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Analytical Methods

Spectrophotometric determination of total serotonin derivatives in the safflower seeds with Ehrlich's reagent and the underlying color reaction mechanism

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Abstract

A new spectrophotometric method for the determination of total serotonin derivatives in the safflower (*Carthamus tinctorius* L.) seeds is described. The determination is based upon a color reaction between serotonin derivatives and *p*-dimethylaminobenzaldehyde (Ehrlich's reagent), which follows the electrophilic substitution reaction mechanism at the indole ring. The main factors affecting correct measurement of total serotonin derivatives concentration were studied. The maximum absorption wavelength of the complex was determined at 625 nm. Lambert-Beer's law is obeyed in the concentration range of 0.025–0.5 mmol/l, with a correlation coefficient (R^2) of 0.9996, a recovery of 99.7%, and a relative standard derivation (RSD) of 1.5%, respectively. The proposed method presented satisfactory results in the determination of total serotonin derivatives in the extract from a strain of safflower seeds, and thus is recommended as a routine method for total serotonin derivatives quantitation.

Keywords: Safflower seeds; *Carthamus tinctorius*; Serotonin derivatives; Spectrophotometry; Ehrlich's reagent; *p*-Dimethylaminobenzaldehyde

1. Introduction

Serotonin derivatives in the safflower seeds are a family of molecules containing seven to ten members featuring a serotonin moiety bound to a phenylpropanoid moiety via an amide bond (Sakamura, Terayama, Kawakatsu, Ichihara, & Saito, 1978; Sato et al., 1985; Zhang, Nagatsu, Watanabe, Sakakibara, & Okuyama, 1997). The structures are exemplified in Fig. 1a.

There are a number of biological effects attributable to these compounds (Pavlık et al., 2002; Yamamotová, Pometlová, Harmatha, Rasková, & Rokyta, 2007), e.g., cathartic effect, antioxidative activities, radical-scavenging activities (electron donating ability), anti-tyrosinase activity, melanine production inhibitory activity, anti-tumor activity, fibroblasts growth promoting activities, proinflamatory cytokine production inhibitory effects as well as pain and anxiety modulating activity.

Although serotonin derivatives possesses so many bioactivities, few simple yet accurate methods are available for the assay of this category of molecules, with the intensively cited determination methods using high-performance liquid chromatography (HPLC) (Kang, Chang, & Choi, 1999; Kang, Jang, Kang, & Back, 2005; Niwa, Etoh, Shimizu, & Shimizu, 2000). The HPLC method, although suitable for determination of the serotonin derivative in a structure-specific manner, is time-consuming due to demand for complicated operation skills and multi-standard substances; besides, in many cases the total rather than the structure-specific quantity is needed, so that the HPLC method accuracy becomes unnecessary. A convenient quantitative routine is thus necessitated for the determination of total serotonin derivatives in the safflower seeds.

Spectrophotometric methods are commonly used today for a broad spectrum of determination purposes. But in the

