Journal of Chromatography, 384 (1987) 371–382
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 989

STRUCTURAL CLASSIFICATION OF FLAVONOIDS IN BEVERAGES BY LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET–VISIBLE AND ELECTROCHEMICAL DETECTION

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SUMMARY

Liquid chromatography with both UV–VIS and electrochemical detection is used to structurally classify flavonoid compounds in wine and grape juice without isolation of the pure compound. Compounds are classified as flavonols, proanthocyanidins, or anthocyanidins, based on their absorption maxima. Catechol substituted compounds are identified using a dual electrode detector.

INTRODUCTION

Flavonoids are a large group of plant secondary metabolites based on the structure of 2-phenylbenzopyrone (Fig. 1). Thousands of flavonoids are known to exist in nature[1]. They differ from one another in the degree of unsaturation, the pattern of hydroxylation or methylation, and type of sugar attached. The most common flavonoids fall into three general classes: proanthocyanidins, flavonols, and anthocyanidins (Fig. 2).

Standards are not available for many of the flavonoids, making identification by direct comparison difficult. The traditional method for flavonoid identification is UV–VIS absorption spectroscopy following isolation by preparative chromatography[1–3]. Each class of flavonoid has unique spectral characteristics. Specific reagents are used in order to determine hydroxyl substitution. For example, orthohydroxylated (catechol) compounds exhibit a bathochromic shift in the presence of aluminum chloride[2].

Photodiode array detectors make it possible to obtain the spectrum of an unknown flavonoid during a single chromatographic run[4]. Flavonoids can be classified

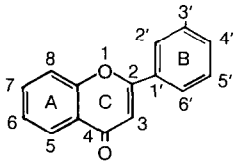

Fig. 1. 2-Phenylbenzopyrone: basic structure of flavonoids.

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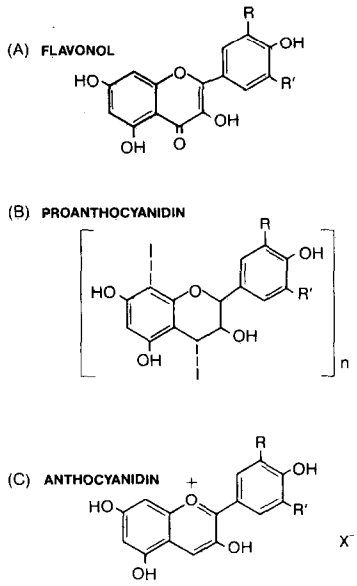

Fig. 2. Three types of flavonoids most commonly found in beverages: (A) flavonols, (B) proanthocyanidins, (C) anthocyanidins. R,R' = H, OH or OCH_3.

as proanthocyanidins, flavonols, or anthocyanidins without isolation of the pure compound, based on their absorption spectrum. However, it is difficult to determine the hydroxyl substitution of a flavonoid from the UV–VIS spectrum alone. Hostettmann *et al.*[4] have used a photodiode array detector with post-column mixing of shift reagents to determine the pattern of hydroxylation.

Liquid chromatography with electrochemical detection (LC–ED) has been used previously for the determination of phenolic compounds[5–9] in foods and beverages. Two electrodes in series can be used to enhance the selectivity for chemically reversible species such as catechols (Fig. 3)[10–15]. The ratio of the response of the downstream electrode to the upstream electrode is dependent on the electrochemical properties of the analyte[14]. In the case of phenols, the electrochemical behavior is directly related to the structure of the compound. Presented here is the combined use

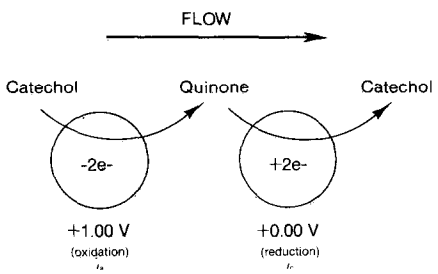

Fig. 3. Dual-electrode detection of catechol substituted compounds.

of a photodiode array detector and an electrochemical detector for the classification of flavonoids in beverage samples[16,17].

