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Impact of four inorganic impurities – iron, copper, nickel and zinc - on the quality attributes of a Fc-fusion protein upon incubation at different temperatures

Supervisors:

Prof. Mariano Stornaiuolo

Dr. Roberta Verani

Dr. Irene Cecchini

Candidate:

Dr. Alessandra Pistacchio

Coordinator: Prof. Maria Valeria D'Auria

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Abstract

A key aspect that must be supervised during the development of a biotherapeutic is the presence of elemental impurities in the final drug product: they must be quantified as to ensure that their concentrations does not affect patients' safety. Regulatory guidelines such as ICH Q3D provides Permitted Daily Exposure (PDE) limits for those impurities considered having a higher potential safety risk. However, one of the limits of such PDE values is that they account for the safety risk, without considering the "quality" one, intended as which are the alterations to the Quality Attributes (QA) of a biologic.

The aim of this Ph.D. project was to examine how four commonly observed elemental impurities could impact the QAs of a Fc-fusion protein, in order to understand how they could affect not only the safety of patients, but also the physicochemical properties of biotherapeutics. To this aim a Fc-fusion protein was treated with increasing concentrations of Ni^{2+}, Cu^{2+}, Zn^{2+} and Fe^{3+} and analysed under normal storage conditions, after 6 and 44 weeks of incubation at different temperatures (+5°C, +25°C, +40°C). The potential changes in conformation, oxidation, aggregation, and fragmentation were monitored. Our data suggest that keeping the levels of these impurities under the safety threshold limits does not guarantee the product quality. While nickel and zinc slightly altered the physicochemical properties of our Fc-fusion protein, iron and copper appeared to be more harmful for the QAs stability. Indeed, these latter elements might cause significant alterations of the product quality such as to potentially alter its efficacy.

The results presented in this thesis have been submitted to Plos One Journal, and are currently under review:

1. *Impact of four inorganic impurities – iron, copper, nickel and zinc - on the quality attributes of a Fc-fusion protein upon incubation at different temperatures*.

Alessandra Pistacchio[1,2], Fabio Baroni[2*], Irene Cecchini[2], Roberta Verani[2], Mariano Stornaiuolo[1], Angelo Palmese[2], Carlo Pergola[2].

[1]Department of Pharmacy, University of Naples Federico II, Naples, NA, Italy

[2]Analytical Development Biotech Department, Merck Serono S.p.a, Guidonia, RM, Italy; an affiliate of Merck KGaA.

1. Introduction:

The aim of this Ph.D. project was to evaluate the immediate and long-term effects of different chemical impurities (leachables) that can be detected in Drug Products (DP) on the Quality Attributes of an Fc-fusion protein.

Fc-fusion proteins are composed of the Fc domain of IgG genetically linked to a peptide or protein of interest [1]. This class of biotherapeutics, together with monoclonal antibodies (mAbs), are widely used to treat different diseases and in the last 30 years have experienced a constant development due to their versatile applications. In this study a Fc-fusion protein developed by Merck KGaA was used.

The alteration of the structure and the formation of related structural variants such as oxidized forms and aggregates are considered critical quality aspects to be carefully considered and controlled during biopharmaceuticals development, in order to guarantee the product efficacy and safety [2]. Immunogenicity, in particular, is one of the safety's aspects of major concern since closely related to such alterations. Indeed, a common drawback of biotherapeutics is the capacity to potentially provoke immune responses; immunogenicity is basically a patient's undesired immune response to a biologic, generally leading to the formation of anti-drug-antibodies (ADAs) capable to inactivate the drug or, in the worst scenario, to induce a severe adverse effect.

Such alterations may depend, among other factors, on the interaction of the molecule with chemical impurities, potentially present in the drug product (DP) as residual manufacturing impurities or leachables [3].

Leachables [3] are chemical entities, both organic and inorganic, that migrate from contact surfaces such as components of container and closure systems of the manufacturing process and of drug product delivery systems. The migration of these

compounds may occur under normal conditions of exposure or during stability studies as a result of a direct contact with the drug product. The above-mentioned substances are identified as “primary leachables”, while “secondary leachables” are identified as new compounds that may be formed upon interaction of a primary leachable with the DP, or the excipients. While major organic leachables arise from plastics and elastomers, inorganic ones are mainly represented by metal ions (such as iron, copper, zinc, nickel, manganese, aluminum, etc.) deriving from various sources, such as stainless steel [4-5], glass vials and rubber stoppers. Metal ions may also be present in the product as process-related residual impurities - being widely used in the cell culture media and in the feed during the manufacturing process - and/or as impurities of buffers and excipients employed.

Critical Quality Attributes (CQA) are physical, chemical, biological, or microbiological properties or characteristics that should be within an appropriate limit, range, or distribution to ensure the desired product quality [6]: their definition and characterization are key components of pharmaceutical development under the Quality by Design (QbD) paradigm. It is known that metal ions may alter those of Fc-fusion proteins and mAbs affecting safety, efficacy and stability of drug products in many ways [5]. For instance, they can alter pH, induce protein precipitation, aggregation, fragmentation or oxidation and the overall consequences of such alterations may affect the biological activity of the drug or potentially induce immunogenicity [7].

For example, a study in 2009 [8] highlighted that microparticles of tungsten leached inside prefilled syringes (tungsten pins are commonly used to create the holes for placement of the staked-in needles). Such microparticles, through electrostatic interactions, were able to induce aggregation and formation of particles in the Fc-fusion protein tested.

Another study [9] demonstrated that the interaction of copper (Cu^{2+}) with the histidine residues of an IgG_1 induced fragmentation at the hinge region of the molecule in a pH range 5-7.

Silicon-oil is commonly used as lubricant or coating agent in syringes, needles, rubber stoppers and other pharmaceutical containers: Thirumangalathu et al. [10] investigated the relationship between mAb aggregation and presence of silicon-oil under shaking (mimicking shipping and handling conditions). Their results demonstrated that the combination of silicon oil and agitation increases the air–water interfacial area available for adsorption of proteins, leading to structural perturbations and eventually to aggregation.

Kumar et al. [11] focused on Metal Catalyzed Oxidation (MCO), since protein oxidation may be highly impacted by elements such as Mg^{2+}, Zn^{2+}, Fe^{2+} and often precedes or occurs simultaneously to aggregation. The study highlighted that cysteine, histidine and methionine were the most susceptible residues to MCO, while tryptophan and tyrosine were significantly impacted by both MCO and light.

In addition, one case of immunogenicity resulting from the interaction between leachables and biotherapeutics has also been reported in literature. Amongst patients administered with Eprex [12], a human erythropoietin (i.e., epoetin-α) used in anemia disease, an increased incidence of pure red cell aplasia (PRCA) was reported. Sharma et al. [13] observed that the cause was the formation of a compound upon interaction of Polysorbate 80, an excipient of the DP formulation, and other chemical impurities originating from uncoated rubber stoppers.

Given the potential unwanted effects of leachables, it is not surprising that both FDA [14-15] (Food and Drug Administration) and EMA [16] (European Medicines Agency) have issued regulatory requirements to monitor and control them as part of the

requisites for the documentation of drug products' primary packaging. Among these regulatory requirements the International Conference on Harmonization (ICH) Q3D [17] classifies elements based on their inherent toxicity and provides Permitted Daily Exposure (PDE) limits for those considered having a higher potential safety risk. This index is used to assess the *Concentration Limit* of each elemental impurity that may be present in the final product without risk for patients' safety.

One of the limits of the PDE value is that it only accounts for the safety risk, without considering the "quality" one, intended as which are the alterations to the QAs of a biologic. This work is therefore focused at clarifying the impact that commonly observed elemental impurities (metal ions) have on selected Quality Attributes (QAs) of an Fc-fusion protein in order to understand how they can affect not only the safety of the patients, but also the quality of a biotherapeutic. To this aim, we evaluated the immediate and long-term effects (under thermal stressed conditions) of four inorganic leachables (i.e., nickel, zinc, copper and iron ions) on conformation (secondary and tertiary structure), oxidation, aggregation and fragmentation.

1.1 Study Overview

An Fc-fusion protein - comprised of an IgG1 with an extracellular domain (ECD) protein fused to the C-terminus via a linker (**Figure 1**)- has been treated with three different concentrations of Ni^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+} (**Table 1**) and the effects on the quality attributes of the molecule have been evaluated after incubation of samples at time zero at +5°C (to simulate the long-term storage temperature), after 6 weeks at both +25°C (to reproduce accelerated stability conditions) and +40°C (to reproduce stressed stability conditions). The incubated samples after 6 weeks were monitored only at +25°C and +40°C, since +5°C is the normal storage temperature of the drug product and

in such a short time it is not expected any change in QAs based on our accumulated experience on the molecule.

Figure 1. Graphical representation of Fc-fusion protein's structure.

