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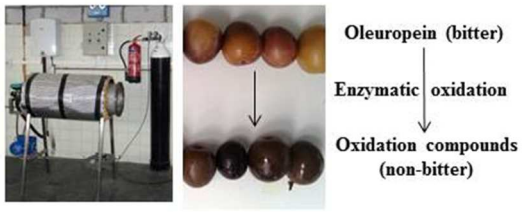
Graphical Abstract



Graphical Abstract
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Evaluation of chemical components of debittered olives undergone preservation and polyphenol oxidation

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Summary

Olives can reduce their bitterness by oleuropein oxidation via an enzymatic reaction. In this study, olives of the Manzanilla and Hojiblanca varieties preserved in acidified brine were submitted to oxidation tests under overpressure of oxygen (0.3 bars) for 3 days. It was found that the oxidation of fruit preserved for more than 3-4 months showed a reduction in the concentration of oleuropein but with great variability (28-100 %). By contrast, when olives were preserved for only one month the oxidation treatment was able to decrease more than 90% of the content of oleuropein in the fruit. These oxidized olives were stored again in the acidified medium for 6 months and no spoilage or organoleptic problems were detected. Oxidation gave rise to darker olives (*L** parameter diminished from 40 to 34). Moreover, the oxidized fruit was enriched with free phenols such as hydroxytyrosol and tyrosol as a consequence of the acid hydrolysis of phenolic glucosides during the preservation step.

Keywords: olive, bitter, oleuropein, phenolic, oxidation

Introduction

Table olives are highly appreciated for their sensory characteristics and nutritive value, and they are used as an appetizer accompanying beverages or in salads, pizzas and Mediterranean-style meals. The most significant production methods of table olives are the Spanish and the Californian styles, both involving a debittering process with sodium hydroxide solutions. Besides these two elaboration methods, alternative processes are also used to debitter the olives without the alkaline treatment. Among them, the most common method worldwide consists of putting the olives directly into a brine solution where they undergo complete or partial fermentation but debittering is slow and takes place for months or even years depending on the olive variety and the initial oleuropein concentration in the fruit. According to "Trade standard applying to table olives" (IOC, 2004) these fruit are labelled as natural (green, turning colour or black), and they are very appreciated in local markets. Besides, they are rich in bioactive substances such as phenolic compounds and triterpenic acids (Romero *et al*., 2004; 2010).

Oleuropein is formed by glucose, elenolic acid and hydroxytyrosol, the latter compound being linked to the rest of the moiety by an ester bond. The cleavage of this bond gives rise to non-bitter substances (Brenes, & De Castro, 1998). It has been proposed that natural olives lose their bitterness due to the diffusion of the oleuropein from the fruit into the surrounding brine and further acid hydrolysis of the glucoside with time (Brenes *et al*., 1993). Ciafardini *et al*. (1994) suggested that microorganisms, particularly lactic acid bacteria, can contribute to the hydrolysis of oleuropein by means of β-glucosidase production, although the growth of these microorganisms in brine may be inhibited by products of the oleuropein hydrolysis (Medina *et al*., 2010). Recently, it has also been reported that the endogenous β-glucosidase of the fruit participate to a large extent in the debittering process of the natural olives (Medina *et al*., 2009).

Consequently, processors must wait for months to market natural olives, and researchers have explored new systems to accelerate the debittering step. Yeasts and lactic acid bacteria are the predominant microorganisms in the brines of natural olives, and species isolated from olive brines have shown the ability to produce β-glucosidase (Ciafardini *et al*., 1994; Psani & Kotzekidou, 2006; Tofalo *et al*., 2013; Bleve *et al*., 2015). Moreover, the use of lactic acid bacteria and yeasts starters to ferment natural olives seems to shorten the debittering process (Servili *et al*., 2006; Pistarino *et al*.,

2013; Bevilacqua et al., 2013), although it depends on the olive variety (Servili *et al*., 2008).

Olives can also lose their bitterness due to oleuropein oxidation, as reported for black dry-salted olives (Ramírez *et al*., 2013). Oleuropein is oxidized to its quinone (Antolovich *et al*., 2004), which rapidly polymerizes to non-bitter brown pigments, which can also affect the quality of the product (Sánchez *et al*., 2013; Ramírez *et al*., 2015). It has recently been discovered that natural fruit in brine can lose its bitterness when olives are oxidized under an overpressure of oxygen (García *et al*., 2008). However, this method has failed with some batches of fruit in several seasons and no reasons were found for this phenomenon. The aim of this work was to study the debittering of olives by oleuropein oxidation in depth, and to optimize the promising new method.

