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▶ During an investigation involving the isolation of urinary steroids which react with isonicotinic acid hydrazide to form colored hydrazones, a compound, later identified as hesperetin, gave a brilliant yellow fluorescence when sprayed with isonicotinic acid hydrazide reagent on filter paper. This led to an investigation of the use of this reagent as a spray for other types of flavonoids. In combination with other techniques it is employed in a scheme for the identification of various groups of flavonoids. An accurate quantitative micromethod for the spectrophotometric determination of certain members of this class of compounds is presented.

MANY reagents are available for use as sprays in the paper chromatographic separation of mixtures of flavonoids (*1*, *2*). To the authors' knowledge only one of these, the borocitric reagent (*11*), has the properties for use as a spray reagent for the purpose of distinguishing between various groups of flavonoids (*5*).

Almost all flavonoids react positively to the cyanidin test (*10*), yielding colors varying from orange to purple. The borocitric and cyanidin reactions have also been employed as quantitative methods for the determination of certain flavonoids. The limitations of these reactions are that the borocitric test must only be performed under anhydrous conditions and that the cyanidin reaction is difficult to carry out under strictly uniform conditions.

Isonicotinic acid hydrazide (INH) has been used for the quantitative determination of Δ^4-3-ketosteroids under various conditions. The possible use of INH as a spray reagent was suggested by Weichselbaum and Margraf (*8*). The finding that hesperetin, isolated from human urine in the course of certain metabolic studies, formed a typical yellow hydrazone on a paper chromatogram suggested the present study.

The INH reaction as described provides an easily reproducible, accurate, and relatively simple micromethod for the quantitative estimation of certain flavonoids. The sensitivity of this method is such that even with the small quantities generally used in paper chromatographic separations, the spots obtained can be eluted, rechromatographed, and finally re-eluted, and the quantities thus isolated can be quantitatively determined by the INH method. Certain data in Table I, summarized from a recent publication of Geissman (*3*), show that various color reactions have been used to distinguish qualitatively among various groups of flavonoids, or even between certain derivatives within these groups. The INH reagents (*6–8*) used as a qualitative tool in two concentrations have similarly the distinct property to distinguish among several groups of flavonoids. The possibility of combining several of these reactions to identify certain unknown mixtures was recognized as a form of a scheme of qualitative analysis (Figure 1).

Table I. Summary of Color Reactions of Flavonoid Compounds in Solution

Reaction	Flavone	Flavonol Aglycone	Flavonol Glycoside	Flavanone	Flavanol	Chalcone
Cyanidin	+	+	+	+	+	−
Borocitric[a]	±	+	+	−	−	±
Alkaline (NaOH and others)	+	+	+	−	−	+
Sulfuric acid	+	+	+	+	+	+
Antimony pentachloride (in carbon tetrachloride)	+	+	+	+	+	+
Nitric acid	−	−	−	+	+	−
Ferric chloride[b]	±	+	+	±	+	±
Neutral and basic lead acetate	+	+	+	−	−	+

[a] Flavones and chalcones react positively only when an OH group is present in position 5.

[b] Positive if an OH group is present at positions 3, 5, or 8; otherwise, compound reacts negatively.

APPARATUS AND REAGENTS

Centrifuge tubes, with screw caps and Teflon washers or standard tapered glass stoppers, 12- to 15-ml. capacity.

Standard low-pressure chromatography sprayer (atomizer), 10-ml. capacity.

Isonicotinic acid hydrazide (INH), Taylor Chemical Co., St. Louis 19, Mo., recrystallized twice from 95% USP ethyl alcohol (melting point 172° C.).

Dimethyl sulfoxide, Stepan Chemical Co., Chicago 6, Ill.

WEAK REAGENT. A-1 (Blank reagent), 0.625 ml. of concentrated hydrochloric acid (37%) made up to 1 liter with absolute ethyl alcohol. A-2 (Reagent), 25 mg. of INH dissolved in 50 ml. of reagent A-1.

STRONG REAGENT. B-1 (Blank reagent), 5.0 ml. of concentrated hydrochloric acid made up to 1 liter with methanol, analytical reagent grade redistilled. B-2 (Reagent), 400 mg. of INH dissolved in 100 ml. of reagent B-1.

When refrigerated, the INH reagents are stable for several weeks.

