

A study of the IR spectra of copigments formed by malvin chloride with flavones

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Research into the essence of the process of copigmentation of anthocyan molecules was continued by applying IR spectroscopy, in an attempt to elucidate the formation mechanism of copigment molecules between malvin chloride and several variously substituted flavones. Analysis of the IR spectra of the formed copigments revealed that the process of copigmentation is achieved *via* the formation of hydrogen bonds. The strength of the formed hydrogen bonds was correlated with the equilibrium constants of the processes of copigmentation of these molecules. The found correlation was in accordance with the supposed mechanism of the copigmentation reaction.

Keywords: malvin, flavones, copigmentation, IR spectra.

INTRODUCTION

Wilstätter and Zollinger¹ observed in 1916 that the color of isolated anthocyanins can significantly vary in the presence of some substances. In 1931 Robinson² called the effect copigmentation. The process of copigmentation is, in addition to complexing with metals and autoassociation, one of the most significant factors stabilizing the structure, *i.e.*, the coloring of anthocyan molecules in a natural environment. It has already been established that the compounds capable of copigments formation include the flavonoids themselves, alkaloids, organic acids, aminoacids and many other organic compounds present under *in vivo* conditions. The structures of the copigments have not been completely elucidated, but it is believed by many authors³⁻⁷ that molecular bonding proceeds *via* hydrogen bonding.

Since the copigmentation process *in vivo* is still not completely understood, studies associated with it are still of high interest. A detailed study of the copigmentation process under *in vitro* conditions would improve the knowledge of the protection of anthocyan molecules, significant for their applications.

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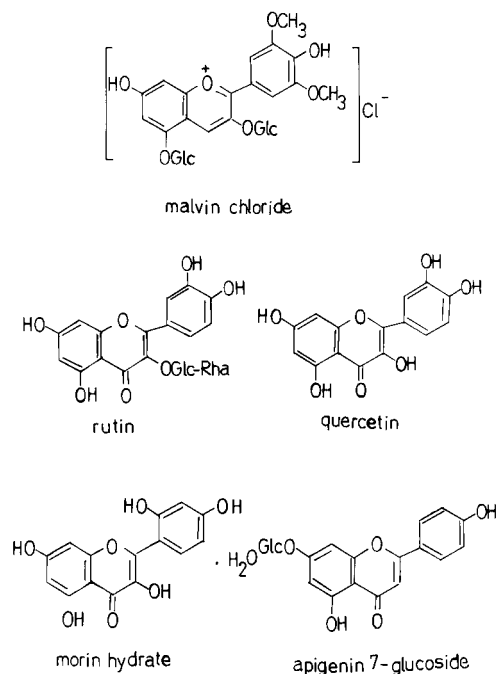


Fig. 1. Structural formulae of the compounds.

The objective of the present work was to utilize IR spectroscopy to obtain an insight into the buildup of the copigment molecules formed between malvin chloride with flavones, and correlate the results with the previously determined equilibrium constants of the processes.^{8–11} According to the literature available to us, this is the first attempt of its kind.

EXPERIMENTAL

Materials

The following substances were used in the experiments: malvin chloride (malvin, in the subsequent text) (97 %, Aldrich Chem. Co), rutin (95 %), quercetin (97 %), morin (98 %) and apigenin 7-glucoside (98 %) (Fluka Biochemika).

Solutions

The copigmentation reaction was conducted in a buffer solution of pH 3.65, which was chosen on the basis of our earlier studies.⁹ The buffer solution of constant ionic strength (0.02 M) was obtained by mixing 0.02 M sodium acetate (p.a. Merck) and 0.06 M phosphoric acid (85 %, BDH Poole-England).⁸ The ionic strength was controlled by adding 0.02 M sodium chloride (p.a., Merck). Mother solution of malvidin 3,5-diglucoside of $c = 2 \times 10^{-3}$ M was prepared in methanol (Uvasol., Merck) and 0.1 % HCl and left to equilibrate in the dark for 1 h. The mother solutions of the flavone compounds, $c = 2 \times 10^{-3}$ M, were prepared in ethanol (Uvasol, Merck). These solutions were diluted to the original concentration by addition of the buffer pH 3.65.

