Multivariate Calibration for the Determination of Total Azadirachtin-Related Limonoids and Simple Terpenoids in Neem Extracts Using Vanillin Assay

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Two-component and multivariate calibration techniques were developed for the simultaneous quantification of total azadirachtin-related limonoids (AZRL) and simple terpenoids (ST) in neem extracts using vanillin assay. A mathematical modeling method was also developed to aid in the analysis of the spectra and to simplify the calculations. The mathematical models were used in a two-component calibration (using azadirachtin and limonene as standards) for samples containing mainly limonoids and terpenoids (such as neem seed kernel extracts). However, for the extracts from other parts of neem, such as neem leaf, a multivariate calibration was necessary to eliminate the possible interference from phenolics and other components in order to obtain the accurate content of AZRL and ST. It was demonstrated that the accuracy of the vanillin assay in predicting the content of azadirachtin in a model mixture containing limonene (25% w/w) can be improved from 50% overestimation to 95% accuracy using the two-component calibration, while predicting the content of limonene with 98% accuracy. Both calibration techniques were applied to estimate the content of AZRL and ST in different parts of the neem plant. The results of this study indicated that the relative content of limonoids was much higher than that of the terpenoids in all parts of the neem plant studied.

Keywords: *Azadirachtin; neem; vanillin assay; multivariate calibration; two-component calibration; mathematical modeling; total limonoids; total terpenoids*

INTRODUCTION

The neem tree, *Azadirachta indica*, is a tropical plant that is known for its pesticidal properties (*1*, *2*). Studies have shown that the seed contains abundant limonoids and simple terpenoids which are responsible for its biological activity (*3*−*7*). In a previous study (*8*) we reported the development of a vanillin-based colorimetric assay for estimating the content of total azadirachtin-related limonoids (AZRL) in the seed-kernel extracts. The quantification was carried out based on the calibration with commercial azadirachtin as the standard. The colored complexes formed upon the interaction of vanillin with azadirachtin had an absorption maximum at 577 nm. HPLC analysis has indicated that more than 50% of the response of the vanillin assay was due to azadirachtin skeleton (*8*). The applicability of this method was tested with other parts of the neem plant (neem leaf, leaf stem, and seed shell) that contain higher amounts of phenolics and terpenoids that can potentially interfere with the vanillin assay. The preliminary data have indicated that the method should be refined in order to accommodate for the interference of phenolics and terpenoids with the vanillin assay. Although the colored complexes formed upon the interaction of vanillin with phenolics and terpenoids show different absorption maxima (phenolics at ∼500 nm and terpenoids at ∼625 nm), at relatively high concentrations compared with the seed content, they may cause unacceptable errors in the quantification of AZRL in other parts of the plant, due to peak broadening. One of the most commonly used techniques to eliminate this interference is to purify the extracts by removing the interfering components (*9*). However, in most cases, the interfering components are not easy to remove completely through simple purification methods. Consequently, we have developed a multivariate calibration to eliminate interference from both simple terpenoids and phenolics during the vanillin assay.

MATERIALS AND METHODS

Materials. Fresh neem plant was collected from Bangalore, India during May 1998. The seeds were removed from their shells and the leaves were separated from the stems. The different parts of the plant were then blended with a coffee bean blender and stored at below 0 °C.

Chemicals. Azadirachtin A (∼95% purity) was purchased from Sigma Chemical Co. (Ontario, Canada). A stock solution

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(0.1 mg/mL) in dichloromethane was stored at 0 °C. HPLC-grade methanol and dichloromethane were purchased from Fisher Scientific (Montreal, Canada); petroleum ether (60−80 °C) was purchased from ACP Chemicals Inc. (Montreal, Canada). Vanillin and concentrated H_2SO_4 were obtained from Fisher Scientific (Montreal, Canada). Limonene and tannic acid were obtained from Sigma-Aldrich Canada Ltd. (Ontario, Canada).