Materials and methods

Industrial olive samples for oxidation assays

Olives of the Hojiblanca and Manzanilla varieties were obtained from two Table Olive Cooperatives located in the province of Seville (Spain) during the 2012/2013 and 2013/2014 seasons. On arrival to the factories, leaves and small branches were removed and the fruits with green-yellow colour on the surface were washed and put into fiberglass underground tanks which contained about 9500 kg of fruit and 5500 L of acidified brine (6% NaCl and 1 % acetic acid) (De Castro *et al*., 2007). Samples of olives were taken from the tanks after 4, 6 and 7 months of brining during the 2012/2013 season and one month of brining during the 2013/2014 season. Subsequently, they were transported to the laboratory for oxidation experiments.

Oxidation experiments

Oxidation tests were performed with olives preserved for months during the two studied seasons. Five kilograms of fruit were put into a cylindrical stainless container (30 cm Ø x 80 cm length) that was hermetically sealed. Subsequently, oxygen was introduced through an inlet valve and expulsed through an outlet valve for 5 min. The outlet valve

was closed, and an overpressure of 0.3 bars was obtained in the container. The drum was rotated one revolution per hour. The oxygen was released after three days of oxidation and the olives were analysed.

Pilot plant assays with Manzanilla olives

Fruit of the Manzanilla variety harvested during the season 2013/2014 with green-yellow colour on the surface were put into 3 L PVC vessels and covered with a brine (9% w/v, NaCl) acidified with acetic acid (0.3 % w/v). After one month of brining, the olives were submitted to oxidation under an overpressure of 0.3 bars for 3 days. Subsequently, the oxidized fruits were put again into the PVC vessels and covered with the same preservation brine for 6 months (Figure 1). All experiments were run in duplicate. Chemical compounds were purchased from Panreac (Barcelona, Spain).

Microbiological analyses

Samples of the preservation solutions from oxidized olives were taken with a sterile pipette from the geometric centre of the PVC vessels. Solutions and appropriate decimal dilution (in sterile 0.1 % peptone water) were plated using a Spiral System model DS (Interscience, Saint Nom La Breteche, France). Enterobacteriaceae were counted on crystal violet neutral-red bile dextrose agar (Merck, Darmstadt, Germany) incubated at 37 ºC, lactobacilli were counted on MRS agar (Oxoid Ltd Basingstoke, UK) with azide (0.02 %), and yeasts on GYE (Glucose Yeast Extract) (Oxoid). Except Enterobacteriaceae, all media were incubated at 32 ºC.

Polyphenol analysis

Pitted fruits were crushed with an Ultraturrax homogenizer, and 3 g of the paste were mixed with 10 mL of dimethyl sulfoxide (DMSO) (Sánchez *et al*., 2013). After 1 min of agitation by vortex, the mixture was centrifuged at 6000 g for 5 min, and the supernatant was filtered through a 0.22 µm pore size nylon filter. An aliquot of 250 µL was mixed with 250 µL of internal standard (0.2 mmol/L of syringic acid in DMSO)

and 500 µL of DMSO. Finally, 20 µL of the mixture were injected into the chromatograph. A Spherisorb ODS-2 (5 µm, 250 x 4.6 mm, Waters Inc.) column was used. The HPLC system consisted of a Waters 2695 Alliance (Waters Inc., Mildford, MA, USA) with a pump, column heater and auto sampler and the detection was performed with a Waters 996 diode array detector at 280 nm. Separation was achieved using an elution gradient with an initial composition of 90 mL of water (pH adjusted to 2.3 with phosphoric acid) and 10 mL of methanol. The concentration of the latter solvent was increased to 30/70 mL (methanol/water) over 10 min and maintained for 20 min. Subsequently, the methanol concentration was raised to 40/60 (methanol/water) over 10 min, maintained for 5 min and then increased to 50/50 (methanol/water). Finally, the methanol concentration was increased to 60/40, 70/30 and 100% in 5 min intervals. The flow rate of 1 mL/min and a temperature of 35 ºC were used (Ramírez *et al*., 2015). DMSO and methanol were obtained from Panreac (Barcelona, Spain).

