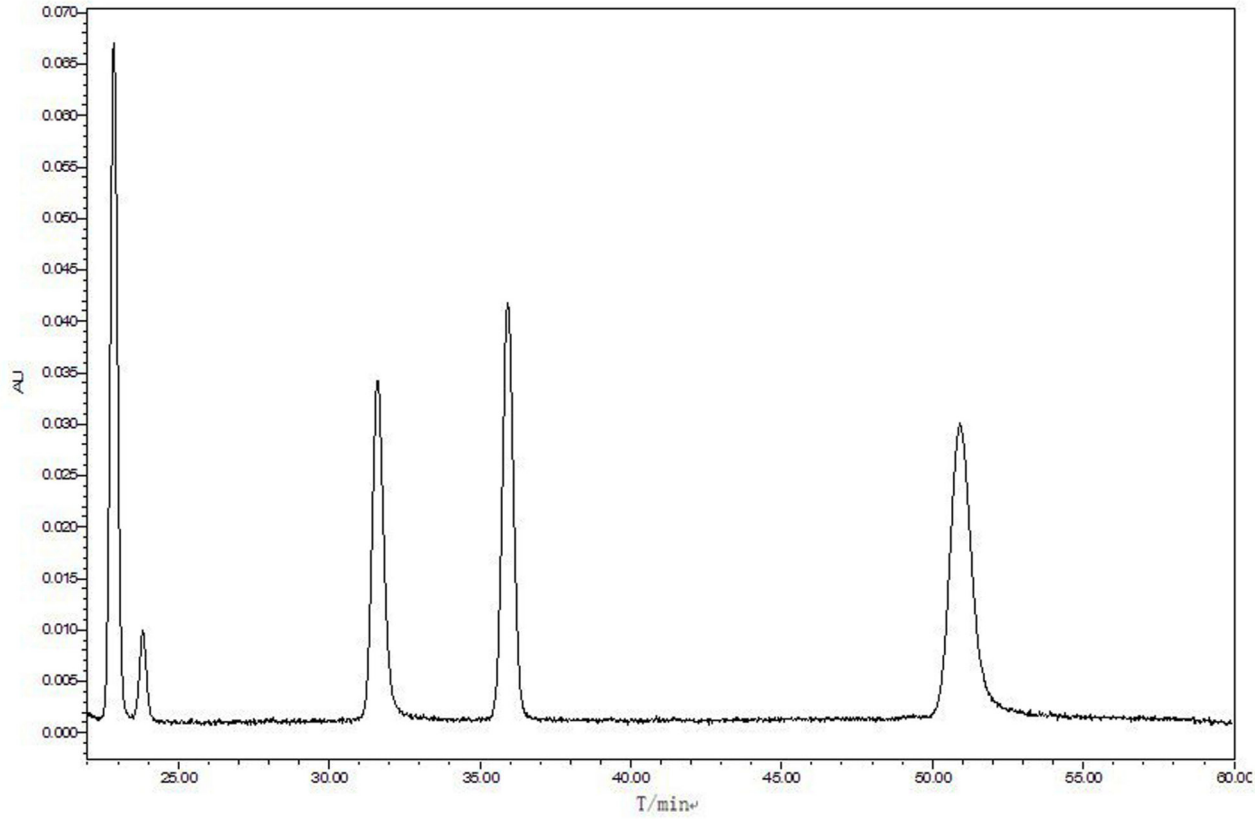

FIGURE 3
Chromatography of five flavan-3-ol standards

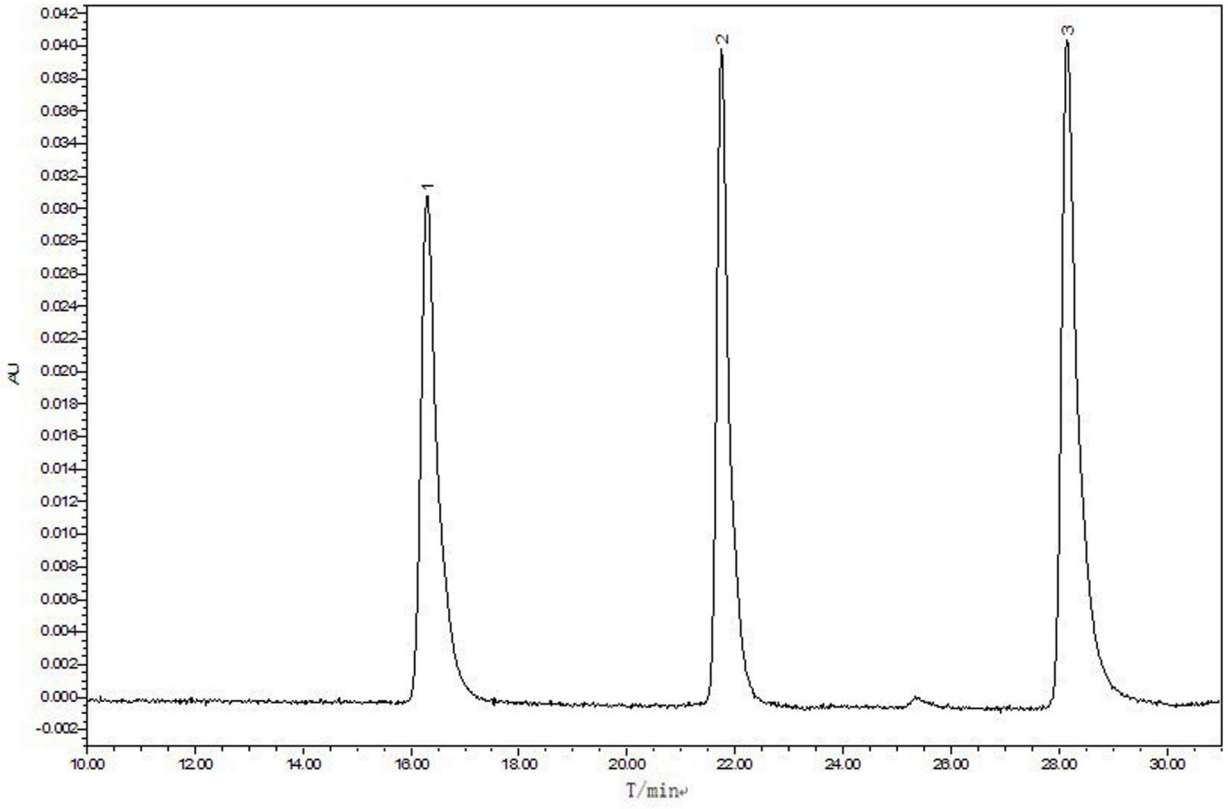

FIGURE 4
Chromatogram of three flavanol-ol standards

min, 20 min, 30 min, 45 min and 60 min at 500 MPa. All samples were filtered through a 0.45 μm organic Millipore filter (Merck Millipore, 290 Concord Road, Billerica, MA, USA). Tests were repeated in triplicate and the results were analysed by Sigmaplot 11.0 and SPSS 17.0.

RESULTS AND DISCUSSION

Effects of UHP treatment on phenolic acids in young red wine

The content of total phenolic acids in the wine after UHP treatment is shown in Fig. 5. At a pressure of 100 MPa, the total content of phenolic acids increased rapidly and reached its highest point at a pressure of 200 MPa. After this point, the total content of phenolic acids declined as the pressure increased to 400 MPa. No drastic changes were recorded at pressures higher that 400 MPa. Treatment at 500 MPa for 5 min resulted in an increase in the total phenolic content, followed by small fluctuations as time continued. The phenolic content stabilised after 30 min.

The 11 phenolic acids changed with the range of pressures and treatment times ($P < 0.05$; Fig. 6). Besides P-coumaric acid, ferulic acid changed to a lesser extent, and the ranges of the other nine phenolic acids were similar to an extent, but the trends varied greatly. Gallic acid content reached its highest level at 100 MPa, followed by mild fluctuations. The remaining phenolic acids also reached a maximum at 100 MPa or 200 MPa, followed by slight fluctuations.

With the treatment time prolonged, the content of the other 10 phenolic acids, in addition to gentisate, changed significantly ($P < 0.05$). Gallic acid and syringic acid, which were present at a higher concentration, changed the most. Chlorogenic acid, caffeic acid and sinapic acid also showed a more significant change, while P-coumaric acid and ferulic acid did not change that much. Among the 11 phenolic acids, the gallic acid content was the highest, as observed in previous studies (Minussi *et al*., 2003). At processing for 5 to 10 min, the concentration of gallic acid and syringic acid increased substantially, and as the time increased to 30 min, they gradually dropped to levels close to those recorded before processing. The levels of chlorogenic acid increased rapidly after 5 min, followed by a stable increase.

