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Maltose-mediated long-term stabilization of freeze- and spray-dried forms of bovine and porcine hemoglobin

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Abstract: Slaughterhouse blood represents a valuable source of hemoglobin, which can be used in production of heme-iron based supplements for prevention/treatment of iron-deficiency anemia. In order to obtain stable solid-state formulation, we have investigated the effect of maltose addition (30 % (w/v)) on stability and storage of bovine and porcine hemoglobin in the powders obtained by spray- and freeze- drying (without maltose: Hb; with maltose: HbM).

Differential scanning calorimetry of spray- and freeze-dried powders pointed toward satisfying quality of the formulation prepared with maltose on dissolving back into solution. After two-year storage at room temperature (20±5 °C) in solid forms, protected from moisture and light, rehydrated spray- and freeze-dried HbM were red, while Hb were brown. Dynamic light scattering showed presence of native hemoglobin monomers in rehydrated spray- and freeze- dried HbM, but their agglomerates in Hb samples. UV-VIS spectrometry confirmed an absence of a significant hemoglobin denaturation and methemoglobin formation in HbM freeze-dried powders. In spray-dried HbM, an increased level of methemoglobin was detected. The results confirmed stabilizing effect of maltose, and suggested its use in production of long-term stable solid-state formulations of hemoglobin, along with drying processes optimization.

Keywords: slaughterhouse blood; heme-iron protein; dynamic light scattering; UV-VIS spectroscopy

INTRODUCTION

Iron deficiency continues to be the leading cause of anemia worldwide, and has a substantial effect on the people’s quality of life in both low-income and
developed countries. Most of the commercial products (supplements and drugs) for prevention and treatment of iron-deficiency anemia are based on non-heme iron. A necessity for development of a new, heme-iron supplement is based on the following facts: 1) bioavailability of heme-iron is higher than non-heme iron (20-30% compared to 3-8%, respectively); 2) absorption of heme-iron is not affected by other dietary components and 3) heme-iron treatment increases serum iron level 23 times higher than non-heme-iron. The main sources of heme-iron are meat and seafood, i.e. hemoglobin and myoglobin as their constituents. In any slaughter, 4 to 5 L of blood per 100 kg of bovine/porcine mass could be obtained. Knowing that 1 L of bovine blood contains 110 g of hemoglobin and 0.4 g of heme iron, we calculated that in a technological process with an yield of 70% from blood of only one cattle, with an average weight of 500 kg, it is possible to isolate 1.7 kg of hemoglobin protein, containing 6 g of heme iron. Unfortunately, slaughterhouse blood is mainly discarded and treated as a waste exposing high pollutant capacity. If this blood is properly collected and processed, it can be used to generate high-added-value food ingredients due to its exceptional nutritive value and functional properties. It has been already shown that heme iron–rich blood products improve the iron status of animals and human subjects with anemia. From an industrial point of view, it is simpler and more cost effective to transport, store, and handle solids than liquid functional food products. However, the development of a process for hemoglobin isolation and its further conversion to long-term stable solid state is still a challenge due to susceptibility of hemoglobin to denaturation. Regarding the techniques for production of active ingredients’ solid forms on industrial scale, the most commonly used are freeze-drying (lyophilization) and spray-drying. On the other hand, these processes can cause irreversible damage to proteins, manifested as structural denaturation and loss of biological efficacy. A wide variety of agents, including sugars, polyols, amino acids, and other polymers can offer thermodynamic stabilization to proteins in liquids. The usage of sugars - whose solid state interactions with proteins and applications in food and pharmaceutical industry have been extensively described - might be the most acceptable approach to the design of hemoglobin stable forms. Nowadays trehalose is accepted as an exceptional and the most commonly used protein stabilizer. However stabilizing effect of lower-priced maltose on proteins has not been intensively studied yet. Chung et al. showed that methemoglobin formation in human maltose-embedded hemoglobin air dried films has been successfully suppressed due to maltose reducible property. Besides, retained ability of deoxyhemoglobin film to converse into oxyhemoglobin suggested strong and unexploited potential of maltose monohydrate to preserve hemoglobin structure and function. Additionally, maltose possesses the characteristic of relatively fast dissolution, sweet taste and low viscosity which provides ‘smooth melt feeling’. In this work, we investigated the effect of maltose addition to bovine and porcine hemoglobin.
solution isolated from slaughterhouse blood (bHb and pHb, respectively) on long-term stability of hemoglobin powders obtained by freeze- and spray-drying. Produced formulations were characterized using differential scanning calorimetry (DSC), dynamic light scattering (DLS) and UV-VIS spectroscopy.