MATERIALS AND METHODS

Equipment

The liquid chromatographic system was a Varian Vista 5500 (Walnut Creek, CA, U.S.A.). Photodiode-array detection was accomplished with a LKB 2140 rapid spectral detector (Bromma, Sweden) which was interfaced to an IBM computer (Boca Raton, FL, U.S.A.). For single-wavelength detection a Kratos Spectroflow 773 UV–VIS variable-wavelength detector (Ramsey, NJ, U.S.A.) was used. Dual-electrode LC–ED experiments were performed with a Bioanalytical Systems LC-4B-17 amperometric detector (West Lafayette, IN, U.S.A.). Two glassy-carbon working electrodes were used, and all potentials are reported with reference to a Ag/AgCl reference electrode. Cyclic voltammetry experiments were performed with the BAS-100 electrochemical analyzer (Bioanalytical Systems).

Liquid chromatographic conditions

Flavonoids were separated by gradient elution. Solvent A was 0.05 *M* ammonium phosphate buffer at pH 2.5, solvent B was acetonitrile. The gradient was linear from 5 to 25% B over 50 min. The mobile phase was sparged with helium prior to and throughout the analysis to prevent bubble formation. The flow-rate was 1.5 ml/min. The Beckmann Altex C_{18} 5-μm column (25 cm × 4.6 mm I.D.) (Berkley, CA, U.S.A.), employed in all studies, was protected by a Brownlee C_{18} 5-μm pre-column (Santa Clara, CA, U.S.A.) and was thermostatted at 35°C. A 100-μl sample loop was used.

Chemicals

Chemicals were purchased from the following sources: catechin, epicatechin, quercetin, rutin, kaempferol were from Sigma (St. Louis, MO, U.S.A.); myricetin, myricitrin, kaempferol 3-rhamnosylglucoside, quercetin 3-arabinoside, kaempferol 7-neohesperidoside from Carl Roth (Karlsruhe, F.R.G.); orthophosphoric acid from E.M. Science (Cherry Hill, NJ, U.S.A.); ammonium hydroxide from J. T. Baker (Phillipsburg, NJ, U.S.A.). Procyanidins B-2, B-1, and C-1 were generous gifts from A. G. H. Lea (Long Ashton Research Station, Bristol, U.K.) and procyanidin B-4 was a kind gift from E. Haslam of the University of Sheffield (Sheffield, U.K.).

High-performance liquid chromatographic (HPLC) sample preparation

Ernest and Julio Gallo Burgundy and Welch's Grape Juice were used to illustrate this method. Flavonoids were separated from the phenolic acids using a C_{18} Sep-Pak cartridge (Waters Assoc., Milford, MA, U.S.A.). After applying 1 ml of beverage to the Sep-Pak cartridge, the sample was washed with 3 ml of water in order to remove sugars and organic acids. The phenolic acids were eluted with 1 ml of 1 *M* ammonium hydroxide and flavonoids were eluted with 1 ml of methanol. The resulting sample was diluted with an equal volume of mobile phase prior to injection. Untreated beverage samples were filtered through a 0.45-μm cellulose acetate filter prior to injection.

Cyclic voltammetry experiments

Cyclic voltammetry experiments were performed in a 0.05 *M* ammonium phosphate buffer (pH 2.5)–methanol (1:1, v/v) solution. Voltammograms were obtained at a scan-rate of 100 mV/s. Sample concentrations were approximately 1 m*M*. The three-electrode system consisted of a glassy-carbon working electrode, a Ag/AgCl reference electrode, and a platinum auxiliary electrode.

RESULTS

Electrochemical behavior of flavonoids

Cyclic voltammograms of quercetin 3-rutinoside (rutin) and kaempferol 3-rhamnosylglucoside are shown in Figs. 4 and 5. Rutin exhibits an anodic wave for the chemically reversible oxidation of the catechol moiety on the B ring to the quinone. On the reverse scan, a cathodic wave due to the reduction of this quinone is seen (Fig. 4). Kaempferol 3-rhamnosylglucoside, which is not chemically reversible, exhibits only an anodic wave for the oxidation of phenol to a radical species (Fig. 5). No cathodic wave is seen on the reverse scan for this compound, because the free radical reacts rapidly with solution components to give a product which is not electroactive. The oxidation of the 3′,4′,5′-trihydroxy derivative (myricetin) is also chemically irreversible.

