INFLUENCE OF FRUIT RIPENING PROCESS ON THE NATURAL ANTIOXIDANT CONTENT OF CHEMLALI OLIVE CULTIVAR

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ABSTRACT: A quantitative study of monomeric phenols and flavonoids based on separation by high performance liquid chromatography (HPLC) was carried out to examine phenolic profile during maturation of chemlali olive cultivar. The extracts were dominated by oleuropein. Its concentration decreases with fruit maturity and is accompanied by the increase of other phenols such as hydroxytyrosol. A weak change in the amounts of flavonoids and other phenolic monomers was observed. The total phenolic content determined by colorimetric measurement ranges from 6 to 16 g Kg^{-1} of pyrogallol equivalent. The level of free phenols in the hydrolysate solution after enzymatic and acid hydrolysis was higher than their level in the extracts. The antioxidant capacity of Chemlali olive fruit extract before and after hydrolysis was also investigated. The radical scavenging effect was evaluated using DPPH experiment. An inverse relationship between total phenols and IC_{50} value during maturation was established. An increase of antioxidant potential after enzymatic and acidic hydrolysis was also observed.

Key-words: Olive, Phenolic compounds, Maturation, Enzymatic hydrolysis, Antioxidant

RESUME: L'identification et la quantification des composés phénoliques au cours de la maturation des olives de la variété Chemlali d'*olea europaea* L. ont été réalisées. L'oleuropéine en est le composé majeur. C'est un ester de l'oleoside 11-méthylester et du 3,4 dihydroxyphényléthanol (hydroxytyrosol) qui confère une saveur amère à l'olive. Les analyses chromatographiques par HPLC montrent une diminution significative de la concentration en oleuropéine au cours de la maturation des olives en faveur d'une augmentation de la concentration en hydroxytyrosol. Les dosages des phénols totaux par la méthode de Folin montrent une augmentation au cours de la maturation des olives. La concentration varie de 6 à 16g/Kg d'équivalent pyrogallol. Par ailleurs, et suite à l'hydrolyse acide et enzymatique des extraits d'olives, la teneur en monomères phénoliques augmente. De plus l'activité antioxydante des extraits d'olive mesurée par la méthode de DPPH croit au cours la maturation, avec la concentration en phénol totaux et par hydrolyse des extraits.

Mots clefs : Olive, Composés phénoliques, Maturation, Hydrolyse, Activité antioxydante

INTRODUCTION

Nowadays olive culture is one of the most important agricultural activities, mainly in the Mediterranean area where ~8 million ha of cultivated olive trees are found (98% of the world crop) [1]. Tunisian olive trees count about 57 million units covering 16000 hectares. *Olea europaea* L. Chemlali cultivar is the most abundant olive variety which representing more than 60% of the total olive plantation in Tunisia [2]. Olives are rarely consumed as natural fruit due to their extreme bitterness. Instead, they are consumed as oil or table olives. In the Mediterranean diet, olive oil is the main source of polyphenols that constitute a complex mixture in both olive fruit and its derived product

A large body of epidemiological studies have shown that the incidence of coronary heart disease (CHD) and certain cancers in the mediterranean countries is low as compared with more northern European societies [3]. It was suggested that this is largely due to the relatively safe and even protective dietary habits of this area where olive and olive oil are the principal source of fat [3]. The

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nature of the mediterranean diet [4]. These compounds act mainly as antioxidants and radical scavengers and could be used as sources of potentially safe natural antioxidants for food industry [5]. Olive oil extracted from olive fruits is known as the only stable oil during its storage and processing [6]. The phenolic compounds increase the shelf-life and nutritional quality of oil [7].

