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Characterization of nine traditional Chinese plant extracts with specific acid dissociation constants by UV-Vis spectrophotometry†

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A UV-Vis spectrophotometric method for characterization of plant extracts with specific acid dissociation constants was developed and nine traditional Chinese plant extracts were used in the analysis workflow. The ionization of plant extracts gave intensive pH-dependent spectral shifts and their spectral changes differed between wavelength ranges. The simulation with the one-step and two-step ionization processes showed that the acid dissociation constants distributed steadily at some wavelength ranges. These specific acid dissociation constants might be used as markers for characterization of plant extracts. This study suggests that the proposed method has potential applications for quality assessment and mass quality control of plant extracts used in food and pharmaceutical industries.

1 Introduction

Plant extracts are a collection of crude mixtures extracted from different parts of plants, which have been widely applied in functional foods, dietary supplements, medicines and pesticides throughout the world due to their complex bioactive phytochemicals such as polyphenols, polysaccharides, flavonoids and organic acids.[1–4] In China, plant extracts are widely consumed as tonic foods and herbal medicines.[5] Phytochemical analysis of plant extracts is an important scientific research area. Isolation of bioactive ingredients from plant extracts in adequate quantities for spectral and biological analysis is the basis of this research.[6,7] However, compound isolation and identification are very tedious and normally rely on a number of rather laborious and time-consuming techniques.[8]

Currently, due to the complexity of plant extracts, a common practice among natural product analysts is to select one or more compounds as either active or "markers" for the purpose of identification and quality assessment.[9–12] However, the efficacy of plant extracts is often attributed to the additive or synergistic effects of all components.[13,14] Sometimes, the efficacy is weaker or even lost in purified fractions.[15,16] Besides, selecting one or more compounds to reflect the complex characteristics of plant extracts can provide opportunities for adulterants by adding the corresponding exogenous substances.[17,18] Therefore, chemical fingerprint analysis is applied for quality assessment of plant extracts to construct specific patterns of recognition for multiple compounds with techniques, such as GC, HPLC and HPLC-MS.[9,18–20] The entire pattern of compounds can then be evaluated to determine not only the absence or presence of desired markers or actives but also the complete set of ratios of all detectable analytes. It represents a comprehensive qualitative approach for the purpose of species authentication, evaluation of quality, and ensuring the consistency and stability of plant extracts and their related products.[9,21,22] These renowned methods mainly concern the separation and identification of specific components in plant extracts. In this paper, the proposed method tried to explore the specific overall properties of plant extracts.

UV-Vis spectrophotometry is generally considered the main alternative to potentiometric titration for pK_a value determination of compounds because of its high sensitivity to concentrations of compounds as low as 10^{-6} M.[23–25] The absorption spectra of the sample changes with respect to pH values reflect the concentration of neutral and ionized species. The largest change in absorbance occurs at the pH corresponding to the pK_a values.[23]

Plant extracts are a complex mixture of different compounds and often the identity of the compounds is only partially known.[26,27] Therefore, the acid dissociation constant of plant extracts is related to all ionic species with absorption at the analytical wavelength, that is, the "mixed" dissociation constant (pK_a^{mix}).[28,29] In this paper, Cape Jasmine Fruit (*Gardenia jasminoides*), Chinese Angelica Root (*Angelica sinensis*), Orange Peel (*Citrus sinensis*), Eucommia Bark (*Eucommia ulmoides*), Ginseng Root (*Panax ginseng*), Licorice (*Glycyrrhiza uralensis*), Pilose Asiabell Root (*Codonopsis pilosula*), Safflower (*Carthamus*

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† Electronic supplementary information (ESI) available: Fig. S1: the linearity between absorbance and concentration at different wavelengths in different plant extracts. Fig. S2: simulation of the two-step ionization process in plant extracts. Fig. S3: acid dissociation constants of plant extracts in the two-step ionization process. See DOI: 10.1039/c3ay40946e

tinctorius) and White Paeony Root (*Paeonia lactiflora*) were chosen. These plant parts have been served as tonic foods and herbal medicines for centuries in China, usually in the form of aqueous or organic (mainly liquor) extracts. Then, the analysis workflow of the proposed method was displayed in hydro-alcoholic extracts of these plant parts. The purpose was to develop an easy way for quality assessment of plant extracts.

