Sensitive Spectrophotometric Assay for 3-Hydroxy-Substituted Flavonoids, Based on Their Binding with Molybdenum, Antimony, or Bismuth

Palaniswamy Viswanathan, Venkataraman Sriram, and Ganesa Yogeeswaran*

Medical Biotechnology, Tamilnad Hospital Academic Trust, Cheran Nagar, Perumbakkam, Chennai 601 302, India

A sensitive spectrophotometric assay has been developed for flavonoids based on their binding with molybdenum, antimony, or bismuth. Acetylation of the hydroxyl group of flavonoids abolished metal binding, thus suggesting a direct role of the hydroxyl groups. From a comparison of several related flavonoids differing in the position of hydroxyl substitutions, the hydroxyl group at position 3 was found to be an important requirement for the formation of a yellow complex. This flavonoid metal complex showed that a specific and significant bathochromic shift in the visible spectrum of the native flavonoid and the corresponding λ_{max} value was used for the colorimetric assays with different metal salts. The molybdenum complex was found to yield higher absorbance compared to antimony and bismuth complexes of various flavonoids. The present method offers a sensitive assay in the 5−25 nM range for these flavonoids and gave comparable results with HPLC quantitative determination.

Keywords: *Flavonoids; fisetin; quercetin; metal complex; spectrophotometer; estimation; bathochromicity*

INTRODUCTION

Flavonoids belong to a diverse group of plant phenolics with ~4000 different compounds being identified so far. These compounds have a 15-carbon skeleton to which hydroxyl groups are substituted at different positions in the three rings, resulting in different structural variants (Figure 1). Their chemistry, metabolism, and distribution in higher plants have been well-reviewed (Cook and Samman, 1996). Different colors imparted by flavonoids were produced by minor changes in their chemical structures within the basic flavonoid structure. The phytopharmaceutical importance of flavonoids was widely investigated for its anti-inflammatory, antibacterial, antiviral, antimutagenic, antiallergenic, antioxidant, and anticancer properties (Middleton, 1993; Hanasaki, 1986; Hertog, 1995). In the past, certain flavonoids had been extensively used as sensitive probes for both detection and determination of several metal ions (Kathyal, 1968; Filer, 1971a−c; Rudolph and Ambrose, 1963). Flavonoids were also used for sequestering trace metals in the detoxification process (Middleton and Kandaswami, 1982).

Separation and quantitative determination of flavonoids require expensive instrumentation such as HPLC and GLC. Despite the many suggested biological functions of flavonoids, no specific colorimetric assay is widely available thus far to researchers to quantify flavonoids in phytochemical extracts and other food products. This might be in part due to the structural complexity of the diverse types of flavonoids. Although a general phenolic assay (Price and Butler, 1977) is available, it is subject to interference by many other compounds, such as antioxidants, often added to plant extracts, such as ascorbic acid and other phenolic acids. Furthermore, this method relies on the redox potential of the phenolic group and is, thus, nonspecific to flavonoids. Hagerman (1994) reviewed the use of an iron alum assay for the quantitative determination of cyanidins, which was also a spectrophotometric redox based assay and, hence, was fraught with the same shortcomings. Intrigued and inspired by the classical detection methods for flavonoids on thin-layer chromatography, which uses basic salts of molybdenum, antimony (Hellmut, 1994), or bismuth as detection reagents, we report here the successful development of a metal ion complexation assay for the specific quantitative determination of 3-hydroxy-substituted flavonoids as compared to other hydroxy-substituted flavonoids. The 3-hydroxy-substituted flavonoids have been implicated as putative phytochemical drugs of importance in certain medicinal plants, and hence this new assay might be of value.

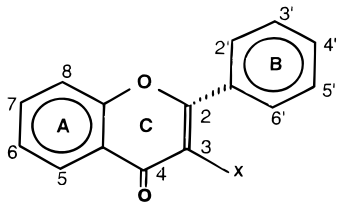

Figure 1. Generic structure of flavonoids. Compound position of OH group: **1**, flavone, unsubstituted; **2**, dihydroxyflavone, 3 and 6; **3**, resokaemferol, 3, 4′, and 7; **4**, fisetin, 3, 3′, 4′, and 7; **5**, quercetin, 3, 3′, 4′, 5, and 7; **6**, gossypetin, 3, 3′, 4′, 5, 7, and 8; **7**, apigenin, 4′, 5, and 7; **8**, luteolin, 3′, 4′, 5, and 7.