The substances were weighed to a precision of 2×10^{-5} g.

IR spectra

IR spectra were recorded on a Perkin Elmer 983 G IR spectrophotometer. The samples of pure components were recorded using the KBr technique with a ratio of 1 mg: 150 mg (sample: KBr). The pure buffer solution as well as the buffer solutions containing the copigments were lyophilized by standard procedure.¹² The IR spectra of the obtained solids were recorded using the KBr technique, as in the case of the pure substances.

The spectra were recorded under conditions generally applied in quantitative work. The spectra were obtained in the region from 4000–250 cm^{-1} , but the bands analyzed in detail were in the region 4000–2000 cm^{-1} . Since the bands in this region are wide and complex, they were resolved using the Lorentz-Gauss band shapes. After deconvolution, the bands belonging to OH valence stretching vibrations, characteristic to the appearance of hydrogen bonds, were analyzed.

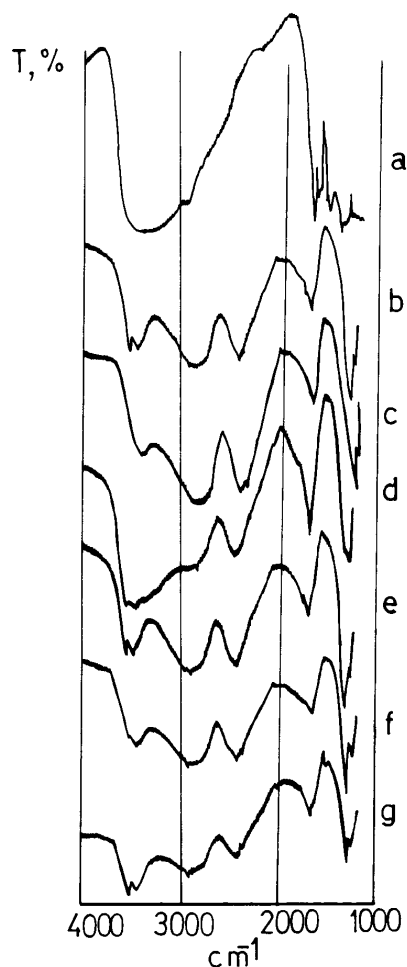


Fig. 2. IR spectra in the region 4000–1000 cm^{-1} of the pure components: a) malvin, b) buffer, c) malvin-buffer and of the copigments: d) malvin-rutin, e) malvin-quercetin, f) malvin-morin, g) malvin-apigenin 7-glucoside.

pH Measurements

The pH of the solutions was measured by an Iskra MA 5730 pH meter with a combined electrode. The standard buffer solution of potassium biphthalate (p.a., Merck) was used for the pH meter calibration.

RESULTS AND DISCUSSION

According to the literature available to us, the results of the present work represent the first confirmation of the assumptions made by many authors that molecular bonding in the copigments proceeds through the formation of hydrogen bonds. As IR spectroscopy is exceptionally suitable for hydrogen bond analysis of such systems, the IR spectra of the pure components, buffer, malvin in buffer solution, and of the copigmentation products were recorded and separately analyzed (Fig. 2a–2g).

IR spectra were recorded in the region from $4000\text{--}250\text{ cm}^{-1}$, but the bands in the region $4000\text{--}2000\text{ cm}^{-1}$ were analyzed in detail, since they are characteristic to OH groups of various protonic species that undergo hydrogen bonding interaction. Another region of interest was also the region from $1800\text{--}1600\text{ cm}^{-1}$, characteristic to the bending vibrations of the same group.

The analysis of the above region revealed that the malvin molecule itself forms intramolecular hydrogen bonds. It is evident from Fig. 2a, which shows the IR spectrum of malvin, that a strong band appears at 3380 cm^{-1} , characteristic to a hydrogen bond, according to its structure and position. The IR spectrum of the acetate buffer made of sodium acetate and phosphoric acid is presented in Fig. 2b. In this system too, strong wide bands characteristic to hydrogen bond formation appear in the region $4000\text{--}2000\text{ cm}^{-1}$. The IR spectrum of malvin in the acetate buffer at pH 3.65 is presented in Fig. 2c. The bands in this region of this system (Fig. 2c) show certain differences in comparison to pure malvin (Fig. 2a) and the buffer alone (Fig. 2b). The general shape of the spectrum, as well as of the position, intensity and half widths of the characteristic bands are changed. The greatest change is that bands below 3000 cm^{-1} , characteristic to the oxonium ion, are observed in the spectrum of the malvin-buffer system (Fig. 2c). This indicates that the buffer, besides adjusting the pH value of the medium, also protonizes the malvin, and "prepares" it for the process of copigmentation. In the further text this is referred to as the reference system, serving as a reference for observing the changes in the spectra of the formed copigments.

