Application of High Performance Liquid Chromatography to Analysis of Flavonoids and Phenyl Propenoids[1]

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INTRODUCTION

Phenyl propenoids and flavonoids are widely distributed in the plant kingdom (Harborne 1964). Many diverse structures have been reported (Harborne and Mabry 1982). For simplicity this paper will be limited to a discussion of the hydroxycinnamates, catechins, related proanthocyanidins, the anthocyanins, and their analysis by high performance liquid chromatography (HPLC). No attempt will be made to give an exhaustive coverage of the literature relating to this topic. Instead, examples of methods that can be used for analysis of each class of compounds will be presented.

The basic structure of the hydroxycinnamates is shown in Figure 1. As with most of the phenolic compounds, the phenyl ring can have substitution groups consisting of hydroxyl or methoxyl groups. Thus the basic cinnamate can either be *p*-coumaric, caffeic, ferulic, or sinapic acid. Generally the acids do not occur in the free form, but instead are esterified. One of the most common ester groups is quinic acid. This compound is called chlorogenic acid when the cinnamate is caffeic acid and is the most widely distributed form (Van Buren 1970). However, the cinnamates can be esterified to other compounds such as tartaric acid, glucose (Van Buren 1970), glycerol, and hydroxy fatty acids (Daniels and Martin 1967, 1968). In addition, it has been shown that some of the cinnamate hydroxyls can be glycosylated, usually with glucose (Chu et al 1973, Marwan and Nagel 1982).

There are two major isomers of catechin (Fig. 2), catechin and epicatechin, the former being in the trans form and the latter representing the cis form. Related to the catechins are the proanthocyanidins, or condensed tannins (Gupta and Haslam 1980), usually consisting of either catechin and/or epicatechin as structural units (Fig. 3). Not only procyanidins but prodelphinidin and propelargonidin have also been demonstrated to occur in some plant materials (McMurrough et al 1983). The proanthocyanidins are receiving considerable attention because of their involvement in haze problems in fruit juices and beers (Jende-Strid and Moller 1981) and because of their possible antinutrient effect, or inhibition of digestion of proteins (Bressani and Elias 1980).

There are a number of anthocyanidins based upon substitutions of either hydroxyl or methoxyl groups in the B ring (Fig. 4). The anthocyanidins do not occur in the free form but are usually glycosylated. The most common substitution is β-D-glucose at carbon 3, but other monosaccharides, and in some cases disaccharides or trisaccharides, may be found. In addition glycosylation can also occur at carbon 5. Thus in *Vitis labrusca* grapes the 3,5-diglucoside is found. Furthermore, the sugars themselves can be acylated, usually with acetic acid or one of the hydroxycinnamates (Van Buren 1970).

EXTRACTION

Often minimal sample preparation is required prior to HPLC analysis. However, if the material has a high content of fats or waxes it may be useful to remove them by extraction with a

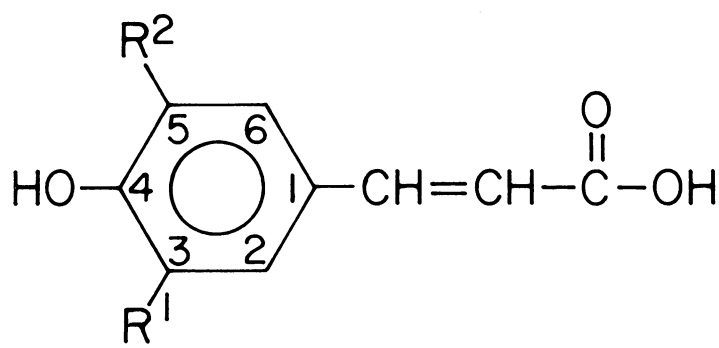

Fig. 1. Basic structure of the hydroxycinnamates. R^1,R^2=H, *p*-coumaric acid; R^1=OH, R^2=H, caffeic acid; $R^1=OCH_3$,R^2=H, ferulic acid; $R^1,R^2=OCH_3$, sinapic acid.

