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Influence of crystal modification on the photoinduced color change in riboflavin

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Two different qualities of riboflavin (RF) i.e., synthetic (RFs) and biosynthetic riboflavin (RFbs) have been investigated with respect to photoinduced color change in the solid state. Several methods (XRD, FT-IR, VIS-, NIR- and fluorescence spectroscopy) were employed to elucidate the properties of the crystalline structure of RFs and RFbs and the influence of irradiation on the color and structural changes of the samples in the solid state. It was shown that RFs an RFbs represent two different crystal modifications of riboflavin and that RFbs can easily be transformed into a dihydrate upon exposure to humidity. Based on the observed irreversible color change and reduction in fluorescence intensity upon irradiation, an irreversible photoreduction of the molecule was assumed in case of RFs. A more pronounced, reversible color change and reversible reduction in fluorescence intensity indicated a reversible photoreduction process in the case of RFbs. The mechanism of these processes was further investigated by means of NIR and FT-IR spectrophotometry. It is apparent from the current study that the crystal modification of RF can strongly influence the solid state photochemistry of this molecule.

1. Introduction

Riboflavin (Fig. 1) is an important component of human nutrition, present in a wide variety of foods and included in many multivitamin dietary supplements. It is known to be photolabile which may raise problems during the production, storage and use of, e.g., vitamin tablets. Two different qualities of riboflavin (RF) are available on the market, i.e., synthetic (RFs) and biosynthetic (RFbs), produced by means of chemical synthesis and microbial fermentation, respectively. The method of production can determine physical properties such as crystalline form, particle shape and surface area. While particle shape and surface area affect the flow and packing properties of the powder, crystal modification can influence the drug bioavailability and stability of the final product including the photoreactivity of both the pure substance and the final preparation (Hilfiker 2006). It has previously been reported that batches of RF tablets produced from RFbs undergo severe color change and turn green upon inadvertent exposure to light although the tablets were quantitatively sound (Sue-Chu et al. 2009). The color change was observed after a short exposure at low light intensity, e.g., after a few minutes under a wolfram lamp or on the windowsill. Knowledge about the mechanism leading to the rapid color change in case of RFbs is required in order to reformulate and stabilize the tablets. The present work tries to offer an explanation for the high light sensitivity and the formation of a green color in the case of RFbs compared to RFs with the aim to optimize the tablet formulation.

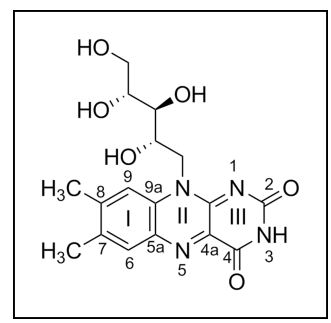

Fig. 1: Structural formula of riboflavin.

Photoinduced reactions of RF can conveniently be divided into three groups: photoreduction, photoaddition and photodealkylation (Heelis 1991). Photoreduction and photoaddition can occur as both intermolecular and intramolecular processes, whereas photodealkylation can take place only as an intramolecular reaction. The vast majority of the photochemical studies refer to RF in solution, while only few reports cover the solid state photochemistry of RF. Photodegradation of the drug in the solid state and in solution may, but does not have to, follow the same pathway (Tønnesen 2004).

In our previous report on the RF tablets it was proposed that RFs and RFbs represent two different crystalline forms, but a

Abbreviations: AA, Amino acids; FT-IR, Fourier transform infrared; HPLC, High-performance liquid chromatography; NIR, Near infrared; RF, Riboflavin; RFbs, Biosynthetic riboflavin; RFdh, Riboflavin dihydrate II; RFs, Synthetic riboflavin; RH, Relative humidity; SEM, Scanning electron microscopy; TLC, Thin layer chromatography; VIS, Visible; XRD, X-ray diffractometry.

detailed study on the physicochemical and photochemical properties of these forms was not included (Sue-Chu et al. 2009). The nature of the crystalline form can govern the course of the solid state photochemical reaction (Ramamurthy and Venkatesan 1987). Further, crystallization conditions can influence the color of the resulting compound. It has been shown that crystal structure and particle size are the main factors controlling the apparent color of the substrate (Mohnicke 2007). Correlation between the photoreactivity and the packing of the reactants has been established for the series of compounds (Ramamurthy and Venkatesan 1987; Hadjoudis et al. 1986). However, to our knowledge the relationship between the crystal structure of RF and its photoreactivity has not previously been reported.

Seven crystalline RF forms have been described in the literature so far (Mohnicke 2007; Gloor 2005). Based on similarities in the powder XRD patterns, these forms have been divided into three structural groups. The thermodynamically stable form anhydrate I is structurally unique, the metastable forms monohydrate II, anhydrate II and dihydrate II belong to the structure group II while anhydrate III, hydrate III and tetrahydrate III belong to the structure group III (Mohnicke 2007). Furthermore, it has been shown that the phase transitions between the forms within the structure group II depend upon relative humidity (RH) (Gloor 2005). Riboflavin monohydrate II is stable in the range between 20% and 75% RH. Although the crystal lattice is preserved in this range, the water content increases with increasing humidity. It was suggested that monohydrate II and anhydrate II may be arranged in layers or tunnel structures, which can be easily refilled with water molecules from the surroundings (Mohnicke 2007; Gloor 2005). Above 75% RH the water molecules are rapidly incorporated into the structure of monohydrate II transforming it into the dihydrate II (Gloor 2005).

Another interesting issue is the presence of trace compounds originating from the production of RF. The patent describing the purification of RFbs states that a trace amount of amino acids (< 0.1%) would be present in a "typical" batch (Wagner 2000). It is well described in the literature that certain amino acids (AA) induce a photoreduction of RF with a quite high rate constant leading to the formation of green products (Heelis 1991). The process is reversible in the dark. Because it is unlikely that RFs is containing trace amounts of amino acids this could explain the rapid photoinduction of a green color in the case of RFbs.

To understand the relationship between crystalline structure and photochemical behaviour of RFs and RFbs X-ray diffractometry (XRD), Fourier-Transform Infrared Spectroscopy (FT-IR) and scanning electron microscopy (SEM) were applied for identification of the crystalline forms. Further, the influence of irradiation on the induced color change and changes in the structure of RF was studied by several spectroscopical metods (i.e., visible (VIS)-, near Infrared (NIR)-, FT-IR and fluorescence reflectance spectroscopy). Qualitative thin layer chromatography (TLC) and HPLC were applied for the detection of amino acids and degradation products, respectively. Finally, solid samples of RFs were spiked with trace amounts of selected amino acids and exposed to radiation in order to evaluate the effect of trace components from the biosynthesis.

