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Methoxychalcones and methoxyflavanones in analytical studies

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Wide application of extracts and raw plant materials places high requirements on quality of these materials. The aim of the study was to describe problems with obtaining and determination of chalcones and flavanones common in plants. The results may be used for creation of a method for quality assessment of plant drugs containing flavonoid compounds.

1. Introduction

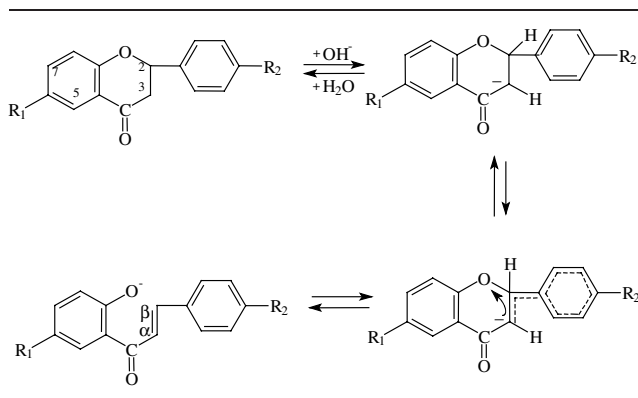
Plant raw materials containing flavonoids are extracted in an alkaline environment by solutions of sodium hydroxide or potassium hydroxide of increasing concentration. In order to optimize experimental conditions and avoid side reactions such as decomposition of initial substrates, the influence of environment on these compounds must be known. One of common processes which take place in the environment is isomerization, i.e. reversible transformation of flavanones into chalcones. Knowledge of this fact enables appropriate management of the reaction course.

In weakly alkaline environment two mechanisms of isomerization have been suggested: E1 cB [1–3] and E2 [4, 5], both discussed by Bulton and Taylor [6]. In E1 cB mechanism the monoanionic form and 2'-hydroxychalcone isomerises to the enolate form of the flavanone, which in water solution transforms into closed form of the flavanone, in protonation constant similar to enolen – (pK about 10).

In E2 mechanism the effect of acid, i.e. water on the ionized form of the particular hydroxychalcone caused rotation around the bond CO–C_α and gradual elimination of chalcone with simultaneous production of flavanone, and the ultimate reaction rate depends on the final process of closing of the chromanone ring.

In strongly alkaline environment of pH above 11 another anion mechanism E1 comes into play for the reaction of opening the flavanones ring, or pseudo-acidic (carbonic) one, from the position of chalcones cyclic transformation [7, 8]. This mechanism has been confirmed on the system chalcone-flavone with free hydroxyl groups (see Scheme). In the environment complex systems are present, thus the range of the studies has been extended to comprise flavanones and chalcones with methoxyl substituent. In the suggested mechanisms an intermediate form exists. Information of its structure was obtained by indirect methods,

Scheme



including numeric methods of quantum chemistry [9]. So far quantum chemistry methods have not been employed for the studies on isomerization process, though ab initio this method was used to determine the dependencies between flavanones and flavylium ion [10].

The study comprised 2'-hydroxy-5'-methoxychalcone (I) and its isomer 6-methoxyflavanone (II) and 2'-hydroxy-4-methoxychalcone (III) and its isomer 4'-methoxyflavanone (IV).