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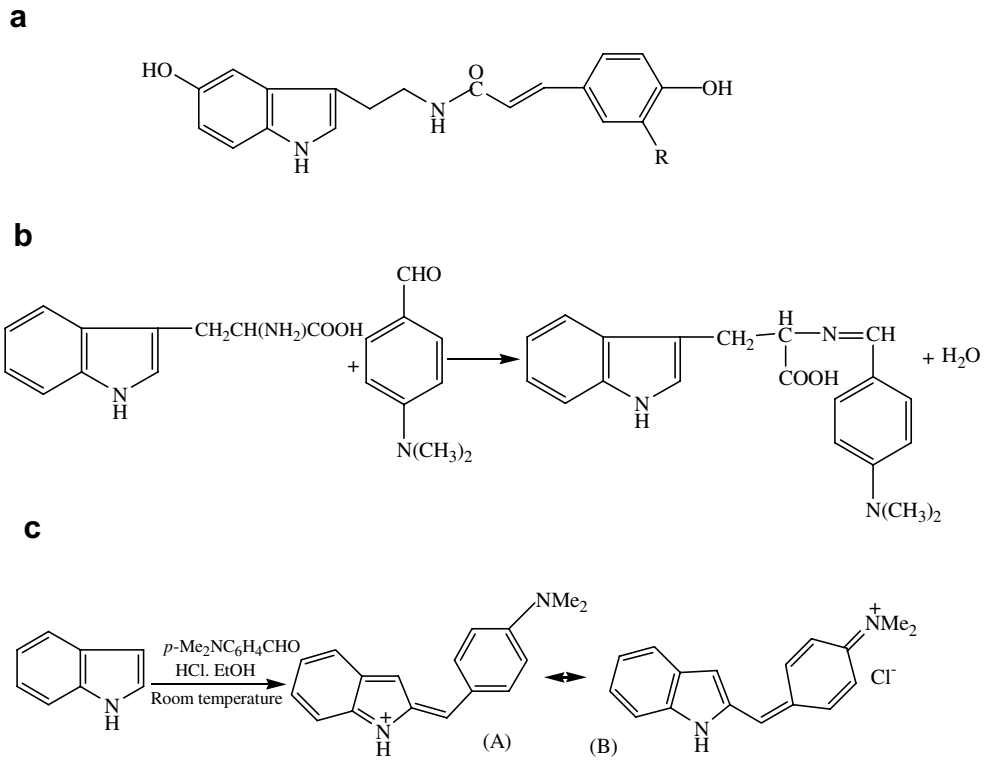

Fig. 1. (a) Chemical structures of feruloylserotonin (R = OCH_3) and *p*-coumaroylserotonin (R = H) in safflower seeds; (b) Condensation of tryptophan with Ehrlich's reagent; (c) Reaction of indole ring derivatives with Ehrlich's reagent.

case of spectrophotometric analyses for some molecules, such as serotonin derivatives, an appropriate chromophoretic substance is needed to render the matter to be determined specific and quantitative absorption in the desired wavelength range. The indole derivatives are known for their specific color reactions, for example, with Ehrlich's reagent in acidic media to produce a violet water-soluble complex that exhibits maximum absorption in the visible region obeying the Lambert-Beer's law over a wide concentration range. This color reaction forms the basis for the efficient, rapid and simple spectrophotometric assay of serotonin derivatives without expensive instrumentation.

The aim of this study was to develop a spectrophotometric method for the determination of serotonin derivatives in the safflower seeds based on the afore-mentioned reaction, and discuss the underlying reaction mechanism. The method thus established was also testified in the determination of serotonin derivatives in the extract from a strain of safflower seeds.

2. Experimental procedures

2.1. Apparatus and reagents

Defatted safflower (*Carthamus tinctorius* L.) seeds were from Xinjiang municipality, PR China. Feruloylserotonin standard was prepared using established methods (Zhang et al., 1997) with a purity of 98% according to HPLC results. A stock standard solution containing 2.84 mol/l feruloylserotonin was prepared: 20 ± 0.1 mg feruloylserotonin standard was dissolved in appropriate volume of methanol, the resulting solution was transferred into a 20 ml calibrated flask, and finally the solution was diluted to the mark with methanol.

0.20 mg/ml Ehrlich's reagent were prepared by dilution of 20±0.1 mg *p*-dimethylaminobenzaldehyde in 100 ml 17:3 (v/v) glacial acetic acid-hydrochloric acid mixture, and then kept under refrigeration before use.

All the solutions were filtered and degassed under reduced pressure.

Glacial acetic acid, hydrochloric acid, methanol, ethanol, *iso*-octane, *n*-hexane, ethylacetate, and other substances, unless otherwise specified, were of analytical grade and purchased from Shanghai Chemical Reagents Ltd. Doubly distilled water was used all through this work.

All spectral measurements were conducted on a UNICO UV-2100 UV–Vis spectrophotometer (Unico Instrument Co., Ltd. Shanghai) in the wavelength range of 200–1000 nm with a 1 cm path length cell.