Determination of collection efficiencies

The dual-electrode thin-layer detector cell was used in the series configuration, as illustrated in Fig. 3. The upstream electrode was operated at a potential of +1.00 V. At this potential all of the flavonoids studied are oxidized. The downstream electrode was operated at a potential negative enough to reduce the oxidation products produced at the upstream electrode. A potential of 0.00 V was chosen based on cyclic voltammetry experiments (Fig. 4) and selectivity considerations.

Collection efficiency, N_0, is defined as the fraction of upstream products which

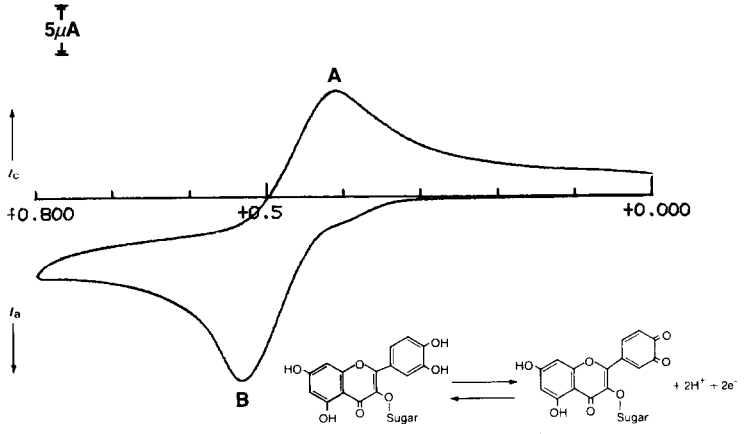

Fig. 4. Cyclic voltammogram of quercetin 3-rutinoside. (A) Oxidation to quinone; (B) reduction of quinone.

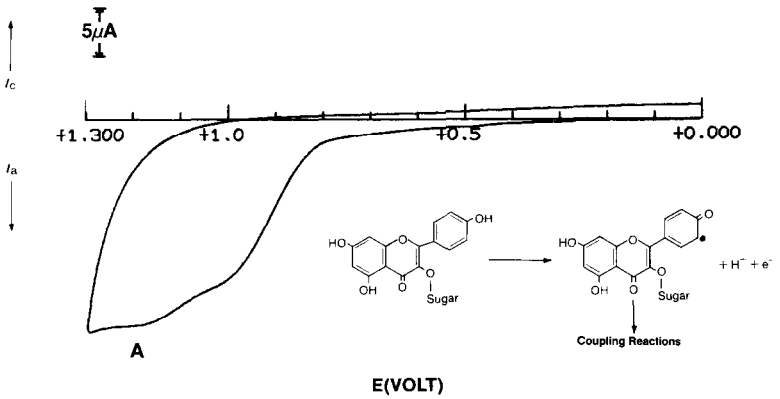

Fig. 5. Cyclic voltammogram of kaempferol 3-rhamnosylglucoside. (A) Oxidation to radical species.

are converted at the downstream electrode. It can be measured by taking the ratio of the current response at the upstream electrode to that at the downstream electrode[14]. Compounds which are chemically reversible, such as catechols and hydroquinones, exhibit high collection efficiencies. The compound produced by oxidation at the upstream electrode is stable and does not undergo further chemical reactions prior to its detection at the downstream electrode. In this case, the amount of product reaching the downstream electrode is entirely dependent on mass transport. The maximum collection efficiency attainable with this thin-layer cell design is 37% (ref. 14).

Compounds, like monophenols, that form chemically unstable products on oxidation exhibit low collection efficiencies. The product produced by oxidation at

TABLE I

COLLECTION EFFICIENCIES FOR SOME REPRESENTATIVE FLAVONOIDS

N_0 = Percent collection efficiency = (downstream/upstream) × 100. S.D. = Standard deviation of three trials.