2. Investigations, results and discussion

The protonation constant for 2'-hydroxy-5'-methoxychalcone was determined in solutions water-dioxan (9:1) log K = 8.95 [1]. By a standard spectrophotometric method [11, 12] the protonation constant was determined as log K = 9.55 ± 0.05 for water solutions of 2'-hydroxy-4-methoxychalcone in a concentration of 5 · 10⁻⁶ mol/l with about 4% v/v of methanol. The protonation constant cannot be determined by a potentiometric method due to the instability of the monoanionic form in weakly alkaline solutions and the low solubility of the neutral form. Also, the Krebs and Speakman method [12] cannot be used to determine the protonation constant due to an increasing rate of isomerization of chalcone to flavanone and a long time of solution saturation. In the pseudoacidic mechanism the key role is played by solubility of all forms present in particular concentration ranges. Solubility of particular isomeric forms with the composition of sediment in saturated solutions is presented in Table 1. Based on the experimental data obtained, the following solubility values were adopted for particular forms: S_{6MFI} = 1.0 · 10⁻⁵ mol/l, S_{5'MChO⁻} = 2 · 10⁻⁴ mol/l, S_{4MFI} = 3 · 10⁻⁵ mol/l, S_{4MChO⁻} = 6.5 · 10⁻³ mol/l. The solubility of neutral forms of chalcones was determined in water saturated solutions of these compounds, and are as follows: S_{5'MChOH} = 1.0 · 10⁻⁶ mol/l and S_{4MChOH} = 2 · 10⁻⁶ mol/l.

The solubility of chalcones and flavanones studied was known, and thus appropriate experimental conditions were determined according to the isomerisation equilibrium in non-saturated solutions. In the course of the study we have noted that the rate of cyclic reaction significantly increased in strongly alkaline solution at pH values of about 14, having reached a plateau within pH 10–12.

In strongly alkaline solutions irrespective of the substrate, flavanone or chalcone, the equilibrium condition is established, between a flavanone and appropriate isomeric chalcone. The composition of isomers: anion 2'-hydroxy-4-methoxychalcone (4MChO⁻) and 4'-methoxyflavanone (4'MFI) and anion 2'-hydroxy-5'-methoxychalcone (5'MChO⁻) and 6-methoxyflavanone (6MFI) can be described by the Henderson equation used for acid-base bal-

Table 1: Composition of solution and sediment in saturated solutions of various pH values

Substrate 4'-methoxyflavanone (4'MFI)

| pH | Composition of solution | | Composition of sediment | Calculated value $pH_{1/2}^S$ |
|-------|--|--------------------------------|--------------------------------------|-------------------------------|
| | Concentration of 4 MChO ⁻ (mol/l) | Concentration of 4'MFI (mol/l) | | |
| 2.40 | — | $2.8 \cdot 10^{-5}$ | 4'MFI | — |
| 9.18 | — | $3.0 \cdot 10^{-5}$ | 4'MFI | — |
| 12.90 | $1.9 \cdot 10^{-3}$ | $3.0 \cdot 10^{-5}$ | 4'MFI | 11.10 |
| 13.24 | $3.9 \cdot 10^{-3}$ | $3.0 \cdot 10^{-5}$ | 4'MFI | 11.13 |
| 13.56 | $6.0 \cdot 10^{-3}$ | — | 4 MChO ⁻ 94% and 4'MFI 6% | 11.22 |
| 14.00 | $6.5 \cdot 10^{-3}$ | — | 4 MChO ⁻ | — |
| 14.30 | — | — | 4 MChO ⁻ | — |

$pH_{1/2}^S = 11.1 \pm 0.1$

Substrate 6-methoxyflavanone (6 MFI)

| pH | Composition of solution | | Composition of sediment | Calculated value $pH_{1/2}^S$ |
|-------|--|--------------------------------|--|-------------------------------|
| | Concentration of 5'MChO ⁻ (mol/l) | Concentration of 6 MFI (mol/l) | | |
| 2.40 | — | $0.8 \cdot 10^{-5}$ | 6 MFI | — |
| 9.00 | — | $1.0 \cdot 10^{-5}$ | 6 MFI | — |
| 10.03 | — | $1.2 \cdot 10^{-5}$ | 6 MFI | — |
| 11.56 | $1.0 \cdot 10^{-5}$ | $1.0 \cdot 10^{-5}$ | 6 MFI | 11.56 |
| 11.72 | $1.2 \cdot 10^{-5}$ | $1.2 \cdot 10^{-5}$ | 6 MFI | 11.79 |
| 12.00 | $2.5 \cdot 10^{-5}$ | $1.2 \cdot 10^{-5}$ | 6 MFI | 11.68 |
| 13.50 | — | — | 6 MFI | — |
| 14.00 | $3.2 \cdot 10^{-4}$ | — | 5'MChO ⁻ 30% oraz 6 MFI 70% | — |
| 14.50 | $2.0 \cdot 10^{-4}$ | — | 5'MChO ⁻ | — |