Several studies have been focused on the phenolic content of olive oil using high resolution techniques [6, 8, 9]. However, less work has been conducted on the phenolic composition of fruit [1, 10]. There are some notable differences in phenolic composition between the mature and immature fruit that are attributed to a series of chemical and enzymatic alterations of some phenolics during maturation phase [11]. Olive attains its maximum fruit weight after eight months following the flowering period. This is followed by physiological modifications and changes in fruit colour with the appearance of the purplish-black olive fruit indicating the end of olive development [12]. Changes in phenol content during fruit development are important and it is desirable to have a maturation index which can be related to fruit composition. Most of the works on the relationship between phenolics and olive development concerns oleuropein which is known to be the most prominent individual phenolic component of olive fruits and the responsible for their intense bitterness [10, 12-14]. The concentration of oleuropein varies with olive varieties and drops with the physiological development of fruit [12-14]. A relationship between oleuropein content in olive and other phenols such as certain flavonoids, dimethyloleuropein and hydroxytyrosol has been revealed in many varieties [12].

This study had a four fold objective: (1) the identification and the quantification of the main phenolic compounds present in the olive extract obtained from *Olea europaea*.L of Chemlali olive cultivar, by using reverse-phase high performance liquid chromatography (HPLC) system; (2) the evaluation of the concentration of oleuropein, hydroxytyrosol, flavonoids and other phenolics at different stages of fruit development; (3) the production of highly valuable products as the aglycone and hydroxytyrosol by acid and enzymatic hydrolysis of the extracts and (4) the study of the antioxidant potential of olive extract during all these stages of maturation.

MATERIALS AND METHODS

1-Olive preparation and sampling strategy

Chemlali olive cultivar was harvested from Sfax (Tunisia). The Olive samples were collected from the beginning of fruit development (01/07/2002) until the end of fruit development (20/02/2003). Fruit (200 g) were selected randomly from different parts of an olive tree and then they were immediately transferred to the laboratory and freeze dried. After that, the olives were blended into a homogenous powder, which was stored for extraction. The phenolic content of olive fruit was profiled as a function of fruit development, harvest date and fruit colour (green, spotted, purple and black). This method is believed to be more suitable for such investigation since different coloured olives are known to be chemically distinct, particularly with respect to phenolic compounds. From each sample, five olives were weighed to determine the growth curve.

2-Extraction

The extraction method adopted for phenolic compounds must be able to achieve the extraction of interest compounds and must avoid chemical modification. The most used solvents to extract polyphenolic compounds from olive are methanol and mixtures of methanol water [15]. Olive (200 g) was blended and then was extracted twice times with methanol and one time by mixture of methanol-water 80/20 (v/v). Thus, a multiple extraction procedure is essential for quantitative analysis of phenolic compounds. The solution was left at rest for 24 hours, filtered using GF/F filter paper and then transferred to a 500 ml flask which was evaporated to dryness. The residue was redissolved in methanol and stored in glass vials at 0°C in darkness for chromatographic analysis.

Extreme care was taken to ensure correct extraction, devoid of chemical and enzymatic modifications, which will invariably result in artefacts [16].

3-Reagents and standards

Phenolic compounds including benzoic acid, caffeic acid, coumaric acid, ferulic acid, tyrosol, vanillic acid and vanillin were obtained from Sigma-Aldrich (Chemie GmbH Steinheim, Germany). Oleuropein was purchased from Extrasynthèse (Genay, France). Hydroxytyrosol was prepared as described previously [17]. Luteolin, luteolin 7-O-glucoside, quercetin, rutin and quercetin 3-arabino-glucoside were obtained from Apin (Abingdon, UK). Apigenin was obtained from Sigma. All phenolic compounds solutions were made in a mixture methanol/water 80/20 (V/V). The double distilled water was used in the HPLC mobile phase. Pure HPLC solvents were used in all cases.

