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Mathematical calculations of iron complex stoichiometry by direct UV–Vis spectrophotometry

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ABSTRACT

The effects of iron-chelating agents on miscellaneous pathologies are currently largely tested. Due to various indications, different properties for chelators are required. A stoichiometry of the complex in relation to pH is one of the crucial factors. Moreover, the published data on the stoichiometry, especially concerning flavonoids, are equivocal.

In this study, a new complementary approach was employed for the determination of stoichiometry in 10 iron-chelating agents, including clinically used drugs, by UV–Vis spectrophotometry at relevant pH conditions and compared with the standard Job's method.

This study showed that the simple approach based on absorbance at the wavelength of complex absorption maximum was sufficient when the difference between absorption maximum of substance and complex was high. However, in majority of substances this difference was much lower (9–73 nm). The novel complementary approach was able to determine the stoichiometry in all tested cases. The major benefit of this method compared to the standard Job's approach seems to be its capability to reveal a reaction stoichiometry in chelators with moderate affinity to iron.

In conclusion, using this complementary method may explain several previous contradictory data and lead to a better understanding of the underlying mechanisms of chelator's action.

1. Introduction

In the past decades, there has been a rapid development of novel iron-chelating agents. Many of them have shown a promising potential in the therapy of iron overload and non-iron overload pathologies [1,2]. Despite of the enormous interest of investigators, to date, a limited number of iron chelators is clinically used. Deferoxamine, deferasirox or deferiprone are indicated in patients, who require a long-term treatment with blood transfusions in haematological disorders [3,4]. Moreover, in epidemiologically rare cases, deferoxamine can be clinically used for the management of acute iron intoxication [5]. Dexrazoxane is an approved cardioprotective agent that effectively protects against anthracycline-induced cardiotoxicity although the involvement of its iron-chelating product has recently been questioned [6,7].

Apart from the above mentioned clinically used iron chelators, there is a wide spectrum of structurally different substances of the both natural and synthetic origin sharing the ability for chelation of Fe(II) and/or Fe(III) [8]. Beyond iron overload conditions, iron-chelating agents are experimentally tested in the prevention/treatment of acute myocardial infarction, neurodegenerative diseases and cancer [1,4,7,9–11].

Thus, in relation to the mentioned approved or examined indications, different properties for iron chelators are required. Activity of iron chelator may be affected by many factors, e.g. pH. Differences in pH due to both physiological (e.g. in upper part of the intestine, where the absorption of iron occurs) and pathological (e.g. ischaemic myocardial tissue and cancer) aspects may significantly influence chelation of iron [12–14].

Moreover, low stability of the complex may allow or even potentiate the reaction of iron with hydrogen peroxide (Fenton chemistry) with the known generation of the most harmful biological oxidant, hydroxyl radical [15]. In order to prevent this reaction, the complex chelator-iron has to be very stable. Complexes with a lower stoichiometry, where all coordination sites are not fully occupied, are more prone to the production of hydroxyl radical, but on the other hand, such complexes may be useful in the therapy of cancer [1,15]. Hence the knowledge of the stoichiometry of the complex may be also of potential clinical significance.

To date, the characterizations of Fe(II)/Fe(III) complexes at different pH conditions are rather scarce. This may be likely associated with difficulties in a methodological approach, e.g. apparently contradictory findings have been published concerning the stoichiometry of the complexes in flavonoids [16,17]. Therefore, the aim of

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this study was to develop a simple, precise and rapid UV–Vis spectrophotometric approach usable at different (patho)physiologically relevant pH. Novel mathematical calculations of the stoichiometry, and a standard method of continuous variation, also known as the Job's method, were employed and their advantages and disadvantages disclosed.

2. Materials and methods

2.1. Reagents

Deferoxamine was purchased from Novartis (Switzerland). Deferasirox was isolated from Exjade tablets (Novartis, Switzerland) by extraction with hot ethanol and then precipitation by water. NMR (Varian Mercury-Vx BB 300 instrument, operating at 300 MHz for 1H, 75 MHz for 13C, Palo Alto, CA, USA) and MS spectra (Agilent 500 Ion Trap LC/MS, Santa Clara, CA, USA) of the product were in accordance with literature and elemental analysis (Fisons EA 1110, Milano, Italy) revealed its sufficient purity (calcd: C 67.56, H 4.05, N 11.25; found: C 67.46, H 4.14, N 11.29) [18]. Deferiprone was a kind gift from ApoPharma Inc. (Apotex Inc., Canada), ethylenediaminetetraacetic acid disodium salt (EDTA), 8-hydroxyquinoline, chloroxine, quercetin and rutin were purchased from Sigma–Aldrich Inc. (USA). Pyridoxal isonicotinoyl hydrazone (PIH) and salicylaldehyde isonicotinoyl hydrazone (SIH) were synthesized as was described previously [19]. The tested iron-chelating agents are depicted in Fig. 1.

3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazinedisulphonic acid sodium salt (ferrozine), ferrous sulphate heptahydrate, ferric chloride hexahydrate, ferric tartrate, hydroxylamine hydrochloride, sodium acetate, acetic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), HEPES sodium salt were purchased from Sigma–Aldrich Inc. (USA) and methanol from J.T. Baker (Avantor Performance Materials, Inc., USA). Ultrapure water was used throughout this study.