Standard	N_0 *(%)*	*S.D.*
Myricetin 3-rhamnoside	7.4	0.5
Quercetin 3-arabinoside	32.5	1.3
Quercetin 3-rhamnoside	34.0	4.2
Quercetin 3-rutinoside	29.8	1.1
Quercetin	11.3	0.9
Kaempferol 3-rhamnosylglucoside	1.1	0.20
Kaempferol 7-neohesperidoside	2.8	0.40
Kaempferol	1.7	0.29
Myricetin	1.8	0.15
Catechin	12.1	1.0
Epicatechin	11.4	1.2
Procyanidin B-2	10.6	2.1
Procyanidin B-4	10.4	0.84
Procyanidin C-1	9.6	0.23
Procyanidin B-1	12.6	0.15
Pelargonidin	7.6	0.51

the upstream electrode is unstable and reacts to form a second compound which is not reducible. The decrease in magnitude of the collection efficiency is dependent on the decomposition rate of the reactive species. In the case of an extremely fast reaction, there will be no response at the downstream electrode[14].

The collection efficiency of a compound can provide information about the hydroxyl substitution of that compound. Table I gives the collection efficiencies for several different flavonoids. Quercetin glycosides show the highest degree of reversibility with collection efficiencies of approximately 30%. These compounds are all reversibly oxidized to quinones. The sugar-substituted quercetins exhibit higher collection efficiencies than their corresponding aglycones. The sugar moiety increases the hydrophilicity of the quinone produced at the upstream electrode, reducing the amount of adsorption on that electrode.

Catechins and procyanidins have collection efficiencies of *ca.* 10%. This is in the same range as quercetin aglycone. Quinones produced by the oxidation of these compounds are more hydrophobic than the corresponding glycosides and are adsorbed on the carbon electrode. Cyclic voltammetry experiments of aglycones show them to be less reversible than the corresponding glycosides. Procyanidin polymers have approximately the same collection efficiency values as the corresponding monomers.

Kaempferol, myricetin, pelargonidin, and their derivatives exhibit the lowest degree of chemical reversibility, with values of less than 8%. These flavonoids are mono- or trihydroxyphenols. Each is oxidized to an unstable radical species, which can react with solvent components.

Dual-electrode LC–ED of flavonoids

The high collection efficiencies exhibited by catechol-substituted flavonoids can

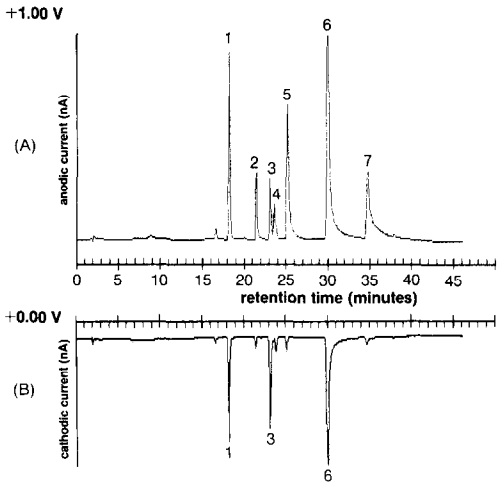

Fig. 6. Use of dual-electrode LC–ED to identify catechol substituted compounds. (A) Upstream electrode +1.00 V *versus* Ag/AgCl (500 nA f.s.). (B) Downstream electrode +0.00 V *versus* Ag/AgCl (125 nA f.s.). Identities: (1) epicatechin, (2) myricetin 3-rhamnoside, (3) quercetin 3-arabinoside, (4) kaempferol 7-neohesperidoside, (5) myricetin, (6) quercetin and (7) kaempferol.

be used in their identification. As an example, chromatograms of a mixture of epicatechin, three flavonol glycosides, and three flavonol aglycones are shown in Fig. 6. The upstream electrode of the dual-electrode detector is operated at +1.00 V. At this potential, all of the flavonoids present in the mixture are oxidized (Fig. 6A).