2.2. Procedure for calibration curve

Working standard solutions were prepared by further dilution of the stock standard solution with methanol. An aliquot of working standard solutions (2 ml) were transferred, respectively into seven 10 ml volumetric flasks, and then 5 ml Ehrlich's reagent solution was added into each flask. The flasks were shaken to achieve homogeneity

before being heated in a 50 °C water bath for 30 min, and then the reaction mixtures were cooled to room temperature and diluted to the mark with 0.1 mol/l hydrochloric acid. After re-shaking to homogeneity, the mixtures were measured in a 1 cm cell at 625 nm for absorbance. A reagent blank, prepared in the same manner as the reaction mixture except for the working standard solution being replaced with an equal volume of 0.1 mol/l hydrochloric acid, was used as the reference. The calibration curve was plotted in the range of 0–0.5 mmol/l feruloylserotonin.

2.3. Sample preparation and analysis

Ten grams ground defatted safflower seeds (20 meshes) were reflux extracted with 100 ml methanol. The extract was filtered through a filter paper (Xinhua No. 2) and vacuum dried at 40 °C, the residue was dissoloved in methanol and fractionated with *iso*-octane, and then the methanol fraction was partitioned between *n*-hexane and 80% methanol. The 80% methanol fraction was further partitioned with ethylacetate and H_2O. The vacuum dried ethylacetate extract was used as the final sample for spectrophotometric analyses.

Samples of desired amounts were, respectively diluted to the mark with methanol in 10 ml volumetric flasks. 2 ml of the sample solution was transferred into a 50 ml volumetric flask, diluted to the mark with methanol. Two milliliter aliquot of diluted sample solution was reacted with Ehrlich's reagent solution, and finally the reaction mixture was measured for absorbance following the steps as described in Section 2.2. The relevant serotonin derivatives concentration was calculated using the calibration curve obtained in Section 2.2.

All standard solutions and samples were measured in triplicates and the mean values were used for data acquisition.

In order to determine the precision and accuracy of the methods, known amounts of feruloylserotonin standard added to a separate set of samples were subjected to recovery studies. Accuracy was assessed by the found recovery mean. Precision are assessed by the relative standard deviations (RSD).

3. Results and discussion

3.1. Absorption spectra

The detection wavelength was optimized with respect to the absorption maximum (λ_{max}) of the reaction product of serotonin derivatives with Ehrlich's reagent. The absorption spectra of the Ehrlich's reaction products of both feruloylserotonin standard solution (reagent concentration $\leqslant$0.5 mmol/l) and the sample solutions, as recorded in a wavelength range of 570–640 nm, indicates a maximum absorbance at 625 nm. Thus the optimum detection wavelength was determined at 625 nm.

3.2. Proposed reaction mechanism

Some primary amines compound such as tryptophan, 5-hydroxytryptamine and tryptamine, etc., react with *p*-dimethylaminobenzaldehyde to yield a Schiff base, a stable imine which gives a coloured complex with a maximum absorption at 590–600 nm as shown in Fig. 1b.

Schiff base is formed by addition to the carbonyl group of the nucleophilic amine, followed by loss of water from the amino alcohol addition product. This reaction is particularly useful and proposed as a means of quantitation for tryptophan (Gao & Fang, 1999).

In contrast to primary amines, serotonin derivatives in the safflower seeds with an indole-based system are secondary heterocyclic amines compounds in which two nitrogen atoms occur, respectively in the amide and the ring. The structural differences will cause differences in reactivity of serotonin derivatives in the safflower seeds from the primary amines. Therefore, serotonin derivatives do not react with Ehrlich's reagent following the route of producing Schiff base. Fortunately, serotonin derivatives are susceptible to electrophilic substitution reaction thanks to the indole rings in the molecular structure. When indole derivatives with active hydrogens both at C-2-position and C-3-position react with Ehrlich's reagent in an acidic medium, a water-soluble coloured cation complex is formed (Joule & Mills, 2004; Lin, 1977), as shown in Fig. 1c.

