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Studies on complex formation between curcumin and Hg(II) ion by spectrophotometric method: A new approach to overcome peak overlap

Research Article

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Abstract: Complex formation between curcumin and Hg(II) ion MeOH/H_2O (1: 1 v/v) was investigated and monitored by the spectrophotometric method. The absorption peak of unreacted curcumin which was close and overlapped with that of the complex, was removed by calculation using Microsoft Excel, thereby, allowing determination of the stoichiometry of the complex by the mole-ratio and the Job's continuous variation methods. Both methods indicated that a 1:1 complex of curcumin and Hg(II) was formed in solution. The formation constant of the 1:1 Hg(II) complex was obtained from two methods, the equilibrium concentration calculation and the linear plot of Benesi-Hildebrand equation, as log K = 4.44 ± 0.16 and 4.83 ± 0.02, respectively. The structure is proposed as a tetrahedral complex of Hg(II) with one curcumin and two chloride ions as ligands.

Keywords: *Metal curcumin complexes • Mole-ratio method • Job's method • Benesi-Hildebrand • Formation constant*

1. Introduction

Curcumin, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a yellow pigment of turmeric, a spice manufactured from the root of *Curcuma longa* which is a member of the *Zingiberaceae* (ginger) family cultivated extensively in India, China, and other countries with a tropical climate. Turmeric, besides extensively used in flavoring and coloring foods, has a long tradition of use in the Chinese and Ayurvedic systems of medicine. Turmeric is oftenly referred to as *the „Multi-Anti" Spice*. In herbal medicine, turmeric has been found to have the following effects: antihepatotoxic, antihyperlipidemic, anti-inflammatory, antioxidant, antitumoral, antimicrobial, antifertile, anti-insect, and anti-Alzheimer's disease [1,2]. In this regards, it is noteworthy to mention an interesting statistic that the elderly (aged 70-79) residents of rural India, who eat large amounts of curry, appear to have the lowest incidence of Alzheimer's disease in the world *ca.* 4.4 times lower than that of Americans [3].

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Curcumin is among the more successful chemopreventive agents investigated in recent years, and is currently in human trials to prevent cancer. It has been observed that curcumin strikingly modulates proteins of iron metabolism in cultured cells and in mouse liver: curcumin represses ferritin protein, increases transferrin receptor, and activates IRP, all indicators of iron depletion. These results suggest that iron chelation may be a mode of action of curcumin, a result that may have important implications for the use of this and other agents in cancer chemoprevention [4].

Studying metal-curcumin complexation is one interesting research topic and is appropriate for those countries in the tropical climate where local medicinal plants are abundant. It helps link the basic chemistry knowledge to medicinal applications. The interaction between the active ingredient of medicinal plants and metal ions in the body frequently involving *complexation* and most of them through the process called *metal chelation*. Many metal ions are dissolved, as free ions, in all of our bodily fluids. They are vital to life but sometimes they can be toxic to body and we must find some way to get them out. Medicines given to the patients for this purposes usually bind with metal ions, *i.e*. metal complexation, rendering them less toxic or easier to be removed from the body. On the other hand, some agents by themself can be used as medicine in free form but in the form of metal complexes they were found to be more effective than the free form. Examples are: vanadyl curcumin and copper curcumin complexes, the former by experimental evaluations, the latter by calculations [5,6].

The *β*-diketone functional group of curcumin can be a powerful natural chelating agent which is safe even administered at high dose in human [7-9]. The stability of curcumin chelation to the metal ion can be estimated by the complex formation constant (K for ML; or β for ML_n, where $n \geq 2$). Complex formation constants are usually expressed as the logarithm of K or log K. The higher the value of log K, the stronger the metal ion is bound to the chelating agent. Thus, log K values are a useful parameter for the chelation therapy.

A number of research groups have previously used UV-Vis spectroscopy to study complex formation [10-12]. Their spectrophotometric data were fitted with commercial fitting programs, such as SQUAD and LETAGROP-SPEFO, to generate the K values. The binding of curcumin to Fe(II) and Fe(III) ions was studied in solution and the complex formation constants were determined by potentiometric and spectrophotometric methods.