2.2. Assessment of iron concentration in stock solutions

Before each experiment, a concentration of iron in stock solutions was routinely checked by a spectrophotometric reagent – ferrozine. Ferrozine specifically reacts with Fe(II) and gives a stable magenta coloured complex with a single absorption maximum at 562 nm [20]. Therefore, an aqueous solution of ferrozine (final concentration 1.67 mM) was used for a direct determination of Fe(II) concentration, which linearly corresponds to the absorbance of the formed complex with ferrozine.

Moreover, the above-mentioned approach was slightly modified for an assessment of Fe(III) by an addition of a reducing agent – hydroxylamine (final concentration 3.33 mM). Afterwards, Fe(III)

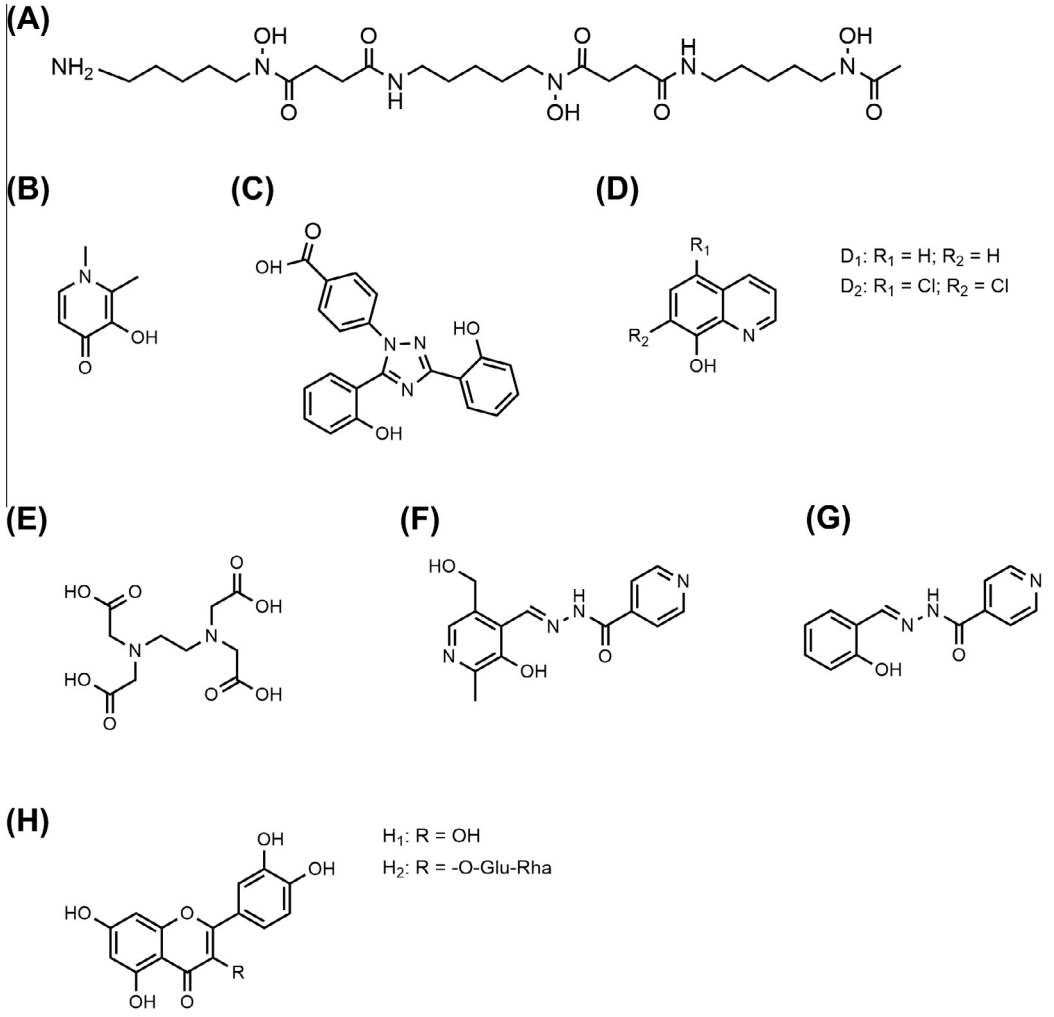

Fig. 1. Iron-chelating agents tested in this study. Deferoxamine (A), deferiprone (B), deferasirox (C), 8-hydroxyquinoline (D_1), chloroxine (D_2), EDTA (E), PIH (F), SIH (G), quercetin (H_1) and rutin (H_2).

was reduced into Fe(II) and its concentration was subsequently determined by ferrozine.

2.3. Iron and pH conditions

The assessment of stoichiometry was performed at four (patho)physiologically relevant pH values (4.5, 5.5, 6.8 and 7.5). For the both lower pHs, 15 mM acetate buffers were used, while 15 mM HEPES buffer was used for pH 6.8. Because oxidation of Fe(II) significantly raises in the course of time at pH 7.5, hydroxylamine was added in the final concentration of 5 mM to the HEPES buffer in order to prevent Fe(II) oxidation [21]. For the determination of Fe(III) chelation at pH 7.5, HEPES buffer without hydroxylamine was used.

Two kinds of ferric solutions, i.e. ferric chloride hexahydrate and ferric tartrate, were tested because of low solubility of Fe(III) at higher pHs. In this study, ferric chloride hexahydrate was used at pHs 4.5 and 5.5 and ferric tartrate at pHs 6.8 and 7.5.