PROCEDURES

Qualitative Detection. The flavonoid compounds used were dissolved in absolute ethyl alcohol, and in a few cases, when their degree of solubility had to be increased, in absolute methanol. When the solubility in either alcohol was insufficient, a few milliliters of dimethyl sulfoxide were added. All compounds were spotted on Whatman No. 1 filter paper strips in quantities ranging from 5 to 100 γ. The area covered by the spots was within 1 cm. in diameter. After drying, the papers were sprayed on both sides with reagents A-2 and B-2, respectively. Following quick drying with warm air from a hair dryer, the paper strips were viewed for visible color, ultraviolet absorption, and fluorescence immediately, 1 hour later, and at various intervals for 24 hours thereafter.

Quantitative Determination. The previously identified flavonoid compound, which is to be determined quantitatively, is transferred in duplicate and in a minimum amount of solvent to a 12- or 15-ml. centrifuge tube. The solvent is then evaporated under a stream of air while placed in a warm water bath. One milliliter (more or less depending on the quantity of material to be assayed) of reagents B-1 and B-2, respectively, is

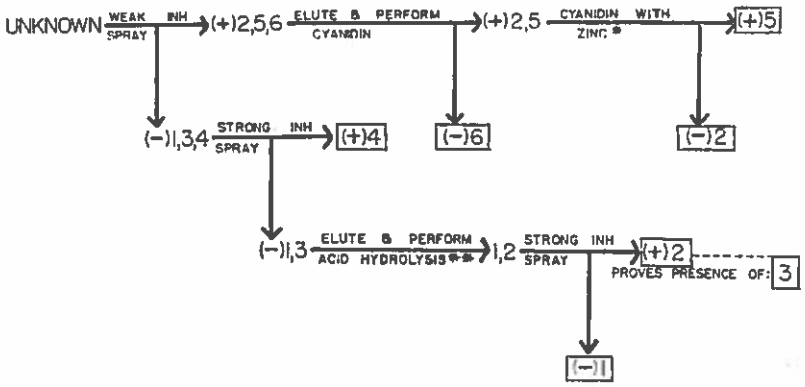

Figure 1. Scheme of qualitative analysis

1. Flavones
2. Flavonol aglycones
3. Flavonol glycosides
4. Flavanones
5. Flavanols
6. Chalcones

* Ref. (4)

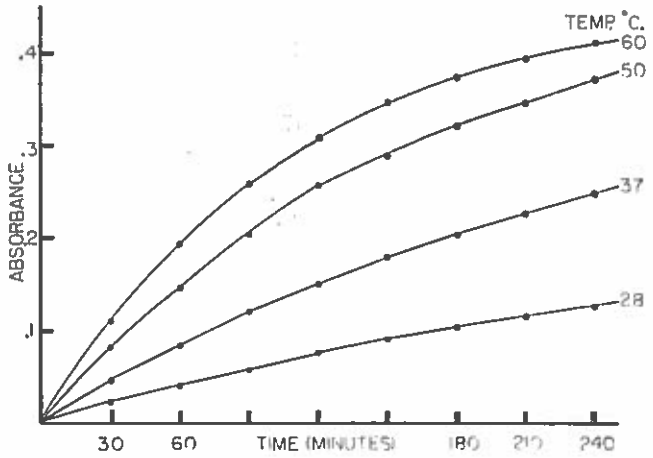

Figure 2. Effect of time and temperature on rate of reaction

added to the dry residues in the two test tubes. The tubes are tightly closed with screw caps and Teflon washers and placed in a constant temperature water bath at 50° C. for 150 minutes. At the end of this period the tubes are immediately cooled and the contents transferred to suitable cuvettes. The colors are read in a spectrophotometer at 405 mμ, within 10 minutes, against reagents B-1 and B-2. The absorbances obtained with reagent B-2 are corrected for those obtained with reagent B-1 (blank).

If desired and if quantitative measurements of the same compound are to be made frequently, a standard plot of concentration *vs.* absorbance could be prepared and used for conversion of blank-corrected absorbancies to actual concentration of the compound being determined.

RESULTS

Sixteen compounds, representatives of six different groups of flavonoids, were tested; 11 had characteristic yellow fluorescence with either one or the other of the INH reagents. As shown in Table II, only two groups, the flavones and flavonol glycosides, reacted negatively toward both reagents. A positive reaction is one which shows a characteristic yellow fluorescence on exposure to short-wave ultraviolet light. While the flavanones under the condition of immediate observation reacted negatively to the weak INH reagent, the same group was re-examined at frequent intervals under short-wave ultraviolet light during a period of 6 hours after spraying and then found to give a positive fluorescent reaction only after approximately 6 hours.

The aglycone forms of flavonols must be considered to react positively, in spite of the fact that some of them show a yellow fluorescence before spraying with INH reagents. The increase in intensity of such fluorescence is easily detectable. Flavanones react positively to the strong INH reagent, but negatively to the weak reagent. Both flavanols and chalcones exhibit strong yellow fluorescence when sprayed with either INH reagent.