**EXPERIMENTAL**

**Blood samples, hemoglobin isolation and purification**

Porcine and bovine blood was obtained from the slaughterhouse “PKB Imes” in Belgrade, Serbia. Blood collection, transportation and handling was carried out according to the protocol given in Kostić et al. Hemoglobin (Hb) was isolated by gradual hypotonic hemolysis process, also described in Kostić et al., but conducted in aseptic conditions. Hemolysates were partially purified by tangential ultrafiltration through the filters with pore size 0.2 µm and 100 kDa (Viva Flow®50, Sartorius AG, Germany). Hb concentration was determined by cyanmethemoglobin method and then adjusted to 10 gL⁻¹. In order to obtain formulations with maltose, maltose monohydrate was dissolved in the purified hemolysate (below indicated as Hb solution) to the final concentration of 30 % (w/v).

**Electrophoretic analysis**

The protein contents in Hb solution before addition of maltose were analyzed by 1) isoelectric focusing (LKB 2117 Multiphor II, LKB Instruments Ltd., UK) on a 7.5 % polyacrylamide gel with 3 % ampholyte solution on a pH gradient from 3.5 to 10 and 2) reducing SDS-PAGE (SE 260 Mighty Small II Vertical Slab Electrophoresis Unit (GE HealthCare LifeScience, USA) on 12 % gel. Proteins are visualized by Coomassie Brilliant Blue staining.

**Spray drying of hemoglobin**

Aliquots of bHb and pHb (with or without maltose) were spray-dried using a Büchi Mini Spray Dryer B-290 (Büchi, Switzerland) according to the protocol of Salvador and co-workers, under the following conditions: inlet temperature 140 °C, outlet temperature 68 °C; and flow rate, 8 mLmin⁻¹. Obtained powders were transferred to polyethylene micro tubes which were sealed with parafilm and then kept for two years in dark, at room temperature (20±5 °C), in a silica gel desiccators.

**Freeze-drying of hemoglobin**

Aliquots of bHb and pHb (with or without maltose) were lyophilized in Petri dishes with BETA 1-8 LD plus liophylizator (Martin Christ, Germany). After cooling to -70 °C, Petri dishes were transferred to the shelf of a freeze-drying apparatus. The primary drying was conducted with a shelf temperature of -60 °C for 24 h followed by -65 °C for 2 h. Obtained samples were kept the same way as described for spray-dried samples.

**Differential scanning calorimetry (DSC)**

DSC was used to measure basic thermostability or ‘susceptibility’ of proteins to thermal denaturation. DSC aluminum pans (30 µl, D. 6.7×3 mm, 08/HBB37408) with10-12 mg of the samples were hermetically sealed and analyzed using a DSC131 Evo (SETARAM Instrumentation, Caluire, France), previously calibrated with indium. An empty sealed pan was used as a reference. Both pans were placed in a chamber, kept at 30 °C for 5 min and subsequently heated from 30 to 110 °C with constant heating rate of 2 °C min⁻¹. The nitrogen flow was 20 mLmin⁻¹.
UV–VIS Spectroscopy

The UV–VIS absorption spectra of Hb samples were recorded at UV–1800 UV–VIS spectrophotometer (Shimadzu, Japan). Before analysis, solid forms of Hb were reconstituted in PBS and centrifuged 10 min at 800xg, at 4 ºC and the absorbance at 415 nm was adjusted to 1 by diluting the samples with PBS.