$pH_{1/2}^S = 11.7 \pm 0.1$

ance calculations. In this equation the acid is methoxyflavanone and the base is an appropriate anion of the isomeric chalcone. The value of $pH_{1/2}$ means reaction rate constant, though here it does not concern isomerization and determines the pH at which in equilibrium state there is 50% of flavanone and 50% of isomeric chalcone: $pH = pH_{1/2} - \log [4'MFI] + \log [4MChO^-]$; $pH =$

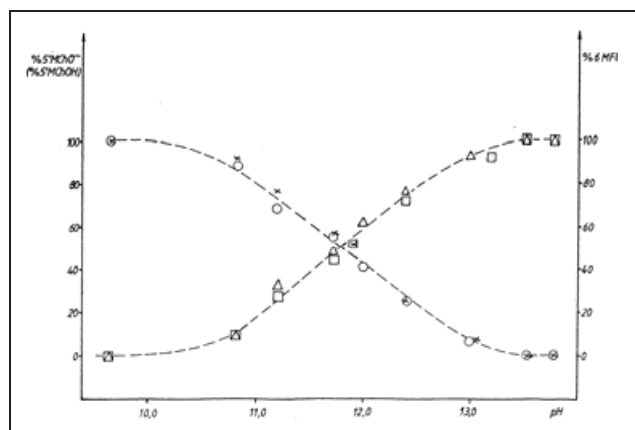


Fig.: Relative composition of the system 5'MChO⁻/6MFI (5'MChOH/6MFI) in non-saturated solutions in the state of final equilibrium as a function of pH, where:
 Δ - % of 5'MChO⁻
 ○ - % of 6 MFI in alkaline environment
 × - % of 5'MChOH
 □ - % of 6 MFI after "freezing" of final equilibrium

Table 2: Molar fractions 4'MFI and 4MChO⁻ calculated by spectrophotometry and $pH_{1/2}$ determined by spectrophotometry in alkaline environment in equilibrium state in non-saturated solutions at various pH values

Substrate 4 MChOH

| pH | 4'MFI | 4MChO ⁻ | $pH_{1/2}$ |
|-------|-------|--------------------|------------|
| 10.56 | 0.71 | 0.29 | 10.95 |
| 10.82 | 0.50 | 0.50 | 10.82 |
| 11.12 | 0.33 | 0.67 | 10.81 |
| 11.76 | 0.08 | 0.92 | 10.70 |

$pH_{1/2} = 10.8 \pm 0.1$

Substrate 4'MFI

| pH | 4'MFI | 4MChO ⁻ | $pH_{1/2}$ |
|-------|-------|--------------------|------------|
| 10.56 | 0.70 | 0.30 | 10.93 |
| 10.82 | 0.50 | 0.50 | 10.82 |
| 11.10 | 0.33 | 0.67 | 10.81 |
| 11.71 | 0.08 | 0.92 | 10.70 |

$pH_{1/2} = 10.8 \pm 0.1$

$pH_{1/2} - \log [4'MFI] + \log [5'MChO^-]$. The results have been presented in the Fig. and Table 2.

