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Colorimetric Method for the Estimation of Total Limonoid Aglycones and Glucoside Contents in Citrus Juices

ANDREW P. BREKSA, III* AND PHIL IBARRA, JR.

Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan Street, Albany, California 94710

A method for estimating the total limonoid aglycone and glucoside concentrations in *Citrus* samples in terms of limonin and limonin glucoside equivalents is presented. The method consists of extraction followed by colorimetric quantification. The colorimetric quantification was based on the formation of red to orange colored derivatives resulting from the treatment of limonin, limonin glucoside, or a fruit extract with 4-(dimethylamino)benzaldehyde (DMAB) in the presence of perchloric and acetic acids. Absorbance maxima for the limonin and limonin glucoside derivatives were found to be 470 and 503 nm, respectively. The influence of DMAB concentration, reaction time, and solvent composition on color development and sensitivity were investigated and optimal assay conditions established. With a microplate format under these conditions, the limits of detection and quantification were determined to be 0.25 and 0.50 μg/mL for limonin and 0.50 and 1.0 μg/mL for limonin glucoside.

KEYWORDS: Limonoids; citrus; solid-phase extraction; juice analysis

INTRODUCTION

The recognition that citrus fruits and juices are one of the most healthful components of the human diet has led to a growing cognizance that much of the positive contribution to human health, nutrition, and consumer acceptance may be attributed to the presence of plant secondary metabolites in these fruits and juices. Due to the complexity and diversity within these families of secondary metabolites, the analyses are generally conducted in a two-step process in which the total concentration of a given family of metabolites is first estimated in equivalents of the predominate metabolite through a colorimetric assay followed by, if needed, a more complete analysis by HPLC or LC-MS to identify individual metabolites and establish their relative concentrations. The goal of this study was to establish a simple and rapid colorimetric method for estimating limonoid aglycone and glucoside concentrations in citrus juices. On the basis of a review of the colorimetric methods used for other plant secondary metabolites (e.g., flavonoids (flavanones (*1*, *2*), anthocyanins (*3*, *4*)) simple phenolics (*1*, *2*), and carotenoids (*5*)) we established the following design criteria: the method must be simple, rapid, solution-based so a spectrophotometer could be used for quantification, amendable to the simultaneous analysis of multiple samples, and able to incorporate a commercially available standard or established extinction coefficient.

To date, no methods for the spectrophotometric determination of limonoid glucosides have been reported, whereas three methods, two colorimetric and one fluorometric, have been reported for the analysis of citrus limonoid aglycones. None of the three assays, examined in their present form, meets all of the criteria established above. The colorimetric method of Wilson and Crutchfield (*6*) and the fluorometric (*7*) method do not meet the rapid and simple criteria because the chromophore and fluorophore forming reactions are nonspecific and require multistep extractions to remove interfering components. The colorimetric method reported by Vaks and Lifschits (*8*), as part of their study of the bacterial degradation of limonin, overcame the lack of specificity by utilizing 4-(dimethylamino)benzaldehyde (DMAB), a furan, thiofuran, and indole ring-specific indicator that was also the same indicator used in the Ehrlich reagent. While they were able to estimate limonoid metabolite concentrations by directly combining chloroform extracts of bacterial cultures with an indicator solution composed of DMAB dissolved in a mixture of perchloric and acetic acids, the resulting biphasic solution precluded use of a plate reader and required that the volume of solution be matched to the optical path of the spectrometer. However, even with that principal drawback, there have been at least three reports (*9*−*11*) of this method having been used to estimate limonin concentrations in citrus juices, including one in which results obtained were found to be comparable to those obtained by HPLC (*11*). These reports warranted further evaluation of the method of Vaks and Lifschits as the basis of developing a standard protocol for estimating total limonoid aglycone and glucoside concentrations. Reported herein are the results from our evaluation of solvent composition, reaction time, and DMAB concentration, and the ultimate resulting methods for estimating limonoid aglycone and glucoside concentrations in citrus juices that meet the criteria described above.

* Corresponding author. Phone:510-559-5898. Fax:510-559-5849. E-mail: apb3@pw.usda.gov.

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MATERIALS AND METHODS

Materials. Water was distilled and deionized. Solvents (Fisher, Pittsburgh, PA) were HPLC grade. Perchloric acid (70%, ACS Reagent) and glacial acetic acid (ACS PLUS) were purchased from Fisher, and DMAB (Ehrlich's Reagent) was purchased from Sigma (St. Louis, MO). Strata-X (30 mg, 1 mL) solid-phase extraction (SPE) columns were obtained from Phenomenex (Torrance, CA). All other reagents were analytical grade.