The response at the downstream electrode is shown in Fig. 6B. Only three compounds exhibit high collection efficiencies at the second electrode. Epicatechin, quercetin 3-rutinoside and quercetin are oxidized to quinones, which are reduced at the downstream electrode. Myricetin 3-rhamnoside, myricetin, and kaempferol produce unstable radical species upon oxidation, and show very little response at the downstream electrode.

Liquid chromatography with combined electrochemical and UV–VIS detection

As is apparent from the preceding section, the collection efficiencies can be used to obtain information regarding the hydroxylation pattern of flavonoids. When used in combination with a UV–VIS or photodiode-array detector, the structure of the parent flavonoid can be determined.

Liquid chromatography can be combined with UV–VIS detection and ED for the classification of flavonoids as follows. The flavonoids are divided into proanthocyanidins, flavonols, and anthocyanidins, based on their absorption maxima. Proanthocyanidins have one absorption maximum at 280 nm. Flavonols have maxima at both 280 and at 360 nm. Anthocyanidins are readily distinguishable by their absorbance in the visible region around 525 nm[1–3,18–21]. Table II gives the ranges for the absorption maxima of proanthocyanidins, flavonols, and anthocyandins.

ED is used to distinguish the orthodihydroxylated flavonoids from the mono- and trihydroxy compounds. Catechol derivatives have high collection efficiencies (10–39%), while mono- and trihydroxylated flavonoids have very low collection efficiencies (less than 10%). In general, the methoxylated derivatives exhibit lower collection efficiencies than their hydroxylated analogs[14]. In many cases, monophenols can be distinguished from trihydroxy compounds on the basis of their hydrophobicities, as inferred from relative retention times[18–25].

Grape juice analysis

As an example of the use of a photodiode-array detector in conjunction with an electrochemical detector, the analysis of a neutral extract of grape juice is shown in Figs. 7 and 8. Three flavonols were detected at 360 nm (Fig. 7). These three compounds were further classified on the basis of their electrochemical behavior. Fig. 8 shows the LC–ED chromatogram obtained for this sample. The response at the upstream electrode is shown in Fig. 8A. Fig. 8B is the chromatogram obtained at the

TABLE II

ABSORPTION MAXIMA FOR FLAVONOID COMPOUNDS

Compound	*Max (1) (nm)*	*Max (2) (nm)*
Anthocyanidins	270–280	475–560
Flavonols	250–270	350–390
Proanthocyanidins	275–290	–

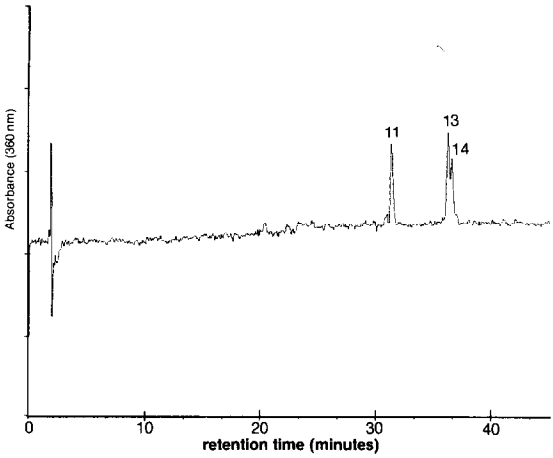

Fig. 7. Chromatogram of grape juice neutral extract at 360 nm.

downstream electrode. Based on spectral information and collection efficiency values, several flavonoids are identified in the grape juice sample. The spectrum obtained with the photodiode-array detector shows that peaks 11, 13 and 14 are flavonols. Peak 11 is a chemically irreversible flavonol glycoside. Based on the retention characteristics, it is probably a myricetin glycoside or a kaempferol diglycoside. Peaks 13 and 14 are quercetin glycosides with collection efficiencies of 26 and 25%, respectively.

Table III shows the data for several of the peaks present in the grape juice neutral extract. Peaks 3 and 8 are identified as catechin and epicatechin. They have collection efficiencies of about 10%, absorption maxima of 278 nm and are inseparable from standard compounds. Peaks 9 and 10 are procyanidins. They have absorption maxima of *ca.* 280 nm and collection efficiencies of *ca.* 10%.