Dynamic light scattering (DLS)

The size distribution based on particles number of bHb and pHb preparations was analyzed by DLS using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Prior to analysis solid forms of Hb were dissolved in 0.22 µm filtered PBS and centrifuged 10 min at 800xg, at 4 ºC. All measurements were repeated three times.

Morphological examination of Hb solid forms

Morphological examination of the obtained hemoglobin solid forms by field-emission scanning electron microscopy (FE-SEM) was performed as described in Kostić et al.23

Statistics

Statistical analysis was performed with the use of Microsoft Office Excel 2007 software (Microsoft Corporation, Wash., USA). Differences between groups were tested for statistical significance (p < 0.05) by Student t-test.

RESULTS AND DISCUSSION

Prior to production of solid Hb formulations, purity of starting porcine and bovine Hb solution was estimated by SDS-PAGE and isoelectric focusing (Fig. 1A and 1B). As it can be seen from the Fig. 1A, SDS-PAGE ran under reducing condition revealed that 91±2 % of total proteins represent globin chains at 16 kDa. Besides this 16 kDa band, two weak protein bands with apparent molecular mass of 30 kDa and 70 kDa were found in prepared Hb solutions. Isoelectric focusing showed the existence of two intensive and several weak Hb bands at isoelectric point (pI) from 6.5 to 7.326,27. A weak protein band at pI 5.85 was also present (Fig. 1B). These data revealed relatively high level of the protein purity, even though we didn’t use any additional purification technique such chromatography. DLS analysis (Fig. 1C) demonstrated presence of Hb molecules with hydrodynamic diameter of ~7 nm, which corresponds to hydrodynamic diameter of native hemoglobin monomer (i.e. α2β2 globin tetramer)28, without presence of aggregates or decomposed molecules. UV–VIS absorption spectroscopy has been employed as a universal method for investigating the structural changes of the proteins29. The absorption bands at 275, 350, 413–415, 541, 576 and 630 nm, characteristic for native oxyhemoglobin, are identified (Fig. 1D). Absorption maximum at 275 nm originates from aromatic heterocyclic rings of tryptophan and tyrosine residues30 from globin chains. Coordinated-covalent bond between iron and globin chains via proximal histidine maximum is responsible for the absorption maximum at 350 nm, while porphyrin ring of heme group shows intensive absorption maximum at 413–415 i.e. Soret band31. The two absorption maxima at 541 and 577 nm (α and β) derived from oxyhemoglobin32 were detected as well. Absorbance at 630 nm, originating from methemoglobin33 was very low. Besides, UV–VIS spectra analysis
showed that minimal individual variation in Hb molecules existed (inset table in Fig. 1D), which allowed us to prepare solid formulation from pooled samples.

Figure 1. Physico-chemical characterization of bovine (bHb) and porcine hemoglobin (pHb) solution isolated from slaughterhouse blood by gradual hypotonic hemolysis and purified by tangential ultrafiltration: A SDS-PAGE under reducing conditions (sample: 25 μg protein); B: Isoelectric focusing (sample: 100 μg protein); C: Size distribution by particles number; D: representative UV-VIS spectra. Data in the inset table represent mean ±SD of four different hemoglobin samples of both species.

Spray- and freeze-drying are two the most frequently used methods of drying protein solutions in food and pharmaceutical industry. However, these techniques have also some shortcomings: they cause many destabilizing stresses that could result in irreversible protein denaturation/oxidation, if protective agents are not added. Protective effect of disaccharides such as sucrose and glucose during spray- and freeze-drying of human and porcine Hb have been described; however data on maltose efficacy to mediate stability of dried hemoglobin by these techniques are scarce. Suppression of methemoglobin formation in Hb containing maltose has been reported only for human hemoglobin films dried at room temperature (22 °C).

