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A simplified 2, 4-dinitrophenylhydrazine assay for Flavonoids and its comparison with a Standard flavonoid assay

S. A. Mir[1]*, A. S. Bhat[1], A. A. Ahangar[1]

[1]Division of Veterinary Pharmacology & Toxicology, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir, F.V.Sc. & A.H., Shuhama, Srinagar-190001, J & K, India.

***Corres.author: mirsamir.19@rediffmail.com**
Telephone/Telex No: 91-0194-2262216

Abstract: A simplified version of the 2, 4-DNPH assay for flavonoids has been improvised. The assay includes incubating test sample with DNPH in a 4.5 mL reaction volume in hot water at about 80 ^{0}C containing 2 mL methanol at about 0.2 N HCl, cooling samples to room temperature, adding 3 mL 1M NaOH, and monitoring red color at 470 nm at 10-15 minutes. The reagent has been found capable of assaying both flavonols (rutin, quercetin and morin) and flavones (diosmin and diosmin rich bioflavonoid, daflon) within 1 to 4 µmoles of flavonoid compared to HCl method that responds within 0.15 µmoles of flavonoids. The linearity range for various flavonoids with DNPH assay, in µmoles has been 0.2 to 1 (quercetin), 0.5 to 2.0 (rutin, daflon), 0.5 to 3.75 (diosmin) and 1 to 4 (morin) ($r > 0.99$). The observations are contrary to the recommendation that the DNPH assay is rather specific for flavonone and not for flavone and flavonol type flavonoids. A comparison of regression coefficients obtained with DNPH assay and HCl assay have revealed that the DNPH assay is 8 to 76 times less sensitive than HCl assay for assaying test flavonoids.
Key-words: 2, 4-Dinitrophenylhydrazine, Flavonoids, Sugars, Phenolics, Chloral hydras, Assay.

Introduction

Flavonoids constitute an important group of anti-oxidants widely distributed in plants[1,2] with potential implications in health and disease[3]. Of these, diosmin, rutin and daflon, besides their anti-oxidant potentials have been therapeutically employed in a variety of human disorders including amongst others management of chronic venous insufficiency and hemorrhoids[4]. Generally colorimetric methods for flavonoids are considered reliable, accurate and time-saving compared to more advanced chromatographic methods that require special expertise and instrumentation and are more expensive and time consuming. Aluminium chloride method is most often the colorimetric method of choice for general determination of flavonoids[5-7]. Recently a simple alternative colorimetric assay for flavonoids using 75 % HCl has been found to be equally effective and much simpler in execution than aluminium chloride based assay[7]. Additional colorimetric assays employing metallic salts including those of copper, lead, molybdenum and tungsten too have been found to be useful for assaying flavonoids with varying sensitivities[8].

It has been recommended that determination of flavonoids from botanicals must include assays like DNPH that are rather selectively interacting with flavanone type flavonoids with least selectivity for flavone and flavonol

type flavonoids[6]. The assay has been viewed as a complementary assay to routine assay for determination of total flavonoids.

The standard DNPH assay in practice includes incubating test sample in methanol in presence of DNPH for 50 minutes at 50 ^{0}C, then addition of KOH in methanol, and centrifugating an aliquot of colored mixture with methanol to separate out the precipitate, and reading the samples at 495 nm following dilution in methanol The technique is considered selective for flavanones including naringin, ± naringenin and hesperitin, and 1 mg of each has yielded mean absorbance, respectively, as 0.113, 0.240, and 0.258[6]. DNPH reagent *per se* is a non-specific reagent being used to detect aldehyde/ketonic moieties that are prevalent in non-flavonoids so are cause of interference and concern. In fact, the technique is employed for determination of pyruvic acid, monitoring color at 520 nm[9]. Since flavonoids exhibit phenolic functional moieties with and without sugar components, this necessitated investigating response of these phenolics and sugar representatives such as glucose and sucrose *in vitro* to DNPH to assess their contribution if any to color reaction under test conditions.

