

## Improved Method for Isolation of Bacterial Inhibitors from Oleuropein Hydrolysis

FEDERICO FEDERICI<sup>1\*</sup> AND GUIDO BONGI<sup>2</sup>

*Istituto di Biologia Vegetale, Sezione Microbiologia Agraria,<sup>1</sup> and Centro di Studio per l'Olivicoltura (CNR),<sup>2</sup> University of Perugia, I-06100 Perugia, Italy*

Received 17 February 1983/Accepted 9 May 1983

A new high-pressure liquid chromatography multidetection quantitative method for the isolation of the products of oleuropein hydrolysis is described. A single analysis yields sufficient amounts of the compounds to test their inhibitory effect on bacterial growth.

The fermentation of Spanish-type green olives is a complex process affected by several limiting factors such as bleaching of flavors and sugars after lye treatment, pH, presence of inhibitors, and other things (4).

The fermentation rate, in particular, is mainly influenced by the content of inhibitors of the different olive cultivars (1, 2). These inhibitors have been identified as elenolic acid and oleuropein (OLP) aglycone, both hydrolysis products of OLP (6). Exact knowledge of the level of OLP and related compounds in the olive brines during processing is, therefore, of great interest.

Preparative thin-layer chromatography, countercurrent distribution, and paper chromatography are the only isolation methods so far in use; a subsequent analytical thin-layer chromatography method is also needed for identification of the inhibitors (2, 6). Microbiological assays, however, cannot be performed with the small amounts of products which a single analytical thin-layer chromatography preparation yields. As a consequence, bioassays and chemical analysis are presently separate steps of different procedures (6).

This paper describes a simple and reliable high-pressure liquid chromatography (HPLC) method for the rapid quantitative detection of the products of OLP hydrolysis.

OLP was extracted from *S. Agostino* and *Picholine* green olives by the procedure of Walter et al. (6) and purified by means of an RP-8 Lichroprep 40-70  $\mu\text{m}$  (Merck) preparative column ( $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ , 30:70 [vol/vol]; 4 ml/min; 200 mg per injection). The major band collected (from 600 to 650 ml) was OLP at 80% purity. The hydrolysis products were also prepared as indicated by Walter et al. (6).

A semi-preparative all-glass steel-jacketed HPLC column (5) was used for the identification test. The column was packed by the slurry method with 6.5 g of Riedel-DeHaën Kieselgel

60 (C8, 5  $\mu\text{m}$ ). The eluent (isocratic) was  $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ , 30:70 (vol/vol). Column loading trials were performed with a suitable mixture of phenols and resulted in a maximum loading capacity of 2.0 mg per injection. This 2.0 mg corresponds to the quantity (dry weight) of a  $\text{CHCl}_2$  lipid-free extract from approximately 50 ml of fermentation brine. The detection was

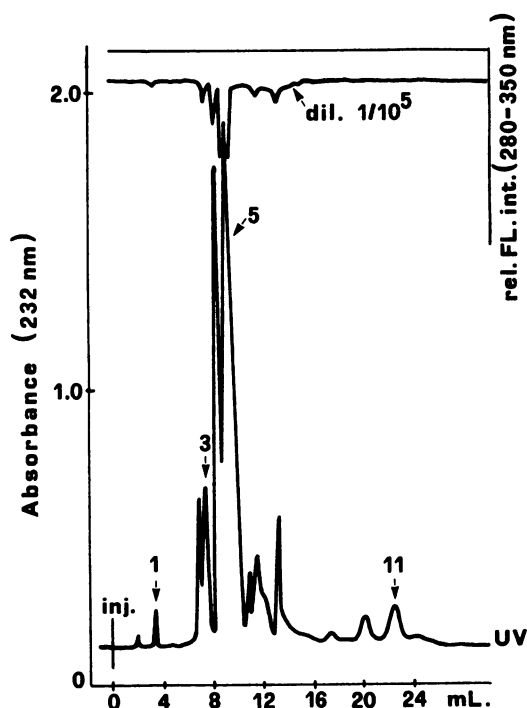


FIG. 1. HPLC chromatogram of an OLP acid (0.5 N  $\text{H}_2\text{SO}_4$ , 15 min at 100°C) hydrolysate (UV, 232 nm, and fluorescence detectors). Solvent system,  $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ , 30:70 (vol/vol); pressure, 3,100 lb/in<sup>2</sup>. Fraction 1,  $\beta$ -3,4-dihydroxyphenylethyl alcohol; fraction 3, OLP; fraction 5; OLP aglycone; fraction 11, elenolic acid.