2. Investiagations and results

2.1. Investigations of the crystalline forms of riboflavin

X-ray diffractograms of RFs and RFbs clearly indicate the differences in crystal structure between the two samples (Fig. 2). RFs and RFbs were identified as the previously described forms anhydrate I and monohydrate II, respectively, by comparison with reference XRD patterns reported in the literature (Gloor 2005).

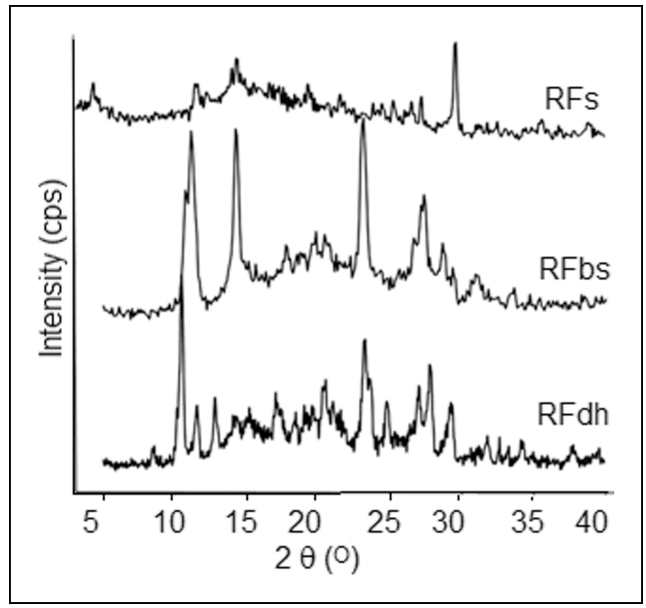

Fig. 2: XRD patterns of RFs, RFbs and RFdh.

The previously described form dihydrate II (RFdh) (Gloor 2005) was prepared by exposing RFbs to elevated humidity. The sample was identified by XRD (Fig. 2). An attempt was also made to prepare anhydrate II by drying RFbs in the presence of P_2O_5. The resulting compound was, however, very hygroscopic and adsorbed water immediately unless it was stored under a dry atmosphere. The use of anhydrate II as a reference compound was thereby restricted under the current experimental conditions and the further investigations were limited (see below).

The FT-IR spectra of RFs and RFbs are shown in Fig. 3. The two RF samples can easily be distinguished by their IR spectra. Identification of the individual bands (see discussion) is based on reference data given in the literature (Abe et al. 1986). The differences in the morphology of RFs and RFbs when studied by use of SEM are also evident (Fig. 4). RFs is made up of needle-like crystals, aligned to form blocks, 10 - 50 μm long. RFbs consists of much smaller, irregularly shaped crystals, forming compact spherical agglomerates with a particle size of about 20 μm. Finally, RFs and RFbs can be differentiated by simple visual inspection; RFs is a yellow powder with poor flowability whereas RFbs appears as a free flowing bright orange powder.

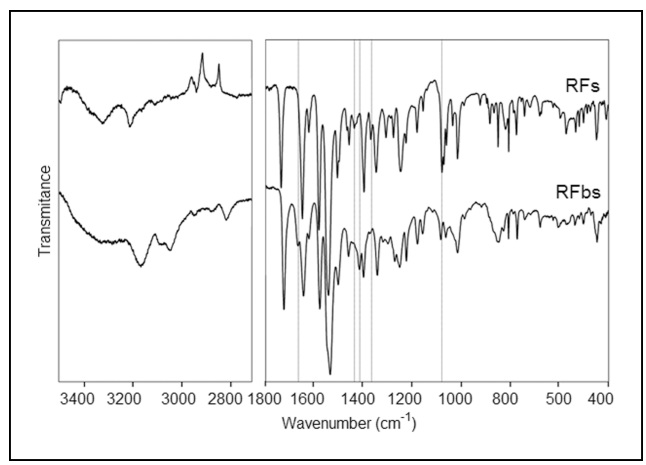

Fig. 3: FT-IR spectra of RFs and RFbs. Characteristic bands are pointed out.

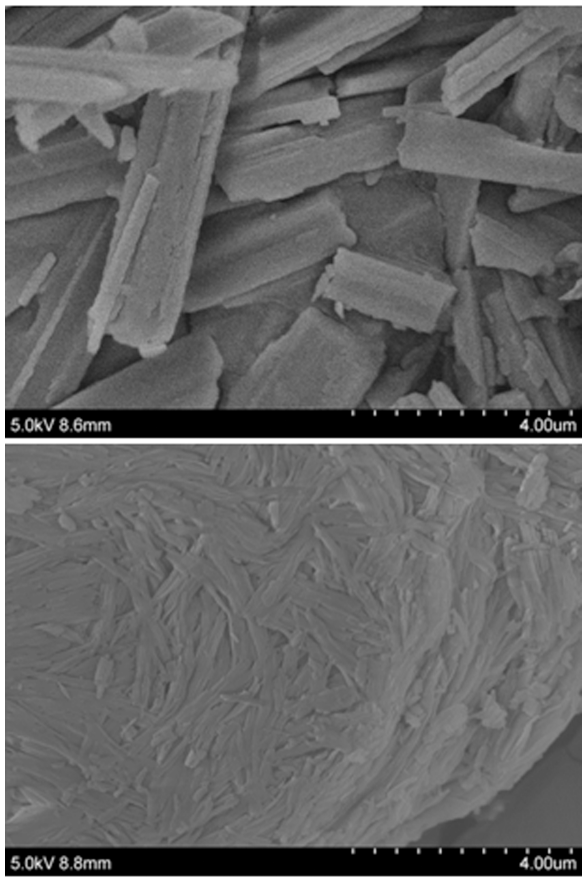

Fig. 4: SEM micrographs of RFs (upper) and RFbs (lower).

2.2. *Effect of irradiation*

A low exposure dose at a moderate intensity was applied in most of the studies in order to mimic the condition that a 'typical' sample would experience during the production, shelf-life and use. For practical reasons different, but comparable light sources were applied in the studies described below.