Preparation of Limonin and Limonin Glucoside Stock Solutions and Standards. Limonin and limonin glucoside, isolated and evaluated for purity as previously described (12), were used to prepare 500 μg/mL stock solutions in acetonitrile. Stock solutions were stored at −20 °C until needed. From the stock solutions, limonin standards (0.5−100 μg/mL) were prepared in acetonitrile, whereas limonin glucoside standards (0.5−100 μg/mL) were prepared in 30% acetonitrile (aq).

Preparation of DMAB Indicator Reagent. The DMAB indicator reagent was prepared based on the methods of Vaks and Lifschits (*8*) and Abbasi et al. (*11*) with some modifications based on the results from our investigation. Perchloric (70%, 240 mL) and acetic (glacial, 300 mL) acids were combined to prepare a stock acid solution. The DMAB indicator reagent was freshly prepared prior to use and formed by dissolving DMAB (1.11 g) in the stock acid solution (30 mL).

Determination of Limits of Detection and Quantification. Limits of detection (LOD) and quantification (LOQ) were estimated in terms of baseline noise and instrument response. To determine the LOD and LOQ, serial dilutions of limonin and limonin glucoside from 0 to 100 ppm were evaluated. LOD was established as the limonoid concentration that yielded an absorbance greater than the sum of the mean plus 3× the standard deviation of the zero ppm standard ($n = 3$), whereas LOQ was defined as the concentration that yielded an absorbance greater than the sum of the mean plus 10× the standard deviation of the zero ppm standard ($n = 3$).

Juice Samples. Valencia oranges, Eureka lemons, Washington navel oranges, Honey tangerines, and Rio Star grapefruits were purchased from a local market in early May 2006. In preparation for analysis, pieces of fruit were cut in half and juiced by hand. Hand juicing was accomplished using a plastic juice reamer, taking care not to disrupt the albedo. For each sample, juice from a minimum of two fruits was combined. Prior to extraction, juice samples were clarified by centrifugation (16000*g*, 5 min, 10 °C), and the supernatant was collected and filtered through filter paper (Whatman #1, Whatman Inc., Clifton, NJ).

Extraction and Determination of Limonin Equivalents. The selective extraction of aglycones from juice was accomplished by chloroform extraction (*13*). A 1:2 mixture of clarified juice (1 mL) and $CHCl_3$ (2 mL) was vortexed (2 min) and then centrifuged (2800 rpm, 5 min) to expedite the separation of the phases. An aliquot of the chloroform phase (1 mL) was collected and evaporated to dryness. The samples were reconstituted in acetonitrile (0.5 mL). Extractions were conducted in triplicate.

The assay was conducted using a clear flat bottom 96-well assay plate with samples and standards organized as follows. The standards (110 μL) were placed in a single column. Samples (110 μL) were plated in duplicate in adjacent columns to give sample and blank columns. A single point calibrator (generally the 75 μg/mL standard) was included with each column in order to account for the time required for the addition of the DMAB indicator or stock acid solutions. Beginning with the standards, the DMAB indicator (165 μL) and stock acid solutions (165 μL) were added rapidly column-by-column using a multichannel pipet. Following incubation at room temperature for 30 min the absorbance at 470 nm was read on a Molecular Devices Spectromax 384-Plus plate reader (Sunnyvale, CA). Samples with absorbance values in excess of the standards were diluted and re-evaluated. Software supplied with the instrument was used to calculate the gross limonin equivalence for each well based on the limonin calibration curve. Using these values, the limonin equivalence (μg/mL) of the sample was calculated using the equation

$$\text{limonin equivalence } (\mu\text{g/mL}) = [(E_{\text{DMAB}} - E_{\text{Blank}})] \times \text{DF} \times N$$

where E_{DMAB} = gross limonin equivalents of the sample from calibration curve, E_{Blank} = gross limonin equivalents of the blank from calibration curve; DF = dilution factor, and N = limonin equivalence of the single point calibrator (known value/ measured value).