„Freezing” of the equimolar state by acidising the solution (previously alkalinized) to about $pH = 1$, where isomerization is extremely slow, allows for examining the composition of the mixture containing neutral isomeric forms in the function of pH and calculation of $pH_{1/2}$ value from the Henderson equation for neutral forms of flavanone and chalcone. The results of these calculations are presented in Table 3. Mean value of $pH_{1/2}$ for the system 2',5'-methoxychalcone/6-methoxyflavanone is 11.7 and for the system 2',4'-methoxychalcone/4'-methoxyflavanone 10.8. However, $pH_{1/2}$ obtained for the system 2',4'-methoxychalcone/4'-methoxyflavanone indicates a tendency to decrease with increasing alkaline solutions which probably is a result of small distance between $pH_{1/2}$ and $\log K = 9.55$.

The value of $pH_{1/2}^S$, i.e. $pH_{1/2}$ was calculated from the solubility of chalcone salts determined at known pH values, even for concentrated solutions, from the modified equation:

$$pH_{1/2}^S = pH - \log c_{MChO^-} + \log S_{FI}$$

Table 3: Composition of mixture obtained by "freezing" the equimolar state obtained at various pH values and calculated $pH_{1/2}$ values

Substrate 4 MChOH

| pH | 4'MFI | 4MChOH | $pH_{1/2}$ |
|-------|-------|--------|------------|
| 10.82 | 0.63 | 0.37 | 11.05 |
| 11.12 | 0.56 | 0.44 | 11.22 |
| 11.76 | 0.46 | 0.54 | 11.69 |

$pH_{1/2} = 11.3 \pm 0.1$

Substrate is 4'MFI

| pH | 4'MFI | 4MChOH | $pH_{1/2}$ |
|-------|-------|--------|------------|
| 10.82 | 0.63 | 0.37 | 11.05 |
| 11.10 | 0.57 | 0.43 | 11.22 |
| 11.71 | 0.48 | 0.52 | 11.67 |

$pH_{1/2} = 11.3 \pm 0.1$

where $S_{6\text{MFI}}$ is $1.0 \cdot 10^{-5}$ mol/l for $5'\text{MChO}^-$ and $S_{4\text{MFI}}$ is $3.0 \cdot 10^{-5}$ mol/l for 4MChO^- (with flavanone present ion the solid phase). The results are presented in Table 1.

The structure of all compounds undergoing isomerization were optimized geometrically by the AM1 method to determine the value of the following parameters: binding energy, dipole moment, heat of formation in energetic minimum. The assumption was that the rationale of this reaction is drive of the matter to reach thermodynamic stability, and internal stability of compounds manifests as low energy of bonds and lack of polarization of a molecule [13–15].

The determined values of parameters are presented in Table 4.

According to earlier studies [1, 3, 7, 8] the rate of cyclisation reaches a plateau in the weakly alkaline range of pH 10 to 12, which points to two mechanisms operating in this pH range. In a strong alkaline environment another mechanism is dominant, where a characteristic measurable value of $\text{pH}_{1/2}$ can be seen corresponding to a logarithm of isomerization constant in the system flavanone-chalcone. In this case, a stable isomerization equilibrium may occur only between forms of various degree of ionization connected with detaching of a proton in alkaline environment:

Table 4: The value of binding energy, dipole moment, heat of formation and electric charges on carbon C-2 and C-3 of expected structures of compounds in the mechanism of isomerization calculated by a semi-empirical quantum AM1 method

Flavanone derivatives

| Parameters | 6MFI | Carboanion with negative charges at C-2 6MFI | Carboanion with negative charges at C-3 6MFI | 4'MFI | Carboanion with negative charges at C-2 4'MFI | Carboanion with negative charges at C-3 4'MFI |
|--------------------------------|----------|--|--|----------|---|---|
| Binding energy (kcal/mol) | -3696.29 | -3659.88 | -3660.94 | -3697.94 | -3661.61 | -3661.38 |
| Dipol moment D | 3.09 | 6.65 | 6.22 | 2.40 | 3.65 | 9.41 |
| Heat of formation (kcal/mol) | -53.94 | -69.64 | -70.70 | -55.60 | -71.37 | -71.14 |
| Log P | 2.59 | 2.77 | 2.87 | 2.59 | 2.77 | 2.87 |
| Electric charges on carbon C-2 | 0.080 | -0.379 | 0.193 | 0.085 | -0.361 | 0.199 |
| Electric charges on carbon C-3 | -0.239 | -0.085 | -0.603 | -0.240 | -0.090 | -0.618 |