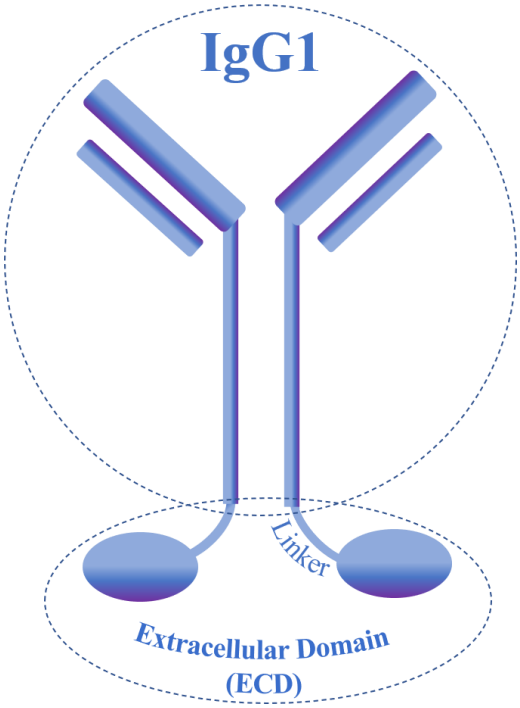


Table 1. Overview of the selected treatments

Element	**Concentration 1**	**Concentration 2**	**Concentration 3** [(d)]
Fe^{3+}	2.2 ppm	2.7 ppm [(b)]	5.5 ppm
Cu^{2+}	0.050 ppm	0.40 ppm [(a)]	1.0 ppm
Ni^{2+}	0.026 ppm [(a)]	0.50 ppm	1.5 ppm
Zn^{2+}	0.055 ppm [(c)]	2.0 ppm	5.5 ppm

[(a)] *Concentration limit* based on PDE as per ICH Q3D – Guideline for elemental impurities Q3D, ICH Harmonized Guideline

[(b)] *Concentration limit* based on PDE calculated based on "European Medicines Agency – Assessment report Ferric, Ferric citrate coordination complex. Procedure No. EMEA/H/C/003776/0000"

[(c)] 30% PDE calculated based on "U.S. Environmental Protection Agency – Toxicological Review of Zinc and Compounds (CAS No. 7440-66-6)"

[(d)] Minimum concentration that caused an alteration to the product, as per preliminary study (see section 1.2 Preliminary study).

Eventually, incubated samples at +5°C, +25°C and +40°C were analysed also after 44 weeks, with a reduced analytical panel, revised upon the evaluation of the results obtained in previous time points.

Circular Dichroism (CD) and Fourier Transform-IR (FT-IR) were chosen to monitor the protein's conformation, Reverse Phase-UPLC (RP-UPLC) to monitor oxidation, Size Exclusion-UPLC (SE-UPLC) to monitor aggregation and fragmentation.

Additional tests were employed as well on selected samples to gain further insights into the obtained results: methionine quantification by Reverse Phase-UPLC, peptide mapping and Intact Molecule analysis by LC-MS, ICP-MS, surface plasmon resonance (SPR) by Biacore and SEC-MALS. An overview of the analytical panels applied at each time point is reported in **Table 2**.

Table 2 – Overview of the analytical panels.

Time Point	Analytical Technique
Time zero	CD; FT-IR, RP-UPLC (oxidation); SE-UPLC.
After 6 weeks	CD; FT-IR, RP-UPLC (oxidation); SE-UPLC. Additional tests on selected samples: RP-UPLC (amino acids quantification); SEC-MALS, Peptide Mapping by LC-MS; Intact Molecule analysis by LC-MS; SPR by Biacore
After 44 weeks	RP-UPLC (oxidation); SE-UPLC; Visual Inspection; pH measurement

The choice of the four elements was based on several reasons. Zinc, copper and iron are intentionally added in the cell culture media and in the feed during the

manufacturing process: despite an efficient product purification process is in place, inevitably some traces persist and consequently are found in the DP. The choice of copper and iron was also based on the preliminary evidence of their impact on the QAs of a biologic, as reported in literature [9-13].

Nickel, instead, (together with iron) was chosen as it is one of the main components of stainless steel [18] and therefore very commonly found in the final DP, even if in very low quantities.

The four inorganic impurities have been added in the form of salts. Zinc (II) sulphate monohydrate, copper (II) sulphate anhydrous and ammonium iron (III) citrate were chosen as used in the manufacturing process, while in case of nickel (not included in the media composition), nickel (II) sulphate hexahydrate was added as it presents the same counter-ion selected for the other target elements.

For each metal ion the concentration levels, reported in **Table 1**, were chosen based on different reasons. The first criteria for the choice was based on their PDEs [17] levels (the *Concentration Limit* was calculated as 0.3x PDE/daily dose), considering the safety aspect. However, frequently such levels are too low to cause any observable effect on the product therefore preliminary experiments (see **Appendix I**) were carried out to determine for each element which was the minimum concentrations that could cause a measurable effect on the examined protein. This concentration corresponds to the highest level applied for each element (Concentration 3). In addition to these two concentration levels, a third one was established for each element considering the concentration intervals: in some cases this was an intermediate value, in other cases this was a lower one.

It should be noted that while for nickel and copper a PDE is established by the guideline, for zinc and iron it is not: for these elements PDEs were therefore calculated

according to ICH Q3D's Appendix 1 (Method for establishing exposure limits) employing suitable documents [17]. Knowing that the daily dose of the tested Fc-fusion protein was established as 2550 mg/day (DP concentration 10 mg/ml) and considering its parenteral administration, the *Concentration Limit* values (Fe^{3+} = conc 2; Cu^{2+}= conc 2; Ni^{2+}= conc 1; Zn^{2+} conc 1) were calculated as follow.

- IRON:
    - PDE calculation:

$$PDE = \frac{NO(A)EL \text{ x } Mass\ Adjustment}{[\text{F1 x F2 x F3 x F4 x F5}]}$$

Where:

NO(A)EL = No-Observed-Adverse-Effect Level

Mass Adjustment = The mass adjustment assumes an arbitrary adult human body mass for either sex of 50 kg.

F1 = A factor to account for extrapolation between species (1 for human data; 5 for extrapolation from rats to humans; 2 for extrapolation from dogs to humans etc.)

F2 = A factor of 10 to account for variability between individuals.

F3 = A variable factor to account for toxicity studies of short-term exposure (10 for studies of a shorter duration; 2 for a 6-month study in rodents, or a 3.5-year study in non-rodents etc.)

F4 = A factor that may be applied in cases of severe toxicity, e.g., non-genotoxic carcinogenicity, neurotoxicity or teratogenicity.

F5 = A variable factor that may be applied if the NOEL was not established

The NOAEL of Ferric (III) Citrate reported on *European Medicines Agency – Assessment report Ferric, Ferric citrate coordination complex.*

Procedure No. EMEA/H/C/003776/0000 was established as = 400 mg/kg/day. Therefore the NOAEL of Fe^{3+} is = 91,19 mg/kg/day

$$PDE\ oral = \frac{91.19\ mg/Kg/day\ \ x\ \ 50\ Kg}{[2\ x\ 10\ x\ 10\ x\ 1\ x\ 1]} = \ 22.8\ mg/day$$

As the bioavailability of Iron citrate is 31% the modifying factor is 10

$$PDE\ parenteral = \frac{22.8\ mg/day}{10} = \ 2.28\ mg/day$$

- $PDE_{Parenteral}$: 2280 µg/day

$$Concentration\ Limit\ = \frac{30\%\ of\ PDE}{Daily\ amount\ of\ drug\ product}$$

$$Concentration\ Limit\ = \frac{0.3\ x\ 2280\ \mu g/day}{2.55\ g/day} = \ 268.2\ \mu g/g$$

Related to the DP concentration:

$$Concentration\ limit = \ 268.2\ \mu g/g\ \text{x}\ 10\ \text{g/L} = \ 2682\ \mu g/L\ (ppb) =$$

$$= 2.7\ mg/L\ (ppm)$$

- COPPER:

$PDE_{Parenteral}$: 340 µg/day

$$Concentration\ Limit\ = \frac{30\%\ of\ PDE}{Daily\ amount\ of\ drug\ product}$$

$$Concentration\ Limit\ = \frac{0.3\ x\ 340\ \mu g/day}{2.55\ g/day} = \ 40\ \mu g/g$$

Related to the DP concentration:

$$Concentration\ limit = \ 40\ \mu g/g \text{ x } 10 \text{ g/L} = \ 400\ \mu g/L\ (ppb) =$$

$$= 0.4\ mg/L\ (ppm)$$

- ZINC:
    - PDE calculation:

$$PDE = \frac{NO(A)EL \text{ x } Mass\ Adjustment}{[\text{F1 x F2 x F3 x F4 x F5}]}$$

Where:

NO(A)EL = No-Observed-Adverse-Effect Level

Mass Adjustment = The mass adjustment assumes an arbitrary adult human body mass for either sex of 50 kg.