Extraction and Determination of Limonin Glucoside Equivalents. Clarified juice (0.5 mL) was applied to a Strata-X SPE column that had been washed with MeOH (1 mL) and equilibrated in water (1 mL). The flow through and a water wash (2 mL) were discarded. After drying under vacuum for 1 min the column was eluted with MeOH (1 mL). The resulting effluent was evaporated to dryness. The sample was reconstituted in 30% acetonitrile (0.5 mL) and further diluted 5:1 with 30% acetonitrile prior to quantification. Extractions were conducted in triplicate. The assay and subsequent data analysis were conducted as described for limonin with the exception that the limonin glucoside standards were used in place of the limonin standards and the absorbance was read at 503 nm. The LG equivalence (μg/mL) of the sample was calculated using the equation

$$\text{LG equivalence } (\mu\text{g/mL}) = [(E_{\text{DMAB}} - E_{\text{Blank}})] \times \text{DF} \times N$$

where E_{DMAB} = gross LG equivalents of the sample from calibration curve, E_{Blank} = gross LG equivalents of the blank from calibration curve; DF = dilution factor, and N = LG equivalence of the single point calibrator (known value/measured value).

Spike Recovery Experiments. Spike recovery experiments were conducted using valencia orange juice spiked with either limonin or limonin glucoside. For limonin recovery experiments, orange juice (9.9 mL) that had been clarified and filtered was spiked with acetonitrile (0.1 mL) containing 0, 500, 1000, or 2000 μg/mL of limonin. For limonin glucoside spike experiments, the clarified and filtered orange juice (9.0 mL) was spiked with water (1.0 mL) containing 0 or 400 μg/mL of limonin glucoside. Three independent experiments were conducted at each level of spike following the extraction and quantification procedures described above.

RESULTS AND DISCUSSION

Initiating our studies from the conditions described by Vaks and Lifschits (*8*) we have developed a method for estimating the total limonoid aglycone and glucoside concentrations in *Citrus* samples in terms of limonin and limonin glucoside equivalents. Limonin and limonin glucoside were chosen as the standards since for most citrus species they are the most abundant aglycone and glucoside and both are commercially available. In addition, for the majority of citrus limonoids the B−C−D-ring structure (Figure 1) remains intact while the biosynthetic effort focuses on the conversion of the A-ring of nomilin to the A−A′-ring structure of limonin (*14*). The majority of other aglycones isolated from citrus are intermediates possessing variations in the structure of the A-ring (e.g., obacunone, ichangin). Akin to the aglycones, the glucosides isolated thus far vary in the structure of their A-ring, while uniformly maintaining the same B−C ring structure with the D-ring opened to a carboxylic acid and a single glucose moiety attached to the C-17 hydroxyl.

Having these standards in hand, we developed a method that is simple, rapid, and amendable to the simultaneous analysis of multiple samples. Quantification is accomplished with a homogeneous solution, which subsequently allows a plate reader to be utilized for quantification. Analysis consists of extraction, and chloroform liquid extraction for aglycones and solid-phase extraction for glucosides, followed by evaporation and reconstitution in acetonitrile for aglycones, or 30% acetonitrile (aq) for glucoside samples prior to colorimetric quantification. The colorimetric quantification is based on the formation of red to orange colored derivatives resulting from the treatment of the limonoid standards or samples with DMAB in the presence of perchloric and acetic acids. Absorbance maximums for limonin