Extraction Procedures. *Procedure 1. Extraction and Partition of Neem Seed Kernel.* A suspension of blended neem seed kernel (2.0 g) in petroleum ether (60 mL) was stirred at room temperature for 12 h. The de-fatted sample was extracted again with methanol (3 × 20 mL) by stirring at room temperature for an additional 12 h. The extract was evaporated under vacuum to obtain a yellow oil. The methanol extract was redissolved in methanol (10 mL) and water (10 mL) followed by the addition of 5% sodium chloride solution (1.0 mL). This mixture was extracted again with petroleum ether (6 × 20 mL) to remove any remaining nonpolar impurities (termed component X). The residue was then extracted with dichloromethane (3 × 20 mL). The combined dichloromethane layers were dried over Na_2SO_4 and the solvent was evaporated under vacuum using a Büchi Rotovapor R114 (Fischer Scientific, Montreal, Canada) to obtain a light yellow solid. The product was dissolved in dichloromethane for further colorimetric analysis. The process was repeated to obtain three replicates.

Procedure 2. Extraction and Partition of Neem Seed Shell. Neem seed shell (2.0 g) was suspended in methanol (2 × 20 mL) for 12 h. The solution was filtered into a flask and washed with methanol (10 mL). The combined methanol solutions were vacuum-evaporated to dryness. The partition method was the same as in the Procedure 1 above except for the amount of solvents used. For redissolving in aqueous methanol, 5 mL of methanol and 5 mL of distilled water was used instead of 10 mL. For the de-emulsification, only 0.5 mL of 5% NaCl solution was added. For partitioning in petroleum ether and dichloromethane, 10 mL of petroleum ether or dichloromethane was used instead of 20 mL. The dichloromethane layer was evaporated under vacuum and weighed, and was dissolved in dichloromethane solution for further analysis. The process was repeated to obtain three replicates.

Procedure 3. Extraction and Partition of the Neem Leaf and the Leaf Stem. Neem leaf (2.0 g) or leaf stem (5.0 g) was stirred in methanol (2 × 20 mL) for 12 h. After extraction, the solvent was evaporated under reduced pressure. The extracts were redissolved in 5 mL of methanol, followed by the addition of 10 mL of distilled water and 1 mL of 5% NaCl solution. The mixture was extracted with petroleum ether (6 × 15 mL) to remove the chlorophyll, then the aqueous methanol layer was extracted with dichloromethane (3 × 15 mL). The combined dichloromethane layers were dried over Na_2SO_4 and evaporated under vacuum to dryness. The product was then dissolved in dichloromethane for further colorimetric analysis.

Colorization Procedure with Vanillin. The colorization procedure of azadirachtin, limonene, tannic acid, and the component X was the same as that reported by Dai et al. (*8*). The component X obtained by the evaporation of the petroleum ether layer in Procedure 1, described above, was dissolved in dichloromethane (0.24 mg/mL) before the vanillin assay. The concentration of tannic acid was 0.01 mg/mL in dichloromethane. After the colorized solutions were stabilized for 5 min, they were scanned over the wavelength range of 400 to 700 nm, using a Beckman DU-64 spectrophotometer equipped with a 10-mm quartz cell and operating at a resolution of 1 nm. The blank solution was obtained by substituting the test solution with an equal volume of dichloromethane. The recorded spectra were converted into xy data files (absorbance vs wavelength) for further mathematical treatment.

Calibration of Azadirachtin and Limonene. Calibration curves were obtained with standard azadirachtin (0.01−0.10 mg/mL, at 577 nm) and limonene (0.002−0.020 mg/mL, at 625 nm) solutions in dichloromethane using the above procedure for vanillin assay. From the calibration curves, response coefficients were obtained: $g_{Az,577\ nm} = 9.476\ (mg/mL)^{-1} \times cm^{-1}$; $g_{Limonene,577\ nm} = 52.14\ (mg/mL)^{-1} \times cm^{-1}$ using Beer's law.

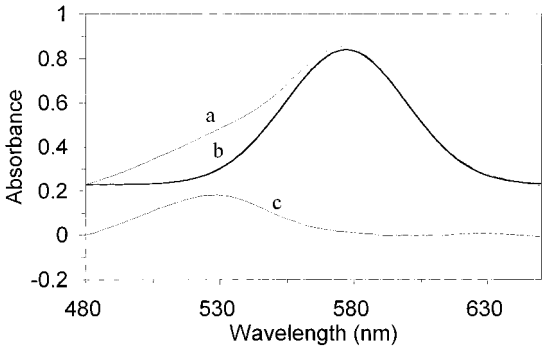

Figure 1. (a) Spectrum of azadirachtin following the vanillin assay; (b) linear regression of the Gaussian distribution curve applied to spectrum 1a; (c) subtraction result of spectrum a from spectrum b.

