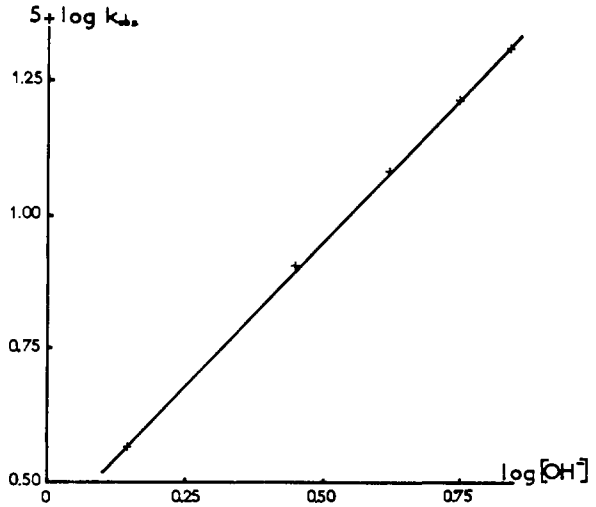

Figure 3. Plot of the logarithms of the observed rate constants vs. the logarithms of hydroxide ion concentration for the hydrolysis of STB to BBU at 25° C in water-methanol.

$[OH^-]$ is essentially 1.0 (Figure 3), indicating that the reaction has a simple first-order dependence on hydroxide ion concentration. The opening of the triazine ring of STB results in BBU formation in the reaction medium. This interpretation is consistent with the observations of White et al. (1973). The nucleophilic attack of hydroxide ion at the carbonyl next to the nitrogen atom N_1 of the benzimidazole ring causes the opening of the triazine ring of STB. This reaction is much slower than the cyclization of benomyl to STB: when $[OH^-] = 1.0$, $k_{obsd} = 0.2 \times 10^{-4}$ s^{-1}; $[OH^-] = 7.0$, $k_{obsd} = 2 \times 10^{-4}$ s^{-1}.

CONCLUSIONS

These results are of theoretical as well as of practical value and allow a better understanding of the chemical fate of benomyl in various conditions.

Benomyl can, in some circumstances, be submitted to alkaline conditions. Some anticryptogamic agents, such as the Bordeaux mixture, are alkaline. Local alkaline conditions can prevail in soils after recent or excessive liming. Heat treatments and alkaline peeling solutions are often used in fruit processing. In these various cases, the involvement of the reaction pathways leading to STB and BBU is quite possible. The environmental impact of either STB or BBU is not well known to date. However, it seems that, in normal practical use situations, the residue levels of benomyl or its derivatives are very low. As for the aerial parts of plants, even after alkaline treatments, intact benomyl constitutes the major component of the local residue on the leaves (Baude et al., 1973); the fast drying-out of the spray droplets prevents noticeable further degradation of benomyl.

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Specificity of the Vanillin Test for Flavanols

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The reaction with vanillin in acidic solution was previously considered to be a specific test for flavanols. In this work the specificity of the vanillin reaction was reexamined by testing the reactivity of 15 flavonoid and two chromone compounds. In addition to flavanols, the dihydrochalcones phloretin and phloridzin gave significant color development. Flavanone and flavanonol aglycones reacted weakly. The structural requirements for a positive reaction have been deduced. The vanillin–HCl screening test, used by plant breeders, has been modified to prevent the possible occurrence of a false positive interpretation due to the presence of anthocyanins in plant materials. The anthocyanidins cyanidin, pelargonidin, and peonidin were identified in alfalfa (*Medicago sativa* L.) herbage, and several other herbaceous legumes were examined for the presence of anthocyanins. The possibility of interference by dihydrochalcones or anthocyanins should be considered when the vanillin–HCl reaction is used for the detection and quantitative analysis of flavanols in plant materials.

Alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), and white clover (*T. repens* L.) are legume forages which may cause ruminant bloat when they are grazed by cattle or sheep, but sainfoin (*Onybrychis viciaefolia* Scop.) and birdsfoot trefoil (*Lotus corniculatus* L.) are legume forages which do not cause bloat. The observation that protein precipitants are present in sainfoin and birdsfoot trefoil herbage, but absent from alfalfa, red clover, and white clover herbage, has led to the conclusion that protein precipitants are responsible for the nonbloating property of sainfoin and trefoil (Gutek et al., 1974; Jones and Lyttleton, 1971). Hence it would be of great benefit if plant breeders could introduce protein precipitants into alfalfa, red clover, and white clover to

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prevent the occurrence of bloat in ruminant animals grazing these species.

The ability to precipitate proteins is a property of tannins. Unfortunately it is difficult to define the term "tannin" and this has been a cause of confusion in the scientific literature. In this report we use the term according to the definition given by Swain and Bate-Smith (1962), i.e., water-soluble, phenolic compounds, with molecular weights between 500 and 3000, and having the ability to precipitate proteins. The tannins of herbaceous legumes are flavolans which are polymers of flavan-3-ols and flavan-3,4-diols (Bate-Smith, 1973; Jones et al., 1973).

In a survey of legume forages Jones et al. (1973) detected tannins by extraction of proteins from herbage in the presence and absence of polyvinylpyrrolidone (PVP). The presence of tannins was indicated by extraction of a larger amount of protein in the presence of PVP compared to extraction in the absence of PVP. In the same study they obtained a positive vanillin–HCl test for flavanols in every species which contained tannins as judged by the PVP test. Similar results have been obtained in this laboratory (Howarth and Goplen, 1974). Jones et al. (1973) obtained a negative result when alfalfa was examined by the vanillin–HCl test. However, Milić (1972) and Delić (1972) used the vanillin–HCl reaction and reported the presence of flavanols in alfalfa. Lehman (1974) has selected alfalfa strains on the basis of response to the vanillin–HCl test. These conflicting results led us to examine the specificity of the vanillin–HCl test, particularly concerning its application to crude leaf extracts of forage legumes for plant breeding purposes.

The vanillin reagent reacts in an approximately stoichiometric manner with compounds containing meta-oriented di- or trihydroxy substituents on the benzene rings (Swain and Goldstein, 1963). A positive reaction is indicated by the appearance of a light pink to deep cherry red coloration. In the case of flavonoids, 5,7-dihydroxy compounds are deactivated by the presence of a carbonyl group in the C-4 position (Ribéreau-Gayon, 1972). Therefore, the reaction has been applied as a specific test for flavanols (Swain and Hillis, 1959) because they lack the carbonyl group at the C-4 position. The reaction has been adapted to a screening test for plant breeding purposes by crushing leaves between two layers of filter or chromatography paper and then applying the reagent to the imprint on the paper (Burns, 1963; Jones et al., 1973).

EXPERIMENTAL SECTION

The specificity of the vanillin reaction was investigated by testing a number of flavonoid compounds which varied in the oxidation level of the middle heterocyclic ring. Several chromone compounds which lack the B ring of the flavonoid nucleus were also tested. The test compounds were obtained from K and K Rare and Fine Chemicals, Pfaltz and Bauer Inc., and Fluka AG.

The test compounds were dissolved in distilled water at concentrations of 8 to 33 μg/ml. The vanillin reagent contained 1% vanillin in 70% (v/v) sulfuric acid (Swain and Hillis, 1959). Four milliliters of vanillin reagent was mixed with 3.0 ml of sample solution. Absorbance readings were taken at 500 nm with the spectrophotometer zeroed against a reagent blank.

Plant material was tested for flavolans by crushing leaves or stems between two layers of 3MM chromatography paper and applying vanillin solution to the imprint on one of the layers (Jones et al., 1973). The vanillin solution contained 2 vol of 10% w/v vanillin in ethanol mixed with 1 vol of concentrated HCl. In this work it was necessary to apply a control solution (2 vol of ethanol in 1 vol of concentrated HCl) to the imprint on the second paper layer to avoid the possibility of obtaining a false positive response.