F1 = A factor to account for extrapolation between species (1 for human data; 5 for extrapolation from rats to humans; 2 for extrapolation from dogs to humans etc.)

F2 = A factor of 10 to account for variability between individuals.

F3 = A variable factor to account for toxicity studies of short-term exposure (10 for studies of a shorter duration; 2 for a 6-month study in rodents, or a 3.5-year study in non-rodents etc.)

F4 = A factor that may be applied in cases of severe toxicity, e.g., non-genotoxic carcinogenicity, neurotoxicity or teratogenicity.

F5 = A variable factor that may be applied if the NOEL was not established

The NOAEL of Zn^{2+} reported on *U.S. Environmental Protection Agency – Toxicological Review of Zinc and Compounds (CAS No. 7440-66-6)* was established as = 0,94 mg/kg/day.

$$PDE\ oral = \frac{0.94\ mg/Kg/day\ \ x\ \ 50\ Kg}{[1 \text{ x } 10 \text{ x } 10 \text{ x } 1 \text{ x } 1]} = 0.47\ mg/day$$

As the bioavailability of Zinc is 10-40% the modifying factor is 10

$$PDE\ parenteral = \frac{0.47\ mg/day}{10} = 0.047\ mg/day$$

- $PDE_{Parenteral}$: 47.0 µg/day

$$Concentration\ Limit\ = \frac{30\%\ of\ PDE}{Daily\ amount\ of\ drug\ product}$$

$$Concentration\ Limit\ = \frac{0.3\ x\ 47.0\ \mu g/day}{2.55\ g/day} = 5.53\ \mu g/g$$

Related to the DP concentration:

$$Concentration\ limit = 5.53\ \mu g/g \text{ x } 10\ \text{g/L} = 55.3\ \mu g/L\ (ppb) =$$

$$= 0.055\ mg/L\ (ppm)$$

- NICKEL:

  $PDE_{Parenteral}$: 22 µg/day

$$Concentration\ Limit\ = \frac{30\%\ of\ PDE}{Daily\ amount\ of\ drug\ product}$$

$$Concentration\ Limit\ = \frac{0.3\ x\ 22\ \mu g/day}{2.55\ g/day} =\ 2.59\ \mu g/g$$

Related to the DP concentration:

$$Concentration\ limit =\ 2.59\ \mu g/g\ \text{x}\ 10\ \text{g/L} =\ 25.9\ \mu g/L\ (ppb) =$$

$$= 0.0259\ mg/L\ (ppm)$$

2. Materials and methods:

2.1 Sample preparation

In order to keep constant the concentration of the Fc-fusion protein in all samples (9 mg/mL), the treatment of the selected elements was performed by adding to 24.3 mL of the protein solution, 2.7 mL of different stock solutions of the four elements, each one corresponding to one of the titration points.

It is important to note that these stock solutions were prepared starting from a *Main Stock Solution*, analysed by ICP-MS (Inductively Coupled Plasma Mass Spectrometry) to determine the exact content of the metal ion. Three dilutions of this *Main Stock Solution* were performed in order to prepare the three specific concentrations stock (*Final Stock Solutions*), as reported in **Table 3.**

Table 3. Sample Treatments - Stock solutions and concentrations used to treat samples

Element	**Concentration of Main Stock solution tested by ICP-MS**	**Final Stock Solution of each concentration**	**Final Concentration in Protein/Placebo Samples***
Fe^{3+}	210 ppm	Stock Iron conc 1 = 22 ppm	2.2 ppm
		Stock Iron conc 2 = 28 ppm	2.8 ppm
		Stock Iron conc 3 = 55 ppm	5.5 ppm
Cu^{2+}	420 ppm	Stock Copper conc 1 = 4 ppm	0.40 ppm
		Stock Copper conc 2 = 0.5 ppm	0.050 ppm
		Stock Copper conc 3 = 10 ppm	1.0 ppm

Zn^{2+}	418 ppm	Stock Zinc conc 1 = 0.55 ppm	0.055 ppm
		Stock Zinc conc 2 = 20 ppm	2.0 ppm
		Stock Zinc conc 3 = 55 ppm	5.5 ppm
Ni^{2+}	238 ppm	Stock Nickel conc 1 = 0.26 ppm	0.026 ppm
		Stock Nickel conc 2 = 5 ppm	0.50 ppm
		Stock Nickel conc 3 = 15 ppm	1.5 ppm

* 2.7 ml of *Final Stock Solution* + 24.3 ml protein or placebo solution

The tested concentrations are reported in **Table 1**. The placebo (same composition of DP without the Fc-fusion protein) of the DP (10mM histidine, 5mM methionine, 6% trehalose, 40 mM NaCl and 0.05% Tween20 pH 5.5±0.1) was treated as well with the same rationale used for the samples.

Each sample was prepared in triplicate and then incubated at +5°C, +25°C and +40°C. A volume of untreated Fc-fusion protein and untreated placebo was incubated as well at the same storage conditions, as reference materials.

Samples were stored in Fiolax clear TopLine (Schott) vials – same ones employed for the DP storage - to ensure no influence by glass components. Additionally, all samples were stored in sealed boxes in order to protect each vial from light.

2.2 Inductively coupled plasma mass spectrometry - ICP-MS

To verify the exact concentration of each *Main Stock Solution* a NexION XX Perkin Elmer ICP/MS equipped with an autosampler ESI Mod. SC-2 DX was used. This technology couples ICP (Inductively Coupled Plasma) and Mass Spectrometry (MS) for elemental analysis by generation of ions. The ICP is involved in generation of a high temperature plasma source, through which the pre-treated sample is passed.

Elements in a sample at such high temperature are ionized and directed further into the MS that, according to their mass/charge ratio, identifies and quantifies each ion. The instrument was optimized following the standard procedures recommended by the vendor.

Stock solutions were submitted to a mineralization with a microwave digestion procedure before proceeding with the ICP/MS analysis. To samples contained within a teflonated MARSXpress vessel was added Nitric acid (HNO3 65%), Hydrogen Peroxide (H2O2), Hydrochloric Acid (HCl 30%) and deionized water (MilliQ). Samples were then placed in the microwave oven for 25 min, at +160°C, 1800 W. Eventually, the internal standard stock solution was added. The operating conditions for ICP-MS are summarized in **Table 4**:

Table 4. ICP-MS Operating conditions

Component / Parameter	Type / Value / Mode
RF Power	1600 Watts
Plasma gas flow	16 L min-1
Auxiliary gas flow	1.2 L min-1
Nebulizer gas flow	0.80 - 1.0 L/min
Nebulizer	Meinhard® PTFE micro-concentric
Spray chamber	Refrigerated PC3 Glass Cyclonic
Operative mode	KED mode (collision using He gas flow)
Triple Cone Interface Material	Nickel / Aluminium / Platinum
PC^3 temperature	+ 2°C ÷ + 5 °C
No. of Sweeps/ Readings / Replicates	30/1/3

2.3 Circular Dichroism spectroscopy

Circular Dichroism (CD) is a spectroscopic technique that can provide a fingerprint of the overall conformation by measuring the optical activity of asymmetric molecules in solution, in terms of absorption of circularly polarised light.

CD measurements in the Far-UV (190 – 250 nm) yields information on secondary structure as this region is dominated by the absorbances of the peptide bonds while measurements in the Near-UV (260-340 nm) yields information on the tertiary structure as the region is dominated by the contributions of aromatic amino acids and disulphide bonds [19].

In case of our Fc-fusion protein only Near-UV measurements were performed in the stability study as in the course of our preliminary one (**Appendix I**) we observed high variability and non-reproducibility of the Far-UV data. An orthogonal technique – FT-IR spectroscopy – was instead used to monitor the molecule's secondary structure.

The CD spectra in the Near-UV region (250-340 nm) were collected on a Chirascan V100 Spectropolarimeter (Applied Photophysics Ltd) at room temperature using cuvettes with a path length of 10 mm. Spectra have been collected at a step size of 0.5 nm, a bandwidth of 1 nm and a response time of 2 s with a 3-scan average. Samples, prepared as described in the *sample preparation* section, were diluted to 1 mg/ml in water MilliQ. After subtraction of the baseline (DP placebo diluted in water MilliQ with the same samples' dilution factor) the spectra were normalized for the value of absorbance at 280 nm.

The normalized CD spectra have been compared by means of the "Chirascan qBiC Comparison Suite" software, provided by the instrument supplier, that allows to calculate a numeric value expressing similarity among spectra: the Weighted Spectral Difference (WSD) [20,21]. This parameter accounts for the absolute differences in

signals between spectra, taking into account both intensity and profile variations. Treated samples were considered comparable to the reference material if their WSD was between 0 (identity) and WSDmean+2STD, where WSDmean is the average of the WSDs calculated for four replicates of the untreated samples incubated at the same temperature.