2.4. Ultraviolet–visible spectrophotometry

All experiments were performed in semi-micro polystyrene or ultraviolet-transparent cuvettes (BrandTech Scientific Inc., The United Kingdom) and absorbance was measured by the use of spectrophotometer Helios Gamma equipped with VisionLite Software 2.2 (ThermoFisher Scientific Inc., USA).

2.4.1. Assessment of absorption maxima of iron-chelating agent and its complex

Firstly, absorption spectra ranging from 220 to 800 nm with wavelength(s) of absorption maximum(a) of a tested substance (λ_{Smax}) were determined at all pH conditions. Molar absorption coefficients of the substance (ε_S) were calculated according to the Lambert–Beer law. Similarly, a determination of the wavelength(s) of absorption maximum(a) of the complex (λ_{Cmax}) and the corresponding molar absorption coefficients (ε_C) were accomplished by the use of iron excess at different concentration ratios ranging from 1:6 to 1:50 (substance:iron). The blank was composed from a buffer and a solvent of the substance (methanol or water) at the ratio 2:1 in the case of Fe(II). Because the absorbance of Fe(III) disturbed the measurement, the assessment of ferric complexes was slightly modified by an inclusion of ferric aqueous solution into the blank.

2.4.2. Job's method

The Job's method, also known as the method of continuous variation, is a simple analytical approach which is used to the determination of stoichiometry of two interacting components. In this method, the total molar concentration of two reactants is kept constant while their molar concentration ratios are continuously varied throughout the series of samples (Fig. 2A) [22].

Briefly, an aqueous solution of Fe(II) or Fe(III) was mixed for 3 min with a methanolic/aqueous solution of a tested substance at different molar concentration ratios ranging from 1:3 to 6:1 (substance:iron) at all tested pHs and afterwards absorption spectra were immediately measured. The blank was composed from a buffer and a solvent at the ratio 2:1, respectively.

2.4.3. Complementary methods based on mathematical calculations of the stoichiometry

In addition to the standard Job's approach, complementary mathematical calculations were employed. Compared to the Job's method, the total molar concentration of the tested substance was continuously varied, while the molar concentration of Fe(II) or Fe(III) was kept constant throughout the series of samples (Fig. 2B).

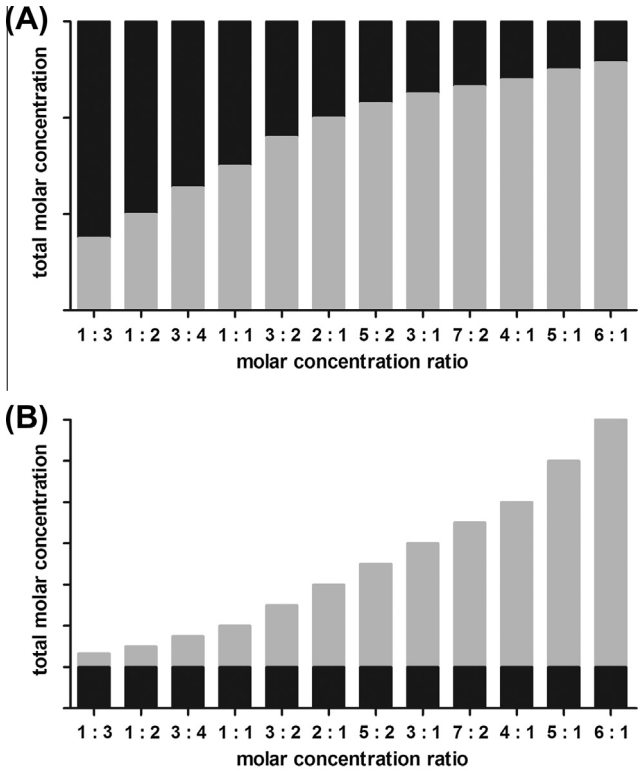

Fig. 2. A schematic depiction of the Job's method (A) and the complementary method (B). The grey columns correspond to the molar concentration of a substance and the black columns correspond to the molar concentration of iron. In the Job's method, the molar concentration ratios of the substance to iron are continuously changing while the total molar concentration is kept constant. In the complementary method, there was a constant molar concentration of iron while a molar concentration of the substance continuously varied. The ratios signify substance to iron.

The preparation of different molar concentration ratios ranging from 1:3 to 6:1 (substance:iron) was identical to the above described protocol.

2.4.3.1. Absorbance at absorption maximum of the complex (Method I). A determination of the stoichiometry according to the Method I was based on a simple evaluation of absorbance of a series of samples at the wavelength of absorption maximum of the complex λ_{Cmax}.

2.4.3.2. Symmetry of the absorption maximum of the complex (Method II). This method was based on a simple assumption that in the theory the absorption maximum of a complex is symmetric, if there are no interfering proximal absorption maxima (Fig. 3). Thus, at similar distance (d) from λ_{Cmax} to the left (λ_{sym1}) or to the right (λ_{sym2}), the absorbance of the complex $A_{C_{\lambda sym1}}$ was proposed to be the same as the absorbance of the complex $A_{C_{\lambda sym2}}$ (Eqs. (1)–(3)).

$$\lambda_{sym1} = \lambda_{Cmax} - d \quad (1)$$

$$\lambda_{sym2} = \lambda_{Cmax} + d \quad (2)$$

$$A_{C_{\lambda sym1}} = A_{C_{\lambda sym2}} \quad (3)$$

According to the known additive character of absorbance, a measured absorbance was additively composed from the absorbance of the formed complex and the non-reacted substance at any wavelength. Therefore in the theory, if the absorbance of the