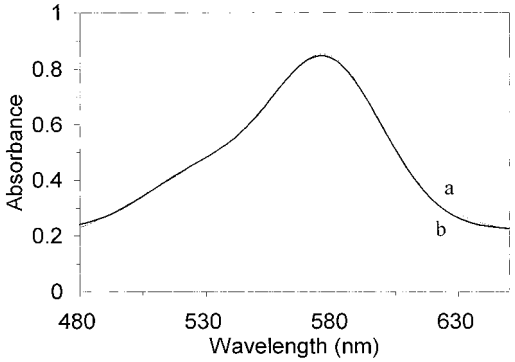

Figure 2. Acquired (a) and simulated (b) spectra of azadirachtin subjected to vanillin assay.

Mathematical Modeling of the Spectra of Azadirachtin and Major Interfering Constituents of Neem Extracts. A Gaussian model was applied to the right-hand half of the spectrum of vanillin-treated azadirachtin solution (see Figure 1a) to obtain eq 1. The exponential function was converted into a logarithmic function through a linear transformation to produce eq 2. A linear regression with the data points from 577 to 650 nm with 1 nm interval (R^2 of 0.9992) generated eq 3.

$$y = y_0 + ae^{-[(x-577)^2/b]} \tag{1}$$

$$p = \ln a - \frac{1}{b}q \tag{2}$$

where $p = \ln(y - y_0)$; $q = (x - 577)^2$; $y_0 = y$ intercept obtained graphically.

$$A = 0.23 + 0.61e^{-(\lambda-577)^2/1036.5} \tag{3}$$

where A = absorbance and λ = wavelength.

The eq 3 was plotted in the wavelength range of 480−650 nm with 1 nm interval along with the original as shown in Figure 1b. Subtraction of spectrum 1b from spectrum 1a resulted in spectrum 1c (see Figure 1c). By applying a Gaussian regression to the data of spectrum 1c, eq 4 was obtained which represents the left-hand half of the spectrum of azadirachtin.

$$A = -0.0067 + 0.1885e^{-(\lambda-527)^2/955.07} \tag{4}$$

where A = absorbance and λ = wavelength.

Combining eqs 3 and 4, a mathematical model for the complete spectrum of azadirachtin over the wavelength range of 480−650 nm can be obtained (see eq 5). The original and the simulated spectra were plotted in Figure 2. A very good

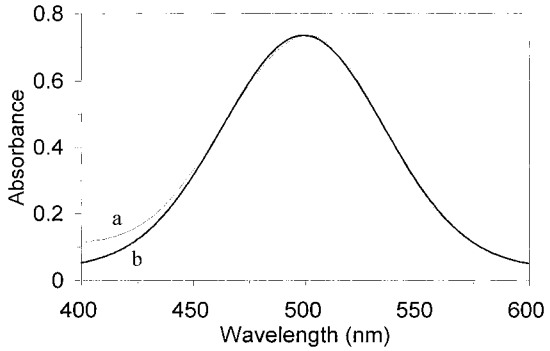

Figure 3. Acquired (a) and simulated (b) spectra of tannic acid subjected to vanillin assay.

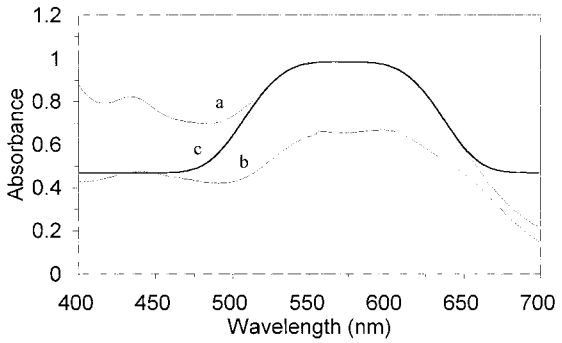

Figure 4. (a) Spectrum of component X of neem seed kernel extract subjected to vanillin assay ;(b) mathematically corrected spectrum of the leaf extract; and (c) the simulated spectrum of the component X.