The above-mentioned reaction of indole ring derivatives is known as RpeXeM reaction. This reaction occurs between indole derivatives and aromatic aldehydes to produce a cation which is stable and separable with a maximum absorption in the visible region (Li & Wen, 2002). Lambert-Beer's law was obeyed over a wide concentration range. Hereby, visible spectrophotometry can be used to determine sample concentrations (Satınsky, Sklenarova, Huclova, & Karlicek, 2003; Banerjee & Mashru, 1989).

Though RpeXeM reaction generally occurs at a C-3-position of an indole moiety, an attack at C-2-position (the position next to nitrogen) can take place if C-3-position is blocked, e.g., in the case of serotonin derivatives.

The above-mentioned reaction involves a typical electrophilic aromatic substitution process, in which the positively charged carbonyl group of *p*-dimethylaminobenzaldehyde acts as the electrophile that reacts with the electron-rich ring of indole derivatives to form an intermediate with a hydroxyl group bonded to the original aldehyde carbon atom of *p*-dimethylaminobenzaldehyde. The intermediate is quickly converted to a cation (A) stabilized by electron delocalization within π system (Miller, 2005). A critical step of this reaction requires the nitrogen in the ring to be protonated, and charges on the indole ring to be delocalized, which is fundamental to their chemical and spectroscopic natures.

Actually, more correct structural representation of A might be drawn as B (Fig. 1c), i.e., there are two resonance forms and the true structure of the species is a hybrid of these, each has 18 π-electrons in a conjugated system.

In compliance with the selection rules, the degrees to which the electrons are attached to the nuclei determine the energy of each electronic transition and therefore the wavelength of absorption. The more resonance forms can be drawn for a resonance system and the more atoms involved in a resonance system, the more significant the electron delocalization within the π system is; and hence electronic transition occurs with a higher probability at lower energy level and as a result shifts the λ_{max} to longer wavelengths. The maximum absorption of the complex of indole ring of serotonin derivatives with Ehrlich's reagent is located at 625 nm, compared with that of the complex of tryptophan with Ehrlich's reagent at 590 nm. The red-shift of the λ_{max} in the former case as compared to the latter is partially attributable to the broader range of delocalization in the former.

Similarly, the interaction in the delocalized π system formed among adjacent residues is an important contributor to the overall stability of the cation. Thanks to the existence of the hybrids of two resonance forms (A and B), the cations are stable enough to make the absorbance determination free of major disturbance by fluctuations of environmental conditions.

3.3. Effects of reagent amounts, reaction temperature and time

The amounts of Ehrlich's reagent should be enough yet not excessive to ensure a complete electrophilic substitution reaction at the indole ring of serotonin derivatives. It was proved that the optimum amount of 0.2 mg/ml Ehrlich's reagent solution was in the range between 4 ml and 8 ml with best analytical results observed using 5 ml. Excessive Ehrlich's reagent had disadvantageous effect since the absorbance of the reagent blank increases so remarkable that the detection sensitivity was decreased.

The stability of the system is related to the temperature. The higher the temperature is, the faster the color reaction rate becomes and the shorter the time the completion of the reaction consumes, although the stability of the system may be decreased at extremely high temperatures.

Even though the color reaction takes place under room temperature with high yields (Li and Wen, 2002), the reaction rate is normally rather slow. However, the results of the present study showed that completion of the reaction can be achieved at satisfactory rates using temperatures in the range of 40–50 °C, as judged following the absorbance of the reaction mixture. Therefore, the optimum reaction temperature was determined at 50 °C.

In the course of kinetic study of reaction between serotonin derivatives and Ehrlich's reagent, a violet water-soluble compound was formed as soon as the standard or samples were contacted with Ehrlich's reagent. With the extension of reaction time, the color of the system became darker, indicating that the amount of the coloured product was increased. A reaction time of 30 min, where the absorbance of the reaction mixture reached its maximum and keep essentially unchanged thereafter, was determined as the optimum parameter for determination.

