Phytochemistry, Vol. 28, No. 1, pp. 67–69, 1989.
Printed in Great Britain.

0031–9422/89 $3.00 + 0.00
Pergamon Press plc.

ACCUMULATION OF OLEUROPEIN DERIVATIVES DURING OLIVE MATURATION

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(*Received* 3 *March* 1988)

Key Word Index—*Olea europaea*; Oleaceae; olive; maturation; oleuropein derivatives; demethyloleuropein; elenolic acid glucoside; esterase.

Abstract—Elenolic acid glucoside and demethyloleuropein are glucosylated derivatives of oleuropein which accumulate during olive (*Olea europaea*) maturation. These compounds appear simultaneously with a fall in oleuropein content and an increase in esterase activity. This enzyme may thus be responsible for the formation of the two oleuropein derivatives.

INTRODUCTION

Seasonal variations in levels of phenolic compounds are well-known phenomena in plants in general and in fruits in particular [1, 2]. They are the result of either active turnover or catabolism of these compounds [3].

In the olive fruit (*Olea europaea* L.), the main phenolic compound is oleuropein (**1**), a heterosidic ester of elenolic acid and 3,4-dihydroxyphenylethanol [4]. Its secoiridoid phenolic structure makes it of considerable technical importance both because of its browning capacity [5, 6] and its intense bitter taste [7, 8]. This compound is very plentiful in young fruits but its levels decline rapidly during maturation [9]; this decline is accompanied by an increase in other phenolic compounds, such as certain flavonoids [10, 11] and verbascoside, a heterosidic ester of caffeic acid and of 3,4-dihydroxyphenylethanol previously identified in olive [12]. A part of the verbascoside molecule is thus the same as in the oleuropein molecule.

The degradation of oleuropein can be carried out *in vitro* [4, 13] to give several derivatives (3,4-dihydroxyphenylethanol, elenolic acid, oleuropein aglycone, etc.), whose biological properties are clearly different from the parent molecule [13–15]. Nevertheless, none of these compounds has been reported as being a natural constituent of olive fruit during its physiological development. In contrast, in ripe olives, demethyloleuropein (**2**), a derivative of oleuropein, has been identified [16, 17].

The accumulation of two compounds which are very close to oleuropein is reported here during development of the fruit of different olive cultivars. These variations and those of the esterases, a group of enzymes likely to contribute to the degradation of oleuropein, form the first stage in a study of the biochemical mechanisms which lead to the reduction in the oleuropein content during maturation of olive fruits.

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RESULTS AND DISCUSSION

It is usual to consider that there are three phases in the life of the olive fruit [18]: a growth phase during which accumulation of oleuropein occurs, a green maturation phase coinciding with a reduction in the levels of chlorophyll and oleuropein, and a black maturation phase characterized by the appearance of anthocyanins and during which the oleuropein level continues to fall.

A reduction in oleuropein levels during fruit maturation was found for all the cultivars studied, e.g. Cailletier, Picholine and L11 (Fig. 1). It was accompanied by the accumulation of two compounds, demethyloleuropein and elenolic acid glucoside (**3**), which were separated by HPLC and identified by their spectral (in particular FAB-MS) and chromatographic characteristics. Demethyloleuropein has already been shown in the mature olive [17]. Elenolic acid glucoside is not phenolic and only corresponds to the secoiridoid part of oleuropein. It is close to the compound previously isolated from olive leaves under the name of oleoside [15]. Elenolic acid glucoside was present in all the cultivars studied, whereas demethyloleuropein was only detected in Cailletier and L11.

Elenolic acid glucoside and demethyloleuropein (when present) appeared at the beginning of green maturation as the oleuropein levels declined (Fig. 1). They then accumulated and reached their maximum during black maturation and became more plentiful than the residual oleuropein in L11 and Cailletier. Nevertheless, the quantity of oleuropein which disappeared between September and November (33 mg/g dry wt in Cailletier for example) was much greater than the amount of demethyloleuropein and elenolic acid glucoside (8 mg/g dry wt) which appeared.

It is difficult to pinpoint the beginning of green maturation in the olive as it takes place very slowly; elenolic acid glucoside and demethyoleuropein (the latter in cv Cailletier and L11) thus appear to be good biochemical markers of this physiological stage insofar as they are not

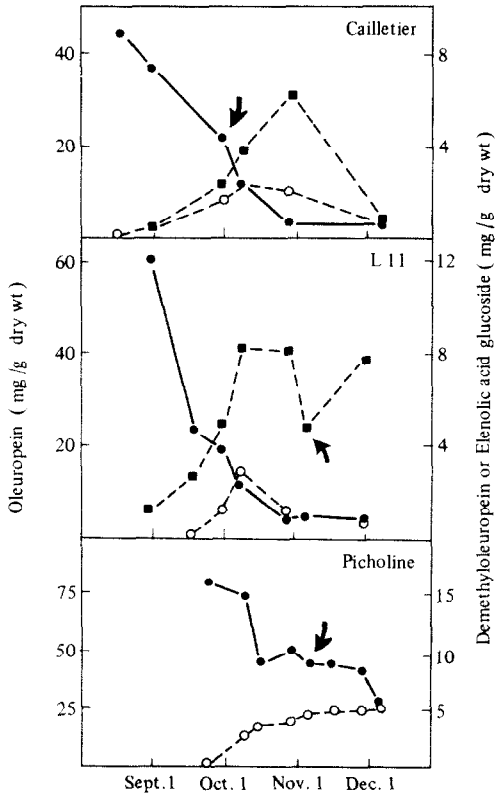


Fig. 1. Comparison of the changes in oleuropein (●), demethyloleuropein (■) and elenolic acid glucoside (○) during maturation of three olive cultivars. Arrows indicate the beginning of black maturation. Each value is the mean of three replicate samples (s.e. was lower than 10% of the mean values).

present in the fruit before the start of the process. In addition, demethyloleuropein, which is naturally present in two cultivars only, could be used as a varietal marker.