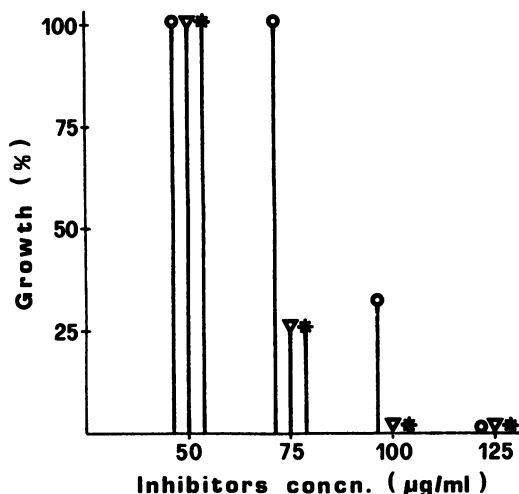


FIG. 2. Effect of inhibitor concentrations on the growth of *L. plantarum* (as percentage of growth of a 24-h nontreated culture). Symbols: O, OLP acid hydrolysate in toto; ∇, OLP aglycone; \*, elenolic acid.

carried out by means of a Perkin Elmer LC 75 UV spectrophotometer (232 nm) and a Hitachi-Perkin Elmer 204S spectrofluorimeter (280-nm excitation, 350-nm emission). Chromatography of an acid OLP hydrolysate is shown in Fig. 1.

The weights of the single fractions were determined with a Sartorius 4503 microbalance by drying 1/10 of the volume in calibrated aluminium caps. The remainder was freeze-dried and redissolved in 200  $\mu$ l of CH<sub>3</sub>OH immediately before bioassay.

Single fractions from single injections were then tested for their antimicrobial activity by using a culture of *Lactobacillus plantarum* as a test organism.

The inhibition of the bacterial growth by the different products of OLP hydrolysis was tested in MRS Broth (Biolife) (diluted 1:3 with distilled water and with 2.0% NaCl, wt/vol). The broths were inoculated with 24-h-old cells to give an initial optical density of 0.050 on a Bausch & Lomb Spectronic 20 spectrophotometer (546 nm) against an uninoculated medium blank and were incubated at 28°C for 3 days. Growth was followed by turbidimetric measurements every 2 h.

Fractions 5 and 11 appeared to be biologically active: the growth of *L. plantarum* was greatly inhibited at a concentration of approximately 75  $\mu$ g of either of the two fractions per ml (Fig. 2). The same inhibitory effect was observed when the whole hydrolysate was used at a concentration of fraction 5 plus fraction 11 equal to approximately 80  $\mu$ g/ml. By this analytical technique, therefore, no other active compound appeared to be retained.

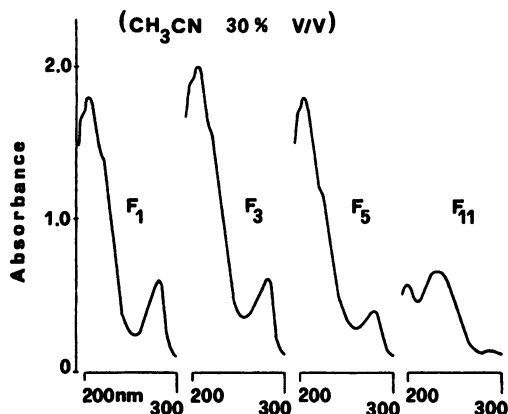


FIG. 3. UV spectra (Perkin Elmer LC 75 + Auto-control) obtained by the stop-flow technique on HPLC fractions 1, 3, 5, and 11. The spectrum of the solvent was previously kept in memory and then subtracted.

Fraction 5 cochromatographed with an authentic OLP aglycone, and fraction 11 cochromatographed with elenolic acid. Partial identification of these compounds was also carried out by UV spectroscopy with the stop-flow technique (Fig. 3).

Chromatograms obtained with a stainless steel column with same stationary phase showed a less resolved region between fractions 2 and 6; the higher resolution of the all-glass system depended on its superior inertness.

The HPLC method reported here could be of help for the optimization of lye concentration and treatment times during the processing of Spanish-type green olives. The procedure could also be used as an aid in the selection of microorganisms resistant to the antimicrobial action of the OLP hydrolysis products (3, 4).

We thank Jone Rossi of the Istituto di Microbiologia Lattiero-Casearia, University of Perugia, for providing the strain of *L. plantarum* used in this study.

#### LITERATURE CITED

1. Etchells, J. L., A. F. Borg, I. D. Kittel, T. A. Bell, and H. P. Fleming. 1966. Pure culture fermentation of green olives. *Appl. Microbiol.* 14:1027-1041.
2. Fleming, H. P., W. M. Walter, Jr., and J. L. Etchells. 1969. Isolation of a bacterial inhibitor from green olives. *Appl. Microbiol.* 18:856-860.
3. Fleming, H. P., W. M. Walter, Jr., and J. L. Etchells. 1973. Antimicrobial properties of oleuropein and products of its hydrolysis from green olives. *Appl. Microbiol.* 26:777-782.
4. Garrido-Fernández, A., and R. H. Vaughn. 1978. Utilization of oleuropein by microorganisms associated with olive fermentations. *Can. J. Microbiol.* 24:680-684.
5. Stahl, K. W., E. Schuppe, and H. Potthast. 1973. GIT-Fachreitschrift für das Laboratorium, p. 563-565. (DP 2329286).
6. Walter, W. M., Jr., H. P. Fleming, and J. L. Etchells. 1973. Preparation of antimicrobial compounds by hydrolysis of oleuropein from green olives. *Appl. Microbiol.* 26:773-776.