Guided by the optimized protocols for susceptible proteins dehydration by spray- and freeze-drying, we have prepared formulations of bovine and porcine
hemoglobin with and without maltose. As stabilizing agents, sugars are commonly used in concentration of 10-20% (w/w)\textsuperscript{21,34,36}. Nevertheless, recent study showed that even higher sugar concentration (up to 43.6% (w/v)), still have measurable effect on protein structure and stability, and more importantly, can shift the mechanism of protein stabilization from preferential exclusion (preferential hydration) to the neutral solvation (partial penetration of sugar into the hydration shell region).\textsuperscript{19} Accordingly, in this study we have examined \~1 M maltose (30% (w/v)) as bovine and porcine Hb stabilizer during freeze- and spray-drying.

Both spray-dried bHb and pHb appeared as homogeneous fine powders, while freeze-dried samples of Hb came out as a slightly clumpy powder. Maltose addition during spray drying resulted in reddish blush like color. In the freeze-dried form of Hb red color remained more prominent in the presence of maltose when compared to spray-dried powders (Fig. 2A and B). Although residual humidity of obtained powders was not determined, spray-dried powders were obviously much less hygroscopic than those obtained by freeze-drying, allowing even direct visualization by FE-SEM (Fig. 2A inset).

Figure 2. Representative photographs of: A) spray-dried and freeze-dried bovine Hb with maltose (inset represent FE-SEM micrograph of spray-dried bovine Hb with maltose), B) reconstituted spray-dried and freeze-dried bovine and porcine Hb without or with maltose (bHb, pHb, bHbM and pHbM) after two years of storage at room temperature.

Thermal unfolding and denaturation profile of maltose monohydrate and pHb and bHb solid formulations are presented in Supplementary Material as Figure S1, S2 and S3, respectively. The sample of maltose (Supp. Mat. Fig. S1) showed typical endothermic glass transition according to literature data with glass transition temperature $T_g = 122$ °C,\textsuperscript{37} $C_p = 11.5$ J g\textsuperscript{-1} K\textsuperscript{-1} at $T_g$ and $\Delta H_{relax} = 100.36$ J g\textsuperscript{-1}. Thermogram of bHb and pHb formulations obtained by freeze-
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drying (Supp. Mat. Fig. S2 A and C) demonstrated only one nearly symmetrical endothermic peak with no detectable shoulders or minor peaks, indicating that these samples are thermally homogenous. $T_m$ value as an indicator of thermal stability and $T_o$ value as a starting point of denaturation, indicated significant shifting toward higher values for bHb and pHb freeze-dried formulations with maltose (Supp. Mat. Fig. S2 B and D). DSC thermograms of spray-dried bHbM and pHbM formulations revealed the existence of peaks with two “shoulders” (Supp. Mat. Fig. S3 B and D). This is commonly seen in DSC analysis of mixtures, but can also indicate some deteriorating effects of spray- drying process on Hb stability. However, since $T_m$ value is an established parameter that can indicate potential shelf life of proteins in pharmaceutical formulation, DSC analysis preliminary pointed toward satisfying viability of Hb formulations with maltose on dissolving back into solution.

Dried Hb samples, both with or without maltose dissolved easily after two years storage. However, reconstituted spray- and freeze- dried forms of bHbM and pHbM showed significant difference even at visual examination in comparison to the samples without maltose. Formulations with maltose possessed bright red color, same as starting Hb solutions, while reconstituted powders produced without maltose were brown (Fig. 2 C and D).

The DLS results (Fig. 3A and 3C) showed that the hydrodynamic diameter of the reconstituted stored pHbM was 6.19±1.41 nm and 6.97±1.49 nm after freeze- and spray- drying, respectively. In the case of bHb, the hydrodynamic diameter of the samples prepared with maltose also kept unchanged over storage of two years, having 6.06±1.32 nm and 6.10±1.40 nm after freeze- and spray-drying, respectively. These data indicated that Hb in the presence of maltose remained in the form of native protein (i.e. undecomposed and non-aggregated α2β2 globin tetramer), regardless of the used drying method, and that good stability of the Hb complex with maltose was kept during two-years storage. On the other hand, rehydrated bHb and pHb prepared by these two drying techniques without the addition of maltose revealed the presence of agglomerates with hydrodynamic diameter of 200-500 nm (Fig. 3B and 3D).