The experiments were accordingly designed to pursue the objectives aimed to: (i) simplify the DNPH assay protocol so as to minimize the steps required to develop the soluble color reaction; (ii) assess applicability of the reagent to assay flavone and flavonol type flavonoids, (iii) compare sensitivity of the assay with already standardized assay for flavonoids using HCl, and (iv) assess whether or not phenolic and sugar residues are capable of contributing to over all color reaction of DNPH assay.

Experimental

The experiments were carried out at an ambient temperature of 27.6±0.4 ^{0}C. The drugs and chemicals used were of standard purity and quality obtained from reputed sources in India. Spectrophtometric measurements were made with UV-Visible Spectrophotometer, Model UVmini-1240 (Shimadzu Corporation, Japan).

Reagents: DNPH reagent: 0.1 % (w/v) DNPH in 15 % HCl in water was prepared by triturating 250 mg crystals of DNPH with pestle and mortar along with 37.5 mL HCl and water was added to make final volume 250 mL. The dissolution was allowed at room temperature over 3 days. The solution was filtered over Whatman Fliter Paper No.42, washed with 15 % HCl, and discarded first few milliliters of the reagent.

NaOH solution: A 4 % (w/v) sodium hydroxide pellets in water served as 1 M solution. Diluted strength (0.1M NaOH) was prepared by dilution in water as per need.

Rutin trihydrate: 0.2 % (w/v) rutin in methanol with consideration to minimum labeled purity as 90%. The requisite dilutions were made in methanol. The requisite dilutions were made in methanol. The molecular weight of rutin was taken as 664.55 for estimating molar equivalents

Quercetin dihydrate: 0.1 % (w/v) in methanol with consideration to minimum labeled purity as 98%. The requisite dilutions were made in methanol. The requisite dilutions were made in methanol. The molecular weight of quercetin was taken as 338.27 for estimating molar equivalents

Morin hydrate: 0.1 % (w/v) morin in methanol with consideration to minimum labeled purity as 95%. The requisite dilutions were made in methanol. The molecular weight of morin was taken as 329.24 for estimating molar equivalents.

Diosmin: Laboratory standard for synthetic diosmin was prepared from Venex-500 mg tablets (Elder Pharmaceuticals Ltd., Mumbai) as per standard procedure already outlined[5]. The concentration of diosmin was adjusted to 0.25 % (w/v) labeled diosmin in 0.1 M NaOH. The stock solution was stored well stoppered in refrigerator. The molecular weight of diosmin was taken as 608.54 for estimating molar equivalents.

Daflon: The flavonoid mixture contained in Daflon tablets was extracted from the powder in the manner applied to Diosmin tablet powder[5]. The final strength was adjusted to 0.25 % (w/v) flavonoid mixture (90 % diosmin and 10 % hesperidin as per label) in 0.1 M NaOH. The concentration was adjusted following its assay with laboratory standard diosmin. The molecular weight of the mixture was estimated as 608.74 for estimating molar equivalents.

Chloral hydras solution: Working solutions (10 µmoles of chloral hydras mL^{-1}) was made in methanol by dilution of stock solution 1.8 % (w/v) in water equivalent to 98.1 µmoles of chloral hydras mL^{-1}.

Sucrose Working solution (10 µmoles of sucrose mL^{-1}) was made in methanol by dilution of stock solution 1.0 % (w/v) sucrose (equivalent to 29.2 µmoles mL^{-1}) in 0.01N HCl.

Glucose: Working solutions (10 and 100 µmoles of glucose mL^{-1}) were made in methanol by dilution of stock solution 5.0 % (w/v) glucose (equivalent to 277.8 µmoles of glucose mL^{-1}) in 0.01N HCl.

Phenolic solutions: Appropriate working solutions of resorcinol, catechol and phenol were made in methanol from respective stock solutions as 0.5 or 1 % % (w/v) prepared in 0.01 N HCl. Working solution of guaiacol was made from stock solution 4.68 % (w/v) in methanol equivalent to 373 µmoles of guaiacol mL^{-1}.