* Address correspondence to this author at the Department of Medical Biotechnology Research and Development, Sri Ramachandra Medical College and Research Institute, 1 Ramachandra Nagar, Porur, Chennai 600 116, India.

10.1021/jf990357q CCC: $19.00
Published on Web 06/10/2000

Table 1. Absorption Spectrum of Hydroxyflavone Complexes with Molybdenum, Antimony, and Bismuth Salts

compound	position of OH group	absorption maxima (nm)			
		native	metal complex flavonoids[a]		
			molydenum	antimony	bismuth
flavone		253, 293	290, 343	257, 293	
dihydroxyflavone	3,6	270, 304	300, 347	275, 303	267
resokaemferol	3,4′,7	267, 350	290, 423	263, 430	300, 420
fisetin	3,3′,4′,7	263, 360	290, 423	273, 430	300, 417
quercetin	3,3′,4′,5,7	260, 373	290, 453	273, 438	277, 433
gossypetin	3,3′,4′,5,7,8	267, 387	290, 403, 470	277, 383, 457	277, 385, 450
apigenin	4′,5,7	260,327	327	327	327
luteolin	3′,4′,5,7	260,335	335	340	338

[a] Spectral shifts of hydroxyflavones and their absorption maxima after complexation with metal ion reagents.

MATERIALS AND METHODS

Chloroform, ethyl acetate, methanol, and butanol were analar grade solvents, and antimony trichloride, bismuth nitrate, and ammonium molybdate were obtained from Merck. Flavone was obtained from Lancaster Chemical Co. Dihydroxyflavone, resokaemferol, apigenin, fisetin, luteolin, quercetin, and gossypetin were obtained from Arrow Chemicals and Pharmaceuticals, Chennai, India.

Extraction of Flavonoids. Bulbs of onion, seeds of black grapes, sun-dried tea leaves, and fresh leaves of a model medicinal plant, *Bryophyllum pinnata*, were taken for flavonoid analysis. The materials were fixed with absolute alcohol and were homogenized for 2 min at high speed using a glass/Teflon pestle polter−Elvejehm homogenizer. The homogenate was then filtered and dried in a vacuum. The dried ethanol extracts of all specimens were redissolved in 2 volumes of water by sonication and phase-partitioned with 10 volumes of petroleum ether/benzene (1:1 v/v) to remove soluble compounds. The aqueous fraction was then mixed with 10 volumes of ethyl acetate (EtOAc) and phase partitioned, and the cycles of processing were repeated two times. The organic fraction (EtOAc fraction) was separated out and used for further analyses. The EtOAc fraction rich in flavonoids was further purified by using C-18 reverse phase cartridges (Analytichem International) according to the method of Williams and McCluer (1980), and the bound flavonoids were eluted with chloroform/methanol (2:1 v/v). Such reverse phase purified flavonoid extracts were hydrolyzed with 0.1 N sulfuric acid for 2 h at 100 °C. The samples were neutralized with 0.1 N NaOH and phase partitioned with chloroform/methanol (2:1 v/v) to obtain the aglycon forms of flavonoids in the organic phase. For the study of the role of hydroxyl groups, nonspecific acetylation of the hydroxyl groups was performed using acetic anhydride in pyridine (Montreuil et al., 1994).

Stock Reagents. Millimolar stock solutions of all flavonoids tested were prepared in dry methanol (distilled analar grade methanol stored over molecular sieve) containing 0.01% butylated hydroxytoluene as antioxidant. Stock solutions of antimony(III) chloride were prepared in chloroform (0.2 g/mL). Bismuth nitrate stock (0.2 mg/mL) was prepared in anhydrous methanol. A 0.02% solution of ammonium molybdate stock was prepared in 50% methanol containing 0.1 N sulfuric acid. All stock solutions were stored at −20° C for up to 3−6 months. Working solutions were prepared by diluting stock solutions appropriately at the time of assay.