2 Materials and methods

2.1 Model construction

The phytochemicals of plant extracts involved in the ionization process are supposed to be unity (M). The pH of the solution is related to the concentration of hydrogen ions. In contrast, the experimentally determined effective mobilities are related to the molar concentrations of ionic species, therefore it is convenient to introduce the "mixed" dissociation constant K_a^{mix}. All acid–base equilibria are treated uniformly in this paper as proton liberation processes. Hence, the protonation of any basic functional group is described as dissociation of the conjugated acid. For instance, the first dissociation step of the fully protonated form of the unity (M) is represented by the following equation.

$$HM^+ \xleftrightarrow{K_{a1}^{mix}} H^+ + M \tag{1}$$

$$K_{a1}^{mix} = \frac{c(H^+)c(M)}{c(HM^+)} \tag{2}$$

Then the distribution functions of HM^+ (X_{HM^+}) and M (X_M) are expressed as:

$$X_{HM^+} = \frac{c(HM^+)}{c(HM^+)+c(M)} = \frac{1}{1+\frac{K_{a1}^{mix}}{c(H^+)}} = \frac{c(H^+)}{c(H^+)+K_{a1}^{mix}} \tag{3}$$

$$X_M = \frac{c(M)}{c(HM^+)+c(M)} = \frac{K_{a1}^{mix}}{c(H^+)+K_{a1}^{mix}} \tag{4}$$

Therefore, the absorbance (A) of analytical solution can be written as:

$$A = A_0 X_{HM^+} + A_1 X_M = \frac{A_0 c(H^+)}{c(H^+)+K_{a1}^{mix}} + \frac{A_1 K_{a1}^{mix}}{c(H^+)+K_{a1}^{mix}} = \frac{A_0 c(H^+) + A_1 K_{a1}^{mix}}{c(H^+)+K_{a1}^{mix}} \tag{5}$$

A_0 and A_1 represent the initial absorbance and the ultimate absorbance in the first step ionization process, respectively. Eqn (5) could be applied to the research of the one-step ionization process. The neutral species (M) would undergo a further deprotonation step.

$$HM \xleftrightarrow{K_{a2}^{mix}} H^+ + M^- \tag{6}$$

$$K_{a2}^{mix} = \frac{c(H^+)c(M^-)}{c(HM)} \tag{7}$$

Similarly, the absorbance (A) in the second deprotonation step is shown as follows.

$$A = \frac{A_1 c(H^+)}{c(H^+)+K_{a2}^{mix}} + \frac{A_2 K_{a2}^{mix}}{c(H^+)+K_{a2}^{mix}} \tag{8}$$

A_2 represents the absorbance of the solution just with chromophore-containing compounds (M^-). Then, the absorbance in two-step ionization process can be written as:

$$A = \frac{A_0 c(H^+)}{c(H^+)+K_{a1}^{mix}} + \frac{K_{a1}^{mix}}{c(H^+)+K_{a1}^{mix}}\left(\frac{A_1 c(H^+)}{c(H^+)+K_{a2}^{mix}} + \frac{A_2 K_{a2}^{mix}}{c(H^+)+K_{a2}^{mix}}\right) = \frac{A_0 c(H^+)}{c(H^+)+K_{a1}^{mix}} + \frac{K_{a1}^{mix}}{c(H^+)+K_{a1}^{mix}} \times \frac{A_1 c(H^+) + A_2 K_{a2}^{mix}}{c(H^+)+K_{a2}^{mix}} \tag{9}$$

In this work, considering the algebraic complexity of the multi-step ionization process, only eqn (5) and (9) are applied to acid dissociation research in plant extracts. The simulation between the absorbance (A) and the hydrogen ion concentration ($c(H^+)$) based on eqn (5) and (9) reveals that the tendency of both normal curves is a V-type like (Fig. 1a and c), while the tendency of semi-logarithmic curves is an S-type like (Fig. 1b) and a double-S-type like (Fig. 1d) respectively. The shape of the curve is very helpful for choosing the suitable mathematical model.

In eqn (5) and (9), A_0, A_1, A_2, K_{a1}^{mix} and K_{a2}^{mix} are the parameters, while A and $c(H^+)$ can be measured. Therefore, parameters K_{a1}^{mix} and K_{a2}^{mix} can be estimated by using nonlinear fitting between A and $c(H^+)$.

2.2 General analysis flow

The general analysis flow for characterization of plant extracts comprises three portions.