When the spectrophotometric method is chosen as the tool to study curcumin-metal ion system one may run into one difficult problem due to peaks overlapping. Curcumin itself has an intense absorption peak at 428 nm (Fig. 1) and when it forms complex with metal ions significant amount of the unreacted curcumin still remains in solution, so this peak remains prominent. The metal complex that forms may have its own absorption close to the characteristic peak of curcumin, hence, causing severe overlap of these peaks. In this report we came around this problem by using the Microsoft Excel as an aid in calculation and help removing the overlapping peak of the unreacted curcumin. This made the next step of calculations possible and the stoichiometries and formation constants of the complexes could be obtained. The metal ion chosen was Hg(II) due to its well known toxicity and environmental importance.

2. Experimental Procedures

2.1 Chemicals and equipment

Curcumin (Sigma, catalog № C-1386), $HgCl_2$ (Carlo Erba, catalog № 46100) and absolute methanol (Merck, catalog № 1.06009.2500) were used as received. All samples were prepared in $MeOH/H_2O$ (1 : 1 v/v). Throughout the experiment, all samples were prepared at room temperature (25°C) using the amber volumetric flask to protect from light degradation.

Absorption spectra were recorded on SPECORD S100 spectrophotometer (Analytik Jena GmbH; Germany) with 1 cm quartz cell. The cell-holder was connected to thermocirculating bath (Lauda A100; Germany) to keep temperature constant at 25 ± 0.1°C.

2.2 Procedure

2.2.1 Sample preparations

Stock solution of curcumin (2.0×10^{-4} M) in $MeOH/H_2O$ (1 : 1 v/v) was freshly prepared before use. Since the solubility of curcumin is low in water, it was first dissolved in absolute methanol and then deionized water was added to make the final solution of 1 : 1 v/v. In order to minimize the effects of light on these solutions, all the experiments were carried out in the dark.

A stock solution of Hg(II) ion (2.0×10^{-4} M) was prepared by dissolving the metal salt in $MeOH/H_2O$ (1 : 1 v/v) then diluted to 100 mL with the same solvent.

From the stock solutions of curcumin and Hg(II) ion, various solutions of lower concentrations were prepared subsequently.

The pH of curcumin solution was 6.6 which gradually decreased upon addition of Hg(II) solution and remained at 5.3 - 5.4 after addition was complete.

2.2.2 Curcumin absorption peak: simulation and subtraction

Absorption peak of free curcumin that remained in the solution was obtained by calculation and used to subtract from solution spectrum to give solely the absorption spectrum of the complex that formed. The calculation process composed of preparing curcumin solutions at various concentrations ranging from 4.0×10^{-6} to 4.0×10^{-5} M covering the working range in the experiment. Then the correlation between concentration and absorbance at each wavelength was constructed by using Microsoft Excel. Once the correlation was established, the absorbance at any wavelength could be obtained by inputting the "expected" concentration, or vice versa. By this method, the entire spectrum of free curcumin at any concentration in the solution could be simulated and used to subtract from the true spectrum obtained from the spectrophotometer. The result was taken as the absorption spectrum of the complex that was being formed in the solution and was free from any overlapping with the absorption from free curcumin. (Details of the method can be found in the Supplementary document.)

2.2.3 The mole-ratio method

In the mole-ratio method, mixtures of solutions were prepared with a constant concentration of curcumin (2.0×10^{-5} M) and variable Hg(II) concentrations (from 0 to 1.4×10^{-4} M). The absorption spectra of these mixtures were recorded at 25 ± 0.1°C in the 200 - 800 nm spectral range, using MeOH/H_2O (1:1 v/v) as the reference.

2.2.4 The Job's continuous variation method

In the Job's method, solutions of curcumin and Hg(II) ion (0 to 2.0×10^{-5} M) were prepared and mixed to give solutions of mole fraction (excluding water) of curcumin varying from 1 to 0. The absorption spectra were recorded in the same manner as described above.