Metal Complexation with Flavonoids. Flavonoids with different hydroxyl groups (Figure 1) were used as standards to determine the role of the number of hydroxyl groups involved in metal complexation with flavonoids. Equimolar concentrations of all flavonoids were treated with calculated reagent excess (10-fold excess) for determining the spectral properties of the organometallic complex thus formed. The metal complexes were further studied by TLC analysis for the content of bound and unbound flavonoids. Precoated TLC plates (with F-254 fluorescent indicator) were purchased from Merck, Darmstadt, Germany. The solvent system used for separation was *n*-BuOH/HOAc/H_2O (4:1:5 upper phase). The plates were studied under a UV light source (254 nm).

HPLC Analysis of Flavonoids in Different Plant Sources. The aglycon fractions of flavonoids extracted from onion, tea leaves, grape seeds, and *B. pinnata* leaves were analyzed quantitatively by HPLC. HPLC separations were performed on an octadecylsilane C-18 column (3.9 × 150 mm, 3 μm) from Shimadzu Corp. The chromatographic system consisted of a Shimadzu (SCL-6B) system controller and a UV−visible detector set at a fixed wavelength of 370 nm. A mobile phase of methanol/water (7:3) was used at a flow rate of 0.9 mL/min. Peak identity was defined by the retention time observed for standard quercetin.

RESULTS AND DISCUSSION

Spectral Studies on the Role of Hydroxyl Groups. A comparative spectral analysis with different standard flavonoids (Table 1) showed that hydroxyl substitution at C-3 in the C-ring was essential for a discernible spectral shift in the visible region, after binding with the metal ions. At the outset of these studies, when metal complexation studies were done with glycated flavonoids (rutin, gossypin, etc.), there was no observable bathochromatic shift. When these flavonoid glycosides were acid hydrolyzed to form aglycons, the metal ion was found to bind with these aglycosidic flavonoids. In some of the flavonoid glycosides tested (position 3 glycation), the glycan moiety bound to the 3-OH position may directly inhibit the formation of metal complex.

Absorption maxima of native (before metal complexation) compounds were found to be in the UV region. Upon binding with antimony or bismuth salts, the maxima were found to shift only to a small extent in the UV region with respect to unsubstituted flavone and dihydroxyflavone (Figure 2A,B). No change in spectral maxima was found in these compounds after reaction with molybdenum salts. On the other hand, tri-, tetra-, penta-, and hexaflavonoids (with a 3-OH in the C-ring) exhibited a bathochromic shift from the UV to the visible region after their reaction with these metal salt reagents (Figure 2C−F). The formation of a yellow fluorescent color complex was observed in all flavonoids (except flavone and dihydroxyflavone) upon binding with metals. The TLC analysis of metal−flavonoid complexes revealed that the metal-bound complex stayed in the origin of the plate, whereas there were only negligible unbound native flavonoids, suggesting near completion of the reaction (data not shown). The absence of any bathochromic shift with native flavone (with no hydroxyl groups) even after prolonged treatment with metal reagents proves the importance of hydroxyl substitution for metal complexation.

Protection of hydroxyl groups by nonspecific acetylation also inhibited the formation of metal−flavonoid complexes and was further confirmed by the change in