fit was observed for the simulated spectrum. Similar procedures were applied to the spectra of limonene (eq 6), tannic acid (eq 7), and component X (eq 8). Again, a good fit was obtained for the experimental and the simulated spectra. The R^2 values for the last two Gaussian regressions were 0.9994 and 0.9993, respectively, and the experimental and the simulated spectra are shown in Figures 3 (a, b) and 4 (a, c).

$$A = 0.223 + 0.1885e^{-(\lambda - 527)^2/955.07} + 0.61e^{-(\lambda - 572)^2/867.5} \quad (5)$$

$$A = 0.165 + 0.7234e^{-(\lambda - 625)^2/1300.4} + 0.1522e^{-(\lambda - 572)^2/867.5} \quad (6)$$

where A = absorbance and λ = wavelength.

$$A_{\text{Tannic acid}} = 0.0732 + 1.3965e^{-0.5(\lambda - 499/36.1591)^2} \quad (7)$$

$$A_{\text{Unknown}} = 0.4697 + 0.5155e^{-0.5(\lambda - 573.12/58.7282)^{3.8}} \quad (8)$$

where $A_{\text{tannic acid}}$ = absorbance of the tannic acid and A_{unknown} = absorbance of component X subjected to vanillin assay.

The models for azadirachtin and limonene were further calibrated using standard solutions (see eqs 9 and 10). Because the aim of this work was to eliminate the interference caused by the phenolics and component X, it was not necessary to calibrate for these two components.

$$A_{\text{AZ}}^{\text{mg/mL}} = 2.496 + 6.826e^{-(\lambda - 577)^2/1036.5} + 2.109e^{-(\lambda - 527)^2/955} \quad (9)$$

$$A_{\text{Limonene}}^{\text{mg/mL}} = 9.46 + 41.56e^{-(\lambda - 625)^2/1300.4} + 8.74e^{-(\lambda - 572)^2/867.5} \quad (10)$$

where $A_{\text{AZ}}^{\text{mg/mL}}$ = absorbance for azadirachtin at the concentration of 1 mg/mL and $A_{\text{Limonene}}^{\text{mg/mL}}$ = absorbance for limonene at the concentration of 1 mg/mL.

Two-Component and Multivariate Calibrations for the Simultaneous Determination of Concentrations of Limonoids and Terpenoids in the Extracts of Various Parts of Neem. The concentrations of AZRL and ST were determined using the Beer's law ($A = EC$). The values listed under matrix I were used for two-component calibration and those listed under matrix II were used for multivariate calibration. Where AZ = azadirachtin, ST = simple terpenoid, A = absorbance, E = absorbtivity, C = concentration, $\lambda_1 = 577$; $\lambda_2 = 625$; $\lambda_3 = 550$; and $\lambda_4 = 499$ nm.

Matrix I

$$E = \begin{vmatrix} \epsilon_{\text{AZ,577nm}} & \epsilon_{\text{Limonene,577nm}} \\ \epsilon_{\text{AZ,625nm}} & \epsilon_{\text{Limonene,625nm}} \end{vmatrix} = \begin{vmatrix} 9.476 & 24.367 \\ 3.245 & 52.141 \end{vmatrix}$$

$$C = \begin{vmatrix} C_{\text{AZ}} \\ C_{\text{Limonene}} \end{vmatrix} \qquad A = \begin{vmatrix} A_{\text{577nm}} \\ A_{\text{625nm}} \end{vmatrix}$$

Matrix II

$$A = \begin{vmatrix} A_{\lambda 1}^{\text{AZ}} C_{\text{AZ}} + A_{\lambda 1}^{\text{Limonene}} C_{\text{ST}} + A_{\lambda 1}^{\text{Tannic-acid}} C_{\text{Phenolic}} + A_{\lambda 1}^{\text{PELayerExtract}} C_{\text{Unknown}} \\ A_{\lambda 2}^{\text{AZ}} C_{\text{AZ}} + A_{\lambda 2}^{\text{Limonene}} C_{\text{ST}} + A_{\lambda 2}^{\text{Tannic-acid}} C_{\text{Phenolic}} + A_{\lambda 2}^{\text{PELayerExtract}} C_{\text{Unknown}} \\ A_{\lambda 3}^{\text{AZ}} C_{\text{AZ}} + A_{\lambda 3}^{\text{Limonene}} C_{\text{ST}} + A_{\lambda 3}^{\text{Tannic-acid}} C_{\text{Phenolic}} + A_{\lambda 3}^{\text{PELayerExtract}} C_{\text{Unknown}} \\ A_{\lambda 4}^{\text{AZ}} C_{\text{AZ}} + A_{\lambda 4}^{\text{Limonene}} C_{\text{ST}} + A_{\lambda 4}^{\text{Tannic-acid}} C_{\text{Phenolic}} + A_{\lambda 4}^{\text{PELayerExtract}} C_{\text{Unknown}} \end{vmatrix}$$