All the bands observed are complex, which indicates the presence of several types of hydrogen bonds, differently formed: H–O --- H bonds (bands around 3500 cm^{-1}), and bonds formed *via* H_3O^+ groups. Since such a band structure indicates a superposition of several bands, direct deconvolution was carried out (some of the deconvoluted spectra are presented in Fig. 3) in order to precisely determine the positions of the individual bands (ν) and their characteristic parameters: $\text{R}_{\text{O}\cdots\text{O}}$ distances¹³ (the length of the hydrogen bond, taken as a measure of the bond strength), and half widths of the bands ($\nu_{1/2}$, cm^{-1}). The values of these parameters obtained in the systems under study are given in Table I.

In the deconvoluted spectra, two groups of bands significant for the structures of the molecules in the interaction, malvin and the investigated flavones, are observed. These are the bands appearing in the region around 3500 cm^{-1} , characteristic to the formation of hydrogen bonds *via* terminal OH groups, and bands appearing below 3000 cm^{-1} , characteristic to the oxonium molecular structure. Further analysis of the deconvoluted spectra revealed that the above band groups do not show the same behavior, indicating that the hydrogen bonds formed are not of the same strength, and involve different positions in the molecules. Only the bands that underwent the largest shift towards lower wavenumbers, relative to the reference system, were taken as a criterion for the strength and the position involved in hydrogen bonding. The values of these band shifts, taken as a quantitative measure of the hydrogen bond strengths, could be correlated with the previously calculated values of the equilibrium constants of the processes,^{8–11} *i.e.*, with the stability of the copigments formed.

The first analysis of the IR spectra of the copigments formed showed that the bands around 3500 cm^{-1} are grouped in two ways: in the copigments malvin-quercetin (Fig. 2e) and malvin-apigenin 7-glucoside (Fig. 2g) the bands are grouped similarly as in the buffer (Fig. 2b), while in the systems malvin-buffer (Fig. 2c) and malvin-morin (Fig. 2f) this band, at around 3535 cm^{-1} , is very weak, while the band at around 3439 cm^{-1} is much stronger. The copigment malvin-rutin (Fig. 2d) could be observed as an intermediate compound, producing a band at around 3200 cm^{-1} . The same behavior was observed in the case of the bands around 3500 cm^{-1} after deconvolution (Fig. 3, Table I). In all the copigments formed these bands undergo a shift to higher wavenumbers, indicating a weakening of the hydrogen bonds with respect to the reference system. The magnitude of the shift is approximately the same in the systems malvin-rutin, malvin-quercetin and malvin-morin (about 34 cm^{-1} , Table I). Since some of these molecules contain sugar molecules and some do not (see structural formulae), and all of these systems show the same degree of weakening of the hydrogen bonds, *i.e.*, the same $\Delta\nu$ shift, it can be assumed that these hydrogen bonds are most likely formed only by those free OH groups that do not belong to sugar molecules. This is also supported by the two times smaller shift of the same band in the system malvin-apigenin 7-glucoside, as a consequence of the reduced number of free OH groups in that molecule (see Fig. 1). All of these facts indicate that the bonds in question are not cardinal in the formation of the copigments, and that they, as such, can not be correlated with the equilibrium constant of the process. The fact that really weak hydrogen bonds are involved is supported by the calculated values of the $R_{O\cdots O}$ distances, which are in agreement with the values of the shifts of the same band in all the systems under study ($R_{O\cdots O} > 2.70\text{ Å}$, Table I). Namely, according to the criterion for the hydrogen bond strength¹³, a bond with an $R_{O\cdots O}$ distance greater than or equal to 2.70 Å can be considered weak, whereas one with the same distance smaller than or equal to 2.60 Å can be considered strong.

