High-Performance Liquid Chromatography of Selected Phenolic Compounds in Olive Oils

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A reverse-phase high-performance liquid chromatographic technique with isocratic elution has been developed to separate and quantitate the major phenolic compounds of the hydroalcoholic extracts of olive oils. Hydroxytyrosol, tyrosol, caffeic acid, *p*-hydroxyphenylacetic acid and homovanillic acid were analyzed on a μBonapak C18 column with an acetonitrile/water/acetic acid (20:90:0.18, vol/vol/vol) mixture as a mobile phase. Electrochemical detection provided selectivity as well as sensitivity. The method was applied to the analysis of the most important phenolic compounds in olive oils.

KEY WORDS: Caffeic acid, electrochemical detection, HPLC, homovanillic acid, hydroxytyrosol, olive oil, *p*-hydroxyphenylacetic acid, tyrosol.

Phenolic compounds constitute an important group of naturally occurring compounds in plants. In contrast to other crude oils, virgin olive oil produced from olives of good quality is consumed unrefined. Thus, virgin olive oils contain phenolic compounds that are usually removed from other edible oils in the various refining stages (1-4). Olive oils are low in tocopherols (5); therefore, the presence of other phenolic compounds capable of antioxidant activity is of particular importance (6).

Several studies concerning the composition of olive oil phenolic compounds have been published. Many analytical methods, such as thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) have been used for their analysis. One of the problems associated with GLC is that nonvolatile phenolic compounds require derivatization prior to the quantitation step. This is not required for high-performance liquid chromatography analysis. Ultraviolet detection has been used extensively in the detection of phenolic compounds (6-15). Among the phenolic compounds identified in olive oils were hydroxytyrosol, tyrosol, caffeic acid, homovanillic acid, vanillic acid, *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid, *p*-conmaric acid, syringic acid and protocatechric acid. Phenolics content in virgin olive oil ranges from 50 to 500 mg/kg expressed as caffeic acid (4).

More recently, liquid chromatography with electrochemical detection has been employed for the analysis of compounds that can be oxidized or reduced. The procedure involves separation of sample constituents by liquid chromatography prior to their oxidation at a glassy carbon electrode in a thin-layer electrochemical cell. The technique is selective because three requirements, retention time, redox activity and pH at the selected potential, must be met simultaneously. Many investigators conducted studies using cyclic voltammetry to help determine optimum conditions for amperometric detection of the phenolic compounds (4,16-28).

In the present study, simultaneous determination of hydroxytyrosol, tyrosol, caffeic acid, homovanillic acid and *p*-hydroxyphenylacetic acid in olive oils by electrochemical detection was investigated. These compounds are the major phenolic compounds in olive oil.

EXPERIMENTAL PROCEDURES

Instrumentation. A bioanalytical Systems LC-4B (Lafayette, IN) detector was used for all experiments. The detector consisted of an LC-4B amperometric controller and an LC-17 glassy carbon transducer. The cell is composed of three electrodes: the working electrode, which is a TL-5A single glassy carbon electrode, a silver/silver chloride (model 2020) reference electrode and an auxiliary electrode.

A Beckman pump, model 110A and a 250 × 4.6 mm μBondapak C18 column (Waters Associates, Milford, MA) completed the chromatographic system.

Mobile phase. The mobile phase was prepared with HPLC-grade reagents and consisted of 10% acetonitrile in a solution of 2 mL glacial acetic acid in 1 L water. This mobile phase was filtered and degassed by passing through a 0.45-μm membrane filter.

Reagents. Tyrosol, caffeic acid, homovanillic acid, *p*-hydroxyphenylacetic acid and cellulose were obtained from Sigma Chemical (St. Louis, MO). Hydroxytyrosol was prepared from olive leaves by the method of Panizzi (29).

