APPLICATION OF REFRIGERATION FOR DEBITTERING TABLE OLIVES

Beatriz Diez Izquierdo[1], Jorge Saura Martínez[1], Ana María Ortuño Tomás[2], José Antonio del Río Conesa[2], María Ángeles Ferrer Ayala[3], Amaury Taboada Rodríguez[1] y Fulgencio Marín-Iniesta[1]

1: Grupo de Biotecnología de Alimentos, Facultad de Veterinaria, Universidad de Murcia, Campus deEspinardo, CP 30100 Murcia, España; 2: Departamento de Biología Vegetal (Área de Fisiología Vegetal), Facultad de Biología, Universidad deMurcia, Campus de Espinardo, CP 30100 Murcia, España; 3: Departamento de Ingeniería Agronómica, Universidad Politécnica de Cartagena, Paseo Alfonso XIII 48,30203 Cartagena (Murcia), España

Fulgencio Marín-Iniesta
fmarin@um.es

Abstract: During the process of making table olives, it is necessary to remove bitterness caused by phenolic compounds like oleuropein. This debittering effect is achieved by degrading these compounds into non-bitter ones that are beneficial for health due to their antioxidant properties. One of the most used methods is alkaline debittering using NaOH but the main drawback is the environmental impact generated by wastes and degradation of high-value nutraceutical compounds in olives. Different olive debittering pre-treatments followed by refrigeration storage at 5ºC during 90 days have been performed using Ocal variety olives (*Olea europaea* L.) by combining heat, β- glucosidase and *Lactobacillus plantarum* treatments. Oleuropein and hydroxytyrosol concentrations were determined in olive pulp and brine. The data has been complemented with sensory tests. The treatment with the best results was storage in refrigeration, which achieved significant debittering and greater degradation of oleuropein, due to the action of endogenous enzymes of the olives.

Keywords: olives, oleuropein, hydroxytyrosol, β-glucosidase, *L. plantarum*.

1. INTRODUCTION

The fruit of the olive tree (*Olea europaea. L*) is a natural source rich in nutrients with high nutraceutical value due to its composition rich in unsaturated fatty acids, vitamin E, carotenoids, minerals, pentacyclic triterpenes and phenolic compounds [1]. Phenolic compounds are important in fruit pulp, contributing to color, flavor, texture and playing an important role in chronic degenerative diseases prevention [2]. The predominant phenolic compound in fresh olives is oleuropein [3][4][5][6], a glycoside present in both the fruit and leaf, which obtains a bitter flavor to the freshly harvested green fruit. The oleuropein concentration decreases throughout the ripening of the fruit. For this reason, hydrolyzing oleuropein is necessary in the processing of table olives, which focuses on the elimination of their natural bitterness to make a pleasant and edible product [7].

Oleuropein is an ester of the secoiridoid family formed of hydroxytyrosol and an oleosidic structure of an elenolic acid and a glucose molecule [3][8][9]. Generally, it is the most predominant phenolic compound in the olive fruit. Its concentration depends on the cultivation of the olive tree, its variety, the climate, the stage of maturation, harvesting and the proces-

sing system used. Phenolic compounds of the secoiridoid family break down during storage. The decrease in oleuropein during the olive ripening has been related to the simultaneous increase of hydroxytyrosol. Its presence in olives has been studied as a reference to the ripening of the fruit and its pharmacological properties [1][3][9][10].

Oleuropein hydrolysis can be catalyzed in two ways: chemical hydrolysis, mainly with alkali treatment; or enzymatic hydrolysis with enzymes present in the fruit, such as glucosidases. The main advantage of alkali hydrolysis is shortening the time required to obtain a palatable product. The main drawback is the high environmental impact produced by alkaline wastewater. On the other hand, enzymatic hydrolysis is a less polluting process, but it requires longer production times. Two pathways have been detected in the hydrolysis of oleuropein during natural ripening of the fruit, through the participation of enzymes such as dimethylesterases and β-glucosidases [9].