Colour analysis

Colorimetric measurements on olives were performed using a BYK-Gardner Model 9000 Colour-view spectrophotometer, equipped with computer software to calculate CIE *L** (lightness), *a** (redness) and *b** (yellowness) parameters by scanning the surface from 380 to 720 nm. The data from each measurement were the average of 20 olives.

Chemical parameters of brines

The concentration of NaCl was analysed by titration with a 0.1 N silver nitrate solution, using a potassium chromate solution as indicator. The pH of brines was measured with a Beckman model 45 pH-meter. Free acidity was measured by titration using a Metrohm 670 Titro processor (Herisau, Switzerland) up to pH 8.3 with 0.2 M NaOH and expressed as % (w/v) of lactic acid (Garrido-Fernández, Fernández-Díez, & Adams, 1997).

Statistical analysis

Statistica software version 7.0 was used for data processing (Statistica for Windows, Tulsa, OK, USA). A comparison among mean variables was made by Duncan´s multiple-range tests, and the differences were considered significant when $p< 0.05$.

Results and discussion

García *et al*. (2008) showed that olives preserved directly in brine and submitted to an overpressure of oxygen of 0.3 bars for several days reduced their content in the bitter compound oleuropein due to its enzymatic oxidation. However, oxidation tests carried out in our lab under these conditions (0.3 bars for 5 days) with olives from different varieties have disclosed that in some cases the concentration of oleuropein slightly decreased. All the failed tests occurred with olives preserved for a long time, and it suggested the importance of the preservation time on the successful of the tests. In this study, the oxidation tests were performed on fruit of the Manzanilla and Hojiblanca varieties, which are the two most employed varieties in Spain for processing as table olives. They were preserved in acidified brine for several months before oxidation during the 2012/2013 season. The results presented in Table 1 show that a complete elimination of oleuropein was reached in fruits of the Hojiblanca variety preserved for 180 days due to the oxidation treatment. In contrast, a remaining concentration of this substance was found in all oxidized Manzanilla olives regardless of the preservation time. The initial concentration of the bitter compound was lower in preserved olives of the Hojiblanca than Manzanilla variety, which can be related to the higher concentration reported in raw olives of the latter variety than the former (Ramírez *et al*., 2014).The reduction in oleuropein concentration in oxidized Manzanilla olives ranged from 28 to 98% but it was not correlated with the time of preservation or the acidity conditions of the brine. As can be observed in Table 2, the pH and acidity of the brines did not vary greatly among samples thereby the variability in loss of oleuropein cannot be attributed to the pH of the olives, which ranged from 3.6 to 3.9 units. It must be noted that there is equilibrium between olive and brine so that the pH and acidity of the fruit is the same as the brine.

It has been demonstrated that the oxidation of oleuropein in olives preserved in brine is catalysed by the enzyme polyphenoloxidase (PPO) (García *et al*., 2008). PPO activity increases with olive maturation (Ortega-García *et al*., 2008) but also decreases

largely with the time of the fruit in the acidified brine (Ramírez *et al*., 2015).). A rapid inactivation of PPO obtained from green olives (Toscano *et al*., 2003) and in olive paste during the malaxation step of the olive oil processing (Taticchi *et al*., 2013) have been reported. Pectinesterase and glycosidase activity are lost in cucumbers within the first week of fermentation in brine (Maruvada, & McFeeters, 2009). All these data imply that the activity of endogenous enzymes diminishes to a large extent in fruits preserved in acidified brine with time, particularly that of PPO. Despite PPO activity has been undetectable after one month of brining (Ramírez *et al*., 2015); its effect has been observed on olives submitted to oxidation (García *et al*., 2008). Besides, the reduction of the oleuropein concentration in olives of the Manzanilla variety preserved for 210 days after oxidation (Table 1) depended to a large extent on the batch tested, which is likely due to the great variability of both phenolic compounds and PPO activity found in raw olives (Ramírez *et al*., 2014).