4-High-performance liquid chromatography

Phenolic monomers and flavonoids identification were carried out by HPLC analysis. It was performed on a Shimadzu apparatus composed of an (LC-10ATvp) pump and an (SPD-10Avp) detector. The columns used to analyse monomeric phenols and flavonoids were respectively a C-18 (4.6 x 250 mm) Shim-pack VP – ODS and a C-8 (4.6 x 250 mm) Shim-pack CLC. The temperature was maintained at 40 °C. The mobile phase used was 0.1% phosphoric acid in water (A) versus 70% acetonitrile in water (B) for a total running time of 50 min. The elution conditions applied for monomeric phenols were: 0–25 min, 10–25% B; 25–35 min, 25– 80% B; 35–37 min, 80–100% B; 37–40 min, 100% B, and finally washing and reconditioning steps of the column were included (40–50 min) linear gradient 100-10% B. The flow rate was 0.6 ml / min and the injection volume was 50µl. For the elution program applied for flavonoids, the following proportions of solvent B were used: 0-20 min, 25–70%; 20-25 min, 70 %; and 25-50 min, 70-25%. The flow rate was 1 ml min^{-1} and the injection volume was 40 µl. All gradients used in this study provided adequate separation. C-18 column was more suitable for the resolution of the range of phenols and has been used for routine phenolic profiling at 280 nm [18]. C-8 column provided adequate separation of flavonoids profiling, detected via UV monitoring at 335 nm [19]. The identification and quantification of phenolic compounds in *Olea europaea* L. *Chemlali* olive variety was based on their spectra, on their retention time in comparison with phenolic standards analysed in the same conditions and on the method of standard addition to the samples.

5-Colorimetric evaluation of total phenols

The total phenols were determined by the colorimetric reaction with Folin-Ciocalteau reagent [20]. An aliquot of the olive fruit aqueous methanol extract was mixed with (2 ml) of Folin-Ciocalteau reagent (Prolabo). Sodium hydroxide solution (6% v/v) was added and the mixture was stirred at 220 rpm. The blue colour formed was measured at 727 nm. The total phenols of aqueous methanol olive extracts, as determined by the Folin-Ciocalteau method was reported as pyrogallol equivalents by reference to a standard curve ($y = 0.0017\ x$, $r^2 = 0.99$).

6-Acid hydrolysis

An aliquot (300 mg) of the olive extract was hydrolysed at 100 °C for 1 h using 1 ml of a 2 M solution of HCl. After 1 h, the sample was cooled and diluted with water (1 ml) and the hydrophobic fraction was extracted with ethyl acetate, which was subsequently removed by evaporation.

7-Enzymatic hydrolysis

Enzymatic hydrolysis was carried out using β-glucosidase from almond (Sigma). The reaction was monitored at 37°C for 3 hours in 2 ml of 50mM sodium acetate. β-glucosidase was added to a solution of sodium acetate buffer containing 50mg of concentrated methanolic extract.

8-DPPH radical scavenging effect

The DPPH (2,6-di-*tert*-butyl-4-methylphenol) radical scavenging effect was evaluated according to the method employed by Na Mee *et al.* [21]. Four millilitres of methanolic solution of each test sample was added to 10 ml DPPH methanol solution (1.5 x 10^{-4} M). After mixing the two solutions gently and leaving them for 30 min at room temperature, the optical density was measured at 520 nm using a Shimadzu UV-160 A spectrophotometer. The test samples and positive control BHT were tested over a range of concentrations. The antioxidant activity of each test sample and BHT were expressed in terms of concentration required to inhibit 50% DPPH radical formation (IC_{50} µg per ml) and calculated from the log-dose inhibition curve.

RESULTS AND DISCUSSION

1-Oleuropein and low molecular weight phenols

Fifteen phenolic compounds were identified in *Olea europea* L. Chemlali cultivar. These included classes of phenolic compounds such as hydroxybenzoic acids, hydroxycinnamic acids, flavonoids and phenolic alcohols. Table 1 lists each identified peak in elution order, with its retention time. The external standard method was used to measure the concentrations. All calibration curves were linear over the concentration range tested with correlation coefficients > 0.96. The external standard method was used to measure the concentrations as described by Chamkha et al.[18].