Anthocyanins were extracted from 10 g of herbage by homogenization with 30 ml of methanol–water (1:1). The homogenizer (Sorvall) was cooled in ice and operated for 2 min. Homogenates were filtered through glass-fiber disks; the filtrates were concentrated to about 2 ml in a rotary evaporator, and centrifuged for 3 min at 600g. The supernatant solutions were streaked on Whatman 3MM chromatography paper and the chromatograms were developed in butanol–acetic acid–water (4:1:5, upper layer). The anthocyanins appeared as colored bands on the chromatograms. They were identified by comparison with R_f values reported in the literature and by their absorption spectra.

The anthocyanins from alfalfa herbage were eluted from the chromatograms with 1% HCl in methanol (v/v) and an aliquot was rechromatographed on a cellulose thin-layer chromatogram developed in acetic acid–HCl–H_2O (15:3:82). Another aliquot was heated to 100 °C for 30 min in 3 N HCl to hydrolyze the anthocyanins. The anthocyanidins so obtained were extracted into amyl alcohol, and their R_f values were determined on a cellulose thin-layer chromatogram developed in acetic acid–HCl–water (30:3:10). Another aliquot of the amyl alcohol extract was spotted on Whatman 3MM chromatography paper and developed in 1% HCl. The anthocyanidin bands were eluted into 1% HCl in methanol for measurement of their visible absorption spectra. Standard anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, and peonidin) were chromatographed along with the anthocyanidins from alfalfa.

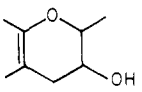
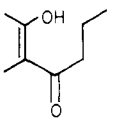
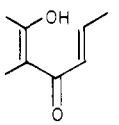
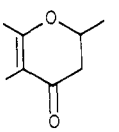
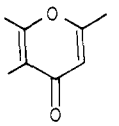
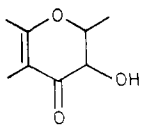
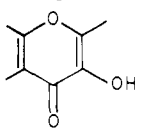
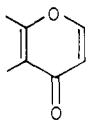
RESULTS AND DISCUSSION

We considered the possibility that the oxidation level of the middle heterocyclic ring may affect the reactivity of flavonoids with the vanillin reagent. The reactivities of flavonoid glycosides and of chromones, which lack the flavonoid B ring, were also tested. Seventeen compounds representing flavanols, flavanones, flavones, flavanonols, flavonols, dihydrochalcones, chalcones, and chromones were examined. The chemical structures of these compounds are shown in Figure 1 and Table I, and their reactions with the vanillin reagent are given in Table I. Six compounds gave positive reactions but there were large differences in the intensities of color development. Molar extinction coefficients of the reactive compounds are shown in Table II.

Our results show that the vanillin reagent is not completely specific for flavanols. Catechin, a flavanol, gave the greatest intensity of color development, but phloretin, a dihydrochalcone, gave a color intensity in the same order of magnitude as catechin. When phloretin concentration in the sample solution was 16 μg/ml a red complex precipitated after addition of the vanillin reagent. There was no visible precipitate at a phoretin concentration of 8 μg/ml. Phloridzin, a dihydrochalcone glycoside, gave an intermediate color intensity while naringenin, hesperetin, and dihydroquercetin gave small amounts of color.