Several studies have identified hydrolytic activity of the glycosidic bond of the β-glucosidase enzyme as an alternative route for processing olives with alkaline solution. This method is known as natural debittering; the olives are kept in a solution with sodium chloride (NaCl) at ambient temperature and the debittering is carried out exclusively through the fermentation process [2]. The activity of β-glucosidase results in two non-bitter compounds: the decarboxymethylated dialdehyde form of oleuropein aglycone (HyEDA) and glucose. Subsequently, the endogenous esterases present in the olives hydrolyze the ester bondof oleuropein, giving rise to hydroxytyrosol and elenolic acid [9].

This enzymatic hydrolysis of oleuropein in olives can be carried out by microbiological processes. Different strains of *Lactobacillus plantarum* have been discovered that are capable of hydrolyzing oleuropeininto non-bitter by-products in natural fermented green olives [8]. However, this microorganism acts better when the phenolic components are found in a smaller proportion, since these phenolic compounds have antimicrobial activity [8]. *Lactobacillus plantarum* is an autochthonous microorganism that was selected as the best bioconverter due to its ability to hydrolyze oleuropein [11]. Different olive debittering pre- treatments followed by refrigeration storage at 5ºC during 90 days were performed using Ocal variety olives (*Olea europaea* L.). The objective is to achieve a better taste and color, as well as a healthier product, reducing the great environmental impact.

2. MATERIAL AND METHODS

2.1. Materials

The study was executed using olives of the Ocal variety (*Olea europaea L.*), harvested in a ripe state in October in Córdoba, Spain during the 2020/2021 campaign, provided by Aceitunas y Encurtidos Guillamón S.L. (Murcia, Spain). The fruit was stored in light brine 4% NaCl. For the microbiological debittering treatment, we selected *Lactobacillus plantarum 299v* due to its oleuropein bioconversion property[12]. The enzyme selected for enzymatic treatment was glucoamylase (Saczyme® Yield) supplied by Novozymes North America, Inc. Saczyme®, which is produced by a genetically modified strain of *Aspergillus niger*.

2.2. Methods

2.2.1. Olive batches

Eight olive batches T1 to T8 were prepared. Olive batches were pretreated as follows. No pretreatment, only cold storage (T1); heat treatment at 37 ºC/24 h (T2); heat treatment at 70 ºC/20 min (T3); β-glucosidase enzymatic treatment (T4); inoculation of *Lactobacillus plantarum* (T5); β-glucosidase and *Lactobacillus plantarum* combined treatment (T6); β-glucosidase treatment and heat treatment 37 ºC/24 h (T7) and treatment β-glucosidase and heat treatment 70 ºC/20 min (T8). For the enzymatic treatment of the samples (T4, T6, T7 and T8), the brine was added

with the commercial preparation Saczyme ® Yield at 0,5 % per gram of fruit. In microbiological treatments (T5, T6), the brine was inoculated at a concentration of $1x10^8$ CFU. Before every treatment, the brine of all samples were stabilized at pH 4.36 with acetic acid. All treated olives T1 to T8 were stored in refrigeration at 5 ºC during 90 days. At 30, 60 and 90 days samples were pitted and stored in refrigeration for analysis. For HPLC determination, samples were stored at -80 ºC prior to lyophilization for determinate phenolic compounds.

2.2.2. Determination of β-glucosidase activity

Saczyme® Yield enzymatic commercial preparation used has cellulase, β-glucosidase and amiloglucosidase activity. The determination of β-glucosidase activity was performed by the maltose hydrolysis method and expressed in Amyloglucosidase Units/g (AGU/g). The average activity was 375 AGU/g (range 422-343).

2.2.3. **Lactobacillus plantarum** *culture*

Lactobacillus plantarum 299v strain was obtained from Laboratorios Salvat S.A. (Barcelona, Spain) A pure colony of *L.plantarum* isolated by streak plate method in MRS agar at 30 ºC was inoculated in 250 mL of liquid MRS medium and incubated at 30 ºC for 24 h. Cell density was determined by total viable count after plating serial dilutions, incubated on MRS agar at 30°C for 48 h.