Taking into consideration the results reported in Table 1 and the previous references on the loss of enzymatic activity with the time of fruit in brine, oxidation tests were performed on 8 batches of olives preserved for only one month in acidified brine during the 2013/2014 season. This time is necessary to reach salt and acidity equilibrium between olive and surrounding liquid. The results presented in Figure 2 show that a great reduction in the oleuropein concentration was reached in all the fruits regardless of the olive variety. Again, the Manzanilla fruit had a higher concentration of oleuropein than Hojiblanca after one month of brine for all the batches. It must be highlighted that less than 10% of oleuropein remained in all the olives submitted to oxidation for several days (Table 3). Other phenolic compounds greatly affected by oxidation were the *o*-phenolic hydroxytyrosol 1-glucoside and the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol. In contrast, the non-orthodiphenolic hydroxytyrosol 4-glucoside, salidroside and tyrosol reduced their concentration in olives during the oxidation treatment to a lesser extent.

These results have demonstrated that the bitter glucoside oleuropein can be eliminated to a large extent in green olives preserved directly in acidified brine but it must be carried out soon after brining. From a practical point of view, the olives can be oxidized just one month from brining and most bitterness would be lost. However, it means that processors should oxidize all their olives in just 1-2 months from brining and they must sell the product immediately. A new experiment was designed to explore the

possibility of preserving the oxidized olives for at least 6 months in the same brine used before the oxidation step. If the experiments were successful processors will have a period of 6 months to pack and sell their product. These assays were carried out with Manzanilla olives at pilot plant scale following the scheme reflected in Figure 1. The results indicated that the pH in brine decreased from 4.2 to 4.0 units during the 6 months of the post-oxidation period, whereas the free acidity increased from 0.4 to 0.7 % expressed as lactic acid, which means that a strong lactic acid fermentation did not occurred. In fact, yeasts were the predominant microbiota in the brines of the oxidized olives although lactobacilli were also detected. Besides, no off-odour or any other symptoms of spoilage were detected during the storage of oxidized fruit by three table olive judges, which are members of the Table Olives Sensory Panel of the Instituto de la Grasa with over 25 years of experience on this field.

With regards to the phenolic compounds, the concentration of hydroxytyrosol increased in the olive pulp with time of preservation, which is probably related to the decrease in hydroxytyrosol 4-glucoside as a consequence of its acid hydrolysis (Figure 3). A similar statement can be made for tyrosol and salidroside, the latter substance being tyrosol glucoside. Luteolin 7-glucoside and rutin concentration also decreased during the post-oxidation step of the fruit in brine. Moreover, the low content of oleuropein in oxidized olives disappeared after 6 months of storage because of the acid conditions (data not shown). Therefore, the olives reduced their concentration in phenolic compounds during the oxidation step but a high concentration of these substances still remained in the oxidized and preserved fruit.

As it has been previously observed (García et al., 2008), the oxidation treatment gave rise to darker olives (Table 4). The lightness (L^*) and yellowness (b^*) parameters were reduced for all olives, regardless of the variety, although the final values were higher for the olives of the Hojiblanca than the Manzanilla variety. Consequently, the fruit changed its colour from yellow-brown to brown. It must also be noted that the colour of the oxidized olives after one month of preservation slightly lightened during the second storage period for 6 months.

Conclusion

The bitterness of olives preserved in acidified brine can be reduced by maintaining the fruit under an overpressure of oxygen for several days. This phenomenon is based on the enzymatic oxidation of the bitter glucoside oleuropein. However, the activity of the enzyme PPO is decreasing with time of brining, and therefore the olives must be submitted to the oxidation treatment as soon as possible from brining. In fact, it was found that olives of the Manzanilla and Hojiblanca varieties lost most of their oleuropein concentration when they were oxidized after one month of preservation. These oxidized olives were preserved again in the acidified brine for 6 months and no sensory defects were detected. As it was expected, the colour of the olives darkened due to the oxidation treatment, and a hydrolysis of the phenolic glucosides occurred during the storage period. This new process must be assayed on many other olive varieties from many countries but the results are very promising to accelerate the debittering step of natural olives non-treated with alkali.

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Table 1

Concentration of oleuropein (mmol/kg) in olives oxidized under an overpressure of oxygen. Fruits of the Manzanilla and Hojiblanca varieties were obtained from industrial tanks where they had been preserved in acidified brine during the 2012/2013 season.