(1) UV-Vis spectra acquisition of the solutions with treatments at different pH values;

(2) Model fittings. The models eqn (5) and (9) related to absorbance (A), hydrogen ion concentration ($c(H^+)$) and mixed dissociation constant (K_a^{mix}) are used for fitting with spectral data. The fittings passing the statistically significant tests can be applied to the calculation of pK_a^{mix} values;

(3) Comprehensive investigation of markers. Numerous pK_a^{mix} values at a wide range of wavelengths form a set and the characteristic pK_a^{mix} values can be approached by cluster analysis of the set. These characteristic pK_a^{mix} values at the specific wavelengths are used as the markers for characterization of plant extracts.

2.3 Extraction procedure

Cape Jasmine Fruit, Chinese Angelica Root, Orange Peel, Eucommia Bark, Ginseng Root, Licorice, Pilose Asiabell Root, Safflower and White Paeony Root were purchased from a local pharmacy. The plant parts were separately soaked in a binary solvent (ethanol–water, 2 : 3, v/v) at a solid–liquid ratio of 1 : 10 (g mL^{-1}). After soaking for 30 days at room temperature, the extracted fluid was clarified by centrifugation at 10 000 × g for 10 min. The supernatant was used as the stock solution and stored in the dark at 4 °C. Working solutions were prepared by dilution of the stock solution using double distilled water.

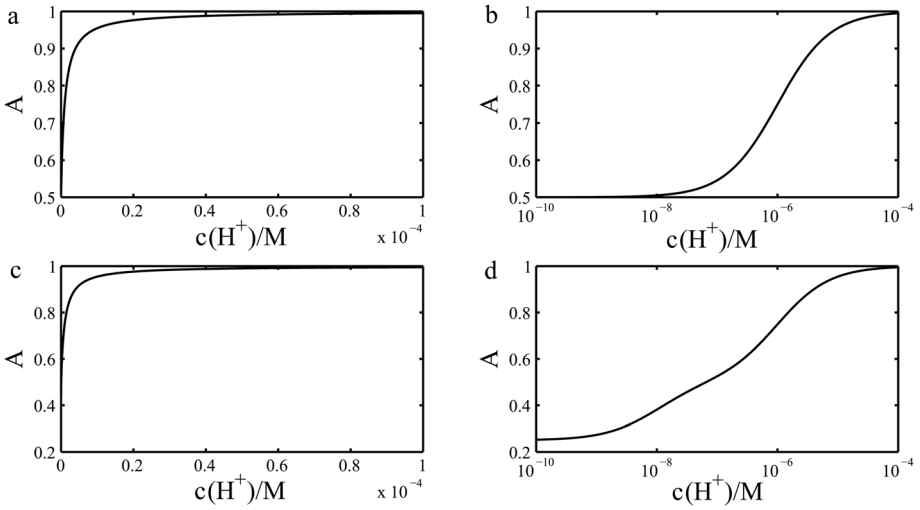

Fig. 1 Simulation of one-step and two-step ionization processes based on eqn (5) and (9). Where $A_0 = 1$, $A_1 = 0.5$, $A_2 = 0.25$, $K_{a1}^{mix} = 10^{-6}$, $K_{a2}^{mix} = 10^{-8}$. (a and c) correspond to eqn (5) and (9) respectively with x-axis of normal coordinate; (b and d) correspond to eqn (5) and (9) respectively with x-axis of logarithmic coordinate.

2.4 Spectra acquisition of plant extracts

UV-Vis spectra of solutions at different concentrations were scanned from 200 to 700 nm at a resolution of 1 nm with a 1.0 cm quartz cell in a UV-Vis spectrophotometer (UV-1700, Shimadzu, Japan) at room temperature, and the corresponding solvent was used as the blank contrast.