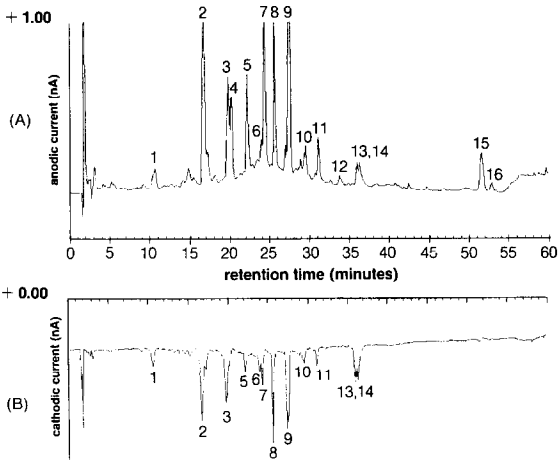

Fig. 8. Dual-electrode LC–ED chromatograms of grape juice neutral extract.

TABLE III

FLAVONOIDS IN GRAPE JUICE (SEE FIG. 8)

N_0 = Collection efficiency; t_R = retention time; ND = not detected.

Peak No.	t_R *(min)*	*UV-Max (1) (nm)*	*UV-Max (2) (nm)*	*LC–ED* N_0 *(%)*	*Identity*
1	10.69	ND		17.0	
2	16.77	276		5.2	Prodelphinidin
3	19.84	(280)		13.0	Catechin
4	20.26	313		1.4	
5	22.19	310		3.4	
6	24.03	280		16.5	Procyanidin
7	24.37	309	525	3.5	Anthocyanidin*
8	25.67	277		10.7	Epicatechin
9a	27.44	278		8.8	Procyanidin
9b	27.64	278		7.8	Procyanidin
10	29.63	280		10.2	Procyanidin
11	31.23	258	370	7.4	Myricetin glucoside
12	33.53	310	525	2.1	Anthocyanidin*
13	36.07	255	355	26.4	Quercetin glycoside
14	36.39	255	355	24.5	Quercetin glycoside
15	51.67	ND		1.1	
16	52.92	ND		0.9	

* Delphinidin-*p*-coumaryl glycoside or methoxy derivative.

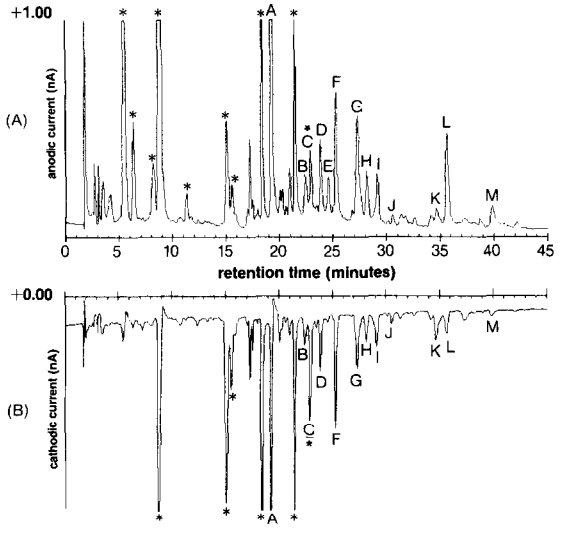

Fig. 9. Dual-electrode LC–ED chromatogram of wine sample.

Peaks 7 and 12 have two distinct absorption maxima. They are classified as anthocyanidins on the basis of their visible absorption at 525 nm. However, they have a second maximum at 310 with a UV spectrum identical with *p*-coumaric acid. Collection efficiencies for both compounds are very low, indicating a mono- or trihydroxylated compound.

Anthocyanidin-*p*-coumaryl glycosides have previously been reported to be present in wine and grape juice[20,21]. Based on spectral and electrochemical data, peaks 7 and 12 are most likely delphinidin or pelargonidin-*p*-coumaryl glycosides. The methoxy derivative, malvidin 3-*p*-coumaryl glycoside, is another possibility, since it should be less chemically reversible than the delphinidin derivative[14].

