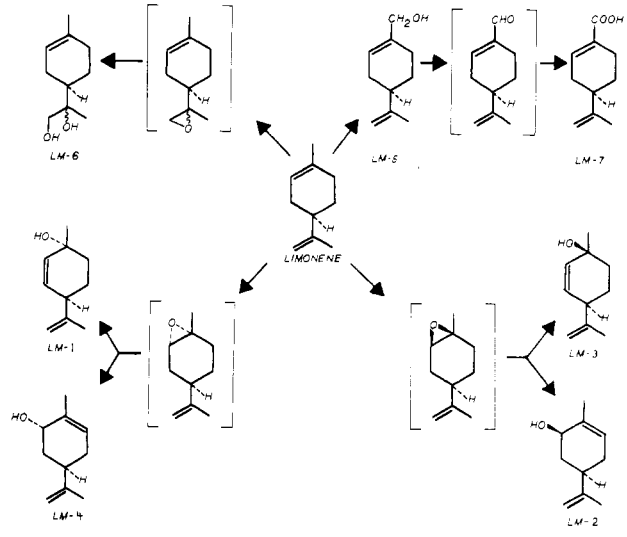
Scheme I. Proposed Metabolic Pathway of Limonene in Rats



unidentified compounds (LM-8, LM-9, and LM-10) comprise 17.5% of the total metabolites. (+)-Limonene was not detected in this analysis.

DISCUSSION

The results of this study have demonstrated the presence of eight neutral metabolites and two acidic metabolites in the urine of limonene-fed rats. Of the ten compounds obtained in the present study only two have been mentioned in the previous metabolic investigations in mammals. A total of 14 different metabolites have now been shown to result from the metabolism of limonene in higher organisms.

The results of the work of Kodama et al. (1974) and of Igimi et al. (1974), as well as the present study, show at least three common metabolites for rats and rabbits; these are uroterpenol, uroterpenol glucuronide, and perillic acid. In addition, the finding of uroterpenol in these three studies corroborates the evidence of Wade et al. (1966) that dietary limonene gives rise to the uroterpenol that occurs in human urine. In addition to the presence of uroterpenol, uroterpenol glucuronide, and perillic acid, Igimi et al. (1974) also report the occurrence of 8,9-dihydroxy perillic acid in rat urine. However, we have no indication of the presence of the latter compound in rat urine.

The metabolites isolated in this study represent formal allylic and direct oxidations of the double bonds of limonene. Perillic acid (LM-7) and perillyl alcohol (LM-5) appear to arise by consecutive, allylic oxidations of C-7 of limonene. Compounds LM-1,3, LM-2,4, and LM-7 are three sets of diastereoisomers, which could be formed from limonene via the appropriate epoxides. These pathways are represented in Scheme I. Proposed intermediates are indicated in brackets and stereochemical assignments for the metabolites are relative. The possible role of epoxides A and B in limonene metabolism is being investigated.

LITERATURE CITED

Dean, F. M., Price, A. W., Wade, A. P., Wildinson, G. S., *J. Chem. Soc. C* **19**, 1893 (1967).
Dhavalikar, R. S., Bhattacharyya, P. K., *Indian J. Biochem.* **3**, 144 (1966).
Igimi, H., Nishimura, M., Kodama, R., Ide, H., *Xenobiotica* **4**, 77 (1974).
Kodama, R., Noda, K., Hiroyuki, I., *Xenobiotica* **4**, 85 (1974).
Mitzner, B. M., Mancini, V. I., Lemberg, S., Theimer, E. T., *Appl. Spectrosc.* **22**, 34 (1968).
Sato, T., *Nippon Kagaku Zasshi* **88**, 252 (1965).
Smith, O. W., Wade, A. P., Dean, F. M., *J. Endocrinol.* **45**, 17 (1969).
Wade, A. P., Wilkinson, G. S., Dean, F. M., Price, A. W., *Biochem. J.* **101**, 727 (1966).

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A New Liquid Chromatography Approach to Plant Phenolics. Application to the Determination of Chlorogenic Acid in Sunflower Meal

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A new technique developed for trace analysis of phenolic compounds combines the advantages of high performance liquid chromatography with thin-layer electrochemistry. Liquid chromatography with amperometric detection at a carbon paste electrode is shown to be ideally suited to the determination of chlorogenic acid in plant matter. Due to the low oxidation potential of chlorogenic acid, it is possible to quantitatively measure a little as 25 pg by chromatography on a polyamide stationary phase (50 cm × 2.1 mm) using 0.1 M citric acid as the mobile phase. A sample of commercial sunflower meal was found to contain 2.6 ± 0.1% (SD) of chlorogenic acid based on the dry weight of defatted meal.