Many factors affect the phenolic content of wine, including grape variety and maturity (Recamales *et al*., 2006). The fermentation process also affects the phenolic content, such as length of soaking time and fermentation temperature. Studies have shown that, if wine ferments at low temperatures, the levels of syringic acid, coumaric acid, sinapic acid and gentisic acid increase (Ramos *et al*., 1999). During the wine fermentation and ageing process, phenolic polymerisation and oxidation reactions occur and the complexity changes. In wine, phenolic acids can combine with anthocyanins and tartaric acid, such as p-coumaric acid and caffeic acid. The combination of the latter may lead to the formation of tartar coumaric acid and tartaric caffeic acid. The hydrolysis of these compounds produces free phenolic acids. Gallic tannins are hydrolysed to gallic acid, especially during the ageing process. Microorganisms participate in the metabolism of hydroxyl cinnamic acid to generate volatile phenols. After malolactic fermentation, the content of caffeic acid, tartaric acid esters and P-coumaric acid decreases, whilst the corresponding free acid content increases. During oak ageing, tannins are hydrolysed to generate ellagic acid, resulting in an increase in ellagic acid levels. At the same time, gentisic acid and caffeic acid levels also increase (Ramos *et al*., 1999; Lee *et al*.. 2002). After UHP treatment, the complexity of the phenolic acids changed, but with an overall upward trend, possibly because of the pressure that promoted the decomposition of certain compounds, which led to the content of the corresponding phenolic acids increasing. However, when the pressure was above 200 MPa, the phenolic acid content declined, probably because oxidation stepped in at the higher pressure.