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nonpolar solvent such as hexane or benzene (Jende-Strid and Moller 1981). If proteins or polysaccharides are a problem, the sample should be extracted with 67–80% (v/v) methanol or ethanol, or 75% (v/v) acetone (McMurrough et al 1981, Jende-Strid and Moller 1981). The extract should then be filtered and evaporated, transferring it to an aqueous environment. If solubility is a problem, one can add 10–20% (v/v) methanol. An alternative method, which may be preferred because it would protect the analytical column, is to extract the materials in an acidified aqueous medium, filter, and pass them through a C_{18} Sep Pak (Waters Associates, Milford, MA). The disposable C_{18} extraction column is then washed with water and the sample eluted with methanol. The sample can then either be diluted with water or evaporated and taken up in water. If the methanol extract is sufficiently concentrated one can inject small amounts (10–15 μl) without affecting retention time severely.

All of the compounds of interest have relatively specific absorption maxima. Thus the catechins and proanthocyanidins can be most easily detected at a wavelength setting of 280 nm, the hydroxycinnamates at 307–333 nm, and the anthocyanins at 510–535 nm. For the hydroxycinnamates we usually use a wavelength setting of 320 nm, and for the anthocyanins, 520 nm. By using these wavelengths one can limit interference by other compounds. The shorter the wavelength the greater the possibility of encountering other compounds that can interfere. Sometimes, depending upon the mixture, it may be necessary to remove the anthocyanins prior to analysis of the catechins and proanthocyanidins. If this is necessary, the catechins and proanthocyanidins, as well as the hydroxycinnamates, can be extracted from an aqueous medium with ethylacetate (Nagel and Wulf 1979, Mulkay et al 1981, Jende-Strid and Moller 1983). Under acetic conditions the anthocyanins will remain in the aqueous phase. The ethyl acetate is then removed by evaporation and the compounds redissolved in water or a small amount of methanol and water.

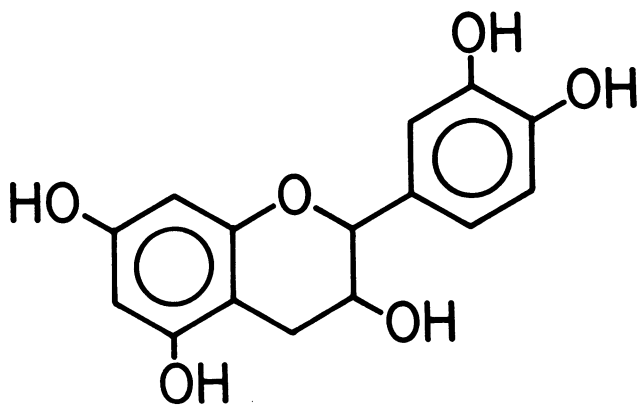

Fig. 2. Structure of catechin.

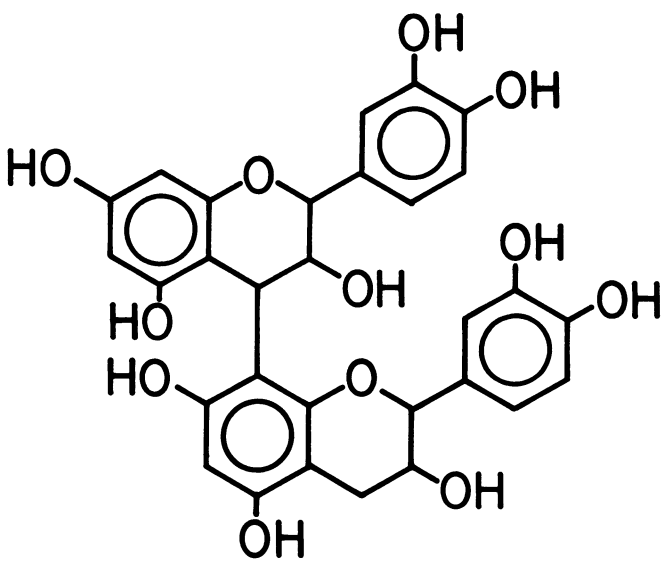

Fig. 3. Structure of procyanidin with two catechin units.

HPLC ANALYSIS

Because the compounds of interest are water soluble with varying degrees of polarity, reversed-phase columns are ideal for