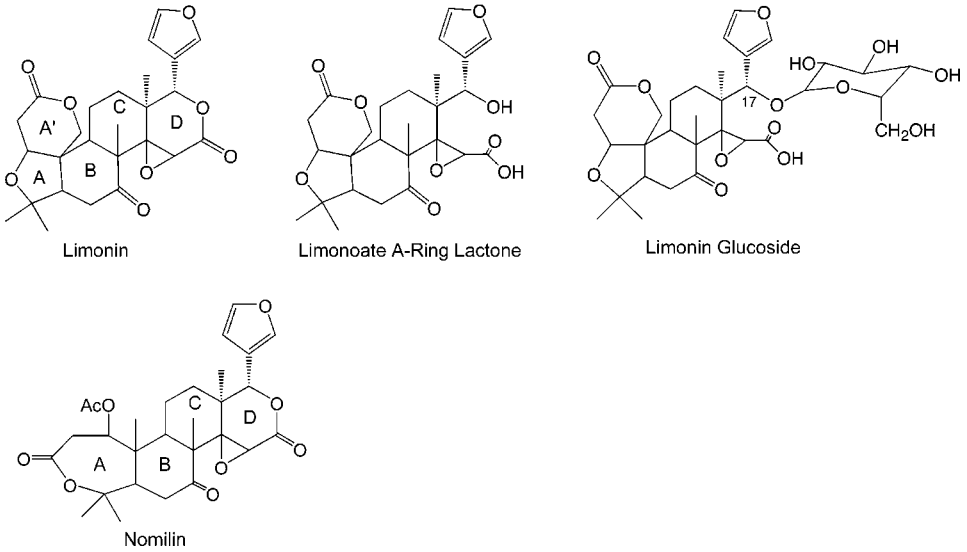

Figure 1. Structures of limonin, limonoate A-ring lactone, limonin glucoside, and nomilin.

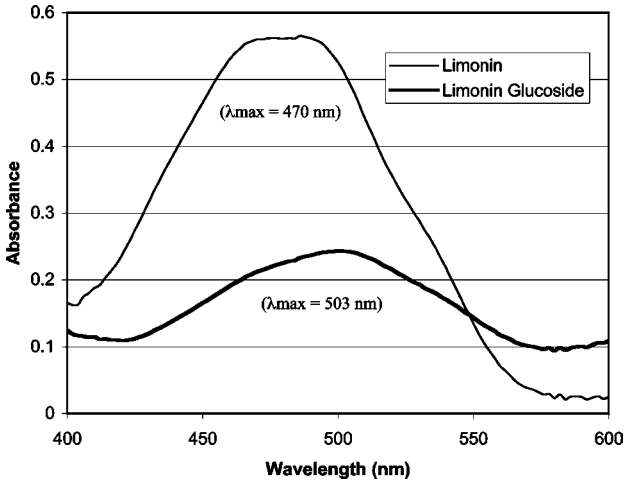

Figure 2. Visible spectra (400–600 nm) of limonin and limonin glucoside reaction products. Limonin and limonin glucoside were dissolved in acetonitrile (100 μg/mL) and reacted with the DMAB indicator reagent. After a 30 min incubation at room-temperature, spectra were obtained using a Hewlett-Packard 8452A diode array spectrophotometer (Palo Alto, CA).

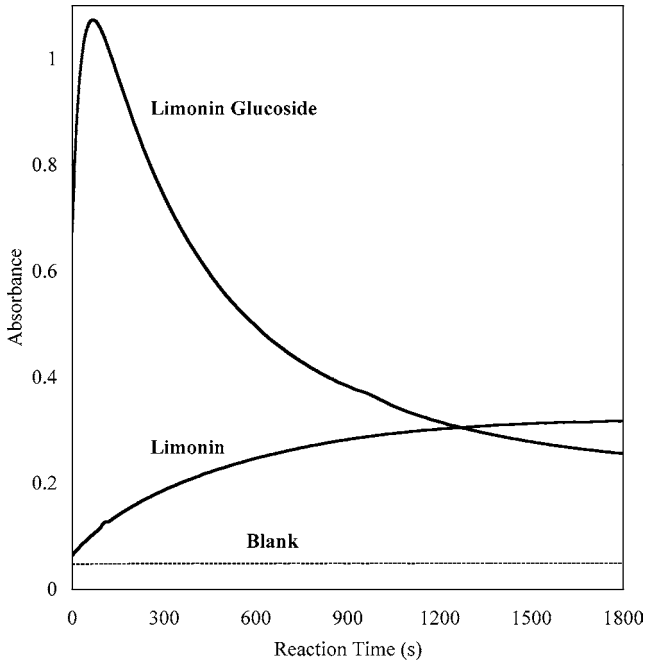

Figure 3. Absorbance vs time (seconds) of limonin and limonin glucoside reaction products.

and limonin glucoside derivatives were found to be 470 and 503 nm, respectively (Figure 2). Limonoate A-ring lactone (LARL, Figure 1) was also tested and found to mirror the properties of limonin, likely due to the rapid conversion of LARL into limonin when treated with the acidic indicator solution (*15*). The spectral characteristics of the limonin glucoside derivative have not been previously reported, whereas Vaks and Lifschits had reported a 503 nm absorbance maximum for limonin. Possibly the differences in the limonin absorbance maximums are a result of the nature of the solutions, homogeneous versus biphasic, or caused by the presence of chloroform. Changes in the acetonitrile content had no effect on the absorbance maximums, but did influence the extent to which the colorimetric derivatives formed.