2.2.5 Calculation for the complex formation constant

The complex formation constant was calculated either from the equilibrium expression (Eq. 1) or the Benesi-Hildebrand plot (Eq. 2).

$$K = \frac{[ML]}{[M][L]} \tag{1}$$

$$\frac{[M][L]}{A} = \frac{1}{K\varepsilon_{ML}} + \frac{1}{\varepsilon_{ML}}[L] \tag{2}$$

Eq. 2 is valid for complex where the metal:ligand ratio is 1:1 where a plot of {[M][L]}/A against [L] will yield a straight line [13-16]. ε_{ML} is the molar absorption coefficient of the complex which is equal to (1/slope) and K is the formation constant equal to (slope/intercept).

3. Results and discussion

3.1 Nature of absorption peaks

3.1.1 Curcumin

Curcumin in methanolic-aqueous solution shows a broad characteristic absorption around 300 - 500 nm with maximum at 428 nm, a shoulder at 360 nm, and a weak absorption at 260 - 280 nm as shown in Fig. 1. The maximum absorption is due to the electronic dipole allowed π - π* type excitation of its extended

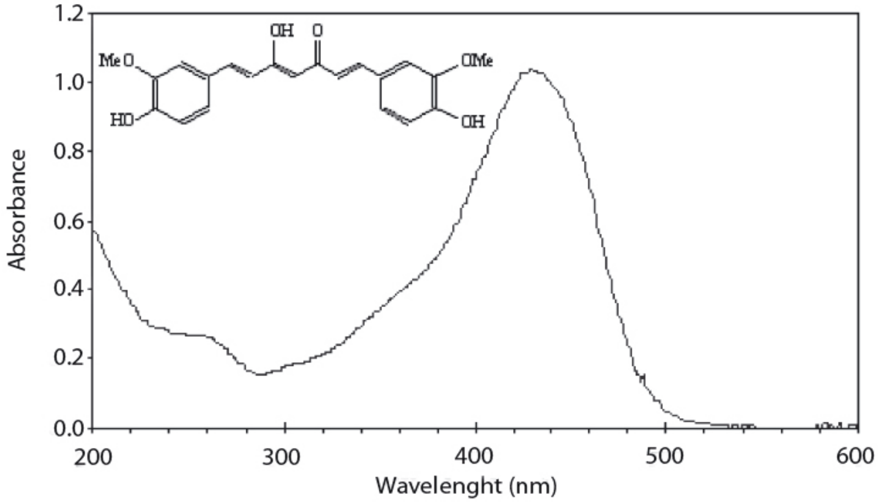

Figure 1. Molecular structure and absorption spectrum of curcumin in MeOH/H_2O (1 : 1 v/v) (2.0×10^{-5} M)

π-conjugation system. Upon light absorption a π electron is excited from the ground state to the first excited state and oscillates from one end of the chromophore to the other. Most likely, the weak, electronic dipole forbidden n - π* band is located somewhere under the main absorption band. The large conjugated framework of curcumin molecule predominates in the enolic curcumin with π electrons are delocalized through the whole molecule between two feruloyl parts which causes a decrease in π - π * transition energy and the absorption band appears at the lower energy (419 nm in acetonitrile) than curcumin in the ketone form (389 nm in acetonitrile) in which there is no conjugation between two feruloyl parts [17-21].

The molar absorption coefficient, ε, of curcumin in MeOH/H_2O (1:1 v/v) obtained in this work was 49,370 M^{-1} cm^{-1} which could be compared to 55,000 M^{-1} cm^{-1} in EtOH [18,19] and 67,680 and 54,954 M^{-1} cm^{-1} in MeOH and EtOH, respectively [22].