Table 1: Phenolic compounds identified in olive fruit [a]

compound	colmun used	retention time (min)
hydroxytyrosol	C_{18}	11.2
tyrosol	C_{18}	17.6
p-hydroxy benzoic acid	C_{18}	19.9
vanillic acid	C_{18}	23.6
caffeic acid	C_{18}	25.8
coumaric acid	C_{18}	29.5
vanillin	C_{18}	34.2
ferulic acid	C_{18}	36.8
oleuropein	C_{18}	39.4
rutin	C_8	8.4
quercitin 3-arabino-glucoside	C_8	9.7
luteolin 7-O-glucoside	C_8	10.9
quercetin	C_8	18.3
luteolin	C_8	18.8
apigenin	C_8	22.3

[a] Identification was founded on retention time and coelution with standards

The oleuropein content in the fruit increased rapidly during the growth of the fruit. Figure 1 shows that a high concentration of oleuropein was obtained during the green maturation sampling. It attained 6.5 g kg^{-1} (All data are reported on a fresh mass basis) for the sample harvested at the end of august (young fruit) with values (600–700mg) per fruit and was far in excess in comparison with the other phenolics. However the dominance of oleuropein was not sustained during the sampling period at various times. The content at initial harvest was 2.5 g kg^{-1} and then increased from the green to purple fruit with a value of 580 mg per olive. After this period, oleuropein concentrations decreased

as the sampling period progressed and reached a minimum at the last stage of maturation (1.5 g kg^{-1}) when fruit reached its maximum weight (1.13 g).

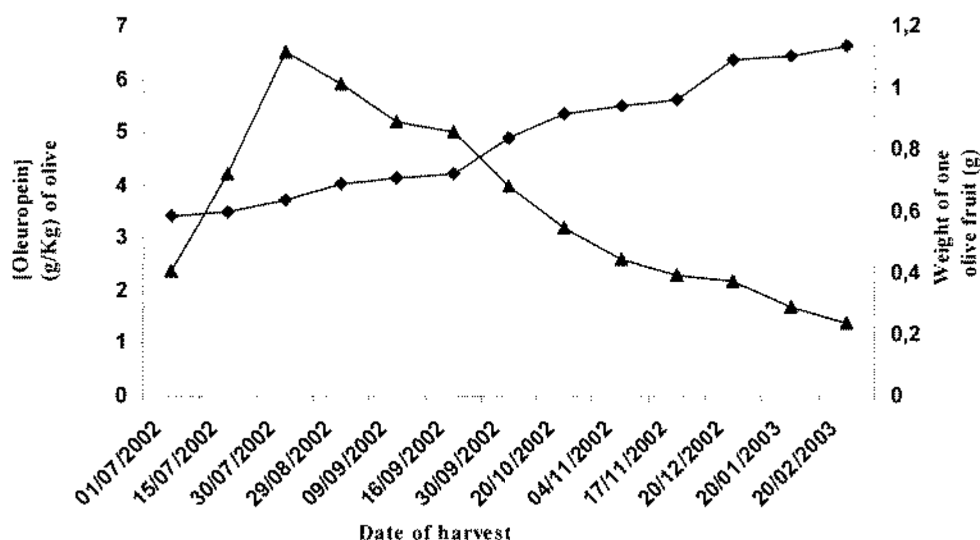

Figure. 1: Oleuropein content and weight of one olive during the maturation of Chemlali olive variety.
(▲): Oleuropein; (♦): weight of one olive. Each point represents the mean of two determinations and two independent experiments (SD <15% of the mean).

The change in oleuropein content may be related to the increased activity of hydrolytic enzymes during maturation. This is in agreement with previous findings [13-14]. An inverse relationship between oleuropein content in olive fruit and hydroxytyrosol was observed from the harvest in October until the last sample. Indeed hydroxytyrosol concentration began at relatively low levels and increased as the sampling period progressed (Figure 2).