3.4. Calibration

Calibration experiments were carried out using seven solutions of the standard of which the concentrations included 0.5, 0.4, 0.3, 0.2, 0.1, 0.05 and 0.025 mmol/l following the serotonin derivatives serial-concentrations as previously described in Section 2.1., the calibration graph was constructed of the absorbances versus the concentrations of the standard solution. The linear regression equation was obtained with a correlation coefficient (R^2) of 0.9996:

$$Y(\text{mmol/l}) = 0.8947A - 0.0044$$

where A is the absorbance of the reaction mixture and Y is the serotonin derivatives concentration.

The linear range of the calibration graph was found to be 0.025–0.5 mmol/l. The limit of detection (LD) was 2.3 μmol/l given by the equation:

$$\text{LD} = kS$$

where k is a numerical factor chosen according to the confidence level desired, and S is the relative standard deviation of blank measurements ($n = 11$). In this study the value of k was set at 3 according to general practices.

3.5. Assay of serotonin derivatives in the samples and interference studies

Four runs of tests gave concentrations of total serotonin derivatives ranged between 0.388 and 0.402 mmol/l, with a mean value of 0.394 mmol/l, which is equivalent to a content of 1.89 mmol/g of total serotonin derivatives in the ethylacetate extract of the safflower seeds. The result is in good agreement with previous reports (Zhang et al., 1997).

Recovery tests were then performed by adding feruloylserotonin standard to the four samples to increase their concentrations by 0.04, 0.06, 0.08 and 0.10 mmol/l, respectively (Table 1). The recoveries were found varying from 97.5% to 103.3 % with a mean of 99.4%, and the corresponding RSDs varied from 2.10% at 0.10 mmol/l to 0.89% at 0.08 mmol/l with a mean of no more than 1.5%, showing no significant difference between the increased and the recovered quantity. The method established herein is proven to have high precision, low accidental error and

Table 1
Recovery and precision of the method established herein ($n = 3$)

Samples No.	Found (mmol/l)	Increased (mmol/l)	Total found mean (mmol/l)	Recovery (%)	RSD (%)
1	0.388	0.10	0.488	100.0	2.10
2	0.402	0.08	0.480	97.5	0.89
3	0.390	0.06	0.452	103.3	1.37
4	0.394	0.04	0.434	100.0	1.45

Table 2
Influences of interfering substances (IFs) on the determination of serotonin derivatives ($n = 3$)

IFs	Ratio IFs/safflower seeds (w/w)	Found amount of serotonin derivatives (%)
Tryptophan	0.1:10	99.71
5-Hydroxy-tryptamine	0.1:10	101.45
Tryptamine	0.1:10	102.50
Indole	0.1:10	100.14
Ferulic acid	0.1:10	99.70

good dependability; and there exists no significant and persistent factors that interfere with the determination.

The specificity of the proposed method was tested in the presence of five interfering substances (IFs) corresponding to the serotonin and phenylpropanoid moiety of serotonin derivatives including tryptophan, 5-hydroxytryptamine, tryptamine, indole and ferulic acid. Roughly 0.1 g of each interfering substance was taken and mixed with 10 g ground defatted safflower seeds, followed by reflux extraction, purification and analyses as described in Section 2.3. Their interferences were not observed (Table 2). It is concluded that the interfering substances such as tryptophan, 5-hydroxytryptamine, tryptamine and indole are certainly removed almost completely because of their solubility in water and organic solvents during extraction and purification, and that ferulic acid itself does not develop a color reaction with Ehrlich's reagent.

4. Conclusion

The color reaction, based on the formation of violet, water-soluble complex of indole rings with *p*-dimethylaminobenzaldehyde in compliance with the electrophilic substitution reaction mechanism, was applied satisfactorily for the assay of total serotonin derivatives in the safflower seeds. In comparison with published HPLC analytical methods, the spectrophotometric technique established in this work is characterized by simplicity, specificity, enough sensitivity and inexpensive instrumentation. Upon further tests using more types of samples, this technique might be confirmed to be an alternative for the routine analysis of total serotonin derivatives.

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