Peaks 4 and 5 are presently unidentified. These compounds have a maximum of *ca.* 310 nm and collection efficiencies of less than 2%. These data indicate that they may be esters and/or glycosides of coumaric acid.

Flavonoids in wine

Fig. 9 shows the LC–ED chromatogram of an untreated wine sample. The compounds marked with asterisks are phenolic acids. These compounds are removed during sample preparation by washing the Sep-Pak with 1 *M* ammonium hydroxide. The acids can be classified separately by a similar method[14,17]. Peaks K, L, and M have characteristic flavonol absorption spectra, with a maximum at 360 nm. A chromatogram of the wine sample at 360 nm is shown in Fig. 10A. The collection efficiencies for the three compounds K, L, and M are 33.3, 5, and 3.9%, respectively. K is a quercetin glycoside. Based on relative retention time, spectrum, and collection efficiency, L is classified as a kaempferol derivative and M appears to be a myricetin aglycone.

A chromatogram of the whole wine sample at 525 nm is shown in Fig. 10B. The peak labelled J absorbs at 525 and 310 nm and has a collection efficiency of 10%. It is probably a delphinidin or pelargonidin-*p*-coumaryl glycoside. Peaks labeled D, G, H, and I are classified by this method as procyanidins. Peak E is classified

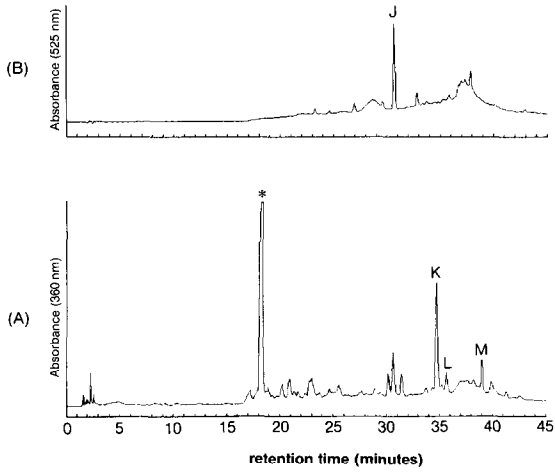

Fig. 10. (A) Chromatogram of a wine sample at 360 nm. (B) Same sample at 525 nm.

TABLE IV

FLAVONOIDS IN WINE (SEE FIG. 9)

Peak No.	t_R *(min)*	*UV-Max (1) (nm)*	*UV-Max (2) (nm)*	*LC–ED* N_0 *(%)*	*Identity*
A	19.44	280		13.4	Catechin
B	22.46	280		11.1	Procyanidin
C	23.00	280		33.0	Caffeic acid
D	23.93	280		10.4	Procyanidin
E	24.63	280		1.9	Prodelphinidin
F	25.37	280		15.9	Epicatechin
G	27.39	280		10.1	Procyanidin
H	28.22	280		12.8	Procyanidin
I	29.21	280		14.3	Procyanidin
J	30.59	310	525	10.0	Anthocyanidin*
K	34.70	280	360	31.0	Quercetin glycoside
L	35.75	280	360	3.7	Myricetin or kaempferol glycoside
M	39.93	280	360	3.9	Myricetin

* Delphinidin-*p*-coumaryl glycoside.

as a prodelphinidin. It is detected at 280 nm and has a low collection efficiency. A list of retention times, absorption maxima, collection efficiencies and tentative identifications for each of the peaks in Fig. 9 is given in Table IV.

CONCLUSIONS

The combination of a dual-electrode amperometric detector and a photodiode-array detector permits classification of complex mixtures of flavonoids on the basis of their conjugation pattern and hydroxyl substitution in a single chromatographic run without need for component isolation. If a photodiode array detector is not available, the same general scheme can be employed using a variable-wavelength detector. Chromatograms at 280, 360, and 525 nm are sufficient to classify the peaks as either proanthocyanidins, flavonols, or anthocyandins. The electrochemical detector can then be used to determine the degree and positions of hydroxylation.

ACKNOWLEDGEMENTS

Special thanks to S. Kirksey (The Procter & Gamble Co., Cincinnati, OH, U.S.A.) for designing the sample preparation step.

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