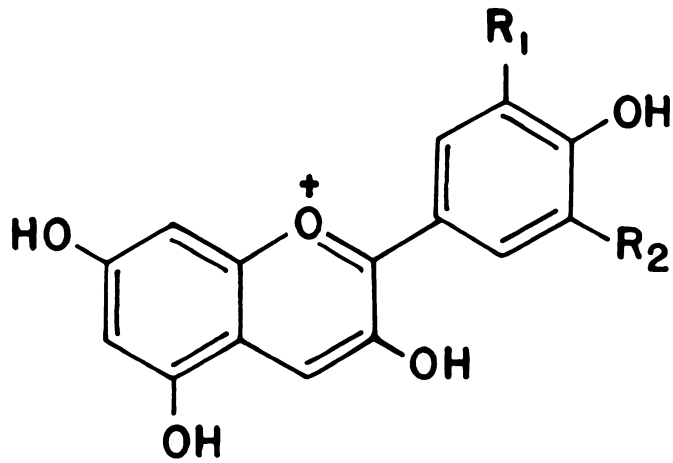

Fig. 4. Anthocyanidins: delphinidin, $R_1=R_2=OH$; petunidin, $R_1=OCH_3, R_2=OH$; malvidin, $R_1=R_2=OCH_3$; cyanidin, $R_1=OH, R_2=H$; peonidin, $R_1=OCH_3, R_2=H$.

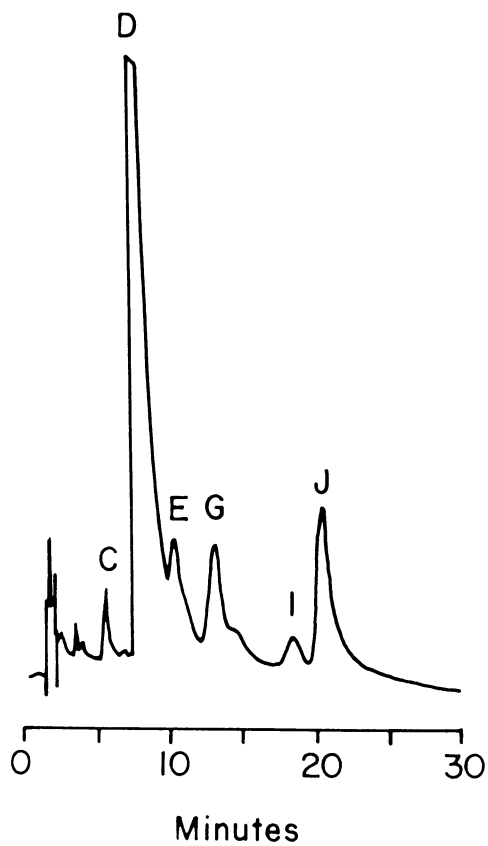

Fig. 5. High performance liquid chromatography separation of White Riesling wine: **(C)** *cis*-caffeoyl tartrate, **(D)** *trans*-caffeoyl tartrate, **(E)** *cis-p*-coumaroyl tartrate, **(G)** *trans-p*-coumaroyl tartrate, **(I)** *trans*-feruloyl tartrate, **(J)** *trans*-caffeic acid. From Baranowski and Nagel (1981).

their separation. Either the C_8 or C_{18} column can be used. Most of the HPLC literature is concerned with use of the octadecyl (C_{18}) reversed-phase column. The solvent system should include an acid to suppress hydroxycinnamate ionization or to ensure that the anthocyanins are in the flavylium form. Because of this, we used up to 10% (w/v) formic acid in the solvent system (Wulf and Nagel 1978), and 10% (w/v) acetic acid has also been used (Williams et al 1978). One concern is that extensive use of solvents more acidic than pH 2 may result in hydrolysis of the aliphatic substitution groups. For this reason we have reduced the concentration of formic acid to less than 2% (w/v), which gives values above pH 2; and in some cases we have attained adequate separation with concentrations as low as 0.1% (w/v). Formic acid is a stronger acid than acetic acid. Therefore, smaller concentrations are necessary to obtain the same pH. In our research with anthocyanins we have used 0.01 *M* trifluoroacetic acid where we wanted to recover the compounds and remove the acid by evaporation.

Aqueous solutions of methanol and acetonitrile are most commonly used with reversed-phase columns. Because of the high viscosity of methanol-water mixtures, we prefer to use acetonitrile. Acetone has been used for separation of the anthocyanins (Nagel and Wulf 1979). Because of its high absorptivity, it cannot be used for analysis of compounds that absorb in the ultraviolet region.

Isocratic conditions are preferred for quantitative analysis because of the greater reproducibility. The problem with gradient elution is that it is difficult to reproduce the conditions, although the reliability of equipment has improved considerably. However, in some cases only gradient elution will result in proper separation of the compounds of interest in a reasonable period of time.