Figure 3 shows the formation of the colorimetric derivatives of limonin and limonin glucoside over 30 min. Limonin shows a gradual increase that eventually stabilizes, whereas limonin glucoside shows a rapid increase followed by a decrease. On the basis of these results we elected to retain the 30 min endpoint suggest by Vaks and Lifschits for limonin and for convenience choose to utilize the same endpoint for limonin glucoside.

Kuroda et al. (*16*) in their study of the reaction of furanoeremophilanes with DMAB determined that the furans reacted with DMAB in a two-step process that ultimately affords condensation products in a ratio of 2:1, furan to DMBA. Furthermore, the first step of the reactions yielded 1:1 colored cationic intermediates. In the second step, reduction of the cation by condensation of a second furan gave 2:1 products that were colorless. The reaction profiles observed for limonin and limonin glucoside suggest that these limonoids likely follow a similar reaction mechanism and lead to the hypothesis that the freedom afforded at C-17 by the open lactone ring of limonin glucoside facilitates the formation of the 2:1 adduct, whereas in the case of limonin, rigidity of the lactone ring may impose steric constraints that retard the formation of the 2:1 product. Our observations that the red color of the limonin test solutions persisted for several days and the limonin glucoside solutions were colorless within several hours add additional credence to this hypothesis. Although not within the scope of this study,

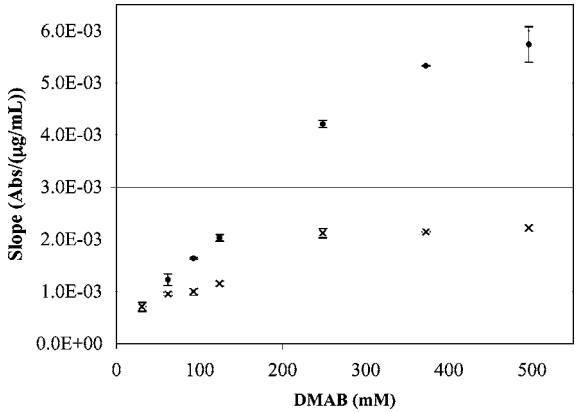

Figure 4. Slope (Abs/(μg/mL)) vs 4-(dimethylamino)benzaldehyde concentration (mM): limonin (●), limonin glucoside (X). Values are means ± SD, $n = 3$.

elucidation of the exact identity of the limonoid condensation products would be essential to confirm this hypothesis and should be a subject of future studies.

Figure 4 shows the effect of DMAB concentration on the overall formation of the colored adducts. DMAB concentrations from 25% to 500% (31−621 mM), the level reported by Vaks and Lifschits, were tested for both limonin and limonin glucoside. An increase in the DMAB concentration up to the 621 mM level resulted in a concurrent increase in the absorbance signal obtained for limonin. For limonin glucoside, the increase reached a plateau at 248 mM (200%). For simplicity the 248 mM level was selected so that a single reagent could be used for both limonin glucoside and limonin. To assess the effects of solvent composition on the formation of the colored adducts, calibration curves of the limonin and limonin glucoside standards were prepared in methanol, ethanol, acetonitrile, and mixtures of these solvents with water (0−70% water v/v). Results for samples prepared in the organic solvents were indistinguishable, indicating that the solvent polarity going from MeOH to acetonitrile had little effect on the reaction kinetics. The same result was found for the aqueous limonoid glucoside solutions. However, in the case of limonin we found that as the concentration of water increased, the intensity of the signal obtained decreased. The presence of water likely retards the formation of the colored cationic intermediate rather than accelerates the formation of the colorless 2:1 adduct. This hypothesis is supported by the observations of Riad et al. (*17*) that the presence of water in the condensation reaction between 2-methylfuran and paraformaldehyde results in significantly reduced yields of the condensation product.