3.1.2 Curcumin-Hg(II) system

The maximum absorption of curcumin at 428 nm decreased in intensity in the presence of Hg(II) ion and a new peak appeared at 359 nm (Fig. 2). For fixed concentration of curcumin and increasing amounts of Hg(II) ion, an increase of the new peak at 359 nm accompanied by a decrease of the peak at 428 nm could be observed. An isosbestic point was detected at wavelength 375 nm. The presence of an isosbestic point indicated that two species were in equilibrium with each other at this wavelength [23]. The occurrence of new peak at 359 nm was due to the formation of Hg(II)-curcumin complex as a result from addition of Hg(II) ion. This peak presumably blue shifted from the original intense band of 428 nm as a result of a breakdown of the π electron delocalization upon complexation. The clean region around 590 nm (Figs. 2 and 6) indicates that the system is free of phenolate species upon addition of Hg(II) ion [12].

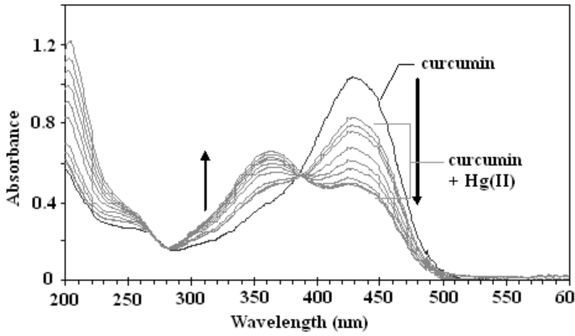

Figure 2. Spectra of the mole-ratio method for curcumin-Hg(II) system with varying Hg(II) concentrations: from 0 to 1.4×10^{-4} M (from top to bottom).

3.2 Resolving the overlapping peaks

3.2.1 The resolving method

The overlapping of relevant peaks made it difficult to obtain accurate absorbance readings of these peaks. For further calculations to be possible, the overlap must be resolved. There are several commercial packages, such as KINFIT and BEST [24,25], that help get around this overlapping problem, but we tried the inexpensive way with the Microsoft Excel software that usually comes with the Windows-based PC.

The original spectrum obtained from this system appeared as curve (a) in Fig. 3. The apparent profile of curve (a) suggested considerable overlapping of unreacted curcumin absorption and another new absorption presumably arose from the new species just formed. The spectrum of free curcumin of unknown concentration that was present in the system was simulated by the method as described in 2.2.2 above and appeared as shaded curve (b) in Fig. 3. The numerical data of curve (b) at each wavelength was correspondingly subtracted from curve (a) to give curve (c) which is the clean spectrum of the complex.

3.2.2 Resolving overlapping peaks and determining stoichiometry of the curcumin-Hg(II) system

The complex formation between curcumin and Hg(II) ion in solution was investigated by using two methods: the mole-ratio and the Job's continuous variation methods. In the mole-ratio method, the ligand concentration was fixed while varying the Hg(II) concentrations. The resulting spectra are shown in Fig. 2 where changes of curcumin spectra were recorded when Hg(II) ion was added. The curcumin absorption peak (at 428 nm) in Fig. 2 was removed by subtraction as described above and the absorption of the new complex could be seen with λ_{max} at 359 nm as shown in Fig. 4. After the overlap was resolved, the absorbances at λ_{max} (359 nm) could be read off directly, and from these new spectra the usual mole-ratio plot (absorbance versus concentration) could

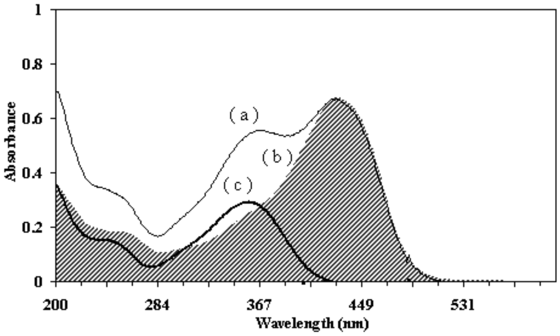

Figure 3. (a) spectrum of mixed curcumin and Hg(II) ion, (b) estimated spectrum of residual unreacted curcumin, and (c) spectrum of the complex (after subtraction).

be constructed as shown in Fig. 5. The stoichiometry was obtained from the intersection of the two straight lines, in this case, it was 2.0 × 10^{-5} M of Hg(II) in equivalent with 2.0 × 10^{-5} M of curcumin, therefore, the stoichiometry is 1:1.