2.2.4. Oleuropein and hydroxytyrosol measurement

To make the extraction, 1g of each lyophilized sample and 10 ml of dimethyl sulfoxide (DMSO) were used. Samples were centrifuged on a magnetic stirrer for 24 h in a room at controlled temperature. Supernatant was collected and filtered through a 0.45 μm pore size nylon membrane filter. For quantification, a Hewlett-Packard Liquid Chromatograph (mod. HP 1050) (Hewlett-Packard Co., Palo Alto, CA, USA), equipped with a diode array detector (range scanned: 220-500 nm) was used. The stationary phase was a LiChroCAR-TR C18 analytical column (Agilent, USA), with a mean particle size of 5 mm (250 x 4 mm id) at 30 °C. The wavelength selected for detection of both compounds was 280 nm. The HPLC- DAD methodology was carried out. The wavelength selected for detection of both compounds was 280 nm. Mobile phase was composed of two solvents, acetonitrile was used for eluent A and H_2O at pH 2, adjusted with formic acid, was used for eluent B. Dilution gradient was as follows: initially 5% eluent A and 95% eluent B; after 30 minutes, 50% A and 50% B; 40 minutes, 80 % A and 20 % B; 45 min, 80 % A and 20 % B; and 5 % of eluent A and 95 % of eluent B at 50 min. The eluent flow was 1 mL/min in all steps. Identification and quantification of oleuropein and hydroxytyrosol was carried out with a comparative study of the retention times and the UV/Vis absorption spectrum with the standards.

2.2.5. Soluble solids measurement

Soluble solids were determined using a PAL-1 digital refractometer to determine it in the brine expressed in °Brix.

2.2.6. CIELAB color space measurement

Color determination of olives was carried out following the coordinates of the CIEL*a*b system, with L* being the luminosity, a* the red/green value and b* the yellow/blue value.

2.2.7. pH measurement

Initial pH was determined and it was monitored throughout the treatment by measuring it in the brine.

2.2.8. Triangle sensory test

A triangular sensory test was carried out to check if significant differences were observed in the organoleptic properties of the olives. Sensory evaluation consisted of a triangle type test, where 3 samples were presented together, two being the same and one different. For the present study, 16 judges participated. Olive batches that had better sensory characteristics, in previous tests, were tested. Finally, the batch with only cold storage (T1) and the batch with enzymatic and microbiological combined treatment (T6) were compared.

2.2.9. Statistical data treatment

The analysis of variance (ANOVA) was applied to determine the significant differences betweendata with a 5 % of variance.

3. RESULTS AND DISCUSSION

3.1. HPLC

In figures 1 and 2 we can see the concentration of oleuropein and hydroxytyrosol determined by HPLCexpressed as mg/g dry weight (DW).

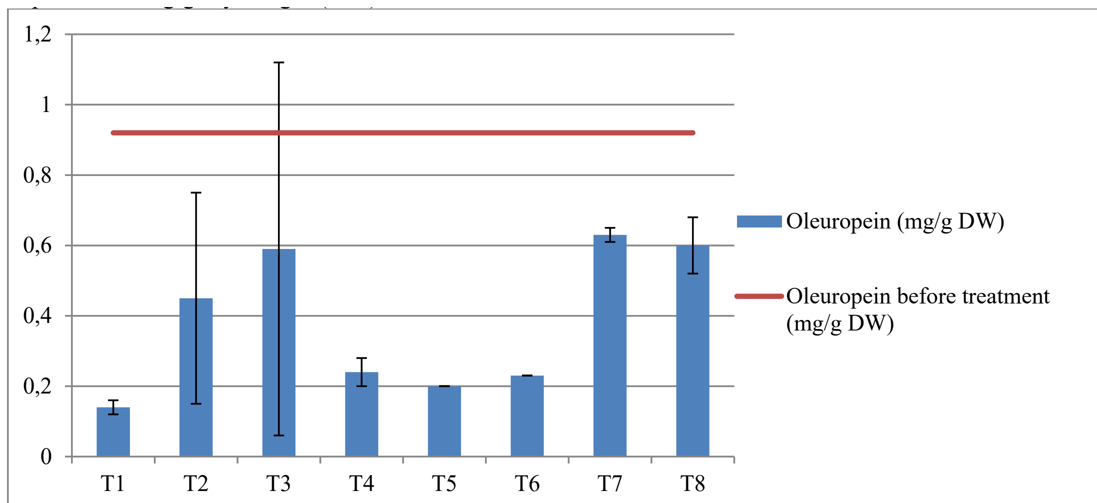

Figure 1. Oleuropein concentrations in olive batches (T1 to T8)