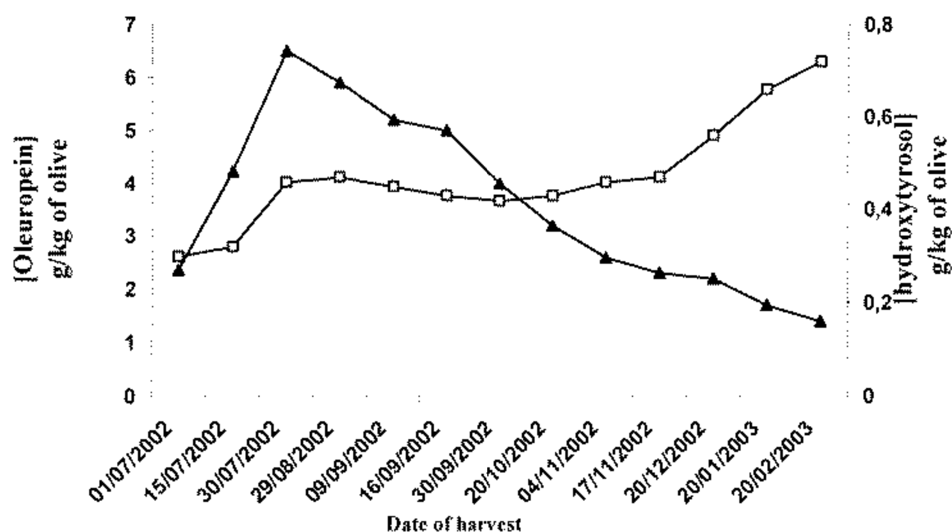

Figure. 2: Oleuropein and hydroxytyrosol levels during the maturation of olive variety.
(▲): Oleuropein; (□): Hydroxytyrosol. Each point represents the mean of two determinations and two independent experiments (SD <15% of the mean).

Chemically, oleuropein is the ester of oleoside 11-methylester and 3,4 dihydroxyphenyl ethanol (hydroxytyrosol). Hydroxytyrosol is the principal product of oleuropein degradation during the maturation of fruit. Many chemical and enzymatic reactions cause the decrease of oleuropein

concentration and the increase of hydroxytyrosol concentration as was shown earlier [11]. It has been reported that glycosidase activities produced hydroxytyrosol, glucose and other derivatives from oleuropein[22-24]. These results agreed with the findings of Amiot *et al.*[12, 13] who observed a decline in oleuropein content with fruit maturity. Indeed, oleuropéin level varies from 3.3 to 45 and from 2.8 to 40 g kg^{-1} of olive fresh mass in *Cailletier* and *Salonenque* cultivars, respectively. Similarly, it has been reported that oleuropein content in the fruit of both cultivars, Gentile (*Larino*) and Gentile (*Colletorto*), varied from 0.87 to 1.45 and from 1.5 to 2.08 g kg^{-1} respectively [14]. Bianco *et al.*[25] have claimed that the concentration of oleuropein falls to zero when olive fruits (*O. europaea, cv. leccino*) are completely black.

HPLC analyses showed that other phenolic compounds were present during the maturation stages of olive and their amounts are higher in black olives than in green ones. Figures 3 and 4 show the chromatograms at 280 nm of phenolic compounds of Chamlali olive extracts of the early harvested fruits and the late ones, respectively. The two profiles are not the same and the phenolic compositions vary quantitatively during the period of green and black maturation of olive fruits.