The effect of UHP treatment on flavan-3-ols in young red wine

The change in the total content of flavan-3-ols in the wine is shown in Fig. 7. As the pressure increased, the content of the flavan-3-ol decreased. The flavan-3-ol levels increased slightly at 100 MPa, and then decreased with the increase in handling pressure, especially at 500 MPa and 600 MPa. The content of flavan-3-ol dropped slightly at 5 min, and then increased to a maximum at 10 min. The lowest point was

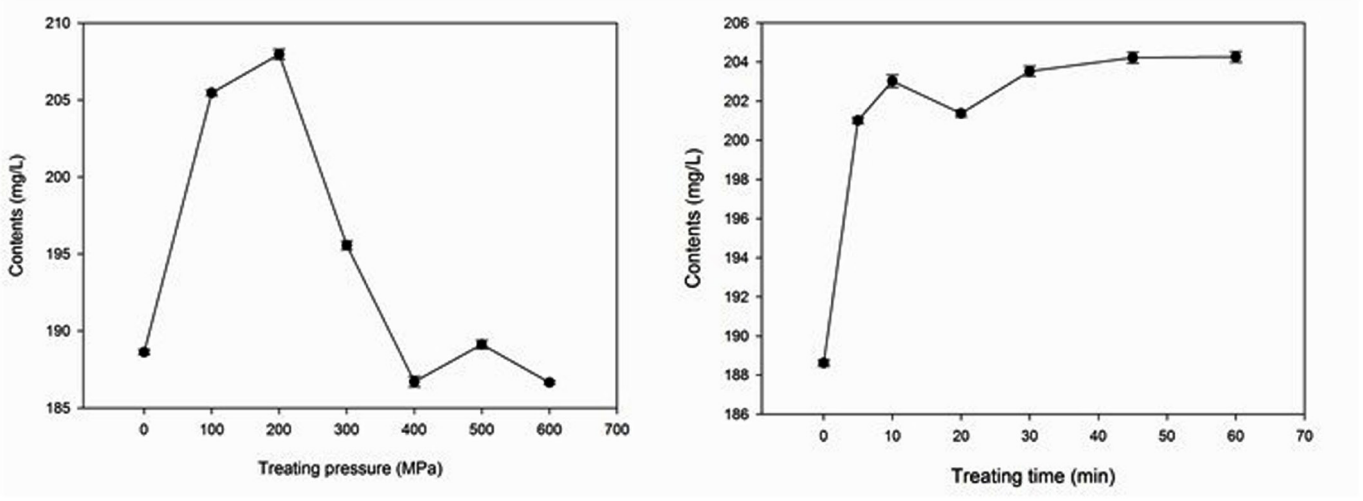

FIGURE 5
Changes in total phenolic acids in red wine treated by UHP

reached at 45 min.

Four of the flavan-3-ols showed significant differences ($P < 0.05$) under different treatment conditions (Fig. 8). With the pressure increasing, the CAT content fluctuated and declined at 100 MPa and 200 MPa. At 400 MPa, the content of EGC, EGCG and EC decreased as the pressure increased. At 500 MPa, with prolonged treatment, the four levels of the flavan-3-ols decreased, with the content of CAT at its lowest at 45 min. The levels recovered at 60 min; EGC and EC showed a tendency to first increase and then decrease. The content of EGCG decreased at 10 min and remained at his level.

CAT and EC are the main flavan-3-ol compounds in wine. ECG cannot be detected in wine samples. Wen *et al*. (2006) studied the impact of oak on the flavan-3-ols and found that ECG can only be detected later in ageing. Flavan-3-ols have a taste of bitterness and astringent. During storage, flavan-3-ols can participate in, or mediate, procyanidin condensation, and combine with anthocyanins to generate polymeric pigments, thus changing the colour of the wine. During ageing, with the help of the acetaldehyde, flavan-3-ols can undergo intense chemical reactions, such as condensation between anthocyanins, flavanols, anthocyanins and flavanols, or they can condensate with anthocyanins without acetaldehyde. Micro-oxygen could cause a slight oxidation of the flavan-3-ols. Flavan-3-ols may also be involved in the decomposition and polymerisation of proanthocyanidins and promote the polymerisation of proanthocyanidins (Vidal *et al*., 2002). At the same time, proanthocyanidins may release free flavan-3-ols. UHP treatment may contribute to the polymerisation reactions, with a decrease in flavan-3-ol content.