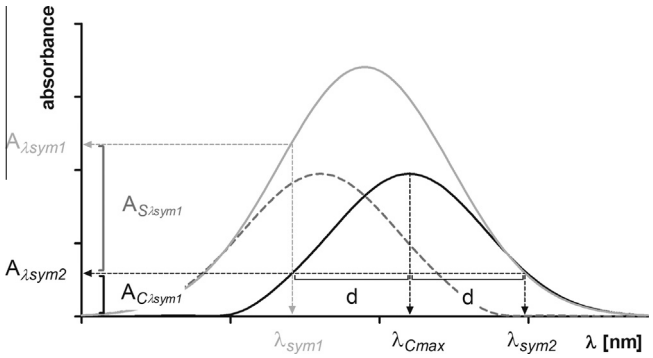

Fig. 3. The method of the symmetry of absorption maximum of the complex. The measured absorption spectrum (grey curve) is the sum of the absorption spectrum of the non-reacted substance (dark grey dashed curve) and the absorption spectrum of the formed complex (black curve).

complex $A_{C_{\lambda sym2}}$ was sufficient (>0.1) and the absorbance of the substance $A_{S_{\lambda sym2}}$ was zero, the measured absorbance $A_{\lambda sym2}$ should be equal to the absorbance of the complex $A_{C_{\lambda sym2}}$ (Eqs. (4) and (5)).

$$A_{\lambda_{sym2}} = A_{S_{\lambda sym2}} + A_{C_{\lambda sym2}} \tag{4}$$

$$A_{\lambda_{sym2}} = A_{C_{\lambda sym2}} \tag{5}$$

Thus, the measured absorbance $A_{\lambda sym1}$ was directly used for the assessment of the molar concentration of the non-reacted substance (Eqs. (6) and (7)):

$$A_{\lambda_{sym1}} = A_{S_{\lambda sym1}} + A_{C_{\lambda sym1}} \tag{6}$$

and because of Eqs. (3) and (5):

$$A_{\lambda_{sym1}} = A_{S_{\lambda sym1}} + A_{\lambda_{sym2}} \tag{7}$$

Considering the Lambert–Beer law (Eq. (8)), the molar concentration of the non-reacted substance (c_S) was calculated as follows (Eq. (9)):

$$A_{S_{\lambda sym1}} = c_S \times \varepsilon_{S_{\lambda sym1}} \times \ell \tag{8}$$

in which ℓ was the known width of cuvette and $\varepsilon_{S_{\lambda sym1}}$ was the molar absorption coefficient of the substance at λ_{sym1},

$$c_S = \frac{A_{\lambda_{sym1}} - A_{\lambda_{sym2}}}{\varepsilon_{S_{\lambda sym1}} \times \ell} \tag{9}$$

Afterwards, the chelation ratio (X) was calculated according to the Eq. (10), in which c_{S_0} was the initial molar concentration of a substance and c_{Fe} was the final molar concentration of iron in the sample.

$$X = \frac{c_{S_0} - c_S}{c_{Fe}} \tag{10}$$

2.4.3.3. Calculation using the absorption maximum of the substance (Method III). A calculation of the stoichiometry using the absorption maximum of the substance was based on the determination of the molar concentration of the non-reacted substance (c_S) likewise in the previous methodology. However, the absorbance at the wavelength of absorption maximum of substance ($A_{\lambda_{Smax}}$) was used (see Supplementary data Fig. S1A). Similarly, $A_{\lambda_{Smax}}$ was the sum of the absorbance of the non-reacted substance and the formed complex (analogously to the Eq. (6)). Hence, considering the Lambert–Beer law, the molar concentration of the non-reacted substance (c_S) was calculated as follows (Eqs. (11)–(13)):

$$A_{\lambda_{Smax}} = c_S \times \varepsilon_{S_{\lambda Smax}} \times \ell + c_C \times \varepsilon_{C_{\lambda Smax}} \times \ell \tag{11}$$

in which $\varepsilon_{S_{\lambda Smax}}$ and $\varepsilon_{C_{\lambda Smax}}$ were the molar absorption coefficients of the substance and the formed complex, respectively, at the wavelength of absorption maximum of the substance λ_{Smax}. The unknown molar concentration of the complex (c_C) was substituted by conversion to the molar concentration equivalents of the substance:

$$c_C + c_S = c_{S_0} \tag{12}$$

And hence the concentration of the non-reacted substance (c_S) was:

$$c_S = \frac{\frac{A_{\lambda_{Smax}}}{\ell} - \varepsilon_{C_{Smax}} \times c_{S_0}}{\varepsilon_{S_{\lambda Smax}} - \varepsilon_{C_{\lambda Smax}}} \tag{13}$$

Afterwards, the calculation of the stoichiometry was accomplished according the Eq. (10).

2.4.3.4. Calculation using the absorption maximum of the complex (Method IV). The calculation of the stoichiometry using the absorption maximum of the complex was analogous to the Method III with one exception that the absorbance was measured at the wavelength of absorption maximum of the complex (λ_{Cmax}) (see Supplementary data Fig. S1B).