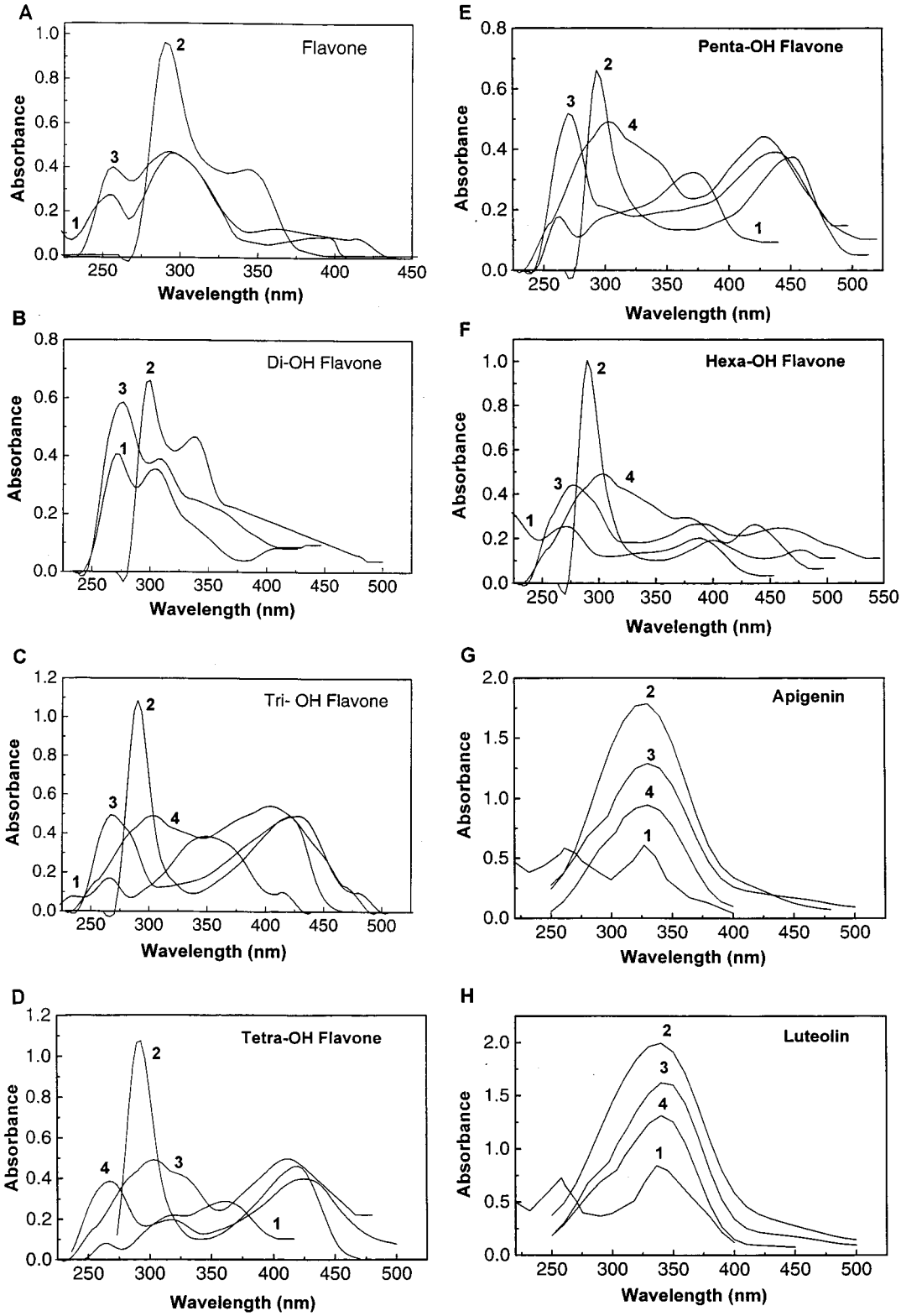

Figure 2. Absorption spectra of hydroxyflavones complexed with metal reagents. The spectra shown are representative of three separate analyses scanned at 20 nm/min intervals: (A) flavone complexed with Sb and Bi; (B) dihydroxyflavone complexed with Sb and Bi; (C−H) resokaemferol, fisetin, quercetin, gossypetin, apigenin, and luteolin complexed, respectively, with Sb, Bi, and Mo; (1) native form of hydroxyflavone; (2) antimony complex of hydroxyflavone; (3) bismuth complex of hydroxyflavone; (4) molybdenum complex of hydroxyflavone.

mobility of the acetylated flavonoid during TLC analysis (unpublished observation). Comparable increases in the bathochromic shift of resokaemferol, fisetin, quercetin, and gossypetin after complexation with metals suggest an involvement of more than one hydroxyl group. The absence of such a spectral shift in either apigenin (4′,5,7-hydroxyflavone) or luteolin (3′,4′,5,7-hydroxyflavone) containing tri- and tetrahydroxy substitutions, respectively, clearly implicates the role of the hydroxyl group at position 3 in the C-ring for metal complexation. However, there was no appreciable bathochromic shift in dihydroxyflavone (3,6-hydroxy flavone). This might

be due to either structural impedance of metal binding due to the orientation of the hydroxyl group at position 6 or insufficiency of other hydroxyl group interaction with the metal cations. In other words, we suggest that a cooperative effect of one or more hydroxyl groups with the hydroxyl group at position 3 is required for effective binding with metal salts to produce a bathochromic spectral shift. It had also been proven earlier that flavonoids, which do not contain a free hydroxyl group at position 3 or 5 in the C-ring, did not form complexes with aluminum chloride (Kathyal, 1968). Furthermore, complexation of the 3-hydroxyl group with metals results in a flavylium structure, which is greatly stabilized by its partly aromatic character (Kathyal, 1968). Hence, our present results are somewhat analogous (with respect to the 3-OH) to the aluminum complexation data of other workers in the past (Kathyal, 1968) and the 5-OH group might possibly be the cooperating hydroxyl group along with 3-OH.