Effect of UHP treatment on the content of proanthocyanidins

The changes in the content of proanthocyanidins after UHP treatment are shown in Fig. 9. As the pressure increased, the proanthocyanidin levels increased. At 100 MPa, the levels went down slightly, and then increased substantially at 200 MPa. At 300 MPa and 400 MPa a slightly decline was observed. The proanthocyanidin levels showed an upward

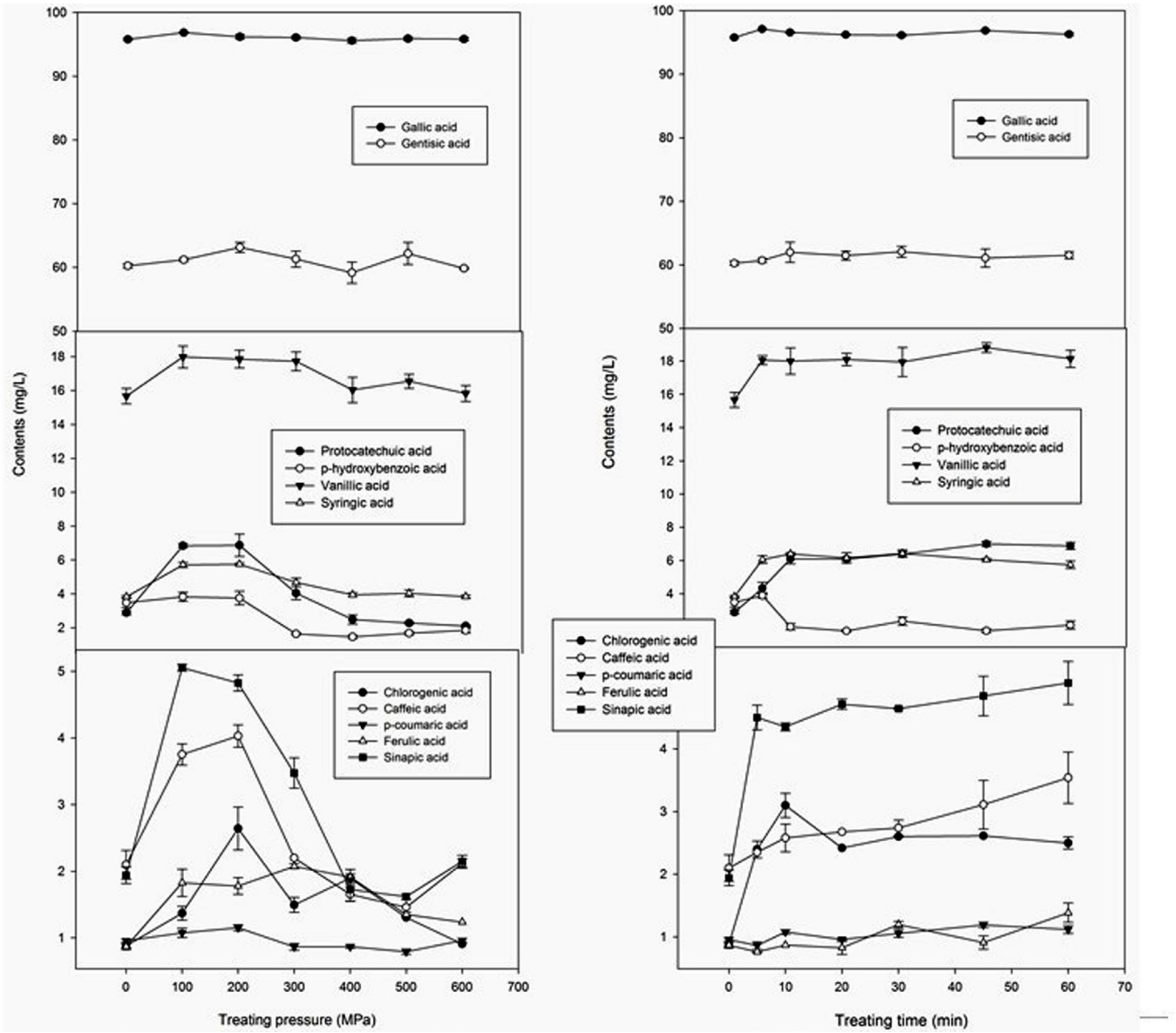

FIGURE 6
Changes in 11 phenolic acids in red wine treated by UHP

tide as treatment continued, but lowered slightly at 10 min. Thereafter the content gradually increased, reached the maximum at 45 min, and decreased at 60 min.