$$= \begin{vmatrix} A_{\lambda 1}^{\text{Total}} \\ A_{\lambda 2}^{\text{Total}} \\ A_{\lambda 3}^{\text{Total}} \\ A_{\lambda 4}^{\text{Total}} \end{vmatrix}$$

$$E = \begin{vmatrix} A_{\lambda 1}^{\text{AZ}} & A_{\lambda 1}^{\text{Limonene}} & A_{\lambda 1}^{\text{Tannic-acid}} & A_{\lambda 1}^{\text{PELayerExtract}} \\ A_{\lambda 2}^{\text{AZ}} & A_{\lambda 2}^{\text{Limonene}} & A_{\lambda 2}^{\text{Tannic-acid}} & A_{\lambda 2}^{\text{PELayerExtract}} \\ A_{\lambda 3}^{\text{AZ}} & A_{\lambda 3}^{\text{Limonene}} & A_{\lambda 3}^{\text{Tannic-acid}} & A_{\lambda 3}^{\text{PELayerExtract}} \\ A_{\lambda 4}^{\text{AZ}} & A_{\lambda 4}^{\text{Limonene}} & A_{\lambda 4}^{\text{Tannic-acid}} & A_{\lambda 4}^{\text{PELayerExtract}} \end{vmatrix}$$

$$C = \begin{vmatrix} C_{\text{AZ}} \\ C_{\text{ST}} \\ C_{\text{Phenolic}} \\ C_{\text{Unknown}} \end{vmatrix}$$

RESULTS AND DISCUSSION

Limonoids are important components in the neem seed kernel, and they are responsible for the pesticidal and medicinal properties of the neem plant (*1*, *2*). The limonoids can be easily extracted using different techniques (*8*). However, determination of the purity of the extracts and quantification of the different limonoids can only be achieved by lengthy HPLC procedures. A recent colorimetric technique (*8*) based on vanillin assay provides a convenient method of determining total azadirachtin related limonoids (AZRL) in these extracts. Although this technique can be used to determine AZRL content in neem seed kernel, extracts originating from other parts of the plant such as seed shell, leaf, and leaf stem are not amenable for such colorimetric analysis because of the presence of other components that also react with vanillin. These extracts contain terpenoids and phenolics that can be considered as the source of interference. Figure 5 indicates that the spectrum of the extract of the seed kernel fits well with that of the pure azadirachtin. A slight band broadening is observed around ~625 nm where simple terpenoids absorb. On the other hand, the extracts from other parts of neem show more severe band broadening on both sides of the λ_{max} of azadirachtin (577 nm). If the true nature of these

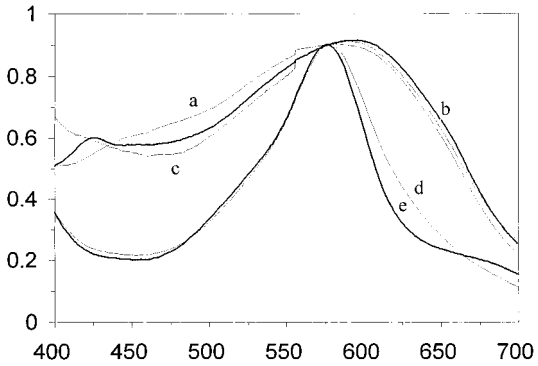

Figure 5. Spectrum of the purified extract of (a) seed shell, (b) leaf stem, (c) leaf, and (d) seed; and (e) standard azadirachtin (0.1 mg/mL) subjected to vanillin assay.