A new approach to the trace analysis of phenolic compounds and aromatic amines has been under development in our laboratory. This approach involves the combination of high performance liquid chromatography with electrochemical (amperometric) detection (LCEC). The emphasis up to this point has been on the analysis of drugs and metabolites in body fluids (Kissinger et al., 1974, 1976; Riggin et al., 1975). This new technique has also proven to be invaluable for the assay of pharmaceutical dosage forms (Riggin et al., 1974), ascorbic acid in pharmaceuticals and food products (Pachla and Kissinger, 1976), and isoquinoline alkaloids in plant matter (Riggin et al., 1976). The fact that many of the phenolic compounds in urine are of dietary origin led us to explore the direct applicability of LCEC to phenolic acids in plant material. In the present paper we briefly review the

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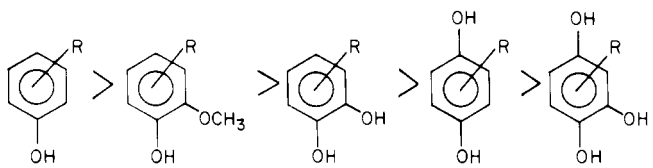
principles of this new technique and describe its use for one of the most ubiquitous plant phenolics, chlorogenic acid.

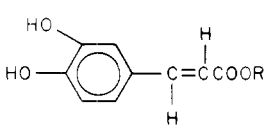
Several designs for electrochemical detectors have been developed recently (Kissinger, 1974; Fleet and Little, 1974) and a number of applications have already been reported. While high performance liquid chromatography has gained some acceptance for trace organic analysis, its applicability has been severely limited in some cases by the lack of a detector which is both sufficiently sensitive and selective for the molecule(s) under study (Kirkland, 1974). It is now apparent that in selected cases electrochemical detection can provide improved sensitivity and selectivity at significantly reduced cost.

The amperometric detectors operate on the principle that some molecules eluted from the chromatographic column can exchange electrons with an electrode surface under controlled conditions, and that the resulting current is proportional to the number of reactive molecules moving past the surface per unit time. The thin-layer amperometric transducer (Kissinger et al., 1974; Shoup and Kissinger, 1976) takes advantage of the fact that electrochemical reactions are often transport limited surface reactions by using a flowing sample stream which is an extremely thin film (typically 50 μm). In this manner the dead volume of the detector can be made extremely low (<1 μl) while the diffusional transport time to the electrode surface is minimized and the sensitivity is enhanced.

The common classes of monocyclic phenolics shown below exhibit a large range of oxidation potentials (ca. 1 V) from the extremely easily oxidized 2,4,5-trihydroxy



compounds to the more stable monophenols. In buffered aqueous media one must also take into account the large pH dependence of the threshold for oxidation. The different classes of compounds can be analyzed more or less selectively depending on their oxidation potential. Those compounds which oxidize very easily can be detected with great specificity by setting the detector potential to a low value. On the other hand, compounds which are difficult to oxidize can not be detected except at a potential where the more easily oxidized compounds will also react. Although chlorogenic acid and its precursor caffeic acid are both catechols, the presence of an acrylic acid group conjugated with the aromatic ring facilitates oxidation to



R = H (caffeic acid)
R = quinic acid (chlorogenic acid)

the corresponding *o*-quinone. It is thus possible to use LCEC to detect the presence of both compounds at very low levels.

EXPERIMENTAL SECTION

Sunflower seed meal (Natural Brand, distributed by the Natural Sales Co.) was purchased locally. The meal was defatted by Soxhlet extraction with petroleum ether (30–60 °C) for 18 h and then air-dried. One gram of the defatted meal was Soxhlet extracted with 80% ethanol for 12 h. The ethanol solution was concentrated to about 10 ml on a rotary evaporator and the residue dissolved in 50 ml of 1 M sodium acetate buffer (pH 4.7). The acetate solution was transferred to a 100-ml volumetric flask and diluted to the mark. An aliquot of this solution was diluted 1 to 20 with distilled water and 5 μl injected onto the chromatographic column. Quantitation was accomplished by peak height measurements compared to an external standard chlorogenic acid solution (Aldrich Chemical Co.).

A 50 cm × 2 mm i.d. commercial glass column (Altex Scientific, Model 251-02) packed with Pellidon polyamide (H. Reeve Angel & Co.) was used with a 0.1 M citric acid mobile phase. The chromatograph and electrochemical detector (Bioanalytical Systems Inc., Model LC-2) have been described elsewhere (Kissinger et al., 1974; Riggin et al., 1975).