Analytical techniques

2, 4-Dinitrophenylhydrazine technique for flavonoids

The analytical technique followed in the study is a modification of the method approved for flavanone type flavonoids[6, 10]. A simplified version of the assay has been improvised for the study. The initial reaction volume has been 4.5 mL. The samples contained in methanol (morin, quercetin and rutin) were added in 2 mL methanol while those contained in alkali (diosmin and daflon) were taken in 2 mL 0.1M NaOH. The samples were matched for alkali and methanol such that each sample contained 2 mL each of methanol and 0.1 M NaOH. The samples were added 0.5 mL of DNPH reagent. Sample controls in corresponding concentration were added 0.5 mL 15 % HCl. The reagent controls contained 2 mL methanol and 2 mL 0.1 M NaOH. The reagent blank was added 0.5 mL 15 % HCl while DNPH blanks were added 0.5 mL DNPH reagent. The samples were gently mixed and incubated in hot water bath for 15 minutes maintained at about 80 ^{0}C. The samples were then cooled to room temperature, added each 3 mL of 1 M NaOH, gently mixed up, and allowed to stand at room temperature for 10-15 minutes and absorbance read at 470 nm.

Hydrochloric acid technique for flavonoids

The method employed for the assay has been described[7]. Test sample in 1 mL solvent was added 3 mL concentrated HCl. The samples were read at 405 nm following standing at room temperature for 30 to 40 minutes.

Results and Discussion

The optimized assay is simpler and faster in execution than recommended DNPH assay[6]. Time is saved by incubating samples at elevated temperature (about 80 ^{0}C) for 15 minutes compared to incubation for 50 minutes at 50 ^{0}C. The final reaction mixture provided transparent color so did not require centrifugation or addition of 25 mL methanol. During optimization, it was observed that incubating the samples in ethanol or acetic-acid ethanol (solvent used for diluting flavonoids like rutin and quercetin) were interfering with the assay and controls developed excessive color possibly due to presence or formation of aldehyde. Thereafter, the solutions of rutin and quercetin were prepared in methanol, and addition of ethanol and acetic acid was avoided. A comparative evaluation of three wavelengths 520, 470 and 495 nm suggested more stable and higher values were observed at 470 nm. Regression estimate for diosmin with 10-minute incubation during optimization studies (0.75 through 3 µmole basis versus corresponding absorbance values, n= 3 each) at 470, 495 and 520 nm were respectively found to be, 0.106 ± 0.001, 0.078 ± 0.003 and 0.058 ± 0.003 ($r > 0.996$). Thus, 470 nm was chosen for the assay under test conditions. Later studies revealed 15-minute incubation was providing better absorbance values than those observed at 10 minute incubation, therefore, 15 minute incubation was employed throughout the studies thereafter. Estimated acidity of the samples at the time of incubation was approximately 0.2 N HCl, while alkalinity at the time of color development was approximately 0.3 N NaOH.

Table 1 Comparative response of selected flavonoids to DNPH assay

Test flavonoid	Mass, µmole	Absorbance[a]	r± s.e.	b ± s.e.
Diosmin	0.50	0.090±0.002	0.9994 ±0.0005	0.143 ±0.002
	0.75	0.125±0.002		
	1.0	0.168±0.001		
	1.5	0.230±0.004		
	2.25	0.355±0.005		
	3.0	0.448±0.008		
	3.75	0.555±0.005		
Daflon	0.5	0.128±0.004	0.9981±0.0022	0.214 ±0.008
	1.0	0.216±0.001		
	2.0	0.445±0.003		
Rutin	0.5	0.091 ±0.001	0.9915±0.0085	0.216 ±0.014
	0.75	0.376 ±0.002		
	1.0	0.409 ±0.003		
	2.25	0.751 ±0.001		
Quercetin	0.2	0.110 ±0.001	0.9999 ±0.0001	0.450 ±0.004
	0.5	0.244 ±0.006		
	1.0	0.470 ±0.005		
Morin	1.0	0.051 ±0.005	0.9998±0.0002	0.057±0.001
	2.0	0.105 ±0..002		
	4.0	0.222 ±0.005		

[a] The values are mean ± S.E. of 5 observations each, and indicate observed absorbance values minus sum of those due to DNPH and that due to corresponding mass of sample in absence of DNPH