2.4.3.5. Theoretical determination of absorbance of the complex at the wavelength of its absorption maximum (Method V). This method was based on a construction of theoretical lines mimicking absorbance of the most probable stoichiometries. Basically, because the molar concentration of iron was stable throughout the complementary approach (Fig. 2B), absorbance was firstly raising dependently on the formation of complex as long as all added substance reacted with iron and formed the complex (Eqs. (14) and (15)):

$$A_{\lambda Cmax} = A_{C_{\lambda Cmax}} \tag{14}$$

$$A_{C_{\lambda Cmax}} = c_{S_0} \times \varepsilon_{C_{\lambda Cmax}} \times \ell \tag{15}$$

In a certain point, at which all iron was exhausted, the absorbance $A_{\lambda Cmax}$ raised only dependently on the absorbance of the further added (non-reacted) substance $A_{S_{\lambda Cmax}}$ (Eqs. (16) and (17)):

$$A_{\lambda Cmax} = A_{C_{\lambda Cmax}} + A_{S_{\lambda Cmax}} \tag{16}$$

$$A_{\lambda Cmax} = c_{eq} \times \varepsilon_{C_{\lambda Cmax}} \times \ell + (c_{S_0} - c_{eq}) \times \varepsilon_{S_{\lambda Cmax}} \times \ell \tag{17}$$

The point of the molar concentration equilibrium (c_{eq}) was at $c_{S_0} = c_{Fe}$ for stoichiometry 1:1, $c_{S_0} = 2 \times c_{Fe}$ for stoichiometry 2:1, etc. The comparison of the measured absorbance with these theoretical lines was able to reveal the searched stoichiometry or

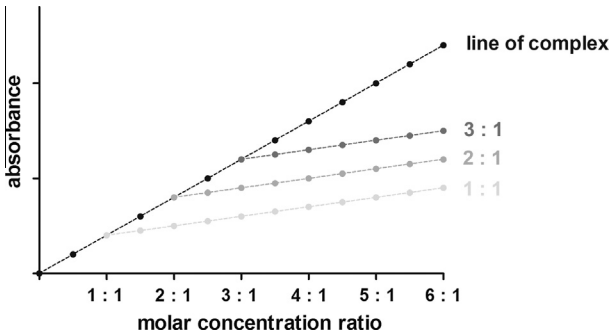

Fig. 4. Theoretical determination of absorbance (Method V). The black line corresponds to the absorbance of the formed complex at the excess of iron. The light grey line mimics the absorbance at the stoichiometry 1:1, the grey line at 2:1 and the dark grey line at 3:1. The ratios signify substance to iron.

Table 1
Summarized wavelengths of absorption maxima of the tested substances and their complexes with iron.

Substance	λ_{Smax} (nm)	λ_{Cmax} (nm)	λ shift (nm)
8-Hydroxyquinoline	240 ± 0 307 ± 1	250 ± 0 355 ± 0 456 ± 0 575 ± 0	10–268
Chloroxine	247 ± 0 278 ± 1 316 ± 2	261 ± 1	14
Deferasirox	246 ± 0 293 ± 1	309 ± 2	14
Deferiprone	280 ± 0	227 ± 1 289 ± 3	9
Deferoxamine	225 ± 1	430 ± 0	205
EDTA	×	256 ± 0	×
PIH	218 ± 2 296 ± 1 342 ± 1	233 ± 3 310 ± 2 367 ± 2 463 ± 2	14–123
Quercetin	370 ± 0	434 ± 2[a] 443 ± 3[b]	64–73[ab]
Rutin	356 ± 2	401 ± 5	45
SIH	218 ± 5 288 ± 0 330 ± 0	231 ± 7 303 ± 0 350 ± 0	13–20

λ shift means the difference between absorption maximum(a) of the tested substance and the complex with iron.
× – no absorption maximum of EDTA was found.
[a] Quercetin: at pH 4.5–6.8.
[b] Quercetin: at pH 7.5.

Table 2
Summarized results of the described methodological approaches. ✓ successful method, (✓) partially efficient method, × unsuccessful method, – the analysis was not performed.

Substance/method	Job's	I	II	III	IV	V	VI
8-Hydroxyquinoline	✓	✓	–	–	–	–	–
Chloroxine	✓	✓	✓	✓	✓	✓	✓
Deferasirox	✓	×	×	×	×	✓	✓
Deferiprone	✓	×	✓	×	×	×	✓
Deferoxamine	✓	✓	–	–	–	–	–
EDTA	✓	✓	–	–	–	–	–
PIH	✓	✓	(✓)	×	×	✓	✓
Quercetin	✓	✓	(✓)	(✓)	(✓)	✓	✓
Rutin	✓	×	(✓)	(✓)	(✓)	✓	✓
SIH	✓	×	(✓)	×	×	✓	✓

Partially efficient method means that the method was able to suggest the correct stoichiometric ratio but the measured points were not in the full agreement with the theoretical lines.

even the reaction stoichiometry at different molar concentration ratios (Fig. 4). At the wavelength of absorption maximum of the substance, the identical approach was used.

2.4.3.6. Theoretical determination of the sum of absorbance of the non-reacted substance and complex at absorption maximum of substance (Method VI). This calculation was similar to the previous methodology based on the construction of theoretical lines mimicking the absorbance of the most probable stoichiometries.