2.2.1. *Total color change*

The total surface color change upon irradiation of RFs, RFbs and RFdh in the solid state was quantified by the ΔEa*b* value (CIELAB Color Space). A specially designed powder sample holder was used for irradiation and the subsequent color measurements. RFbs exhibited the most pronounced initial color change (ΔEa*b* = 2.63). After 24 h storage in the dark the original color was almost entirely recovered (ΔEa*b* = -0.01). The initial color change of the RFs was less pronounced (ΔEa*b* = 1.79) but the change remained nearly constant after 24 h storage in the dark (ΔEa*b* = 1.13). Both these observations are consistent with previous results (Sue-Chu et al. 2009). The initial color change in RFdh (ΔEa*b* = 1.47) was less than observed for RFbs and RFs but the color returned back to the initial value after ≤ 30 min storage in the dark (ΔEa*b* = 0.58), i.e., the profile was similar to RFbs which is the starting material in the preparation of RFdh. The apparent surface color showed some variations upon further storage of the sample, probably due to an uneven drying of the surface and the potential transformation back to the riboflavin monohydrate II (i.e., RFbs) or anhydrate II. One study was also carried out on anhydrate II, following the same procedure as used for the other samples. The result indicated that the dried sample exhibited a color profile comparable to RFbs but with a lower initial change (data not shown). The analyses of the color coordinates in the CIELAB color space revealed that all of the examined samples showed an initial color change towards both green and blue (i.e., an increase in the negative a* and b* values). As the starting color of the substance was yellow or orange-yellow, a change towards blue will visually appear as the formation of a green color.

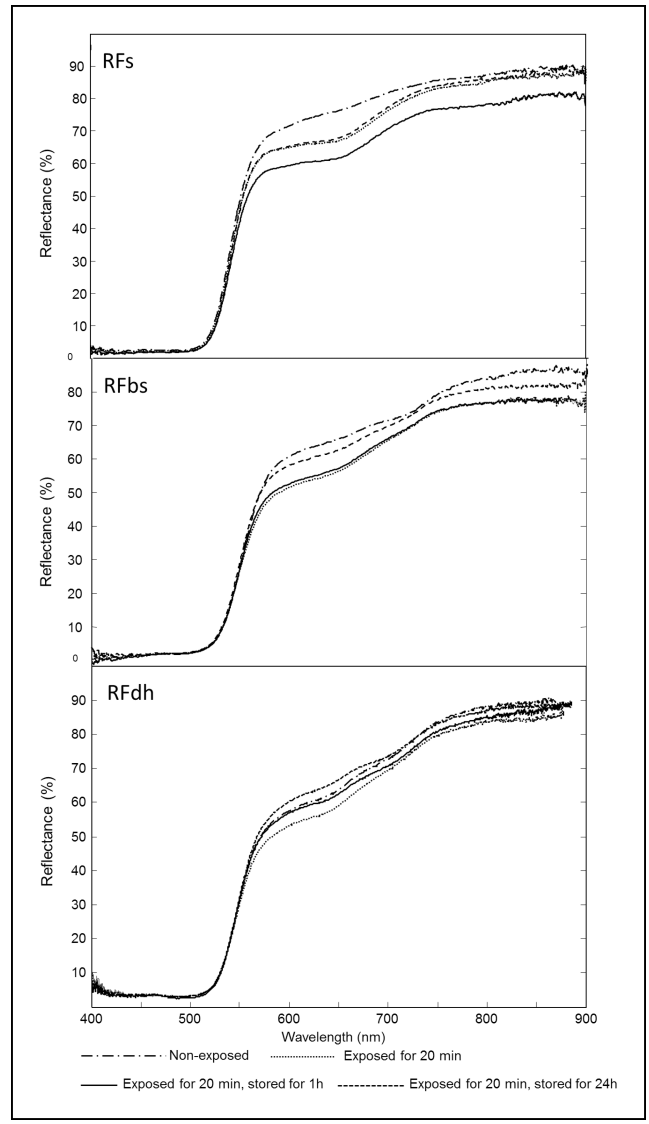

Fig. 5: VIS spectra of RFs, RFbs and RFdh: original (non-exposed) sample, sample exposed for 20 minutes and the same sample after storage in the dark for one and 24 h.

2.2.2. *In situ detection of changes in the visible and near infrared reflection spectra*

A specially modified experimental design allowed for simultaneous recording of visible and near infrared reflection spectra during light exposure of the sample. A disruption of the sample surface, which could be induced when the sample is moved from the light cabinet to the color measuring device, was thereby avoided.

Figure 5 shows the visible (VIS) reflectance spectra of RFs, RFbs and RFdh, before, during and after irradiation. Spectra of RFbs and RFdh exhibited a reversible change upon irradiation. RFbs showed a greater overall spectral change and the recovery of the spectrum after storage in the dark was slower than for RFdh which is consistent with the color measurements. After 24 h the spectrum of the RFdh continued to change as observed above, probably because the surface of the sample was drying. When the spectra of RFbs and RFdh were closely examined it

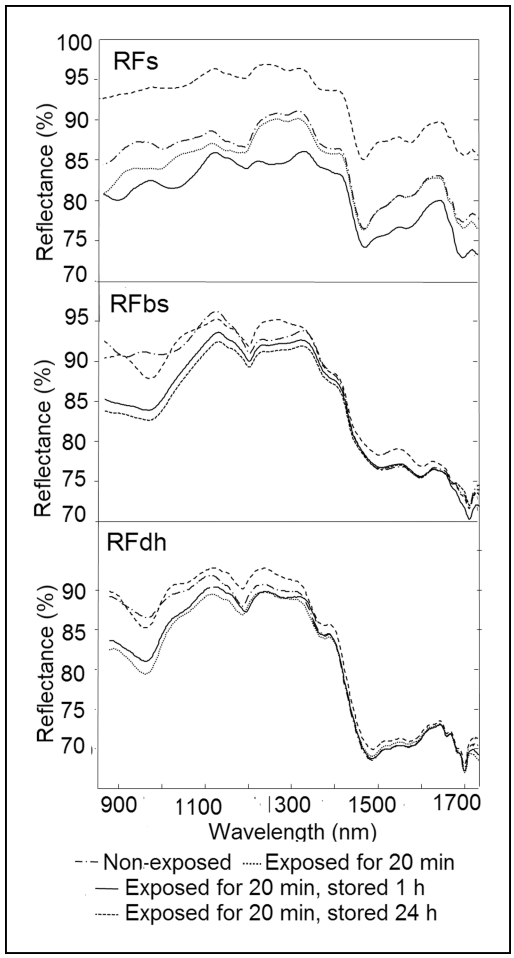

Fig. 6: NIR spectra of RFs, RFbs and RFdh: original (non-exposed) sample, sample exposed for 20 minutes and the same sample after storage in the dark for one and 24 h.