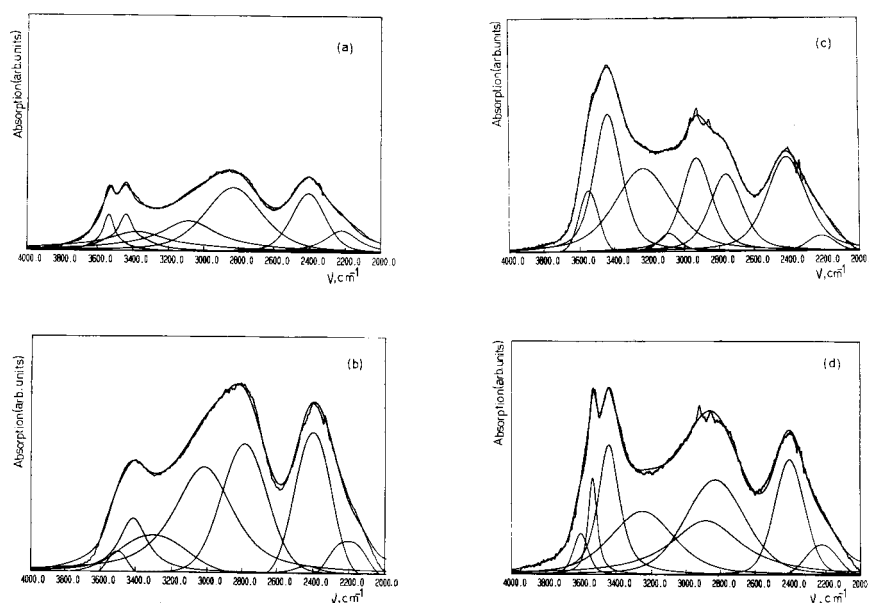


Fig. 3. IR spectra after deconvolution: a) pure buffer, b) malvin-buffer and the copigments, c) malvin-rutin, d) malvin-apigenin 7-glucoside.

The appearances of band groups below 3000 cm^{-1} , at around 2800 cm^{-1} , and around 2400 cm^{-1} indicate the presence of strong hydrogen bonds formed *via* the oxonium groups of the copigments. The proof of the existence of oxonium ions can be found in the region of bending vibrations, bands around 1730 and 1780 cm^{-1} , which can be attributed to the ν_4 vibration of the H_3O^+ ion. It is evident from the IR spectra of this region that the band at around 2800 cm^{-1} , which can be assigned to vibration ν_2 of the H_3O^+ ion, changes most in the spectra of the copigments, *i.e.* the corresponding flavones have the greatest effect on it (Fig. 2d–2g). Its intensity is the greatest in the reference system, malvin in the buffer (Fig. 2c), and it falls when the copigments are formed. From the spectra of this range obtained after deconvolution (Fig. 3, Table I) it is evident that the band, which appears at 2760 cm^{-1} in the copigment malvin-rutin, undergoes the greatest shift towards lower wavenumbers relative to the reference system (see Table I). In the copigment malvin-quercetin the band at 2793 cm^{-1} undergoes a shift that also indicates a strengthening of the hydrogen bond, but the value of the shift, $\Delta\nu$, is significantly lower. The greatest value of the shift of the band at 2760 cm^{-1} in copigment malvin-rutin indicates the formation of the strongest hydrogen bond, which can be considered responsible for the formation of this copigment. This is also supported by the fact that the greatest value of the equilibrium constant is obtained for the process of copigmentation of malvin and rutin ($K = 3300$),⁸ *i.e.*, the greatest stability is obtained in this case. In the copigments malvin-morin and malvin-apigenin 7-glucoside, this band, at around 2800 cm^{-1} , shifts towards higher wavenumbers

relative to the reference system, *i.e.*, the hydrogen bond is weaker, and can be considered not responsible for the formation of these copigments. The value of the $R_{O\cdots O}$ distance of the band at 2800 cm^{-1} is, in all the formed copigments, below or equal to 2.60 Å (see Table I), which indicates that the hydrogen bonds formed *via* the oxonium structure are strong.