The basic structural unit of proanthocyanidins is flavan-3-ol, which plays an important role in the sensory quality of wine. Proanthocyanidins undergo complex reactions during wine storage and ageing. Studies have shown that the content of anthocyanins first increases rapidly, and then declines slowly during bottle storage (Su, 2008). This may be because flavan-3-ol condenses during ageing, thus

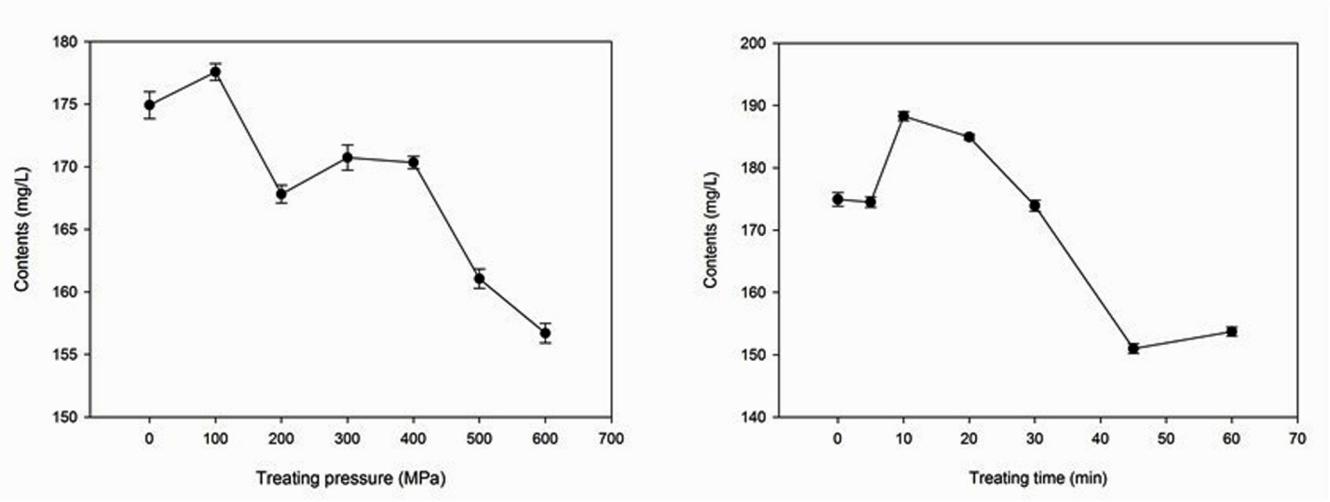

FIGURE 7
Changes in total flavan-3-ols in red wine treated by UHP

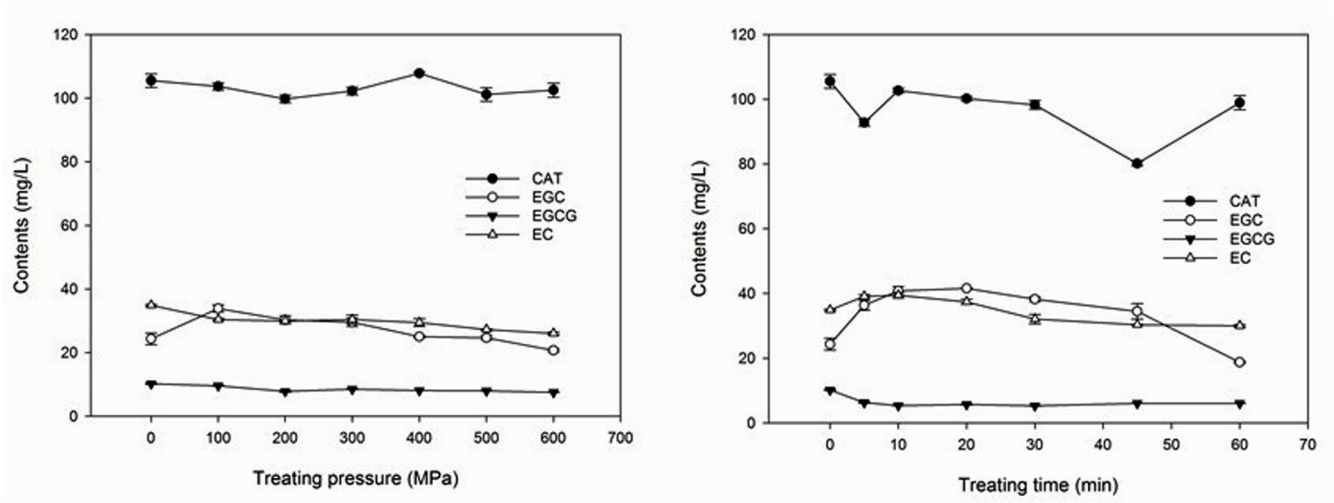

FIGURE 8
Changes in four flavan-3-ols in red wine treated by UHP

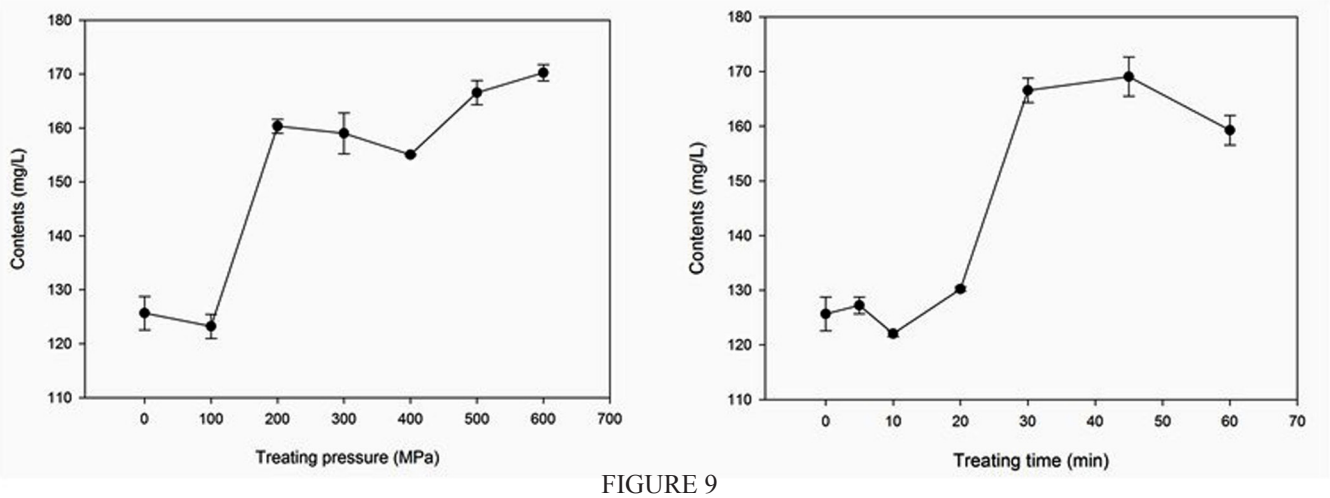

FIGURE 9
Changes in proanthocyanidins in red wine treated by UHP

increasing the concentration of proanthocyanidins. Another study found that proanthocyanidins decrease after oak ageing, especially in European oak. Proanthocyanidins can condensate to generate high-molecular compounds such as tannin analogues (Guadalupe & Ayestarán, 2008) with polysaccharides, proteins, etc. This will lead to a reduced content of proanthocyanidins.

With increasing pressure, the content of proanthocyanidins increased, probably because the pressure narrowed the intermolecular distance while providing