Comparison of Metal Reagents. Spectral scans (Figure 2A−F) of the reaction mixtures were presented for comparing the relative sensitivity and bathochromic spectral shift of antimony, bismuth, and molybdenum complexes of corresponding flavonoid. The reactivity of antimony and bismuth toward different compounds shows only slight variations, despite their valence differences. Molybdenum shows a higher sensitivity compared to antimony and bismuth in their spectral shifting capacities after binding with tri- to hexahydroxy flavonoids. The bathochromic shift in the UV (flavone and dihydroxyflavone alone) and visible region was higher with molybdenum salts compared to either antimony or bismuth. The absence of bathchromatic shift and increasing spectral yield observed in apigenin and luteolin (Figure 2G,H) suggested the importance of 3-position hydroxy substitution. When we tested the efficiency of different soluble salt reagents of arsenic, zinc, copper, and borate to form complexes with various flavonoids, the reaction complex was unstable, with a decrease in absorbance within a few minutes (unpublished observation). Hence, these flavonoid metal (As, Zn, or Cu) complex do not have any bathochromic shift and offer no value for colorimetric assays.

Reaction Stability and Effect of pH and Temperature. The color yield of reaction mixtures was found to be stable for 24 h at room temperature without any change in their absorbance. After 24 h, the molybdenum complex was found to turn blue and the absorption maxima shifted to 540 nm. Variation in pH also affected the color yield in the complexation assay. This was mainly due to the fact that the metal salt reagents could be soluble only in acidic medium, and increasing the pH of the reagents or reaction mixture resulted in precipitation of the metal reagents and the colored complex. The reaction mixture was incubated at 40, 60, 80, and 100 °C for different time periods, and a proportional decrease in the absorbance was observed with an increase in temperature (data not shown). Thus, increasing the temperature of the reaction condition altered the complex formation, suggesting that room temperature (27 ± 2 °C) and acidic pH were ideal for assay conditions.

Linear Regression Analysis. Absorption maxima of the reaction products of fisetin, quercetin, and gossypetin with metal salts range from 410 to 450 nm. Linear regression analyses (Figure 3) with a concentration range of 5−25 nmol for different substrates