Chalcone derivates

| Parameters Isomers* -5'MChOH | trans-s-trans | trans-s-cis | cis-s-cis | cis-s-trans |
|---------------------------------|---------------|-------------|-----------|-------------|
| Binding energy (kcal/mol) | -3687.76 | -3685.65 | -3622.99 | -3625.26 |
| Dipol moment D | 2.31 | 3.30 | 5.59 | 3.16 |
| Heat of formation (kcal/mol) | -45.42 | -43.30 | 19.35 | 17.08 |
| Log P | 3.14 | 3.14 | 3.14 | 3.14 |
| Refractivity | 75.03 | 75.03 | 75.03 | 75.03 |
| Polarizability | 28.60 | 28.60 | 28.60 | 28.60 |
| Hydratation energy (kcal/mol) | -8.53 | -9.04 | -8.04 | -9.73 |
| Surface area (approx) | 433.72 | 441.18 | 427.71 | 407.15 |
| Surface area (Grid) | 480.58 | 481.82 | 490.70 | 481.46 |
| Volume | 781.85 | 782.35 | 787.71 | 779.80 |

Chalcone derivates (cont.)

| Parameters Isomers*** -4MChOH | trans-s-trans | trans-s-cis | cis-s-cis | cis-s-trans |
|----------------------------------|---------------|-------------|-----------|-------------|
| Binding energy (kcal/mol) | -3689.04 | -3687.31 | -3624.85 | -3626.49 |
| Dipol moment D | 4.68 | 4.01 | 4.32 | 3.05 |
| Heat of formation (kcal/mol) | -46.70 | -44.97 | 17.49 | 15.85 |
| Log P | 3.14 | 3.14 | 3.14 | 3.14 |
| Refractivity | 75.03 | 75.03 | 75.03 | 75.03 |
| Polarizability | 28.60 | 28.60 | 28.60 | 28.60 |
| Hydratation energy (kcal/mol) | -8.46 | -9.10 | -7.29 | -9.71 |
| Surface area (approx) | 435.48 | 436.92 | 418.08 | 408.40 |
| Surface area (Grid) | 478.68 | 481.82 | 483.77 | 484.09 |
| Volume | 781.11 | 781.69 | 784.55 | 781.99 |

Chalcone derivates (cont.)

| Parameters Isomers** -5'MChO ⁻ | trans-s-trans | trans-s-cis | cis-s-cis | cis-s-trans |
|--|---------------|-------------|-----------|-------------|
| Binding energy (kcal/mol) | -3672.39 | -3663.54 | -3655.74 | -3655.74 |
| Dipol moment D | 6.15 | 11.02 | 3.87 | 3.88 |
| Heat of formation (kcal/mol) | -82.15 | -73.29 | -65.50 | -65.50 |
| Log P | 4.56 | 4.56 | 4.56 | 4.56 |
| Refractivity | 74.70 | 74.70 | 74.70 | 74.70 |
| Polarizability | 28.15 | 28.15 | 28.15 | 28.15 |
| Hydratation energy (kcal/mol) | -6.41 | -6.46 | -5.51 | -5.57 |
| Surface area (approx) | 438.80 | 448.43 | 393.15 | 398.21 |
| Surface area (Grid) | 476.13 | 476.65 | 483.73 | 478.01 |
| Volume | 772.75 | 773.87 | 768.01 | 768.25 |

Chalcone derivates (cont.)