In the Job's method, several mixtures of curcumin and Hg(II) were prepared at various mole fractions with the total concentration of 2.0 × 10^{-5} M and their absorption spectra are shown in Fig. 6. The heavily overlapping peaks in Fig. 6 did not warrant accurate reading of absorbance of the complex absorption peak at 359 nm. The free curcumin peak at 428 nm was removed by subtraction as described above. The resulting clear spectra of complex are displayed in Fig. 7 and subsequent Job's plot is obtained in Fig. 8. The complex stoichiometry as being read from Fig. 8 is 0.53 : 0.47 or 1:1 and agrees with the result from the mole-ratio method.

3.3 Calculations of the complex formation constant of the curcumin-Hg(II) system

The complex formation constant of 1:1 complex was determined using the equilibrium concentrations (Eq. 1) and the Benesi-Hildebrand plot (Eq. 2). In these calculations the concentration of free or unreacted curcumin in the solution must be known. This was made possible by using the method just described in 2.2.2 and the concentration of the unreacted curcumin could be obtained.

In using Eq. 1, as an example, when solutions of two reactants were mixed, the starting concentration of both curcumin and Hg(II) were 2.0 × 10^{-5} M. At the end of the reaction, the remaining unreacted curcumin concentration was 1.3 × 10^{-5} M. This value was arrived at by inputting the "estimated" concentrations into the calculation model until the best result was obtained after subtraction. Once the concentration of unreacted curcumin in the solution was known, the concentration of other species such as [M], [ML] could be obtained. And finally, with the knowledge of the stoichiometry just obtained from either the mole-ratio or the Job's method, the K value of Eq. 1 could be calculated, in this case it was 4.02 × 10^4 or log K = 4.60. The experiment was carried out in triplicate so the formal value is log K = 4.44 ± 0.16 for the complex formation between curcumin and Hg(II) ion in solution.

The K value can also be obtained by using the Benesi-Hildebrand equation (Eq. 2) where {[M][L]}/A was plotted against [M]. In this plot, the 1:1 complex would yield a straight line which K = (slope)/(intercept)

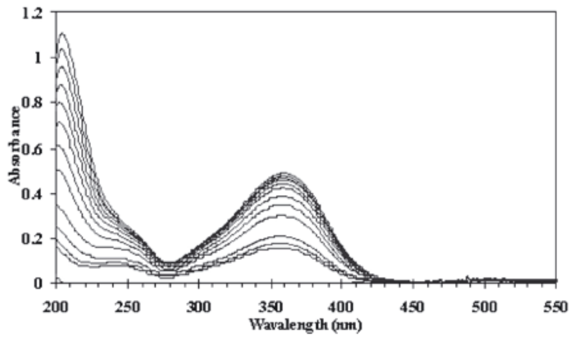

Figure 4. Spectra of the mole-ratio method for the curcumin-Hg(II) system (after subtraction of residual free curcumin from Fig. 2).

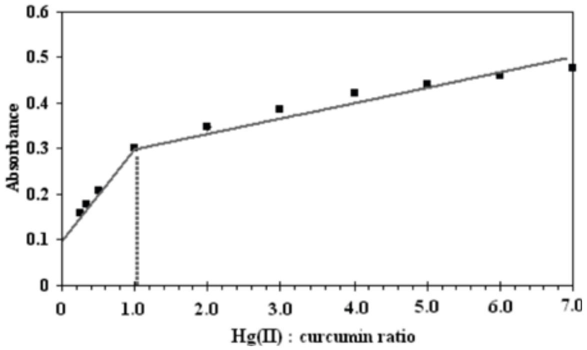

Figure 5. The molar-ratio plot of curcumin-Hg(II) system.