Cyclic voltammetry experiments were carried out using a Bioanalytical Systems Model CV-1 equipped with a Hewlett-Packard Model 7015 XY recorder. A small (3.0 mm diameter) carbon paste electrode was used in a simple cylindrical cell with a side arm for a Ag|AgCl reference electrode. Details of the electrodes and the cell design will be furnished upon request.

Table I. Peak Potentials for the Oxidation of Selected Phenolic Acids at the Carbon Paste Electrode

Compound[a]	1 M acetate (pH 4.7)	1 M $HClO_4$
3,4-Dihydroxycinnamic (caffeic)	+0.35[b]	+0.62
Chlorogenic	+0.39	+0.62
3,4-Dihydroxyphenylpropionic (dihydrocaffeic)	+0.43	+0.60
3,4,5-Trihydroxybenzoic (gallic)	+0.46	+0.63
3,4-Dihydroxybenzoic (protocatechuic)	+0.52	+0.68
4-Hydroxy-3,5-dimethoxybenzoic (syringic)	+0.55	+0.83
4-Hydroxy-3-methoxycinnamic (ferulic)	+0.57	+0.81
4-Hydroxy-3-methoxybenzoic (vanillic)	+0.72	+0.94
4-Hydroxycinnamic (*p*-coumaric)	+0.73[c]	+0.92
4-Hydroxyphenylacetic	+0.77	+1.07
4-Hydroxybenzoic	+0.99	+1.20

[a] All samples were 0.5 mM solutions in the medium indicated. [b] Values reported in volts vs. Ag|AgCl at 200 mV/s. [c] Poorly defined anodic peak.

RESULTS AND DISCUSSION

That chlorogenic and caffeic acids are ideally suited to the LCEC technique is indicated by the cyclic voltammogram shown in Figure 1. Both molecules behave very similarly in that they exhibit almost ideal electrochemical behavior upon oxidation at a carbon electrode. The sharp anodic and cathodic peaks clearly indicate that the electron transfer rate is fast. This is very unusual for phenolic compounds. The well-behaved voltammogram is sufficient to suggest that LCEC will have a good sensitivity for chlorogenic acid. In fact, 25 pg can be quite easily detected for standard solutions. The response is linear at least up to 200 ng and a precision of better than ±3% relative standard deviation is adequate for most agricultural purposes.

Although the mechanistic electrochemistry of phenolic compounds is very poorly understood, the primary consideration for LCEC is the oxidation potential. Table I gives results for two electrolyte solutions commonly used to study phenolic oxidations. The relative ease of oxidation of caffeic acid and chlorogenic acid is clear. This suggests that they might be selectively detected in very complex mixtures of other phenols. Since the two compounds are very easily separated by HPLC they can be monitored simultaneously.

The quantitative assay of chlorogenic acid in sunflower

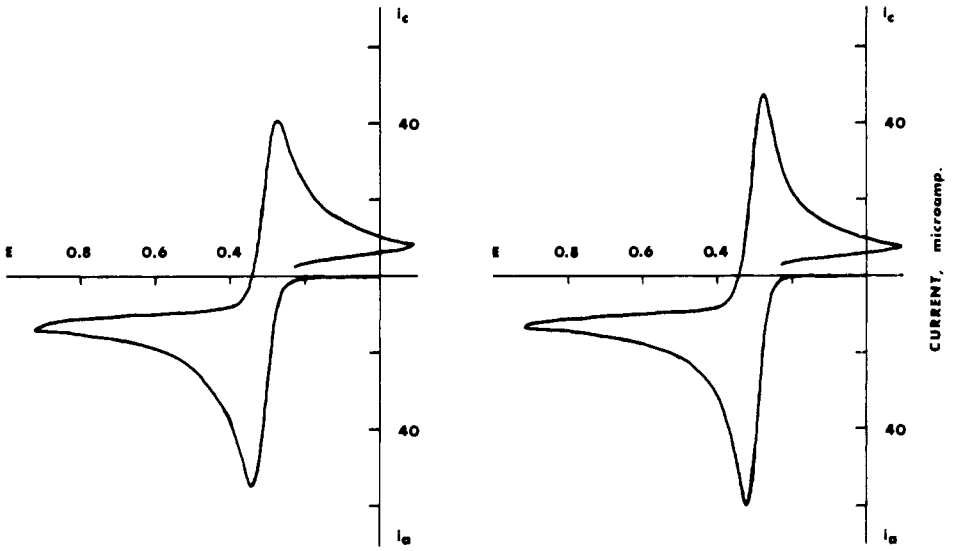


Figure 1. Cyclic voltammetry of chlorogenic acid (left) and caffeic acid (right) at a carbon paste electrode (3 mm diameter) in 1 M acetate buffer: sample concentration, 1×10^{-3} M; scan rate, 300 mV/s; reference electrode, Ag|AgCl (3 M NaCl).