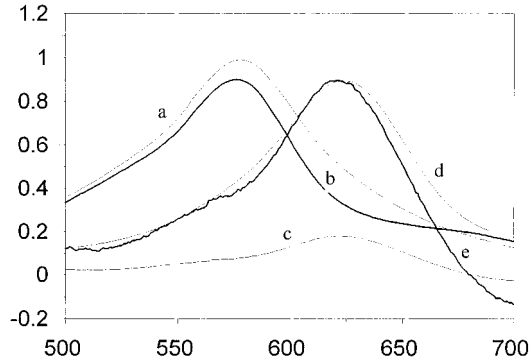

Figure 6. Spectrum of (a) neem seed kernel extract; (b) standard azadirachtin; (c) subtraction result of a from b; (d) limonene; (e) adjusted spectrum c.

interfering components can be verified and they are subsequently eliminated, more accurate values of AZRL content in other parts of neem plant can be obtained with the convenient vanillin assay.

Identification of the Origin of the Interfering Component in the Neem Seed Kernel Extract. Simple terpenoids such as diterpenoids and triterpenoids are also important components in different parts of the neem plant (*10*−*12*). Because of the similarity between the physical properties of limonoids and simple terpenoids, it is therefore likely that these components can be co-extracted along with limonoids and potentially interfere with the vanillin assay. Specifically, they are known to produce colored complexes with vanillin and show an absorbance maximum at 625 nm (*8*). To confirm the presence of terpenoids in the neem extracts originating from seed kernels, the spectrum of the standard azadirechtin was subtracted from the spectrum of the seed extract after the vanillin assay and the residual spectrum was compared with that of limonene (a simple terpenoid) as shown in Figure 6. Multiplied by an appropriate factor, the residual spectrum was found to be very similar to that of limonene (spectra d and e in Figure 6). Because these simple terpenoids are also known to have pesticidal or medicinal properties, it would also be advantageous to determine their amounts in the extracts of various parts of neem.

Evaluation of the Two-Component Calibration Technique for the Simultaneous Determination of AZ and ST in a Model System. A two-component calibration technique was developed based on azadirachtin and limonin as standards (see Material and Methods section). This technique was evaluated

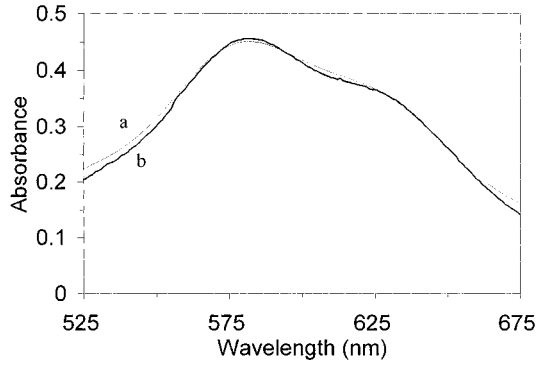

Figure 7. (a) Spectrum of the mixture of azadirachtin (0.040 mg/mL) and limonene (0.0050 mg/mL); (b) the sum of the spectra of azadirachtin and limonene calculated using the two-component calibration method.

for the simultaneous determination of limonoids and terpenoids using known concentrations of azadirachtin and limonene (75:25 w/w) in a model system. The dichloromethane solutions of the two standards were subjected to the vanillin assay and abosrbances at wavelengths 577 and 625 nm were determined. Figure 7a shows the spectrum of the mixture after the vanillin assay. Solving the matrix I (see Material and Methods section) the concentrations of the azadirachtin and limonene were calculated and the results are shown in Table 1. The calculated concentrations were very close to the known values, indicating the validity of this approach. In addition, the experimentally acquired spectrum of the known mixture was compared with the simulated spectrum (Figure 7b) generated by the addition of the two calculated spectra of azadirachtin and limonene. Figure 7 shows that the two spectra fitted well, indicating that the two-component calibration technique can be used for the simultaneous determination of the AZRL and ST in the neem extracts and that there are no interactions between the spectra of AZ and limonene. The data also indicated that the accuracy of the vanillin assay in predicting the content of azadirachtin in a mixture containing limonene (25% w/w) can be improved from 50% overestimation to 95% accuracy using the two-component calibration (see Table 1).