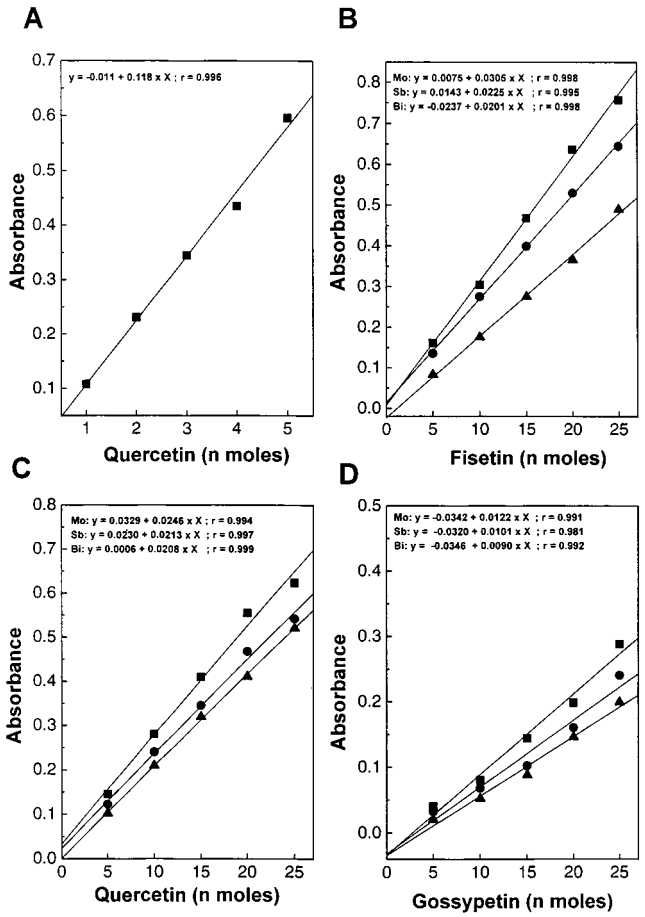

Figure 3. Linear regression analysis of metal assays compared with general phenolic assay: (A) Prussian blue assay for general phenolics (quercetin as standard, λ_{max} = 720 nm); (B) fisetin complex with Sb, Bi, and Mo (λ_{max} = 420 nm); (C) quercetin complex with Sb, Bi, and Mo (λ_{max} = 430 nm); (D) gossypetin complex with Sb, Bi, and Mo (λ_{max} = 450 nm); (■) hydroxyflavone + Mo; (●) hydroxyflavone + Sb; (▲) hydroxyflavone + Bi.

revealed molybdenum complexes to be more sensitive compared to the other reagents. The Prussian blue assay (Figure 3A) was more sensitive than all metal assays (1−5 nmol range), but the interference of other phenolics was high in this assay. Although quercetin is known to bind with molybdenum and related cations in the ratio of 1:1 and 1:2 (Kathyal, 1968), we observed only a binding efficiency of 1:1 in all flavonoids with Sb, Bi, and Mo. Interestingly, all three metal salts used in our metal complexation assay are absent in many biological system and hence might show negligible competition with other metals.

The detection limit and determination limit were found for antimony using quercetin as standard flavonoid. To determine the precision obtained with larger quantities of antimony, a set of 2−100 nmol standards (2 nmol intervals) was individually analyzed. The results indicate a detection limit of 50 nmol of flavonoid with respect to all three metals; a nonlinear increase was observed beyond 50 nmol. The absorbance value for samples containing up to 25 nmol of quercetin was linear within the precision of the procedure (CV percentage = 2), and deviation from linearity was observed beyond 50 nmol quantities. The minimum quantity of flavonoid that can be determined with a precision of 10% is 2 nmol. The molar absorptivities of quercetin−metal

Table 2. Quantitation of Flavonoids in Different Plant Products Using Metal Binding Assay Compared with HPLC

plant products	metal binding assay[a]			quantification by HPLC
	antimony	bismuth	molybdenum	
onion	1324 ± 37	1290 ± 30	1141 ± 87	1453
Bryophyllum	185 ± 6	188 ± 18	178 ± 5	243
black tea	993 ± 29	993 ± 2	993 ± 12	1217
grape seed	149 ± 19	132 ± 3	165 ± 1	201

[a] With reference to quercetin standard, units are expressed as nanomoles per gram of fresh tissue weight. Results are mean ± SD of triplicate values from two individual batches of extracts.

complexes were determined to be 3.1×10^7, 2.5×10^7, and 1.8×10^7 for Mo, Sb, and Bi, respectively.

Estimation of Flavonoids. Reaction efficiency was analyzed by using ethyl acetate fractions of different plant products (Table 2). Precision and accuracy of the metal assays were confirmed by using HPLC for comparison of flavonoid estimation in plant products. In HPLC the retention time of 7.3 min was observed for pentahydroxyflavone (quercetin) in onion, tea, grape seed, and *Bryophyllum* extracts. On the basis of the peak area measurement for quercetin in HPLC, the comparison was made with the metal assay. There was good agreement between the two assays as observed for onion, tea, and *Bryophyllum pinnata* samples (coefficient of variation ranging from 4.5 to 17%). However, a significant variation in the flavonoid content of grape seeds (coefficient of variation ranging from 26 to 33%) was observed. This variation was found to be due to interference of anthocyanidins present in the flavonoid fraction of grape seed. Anthocyanidins were found to interfere in the reaction of flavonoids with metal reagents, decreasing the absorbance values to ~25−50% in a concentration-dependent manner (unpublished observations). In summary, the complexation of molybdenum, antimony, or bismuth with 3- and 5-hydroxy-substituted flavonoids offers a versatile colorimetric method of analysis for these phytochemicals of medicinal value in plant extracts.