The principle was the same, i.e. firstly, the absorbance depended on the formation of complex up to the point, in which the whole iron was consumed for the complex formation, and thereafter the absorbance was dependent only on the added (non-reacted) substance (Eqs. (14)–(16)). But in the contrast to the Method V, it presumed that diverse complexes with different molar absorption coefficients were formed in the excess of iron. Therefore, the absorbance was not rising linearly up to the concentration equilibrium. Thus, the lines depending only on the non-reacted substance were constructed directly from the measured absorbance at the most probable chelation ratios (1:1, 2:1, 3:1, etc.).

The identical approach was used at the wavelength of the absorption maximum of the complex.

2.5. Data analysis

The majority of experiments, in particular those that determined unclear stoichiometry, were performed at least in duplicates with two different stock solutions. On the other hand, some experiments, which gave unequivocal outcomes, were performed as a single measurement after the concentration of both reagents was calibrated.

Data are expressed as means ± SD. In appropriate cases, a single measurement is depicted for better lucidity in figures.

3. Results and discussion

First, the absorption spectra of all 10 analyzed substances and their iron complexes were measured and compared. The tested substances varied markedly in the positions of their absorption maxima and in the arithmetic differences between the absorption maxima of the pure substance and its corresponding complex. In almost all tested cases at pH $\geqslant$ 5.5, there were no apparent differences in absorption maxima between Fe(II) or Fe(III) complexes. This suggests that only one type of iron-substance complex was formed and thus the data were summarized. Since ferrous ions may be oxidized in the complex with strong iron chelators under physiological pH, ferric complexes were likely formed [23].

Quercetin was an exception because there was a marked difference between the absorption maximum of the complex at different

pH conditions (Table 1), which is in agreement with the previously published data [24].

At pH 4.5 the iron-chelating activity differed among the tested substances. 8-Hydroxyquinoline, SIH, PIH, quercetin and rutin had low affinity for Fe(II) but not for Fe(III) at this pH.

In three iron-chelating agents, there were marked shifts of the absorption maximum(a) of the pure substance and the formed complex (deferoxamine and 8-hydroxyquinoline) or the pure substance (EDTA) did not absorb in the measured range of wavelengths. In these cases, the analytical approach was not complicated. The Job's method or Method I was sufficient for the assessment and no additional approaches were necessary (see summarized data in Table 2). Examples are shown for deferoxamine (Fig. 5 and see Supplementary data Fig. S2), EDTA (see Supplementary data Fig. S3) and 8-hydroxyquinoline (see Supplementary data Fig. S4). The EDTA-iron complex's absorption maximum was localized at the low wavelength (256 nm), at which Fe(III) is known to exert some absorbance. But this fact did not interfere with the assessment in low concentrations of iron and EDTA. The resulting stoichiometries are in full accordance with available literary data, since both deferoxamine and EDTA are hexadentate iron chelators, and hence 1:1 ratios, as expected, were confirmed in this study as well [1,15]. 8-Hydroxyquinoline formed 3:1 complexes which is in agreement with the bidentate nature of this iron-chelating agent [25].

In all other tested substances, all described methodological approaches were applied (see summarized data in Table 2).

The Method I, based on the use of the wavelength of the absorption maximum of complex (λ_{Cmax}), was able to successfully determine the chelation stoichiometry in several other cases due to the character of the absorption spectra (chloroxine, quercetin and PIH), but not in other tested chelators (deferasirox, rutin and SIH; Supplementary data Figs. S5, S7 and S9).

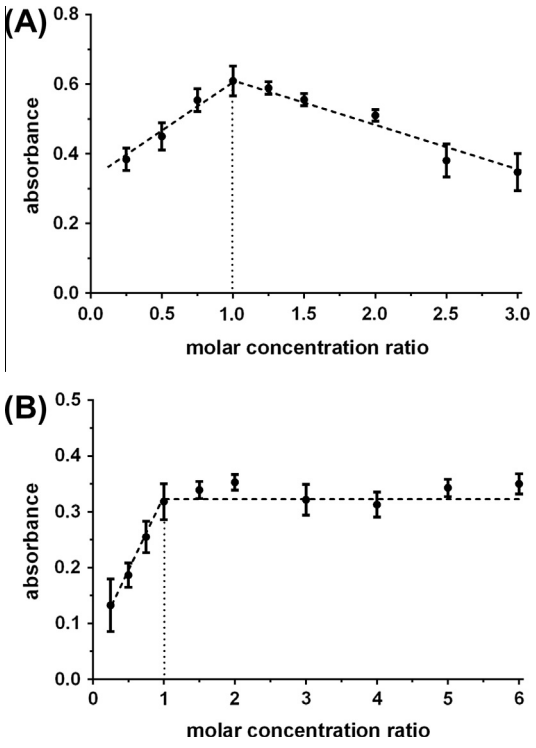

Fig. 5. Assessment of deferoxamine-Fe(II) complex. Figure shows the standard Job's method (A) and simple method I (B) at pH 7.5. Absorbance was read at λ_{Cmax} (430 nm). The ratios signify substance to iron. The total molar concentration of deferoxamine and iron was 0.5 mM for the Job's method. In the Method I, the final molar concentration of deferoxamine was from 0.025 to 0.6 mM while that of iron was constantly 0.1 mM. The assessment was performed in duplicates.

The Method II was able to clearly identify the stoichiometry in chloroxine (Supplementary data Fig. S8E) and deferiprone. In several cases (Table 2), the method suggested apparently the correct stoichiometric ratio, but the resulting graph did not fit in the expected scheme. This was the case of quercetin or SIH, where the ratio 1:1 and 2:1, respectively, was suggested but the chelation lines were not identical with the theoretical lines (Fig. 6A and Supplementary data Fig. S7E). In the case of deferasirox, the Method II completely failed. The Methods III and IV were even less efficient and gave the clear result only in the case of chloroxine (Supplementary data Fig. S8).