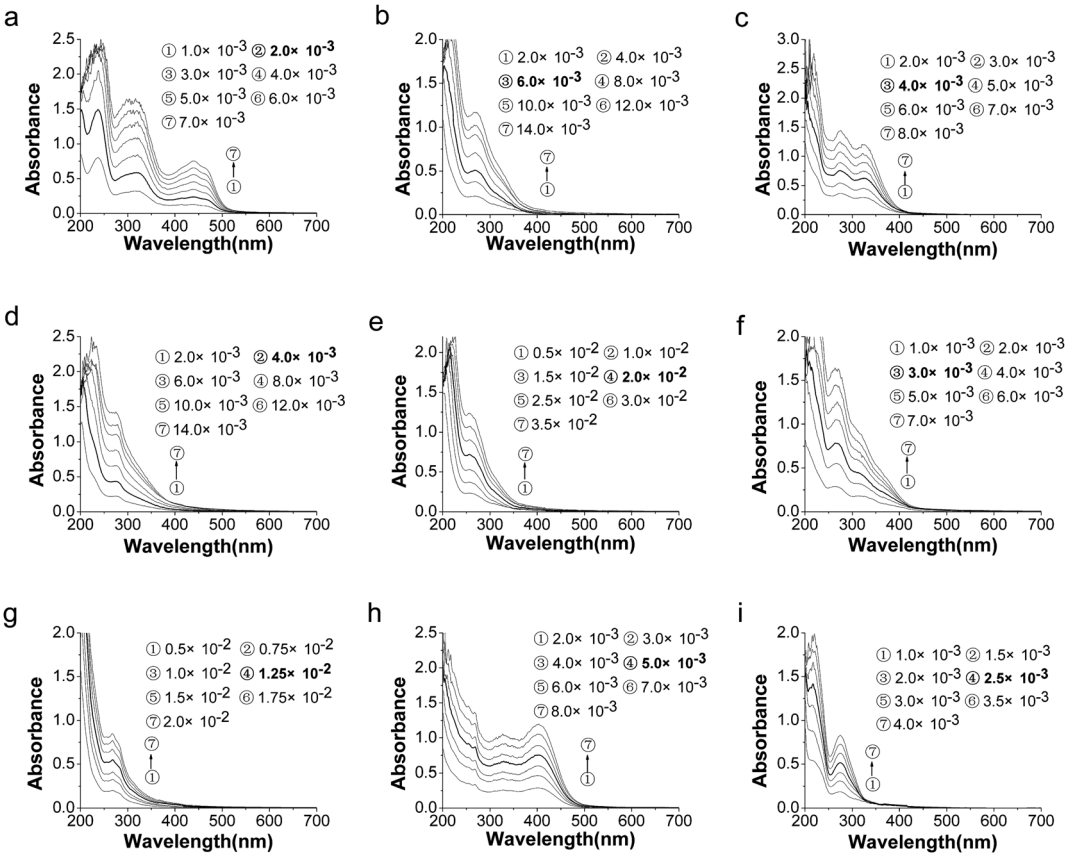

Fig. 2 UV-Vis absorption spectra of nine plant extracts at different dilutions. (a to i) correspond to the spectra of extract solutions of Cape Jasmine Fruit, Chinese Angelica Root, Orange Peel, Eucommia Bark, Ginseng Root, Licorice, Pilose Asiabell Root, Safflower and White Paeony Root respectively. The concentration of plant extracts chosen for following studies is indicated in bold.

2.5 Interaction between plant extracts and hydrogen ion

Buffer systems of 0.1 M citrate–0.2 M disodium hydrogen phosphate and 0.2 M glycine–0.2 M sodium hydroxide were used to supply different pH conditions. Acid dissociation study of plant extracts was performed at pH values ranging from 4.8 to 9.6.

2.6 Data processing

A script based on 1stOpt version1.5 (7D-Soft High Technology Inc., China) was developed for nonlinear fitting of eqn (5) and (9) by using the quasi-Newton algorithm with default parameters. The correlation coefficient (R^2) between the measured absorbance and the calculated absorbance of the fitted model was applied to weigh the fitting.

3 Results

3.1 UV-Vis spectra of nine plant extracts

The appearance of UV-Vis spectra differed between plant extracts (Fig. 2). For example, extracts of Cape Jasmine Fruit exhibited three major absorption bands in the UV-Vis region and these absorption peaks were at about 240 nm, 320 nm and 440 nm respectively (Fig. 2a). The UV-Vis spectrum information was helpful for preliminary qualitative analysis of plant extracts. However, it was apparent that some pairs of plant extracts shared more similarities in their UV-Vis spectra and presented difficulties in distinguishing them. For instance, extracts of Ginseng Root and Pilose Asiabell Root seemed to share the same characteristics in their UV-Vis spectra (Fig. 2e and g) and additional information was needed to differentiate them.

UV-Vis spectrophotometry is a feasible method for analysing plant extracts. One of the critical factors in the analysis is choosing the appropriate concentration of the plant extract.[30] Thus, in this study, the linearity of the absorbance and the concentration in nine plant extracts was analyzed based on the Lambert–Beer law (Fig. S1†) and their concentrations used for the following studies are indicated in Fig. 2.

3.2 Acid dissociation of nine plant extracts

The absorption spectra of the solutions at different pH values revealed that the ionization of some plant extracts gave intensive pH-dependent spectral shifts (Fig. 3). However, two of nine plant extracts, Eucommia Bark and Pilose Asiabell Root, showed inconspicuous changes in their spectra with pH value variation. The spectra of both plant extracts seemed to be insensitive to pH values.