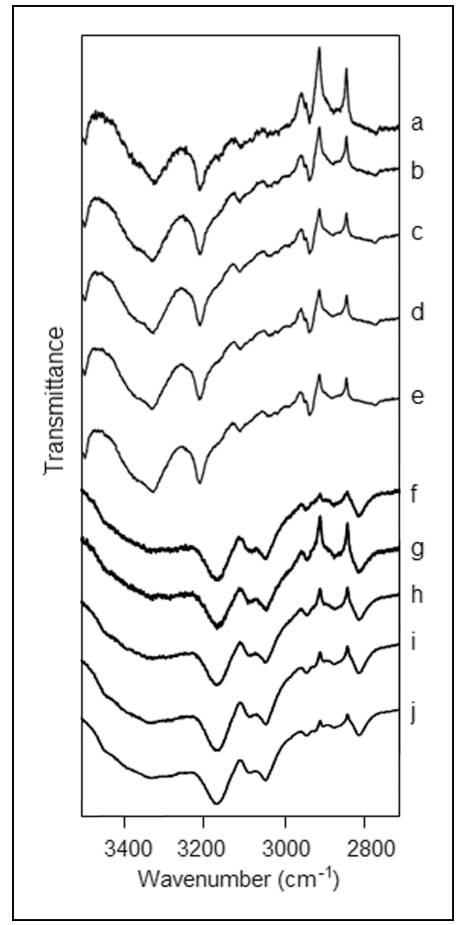

Fig. 7: FT-IR MIR spectra of RFs (5 top spectra) and RFbs (5 bottom spectra): a,f - original (non-exposed) sample, b, g - sample exposed for 10 min, c,h - the same sample after storage in the dark for 5 min, d,i - 15 min and e,j - 60 min.

was observed that they nearly could be superimposed after 24 h in the dark, although they showed a clear initial difference. This supports the assumption that the final color change observed in RFdh originates from the drying of the powder surface and the following transformation into monohydrate II (i.e., the form of RFbs). Furthermore, the spectra of both RFbs and RFdh exhibited an apparent change around 650 nm during the irradiation. The visible spectrum of Rfs did not fully recover to the original shape after 24 h in the dark. This is also consistent with the above color measurements.

Fig. 6 shows the near infrared (NIR) reflectance spectra of RFs, RFbs and RFdh, before, during and after irradiation. The spectral changes of RFs became more pronounced with an increase in the lag-time between the exposure and the spectral recording. This could indicate the occurrence of a dark reaction which possibly could explain the irreversible color change observed above. The most pronounced differences were observed as follows. The band at ~1000 nm was visible in the original spectrum but diminished during storage, while there was a change in the relative intensity of the bands at ~1470 and ~1570 nm. The most pronounced change of the spectra of RFbs could be observed between 900 and 1100 nm. The broad band can be seen in the original spectrum at ~1025 nm. Upon irradiation this band was replaced by sharp and strong band at ~970 nm. The change was not reversible. RFdh did not exhibit pronounced changes in the NIR spectrum upon irradiation. This is in agreement with the very slight photoinduced color changes observed for this sample. The higher reflectance values in the NIR spectra below 1400 nm indicate the shallow penetration depth of the incident light, i.e., the signal results only from the surface of the sample. The part of the spectra above this value consists of a signal that contains reflectance both from the surface and from deeper layers of the sample. These deeper layers might have been shielded from exposure to the irradiation source; i.e., the reflected signal is a mixture of irradiated and non-irradiated RF and is therefore less accurate.

2.2.3. *FT-IR spectra in the mid infrared range (MIR)*

FT-IR spectra of irradiated synthetic and biosynthetic riboflavin were compared to the spectra of non-irradiated samples. Changes were observed only in the range 3500 - 2800 cm^{-1} (Fig. 7). These changes were minor after irradiation in the case of RFs. The band at ~3320 showed an increase in intensity and a shift towards higher wave numbers. Further, an appearance of a shoulder at ~3380 cm^{-1} could be observed. At the same time, the intensity of the band between 2920 and 2840 cm^{-1} decreases. The changes were irreversible after one hour of storage.

In the case of RFbs a more pronounced, reversible change of the spectra was observed upon irradiation. The broad band between 2920 and 2840 cm^{-1} became clearly more intensive after irradiation but almost disappeared after storage in the dark for one hour. The same band exists in the spectrum of non-irradiated RFs, but in that case it decreased upon irradiation.

2.2.4. *Fluorescence emission spectra*

Riboflavin exhibits fluorescence both in solution and in the solid state. The emission wavelength of RF in solution was 537 nm at excitation 350 nm and 374 nm, respectively. The excitation spectrum at emission 537 nm was very broad with a maximum