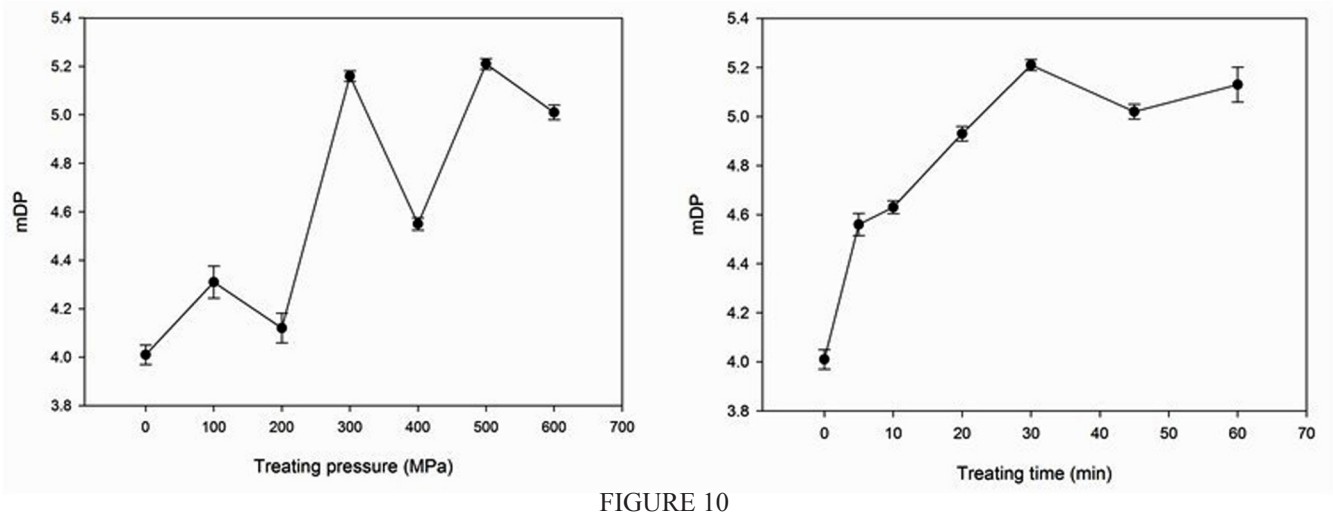

FIGURE 10
Changes in ADP of proanthocyanidins in red wine treated by UHP

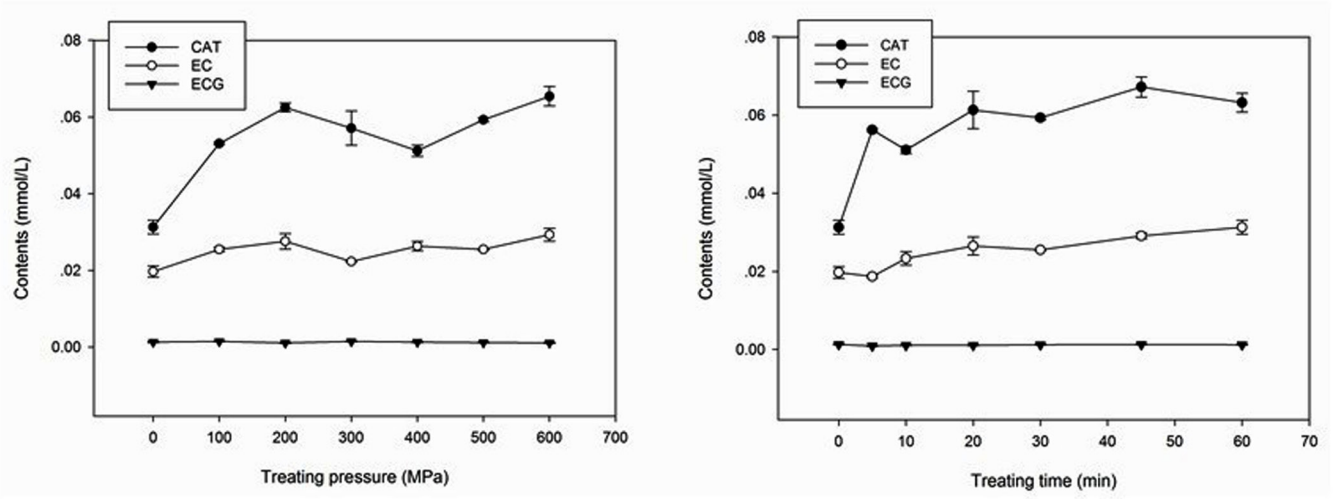

FIGURE 11
Changes in starting units of proanthocyanidins in red wine treated by UHP

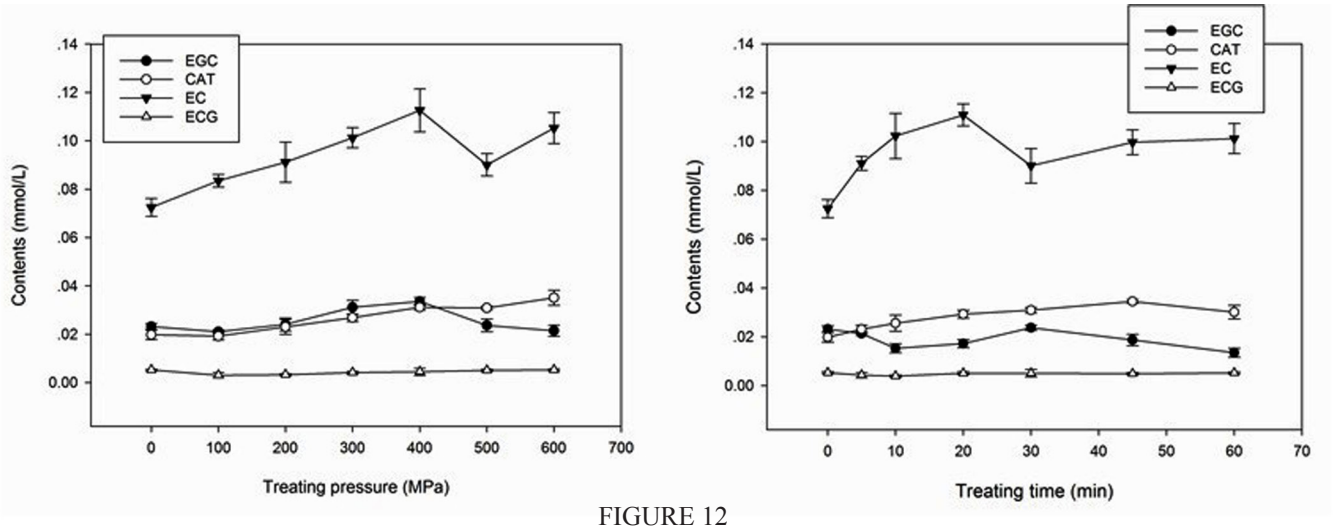

FIGURE 12
Changes in extension units of proanthocyanidins in red wine treated by UHP

energy, which is beneficial to the structural unit of the condensation reaction. On the other hand, the high-pressure conditions can also affect the condensation reaction between proanthocyanidins and other macromolecules. Thus, under certain stress conditions, the content of proanthocyanidins decreased slightly. On the whole, the content dropped. As treatment was prolonged, the proanthocyanin levels also showed a clear upward trend, indicating that continuing pressure is conducive to the formation of proanthocyanins. At 10 min the content tailed off slightly, probably because different processing times influence the condensation reactions that proanthocyanidins are involved in.