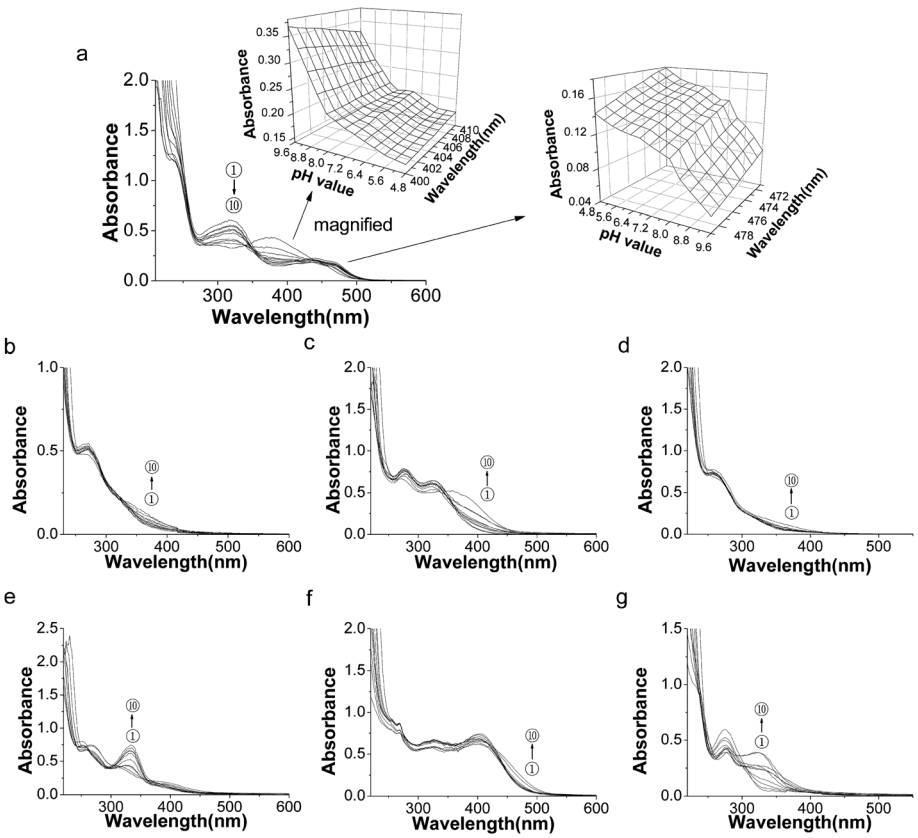

Fig. 3 UV-Vis absorption spectra of extract solutions with treatments at different pH values. (a to g) corresponded to the spectra of extract solutions of Cape Jasmine Fruit, Chinese Angelica Root, Orange Peel, Ginseng Root, Licorice, Safflower and White Paeony Root respectively. ① to ⑩ correspond to the pH values 4.8, 5.6, 6.4, 6.8, 7.2, 7.6, 8.0, 8.4, 8.8 and 9.6 respectively. Three replicates were performed for the extract solutions at each pH point. And the shown spectra at each pH point were the averages of three replicate spectra. Extract of Cape Jasmine Fruit was taken as an illustration and its spectral changes were magnified at wavelengths both around 405 nm and 475 nm.

The spectral changes with pH values provided acid dissociation information for absorbing species in plant extracts. Taking the spectra of Cape Jasmine Fruit as an example, the trend of the absorbance (around 405 nm) *versus* pH was a double-S-type shaped curve (Fig. 3a). It implied that acid dissociation of the Cape Jasmine Fruit extract at these wavelengths was in line with a two-step ionization process, thus the pK_a^{mix} values could be calculated by fitting the spectral data obtained at these detectable wavelengths with eqn (9). Similarly, the tendency of the absorbance changes with pH values at around 475 nm was an S-type like, and thus these spectral data could be fitted with eqn (5) for obtaining the pK_a^{mix} values in a one-step ionization process.

The spectral changes with pH values differed within plant extracts. At some wavelength ranges, the spectra changed greatly with pH values. This may be attributed to the interaction between hydrogen ion and absorbing species of plant extracts. Interestingly, it seemed that each plant extract had some such wavelength ranges that the absorbance was sensitive to pH values. This sensitivity was determined by the components in plant extracts and could be used for characterizing plant extracts.