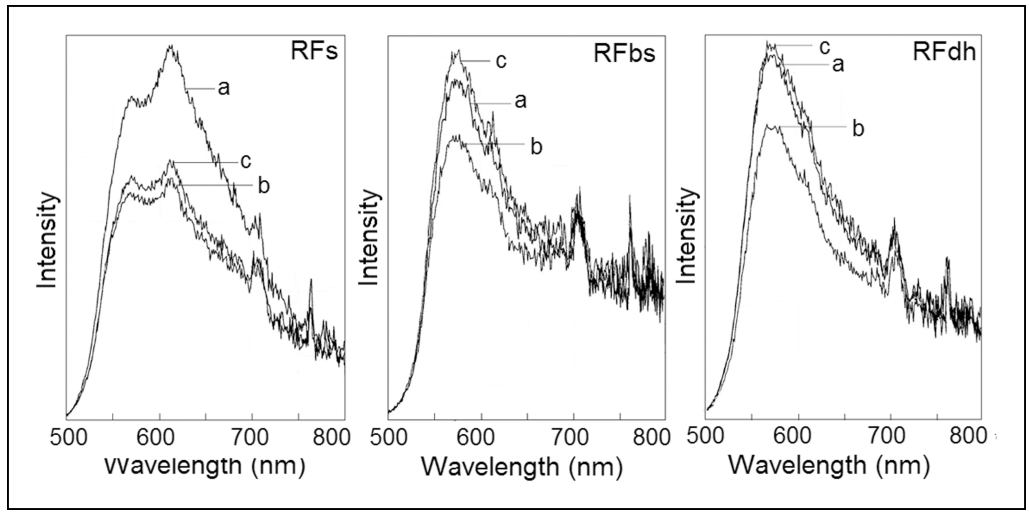

Fig. 8: Fluorescence emission spectra of RFs, RFbs and RFdh: a - original (non-exposed) sample, b - sample exposed for 10 minutes and c - the same sample after storage in the dark for 24 h; Intensity is given in arbitrary units and normalized to the same level.

at approximately 360 nm. The fluorescence was red shifted in the solid state compared to the solution. RFbs in the solid state emits at 574 nm, 711 nm and 765 nm while RFs emitted at 571 nm, 618 nm, 712 nm and 763 nm. The emission spectra were not influenced by a change in excitation wavelength from 350 nm to 374 nm. This was consistent for both qualities of RF. However, the shape of the emission spectra of RFs and RFbs were clearly different (Fig. 8).

Phosphorescence of RF in a PVA film has been reported to occur at 630 nm (Mieloszyk and Drabent 1988). The additional emission peak at 618 nm in the spectrum of RFs might, therefore, be related to phosphorescence but no long-lived component could be identified in the life-time studies of the excited states (see below). The fluorescence intensity of RFs was about 3 times stronger than for RFbs and RFdh. The results again emphasize that the two qualities of RF represent different crystalline forms. It is previously demonstrated for other compounds that when the crystal structure of the forms is sufficiently different, the difference can be manifested in the photophysics of the system (Brittain 2006). The excitation spectra at emission 574 nm consisted more or less of a straight line and it was not possible to identify a maximum. The spectra of Rfdh were quite similar to the spectra of RFbs although the intensity was about 50% higher in the case of the RFdh. The emission peaks above 700 nm are rather sharp compared to the rest of the spectrum. Similar emission peaks observed in a previous work have been assigned to leakage from the light source (Pan et al. 2001). In the current experimental design the peaks above 700 nm could however, not be observed when the sample was replaced by a scatterer. This indicates that these emission peaks originate from the RF samples rather than from the xenon lamp.

The fluorescence was recorded prior to and immediately after exposure to irradiation. A reduction in fluorescence intensity at the emission maximum was observed in all samples. The samples were followed over a time-span of 24 hours storage in the dark. The fluorescence signal returned to the original value during this period in case of RFbs and RFdh, while it remained low in the case of RFs (Fig. 8). This further emphasized that the photoinduced changes in RFbs and RFs were reversible and irreversible, respectively, which is consistent with the observations above. The reduced form of RF is reported to be non-fluorescent in the solid state (Hemmerich et al. 1971). The reduction in fluorescence signal upon irradiation could therefore indicate the formation of an intermediate or a degradation product related to the reduced RF molecule. The characteristic emission peak at 420 nm of lumichrome (Pan et al. 2001) could not be detected (emission filter removed). This is consistent with the HPLC-analysis, where no degradation products could be detected.

An attempt was made to record the excited state lifetime of the samples. The lifetime was either below the detection limit ($\sim$ 1 ns) or the signal was too weak to be detected by the instrumentation. Traces of a long-lived component ascribed to a previously described delayed fluorescence or phosphorescence could not be detected (Mieloszyk and Drabent 1988; Gordon-Walker et al. 1970).

2.3. Qualitative HPLC analysis

RFs, RFbs and RFdh were exposed to irradiation in the solid state and analyzed by HPLC in order to detect the formation of the major photodegradation products lumichrome and lumiflavin. Neither of the products could be detected under the current experimental conditions.

2.4. Interactions with amino acids

Solid samples of RFs were spiked with trace amounts of selected AA under various experimental conditions and exposed to irradiation. The rapid formation of a green color could, however, not be observed. The reason could either be that the AA were located differently in the RFs (spiked) and RFbs particles leading to a difference in photoreactivity, or that the presence of AA was not the reason for the development of a green color in RFbs. Trace amounts of AA in biosynthetic riboflavin could not be detected by use of the TLC/ninhydrin method.

3. Discussion

The above results confirm that RFs and RFbs represent two crystalline forms of RF identified as anhydrate I and monohydrate II, respectively. It was shown within this study that the form monohydrate II can easily transform into the form dihydrate II. Such a transformation could occur under e.g., wet granulation in the preparation of RF tablets or during storage.

Close examination of FT-IR spectra of RFs and RFbs suggested the presence of markedly different hydrogen bonding pattern in the two forms of RF. Two strong bands above 1600 cm^{-1} were assigned to two carbonyl stretching vibrations of riboflavin. The lower frequency band, arising from the C2 = O (Fig. 1) stretching vibration appeared as a doublet in the spectrum of RFs and as a triplet in the spectrum of RFbs. It has been previously shown

(Abe et al. 1986) that this band is sensitive to acetylation of the ribityl side chain, indicating the presence of specific interactions between the C2 = O group and OH groups of the ribityl moiety. Further comparison of the spectra revealed a shift in the frequencies of the OH in-plane bending mode of the ribityl moiety (between 1400 and 1500 cm^{-1}) (Abe et al. 1986) and differences in the shape and intensity of the band assigned to C-O stretching vibrations of the side chain at 1080 cm^{-1}. The band arising from the N3-H bending mode observed in the spectrum of RFs at 1360 cm^{-1} was almost absent in the spectrum of RFbs. This again indicates that inter- and intramolecular hydrogen bonding properties of these two crystal modifications are different.

Visual appearance of RFbs and RFs crystals (shape, size and color) observed within our study is in the agreement with previously described appearance of the crystals of monohydrate II and anhydrate I, respectively (Mohnicke 2007). The difference in particle shape and size, observed for RFs and RFbs could explain the higher overall color change detected for RFbs, as the extent of the photochemical reaction is dependent upon the surface area of the sample (Anderson and Byard 2004). However this could not explain the reversible nature of the discoloration of RFbs.