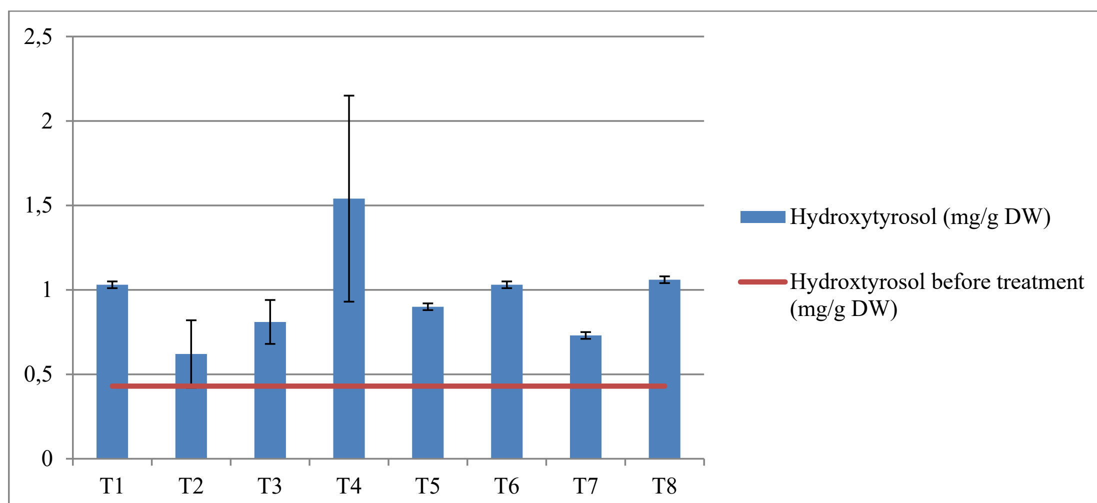

Figure 2. Hydroxytyrosol concentrations in olive batches (T1 to T8)

In the case of oleuropein, the initial concentration in the untreated olives was 0.92±0.10 mg/g DW. After 90 days of treatment, it is observed that batches that were not subjected to an increase in temperature suffer a greater degradation of oleuropein concentration, obtaining values of 0.14±0.02 in T1, 0.24±0.04 in T4, 0.20±0.0 in T5 and 0.23±0.00 in T6 with respect to the initial concentration, with no significant differences between these last three. On the other hand, in batches that were subjected to an increase in temperature, the degradation was lower, obtaining 0.45±0.30 in T2, 0.59±0.53 in T3, 0.73±0.02 in T7 and 0.60±0.08 in T8. On the other hand, the initial concentration of hydroxytyrosol was 0.43 mg/g DW in untreated olives. After 90 days, in all treatments an increase in the concentration of hydroxytyrosol was observed with respect to the untreated olives. A greater increase can be seen in the treatments where the Saczyme® Yield enzyme preparation was added. The high result of oleuropein degradation observed in T1 compared to the rate of hydroxytyrosol appearance could be explained by the effect of the olives' endogenous enzymes, which could be related to an alternative degradation route, leading to a progressive increase of another phenolic compound. Within the alternative pathways that could be generated, we could obtain an oleuropein quinone by oxidation of the secoiridoid [3] and an increase in the intermediate oleuropein aglycone (HyEDA) due to a slower degradation, which would be related to the progressive increase in soluble solids throughout the treatment [13]. We can observe a significant production of hydroxytyrosol in batch T5 that can be attributed to the esterase activity of *Lactobacillus plantarum*. The combination of *Lactobacillus plantarum* and Saczyme® Yield in batch T6 produces more hydroxytyrosol than T5 but less than T4. This could elucidate that hydroxytyrosol formation is due to endogenous action of an esterase enzyme responsible for hydrolyzing the ester bond of the oleuropein aglycone (HyEDA). Esterase enzyme is less sensitive than β-glucosidase to storage temperatures. Comparing the activity of both enzymes, the high sensitivity of β-glucosidase to brine conditions was demonstrated, its activity being undetectable after 3 days of storage at 10 ºC in a brine with 30 g/L NaCl and 3 g /L of acetic acid [14]. Other studies observed that biological debittering in olives carried out by microorganisms such as *L. plantarum* was delayed by the presence of phenolic compounds [6]. Due to the results obtained in HPLC, only the ºBrix and color determinations of batches with the greatest oleuropein degradation effect are shown below.