Effect of UHP treatment on the average degree of polymerisation of proanthocyanidins

Treatment under different conditions and changes in the average degree of polymerisation of proanthocyanidins is are shown in Fig. 10. With an increase in pressure, the average degree of polymerisation showed an upward tendency, similar to a wave effect. At 100M Pa, the degree of polymerisation increased, then decreased slightly. A significant increase was observed at 300 MPa, after which it fluctuated. The average degree of polymerisation of proanthocyanidins showed a clear upward trend as treatment continued, reaching a peak at 30 min. This was followed by a slight decrease.

During the ageing process, proanthocyanidins react with anthocyanins and precipitate, while the flavan-3-ol can combine with oligomer proanthocyanidins to generate poly-proanthocyanidins in the presence of acetaldehyde (Vidal *et al*., 2004). On the other hand, proanthocyanidins may be subjected to reaction with nucleophile agents (e.g. EC) and generate oligomeric proanthocyanidins. The two reactions happen at the same time; the dynamic equilibrium process leads to the volatility of the average degree of polymerisation of proanthocyanidins.

With the increase of pressure, the average degree of polymerisation of the proanthocyanidins fluctuated and changed, but showed an overall upward trend. This may be because high pressure can promote some related reactions, similar to what happens in the ageing process. As treatment increased, polymerisation increased significantly, illustrating that a sustained increase in pressure can promote the polymerisation reaction to generate poly- proanthocyanidins.

Effect of UHP treatment on proanthocyanidins

Anthocyanins also undergo a change after UHP treatment (Fig. 11). With an increase in pressure, the content of CAT and EC changed, while ECG itself had a lower level at the start. At 100 MPa and 200 MPa, the content of CAT and EC increased and then decreased when the pressure increased to above 400 MPa. The content of CAT first increased, declined slightly at 10 min, and then increased. The content of EC decreased slightly at first, but then gradually increased as treatment prolonged. The content of ECG did not change significantly.

During ageing, the composition of proanthocyanidins also changed dramatically. Proanthocyanidins and macromolecules can polymerise, resulting in a decrease in initial concentrations. Flavan-3-ol monomers condensate to generate the proanthocyanidins with acetaldehyde. The processes reach a dynamic equilibrium during ageing.

After pressure treatment, the content of CAT and EC increased. With prolonged pressure a new dynamic equilibrium formed. High pressure may be more conducive to promote reactions and can increase the condensation of flavan-3-ol and the cleavage of proanthocyanidins, leading to an increase in the CAT and EC content. The increase in EC concentration was more obvious when the processing time was longer than 30 min.

Effect of UHP treatment on the extension units of proanthocyanidins

The changes in the extension units of proanthocyanidins after pressure treatment are shown in Fig. 12. With the pressure increasing, the content of EC and CAT showed an upward trend. While the content of EGC first went up and then decreased, the change was less obvious. The highest level of EC was recorded at 400 MPa, but it decreased at 500 MPa. As time passed, the content of EC showed an "up-down-up" trend. This was more obvious when the processing time was less than 20 min. EC is the main "extension unit", and has the highest concentration, followed by CAT and EGC. Similar to the starting unit, the content of the "extension unit" also shows changes in volatility. After bottle storage, the content of the EC and CAT "extension units" in proanthocyanidins increased (Su, 2009).

After the UHP treatment, the content of EC and CAT increased, possibly because the high pressure promoted EC and CAT to participate in the polymerisation of proanthocyanidins, which generated an "extension unit". The content of EC increased, although it fluctuated. The content of EGC first increased and then decreased, but ECG showed no significant changes due to its low level. With an increase in time, the content of EC and CAT increased, illustrating that long periods at high pressure are conducive to promote EC and CA to participate in the formation of proanthocyanidins as structural units.

CONCLUSION

After UHP treatment, the polyphenol levels in wine changed dramatically. The content changes of 11 phenolic acids were totally different, but showed an overall upward trend, possibly because the pressure promoted the decomposition of some compounds. This led to an increase in the content of the corresponding phenolic acids. The content of flavan-3-ols declined, while the content of proanthocyanidins increased. The concentration of the "initial unit" and "extension unit" also increased, probably because high-pressure conditions promoted the formation of proanthocyanidins, which use flavan-3-ols as structural units. At the same time, UHP influenced the polymerisation and cleavage reactions that proanthocyanidins are involved in. Thus, the average degree of polymerisation of the proanthocyanidins and the content of the "composition unit" showed similar fluctuations, just like the natural changes during the ageing process. The effect of UHP on wine compounds is rather complex and more in-depth research will have to be done to attain a theoretical basis for changes taking place during artificial ageing.

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