3.3 Calculation of acid dissociation constants

For comprehensive investigation of acid dissociation of the plant extract, the spectral data were fitted by using eqn (5) and (9). The fitting results implied that the correlation coefficient (R^2) was undulating with wavelengths (Fig. 4 and S2†). Taking the simulation of the one-step ionization process in Cape Jasmine Fruit extracts as an illustration, the fitting varied between wavelengths (Fig. 4a). However, there existed some relatively stable and continuous regions with high correlation coefficients. In this study, the threshold of statistical significance in the fitting was set as $R^2 = 0.9$. Therefore, totally four regions were screened to calculation of the pK_a^{mix} values in Cape Jasmine Fruit extracts. Similarly, the screening criteria in other plant extracts were applied with the same process.

The distribution of pK_a^{mix} values in plant extracts was well-concentrated and exhibited certain regularity (Fig. 5 and S3†). In one-step ionization processes, the plots of pK_a^{mix} values *versus* wavelength were mainly in a parabolic or line shape, which suggested that the pK_a^{mix} values were affected by wavelengths. The pK_a^{mix} values in the two-step ionization process distributed more complexly than those in the one-step ionization process. Most of the regions had values of pK_{a1}^{mix} and pK_{a2}^{mix}, while some regions only had ones because the other pK_a^{mix} values out of experimental range were omitted. It was worth mentioning that all regions had only pK_{a2}^{mix} values in Safflower extracts (Fig. S3†), which indicated that simulation of the two-step ionization process in some regions might be not suitable.

3.4 Comprehensive investigation of markers in plant extracts

It was shown that some pK_a^{mix} values deviated from the centre of cluster in the region. As an example, the pK_a^{mix} values at the two

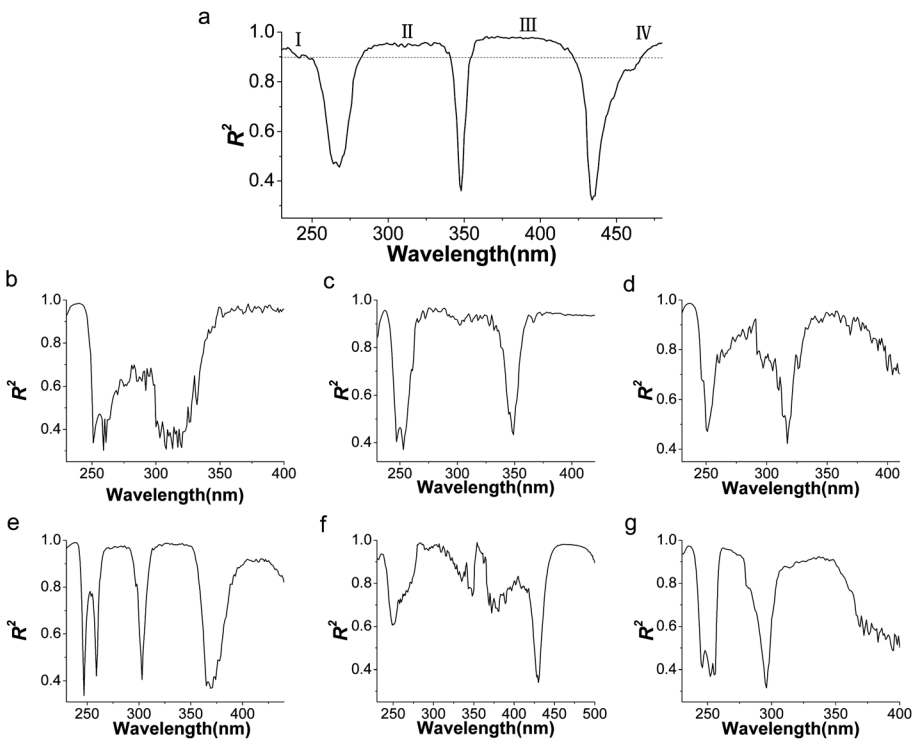

Fig. 4 Simulation of the one-step ionization process in plant extracts. The data were fitted by eqn (5). (a to g) correspond to the fitting results of extracts of Cape Jasmine Fruit, Chinese Angelica Root, Orange Peel, Ginseng Root, Licorice, Safflower and White Paeony Root respectively. Extract of Cape Jasmine Fruit was taken as an illustration. The four regions (indicated by Roman numerals) passing the threshold (over the dashed line, $R^2 > 0.9$) were screened to pK_a^{mix} values calculation.