Chloroform, ethylacetate and water HPLC grades were obtained from VWR Scientific (Chicago, IL), methanol HPLC grade from EM Science (Septech, Wadefield, RI), acetonitrile HPLC grade from Burdick and Jackson Laboratories, Inc. (Muskegam, MI), hexane from Malinckrodt (St. Louis, MO) and acetic acid A.C.S. reagent from Aldrich Chemical Co. (Milwaukee, WI).

Standards. Individual standard solutions and standard mixtures of phenolic compounds were prepared in the mobile phase. Standard curves were generated by means of dilutions of these standard solutions. Five ppm of each compound was added to olive oil (Gondola Brand), and replicate samples were analyzed to determine the recovery of each.

Sample preparation. Four brands of olive oil (Gondola, Olio Sasso, Fillipo Berio and Marca Il Duomo) were purchased from local retail stores. Three samples of each olive oil brand were randomly analyzed. Thirty grams of olive oil were dissolved in 30 mL hexane and extracted three times with 20 mL of a methanol/water (60:40, vol/vol) solution. The mixtures were shaken for two minutes and then allowed to separate. The lower layers were combined and evaporated under reduced pressure. Residues were made up to 5 mL with the mobile phase. Turbid solutions were filtered or centrifuged before injection. All samples were analyzed in triplicate.

HPLC-electrochemical analysis. Twenty μL of each sample was injected onto the column through an ALTEX 210 A injection valve (Beckman Instruments Inc., Berkeley, CA) equipped with a 20-mL sample loop. The sensitivity

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was 10 nA, and the applied potential was 0.8 V *vs.* Ag/AgCl 3M NaCl. Peak areas were recorded on a Spectra-Physics SP4270 integrator (Autolab Division, Spectra-Physics, San Jose, CA). Sample concentrations were calculated based on peak areas compared to those of each of the five external standards.

RESULTS AND DISCUSSION

Varying the acetonitrile content of the mobile phase revealed unexpected selectivity differences. The percentage of acetonitrile was increased until good resolution was achieved. The most suitable mobile phase composition was acetonitrile/water/acetic acid (10:90:0.18, vol/vol/vol). The elution order is typical of reversed-phase chromatography, that is, polar compounds elute first, followed by those of decreasing polarity.

Representative chromatograms of a standard mixture and of an olive oil extract are shown in Figures 1 and 2. Hydroxytyrosol, tyrosol, *p*-hydroxyphenylacetic acid, homovanillic acid and caffeic acid are clearly separated. The standard solutions, when injected separately, gave single peaks with different retention times (Table 1).