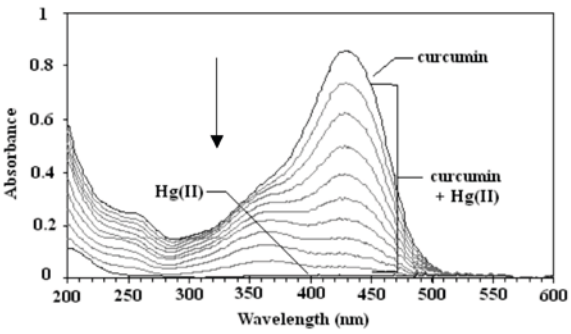

Figure 6. Spectra of the Job's method for curcumin-Hg(II) system with varying curcumin mole fractions: from 1 to 0 (from top to bottom).

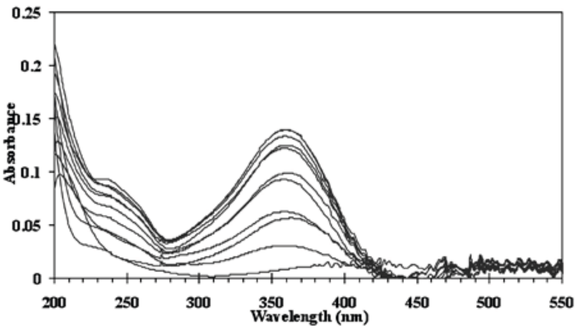

Figure 7. Spectra of the Job's method for the curcumin-Hg(II) system (after subtraction of free curcumin from Fig. 6).

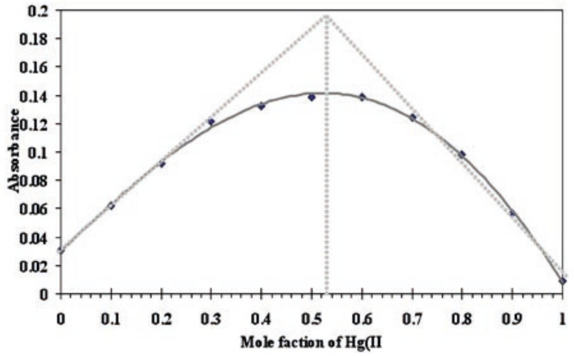

Figure 8. The Job's plot of absorbance at 359 nm versus the mole fraction of Hg(II) ion from Fig. 7.

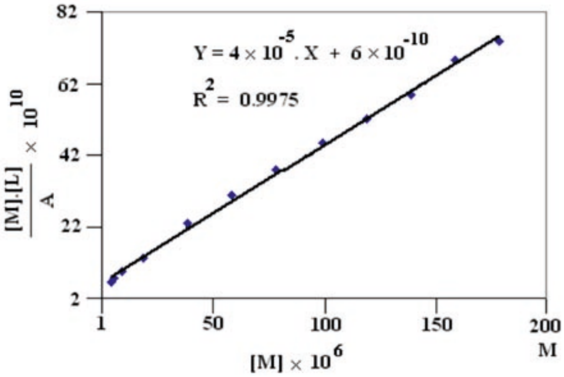

Figure 9. The Benesi-Hildebrand plot for the curcumin-Hg(II) system.

and ε_{ML} = 1 / (slope). A typical Benesi-Hildebrand plot is shown in Fig. 9 and yields log K = 4.83 ± 0.02 and log ε_{ML} = 4.37 ± 0.06.

Up to date, there has been no report on the formation constant of curcumin-Hg(II). Other metal ions that have been reported are Fe(II), Fe(III), and Cu(II). For Fe(II) and Fe(III), using potentiometric method and spectrophotometric method combined with SQUAD software, all of the proposed complexes were of the 1:1 type while for Cu(II), using spectrophotometric method and the LETAGROP-SPEFO software, both 1:1 and 1:2 were possible [10-12].