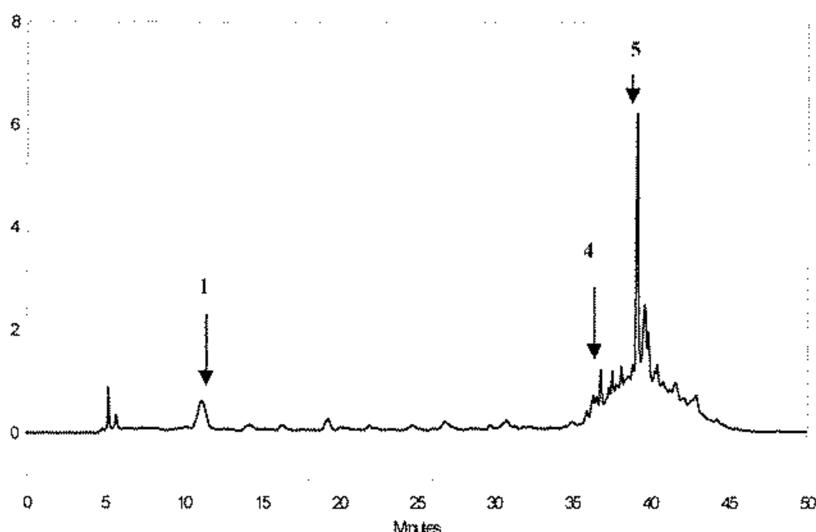

Figure. 3: Chromatogram profile of chemlali olive extract of the early harvested fruits (01/07/2002) at 280nm.
1: Hydroxytyrosol, **4:** Ferulic acid, **5:** Oleuropein.

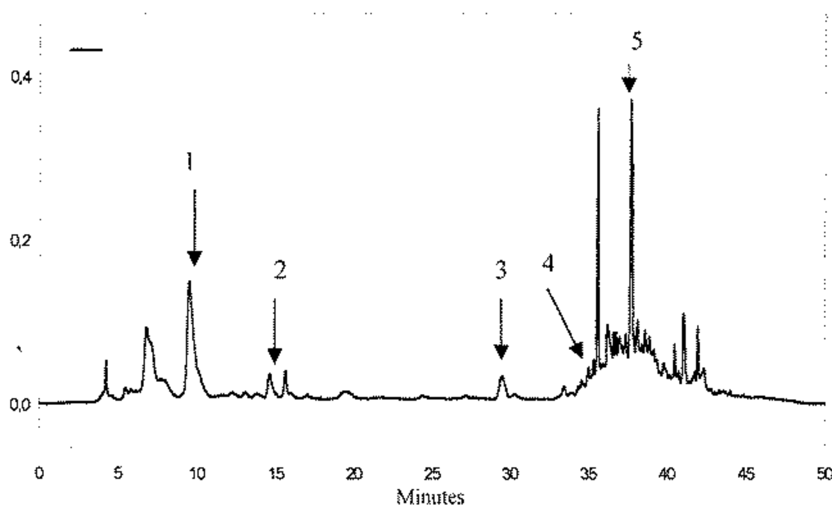

Figure. 4: Chromatogram profile of chemlali olive extract of the late harvested fruits (20/02/2003) at 280 nm.
1: Hydroxytyrosol, **2:** Tyrosol, **3:** *p*-coumaric acid, **4:** Ferulic acid, **5:** Oleuropein.

Tyrosol is the second abundant phenolic monomer compound, after hydroxytyrosol. The tyrosol concentration increased from 75 to 375 mg Kg^{-1} during fruit maturation. This increase may arise from ligistroside transformation or the hydrolysis of other compounds containing tyrosol.
Many hydroxycinnamic acids were identified in this study. They included ferulic, caffeic and *p*-coumaric acids. Ferulic acid remained relatively constant although it increased slightly at the black maturation. *p*-coumaric acid showed a weak variation and decreased in the last phase of maturation. Caffeic acid was present at a very low concentration during all the phases of maturation. Other phenolic monomers including *p*-hydroxybenzoic acid, vanillic acid, and vanillin exhibited low concentrations and were present as minor components in the harvest samples. This is in agreement with previous findings about the Spanish varieties showing that vanillic acid, vanillin, *p*-coumaric and ferulic acids practically remained constant and their concentrations were lower than 2 to 4 ppm during maturation [6]. On the other hand, it was established that the phenolic profile and content of olive drupes can be strongly affected by agronomic parameters such as cultivar, pedoclimatic production conditions, agronomic techniques, and fruit ripening [26]. Phenolic content in the oil can also be conditioned by those factors [27].