The statistical analysis was performed using Minitab 19 software by applying the tool *Basic statistic – Graphical Summary* that provides a report with several descriptive statistic tools, such as mean, standard deviation and graphical representation of the population distribution together with the identified outlier/s (asterisk in the box-plot). More in detail, outliers' calculation was done using a data set of nine data points (three replicates per concentration).

2.4 FT-IR spectroscopy

FT-IR is a spectroscopic technique able to detect changes in the vibrational states of a molecule's bonds. The sample is shined by infrared radiation and a characteristic spectrum is produced based on the absorption at each wavelength.

Comparability of the secondary structure between different samples can be assessed by looking to the bands originating from the vibrations of C=O stretches (amide I band) and to that originating from N-H bends and C-N stretches (amide II band) [22].

The analyses were performed on a Tensor II FT-IR spectrometer (Bruker) equipped with a BioATR II cell, suitable for samples in solution, coupled to an LN-MCT Photovoltaic detector. The 256-scans interferograms were recorded at ambient temperature from 3900 cm^{-1} to 1000 cm^{-1} with a resolution of 4 cm^{-1}, scanner velocity set to 30 kHz and zero filling set to 8. For each sample, the blank spectrum subtracted

was obtained by analysing the eluate collected after centrifuging 200 µl of the sample at 14000 rmp in Amicon vials (cut-off 10 kDa) for 5 minutes.

Bruker OPUS software was used for the calculation of the second derivative spectra (Savitzky-Golay algorithm).

Treated samples were considered comparable to the reference material if their amide I and II values fell within the minimum and maximum values obtained for the three replicates of untreated material incubated at the same temperature.

No normalization was performed.

2.5 Size Exclusion Chromatography-UPLC (SEC-UPLC)

SEC-UPLC is a chromatographic method in which molecules in solution are separated by their size and, in this case, by their molecular weight. The column is filled with material containing many pores of known size, therefore, when dissolved molecules flow into the column smaller ones will pass it more slowly since they penetrate deep into the pores. On the contrary, larger molecules will flow quickly since they do not enter the pores. Consequently, species with larger molecular weight will elute from the column sooner while species with smaller molecular weight will elute later [23].

SEC-UPLC experiments were performed with an Aquity UPLC Waters instrument using an Acquity UPLC Protein BEH SEC Column (200Å, 1.7µm, 4.6 x 150mm, 1/pkg). The column temperature was set at +30°C ± 5°C and the autosampler temperature at +5 ± 3°C. The UPLC was equipped with a 50 µL loop and a 250 mL syringe.

The samples, prepared as described in the sample preparation section, were diluted with Phosphate Buffer Solution 1X (PBS) to a final concentration of 0.25 mg/ml. For each of them were loaded 20 µL and the acquisition was carried out at 214 nm, with

an isocratic gradient of 100% PBS 1x and a flow rate of 0.3 ml/min. The duration of each analysis was 10 minutes with a sampling rate of 10.

Treated samples were considered comparable to the reference material if the value of % Area of High Molecular Weight (HMW), % Area of Low Molecular Weight (LMW) and % Area of Monomer fell within the minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature.

Waters Empower software, provided by the instrument supplier, was employed for the analysis of the data.

2.6 Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS)

To characterize the nature of the LMW detected by SE-UPLC, samples incubated for 6 weeks at +40°C (one replicate per sample) were analysed by SEC-MALS a technique coupling size exclusion chromatography (SEC) to an in-line multi-angle light scattering detector and to an in-line refractive index or UV/Vis detector. The Multi-Angle Light Scattering (MALS) measures the amount of light scattered by particles in solution relative to the angle of the incident light, allowing to directly measure the absolute molar masses of each species detected by SEC-HPLC [24].

SEC analysis was carried out on an Agilent 1260 HPLC system equipped with an XBridge Protein BEH SEC 200Å x 300 mm column (Waters). Separation was achieved at ambient temperature under isocratic conditions using a PBS 1X pH 7.4±0.1mobile phase at a flow rate of 1.0 ml/min; the duration of each run was 20 minutes. MALS analysis was performed continuously on the SEC column eluate, as it passed through a DAWN-HELEOS II MALS detector in series with an Optilab T-rEX refractive index detector (Wyatt Technologies, Inc.).

Samples, prepared as described in the sample preparation section, were diluted in Phosphate Buffer Solution 1X to a final concentration of 0.5 mg/ml. 200 µL of each diluted sample were loaded on the column and UV detection was set to 280 nm as to allow the determination of the molar masses employing the molecule's molar extinction coefficient.

Data were analysed using the Astra software version 7.3.1 (Wyatt) employing a protein concentration determined by the UV detector.

2.7 Reverse Phase-UPLC (RP-UPLC) – Methionine oxidation

RP-UPLC was used to monitor the most relevant oxidation site of the molecule previously localized by peptide mapping studies: a methionine located on the heavy chain of the Fc-fusion protein, in the ECD domain (Met-ECD1). The technique is based on the use of a polar mobile phase - in this case acetonitrile (ACN) - and a stationary phase that is represented by the non-polar material of the column. In these conditions the more polar analytes, *i.d.* oxidized methionines (methionine sulfoxide), elute sooner than non-polar analytes, *i.d.* intact methionine, which are retained longer [25].

Prior to the RP-UPLC analysis all samples and placebos were submitted to a series of pre-treatment steps, ultimately aimed at the enzymatic digestion and de-glycosylation of the Fc-fusion protein.

RP-UPLC experiments were performed with an Aquity UPLC Waters instrument using an Acquity UPLC Peptide BEH C18 column (130Å, 1.7 µm, 2.1 mm x 100 mm, 1/pkg). The column temperature was set at +60°C ± 5°C and the autosampler temperature at +5 ± 3°C. The mobile phases were composed of 2% Acetonitrile, 0.1% Trifluoroacetic acid (Mobile phase A1) and of 80% Acetonitrile, 0.1% Trifluoroacetic acid (Mobile phase B1). The separation was achieved in 20 min linear gradient from 18% to about 24% phase B1. The duration of each analysis was 30 minutes and the acquisitions were carried out

at 214 nm, with a flow rate of 0.6 ml/min. An additional oxidation control sample (OCS) was prepared exclusively for the identification of the oxidized Met-ECD1 by adding 10 µL of hydrogen peroxide solution (1% H_2O_2) to 200 µL of the DP solution. After incubation of the sample at room temperature for 20 minutes, 6 mg of L-Methionine were added to stop the oxidation.

Treated samples were considered comparable to the reference material if the value obtained for the %Area of Met-ECD1 oxidised fell within the minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature.

Waters Empower software, provided by the instrument supplier, was employed for the analysis of the RP-UPLC data

2.8 Reverse Phase-UPLC (RP-UPLC)- Amino acids quantification

Since in the DP placebo of the Fc-fusion protein Methionine is present as antioxidant, RP-UPLC was employed also to quantify this amino acid on selected samples, based on a calibration curve.

These experiments were performed with an Aquity UPLC Waters instrument using an XBridge BEH C8 Column (130Å, 3.5 µm, 3 mm X 100 mm, 1/pkg). The column temperature was set at +35 ± 5°C and the autosampler temperature at +5 ± 3°C. The mobile phases were composed of 0.1% phosphoric acid (v/v) + 5 mM octanesulfonate in water (Mobile phase A1) and of acetonitrile/2-propanol (1/1) (v/v) (Mobile phase B1). The UPLC was equipped with a 50 µL loop and a 250 mL syringe. 100 µL of samples, prepared as described in the sample preparation section, were diluted with 900 µL of mobile phase A1, and then 100 µL of this solution was diluted with 900 µL of mobile phase A1. 20 µL of this solution were loaded in the UPLC for

the analysis, and the acquisition was carried out at 205 nm, with an isocratic gradient of 90% Mobile Phase A1, 10% Mobile Phase B1 (run time 12 min, flow rate 1 ml/min).

The calibration curve was built setting the 5 concentration (mM) of standard injected (134 mM of Methionine USP Reference Standard and 95 mM of Histidine HCl monohydrate EP Reference Standard) , corresponding to 50%, 75%, 100%, 125% and 150% of the target on the x-axis, and the corresponding area on the y-axis. Calculation of the calibration curve intercept and slope values, and the correlation coefficient (R) of the linear fitting was obtained by Empower software.

Waters Empower software, provided by the instrument supplier, was employed for the analysis of the RP-UPLC data.

Treated samples were considered comparable to the reference material if the value obtained for the concentration of Methionine (mM) fell within the minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature.

2.9 Surface Plasmon Resonance by Biacore – Biological characterization

Surface Plasmon Resonance (SPR) assays performed on a Biacore T200 (GE Healthcare) instrument were employed for the biological characterization of selected treated samples (**Table 5**). SPR is a technique that allows to study quantitatively and qualitatively the binding of a mobile reactant to a binding partner immobilized on the sensor surface [26]: therefore the functional characterization of the molecule's Fab portion and of the ECD fused protein was performed in terms of binding affinities and kinetic rate constants.