HPLC OF THE CINNAMATES

The separation of the tartrate esters of the hydroxycinnamic acids of grapes is shown in Figure 5. The elution solvent consisted of 9% (v/v) aqueous acetonitrile in 0.5% (w/v) formic acid (Baranowski and Nagel 1981). The separation of the glucose esters and ethers of the hydroxycinnamates of cranberries is shown in Figure 6 (Marwan and Nagel 1982). In this case 8% (v/v) acetonitrile in 0.5% (w/v) formic acid was used to obtain the separation. Casteele et al (1983) separated a large number of phenolic compounds including derivatives of cinnamic acids on a reversed-phase C_{18} column using a sophisticated gradient elution pattern with a solvent system of formic acid, water, and methanol.

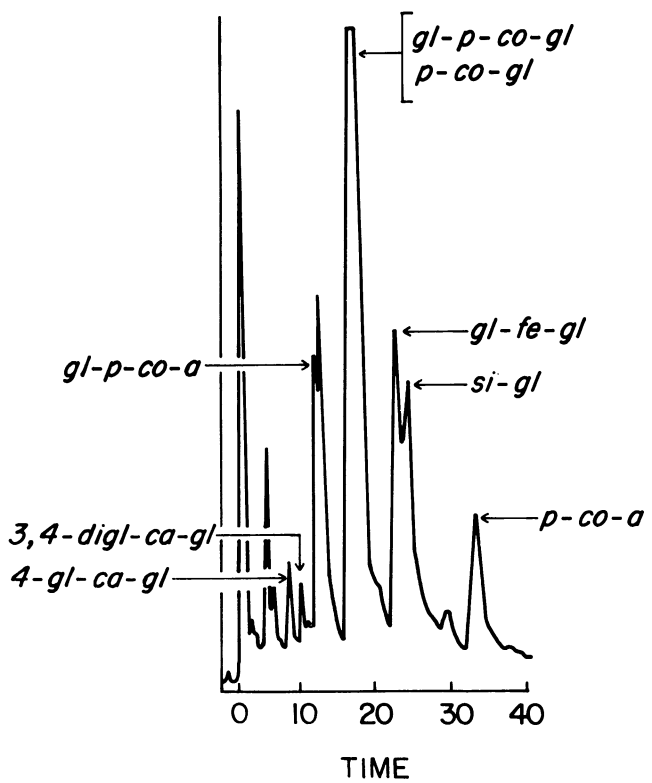

Fig. 6. High performance liquid chromatography separation of the hydroxycinnamic acid derivatives of cranberry. (Reprinted, by permission of the authors and the Institute of Food Technologists, from Marwan and Nagel, 1982. J. Food Sci. ©Institute of Food Technologists.)

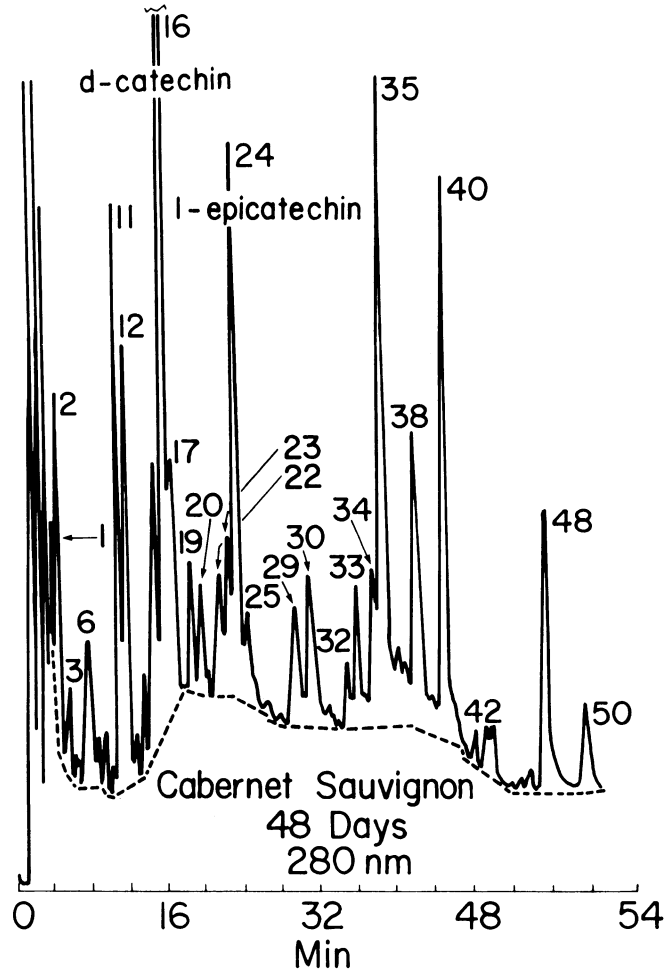

Fig. 7. Separation of the ethyl acetate extract of Cabernet Sauvignon wine. From Nagel and Wulf (1979).