The effects of INH spray reagents on different types of flavonoids were utilized, with other reactions, for a proposed scheme of qualitative group analysis (Figure 1). For example, a mixture consisting of naringenin,

Table II. Immediate Effects of Two INH Paper Spray Reagents on Various Flavonoids under Short Wave-Length Ultraviolet Light

Compounds	Weak INH Spray	Strong INH Spray
Flavones		
Apigenin	−	−
Ponkanetin	−	−
Rhoifolin	−	−
Flavonols (aglycones)		
3-OH flavone	+	+
Morin	+	+
Quercetin	+	+
Flavonols (glycosides)		
Quercitin	−	−
Rutin	−	−
Flavanones		
Hesperidin	−	+
Hesperetin	−	+
Naringenin	−	+
Naringin	−	+
Prunin	−	+
Flavanols		
2,3-Dihydroquercetin	+	+
Chalcones		
Hesperidin methylchalcone	+	+
Tetramethoxy eriodictyol-chalcone	+	+

quercetin, and rutin was analyzed and identified in the following manner: After chromatography (*9*) on several strips of paper the mixture resolved into three distinct spots viewed by fluorescence under ultraviolet light. The spraying of one strip with weak INH reagent resulted in positive reaction of one spot. Figure 1 indicates a choice among flavonol aglycones, flavanols, and chalcones. Elution of corresponding spots from unsprayed paper strips and the performance of a cyanidin test eliminated chalcones as possible identity. A subsequent cyanidin test with zinc (*4*) was negative and left flavanol aglycone as the group to which this material had to belong. Purification and micro infrared analysis proved the compound to be quercetin.

Spraying the same paper strip with strong INH reagent resulted in positive reaction of one of the remaining spots, placing the compound in the group of flavanones. Purification and micro potassium bromide infrared spectroscopy identified it as naringenin.

The one remaining spot which had reacted negatively to both INH reagent sprays was eluted from several strips and a portion hydrolyzed with hydrochloric acid. Part of the hydrolyzate was respotted on paper and sprayed with strong INH reagent. This time the reaction was positive, placing the compound in the group of flavonol aglycones. As hydrolysis had been performed, actually the corresponding glycoside was being dealt with. Purification and infrared spectroscopy proved the compound to be rutin.

Table III. Replicability and Precision of the Quantitative Method of Determination

Quantity, Ml.	1	2	3	4	Mean Values
5	0.133	0.136	0.131	0.133	0.133
5	0.132	0.133	0.133	0.132	
10	0.264	0.266	0.266	0.268	0.266
10	0.265	0.267	0.268	0.266	
15	0.399	0.395	0.404	0.399	0.399
15	0.400	0.395	0.401	0.400	
20	0.538	0.532	0.532	0.527	0.532
20	0.535	0.533	0.532	0.532	

Table IV. Absorptivities (E) of Isonicotinic Acid Hydrazones of Several Flavonoids

(At 405 mμ, incubated at 50° C. for 150 minutes)

Compound	Group	E
Rutin	Flavonol glycoside	3,260
Hesperidin	Flavanone	8,700
Hesperetin	Flavanone	10,960
Naringin	Flavanone	10,900
2,3-Dihydroquercetin	Flavonol	4,700
Hesperidin methylchalcone	Chalcone	7,600

The sensitivity of detection of certain flavonoids on paper chromatograms with the strong INH reagent extends to levels as low as 5 γ. Other reactions involved in the scheme of analysis must necessarily also be applied on a micro scale.

Evidence for the precision and reproducibility of the quantitative method is given in Table III. Excellent agreement on a day-to-day basis has been observed. The effect of temperature and time on color development for hesperetin is shown in Figure 2. Full color equivalence cannot be reached under the proposed reaction conditions. Varying the temperature markedly affected the rate at which the absorbance increased. However, the absorbance obtained under these conditions follows Beer-Lambert's law in a range from 0 to 25 γ of hesperetin per ml. of reagent. Similar standard plots have been obtained with other compounds.

The reaction was not affected by light. There was no evidence, when the reaction was carried out in an open tube at room temperature, that the presence or absence of oxygen affected the reaction in any way. In this laboratory the reaction takes place in tightly stoppered tubes to prevent evaporation of the solvent. Absorptivities of various compounds are presented in Table IV as evidence that color equivalences of different flavonoids even within the same group may vary considerably. The use of standards for comparative purposes or the preparation of standard plots for individual compounds is necessary. The absorption maximum for the isonicotinyl hydrazones was between 404 and 408 mμ. For practical use, a maximum of 405 mμ was chosen.

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