The reasons for the success/failure of the Methods II, III and IV likely consisted in three factors: (a) a small difference between wavelengths of absorption maxima of the substance and its complex with iron, (b) a presence of another absorption maximum in the proximity of analyzed absorption maximum and (c) a low

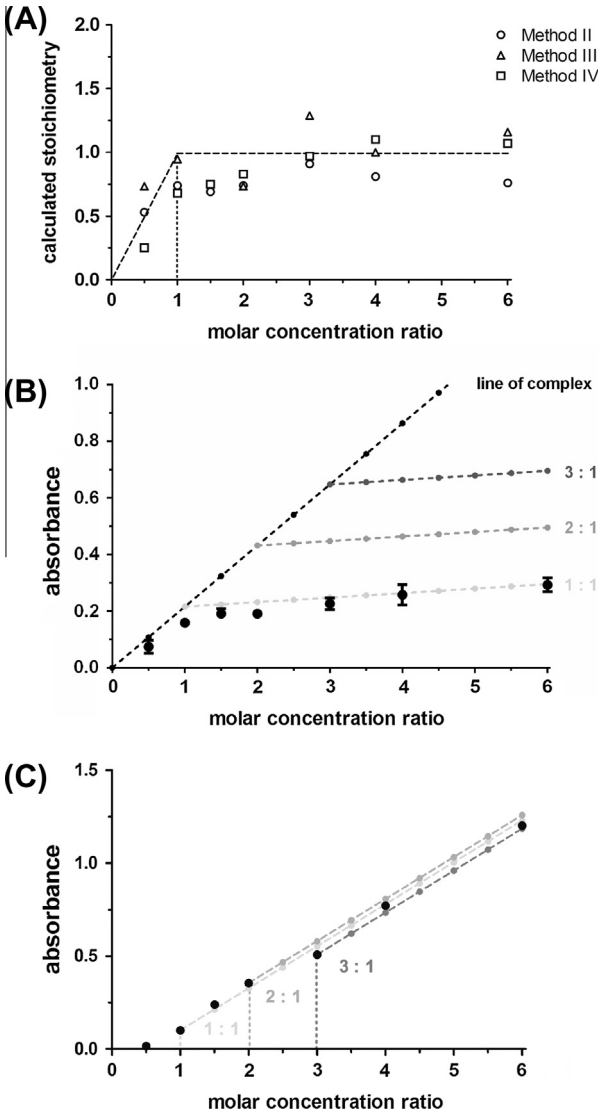

Fig. 6. Quercetin and Fe(III) at pH 7.5. Complementary approach – the plots of Method II, III and IV (A), the plot of Method V (B) and the plot of Method VI (C), in which all lines are almost identical suggesting the formation of the complex at the stoichiometry 1:1. The final molar concentration of iron was 0.01 mM and the final molar concentrations of quercetin were 0.005–0.06 mM. In Fig. 6B, the light grey line mimics the absorbance at the stoichiometry 1:1, the grey line at 2:1 and the dark grey line at 3:1. The ratios signify substance to iron. In this figure, tsingle measurements are depicted for better lucidity. In this figure, single measurements are depicted for better lucidity.

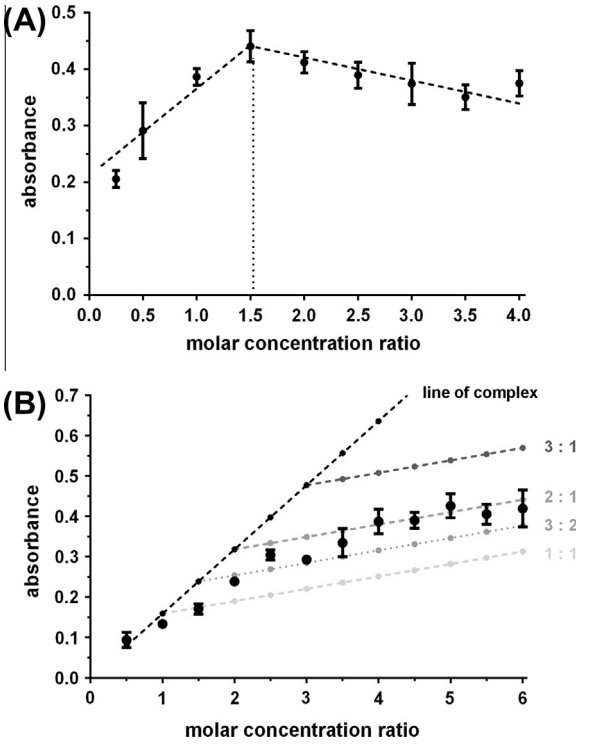

Fig. 7. Rutin and Fe(II) at pH 6.8. Job's method – the Job's plot at λ_{Cmax} (404 nm) (A). The total molar concentration of rutin and iron was 0.05 mM. Complementary approach – the plot of Method V (B). The final molar concentration of iron was 0.01 mM and the final molar concentrations of rutin were 0.005–0.06 mM. In Fig. 7B, the black line corresponds to the absorbance of the formed complex at the excess of iron. The light grey line mimics the absorbance at the stoichiometry 1:1, the dotted grey line at 3:2, the dashed grey line at 2:1 and the dark grey line at 3:1. The ratios signify substance to iron. The assessment was performed with 4 new stock solutions.