Conclusions. A new analytical method , without a need for sophisticated instrumentation, provides a simple colorimetric estimation of 3-hydroxy flavonoids present in plant extracts. The results suggested that the direct measurements of soluble flavonoids in the unknown fractions were in agreement to ~75−80% as compared with results obtained by HPLC. The metal salts of Mo, Sb, and Bi (in decreasing order of affinity) complex with 3-hydroxy-substituted flavonoids, exhibiting a bathochromic shift with the formation of yellow fluorescent color. Anthocyanidins prevented the color formation and, hence, necessitated the removal of the coloring pigments from plant materials prior to the assay. The rapidity and simplicity of the method make it ideal for flavonoid estimation in various native medicinal plant products as well as foods and beverages.

ACKNOWLEDGMENT

HPLC analysis facility was kindly provided by Dr. N. M. Samuel, Professor and Head of Department of Experimental Medicine, Dr. MGR Medical University, Chennai, India.

LITERATURE CITED

Cook, N. C.; Samman, S. Flavonoids—Chemistry, metabolism, cardio-protective effects, and dietary sources. *J. Nutr. Biochem*. **1996**, *7*, 66−76.

Filer, T. D. Fluorometric determination of submicrogram quantities of zirconium. *Anal. Chem*. **1971a**, *43*, 469−473.

Filer, T. D. Fluorometric determination of submicrogram quantities of antimony. *Anal. Chem*. **1971b**, *43*, 725−729.

Filer, T. D. Fluorometric determination of submicrogram quantities of tin. *Anal. Chem*. **1971c**, *43*, 1753−1757.

Hagerman, E. A.; Butler, G. L. Assay of condensed tannins or flavonoid oligomers and related flavonoids in plants. *Methods Enzymol*. **1994**, *234*, 429−437.

Hanasaki, Y.; Ogawa, S.; Fukui, S. The correlation between active oxygen scavenging and antioxidative effects of flavonoids. *Free Radical Biol. Med*. **1994**, *16*, 845−850.

Hellmut, J.; Werner, F.; Walter, F.; Hans, W. *Thin-Layer Chromatography, Reagents and Detection Methods*; VCH: Weinheim, Germany, 1994; Vol. 1B, pp 206−209.

Hertog, M. G. L.; Hollman, P. C. H. Potential health effects of the dietary flavonol quercetin. *Eur. J. Clin. Nutr*. **1996**, *50*, 63−71.

Kathyal, M. Flavones as analytical reagents—A review. *Talanta* **1968**, *15*, 95−106.

Middleton, E., Jr.; Drzewiecki, G. Effects of flavonoids and transitional metal cations on antigen-induced histamine release from human basophils. *Biochem. Pharmacol*. **1982**, *31*, 1449−1453.

Middleton, E., Jr.; Kandaswami, C. The impact of plant flavonoids on mammalian biology: Implications for immunity, inflammation and cancer. In *The Flavonoids: Advances in Research since 1986*; Harborne, J. B., Ed.; Chapman and Hall: London, U.K., 1993; pp 619−652.

Montreuil, J.; Bouquelet, S.; Debray, H.; Lemoine, J.; Michalski, J. C.; Spik, G.; Strecker, G. Glycoproteins. In *Carbohydrate Analysis—A Practical Approach*; Chaplin, M. F., Kennedy, J. F., Eds.; IRL Press: Oxford, U.K., 1994; pp 188−189.

Price, M. L.; Butler, L. G. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *J. Agric. Food Chem*. **1977**, *25*, 1268−1273.

Rudolph, S. B.; Ambrose, T. Fluorometric determination of tungsten with flavonol. *Anal. Chem*. **1963**, *35*, 1910−1912.

Williams, M. A.; McCluer, R. H. The use of sep-pak C18 cartridges during the isolation of gangliosides. *J. Neurochem*. **1980**, *35*, 266−269.

Received for review April 13, 1999. Revised manuscript received March 28, 2000. Accepted April 2, 2000. This work is partially supported by the facilities and funds available from the Research Council of Tamilnad Hospital Academic Trust. Senior Research Fellowships offered by the Council of Scientific and Industrial Research, Government of India, to P.V. and V.S. are gratefully acknowledged.

JF990357Q