Methods for the analysis of limonin glucosides, with the exception of LC-MS (*18*), require that samples be processed through a combination of absorptive and ion exchange chromatographic steps prior to analysis (*19*−*21*). Because of the specificity of the DMAB indicator solution, we sought to reduce the sample preparation to a single styrene divinyl benzene (SDB) solid-phase extraction (SPE) step. The limonoids, both aglycone and glucoside, were captured using a Strata-X SPE; however, we were unable to find suitable conditions to selectively elute limonin glucoside. To determine if the solvent dependent differences in reactivity could be used to selectively measure glucoside concentrations in the presence of aglycones, a set of limonin standards were prepared in 30% acetonitrile and the limonin glucoside equivalents for each standard measured (Figure 5). The differences in reactivity and absorbance maximums combined together resulted in suppressing the aglycone dependent signal by a factor of 7. Considering that

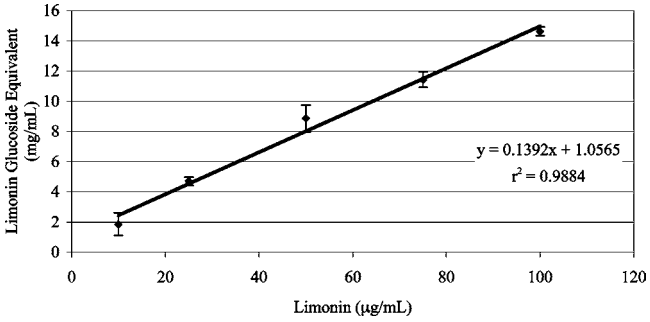

Figure 5. Plot of limonin glucoside equivalence (μg/mL) vs limonin concentration (μg/mL) for the limonin standards demonstrates that the conditions used to determine limonin glucoside equivalents result in selective suppression of limonin dependent signal. Limonin standards (10–100 μg/mL) were dissolved in 30% acetonitrile (100 μg/mL) and reacted with the DMAB indicator reagent Values are means ± SD, $n = 3$.

Table 1. Total Limonoid Agylcone and Glucoside Equivalents in Citrus Juices

sample	aglycone equivs[a,b]	glucoside equivs[b,c]
lemon	7.1 ± 0.8	43.5 ± 3.9
Rio Star grapefruit	13.1 ± 1.4	216.9 ± 17.4
Honey tangerine	1.8 ± 0.2	148.8 ± 9.9
Valencia	5.5 ± 1.5	154.1 ± 5.6
Washington navel	2.4 ± 0.3	141.3 ± 2.4

[a] Expressed as ppm equivalent of limonin. [b] Results presented as the mean ($n = 3$) ± SD. [c] Expressed as ppm equivalent of limonin glucoside.

aglycone concentrations in juice are generally less than 30 μg/mL (*22*), whereas glucoside concentrations are much higher (*22*), it is unlikely that any aglycones present would significantly interfere with the estimation of glucoside concentrations. In fact, when the spiked orange juice samples (final concentration 5, 10, 20 μg/mL) were analyzed for glucoside equivalents, the equivalency results differed less than 1 standard deviation from each other.

Utilizing the conditions described in the Materials and Methods section, the LOD and LOQ values were determined to be 0.25 and 0.50 μg/mL for limonin, and 0.50 and 1.0 μg/mL for limonin glucoside, respectively. Formation of the colorimetric adducts was linear with increasing limonoid concentrations to levels in excess of 100 μg/mL, and typical correlation coefficients for calibration curves were greater than 0.98. Results from spike recovery experiments for limonin (95−104%) and limonin glucoside (99−102%) were comparable to values found in the literature (92−111%) (*11*, *13*, *19*, *20*, *23*, *24*). The total limonoid glucoside and aglycone concentrations were estimated for a number of juice samples using the method described (Table 1). Results for aglycone equivalencies showed that the tangerine sample had the lowest limonin concentration and both the grapefruit and lemon samples exhibit limonin concentrations above the level acceptable to consumers. For the glucoside equivalencies, the lemon sample had the lowest concentration and the grapefruit the greatest.

In conclusion, we have presented a colorimetric method for estimating limonoid concentrations in citrus juices. During optimization, the influence of DMAB concentration, reaction time and solvent composition on color development, and sensitivity were investigated. The limit of quantification and linear nature of the method make it a valuable tool for identifying juices with limonin concentrations in excess of the level acceptable to consumers. Although the assay was only demonstrated for juice in this report, it can be readily adapted

for the analysis of other citrus derived extracts. In addition, the methods described do not require expensive equipment, thus increasing application potential to researchers, juice processors, and citrus growers.

ABBREVIATIONS USED

DMAB, 4-(dimethylamino)benzaldehyde; LOD, limit of detection; LOQ, limit of quantification; LG, limonin glucoside; HPLC, high-pressure liquid chromatography; LC-MS, liquid chromatography with tandem mass spectrometry.

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