Swain and coworkers (Swain and Hillis, 1959; Goldstein and Swain, 1963) tested the specificity of the vanillin reaction using a variety of compounds. Our results confirm their finding that chalcones, flavonols, and flavanone 7-glycosides do not give a color reaction (Swain and Hillis, 1959). However, we have tested a greater variety of flavonoid compounds and our observations provide new information on the structural requirements for a positive

Table I. Responses of Some Flavonoid and Chromone Compounds to the Vanillin Test for Flavanols

Class	Structure of C_3 unit	Compound	Reaction with vanillin
Flavanols		Catechin (5,7,3′,4′-tetrahydroxyflavan-3-ol)	+++
Dihydrochalcones		Phloretin (4,2′,4′,6′-tetrahydroxydihydrochalcone)	+++
		Phloridzin (phloretin 2′-glucoside)	++
Chalcone		Butein (3,4,2′,4′-tetrahydroxychalcone)	–
Flavanones		Naringenin (5,7,4′-trihydroxyflavanone)	+
		Naringin (naringenin 7-rutinoside)	–
		Hesperetin (5,7,3′-trihydroxy-4′-methoxyflavanone)	+
		Hesperidin (hesperetin 7-rutinoside)	–
Flavones		7-Hydroxyflavone	–
		5,7-Dihydroxyflavone	–
Flavanonol		Dihydroquercetin	+
Flavonols		Kaempferol (5,7,4′-trihydroxyflavonol)	–
		Quercetin (5,7,3′,4′-tetrahydroxyflavonol)	–
		Quercitrin (quercetin 3-rhamnoside)	–
		Rutin (quercetin 3-rutinoside)	–
Chromones		Eugenin (2-methyl-5-hydroxy-7-methoxychromone)	–
		2-Methyl-5,7-dihydroxychromone	–

reaction by flavonoid compounds.

Vanillin is protonated in acid solution, giving a weak electrophilic radical which reacts with the flavonoid ring at the 6 or 8 position. This intermediate compound is dehydrated to give a red colored compound (Ribéreau-Gayon, 1972). Table I shows that a single bond between

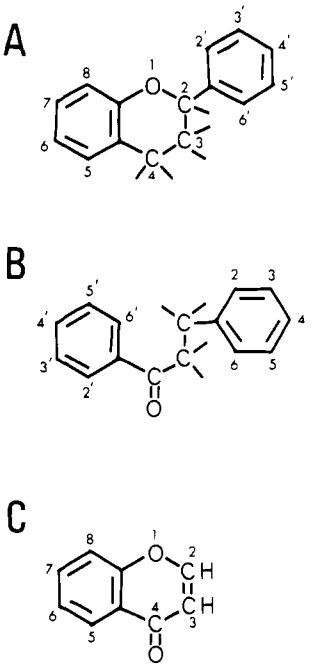


Figure 1. Numbering systems for: (A) most flavonoids; (B) chalcones, dihydrochalcones; (C) chromones.

Table II. Molar Extinction Coefficients of the Colored Products Formed from Flavonoid Compounds with the Vanillin Reagent

Compound	$\epsilon \times 10^{-3}$
Catechin	33.8
Phloretin	25.3
Phloridzin	9.1
Naringenin	2.5
Hesperetin	2.8
Dihydroquercetin	2.5

C-2 and C-3 is an essential requirement for a positive reaction. Chalcones, flavones, flavonols, and chromones are completely inactivated by a double bond in this location. Inactivation is probably due to decreased electron density in the A ring because of electron delocalization. Only small amounts of color were given by naringenin, hesperetin, and dihydroquercetin indicating nearly complete inactivation by the electron-withdrawing effect of the carbonyl group at C-4. However, much less inactivation by the carbonyl occurred with the dihydrochalcones which were activated by an additional free hydroxy group at C-6′. The flavanone glycosides, naringin and hesperidin, did not react, indicating the requirement for a free hydroxy group at C-7. The glycoside phloridzin produced less color than the corresponding aglycone, phoretin, probably because glycosylation decreased electron density at C-2′ or because the glucosyl group sterically hindered substitution at C-3′. In summary, the essential structural requirements for reaction of the flavonoids with the vanillin reagent are a single bond at the 2,3 position and free meta-oriented hydroxy groups on the B ring. Substantial amounts of color development are therefore given by the flavanols and dihydrochalcones.