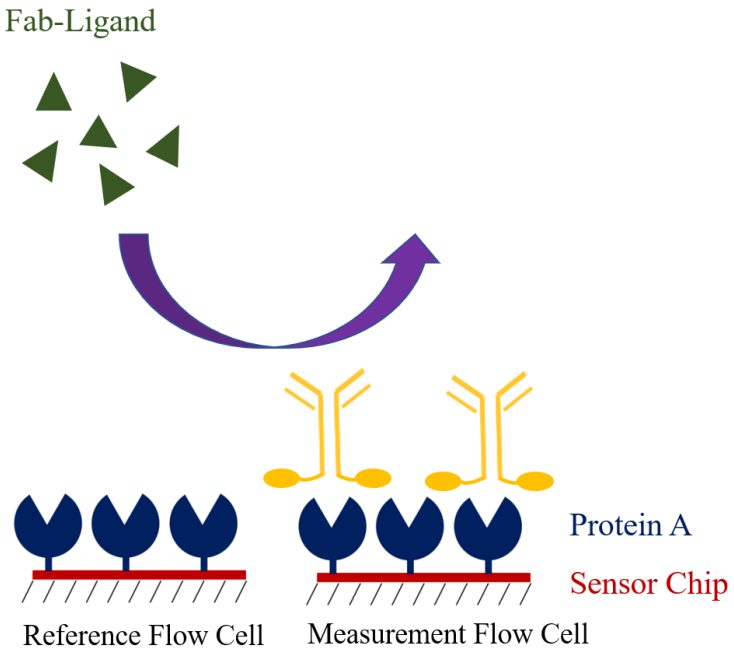
Table 5 – Samples tested by Biacore assays

Samples
Sample reference Time zero
Sample reference 6 weeks +25°C
Sample reference 6 weeks +40°C
Sample Iron conc 1 6 weeks +25°C
Sample Iron conc 1 6 weeks +40°C
Sample Copper conc 1 6 weeks +25°C
Sample Copper conc 1 6 weeks +40°C
Sample Nickel conc 3 6 weeks +25°C
Sample Nickel conc 3 6 weeks +40°C
Sample Zinc conc 3 6 weeks +25°C
Sample Zinc conc 3 6 weeks +40°C

2.9.1 Fab portion binding affinity

In order to determine the kinetic and thermodynamic constants of the Fc-fusion protein/Fab ligand interaction, the Fc-fusion protein was used as ligand: more in detail, as shown in **Figure 2**, the Fc portion was captured by Protein A immobilized by amine coupling reaction onto a sensor chip CM5 (Series S). Different concentrations of Fab-ligand (diluted in a range from 116 to 1.43 nM) were then flowed over the immobilized molecule and the interaction was observed in a “Single Cycle” analysis (sequential injections of samples at increasing concentrations). Each cycle was composed by 5 sample injections (one for each concentration), followed by the dissociation phase. At the end of each cycle, the surface was regenerated.

Figure 2. Measurement of Fc-fusion protein/Fab ligand binding affinity by Biacore assay.



First, the carboxyl groups on the sensor surface were activated by injecting 150 µl of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 150 µl of N-hydroxysuccinimide (NHS). Protein A (400 µg/ml in 10mM Acetate buffer pH 5.0) was injected for 10 minutes at 5 µl/min, over the activated surface, to which it became covalently attached by its primary amines. The excess reactive esters were then blocked with 1 M ethanolamine.

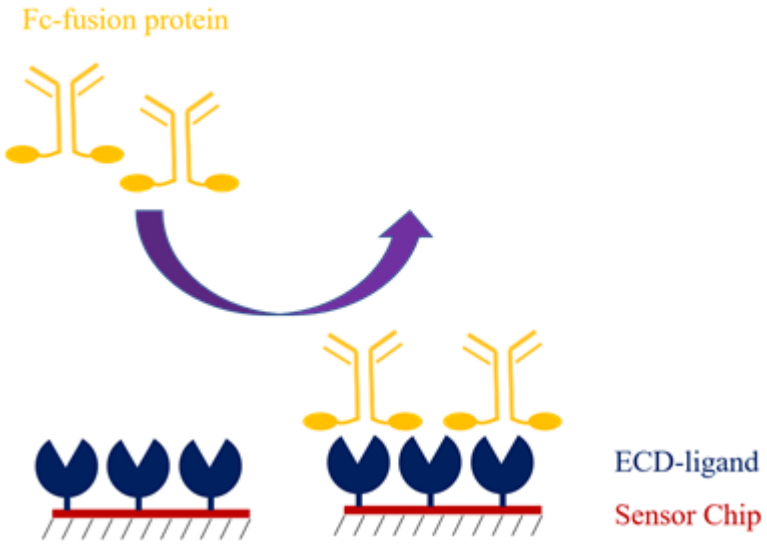
Each sample, prepared as 5nM (0.57 µg/ml) in HBS-EP running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% Surfactant P20, pH 7) was injected only into the measurement flow cell while the reference flow cell was left empty to provide a reference surface. To measure the binding kinetics, the ligand (116–1.43 nM in 3-fold serial dilutions) and a buffer blank for baseline subtraction were sequentially injected, with a regeneration step inserted between each cycle. The protein A surface was regenerated with glycine (pH 1.5) at 30 µl/min. The binding interactions were

monitored over a 3-min association period and a 30-min dissociation period (running buffer only) at 30 µl/min. The interaction was observed in real time and the affinity constant KD was calculated by the Biacore T200 evaluation software (GE Healthcare) from the kinetic constants with a 1:1 binding algorithm.

2.9.2 ECD fused protein binding affinity

In order to determine the affinity constant of the interaction Fc-fusion protein/ECD ligand (**Figure 3**), the latter was directly immobilized by an amine coupling reaction into a sensor chip CM5 (Series S). The Fc-fusion protein, used as analyte, was diluted in a range from 200 to 12.5 nM.

Figure 3. Measurement of Fc-fusion protein/ECD ligand binding affinity by Biacore assay.



The interaction was observed in a “Multi Cycle analysis” (each cycle was composed by the injection of a single concentration followed by the injection of the

regeneration solution) and the binding affinity constant KD was measured at the equilibrium ("Steady state" fitting model).

The sensor surface was activated by injecting 150 µl of EDC and 150 µl of NHS. The ligand (5 µg/ml in 10mM Acetate buffer pH 4.0) was injected over the activated surface - to which it became covalently attached by its primary amines - in order to reach a target level of 500 RU. The excess reactive esters were then blocked with 1 M ethanolamine. Each sample concentration, prepared in a concentration range between 200 and 12.5 nM (serial dilution 1:2) in HBS-EP running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% Surfactant P20, pH 7.4), was injected into both measurement and reference flow cell. To measure the binding affinity, the analyte and a buffer blank for baseline subtraction were sequentially injected, with a regeneration step inserted between each cycle. Binding interactions were monitored over a 15-minutes association period and a 1-minute dissociation period (running buffer only), at 10 µl/min.

2.9.3 SPR Data Elaboration

All data were analysed by the Biacore T200 evaluation software (GE Healthcare).

Each Biacore assay is made of 2 independent analytical runs and the results are generated as average of these two runs. A reference sample is tested, in each analytical run, at the beginning and at the end.

Results are generated for each run as Absolute Binding Affinity (KD, Mn) and Relative Binding Affinity (KD, %) according to the following formula:

$$Relative\ KD\ (\%) = \frac{Average\ Reference\ Samples\ (Absolute\ KD, final\ and\ initial) * 100}{Sample\ Absolute\ KD}$$

The relative binding % of each sample is calculated using the average of the results generated by the reference material time zero tested in each run. Results for each sample are expressed as average of the two independent analytical runs.

Treated samples were considered not comparable to the reference material if their Relative KD (calculated from Absolute KD as reported above) experienced a decrease > 25% in comparison to the Relative KD of the untreated material incubated in the same storage conditions (temperature and weeks).

2.10 Peptide mapping by LC-MS

Reducing Peptide Mapping by LC-MS was used to determine the effect on post-translational modifications (PTMs) of samples treated with iron and copper (all concentrations) stored for 6 weeks at +40°C. Peptide mapping is a technique that can be used to comprehensively identify the primary structure of a protein [27]. Since the primary structure of a recombinant protein is already known, it is possible to predict the fragments that will be generated when the protein is digested using specific enzymes. Using this approach, the Fc-fusion protein will be broken into several separate fragments, then a RP-UPLC will separate these out into a classic “fingerprint” chromatogram. Combining the separation with mass spectrometry detection allows to correlate the actual peaks observed in the peptide mapping chromatogram with the expected fragments predicted by the analysis software.