Response of test flavonoids to DNPH

All test flavonoids reacted with varying intensities (Table 1). Comparison of regression coefficients reveals the order of reactivity followed the following pattern:

quercetin (3.1X) > rutin (1.5X) ≥ daflon (1.5 X) > diosmin (1 X) > morin (0.4 X)

Thus, quercetin was nearly 3 folds more responsive while rutin and daflon were 1.5 times as responsive as diosmin, and morin was least responsive (2.5 times lesser than equimolar diosmin). Increased reactivity to daflon than to diosmin is presumably due to presence of hesperitin (a flavanone constituting 10 % of daflon with 90 % provided by diosmin).

It was mandatory to take control samples in appropriate concentrations in absence of DNPH, as the samples reacted intensely with high alkalinity with least reaction by diosmin and daflon. The order of reactivity of control flavonoids to alkaline pH was found to be in the order based on estimated regression coefficients over the test range (Table1):

rutin (19 X) > morin (11X) > quercetin (5 X) >>diosmin (1 X) ≥ daflon (0.9 X)

The color due to diosmin and daflon has been palish with absorbance range 0.017 through 0.104 (diosmin: r = 0.992, b = 0.028) and 0.018 through 0.055 (daflon: r=0.999, b = 0.025). Rutin and quercetin induced deep yellowish-orange color with absorbance range 0.141 through 1.05 (rutin: r= 0.999, b= 0.520) and 0.034 through 0.146 (quercetin: r = 0.998, b= 0.141). The color formed by morin was intensely golden yellow with range 0.343 through 1.23 (r=0.999, b= 0.296). As evident, diosmin and daflon induced control color is least interfering compared to those with rutin, morin and quercetin in that order. This observation makes it mandatory to take simultaneous control samples treated with matched volume of acid in absence of DNPH and reading DNPH treated samples against control as blank. Regression coefficients of rutin and morin for controls,

respectively, as 0.520 ±0.004 and 0.296 ± 0.003 are much higher than those obtained with DNPH treated samples, correspondingly as, 0.216±0.014 and 0.057±0.001.

Comparative response of test flavonoids to HCl

Compared to DNPH assay, test flavonoids responded quite efficiently to HCl method (Tables 2 & 3). Controls were taken for each concentration but added 3 mL 15 % HCl compared to concentrated HCl. This acidity rendered controls of diosmin, daflon and quercetin colorless, rutin developed a faint negligible tinge while morin showed concentration related faint palish coloration with absorbance range over 0.03 through 0.3 µmole ranging from 0.018 through 0.135 (r±S.E., 0.9991±0.0001, b ± S.E., 0.428±0.009). Each standard sample was uniformly read against sample concentration control. As evident (Table 2), the concentration range over linearity for test flavonoids was in general much lower, within 0.15 µmole (0.0164 to 0.164 µmole) than with DNPH assay, within 4 µmole. Comparison of regression coefficients between two assays (Table 3) reveals mean ± S.E. relative sensitivity of HCl method versus DNPH method has been 31 ± 11: the range being from 8 through 76 folds regression estimate with HCl than with DNPH assay; the least sensitivity is with quercetin and maximum with morin. DNPH assays require flavonoid concentration for assay purpose to be taken in 1 mg[6]. In contrast to DNPH assay, standard flavonoids (15) have responded effectively to 100 µg of flavonoid by $AlCl_3$ method while HCl method for test flavonoids provides linear response over 10 to 100 µg of flavonoid.

Table 2 Comparative response of test flavonoids to HCl method

Test flavonoid	Mass, µmole	Absorbance[a]	r± s.e.	b ± s.e.
Diosmin	0.0164	0.077±0.002	0.9998 ±0.0002	4.73 ±0.05
	0.041	0.193±0.002		
	0.082	0.398±0.004		
	0.164	0.774±0.004		
Daflon	0.0164	0.086±0.001	0.9999±0.0001	4.78 ±0.04
	0.041	0.202±0.001		
	0.082	0.403±0.002		
	0.164	0.791±0.003		
Morin	0.03	0.130 ±0.001	0.9999±0.0001	4.35±0.03
	0.09	0.394 ±0..002		
	0.15	0.663 ±0.002		
	0.30	1.318±0.008		
Rutin	0.015	0.067 ±0.006	0.9999±0.0001	3.53 ±0.01
	0.045	0.173 ±0.001		
	0.075	0.279 ±0.001		
Quercetin	0.03	0.131 ±0.001	0.9991 ±0.0001	3.54 ±0.03
	0.09	0.345 ±0.002		
	0.15	0.556 ±0.002		