Identification of the Interfering Components in the Extracts of Other Parts of Neem and Application of Multvariate Calibration. The spectra of the extracts obtained from the other parts of neem after the vanillin assay were more complicated than the those of the neem seed kernel extracts (see Figure 5). The severe band broadening observed on both sides of the λ_{max} of azadirachtin (577 nm) was initially attributed to the presence of both phenolics (λ_{max} ~500 nm) and terpenoids (λ_{max} ~625 nm) in the extracts because both are common components in plant materials and both classes of compounds react during vanillin assay to produce colored complexes (*9*). As shown in Figure 8a, the peak of the spectrum obtained after the vanillin assay of the leaf extract as an example, was much wider than that of the standard azadirachtin. The maximum absorbance, however, was still at around 577 nm, indicating that AZRL are still the main components. When the spectrum of standard azadirachtin was subtracted from the spectrum of the extract (see Figure 8c) the resulting spectrum indicated the existence of severe interference at both 600−650 and 450−550 nm regions. However, when the standard spectra of azadirachtin, limonene, and tannic acid (a representative phenolic compound)

Table 1. Evaluation of the Two-Component Calibration Technique with Known Concentrations

known concentration		single-component calibration	two-component calibration		multivariate calibration	
C_{AZ} (mg/mL)[a]	$C_{limonene}$ (mg/mL)	C_{AZ} (mg/mL) ± % SD[b]	C_{AZ} (mg/mL) ± % SD[b]	$C_{limonene}$ (mg/mL) ± % SD[b]	C_{AZ} (mg/mL) ± % SD[b]	$C_{limonene}$ (mg/mL) ± % SD[b]
0.019	0.0065	0.036 ± 3.5	0.018 ± 4.8	0.0065 ± 1.8	0.018 ± 5.4	0.0066 ± 2.4
0.038	0.0050	0.052 ± 4.6	0.037 ± 3.4	0.0049 ± 2.2	0.036 ± 2.8	0.0051 ± 3.5

[a] Corrected concentration of the commercial azadirachtin (AZ, 95% purity). [b] On the basis of 5 measurements. AZ = azadirachtin.

Table 2. Percent Distribution of AZRL and ST by Different Calibration Methods in Different Parts of Neem

sample[a]	% AZRL[b] obtained with single-component calibration ± % SD[c]	% AZRL obtained with two-component calibration ± % SD[c]	% ST obtained with two-component calibration ± % SD[c]	% AZRL obtained with multivariate calibration ± % SD[c]	% ST obtained with multivariate calibration ± % SD[c]
seed	1.038 ± 1.36	0.987 ± 0.82	0.015 ± 8.31	0.956 ± 0.90	0.013 ± 6.54
seed shell	0.281 ± 0.57	0.197 ± 1.55	0.016 ± 1.78	0.069 ± 1.25	0.013 ± 1.56
leaf	0.300 ± 0.14	0.222 ± 0.65	0.030 ± 1.36	0.158 ± 0.78	0.020 ± 1.15
leaf stem	0.070 ± 0.59	0.049 ± 0.59	0.008 ± 0.78	0.027 ± 0.87	0.005 ± 1.01

[a] See Material and Methods section (procedures 1−3). [b] AZ = azadirachtin, ST = simple terpenoids. [c] On the basis of 5 measurements.

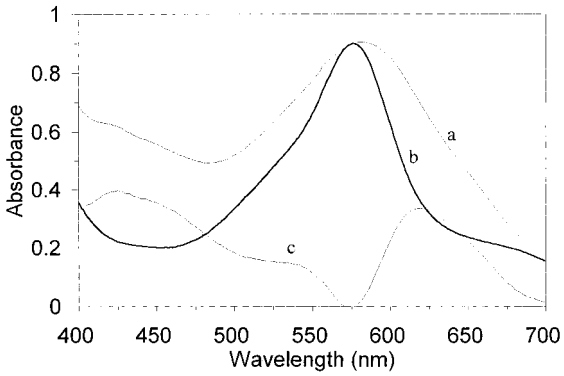

Figure 8. Spectra of purified neem leaf extract (a), standard azadirachtin (b), and difference spectrum (c).