Sample	Time in brine (days)	Before oxidation (mmol/kg)	After oxidation (mmol/kg)	% reduction
Manzanilla 1	120	11.35 (4.48)a[x]	8.13 (0.74)a	28.4
Manzanilla 2	120	7.45 (0.87)a	4.73 (0.16)b	36.5
Hojiblanca 1	180	0.89 (0.11)a	0b	100
Hojiblanca 2	180	0.40 (0.03)a	0b	100
Hojiblanca 3	180	0.27 (0.02)a	0b	100
Manzanilla3	210	0.98 (0.06)a	0.61 (0.13)b	37.8
Manzanilla 4	210	2.18 (0.53)a	1.10 (0.09)b	49.5
Manzanilla 5	210	2.22 (0.13)a	0.05 (0.03)b	97.8
Manzanilla 6	210	3.12 (1.26)a	0.78 (0.41)b	75.0
Manzanilla 7	210	1.10 (0.22)a	0.52 (0.01)b	52.7

[x]Standard deviation is shown in brackets. Different letters in the same mean value row indicates significant differences according to a Duncan´s multiple-range test ($p < 0.05$).

Table 2

Chemical characteristics of the brines containing the olives of the 2012/2013 season submitted to the oxidation treatment. Fruits were obtained from industrial tanks.

Sample	pH	acidity (expressed as % of lactic acid)
Manzanilla 1	3.71	0.40
Manzanilla 2	3.70	0.37
Hojiblanca 1	3.84	0.31
Hojiblanca 2	3.92	0.35
Hojiblanca 3	3.87	0.32
Manzanilla 3	3.70	0.26
Manzanilla 4	3.67	0.32
Manzanilla 5	3.92	0.52
Manzanilla 6	3.75	0.59
Manzanilla 7	3.58	0.46

Table 3

Percentage (%) of phenolic compounds remained in olives after the oxidation test. They were obtained from industrial tanks after one month of brining during the 2013/2014 season.

Compound	Manzanilla	Hojiblanca
Hydroxytyrosol 1-glucoside	6.5 (2.3)[a]	10.1 (1.3)
Hydroxytyrosol 4-glucoside	62.7 (12.1)	70.6 (7.9)
Salidroside	103.2 (9.0)	107.5 (7.3)
Tyrosol	70.2 (5.1)	1.0 (0.1)
HyEDA[b]	ND[c]	15.4 (9.2)
Oleuropein	7.4 (3.1)	10.1 (6.5)

[a]Standard deviation of 4 replicates (Manzanilla 1-4; Hojiblanca 1-4) is shown in brackets; [b]Dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol; [c]Not detected.

Table 4

Changes in the colour parameters of olives submitted to the oxidation treatment. Fruit were obtained from industrial tanks during the season 2013/2014. The fruit were submitted to oxidation after one month of brining.

	L^*		a^*		b^*		ΔE^*_{ab}
Sample	Before	After	Before	After	Before	After	
Manzanilla 1	41.2 (1.7)a[x]	32.0 (0.1)b	6.6 (0.5)a	6.3 (0.3)a	23.2 (1.9)a	9.1 (0.3)b	16.8 (2.3)
Manzanilla 2	36.9 (0.3)a	31.7 (0.3)b	7.0 (0.0)a	6.5 (0.2)b	16.8 (0.3)a	8.6 (0.3)b	9.7 (0.8)
Manzanilla 3	36.9 (0.1)a	31.6 (0.7)b	7.5 (0.3)a	6.4 (0.1)b	16.2 (0.5)a	8.2 (0.4)b	9.6 (1.2)
Manzanilla 4	34.6 (0.2)a	31.1 (0.8)b	7.5 (0.6)a	6.7 (0.4)b	14.3 (0.1)a	8.3 (0.2)b	7.0 (0.6)
Hojiblanca 1	40.9 (0.5)a	35.8 (1.0)b	5.6 (0.1)a	5.7 (0.6)a	19.3 (0.0)a	13.9 (0.9)b	7.4 (0.4)
Hojiblanca 2	42.1 (0.6)a	37.0 (0.4)b	5.4 (0.1)a	5.9 (0.2)b	19.7 (1.9)a	14.6 (0.9)b	7.3 (0.9)
Hojiblanca 3	45.4 (0.5)a	36.4 (0.3)b	4.8 (0.0)a	5.5 (0.2)b	23.4 (0.2)a	14.3 (0.2)b	12.7 (0.3)
Hojiblanca 4	42.8 (0.6)a	37.4 (0.3)b	5.3 (0.3)a	5.5 (0.0)a	22.4 (0.7)a	14.1 (0.2)b	10.0 (0.3)

[x]Standard deviation of duplicates is shown in brackets. Different letters in the same mean value row for each colour parameter indicates significant differences according to a Duncan´s multiple-range test ($p< 0.05$). $\Delta E^*_{ab} = \sqrt{(L^*_b - L^*_a)^2 + (a^*_b - a^*_a)^2 + (b^*_b - b^*_b)^2}$

Figure legends

Figure 1. Flowchart of the oxidation and preservation experiment.