[a] The values are mean ± S.E. of 5 observations each. The concentrations are in the range of 10 to 100 µg (diosmin, daflon and morin) and 10 through 50 µg (quercetin and rutin)

Response of test non-flavonoids to DNPH

A close scrutiny of the chemical structures of test flavonoids indicates that the flavonoids are either glycosides containing a disaccharide rutinose (composed of L-rhamanose (deoxy-mannose) and D-glucose (diosmin, rutin, hesperidin) or aglycones (morin and quercetin). Furthermore, the flavonoids exhibit characteristic phenolic nuclei such as presence of resorcinol (one molecule in each of test flavonoids and two in morin), guaiacol (one molecule in each of diosmin and hesperidin), and catechol (one molecule in each of quercetin and rutin). These

observations necessitated in testing phenolics and sugar responses to DNPH under test conditions. Chloral hydras was selected as a general standard to test its ability to react with DNPH keeping in view specificity of DNPH to C=O function and presence of HC=O in chloral hydras.

As evident in Table 4, all test phenolics baring guaiacol and phenol showed quantitatively a varied response to DNPH with most remarkable response shown by catechol. Regression estimate for catechol 0.513 is more than those obtained with flavonoids (Table 1) which is somewhat more than that of quercetin (0.45) and nearly twice of the value observed with rutin (0.216), two flavonoids that contain catechol function in their structure. Even control samples of the catechol over the concentration range 0.2 through 1 µmole showed perfect linearity (r ± S.E., 0.999±0.002; b± S.E, 0.054±0.002). Response to sucrose (a disaccharide containing aldose and ketose) was nearly 4 times more than to mono-sacharide (glucose). A comparison of regression coefficients (ratios approximated) reveals relative reactivity to DNPH by the test agents in the order:

Catechol (78 X) >> sucrose (2.5 X) > resorcinol (1 X) = chloral hydras (1 X) >>glucose (0.6 X)

Guaiacol up to 20 µmoles and phenol up to 100 µmoles failed to produce any effect. Failure of guaiacol to react suggests it to be an unlikely contributor to color reaction by diosmin and daflon.Contribution of sugar component to color reaction seems marginal and not mandatory. For instance, aglycone type flavonoids such as morin and quercetin reacted effectively with highest regression estimate observed with quercetin, 0.45, and that of its glycoside version rutin being about half, 0.216.(Table 3). Resorcinol function is a common functional moiety in all test flavonoids and results have indicated that regression estimate for resorcinol, 0.0066, is far less than those observed with test flavonoids (0.057 through 0.45) with morin that contains two resorcinol functions with least value of regression estimate (0.057) is nearly 9 times that obtained with resorcinol. Catechol is definitely contributing to the color reaction, as indicated by its high response and those of quercetin and rutin.

A comparative evaluation of observations of net absorbance values with 1 µmole of each test substance has revealed that none of the non-flavonoids baring catechol elicited any response. In contrast, mean net absorbance values (with control values within parenthesis) were respectively observed for catechol, 0.628±0.009 (0.053), rutin, 0.516 ±0.003 (0.402), quercetin, 0.470±0.005 (0.146), daflon, 0.216±0.001(0.029), diosmin, 0.168 ±0.001 (0.027) and morin, 0.051±0.005 (0.343). The control absorbance values reflect effect of alkali coupled to incubated test sample in acidic medium while net absorbance reflects effect in response to DNPH action.

The foregoing observations reveal that color reaction by DNPH is not significantly contributed by phenolics and sugar components baring catechol. However, to obviate their interference to whatsoever extent, it is proper to ensure their elimination from the flavonoid test extracts to enhance specificity of the determination. Chemicals with aldehyde functions (as indicated by experimentation with chloral hydras) must be necessarily be absent from the test samples.