3.2. ºBrix measurement

Table 1. Olive soluble solids measurement results expressed in ºBrix.

Olives batches	T1	T4	T5	T6
30 days	12.50±1.05	14.20±0.10	15.10±0.12	15.40±0.22
60 days	14.57±1.15	16.30±0.20	15.80±0.14	16.10±0.10
90 days	15.23±0.06	15.23±0.12	15.67±0.06	15.63±0.29

At the start of the process (time = 0 days), untreated olive samples had 10.53 ºBrix. At the beginning of storage, soluble solids increase rapidly due to the exchange between the split olives and the brine [14]. As can be seen in Table 1, there was a minor increase in soluble solids in batch T1 compared to other batches. An increase in soluble solids was observed in the first 60 days in all batches, corresponding to oleuropein hydrolysis that forms glucose as a by-product, with a slightly higher increase in T4.

3.3. CIELAB color space measurement

Table 2: Olives CIELAB color space measurement

			90 days		
	Before treatment	**T1**	**T4**	**T5**	**T6**
*L**	57.41±2.19	56.25± 3.48	56.16±2.63	55.16±2.80	54.1±2.44
*a**	-0.76±2.72	-2.31± 2.60	-0.48^{a}±1.44	-2.07±1.17	-3.8^{a}±1.19
*b**	41.17±3.66	41.69± 8.03	37.25±5.66	37.2±3.23	38.04±3.61

After 90 days, treated olives have the green color demanded by the consumer. A favorable evolution was observed in all batches shown in Table 2, highlighting only the cold storage batch (T1) and the combined enzymatic and microbiological treatment batch (T6). T1 has an a* (red-green) value of -2.31 and a b* (yellow-blue) value of 41.69. T6 presents an a* (red-green) lower value of -3.8, therefore greater shade of green and a b*(yellow-blue) value of 38.04.

3.4. pH measurement

Although an increase in pH up to 60 days and a decrease in pH from 60 to 90 days was observed in all batches, no significant differences were observed. *Lactobacillus plantarum* 299v strain is resistant to refrigeration temperatures (5ºC) for 6 months as stated [12]. However, these temperatures may not favor its growth and fermentation process and therefore do not significantly reduce the pH value.

3.5. Triangle sensory test results

11 of 16 judges did not recognize T1 and T6 batches as different in the triangular sensory test andtherefore no significant differences in bitter taste were observed.

4. CONCLUSIONS

The best treatment regarding oleuropein degradation was the olive batch T1 with only cold storage, followed by enzymatic treatment (T4), microbiological treatment (T5) and combined treatment (T6), with no significant differences between these last three. Regarding the color, only cold storage (T1) and combined treatment (T6) showed the green color preferred by the tasters. Regarding the bitter taste, there were no significant differences between only cold storage treatment (T1) and combined treatment (T6). The phenolic compounds present in the olive and low temperatures makes an unfavorable environment for the *Lactobacillus plantarum* 299v strain, preventing its growth and thus hindering its oleuropeinolytic enzymatic capacity. The assayed β-glucosidase enzyme is highly sensitive to low temperatures, as well as to brine components, and past 3 days, its activity is not detected in the brine. Cold treatment allows less salt to be added, which makes olives healthier, and also generates an adequate degradation of oleuropein with a reduction in bitterness, good maintenance of the green color of the raw material and achieving a good sensory properties in the final product.