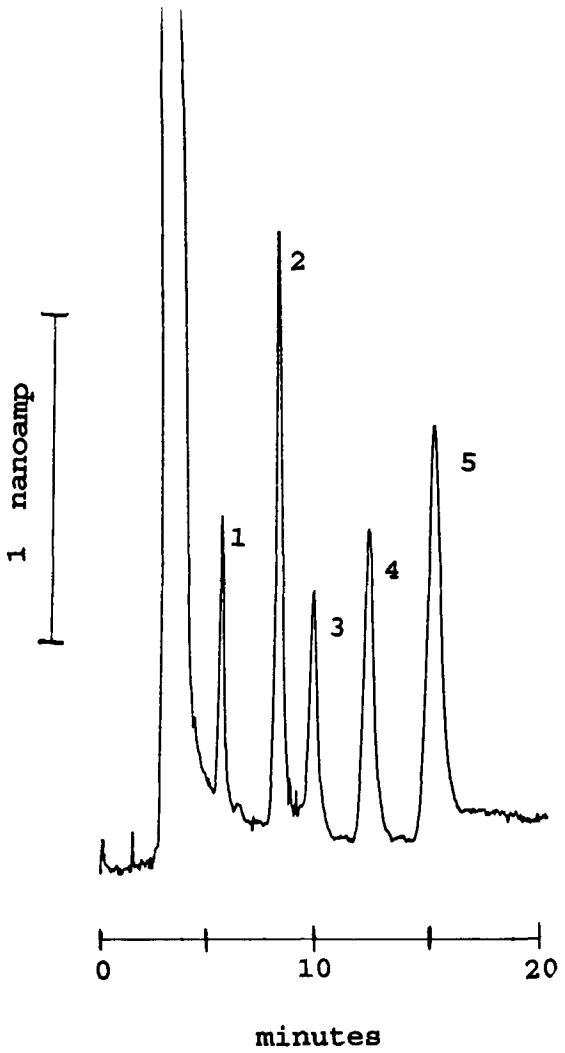

FIG. 1. Chromatogram of a mixture of standards. Peaks: 1, hydroxytyrosol; 2, tyrosol; 3, *p*-hydroxyphenylacetic acid; 4, homovanillic acid; and 5, caffeic acid. Amounts injected were 1.5, 20.2, 29, 1.4 and 2.6 nanograms, respectively.

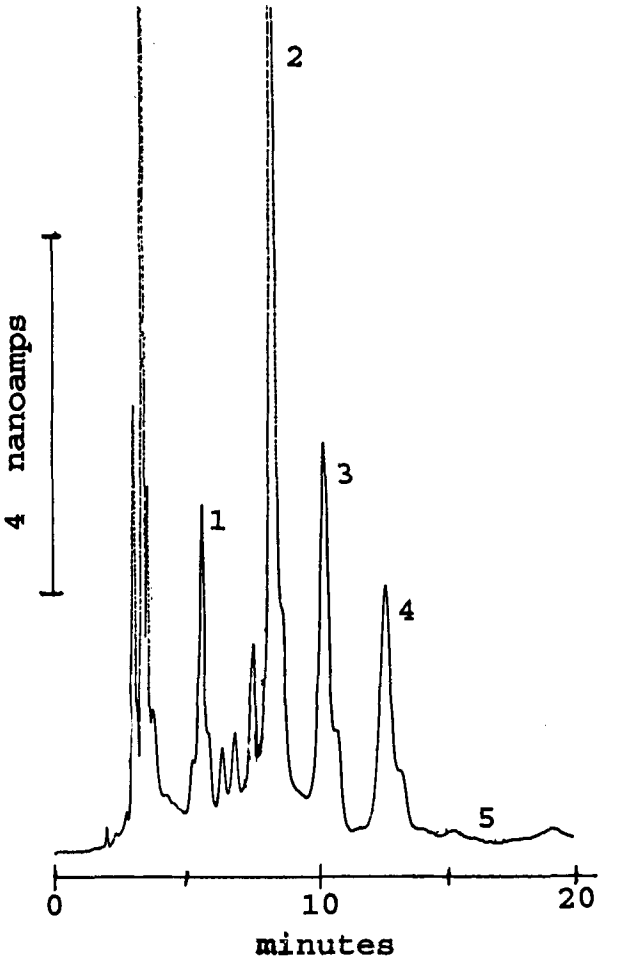

FIG. 2. Chromatogram of an extract of olive oil (Gondola brand) Peaks: 1, hydroxytyrosol; 2, tyrosol; 3, *p*-hydroxyphenylacetic acid; 4, homovanillic acid; and 5, caffeic acid.

TABLE 1

Retention Times of Phenolic Compounds[a]

	Retention time (min)	
	Mean	SD
Hydrotyrosol	5.70	0.01
Tyrosol	8.40	0.02
p-Hydroxyphenylacetic acid	10.11	0.05
Homovanillic acid	12.54	0.06
Caffeic acid	15.32	0.04

[a]Results are expressed as the mean and SD of 12 determinations.