| Parameters Isomers*** -4MChO ⁻ | trans-s-trans | trans-s-cis | cis-s-cis | cis-s-trans |
|--|---------------|-------------|-----------|-------------|
| Binding energy (kcal/mol) | -3673.26 | -3664.89 | -3655.51 | -3655.92 |
| Dipol moment D | 11.48 | 17.09 | 7.00 | 7.97 |
| Heat of formation (kcal/mol) | -83.02 | -74.65 | -65.26 | -65.68 |
| Log P | 4.56 | 4.56 | 4.56 | 4.56 |
| Refractivity | 74.70 | 74.70 | 74.70 | 74.70 |
| Polarizability | 28.15 | 28.15 | 28.15 | 28.15 |
| Hydratation energy (kcal/mol) | -6.32 | -6.50 | -5.43 | -5.41 |
| Surface area (approx) | 441.37 | 445.76 | 391.69 | 391.27 |
| Surface area (Grid) | 475.19 | 475.44 | 478.62 | 475.24 |
| Volume | 772.36 | 774.74 | 767.68 | 765.14 |

flavanone + OH⁻ ⇌ chalconate + H₂O. Thus, these isomerization equilibria are a result of acid-base reaction, where flavanone is a carbonic acid (pseudoacid) and corresponding chalcone is its conjugated base. As a result of this reaction a transient form appears: carbanion of flavanone, and p*H*_{1/2} value corresponds to the logarithm of carbanion protonation constant. The results of calculations done by a semi-empirical method suggest that in case of 6-methoxyflavanone the carbanion with negative load at C₃ is more stable than with load at C₂ (Table 4), while carbanions of 4'-methoxyflavanone do not differ significantly in the binding energy or heat of formation. The studied methoxyflavanones are characterized by a small dipole moment of carbanions with a negative load at C₂, but the highest negative load is present at C₃ (Table 4). Studies of exchange of a proton to deuterium by NMR showed that both during opening of a flavanone and during cyclization only protons at C₃ in flavanone molecule are mobile, i.e. acidic. The so-called methane proton (at C₂) is not exchanged even in 40% deuterated sodium hydroxide [7]. Negative load at carbon C₃ (of created carbanion of flavanone) moves towards carbon C₂ (carbon β in chalcones), then to ether oxygen, which causes its reduction to a phenolate anion with disruption of the double bond α and β, i.e. the formation of a chalconate. This reaction is reversible by nucleophilic attack of phenolate oxygen, which acts as a base on the double bond in the chalcone, reducing it to a carbanion, thus leading to the formation of a chalcone. Although it is known that the calculation does not reproduce absolute values relative stabilities are shown well. Calculations show an important difference between the energies of the *s*-trans and *s*-cis conformers. Unfortunately, the NMR method is not applicable to 2'-hydroxychalcones because it is based on the solvent-induced shifts of protons α and β, which in the spectra of these compounds appear as a complex signal (δ 7.5–8.3) in the aromatic proton zone. The results of calculations done by the semi-empirical method suggest that the *trans-s*-*trans* chalconate form seems to be more stable than others (Table 4). The following theoretical results by method AM1 (without using solvents) have been helpful in elucidating the structural changes that occur in the isomerization process and give support to the proposed initial conformation.

Another interesting fact is that in alkaline environment pure flavanone undergoes sedimentation (Table 1). The analysis of solubility of isomers shows that similarly to the system flavanone/2'-hydroxychalcone [7, 8], the reason of chalcone sedimentation is its poor solubility in combination with the good solubility of the chalconate. If this is the case, at some specific pH values three phases should be present: solid flavanone, solid chalconate salt and a saturated solution of all the three components.