2-Quantification of flavonoides

Six flavonoids were identified, quantified and their quantities were reported as mg $k.g^{-1}$ fresh weight of olives. The flavonoids studied have been divided in two groups: flavones (luteolin 7-O-glucoside; luteolin and apigenin) and flavonols (rutin; quercetin 3-arabino-glucoside and quercetin). It seems that flavonoids glucosides were dominant and exhibited the most significant variation with physiological maturity. The main flavonoid compounds quantified in our study were luteolin 7-glucoside, rutin and quercetin 3-arabino-glucoside. The luteolin 7-O-glucoside amount was noticeably higher between the end of August harvest and the mid September harvest. The maximum concentration reached was at mid September with a value of 70 mg $Kg^{-1}$of fresh fruit. After this period, its concentration decreased until the end of the sampling period. At the same time, luteolin concentrations started at very low levels and increased as the sampling period progressed. The chemical structures of luteolin 7-glucoside and luteolin and their respective levels during fruit maturation suggest that they may be related biochemically. Thus one possibility is that luteolin is a product of luteolin 7-O-glucoside transformation by glycosidase activities. Apigenin remains substantially unchanged and quite low throughout all stages of maturation.

These results corroborate with previous findings. It has been reported that luteolin may originate from rutin or luteolin glucoside and apigenin from apigenin glucosides [6]. The evolution of these two flavonoids, luteolin and apigenin, had an indirect relation with that of their glucoside form derivatives [6].

Profiles of the three flavonols identified in olive during maturation were dominated by quercetin 3-arabino glycoside. The latter flavonol reached its maximum level in the November harvest and then decreased during the last stage of maturation. Rutin concentration increased slowly until the end of December harvest (80 mg Kg^{-1}) and it decreased in the end of the growing period of the fruit. Quercetin concentrations were noticeably lower and showed very little change during the maturation. As was explained earlier [13] this may be related to *Olea europaea* L. fruit which appears to accumulate only glycosylated derivatives because they are probably less toxic than aglycones.

3-Total phenols determination

The total phenols concentration of olive extracts varied from 6 to 16 g Kg^{-1} of equivalent pyrogallol during maturation of the variety. The highest amount of the total phenols was present in black olives (from the harvest in October to February). This could corroborate with the high concentration of phenolic compounds quantified by HPLC (simple phenols and flavonoids aglycone) during this same period of harvest. The lower amount determined by HPLC is due to (i) a lack of quantification of all

phenolic compounds detected in the chromatograms, (ii) the fact that many phenolics are not detected by HPLC in our experimental condition, and (iii) the presence of interactions between Folin reagent and hydroxyl groups of other compounds, such as proteins and sugars[18].

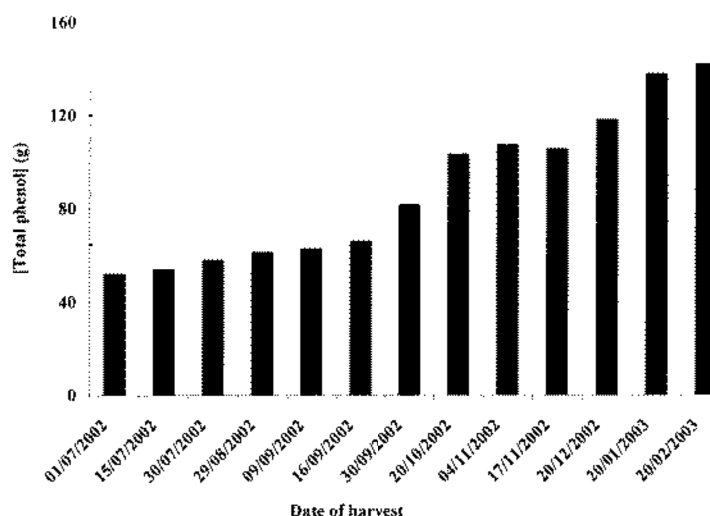

Figure 5: Total phenols concentration after hydrolysis of olive extracts per g of hydrolysis solution from 1 Kg of fresh olive.