3.4 Possible structure of curcumin-Hg(II) complex in solution

Curcumin molecule has two possible functional groups that can coordinate to the metal ion: the central β-diketone and the phenolic group (at both ends). To distinguish between the two sites three compounds were used comparatively: acetylacetone, vanillin, ferulic acid. Acetylacetone has the central β-diketone resembles that of curcumin while vanillin and ferulic acid have the side phenolic group like those at both ends of curcumin. The absorption spectra of these three molecules were studied with and without addition of Hg(II). Only acetylacetone showed change in absorption while those of vanillin and ferulic acid remained unchanged upon addition of Hg(II). From this study, it can be concluded that the functional group of curcumin that favors binding to metal ion is the central β-diketone.

Furthermore, upon addition of Hg(II) the conductivity of solution remained very low, close to zero, as did the curcumin solution. This indicates that the complex in solution under studied is a non-electrolyte type.

The stoichiometric ratio of 1 : 1 for curcumin and Hg(II) ion in solution could lead to the proposed structure as in Fig. 10. Being the d^{10} configuration, the complex of Hg(II) ion always exist in the tetrahedral geometry. Curcumin coordinates to Hg(II) using its β-diketone functional group and two chloride ions take the other two positions to complete the coordination number of 4. This proposed structure with the unionized methylene protons, hence disruption of π electron delocalization, also agrees with the absorption shown in Figs. 2 and 6 where the characteristic absorption of complex blue shifted to 359 nm and without any other absorption nearby (*e.g.* the phenolate absorption at ca. 590 nm).

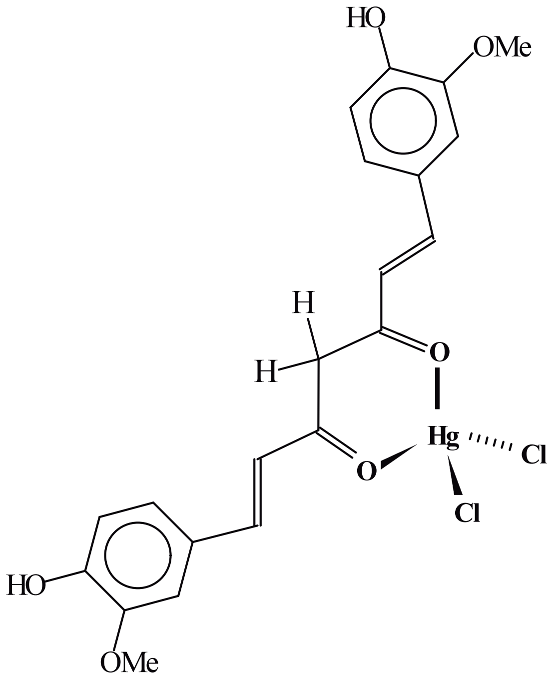

Figure 10. Proposed structure of the curcumin-Hg(II) complex in solution

4. Conclusions

Due to the wide spread use of curcumin as herbal medicine, many researchers have focused their attention to study the interaction of curcumin and metal ions by several methods. When UV-Vis spectrophotometer was used to study this interaction it was found that there was an extensive overlapping between absorptions of the unreacted curcumin and the new complex. This overlapping problem could have hampered the spectrophotometric study, had the spreadsheet method introduced in this article not been used. With this approach, the overlapping peak was removed and a clean spectrum of the complex could be obtained. The spectrum of free curcumin of unknown concentration was obtained by using the simulated calibration curve. The original overlapped spectrum was subtracted numerically with the calculated absorption of the remained free curcumin and the resulting spectrum of the new complex was obtained. Subsequently, the composition of the complex was determined using the mole-ratio and the Job's continuous variation methods. Results from both methods showed that the stoichiometric ratio of Hg(II)-curcumin was 1:1. The complex formation constant then was calculated and the results are given as log K. For Hg(II)-curcumin, the 1:1 complex, the log K from equilibrium concentration method is 4.44 ± 0.16 and 4.83 ± 0.02 from the Benesi-Hildebrand plot.

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