All benzoic acid derivatives, except *p*-hydroxybenzoic acid, have substitutions *meta* to the carboxyl group. The *meta* substituents, *e.g.*, hydroxy and methoxy groups, are electron-releasing, thereby facilitating the oxidation of the *para* OH group (24). Homovanillic acid is easily oxidized at 0.8 V due to its 3-methoxy-4-hydroxyphenyl group. Chemical oxidation of the vinylic side-chain can occur

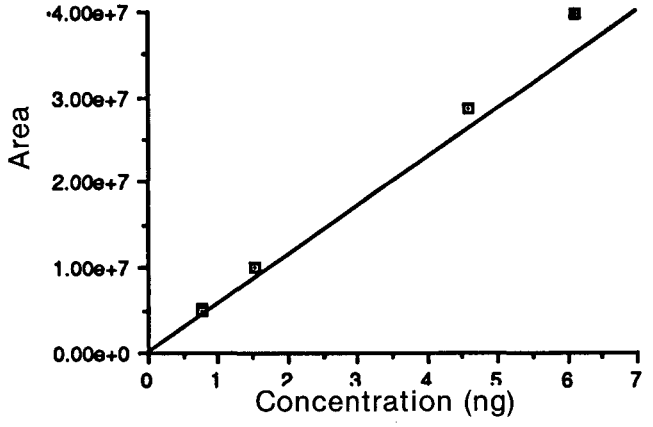

FIG. 3. Standard curve of peak area *vs.* nanograms of hydroxytyrosol injected. $R^2 = 0.999$.

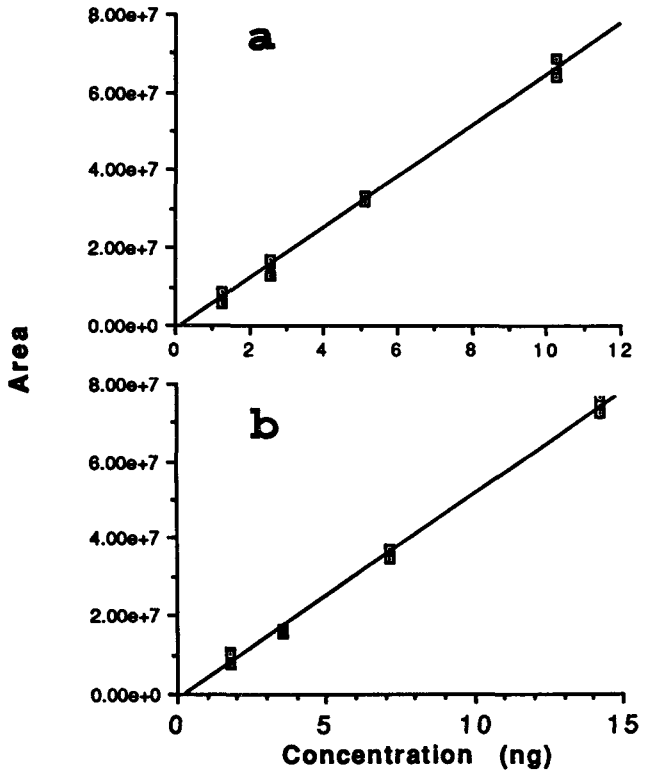

FIG. 4. a: Standard curve of peak area *vs.* nanograms of homovanillic acid injected, $R^2 = 0.996$. b: Caffeic acid, $R^2 = 0.995$.