Acid treatment of aqueous methanolic extracts induced hydrolysis or break down of the more complex phenolic molecules. The most notable effect seen was the increase of the amount of total phenolics (Figure 5). This increase can be attributed to degradation of most species containing phenolic compounds. The hydrolysis of olive extract resulted in an increase in hydroxytyrosol concentration followed by a complete disappearance of oleuropein. In addition, the level of free phenols in the hydrolysate solution was higher than their level in the extract before hydrolysis.

Olives extracts was also hydrolysed by β-glycosidase from almonds. The analysis of degradation products by TLC showed besides the appearance of a spot known for oleuropein, other spots with different migration rates indicating the presence of tow major compounds. TLC of ethyacetate phase of hydrolysate (data not schown) showed the Rf values: 0.8 for oleuropein aglycone, 0.6 for hydroxytyrosol and 0.5 for oleuropein using as eluent $CHCl_3$: CH_3OH (8:2 v/v). This is in agreement with previous findings [28]. The amount of total phenolics increased after enzymatic treatment of aqueous methanolic extract. The different responses of these fractions arise from the high level of identified compounds in hydrolysate solution. However, the concentration of oleuropein decreases and the amount of hydroxytyrosol and oleuropein aglycone increases.

4-Antioxidant potential of crude extracts and their hydrolysis during maturation

The DPPH radical scavenging effect for all the methanol extracts exhibited antioxidant activity which showed correlation between total phenol content and DPPH radical-scavenging activity over all stages of fruit development. The lower IC_{50} values 1.4 µg/ml which indicated higher antioxidant potential were observed for the latest harvested samples (Black olive extract harvested in February). The antioxidant activity of the extract was closely correlated to its content of total phenols. This is probably due to the significant inhibition of the radical formed by a high concentration of *O*-diphenol such as hydroxytyrosol and luteolin.

The DPPH radical scavenging activity of the olive extracts after hydrolysis was measured. The IC_{50} values after hydrolysis of the extracts harvested from July to October were nearly the same. They have almost the same IC_{50} value 1.95 µg ml^{-1}. The last hydrolysed fractions harvested from November to February showed the highest antioxidant potential with IC_{50} value 1.5 µg ml^{-1}. The behaviour difference of the extract and its hydrolysate may be due to the fact that aglycones obtained

after hydrolysis often have a higher antioxidant activity than their respective glycosides. There are also may be other mechanisms operative affecting the antioxidant potency. Acid hydrolysis also caused an increase of concentrations of hydroxylated phenolic compounds such as hydroxytyrosol, tyrosol and other derivatives followed by a complete disappearance of oleuropein. It has been reported that acid hydrolysis causes the degradation of oleuropein to produce hydroxytyrosol and other oleuropein derivatives [29].

In Conclusion, many phenolic compounds have been identified and quantified in Chemlali olive cultivar from Tunisia by using a reverse phase HPLC system. Considerable differences of phenols concentration such as oleuropein and hydroxytyrosol were observed during the stage of fruit development. It seems that the weight of the fruit is not in direct relation with the level of oleuropein concentration. The antioxidant potential of olive extract was found to increase during maturation in correlation with the total phenols content. The harvest at the last phase of maturation led to a decrease of the bitter taste of olive and an increase of the level of flavonoids aglycones and hydroxytyrosol. These phenolic compounds of olive are of great interest as they contribute to sensory characteristics and the long stability of virgin olive oils, and as they may be involved in biochemical and pharmacological effects, including anticarcinogenic and antioxidant properties [4]. The phenolic composition represents an useful contribution to biochemical characterization of chemlali olive cultivar.

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