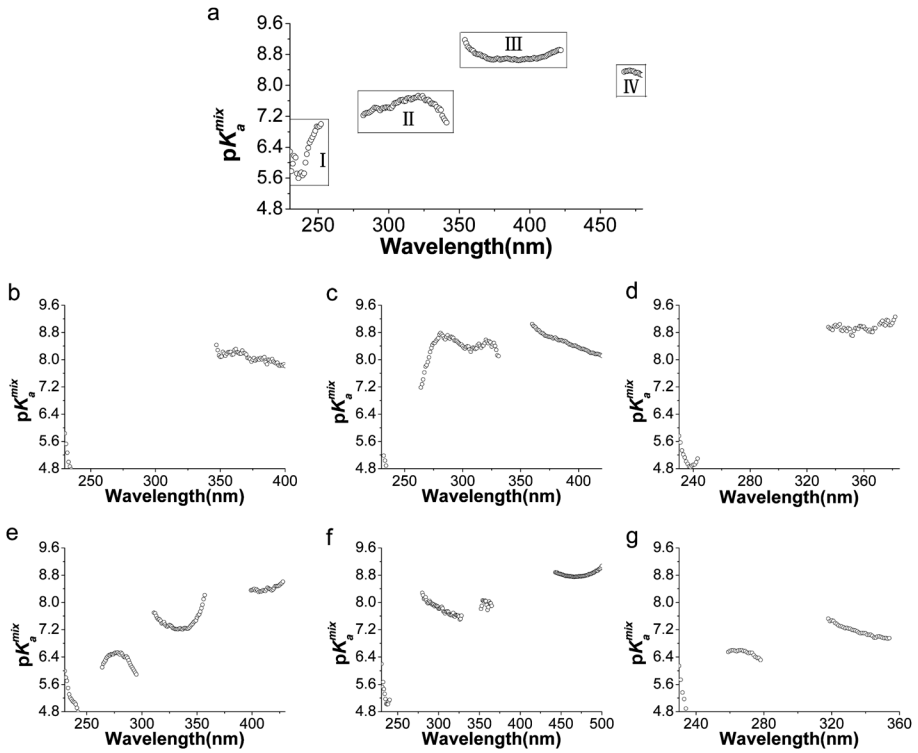

Fig. 5 Acid dissociation constants of plant extracts in the one-step ionization process. Where $pK_a^{mix} = -\lg K_a^{mix}$, the fitted pK_a^{mix} values out of the experimental range were omitted. (a to g) correspond to the acid dissociation constant profiles of extracts of Cape Jasmine Fruit, Chinese Angelica Root, Orange Peel, Ginseng Root, Licorice, Safflower and White Paeony Root respectively. The extract of Cape Jasmine Fruit was taken for an illustration. The pK_a^{mix} values encircled with boxes were calculated from the corresponding regions in Fig. 4a.

ends of a parabolic-shape like region might have a serious deviation from the centre. These pK_a^{mix} values were eliminated in the analysis of characteristics of the pK_a^{mix} values' distribution.

The analysis indicated that the characteristic pK_a^{mix} values at each region were consistent (Table 1). It reflected that the chemical properties of plant extracts involved in acid–base equilibrium. It was shown that the pK_a^{mix} values from the one-step ionization process and two-step ionization process might be slightly different. Interestingly, the pK_a^{mix} value in the one-step ionization process corresponded closely to one of the pK_a^{mix} values in the two-step ionization process, which reflected the consistency of the plant extract in the acid–base equilibrium. The difference was reasonable due to the discrepancy in the applicability of the models and the reaction of plant extracts in the acid–base equilibrium. For example, it seemed that simulation of the two-step ionization process might be more suitable to reflect the reaction of acid dissociation in the Cape Jasmine Fruit extract than that of the one-step ionization process, whereas the Safflower extract was in a reverse situation.

Here, these characteristic pK_a^{mix} values at the specific wavelengths were used as the markers for characterization of plant extracts, which may be applied to qualitative analysis and preliminary quality assessment of plant extracts. For instance, the products without these markers were not in agreement with quality standards of the plant extract. Even for adulterants, it was very difficult to artificially make these makers except the clearness of components and composition ratio.