The present study does not confirm that flavonoids other than flavanones can not be determined by the DNPH assay. In fact, the present study has indicated the method is complementary to determination of flavonoids in general. These findings are contrary to those presented by others[6], who have failed to get absorbance for 12 flavonoids at 1 mg concentration including three flavonols used in the present study viz., morin, quercetin and rutin. The mean absorbance values for 15 flavonoids using 1 mg each has recorded as 0.000 ± 0.000 for 12 flavonoids belonging to flavones(3), isoflavones(2) and flavonols including morin, quercetin and rutin(7) and while flavonones (3) such as naringin, (±) naringenin and hesperetin respectively as 0.113 ±0.005, 0.240± 0.002 and 0.258±0.013. The absorbance values obtained in present study for flavones and flavonols have been comparatively quite high (Table 1). Minimum recorded response to test flavonoids (Table 1) corresponds to about 68 µg of quercetin (linear range: 68 to 338 µg) and about 300 µg of diosmin (linear range: 304 to 2282 µg), daflon (linear range: 306 to 1216 µg), morin (linear range: 329 to 1317 µg) and rutin (linear range: 332 to 1495 µg).

Very high values obtained with morin, which is a flavonol, disregards the assertion that 2, 4-DNPH is not capable of detecting or determining flavones and flavonols[6]. Our observations with response of chloral hydras also confirm that the keto function is critical for its reaction with the reagent[9].

Table 3 Comparative evaluation of HCl versus DNPH assays for select flavonoids

Flavonoid	Regression coefficients over linear ranges[a]		Approximate Relative sensitivity[b]
	DNPH Assay	HCl Assay	
Diosmin	0.143 ± 0.002	4.73 ± 0.05	33
Daflon	0.214 ± 0.008	4.78 ± 0.04	22
Morin	0.057 ± 0.001	4.35 ± 0.03	76
Quercetin	0.450 ± 0.004	3.54 ± 0.03	8
Rutin	0.216 ± 0.014	3.53 ± 0.01	16

[a] In terms of μmoles flavonoid versus absorbance values
[b] Ratio of regression coefficients with HCl versus DNPH assays

Table 4 Comparative response of selected non-flavonoids to DNPH assay

Test flavonoid	Mass, μmole	Absorbance	r± s.e.	b ± s.e.
Catechol	0.2	0.211±0.002	0.9944± 0.0064	0.5134 ± 0.0315
	0.5	0.406±0.004		
	1.0	0.628±0.009		
Resorcinol	5	0.029±0.001	0.9998 ± 0.0002	0.0066 ± 0.0001
	10	0.063±0.002		
	15	0.095±0.003		
Sucrose	5	0.082 ±0.001	0.9979 ± 0.0024	0.0167 ± 0.0001
	10	0.181 ±0.002		
	20	0.335 ±0.001		
Glucose[a]	5	0.011 ±0.001	0.9996± 0.0005	0.0037 ± 0.0001
	15	0.043 ±0.001		
	50	0.176±0.006		
Chloral hydras	5	0.045±0.001	0.9946± 0.0062	0.0069 ± 0.0004
	10	0.069±0.001		
	20	0.146±0.001		
Guaiacol	5 - 20	No response		
Phenol	50-100	No response		

[a]Higher concentrations of glucose 100 and 250 μmole produced absorbance, respectively, as 0.169 ±0.005 and 0.192±0.006 with no significant difference with 50 μmole ($p>0.1$)
The values are mean ± S.E. of 5 observations each.

Conclusions

The study has provided an improvised simplified method for determination of flavonoids by DNPH method. The reagent shows strong interaction with catechol so that may be strongly contributing to the reaction due to quercetin and rutin. Contrary to the reports that the DNPH method is unsuitable for flavones and flavonols, the present investigation with employed protocol is capable of determining all test flavonoids. However, overall sensitivity of DNPH assay has been 8 to 78 times lesser than those observed with standard HCl method.

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