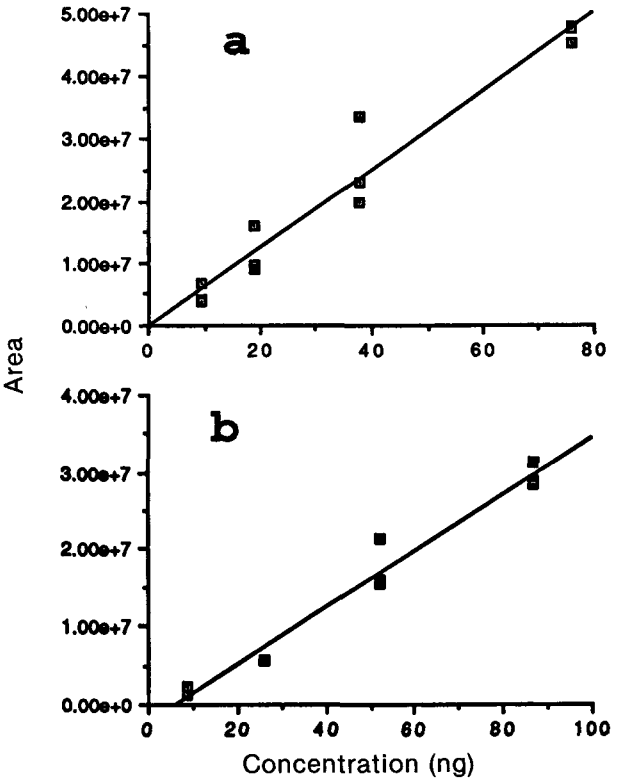

FIG. 5. a: Standard curve of peak area *vs.* nanograms of *p*-hydroxyphenylacetic acid injected, $R^2 = .952$. b: Tyrosol, $R^2 = 0.972$.

under mild oxidation conditions, and the other possibility is oxidation of the hydroxy group attached to the aromatic ring (18).

Peak area *vs.* concentration curves were constructed for each standard. Figures 3, 4 and 5 show linear relationships between concentration and peak areas obtained with the electrochemical detector for the five standards. Data in Table 2 indicate the suitability of this method for quantitative and sensitive determination. Table 3 shows the amounts of the different phenolic compounds actually found in the four brands of olive oil studied.

Isocratic conditions lead to a smooth baseline. Baseline drift often occurs when gradient elution is employed with an electrochemical detector (24). Day-to-day reproducibility is good for several days after polishing the working electrode. However, one disadvantage associated with the use of an electrochemical procedure is the gradual absorption

TABLE 2

Analytical Characteristics of Phenolic Compounds

Phenolic compound	Standard curve[a]				Recovery[b] from olive oil (5 ppm of each added)
	Slope	Int.	R^2	LDQ	
Hydroxytyrosol	6.37[c]	0.289	.9977	0.09[d]	93
Tyrosol	0.367	−2.3	.9724	5.0	97
p-Hydroxyphenylacetic acid	0.692	−0.21	.9516	6.4	100
Homovanillic acid	6.59	−1.3	.9956	0.24	97
Caffeic acid	5.71	−4.9	.9931	0.44	99

[a]Concentrations, three replicates.
[b]Percent − average of six determinations.
[c]Integrator counts × 10^6 per ng injected.
[d]Amount corresponding to y intercept + 2 SD. LDQ, least detectable quantity.

TABLE 3

Phenolic Compounds in Four Different Types of Olive Oil

Brand names	Hydroxytyrosol	Tyrosol	*p*-Hydroxyphenylacetic acid	Homovanillic acid	Caffeic acid
Gondola	0.18 ± 0.007[a]	9.62 ± 1.34	2.79 ± 0.30	0.23 ± 0.005	0.030 ± 0.016
Olio Sasso	0.19 ± 0.015	0.75 ± 0.29	0.19 ± 0.11	0.03 ± 0.008	0.014 ± 0.007
Fillipo Berio	0.57 ± 0.035	2.36 ± 0.45	0.31 ± 0.06	0.03 ± 0.002	nd[b]
Marca Il Duomo	0.74 ± 0.170	2.61 ± 1.25	1.73 ± 0.06	0.14 ± 0.026	nd

[a]Results are expressed as the mean ± SD of three determinations in mg/kg of oil.
[b]Not detected.

of the phenolic compounds and their oxidation products on the electrode. Frequent cleansing was required and carried with it the risk of electrode damage.