Fig. 4 shows the absorption spectra of different dried forms of pHb and bHb, rehydrated after being stored for 2 years at room temperature (20±5 °C) and protected from moisture and light. In order to assess the ability of maltose to stabilize Hb molecules dried by these two methods, data on absorption spectra of these rehydrated formulations were compared to our laboratory “standard”, i.e. Hb solutions kept at -20 °C for the same period of time.

The heme group is hidden in the hydrophobic cavity formed by protein chains to avoid the entry of polar molecules or oxidizing agents to protect its stability. Thus, absorption maximum which originates from heme is an indicator of Hb’s deterioration.
As it can be seen from Fig. 4 only slight hypsochromic shifting of Soret band was detected when stored Hb formulations with maltose were compared with fresh sample kept at -20 °C. The values of Δα/Δβ ratio close to 1 of both spray- and freeze-dried Hb with maltose (Supp. Mat. Table SI) confirmed the low level of transformation of oxygenated to oxidized Hb during storage\(^\text{31}\). This result indicated relatively high quality of maltose dried Hb forms stored for two years at ambient temperature protected from moisture and light\(^\text{41}\). A large increase in the \(A_{\text{Soret}}/A_{\text{275}}\) ratio and a large decrease \(A_{\text{Soret}}/A_{\text{577}}\) ratio which reflect the considerable presence of free heme and breakdown of Hb molecule, respectively\(^\text{31}\) were not detected for freeze-dried Hb with maltose (Supp. Mat. Table SI).

Very high \(A_{\text{Soret}}/A_{\text{577}}\) values for formulation with maltose probably reflect intensive Hb degradation through unstable Hb intermediates (ferryl/ferryl radical) which oxidize residues within globin chains and lead to Hb degradation\(^\text{42}\).

Comparable quality of maltose dried Hb forms with Hb solution stored at -20 °C (Supp. Mat. Table SI) opens the possibility to avoid storage in freezer, which is impractical (or more precisely, almost impossible) on large scale. During spray-drying, as well as freeze-drying, substantial oxidation of Hb to methemoglobin occurred in the absence of maltose (Fig. 4C and 4D, Table SI in Supp. Mat.).
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Figure 4. Representative UV/VIS spectra of freeze-dried: A) and B) and C) and D) spray dried bovine and porcine hemoglobin without (bHb and pHb) and with maltose (bHbM and pHbM) rehydrated after being stored for 2 years at ambient temperature.

At a given concentration, maltose suppressed methemoglobin formation in bovine and porcine samples more effectively during freeze drying than in spray drying (Fig. 4A and 4B, Supp. Mat. Table S1). Similar results were demonstrated by Labrude et al. in the case of sucrose usage as a protectant for spray-dried human Hb. This was somewhat expected since spray drying as dehydration process include exposition of sample to higher temperature than in freeze drying process, accelerating Hb autoxidation and easier access of oxidizing agents to the heme pocket. Several hypotheses have been suggested to explain protective effect of sugars on proteins. According to "water replacement hypothesis" sugars hydrogen-bond to biomolecules during dehydration, acting as substitutes of hydration water molecules, especially when high concentration of sugars (>20%) is used. "Preferential hydration" hypothesis suggests that sugars, rather than directly binding to biomolecules, entrap the residual water at the interface by glass formation, thus preserving the native solvation. And the third "high viscosity" hypothesis considers the large viscosity of the host glassy matrix responsible for protection for low-water systems since it causes motional inhibition and hindering of the dynamic process. Probably all these mechanisms contributed to preserved native structure of pHb and bHb after their conversion to solid forms by freeze- and spray- drying in the presence of maltose.
CONCLUSION

In the present study, we assessed the impact of sugar maltose on the stability of bovine and porcine hemoglobin in the solid state obtained by spray- and freeze-drying. DSC showed that maltose addition in hemoglobin solution shifted starting point of thermally induced denaturation and melting point of dried hemoglobin forms toward higher values, confirming its protective effect. After having been stored for two years at room temperature (20±5 °C), protected from moisture and light, reconstituted solid formulation of bovine and porcine hemoglobin with maltose retained color and physico-chemical characteristics same as the starting hemoglobin solution and demonstrated the absence of biologically inactive methemoglobin, as verified by DLS and UV-VIS spectroscopy. Although encouraging, these preliminary results on maltose use as stabilizing additive indicate the need for more specific optimization of drying processes parameters themselves, with an aim of production of long-term stable solid-state formulations of hemoglobin.