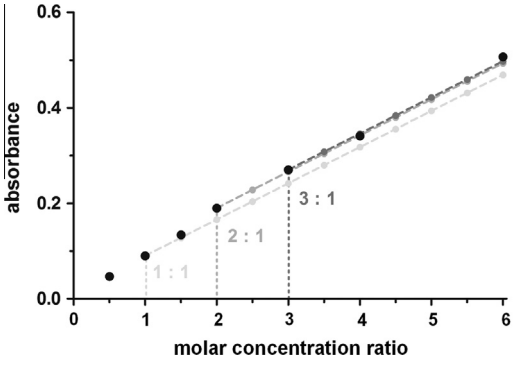

Fig. 8. PIH and Fe(III) at pH 7.5. Complementary approach – the plot of Method V. The final molar concentration of iron was 0.01 mM and the final molar concentrations of PIH were 0.005–0.06 mM. The light grey line mimics the absorbance at the stoichiometry 1:1, the grey line at 2:1 and the dark grey line at 3:1. The line 3:1 is identical with the line 2:1 suggesting the latter complex. The ratios signify substance to iron. In this figure, a single measurement is depicted for better lucidity.

difference between molar absorption coefficients of the substance and its complex at analyzed wavelength(s).

The stoichiometries of the both tested flavonoids, rutin and quercetin, were identified by all Methods II–VI suggesting that the wavelength difference of 45 nm between the tested substance and its complex was sufficient for these methodologies. In all other compounds (chloroxine, deferasirox, deferiprone, SIH and PIH), the difference was much lower ranging approximately from 9 to 25 nm. One exception was PIH, in which a distant absorption maximum at 463 nm (difference 121 nm) was observed as well. But at this wavelength, absorbance of the complex was very low (see Supplementary data Tab. S1) which did not enable the precise calculation. As mentioned previously, the Methods III and IV failed with an exception of chloroxine in all mentioned substances suggesting that the difference between wavelengths (factor a) is the principal factor for the applicability of those methods.

Although the difference between wavelengths in the case of chloroxine was quite small, comparable to deferiprone, and smaller than PIH or SIH, these methods were applicable. The reason likely lay in the steepness of the absorption maxima, i.e. the differences between the molar absorption coefficients (factor c; Supplementary data Tab. S1). The second factor (b), the presence of a close absorption maximum, may substantially contribute to the failure as well. This was apparently true for deferasirox, PIH and SIH (Supplementary data Figs. S5A, S7A and S10A, respectively).

The Methods V and VI were the most efficient and were able to reveal the stoichiometry in all cases. The only exception was deferiprone in the case of Method V. The reason for failure of Methods III, IV and V in the case of deferiprone, in contrast to successful Methods II and VI, can be likely explained by the dependence of the former methods on the assessment of molar absorption coefficients of complex. The probable explanation was the very small difference between the wavelengths of pure deferiprone and its complex (9 nm), which blunted the correct calculation of the molar absorption coefficient of the complex. Indeed, there was almost no difference between the molar absorption coefficient of pure deferiprone and its complex at the λ_{Cmax} (Supplementary data Tab. S1).

Using the Job's method and the complementary mathematical calculations for the determination of chelation stoichiometry, it was disclosed that all tested substances chelated iron in various manners and the obtained ratios were consistent with the previously published data – deferasirox 2:1 (Fig. S5) [15,26]; quercetin 1:1 (Fig. 6 and Supplementary data Fig. S6) [27]; SIH 2:1 (Supplementary data Fig. S7) [28–30]; chloroxine 3:1 (Supplementary data Fig. S8) [25]; rutin 1:1, 3:2 and 2:1 (Fig. 7 and Supplementary data Fig. S9) [16,17]; PIH 2:1 (Fig. 8, Supplementary data Fig. S10) [28–30]; and deferiprone 3:1 (Supplementary data Fig. S11) [28].

Another feature of the theoretical methods, especially of the Method V, was demonstrated within the assessment of rutin's stoichiometry at pH 6.8. Comparing the proposed values for absorbance with the measured ones, it was found that rutin may chelate iron at various chelation ratios (1:1, 3:2 and 2:1, rutin to iron, respectively) depending on its concentration (Fig. 7B). On the other hand, the Job's method showed only one ratio 3:2 (Fig. 7A). Comparing these two different methodological approaches led to a slight superiority of the complementary approach. Therefore, this finding may explain the diverse results in flavonoids from the different studies [16,17].

This method is not able to determine the iron oxidation status in the complex. Notwithstanding this limitation may be of importance for chemical screening of novel specific ferrous chelators, this is of lower importance for a pharmacological study. The main question in the later is the efficacy of a tested substance to chelate ferrous or ferric iron while the oxidation status of the complex is of secondary importance.

4. Conclusion

This study reported the novel calculations for the assessment of stoichiometry of chelators with iron. This approach can be useful in the confirming of the chelation stoichiometry and moreover, it may reveal the reaction stoichiometry in chelators with a moderate affinity to iron. Since the data on stoichiometry of the com-

plexes of several substances were apparently different among miscellaneous studies, particularly in flavonoids, we suggest that for the correct stoichiometry calculation, the both Job's and the complementary approaches should be used.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2013.06.002.

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