HPLC investigations did not reveal the formation of photodegradation products. Moreover, the major photodegradation products to be expected, i.e., lumichrome and lumiflavin are yellow substances (Ahmad and Vaid 2006). Photodegradation could therefore not explain the color change towards green. On the other hand, the reduced forms of RF (1,5-dihydroriboflavin and flavisemiquinone) are known to be green and are prone to re-oxidation in the presence of molecular oxygen (Heelis 1991; Ebitani et al. 1991). The color of the reduced forms depends upon the state of reduction i.e., fully reduced RF (1,5-dihydroriboflavin) is dark green whereas flavisemiquinone is light green. It was therefore logical to assume that photoreduction plays an important role in the observed discoloration of the powder samples of RF upon irradiation. A wide range of external electron donors were reported in the literature to react with flavins in intermolecular photoreduction, e.g., amino acids, α-hydroxycarboxylic acids, thiols, aldehydes and unsaturated hydrocarbons, leading to the formation of 1,5-dihydroflavins or their alkyl derivatives (Heelis 1991). In the case of intramolecular reactions, the activated –CH-OH group of the RF side chain serves as an electron donor, resulting in the in the formation of 2' and 4' keto- derivatives and formylmethyl flavin. Additionally, the formation of the flavosemiquinone radical has been reported in flash photolysis studies of flavins in the absence of added electron donors (Hemmerich et al. 1980). The authors postulated that flavin undergoes triplet-triplet electron exchange in a dye-dye reaction, leading to the formation of the cation and anion radicals. The flavosemiquinone radical could be formed in the reaction between the anion radical and H^+.

The reduced form of the flavins is non-fluorescent (Hemmerich et al. 1971). The observed change of the color towards green and the reduction in fluorescence signal upon irradiation therefore indicated that reduction had occurred in both RFs and RFbs, as well as in dihydrate II during irradiation. The fact that both processes were irreversible in the case of RFs but reversible in the case of RFbs and RFdh pointed towards different reaction mechanisms. It was postulated that the rapid and extensive discoloration observed in case of RFbs might be explained by photoreduction induced by an external electron donor, e.g., trace amounts of amino acids originating from the biosynthesis. However, AA could not be detected in the biosynthetic sample nor could a similar color change be induced when RFs was spiked with AA. Therefore, the further investigations were focused on the possibility of intramolecular photoreduction or intermolecular dye-dye reactions, and the structural differences between the samples that might lead to a difference in reaction mechanisms. The differences in the crystalline structures of RFs, RFbs and RFdh are discussed above. If the solid state photoreactivity is mainly governed by the structural properties of the samples it could be expected that similar processes should occur upon irradiation of RFbs and RFdh since monohydrate II (i.e., RFbs) and dihydrate II belong to the same system, with the assumed structural similarities (Mohnicke 2007). In many ways, this was confirmed in the present study: both forms exhibit reversible discoloration and a reduction in the fluorescence signal upon exposure to light. On the other hand, the form anhydrate I (i.e., RFs) which is described as structurally unrelated to the forms monohydrate II and dihydrate II (Mohnicke 2007) exhibits irreversible color change and reduction in the fluorescence signal upon irradiation. Further, it was apparent that the water present in the structure of RF could influence the ongoing processes in addition to the crystal structure. The observed total color change upon irradiation was larger for RFbs than for RFdh under similar conditions. These two forms of RF have a similar structure but a different hydration level. Similarly, the unstable form anhydrate II which is assumed to have the same structure as RFbs but does not contain crystal water (Mohnicke 2007; Gloor 2005) showed a reversible, less pronounced color change upon irradiation compared to RFbs.

Structural changes upon irradiation were reflected in the mid infrared part of the FT-IR spectra. RFbs demonstrated a reversible increase in the broad band between 2920 and 2840 cm^{-1} while a non-reversible decrease in this band was observed in the case of RFs. The assignment of this band was difficult since more than one structure present in the molecule of riboflavin may contribute to its increase. In this part of the spectrum the following bands may be expected: O-H stretching of strongly hydrogen bonded hydrogen atoms, N^+-H stretching of ammonium salts or C-H stretching in methylene groups belonging to the side-chain (Larkin 2011). Additional spectral changes could be observed in the case of RFs; i.e., an increase in intensity and a shift of the band at ~3320 cm^{-1} and an appearance in the shoulder at ~3380 cm^{-1}. Campos Vallette et al. (1994) have ascribed a band at 3374 cm^{-1} to OH stretching vibrations. The observed changes in the spectrum might therefore indicate changes in OH vibrations due to an alteration in hydrogen bonds. However, amide NH stretching vibration is expected to occur between 3470 and 3200 cm^{-1}, while a secondary amine group should exhibit a band at higher wavenumbers – between 3500 and 3300 cm^{-1} (Larkin PJ. 2011). Furthermore, the IR spectrum of the reduced form of riboflavin (assumed to be a flavosemiquinone) was reported by Ebitani et al. (1991). They assigned the strong, wide spectral band at 3400 cm^{-1} to the newly formed NH groups. Based on this information, the rise of a band at ~3320 cm^{-1} and appearance of the shoulder at a higher wavenumber may occur if a new NH group is established at the position 5 (i.e., flavosemiquinone), or both positions 1 and 5 (i.e., fully reduced riboflavin) (Fig. 1). The more pronounced and reversible spectral changes observed after radiation of RFbs were consistent with the reversible and pronounced color change of the sample. The less pronounced but irreversible spectral changes of RFs were consistent with the irreversible and less pronounced color change of this sample.

Evidence for the formation of the NH group upon irradiation of the RFs could be also found in the NIR spectrum. The band at ~1000 nm can be assigned to the second overtone of OH stretch vibrations (Weyer and Lo 2002). A decrease of the band after irradiation and storage in the dark may suggest changes in the RF side chain. Bands at ~1470 nm and ~1570 nm are assigned to the first overtone of OH and NH stretching vibrations, respectively. The slight decrease of the OH stretching band and the slight

increase of the NH stretching band may suggest the appearance of a new NH group and change in the side chain.