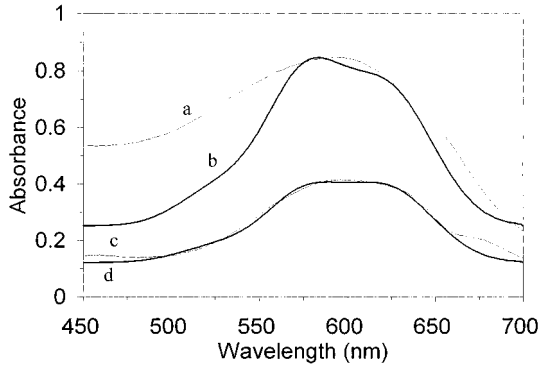

Figure 9. Simulation of the neem leaf extract subjected to vanillin assay with the two-component model before and after removal of interference: (a) neem leaf extract subjected to vanillin assay; (b) simulation curve before multivariate correction; (c) spectrum after multivariate correction; (d) simulation curve (with two-component calibration) after multivariate correction.

were subtracted from that of the extract, the shape of the resulting spectrum was similar to the spectrum of the component X, obtained from the seed (see Materials and Methods section) as shown in Figure 4b. In addition, when the spectrum of the leaf extract was mathematically corrected for the presence of phenolics, the resulting spectrum did not fit well with the simulated spectrum generated from the mathematical modeling using the spectra of azadirachtin and limonene. However, when the spectrum of component X together with that of the phenolics were subtracted from the spectrum of the leaf extract, the resulting spectrum fitted well with that of the model spectrum generated from standard azadirachtin and limonene, indicating that the major interfering factor is due to the presence of component X.

Consequently, with azadirachtin, limonene, tannic acid, and component X as standards, a multivariate technique can be used, as described in the Materials and Methods section, to eliminate the interference and to determine the amount of AZRL and ST in the extracts. The values of the matrix II can be obtained through eqs 7, 8, 9, and 10. With the experimentally obtained values for the absorbance at different wavelengths, the concentrations of AZRL and ST, as well as the relative concentrations of the phenolics and component X, can be calculated. Therefore, to obtain more accurate information from vanillin assay, the spectra of extracts originating from parts of neem other than the seed should be subjected to mathematical correction using multivariate calibration before the application of two-component calibration for the determination of total AZRL and ST. Figure 9 compares the simulated spectra of the leaf extract using the two-component calibration, after vanillin assay with and without multivariate correction. It can be seen clearly that the two-component calibration directly applied to the original spectrum still shows severe interference, especially at the lower wavelengths, whereas after the multivariate correction, the simulated spectrum exhibits a good fit.

Distribution of Total Limonoids and Terpenoids in Different Parts of Neem Plant. The extracts obtained from seed, seed shell, the leaf, and the leaf stem were subjected to vanillin assay. The contents of AZRL and ST were calculated using multivariate and/or two-component calibration techniques and the results are shown in Table 2. According to Table 2, the AZRL content in the neem shows the following increasing order: seed kernel ≫ leaf > seed shell > leaf stem. But for the ST, the leaf had the highest content followed by the seed kernel, the seed shell, and the leaf stem. The content of AZRL relative to ST in different parts of neem is shown in Table 3. According to this table, the seed kernel contains much higher amounts of AZRL relative to ST (∼ 73-fold) compared with other parts of the plant where the relative content was only between 5 and 8-fold higher. This relative distribution might give us a clue as to how these components were biosynthesized

Table 3. Relative Content of AZRL in Different Parts of Neem

sample	AZRL/ST[a]
seed	73
seed shell	5
leaf	8
leaf stem	5

[a] AZ = azadirachtin, ST = simple terpenoids.

and stored in the plant. The AZRL is a class of highly oxidized terpenoids with complex structures. Therefore, ST might represent the raw materials for biosynthesis which can be carried out in the green parts of the tree, mainly the leaves and the green stem. After synthesis, the AZRL is transported to the seeds and stored in the seed kernel.

CONCLUSION

The multivariate calibration method developed, based on the vanillin assay for quantification of AZRL and ST, can effectively eliminate the interference caused by co-extracting components in the extracts of various parts of neem. Because of the difficulty in completely separating ST from AZRL by simple solvent partitioning, the multivariate calibration technique could be considered a useful refinement of the vanillin assay.

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