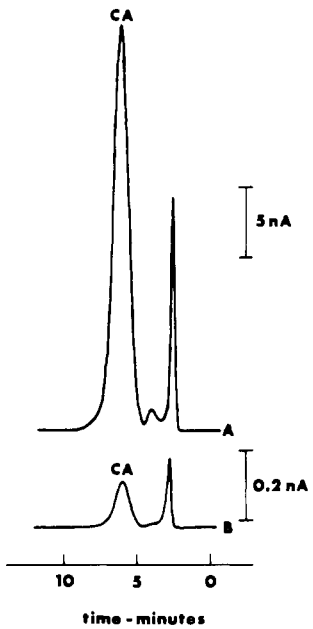


Figure 2. Liquid chromatogram of chlorogenic acid (CA): (A) extract from sunflower meal (100 ng of CA); (B) aqueous standard (500 pg of CA).

seeds, meal, and flour has been reported by several groups (Mikolajczak et al., 1970; Pomenta and Burns, 1971; Sabir et al., 1974). The present LCEC approach eliminates the need for time consuming paper, thin-layer, or gas–liquid chromatography techniques while at the same time providing considerably better sensitivity. Figure 2 illustrates the LCEC chromatogram of chlorogenic acid extracted from sunflower meal. We have only examined a single commercial lot of meal and our results indicate that chlorogenic acid represents 2.6 ± 0.1% (SD) of the dry weight of defatted meal. This value is in good agreement with the literature (Mikolajczak et al., 1970). Recent experiments indicate that the method reported here is also well suited to monitoring chlorogenic acid in coffee, apples, and tobacco products.

The present paper describes only one example of the wide variety of phenolic natural products which are suitable for analysis by both liquid chromatography and electrochemistry. The LCEC approach also has merit for assay of phenolic antioxidants in food products and for analysis of phenolic pesticide residues. In dealing with many such problems, the detector selectivity may not be sufficient due to the higher oxidation potential of the phenols in question. In these cases the combination of TLC with LCEC is very powerful. Both specificity and sample preparation are improved because large numbers of samples can be run in parallel and because the mechanism of separation is often very different than that used in the column. All components necessarily elute between R_f 0 and 1, whereas for LC there is no limitation on the retention time. This means that TLC–LC experiments on a large number of samples can often be carried out more quickly than LC alone because uninteresting components strongly retained on the LC column can be eliminated in the TLC cleanup. Furthermore, the TLC step will often prolong the life of very expensive HPLC packing materials.

LITERATURE CITED

Fleet, B., Little, C. J., *J. Chromatogr. Sci.* **12**, 747 (1974).
Kirkland, J. J., *Analyst* **99**, 859 (1974).
Kissinger, P. T., *Anal. Chem.* **46**, 15R (1974).
Kissinger, P. T., Felice, L. J., Riggin, R. M., Pachla, L. A., Wenke, D. C., *Clin. Chem. (Winston-Salem, N.C.)* **20**, 992 (1974).
Kissinger, P. T., Riggin, R. M., Alcorn, R. L., Rau, L.-D., *Biochem. Med.* **10**, in press (1976).
Mikolajczak, K. L., Smith, C. R., Jr., Wolff, I. A., *J. Agric. Food Chem.* **18**, 27 (1970).
Pachla, L. A., Kissinger, P. T., *Anal. Chem.* **43**, in press (1976).
Pomenta, J. V., Burns, E. E., *J. Food Sci.* **36**, 490 (1971).
Riggin, R. M., McCarthy, M. J., Kissinger, P. T., *J. Agric. Food Chem.* **24**, in press (1976).
Riggin, R. M., Rau, L.-D., Alcorn, R. L., Kissinger, P. T., *Anal. Lett.* **7**, 791 (1974).
Riggin, R. M., Schmidt, A. L., Kissinger, P. T., *J. Pharm. Sci.* **64**, 680 (1975).
Sabir, M. A., Sosulski, F. W., Kernan, J. A., *J. Agric. Food Chem.* **22**, 572 (1974).
Shoup, R. E., Kissinger, P. T., *Chem. Instrum.*, submitted for publication, 1976.

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