4 Discussion

The results suggested that the proposed method might be a quick and simple procedure for characterization of plant extracts. However, spectrophotometric methods are selective. The sample under investigation must possess chromophore(s) in proximity to the ionization center(s), so that the protonated and deprotonated species exhibit sufficient spectral dissimilarity for their identification in mixtures.[23,25] As we noted, two of nine plant extracts did not give changes in their spectra of the studied pH range. Therefore, in order for the plant extract to be suitable for the analysis at least two requirements should be met. First, the plant extract should exhibit a broad absorption in the UV-Vis region. Second, the absorbing species of plant extract should be sensitive to pH, indicating that protonation could cause a spectral shift.

Plant extracts are a complex mixture of numerous substances,[27] and their compositions may be affected by many factors such as geographic origin, collection season, mode of storage, and even harvest technology and conditions. For practical application in quality assessment, however, many samples combining different influencing factors should be tested to find some consistent pK_a markers. Then, these consistent pK_a markers may be used for the discrimination of plant extracts from different categories.

Generally, the methodology described here seems to be a promising tool for characterization of plant extracts used in the food and pharmaceutical industries. The major advantage of

Table 1 Comprehensive markers investigated in plant extracts

		One-step ionization process		Two-step ionization process	
Extract source	Markers	pK_a^{mix} (mean ± SD)	Wavelengths (nm)	pK_a^{mix} (mean ± SD)	Wavelengths (nm)
Cape Jasmine Fruit	I	6.27 ± 0.49	230–252	5.49 ± 0.45, 7.50 ± 0.31[a]	237–253, 230–253[b]
	II	7.48 ± 0.16	282–341	5.06 ± 0.15, 7.76 ± 0.16	294–330, 279–342
	III	8.76 ± 0.11	354–422	6.15 ± 0.21, 8.95 ± 0.12	355–428
	IV	8.34 ± 0.04	467–480	5.39 ± 0.19, 8.42 ± 0.32	454–480
Chinese Angelica Root	I	5.30 ± 0.39	230–234	6.95 ± 0.34[c]	230–240
	II	8.07 ± 0.16	347–400	6.08 ± 0.54, 8.45 ± 0.27	338–396, 338–400
Orange Peel	I	5.04 ± 0.15	232–234	5.92 ± 0.63	230–237
	II	8.47 ± 0.16	271–331	5.93 ± 0.54, 8.52 ± 0.20	265–286, 266–334
	III	8.50 ± 0.25	360–420	6.24 ± 0.22, 8.80 ± 0.15	369–420, 359–420
Ginseng Root	I	5.11 ± 0.28	230–243	5.66 ± 0.54	230–246
	II	8.96 ± 0.11	335–382	5.89 ± 0.33, 9.35 ± 0.28	329–398, 328–398
Licorice	I	5.32 ± 0.35	230–241	5.04 ± 0.28, 6.79 ± 0.13	230–239
	II	6.39 ± 0.13	264–291	6.14 ± 0.44, 7.50 ± 0.26	248–258
	III	7.37 ± 0.15	311–353	6.21 ± 0.18	265–292
	IV	8.41 ± 0.08	399–428	5.05 ± 0.21, 7.44 ± 0.15	332–345, 313–356
	V	—	—	6.09 ± 0.07, 8.06 ± 0.14	367–410
Safflower	I	5.34 ± 0.36	230–240	7.01 ± 0.24	230–243
	II	7.82 ± 0.19	280–328	7.83 ± 0.18	280–325
	III	7.95 ± 0.09	352–365	8.72 ± 0.06	344–350
	IV	8.83 ± 0.08	443–500	8.02 ± 0.07	351–365
	V	—	—	8.82 ± 0.08	444–500
White Paeony Root	I	5.46 ± 0.49	230–234	6.21 ± 0.40	230–234
	II	6.52 ± 0.09	259–278	8.64 ± 0.16	239–242
	III	7.16 ± 0.17	318–354	6.77 ± 0.15	259–278
	IV	—	—	6.68 ± 0.02, 8.65 ± 0.07	314–341

[a] The two pK_a^{mix} values were pK_{a1}^{mix} and pK_{a2}^{mix} in the two-step ionization process respectively. [b] The two wavelength ranges corresponded to pK_{a1}^{mix} and pK_{a2}^{mix} respectively. [c] The other pK_a^{mix} value out of the experimental range was omitted.

the proposed approach lies in its simple operation and low cost. It can serve as an alternative for preliminary quality assessment of plant extracts where advanced instruments (*e.g.* HPLC) are not available for routine analysis. Access to advanced instrumentation is often a problem in developing countries or poor areas. Both the proposed method and the traditional fingerprinting method will be valuable to facilitate the lot-to-lot quality control process for manufacturers prior to processing of plant extracts.

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