5. REFERENCES

[1] Moreno-González, R., Juan, M.E., Planas, J.M., 2020. Table olive polyphenols: A simultaneous determination by liquid chromatography–mass spectrometry. J. Chromatogr. A 1609, 460434. https://doi.org/10.1016/j.chroma.2019.460434

[2] Ambra, R., Natella, F., Bello, C., Lucchetti, S., Forte, V., Pastore, G., 2017. Phenolics fate in table olives (Olea europaea L. cv. Nocellara del Belice) debittered using the Spanish and Castelvetrano methods. Food Res. Int. 100, 369–376. https://doi.org/10.1016/j.foodres.2017.07.027

[3] Charoenprasert, S., Mitchell, A., 2012. Factors influencing phenolic compounds in table olives (Olea europaea). J. Agric. Food Chem. 60, 7081–7095. https://doi.org/10.1021/jf3017699

[4] Hassen, I., Casabianca, H., Hosni, K., 2015. Biological activities of the natural antioxidant oleuropein: Exceeding the expectation – A mini-review. J. Funct. Foods, Natural Antioxidants 18, 926–940. https://doi.org/10.1016/j.jff.2014.09.001

[5] Ozdemir, Y., Guven, E., Ozturk, A., 2014. Understanding the Characteristics of Oleuropein for Table Olive Processing. J. Food Process. Technol. 05. https://doi.org/10.4172/2157-7110.1000328

[6] Romero, C., García, P., Brenes, M., 2020. Chemical hydrolysis of oleuropein affected by the type of organic acid. Food Chem. 316, 126351. https://doi.org/10.1016/j.foodchem.2020.126351

[7] Marsilio, V., Seghetti, L., Iannucci, E., Russi, F., Lanza, B., Felicioni, M., 2005. Use of a lactic acidbacteria starter culture during green olive (Olea europaea L cv Ascolana tenera) processing. J. Sci. FoodAgric. 85, 1084–1090. https://doi.org/10.1002/jsfa.2066

[8] Soler-Rivas, C., Espín, J.C., Wichers, H.J., 2000. Oleuropein and related compounds. J. Sci. Food Agric. 80, 1013–1023. https://doi.org/10.1002/(SICI)1097-0010(20000515)80:7<1013::AID-JSFA571>3.0.CO;2-C

[9] Vidal Castro, A.M., 2021. MEJORA DE LAS CARACTERÍSTICAS ORGANOLÉPTICAS, FUNCIONALES Y NUTRICIONALES DE ACEITES DE OLIVA VÍRGENES. Jaén : Universidad de Jaén.

[10] Garrido Fernandez, A.G., Adams, M.R., Fernandez-Diez, M.J., 1997. Table Olives: Production and processing. Springer US.

[11] Zago, M., Lanza, B., Rossetti, L., Muzzalupo, I., Carminati, D., Giraffa, G., 2013. Selection of *Lactobacillus plantarum* strains to use as starters in fermented table olives: Oleuropeinase activity and phage sensitivity. Food Microbiol. 34, 81–87. https://doi.org/10.1016/j.fm.2012.11.005

[12] Aponte, M., Ungaro, F., d'Angelo, I., De Caro, C., Russo, R., Blaiotta, G., Dal Piaz, F., Calignano, A., Miro, A., 2018. Improving in vivo conversion of oleuropein into hydroxytyrosol by oral granules containing probiotic *Lactobacillus plantarum* 299v and an *Olea europaea* standardized extract. Int. J. Pharm. 543, 73– 82. https://doi.org/10.1016/j.ijpharm.2018.03.013

[13] Ramírez Castro, E.M.R., 2015. Estudio de la degradación enzimática del Glucósido Amargo Oleuropeína durante el procesamiento de aceitunas de mesa (http://purl.org/dc/dcmitype/Text). Universidad de Sevilla.

[14] Arroyo-López, F.N., Durán-Quintana, M.C., Romero, C., Rodríguez-Gómez, F., Garrido-Fernández, A., 2007. Effect of Storage Process on the Sugars, Polyphenols, Color and Microbiological Changes in Cracked Manzanilla-Aloreña Table Olives. J. Agric. Food Chem. 55, 7434–7444. https://doi.org/10.1021/jf070571b

[15] Asehraou, A., Peres, C., Faid, M., Brito, D., 2002. Reducing the bloater spoilage incidence in fermented green olives during storage. Grasas Aceites 53. https://doi.org/10.3989/gya.2002.v53.i3.325