The sample preparation was based on reduction, alkylation, and enzymatic hydrolysis by Lys-C followed by deglycosylation with PNGase F. The hydrolysed samples were then analysed by LC-MS by BioAccord system. The column used for chromatographic separation was a Acquity PREMIER peptide CSH C18 1.7µm, 2.1mm x 150mm, 130Å (Waters) and the mass spectrometer was operated in full scan mode

with fragmentation. The column temperature was set to +60°C ± 2°C and the autosampler one to +7°C ± 3°C. The mobile phases were composed of 0.1% Formic Acid in Milli-Q Water (Mobile phase A1) and of 0.1% Formic Acid in ACN (Mobile phase B1). The separation was achieved by 30 min linear gradient from 5% to 22% phase B1, with a flow rate of 0.4 ml/min. The acquisition was carried out at 214 nm.

The mass spectrometer was set up as follows: MS scan range 50-2000 m/z, scan rate 5 Hz, capillary voltage 1.50 kV, cone voltage 28.0 V and desolvation temperature 550°C. Data were processed by UNIFI Scientific Information System software (Waters).

2.11 UPLC-fraction collector

The LMW species, detected by SE-UPLC, was further investigated by collecting it from Sample Iron conc 3 incubated at +40°C with an UPLC-fraction collector in order to analyse it by LC-MS analysis.

The LMW peak was collected employing an Acquity UPLC Waters instrument using an Acquity UPLC Protein BEH SEC Column (200Å, 1.7µm, 4.6 x 150mm, 1/pkg). The column temperature was set to +30°C ± 5°C and the autosampler one to +5 ± 3°C. The UPLC was equipped with a 50 µL loop and a 250 mL syringe. The acquisition was carried out at 214 nm, with an isocratic gradient of 100% Phosphate Buffer Solution 1x and a flow rate of 0.3 ml/min; the fraction collector was set to collect from 3.950 min to 4.550 min. Sample Iron conc 3 6 weeks +40°C, prepared as described in the sample preparation section, was diluted in PBS 1X pH 7.4±0.1 to a final concentration of 0.5 mg/ml and 192 injections, each one of 200µl, were performed.

The collected LMW was then buffer exchanged (10mM histidine, 5mM methionine, 6% trehalose and 40 mM NaCl pH 5.5±0.1) in 30kDa Amicon filters.

2.12 Intact Molecule by LC-MS

The LMW species detected by SEC-UPLC in samples treated with iron, was further investigated by analysing it by LC-MS analysis [27]. Prior to analysis, samples (LMW collected from sample iron conc 3 6 weeks +40°C and untreated material incubated for 6 weeks at +40°C) were deglycosylated using PNGase F in Tris-HCl 50 mM pH 8.0 buffer for 18h at +37°C and subsequently reduced with DTT 1M (incubated for 30 min at +50°C). Analyses were performed using ultra-performance liquid chromatography coupled to quadrupole-time-of-flight mass spectrometer (XEVO G2-XS QTOF, Waters). The chromatographic column used for the analysis was a Waters BEH C4 (1.7 μm, 2.1 × 50 mm). Compounds were separated using a linear gradient with distilled water containing 0.2% formic acid (eluent A1) and acetonitrile containing 0.2% formic acid (eluent B1) as mobile phases. The separation was achieved in 25 min linear gradient from 5% to 35% phase B1, with a flow rate of 0.2 ml/min. The acquisition was carried out at 214 nm and the column temperature was set to +80°C ± 5°C, while the autosampler one to +8°C ± 3°C. The mass spectrometer, operating in the positive mode (E+), was set up as follows: MS scan range 600-3000 m/z, capillary voltage 3.0 kV, sampling cone 80.0 V, source temperature 150 °C, and desolvation temperature 350°C. Desolvation gas and cone gas flows were 800 L/Hr and 0.0 L/Hr, respectively.

Data have been analysed using the UNIFI Scientific Information System software (Waters). Of note, the theoretical molecular weight, calculated on the basis of the primary structure of the molecule, for both LC and HC was computed considering all the intra- and inter-chain disulfide bridges in their reduced form.

3. Results:

3.1 Semi-quantitative analysis by ICP-MS

The *Main Stock Solutions* were analysed by ICP-MS in order to determine the exact content of each metal ion, prior the preparation of the three *Final Stock Solutions* that were used to treat the samples. The concentrations obtained by ICP-MS were:

- *Main Stock Solution* Fe^{3+}: 210 ppm
- *Main Stock Solution* Cu^{2+}: 420 ppm
- *Main Stock Solution* Zn^{2+}: 418 ppm
- *Main Stock Solution* Ni^{2+}: 238 ppm

3.2 Stability studies – Time zero and 6 weeks

3.2.1 Conformation - Tertiary structure

All samples treated with the four metal ions at time zero and after 6 weeks of incubation at +25°C and +40°C were tested by Near-UV Circular Dichroism.

Near-UV CD was chosen as suitable technique to monitor changes of the tertiary structure of the molecule. This spectroscopic technique provides in fact a fingerprint of the overall conformation as the region (250-340 nm) is dominated by the contributions of aromatic amino acids' side chains and disulphide bonds.

3.2.1.1 Iron

After addition of iron, at time zero (**Figure 4 and Table 6**), only two samples treated with iron fell outside the range determined by the WSDs of the reference spectra: Sample Iron conc 1 rep 1 and Sample Iron conc 3 rep 2. After 6 weeks, at both +25°C (**Figure 5 and Table 7**) and +40°C (**Figure 6 and Table 8**), however, differences were observed for all the tested concentrations when compared to the untreated material incubated at the same conditions of temperature and storage time and were particularly dramatic for samples incubated at +40°C.

Interestingly, CD spectra suggest a different level of modification of the tertiary structure depending on the incubation temperature (**Figures 5 and 6**): at +25°C spectra perturbations were observed only around 255 nm, 275 nm and 285 nm (absorption of phenylalanine, tyrosine and tryptophan) while at +40°C modifications were observed also in the region where disulphide-bridges absorb (310-330 nm).

Figure 4 - Circular Dichroism of samples treated with iron at time zero. A) WSD comparison of the Near-UV CD spectra after incubation with iron at time zero (all replicates). Only one replicate of concentration 3 and one of concentration 1 do not fall within the range determined by the WSDs of the untreated material. Concentration 3 replicate 3 and concentration 2 replicate 3 are not reported as determined as outliers (Box plot detection) B) Overlay of the Near-UV CD spectra of the samples treated with the three concentrations of iron at time zero (one replicate per sample is shown).

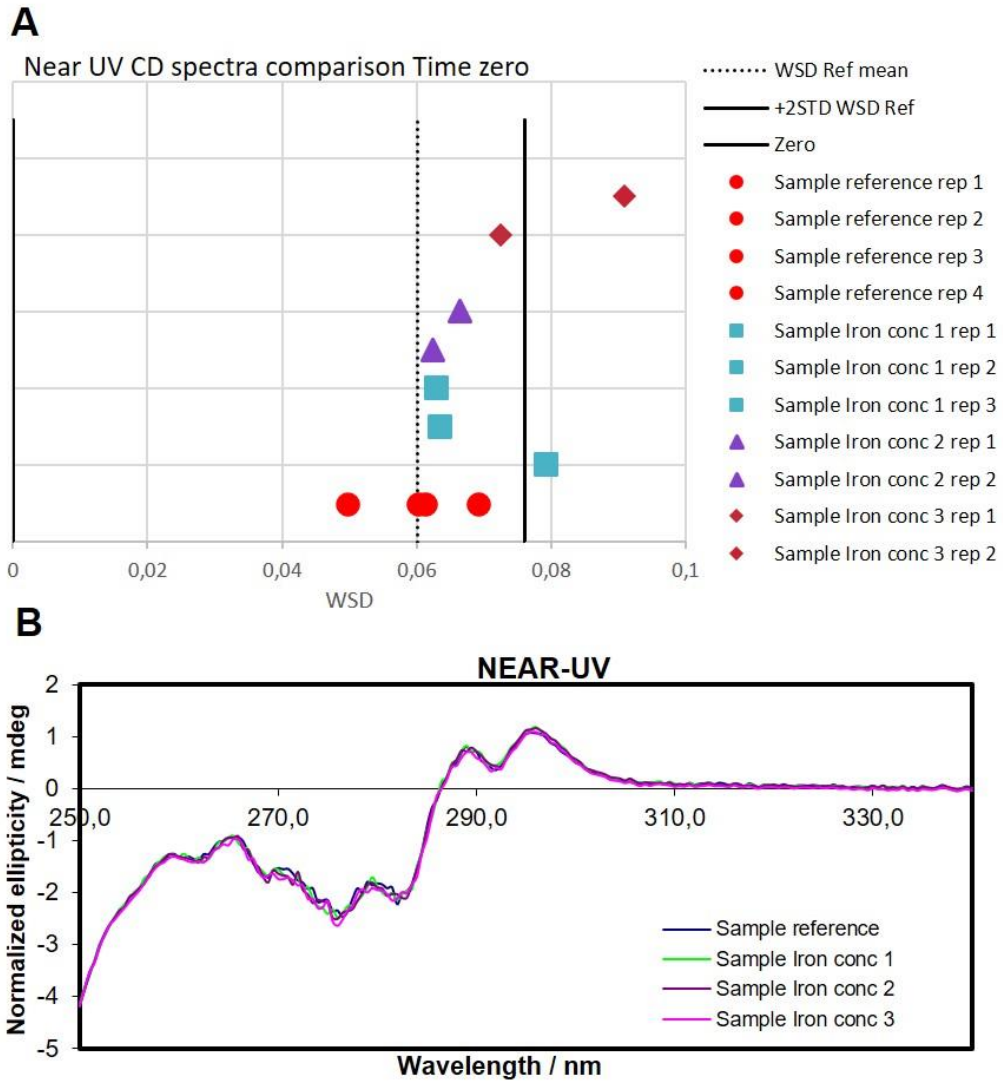


Table 6 – WSD values – Samples treated with iron at time zero.