Figure 2. Content of oleuropein in olives preserved for one month in industrial tanks during the season 2013/2014, and submitted to the oxidation test. Bars mean the standard deviation of duplicates. Different letters on the bars mean significant differences for the same batch according to a Duncan´s multiple-range test ($p< 0.05$).

Figure 3. Evolution of phenolic compounds in the pulp of Hojiblanca olives after one month of brining which were oxidized and put back into their brine for another 6 months. After oxidation; After oxidation and 1 month of brining; After oxidation and 3 months of brining; After oxidation and 6 months of brining. Bars mean the standard deviation of duplicates. Different letters on the bars mean significant differences for the same X value according to a Duncan´s multiple-range test ($p< 0.05$).

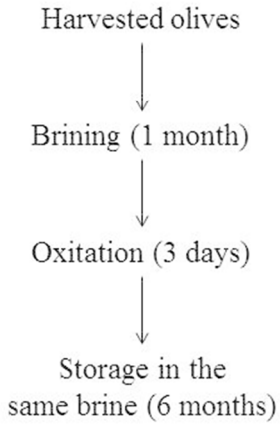

Figure 1
190x254mm (96 x 96 DPI)

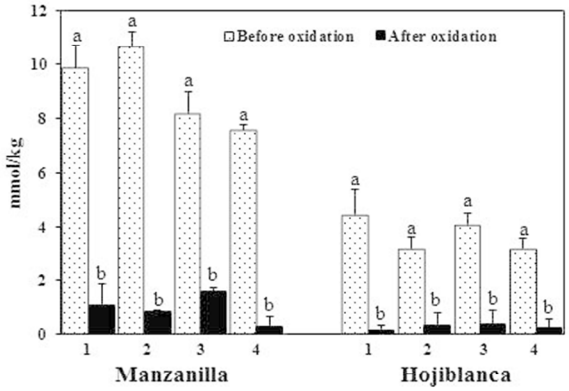

Figure 2
190x254mm (96 x 96 DPI)

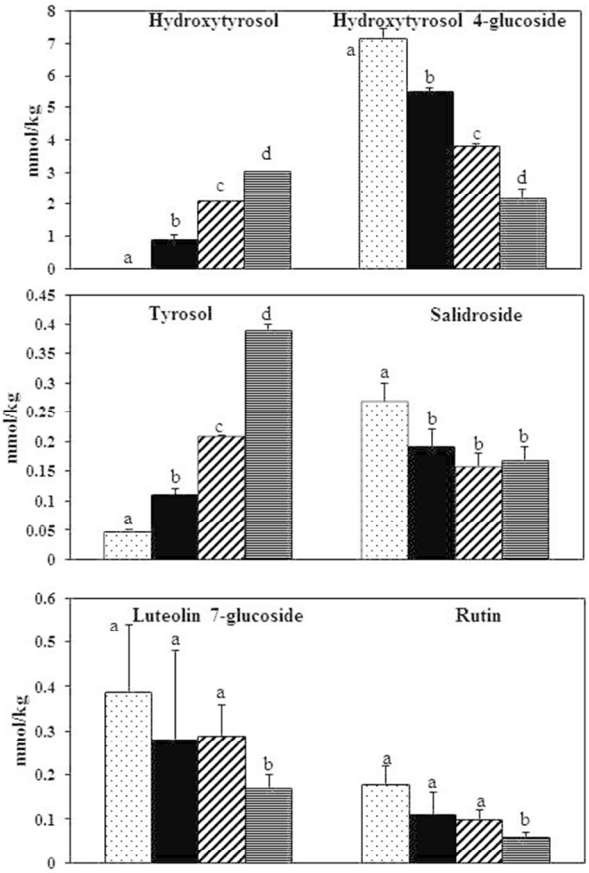

Figure 3
190x254mm (96 x 96 DPI)