This is the specific triple point p*H*_t depending on the solubility of a particular chalcone. Mathematical description of these dependencies allows a quantitative prognosis of the position of equimolarity, which was used in practice for obtaining isomeric chalcones. For p*H* > p*H*_T the following dependency holds true:

$$pH_T = pK_{FL} - pC_{ChO^-} \quad \text{where} \quad pK_{Fl} = pH_{1/2} + pS_{Fl}$$

For 5'MChOH $pK_{FL} = 11.7 + (-\log 1.0 \cdot 10^{-5}) = 16.7$

$pH_T = 16.7 - (-\log 2.0 \cdot 10^{-4}) = 13.0$

For 4MChOH $pK_{Fl} = 10.8 + (-\log 3.0 \cdot 10^{-5}) = 15.3$,
 $pH_T = 15.3 - (-\log 6.5 \cdot 10^{-3}) = 13$.

The calculated values of triple points 13.0 for the system 2'-hydroxy-5'-methoxychalconate anion and 6-methoxyflavanone and 13.1 for the system 2'-hydroxy-4-methoxychalconate anion and 4'-methoxyflavanone show that the chalconate should sediment at p*H* > p*H*_T i.e. at p*H* > 13, which is consistent with experimental data (Table 1). Pure chalcone was obtained by the so called "semi-dry" method by grinding the solid flavanone (1 mmol) with a solution of sodium hydroxide (20 mmol) with small amounts of methanol (1–2 ml), and then acidising with solid sodium hydrosulphate.

The efficiency of the chalcones obtained was not studied and a strong alkaline environment ensured complete transition of flavanone into chalcone. The greatest problem is obtaining a neutral chalcone by acidifying the reaction environment to a pH close to neutral with the possibility of closing chalcone to flavanone in the course of gradual neutralizing or formation of decomposition products in an alkaline environment (benzaldehyde and acetophenone). Theoretical calculations show that the most stable flavanone is the molecule (binding energy), and that the carbanion with negative load at carbon C₃ is only an intermediate form leading to the more stable anion form of chalcone. Confirmation of the isomerization mechanism demonstrated in analytical studies suggests that methods of quantum mechanics may be useful in studies on these compounds.

3. Experimental

6-Methoxyflavanone and 4'-methoxyflavanone were bought from Sigma (St. Louis, MO, USA). Isomeric chalcones: 2'-hydroxy-5'-methoxychalcone were obtained from 6-methoxyflavanone and 2'-hydroxy-4-methoxychalcone from 4'-methoxyflavanone according to an own method developed on the basis of results from this study. The purity of compounds was confirmed by UV/VIS and NMR, and measurements of melting point.

Buffer solutions were prepared from reagents from Polskie Odczynniki Chemiczne (Gliwice).

Stability of the compounds was measured in non-saturated buffer solutions prepared according to standards [11, 16] in a concentration of $5 \cdot 10^{-6}$ mol/l with about 4% v/v of methanol at a pH between 1–10 and $4 \cdot 10^{-5}$ mol/l with about 4% v/v of methanol for 2'-hydroxy-4-methoxychalcone and for 2'-hydroxy-5-methoxychalcone, 6-methoxyflavanone and 4'-methoxyflavanone of $4 \cdot 10^{-5}$ mol/l with about 4% v/v of methanol.

The solubility determinations necessary in the Krebs and Speakman method were made in aqueous buffered solutions. Solutions saturated at 25 °C were prepared by stirring a suspension of solid flavanones or chalcones for several hours in aqueous buffered solutions. After equilibration, the concentration of flavanones or chalcones in the supernatant liquid was determined spectrometrically (after filtration through sintered-glass crucibles, Schott Jena G-5, and washing with the neat solution).

All those methods are described in an excellent manual [11] and details are given elsewhere [7, 8].

Sediment and filtrate were studied spectrophotometrically within UV/VIS by a spectrophotometer PAY-ATI UV-4, and ¹H NMR in DMSO-d₆ using a nuclear magnetic spectrometer Tesla DM 567A.

The temperature during measurements was about 25 ± 1 °C. Calculations of binding energy, dipole moments and heat of formation were done by a semi-empirical method AM1 using the programme Hyper Chem[®] 5.0.

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