Sample	WSD - Similarity
Sample reference rep 1	0.061297
Sample reference rep 2	0.060306
Sample reference rep 3	0.069126
Sample reference rep 4	0.049775
Sample Iron conc 1 rep 1	0.079256
Sample Iron conc 1 rep 2	0.063515
Sample Iron conc 1 rep 3	0.063000
Sample Iron conc 2 rep 1	0.062483
Sample Iron conc 2 rep 2	0.066422
Sample Iron conc 2 rep 3	0.827150
Sample Iron conc 3 rep 1	0.072442
Sample Iron conc 3 rep 2	0.090985
Sample Iron conc 3 rep 3	0.255540

Figure 5. Circular Dichroism spectra of samples treated with iron after 6 weeks at +25°C. A) WSD comparison of the Near-UV CD spectra after incubation with iron for 6 weeks at +25°C (all replicates): the WSDs calculated for the samples treated with iron do not fall within the range determined by the WSDs of the untreated material incubated at the same conditions. B) Overlay of the Near-UV CD spectra of the samples treated with the three concentrations of iron after 6 weeks of incubation at +25°C (one replicate per sample is shown).

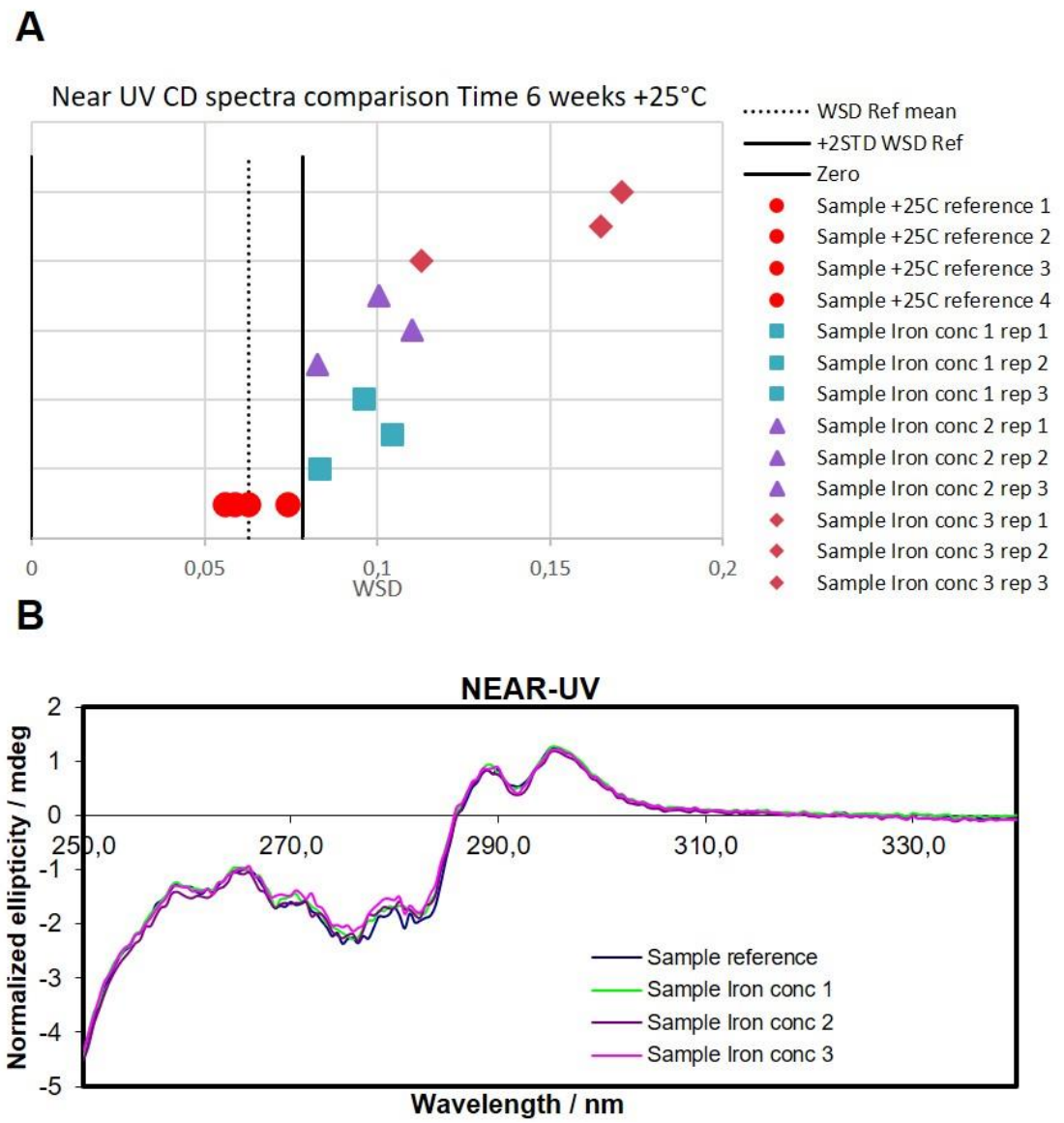


Table 7 – WSD values – Samples treated with iron after 6 weeks at +25°C.

Sample	WSD - Similarity
Sample +25C reference 1	0.055691
Sample +25C reference 2	0.058722
Sample +25C reference 3	0.062456
Sample +25C reference 4	0.073838
Sample Iron conc 1 rep 1	0.083307
Sample Iron conc 1 rep 2	0.10434
Sample Iron conc 1 rep 3	0.096237
Sample Iron conc 2 rep 1	0.082639
Sample Iron conc 2 rep 2	0.11016
Sample Iron conc 2 rep 3	0.10063
Sample Iron conc 3 rep 1	0.11269
Sample Iron conc 3 rep 2	0.16467
Sample Iron conc 3 rep 3	0.17074

Figure 6. Circular Dichroism spectra of samples treated with iron after 6 weeks at +40°C A) WSD comparison of the Near-UV CD spectra after incubation with iron for 6 weeks at +40°C (all replicates): the WSDs calculated for the samples treated with iron do not fall within the range determined by the WSDs of the untreated material incubated at the same conditions. B) Overlay of the Near-UV CD spectra of the samples treated with the three concentrations of iron after 6 weeks of incubation at +40°C (one replicate per sample is shown).