These results raise the possibility of interference by dihydrochalcones when the vanillin–HCl test is used for the detection or quantitative determination of flavanols in plant materials. Although considerable quantities of phloridzin are present in apple and pear leaves (Williams, 1966), the dihydrochalcones are less widely distributed than flavanols (Harborne and Simmonds, 1964). Accordingly, a positive vanillin–HCl reaction is probably

Table III. Anthocyanins from Alfalfa Herbage

Paper chromatography		Thin-layer chromatography, R_f[b]	Identity of anthocyanidins[c]
Band no.	R_f value[a]		
1	0.03	0.90	Cy
2	0.07	0.66 and 0.90	Cy
3	0.17	0.66 and 0.90	Cy, Pg, Pn
4	0.24	0.74 and 0.90	Cy, Pg, Pn
5	0.37	0.08	Cy

[a] Solvent: butanol-acetic acid-water (4:1:5), upper layer. [b] Solvent: acetic acid-HCl-water (15:3:82). [c] Abbreviations: cyanidin (Cy), pelargonidin (Pg), and peonidin (Pn).

attributable to flavanols but additional tests by paper chromatography would be necessary to confirm their presence and to establish that dihydrochalcones are not present.

When we used the vanillin–HCl screening test (Jones et al., 1973) to survey a number of alfalfa cultivars and strains, several plants gave a pink color which appeared to be a positive reaction. However, further examination of these plants by testing for protein precipitants (Jones and Lyttleton, 1971) or by paper chromatography of methanol extracts (Sarkar et al., 1976) failed to confirm the presence of flavanols or tannins. Since anthocyanins give a pink to red color in acidic solutions, alfalfa herbage was examined for the presence of anthocyanins. Paper chromatography of extracts from alfalfa herbage showed the presence of five colored bands (Table III), three of which gave two bands by thin-layer chromatography. When the colored bands were eluted from paper chromatograms and hydrolyzed in HCl, three anthocyanidins were obtained: cyanidin, peonidin, and pelaragonidin. Delphinidin, petunidin, and malvidin occur in alfalfa petals (Cooper and Elliott, 1964) but to the best of our knowledge this is the first report on identification of the anthocyanidins in alfalfa herbage. Several other herbaceous legumes were examined for the presence of anthocyanins. Sericea (*Lespedeza cuneata* Don.), birdsfoot trefoil (*Lotus corniculatus* L.), small hop clover (*Trifolium dubium* Sibth.), and large hop clover (*T. campestre* Schreb.) contained an anthocyanin which was identified as cyanidin 3,5-diglucoside. Identification was on the basis of R_f value and absorption spectrum. Anthocyanins were not detected in rabbit foot clover (*T. arvense* L.) and crownvetch (*Coronilla varia* L.).

We introduced a modification to the vanillin–HCl screening test to avoid a false, positive interpretation due to the presence of anthocyanins in the plant materials. A control solution containing HCl in ethanol was applied to the leaf imprint on the second layer of chromatography paper. If a red coloration results from the presence of anthocyanins, it will appear on the leaf imprint treated with this control solution, as well as on the imprint treated with the complete reagent solution. Red color resulting from the presence of flavolans will appear on the imprint treated with the complete reagent solution but not on the imprint treated with the control solution. The modified vanillin–HCl screening test has been used to survey many alfalfa plants and we have not detected the presence of flavolans (Howarth and Goplen, 1974). Thus in this work, as well as that reported elsewhere (Sarkar et al., 1976), we have been unable to confirm the occurrence of flavanols in alfalfa as reported by Milić (1972) and Delić (1972). Possibly their extracts contained anthocyanins.

In conclusion, the vanillin–HCl reaction is useful for the detection and quantitative analysis of flavanols in plants but the possibility of interference by dihydrochalcones and anthocyanins should be considered.

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