Irradiation of RFbs resulted in apparent changes of the NIR spectra between 900 and 1100 nm. The band at ~1025 nm in the spectrum of the non-irradiated sample is assigned to the second overtone of bonded OH stretch vibrations (Weyer and Lo 2002). The strong band at ~970 nm, visible in the spectra of irradiated and stored samples is assigned to non-bonded OH stretch vibrations (Weyer and Lo 2002). It could therefore be concluded that hydrogen bonds are being broken and OH groups are released upon irradiation of the biosynthetic sample. These OH groups may originate either from the water present in the biosynthetic sample or from the ribityl side chain. No significant spectral changes could be observed upon irradiation of RFdh. The strong band, assigned to non-bonded OH stretch vibrations at ~970 nm can be observed in the spectra of non-irradiated, irradiated and stored sample, and is believed to arise from non-bonded water present in the structure of the RFdh. A slight increase in the intensity of this band could be observed during and after the irradiation.

Analysis of NIR and MIR spectra before and after irradiation indicated that an irreversible intramolecular reduction did take place in the case of RFs. This is supported by the irreversible decrease of the fluorescence signal and the change towards green upon irradiation of the sample. Although the reversible color change and the decrease in the fluorescence signal detected after irradiation of RFbs and RFdh suggested a reversible reduction of the riboflavin molecule, this could not be confirmed by NIR and MIR spectroscopy. One of the possible pathways is the dye-dye reaction leading to the formation of the cation and anion radicals and subsequent formation of flavisemiquinone radical. These species are difficult to identify. Furthermore, as H^+ is required for the formation of the flavisemiquinone radical from the anion radical, presence and amount of water can play an important role in this process.

Features of the crystalline structure e.g., the molecular conformation, the mutual orientation and the separation distance of the reacting groups, are obviously of major importance for the observed photoinduced color change in solid riboflavin. Impurities of amino acids from the biosynthesis of RFbs did not appear to contribute to the process.

Based on the assumption that the crystal modification is a key feature in the context of the photoinduced color change in solid state RF, a stabilization of the product cannot be easily achieved by addition of excipients or reformulation of e.g., RF tablets. A stabile product should only be obtained by exclusion of light, by preparation of a photostable crystal modification or by "locking" the molecule in an inert matrix that prevents intra- and intermolecular photoreduction.

4. Experimental

4.1. Materials

RFs and RFbs were generously provided as a gift by Weifa A/S (Oslo, Norway). RFdh was produced by exposing RFbs to elevated humidity in the following way: A Petri dish containing the powder samples and small containers of water was sealed with cling film and placed in an incubator at 40 °C for 48 h. Riboflavin anhydrate II was obtained by drying RFbs in a desiccator in the presence of P_2O_5 for one week. The reduced form of riboflavin was synthesized according to Ebitani et al. (1991) using RFbs as the starting material. L- Histidine was purchased from Sigma-Aldrich (St. Louis, USA), L-cystein, L-methionine and ninhydrin were purchased from Merck, Darmstadt, Germany. All solvents used were of analytical grade.

4.2. Methods

4.2.1. X-ray diffractometry

The structural properties of synthetic, biosynthetic and riboflavin dihydrate II were investigated by powder x-ray diffraction (Bruker, D8, Billerica, USA) using Cu α_1 radiation ($\lambda = 1.54056$ Å).

4.2.2. FT-IR spectroscopy

The FT-IR spectra were obtained with a Varian FT-IR 670 spectrophotometer (Agilent Technologies, Santa Clara, USA) to acquire the medium-IR-range spectrum (3500 to 400 cm^{-1}). The device has a resolution of 1 cm^{-1} and the attenuated total reflection (ATR) method was applied. Powder samples of non-exposed synthetic and biosynthetic riboflavin were investigated. Irradiated synthetic and biosynthetic RF samples were investigated immediately after exposure and after 5, 15 and 60 min of storage in an open container protected from light.

4.2.3. Scanning electron microscopy (SEM)

The morphology of synthetic and biosynthetic riboflavin was examined by field emission scanning electron microscopy (Hitachi S-4800. Hitachi High-Technologies Inc., Toronto, Canada). Powder samples were spread on an aluminum stub using carbon stickers and sputtered with 5 nm platinum in a sputter coater (Cressington 308UHR. Cressington Scientific Instruments Ltd., Watford, UK). The pictures were taken at an acceleration voltage of 5 kV and a working distance of about 8 mm.

4.2.4. Irradiation of the samples

The powder samples were filled in specially made Plexiglas® sample holders covered with a quartz lid and placed in a light chamber (Polylux PT, Dreve Dentamid GmbH, Unna, Germany) equipped with three fluorescent tubes emitting light in the wavelength range 350–400 nm (Ralutec 9W/171, Radium, Germany) with an emission maximum at 370 nm. The mean irradiance calculated from nine points at the inside of irradiation chamber was 0.017 ± 0.0013 W/cm^{-2}. Samples wrapped in aluminum foil were included as dark controls to evaluate the temperature effect. For fluorescence measurements, the samples were irradiated in a triangle quartz cuvette. The powder samples were irradiated as a thin layer (1–2 mm) prior to the FT-IR measurements.

4.2.5. Total color change

The color of the samples was determined by use of a Konica Minolta spectrophotometer CM-3500d (Minolta, Kyoto, Japan). The sample color was measured using the CIE L*a*b* (CIELAB) color space. The model is based on the assumption that three pairs of opposing color sensations can produce all colors; red and green; yellow and blue; and black and white. (Marcus 1998). The CIELAB color coordinate a* correlates with red (+a*) and green (-a*) while the CIELAB coordinate b* correlates with yellow (+b*) and blue (-b*). L* correlates with perceived lightness in the CIELAB color space. A perfect white would have an L* of 100, and a perfect black would have an L* of 0. Color difference ΔEa*b* in the L*a*b* color space, which indicates the degree of color difference but not the direction, is defined by the following equation:

$$\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL*, Δa*, and Δb* are differences in L*, a* and b* values respectively, between the specimen color and the target (reference) color (Editorial 1998). Each sample was used as its own control, i.e., the samples were measured before and after irradiation and the color difference was calculated. The value for the color difference (ΔEa*b*) measured for the dark control was subtracted from the value measured for the samples. The samples were filled in the above mentioned Plexiglas® sample holder and stored in them protected from light in-between of the measurements The color was determined at 3–5 different points of the sample surface and each point was measured 3 times. Average values of all the measurements were calculated. Three samples of each RF quality were evaluated.