SUPPLEMENTARY MATERIAL

Supplementary Material are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

ОТРАЈНА СТАБИЛИЗАЦИЈА СПРЕЈ СУШЕНОГ И ЛИОФИЛИЗОВАНОГ ГОВЕЂЕГ И СВИЊСКОГ ХЕМОГЛОБИНА МАЛТОЗОМ

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Хемоглобин из еритроцита добијених из отпадне кланичне крви је добро познат, али недовољно искоришћен, богат извор хемског гвожђа. У циљу добијања стабилне формулаје овог протеина у чврстом стању, испитан је ефекат додавања малтозе у говеђи и свињски хемоглобин изолован из кланичне крви током спреј сушења и лиофилизације. Ефекат малтозе је процењен на основу резултата анализе добијених прахова након две године складиштења на собној температури (20±5 °C), заштићено од влаге и светлости. Пре складиштења урађена је анализа формулаја добијених спреј сушењем и лиофилизацијом, анализа распределе подела по величини, а на основу резултата анализе добијених прахова након две године складиштења на собној температури (20±5 °C), заштићено од влаге и светлости. Пре складиштења урађена је анализа формулаја добијених спреј сушењем и лиофилизацијом, а на основу резултата анализе добијених прахова након две године складиштења на собној температури (20±5 °C), заштићено од влаге и светлости.

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значајне денатурације хемоглобина и формирања метхемоглобина у формулацијама са малтозом добијеним лиофилизацијом, док је у чврстим формама спреј осушених хемоглобина са малтозом показала повећано присуство метхемоглобина. Резултати ове студије су потврдили ефекат малтозе као стабилизирајућег адитива и њену потенцијалну употребу у производњи дугорочно стабилних чврстих форми хемоглобина, али указали и на потребу за додатном оптимизацијом параметара процеса сушења.

(Примљено 13. маја; ревијирано 22. јуна; прихваћено 24. јуна 2019)

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On Line First
SUPPLEMENTARY MATERIAL TO
Maltose-mediated long-term stabilization of freeze- and spray-dried forms of bovine and porcine hemoglobin

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Figure S1. DSC thermogram of un-aged amorphous maltose monohydrate showing the glass transition and enthalpy relaxation endotherm at the glass transition temperature

Cp continuous, Jg⁻¹ K⁻¹
Sample temperature, °C
Heat Flow, mW

On Line First
Fig. S2. Thermal unfolding and denaturation profile of freeze-dried A) bovine hemoglobin without maltose (bHb) and B) with maltose (bHbM), C) porcine hemoglobin without maltose (pHb) and D) with maltose (pHbM)

Fig. S3. Thermal unfolding and denaturation profile of spray-dried A) bovine hemoglobin without maltose (bHb) and B) with maltose (bHbM), C) porcine hemoglobin without maltose (pHb) and D) with maltose (pHbM)
Table S1. Spectral characteristics of spray-dried and freeze dried pooled bovine and porcine hemoglobin without (Hb) and with maltose (HbM), rehydrated after 2 years storage at ambient temperature. Hemoglobin stored at -20 °C represents a control of the measurement.

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<th>Soret band, nm</th>
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<td>Hb -20 °C</td>
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<td>3.93</td>
<td>11.41</td>
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<tr>
<td>Hb spray-dried</td>
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<td>0.04</td>
<td>4.27</td>
<td>28.78</td>
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<td>HbM spray-dried</td>
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<tr>
<td><strong>Porcine Hb</strong></td>
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<tr>
<td>Hb -20 °C</td>
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<tr>
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<td>0.90</td>
<td>3.49</td>
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* Δα/Δβ = (A_{577} - A_{560}) / (A_{541} - A_{560})