The chemical relationship between oleuropein, elenolic acid glucoside and demethyloleuropein and their respective levels during fruit maturation suggest that they may be related biochemically. Thus, one possibility, is that the last two compounds are formed from oleuropein by the action of esterases. In keeping with this proposal, esterase activity increased considerably during the first phase of maturation and reached a maximum during black maturation (Fig. 2). However, this implied involvement of esterases in oleuropein metabolism needs to be supported by the determination of (i) the characteristics of *in vitro* degradation of oleuropein by esterases extracted from olive fruit and (ii) their respective subcellular sites and modifications of these during maturation. In addition, the possibility that elenolic acid glucoside and demethyloleuropein might be intermediates in the biosynthesis of oleuropein cannot be completely excluded, i.e. they may accumulate progressively during maturation when biosynthesis slows down.

The fruit of *O. europaea* appears to accumulate only glucosylated derivatives of oleuropein, which are probably less toxic than aglycones [19]. In contrast, 3,4-dihydroxyphenylethanol and non-glucosylated secoiridoids derived from oleuropein were found in the leaf. Nevertheless, among the numerous aglycones produced by *in vitro* hydrolytic degradation of oleuropein [4, 14, 20], 3,4-dihydroxyphenylethanol only appears in fruits [20, 21] when subjected to alkaline treatment to remove bitterness in order to make them edible. It is probable that oleuropein can be converted by the action of glucosidases [15] but the intermediate compounds are probably immediately remetabolized in the fruit.

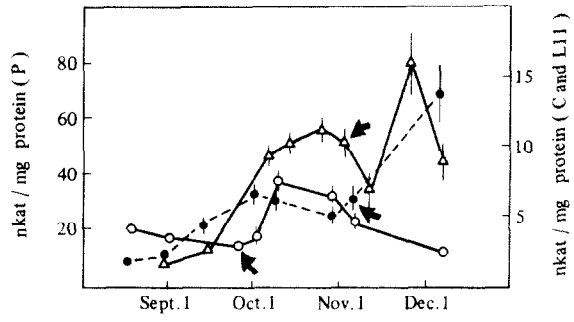
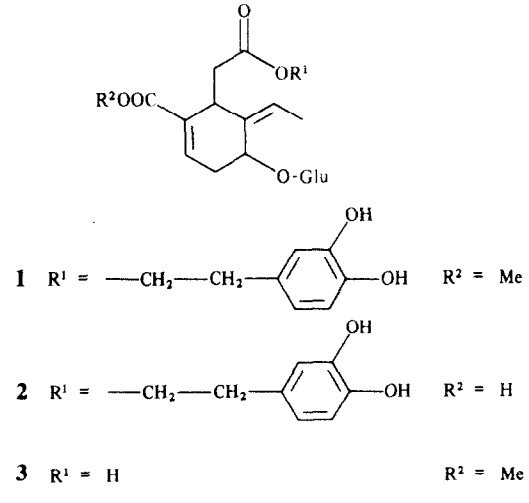


Fig. 2. Changes in esterase activity of three cultivars during olive maturation. Picholine (P), △-△; L 11, ●-●; Cailletier (C), ○-○. Arrows indicate the beginning of black maturation. Each value is the mean of three replicate samples ±s.e. When absent, errors bars do not exceed the dimensions of symbols.

EXPERIMENTAL

Plant material. Fruits of 11 cultivars were picked at different physiolological stages as described previously [9]. They were freeze-dried and ground in liquid N_2. The powder obtained was analysed for phenolic compounds and esterase activity.

Extraction, separation and identification of phenolic compounds. The extraction (80% EtOH) of phenolic compounds and their separation by HPLC have already been described [9]. The R_ts of oleuropein, demethyloleuropein and elenolic acid glucoside were 15.8, 10.3 and 7.0 min respectively. The phenolic extract was also



Scheme 1.

subjected to chromatographic analysis on silica gel 60 (HPTLC aluminium sheet precoated with silica gel $60F_{254}$) with EtOAc–MeOH–H_2O (200:33:27). After spraying with *p*-toluene sulphonic acid (1%)+vanillin (2%) in EtOH, phenolic secoiridoids (oleuropein and demethyloleuropein) turned orange and non-phenolic secoiridoids (elenolic acid glucoside) turned grey. The R_fs of oleuropein, demethyloleuropein, and elenolic acid glucoside were 0.55, 0.44 and 0.22 respectively.

After isolation by prep.. TLC under the above conditions, demethyloleuropein and elenolic acid glucoside were analysed by FAB MS (GOL JNS-BX 300). Samples were suspended in a glycerol and NaCl matrix and bombarded with 5 keV Xe atoms. Demethyloleuropein: $[M+H]^+ = 527$ and $[M+Na]^+ = 549$; elenolic acid glucoside: $[M+H]^+ = 405$ and $[M+Na]^+ = 427$.

HPLC quantification of oleuropein and demethyloleuropein was carried out by internal calibration at 280 nm (coumarin as int. standard). Elenolic acid glucoside was estimated at 240 nm, with the same extinction coefficient as oleuropein. All results are expressed in mg/g dry wt.

Esterase assay. Proteins were extracted from acetone powders according to ref. [22]. Esterase activity was determined spectrophotometrically with *p*-nitrophenylacetate [23]. Results are given in nkat/mg protein. Proteins were estimated by Bradford's method [24].

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