The electrochemical detector offers greater sensitivity and stability at low concentration levels of these phenolic compounds than do other detectors. The method is suitable for the measurements of small quantities of phenolic compounds in olive oils. The simple extraction procedure is rapid (less than 20 min) and could easily be adapted to other oils and numerous fields of application, such as phytochemistry, food quality control and basic research. It is believed that this method is not restricted to the five particular phenolic compounds described here and that it could easily be extended to the analysis of other phenolic compounds.

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REFERENCES

1. Monteduro, G., and C. Cantarelli, *Riv. Ital. Sostanze Grasse 46*:115 (1969).
2. Vasquez, R.A., C. Janer del Valle and M.L. Janer del Valle, *Grasas y Aceites 27*:185 (1976).
3. Vasquez, R.A., *Rev. Franc. des Corps Gras. 25*:21 (1978).
4. Vasquez, R.A., C. Janer and E. Grauani, *Quimica y Technologia de Alimentos 2*:195 (1980).
5. Gutfiner, T., *J. Am. Oil Chem. Soc. 58*:966 (1981).
6. Gutfiner, T., and A. Letan, *Lipids. 9*:658 (1974).
7. Cicheli, A., and M. Solinas, *Riv. Merceol. 23*:55 (1984).
8. Camuvati, F., and E. Fedeli, *Riv. Ital. Sostanze Grasse 59*:623 (1982).
9. Cortesi, N., E. Fedeli and E. Tisurnia. *Ibid. 62*:281 (1985).
10. Cortesi, N., A. Ponziani and E. Fedeli, *Ibid. 58*:108 (1981).
11. Graviani, C.E., V. Colchero and R.A. Vazquez, *Grasas y Aceites 31*:85 (1980).
12. Graciani, C.E., and R.A. Vazquez. *Ibid. 31*:237 (1980).
13. Graciani, C.E., and R.A. Vazquez, *Ibid. 30*:365 (1979).
14. Ragazzi, E., and G. Veronese, *Riv. Ital. Sostanze Grasse 50*:663 (1973).
15. Solinas, M., and A. Cichelli, *Riv. Soc. Ital. Sci. Aliment. 4*:223 (1982).
16. Richards, G.E., P.D. Gluckman and S.C. Manelli, *Life Sci. 41*:1881 (1987).
17. Mayer, G.S., and R.E. Shoup, *J. Chrom. 255*:533 (1983).
18. Chiavari, G., P. Vitali and G.C. Galletti, *Ibid. 392*:426 (1987).
19. Grossi, G., A. Bargossi, R. Battistoni, A. Lippi and G. Sprovieri, *Ibid. 465*:113 (1989).
20. Hiroshi, T., M. Teruhiko and S. Takeshi. *Ibid. 515*:265 (1990).
21. Yu, Z.J., K.D. Lim, R. Hoskins, R.W. Rockhold and I.K. Ho, *Neruochem. Resp. 15*:613 (1990).
22. Masatoxhi, Y., R.I. Matsunaga, K.E. Fukuda and M. Nakamura, *J. Chrom. 416*:278 (1987).
23. Morrison, R.T., and R.N. Boyd (ed.), *Organic Chemistry*, 3rd edn., Alyn and Bacon, Boston, 1978, p. 396.
24. Hayes, P.J., M.R. Smyth and L. McMurrough, *Analyst 112*:1197 (1987).
25. Hayes, P.J., M.R. Smyth and L. McMurrough, *Ibid. 112*:1205 (1987).
26. Wester, P., J. Gottfries, K. Johansson and F. Klinteba, *J. Chrom. 615*:261 (1987).
27. van Maard, P.M.M., J.P.M. Wieleders and J.B.W. Wikkerink, *Biomed. Chrom. 5*:209 (1987).
28. Jun-Ichi, S., A. Watanabe and R. Takahashi, *J. Chrom. 430*:110 (1988).
29. Panizzi, L., M.L. Scarpati and G. Oriente, *Gazz. Chim. Ital. 90*:1669 (1960).

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