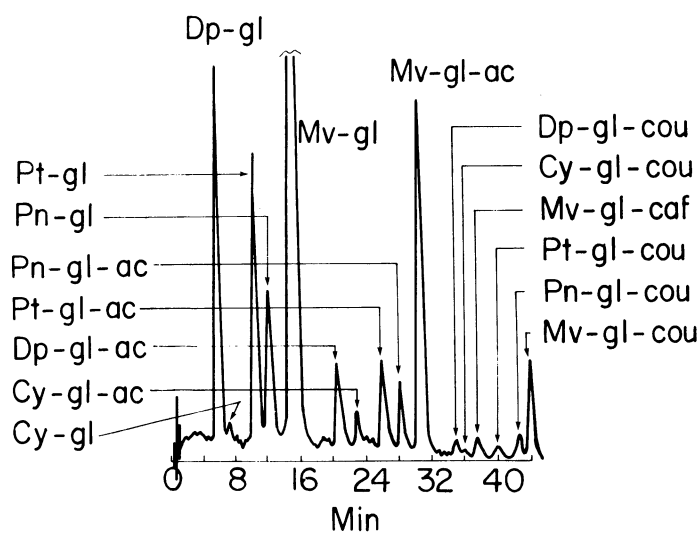

Fig. 8. High performance liquid chromatography separation of the anthocyanins of Cabernet Sauvignon. From Nagel and Wulf (1979).

Court (1977) used gradient elution with methanol and 0.1*M* potassium dihydrogen phosphate to demonstrate the presence of a number of hydroxycinnamic acid derivatives and flavonols in tobacco.

HPLC OF CATECHINS AND PROANTHOCYANIDINS

The separation of more than 50 components of an ethyl acetate extract of Cabernet Sauvignon wine is shown in Figure 7 (Nagel and Wulf 1979). To prevent interference by the hydroxycinnamic acid esters, a 1% acetic acid solution was adjusted to pH 4 with sodium hydroxide. Then a gradient of from 4.8 to 32% acetonitrile in 65 minutes was used, but even with this long, linear gradient the base line drifted. In any case, the sample undoubtedly contained proanthocyanidins and flavonols (Wulf and Nagel 1980). McMurrough (1981) used HPLC to demonstrate the presence of catechin, prodelphinidin, and procyanidin in barley extracts. His elution system consisted of 2.5% acetic acid in water with a linear gradient from 0 to approximately 35% methanol. In the same publication he discusses the HPLC separation and identification of flavonol glycosides in barley and hops. Mulkay et al (1981) present a procedure for extraction of barley polyphenols and subsequent analysis by HPLC using a linear gradient of acetic acid from 2 to approximately 7.5%. They obtained excellent separation of a number of proanthocyanidins.

HPLC OF ANTHOCYANINS

Nagel and Wulf (1979) separated 16 different anthocyanin derivatives from Cabernet Sauvignon using a linear gradient of 5–20% acetone in 10% formic acid (Fig. 8). A similar separation was obtained using 12.5–47.5% methanol in place of the acetone (Wulf and Nagel 1978). No preparation of the sample was required except for filtration. Williams et al (1978) extended the technique to separation of the anthocyanidin 3,5-diglucosides. They used a nonlinear gradient with a solvent system consisting of acetic acid, water, and methanol. Wilkinson et al (1977) developed a technique for the analysis of the anthocyanidins (aglycones) that involved the extraction of the plant materials, followed by hydrolysis in the presence of HCl, adsorption of the anthocyanidins on a polyclar AT column (GAF Corp., NY), and elution with methanol. The concentrated sample was then analyzed by HPLC. This technique has the advantage that it simplifies the number of compounds to be analyzed to just six anthocyanidins. The disadvantage is that the technique used probably does not result in quantitative recovery of the pigments because the aglycones are relatively unstable even under acid conditions. Casteele et al (1983) present data on the HPLC separation of many anthocyanidin derivatives, proanthocyanidins, and other phenolic compounds.

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