4.2.6. In situ visible and near infrared reflection spectroscopy

Visible and near infrared reflection spectra were recorded during irradiation of the samples. A lag-time between irradiation and recording of the spectra was thereby omitted and the surface of the powder was not disturbed. Reflectance spectra were collected in the wavelength range 400–1700 nm using fiber optic spectrometers (NIR512 and SD2000, Ocean Optics, Dunedin, USA) and fiber probes (ZQQAD400–1.5-LIN-4-BX, QR400-7-VIS-BX, Ocean Optics, Dunedin, USA). The light sources used for the measurements were two fiber coupled sources (LS1, Ocean Optics, Dunedin, USA). The samples were placed in a specially constructed sample holder to shield them from unintentional light exposure. The fiber probes were placed in a 45 degrees angle with respect to the surface of the sample, and arranged in such a way that the collection areas of the probes overlapped. The samples were illuminated using a fiber bundle attached to the sample holder. The IR filter of the lamp was removed, and the lamp was used at 80 % of max intensity (Fostec DCR II with a 150 W DDL bulb, Schott, NY, USA). The temperature never exceeded 25 °C inside the chamber. The experimental

setup consisted of an open system, i.e., a sample holder without any protective lid. The samples were measured prior to and during light exposure. Spectra in the range 400 –1700 nm were recorded every 20 s for 20 min. The samples were then kept in the dark inside the chamber for 60 min before they were measured again. Afterwards the samples were removed from the measurement chamber and stored in the dark in an open container over night. The samples were measured again the following day. The total irradiation dose after 20 min of exposure should roughly correspond to 10 min exposure in the Polylux PT light chamber (see above). The estimation takes into account the overlap integral of the output spectrum of the light source and the absorption spectrum of riboflavin, and the value for the estimated irradiance. The data were converted to reflectance by calibration against a white Spectralon tile (WS-1, Ocean Optics, Dunedin, USA). The data recorded were processed using Matlab (MathWorks, Kista, Sweden).

4.2.7. Fluorescence emission spectroscopy

The steady state fluorescence spectra were recorded on a PTI modular Fluorescence System (Photon Technology International, Ontario, Canada) using FeliX32 software. The excitation source was a 75 W xenon lamp and the monochromators were Model 101 with f/4 0.2-m Czerny-Turner configuration. The entrance and exit slits were adjusted to 2 nm. The instrument was equipped with a red-sensitive photomultiplier. An excitation and emission correction was automatically performed. The measurements were performed at $25.0 \pm 0.1\,^{\circ}C$. A filter with a cut-off at 495 nm was inserted at the emission side. The samples were prepared in a triangle cuvette as the RFbs was too free-flowing to fit into a conventional solid sample holder. RFs, RFbs and RFdh were examined, prior and after irradiation, and upon storage protected from light. An aqueous solution of RF in water (10^{-4} M) was used as a reference.
Luminescence lifetime measurements were performed by use of an OBB™ EasyLife® (Optical Building Blocks, New Jersey, USA) excitation 405 nm (short lived species) and an OBB™ EasyLife L® (Optical Building Blocks, New Jersey, USA) (long lived species). In the first case a scatterer Ludox® CL collodial silica (Aldrich, Steinheim, Germany) in water was applied. An emission filter with a cut-off at 495 nm was applied in all measurements.

4.2.8. Qualitative HPLC analysis

The HPLC system consisted of a Shimadzu LC-9A Liquid Chromatograph Pump, a Shimadzu SPD-10A UV Spectrophotometric detector and a Shimadzu 10 ADvp auto sampler. The stationary phase was a Nova-Pak® C18 3.9 × 150 mm (i.d) column (Waters). The mobile phase consisted of water/methanol (13:7). Flow 0.5 ml/min, injection volume 20 µl, detection wavelength 374 nm. Expected retention times were: riboflavin ∼ 5 min, lumiflavin ∼7 min, lumichrome ∼20 min. The samples were prepared by dissolving irradiated samples (RFs, RFbs and RFdh) and the dark controls in distilled water at a theoretical RF concentration of 1.3×10^{-5} M. The standard solutions were prepared in the same way as the samples in the concentration range $1.3 \times 10^{-5} - 1.3 \times 10^{-6}$ M (Reg ≥ 0.996). Powder samples were irradiated in a thin layer (1-2 mm) in a Petri dish protected by cling-film, in a cabinet SUNTEST CPS + (Atlas, Gelnhausen, Germany). The light source was a xenon lamp (1.5 kW) equipped with a 6 mm special glass filter transmitting light corresponding to exposure behind window-glass (cut-off approximately 310 nm). The cabinet was equipped with a SunCoolTM device (Atlas, Gelnhausen, Germany) which maintains a chamber temperature < 30 °C. The intensity was measured by use of a XenoCal Sensor (Atlas, Gelnhausen, Germany) with a spectral range 300–800 nm. Samples were exposed to the irradiation dose of 2.25 kJ/cm^{-2} and dark controls were included.

4.2.9. Qualitative thin layer chromatography

Qualitative thin layer chromatography (TLC) was used for screening of trace amounts of AA present in the biosynthetic riboflavin sample. A saturated RFbs solution in methanol was prepared. Solutions of L-methionine, L-histidine and L-cysteine in methanol (1% w/w) were used as the reference samples. 0.2 % solution of ninhydrin in acetone was used for detection of AA. The TLC stationary phase was silica gel 60 F_{254}, layer thickness 0.2 mm (Merck, Darmstadt, Germany), whereas the mobile phase consisted of n-butanol: acetic acid: water (60: 20: 20). After the development, the plates were dried, dipped in the ninhydrin solution and subsequently heated in the oven at 80 °C for 5 min. The plates were visually examined.

4.2.10. Addition of the amino acids to the synthetic riboflavin

Amino acids (L-histidine and L-cysteine) were added to the powder sample of RFs to obtain mixtures containing 0.1 % of AA (w/w), according to different procedures. In the first procedure 2.5 mg of L-histidine and 2.5 mg of L-cysteine were added to RFs (4.995 g) and the sample was gently ground in an agate mortar to obtain a homogenous sample. In the second procedure L-histidine and L-cysteine (5 mg of each) were dissolved in 20 ml of distilled water. 5 ml of this solution was added to 2.5 g of RFs placed in an agate mortar and gently ground for about 10 min. The resulting paste was air dried.

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