The band at around 2400 cm^{-1} undergoes a shift towards lower wavenumbers in the systems malvin-quercetin, malvin-morin, and malvin-apigenin 7-glucoside. This indicates that the hydrogen bond in these copigments is stronger than in the reference system, and can, as such, be considered responsible for the formation of these copigments. The magnitude of the shift of this band is consistent with the value of the calculated equilibrium constant for the process of copigmentation of malvin and the flavones under study. In the copigment malvin-morin, the formation of the hydrogen bond is observed through the band at 2384 cm^{-1} . The somewhat smaller shift of this band ($\Delta\nu = -24\text{ cm}^{-1}$) than the shift of the corresponding band in the copigment malvin-rutin ($\Delta\nu = -35\text{ cm}^{-1}$) is consistent with the lower equilibrium constant of the process ($K = 2300$)¹⁰ and the lower stability of this copigment than that of the copigment malvin-rutin. In the case of the copigment malvin-quercetin, the hydrogen bond is achieved *via* the oxonium structure of the molecule, and corresponds to the band at 2392 cm^{-1} . The smaller shift of this band ($\Delta\nu = -16\text{ cm}^{-1}$) compared to the corresponding bands in the copigments malvin-rutin and malvin-morin indicates a lower stability of this copigment, resulting in a lower value for the equilibrium constant of the process ($K = 650$).⁹ The smallest shift of the bands at 2405 cm^{-1} ($\Delta\nu = -3\text{ cm}^{-1}$), used to observe the formation of the hydrogen bond in the copigment malvin-apigenin 7-glucoside, is consistent, also in this system, with the value of the equilibrium constant of the process ($K = 137$),¹¹ which is the lowest among the systems studied. The same band, at around 2400 cm^{-1} , in the system malvin-rutin shifts towards higher wavenumbers, indicating a weaker hydrogen bond than in the reference system, this could, as such, be considered irrelevant in the copigment formation. The $R_{O\cdots O}$ values are below 2.60 Å for the band at 2400 cm^{-1} , in all the systems under study (see Table I), which is also a proof of the formation of strong hydrogen bonds.

CONCLUSION

From the IR spectra of the compounds under study (Fig. 2a–2g), the presence of two groups of hydrogen bonds was found. The bonds are formed *via* different structures in the interacting molecules: free hydroxyl groups (bands at around 3500 cm^{-1}) and the oxonium ion (bands below 3000 cm^{-1}). From the magnitude of the shift of the bands it was concluded that the hydrogen bonds formed are neither equal with respect to their strength, nor with respect to the positions in the molecules that take part in the bond.

It was established that in the malvin-rutin system a strong hydrogen bond, responsible for copigment formation, is formed *via* the oxonium structure of the

molecule, and corresponds to the band at around 2800 cm^{-1} , whereas in systems malvin-morin, malvin-quercetin, and malvin-apigenin 7-glucoside it is formed *via* the oxonium structure that corresponds to the band at around 2400 cm^{-1} . This was confirmed by the values of the shifts of the relevant bands that are, in all the systems under study, in agreement with the values of the earlier obtained equilibrium constants of the process of copigmentation of malvin with the corresponding flavones.

The values of the R_{O-O} distance accompanying the shifts were further proof of the strength of hydrogen bonds in the systems under study.

ИЗВОД

ПРОУЧАВАЊЕ ИС СПЕКТРА КОПИГМЕНАТА МАЛВИН-ХЛОРИДА И ФЛАВОНА

УБАВКА Б. МИОЧ, ЈАСМИНА М. ДИМИТРИЋ МАРКОВИЋ и ЈАЛИСАВЕТА М. БАРАНАЦ*

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Проучавање суштине процеса копиментације антоцијанских молекула настањено је, применом ИС спектроскопије, у покушају разјашњења механизма настајања молекула копигмената између малвин-хлорида и неколицине различито супституисаних флавоана. Анализом ИС спектра награђених копигмената констатовано је да се процес копиментације остварује механизмом водоничних веза. Јачина награђених водоничних веза корелирана је са константом равнотеже процеса копиментације испитиваних молекула. Нађена корелација била је у сагласности са претпостављеним механизмом реакције копиментације.

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