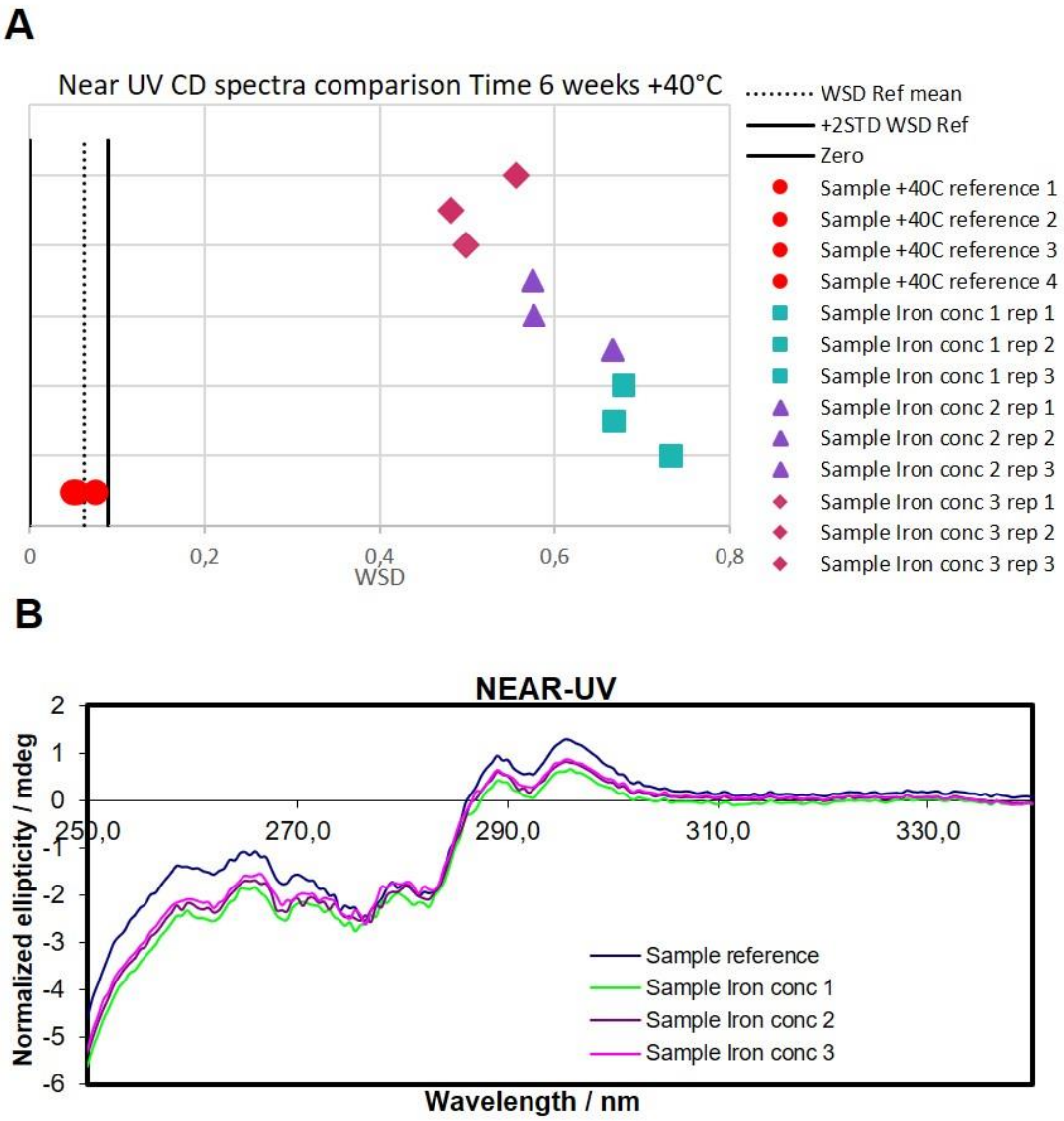


Table 8 – WSD values – Samples treated with iron after 6 weeks at +40°C.

Sample	WSD - Similarity
Sample +40C reference 1	0.074814
Sample +40C reference 2	0.053126
Sample +40C reference 3	0.049130
Sample +40C reference 4	0.074463
Sample Iron conc 1 rep 1	0.73292
Sample Iron conc 1 rep 2	0.66780
Sample Iron conc 1 rep 3	0.67858
Sample Iron conc 2 rep 1	0.66640
Sample Iron conc 2 rep 2	0.57623
Sample Iron conc 2 rep 3	0.57514
Sample Iron conc 3 rep 1	0.49916
Sample Iron conc 3 rep 2	0.48115
Sample Iron conc 3 rep 3	0.55547

3.2.1.2 Copper

In case of copper, we observed perturbations of the tertiary structure only in case of samples treated with concentration 3 (1 ppm). At time zero (**Figure 7 and Table 9**), after 6 weeks at +25°C (**Figure 8 and Table 10**) and after 6 weeks at +40°C (**Figure 9 and Table 11**) all replicates treated with 1 ppm of copper fell outside the range determined by the WSDs of the untreated material incubated at the same conditions, while all samples with concentration 1 and 2 fell inside the range in all conditions tested.

Alterations in case of copper were small and subtle, localized around 250-265 nm and around 275 nm, indicating different absorption of phenylalanine and tyrosine, respectively.

Figure 7- Circular Dichroism of samples treated with copper at time zero. A) WSD comparison of the Near-UV CD spectra of samples treated with copper at time zero. Samples treated with concentration 3 do not fall within the range determined by the WSDs of the untreated material. **B)** Overlay of the Near-UV CD spectra of the samples treated with the three concentrations of copper at time zero (one replicate per sample is

shown). **C)** Zoomed view of Near-UV CD spectra of the samples treated with the three concentrations of copper at time zero (one replicate per sample is shown)

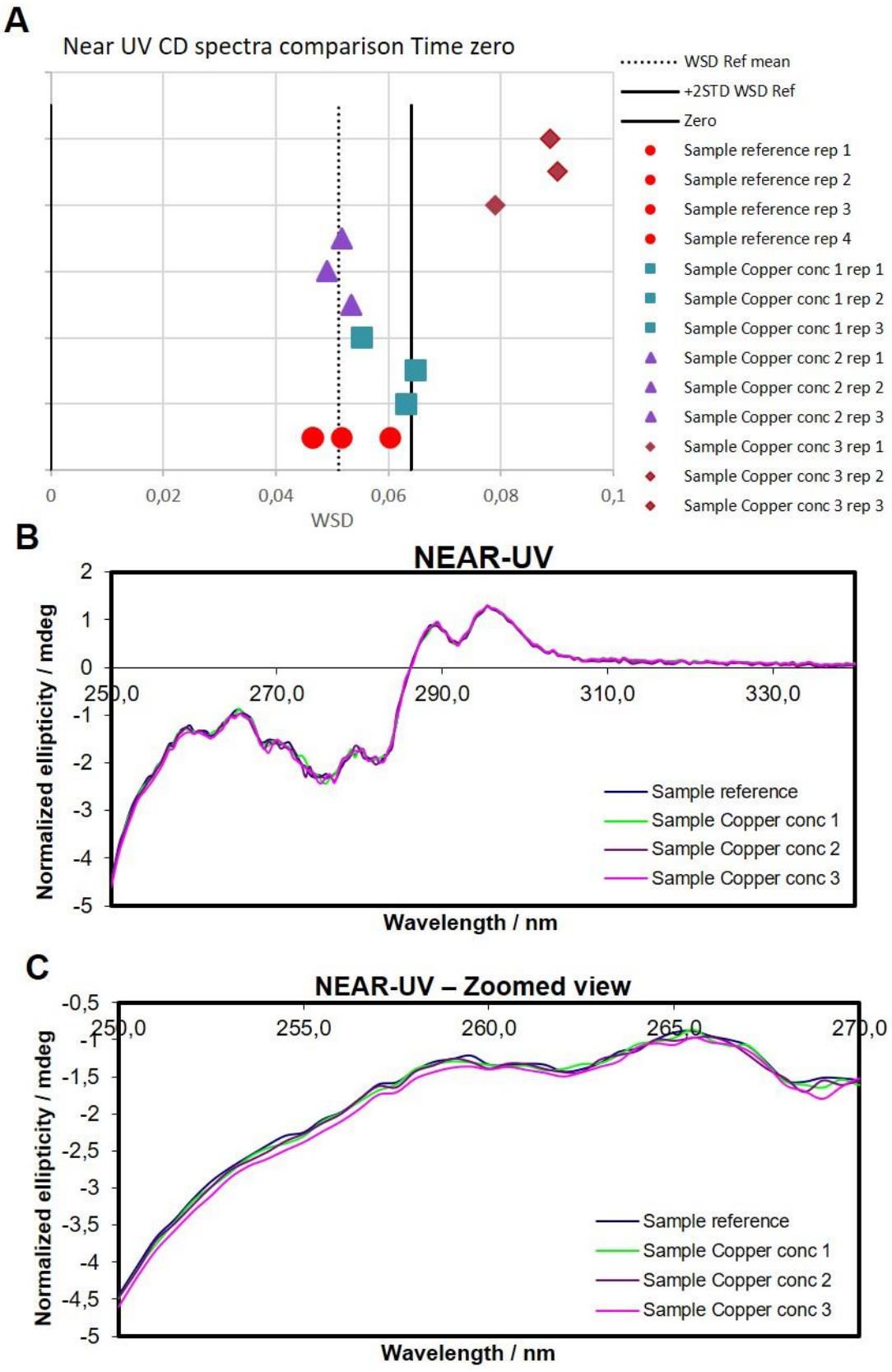


Table 9 – WSD values – Samples treated with copper at time zero.

Sample	WSD - Similarity
Sample reference rep 1	0.046459
Sample reference rep 2	0.04649
Sample reference rep 3	0.051684
Sample reference rep 4	0.060219
Sample Copper conc 1 rep 1	0.063256
Sample Copper conc 1 rep 2	0.064802
Sample Copper conc 1 rep 3	0.055346
Sample Copper conc 2 rep 1	0.053345
Sample Copper conc 2 rep 2	0.049039
Sample Copper conc 2 rep 3	0.05167
Sample Copper conc 3 rep 1	0.078999
Sample Copper conc 3 rep 2	0.090011
Sample Copper conc 3 rep 3	0.088719

Figure 8- Circular Dichroism of samples treated with copper after 6 weeks at +25°C. A) WSD comparison of the Near-UV CD spectra of the samples treated with copper after 6 weeks at +25°C. Samples treated with concentration 3 do not fall within the range determined by the WSDs of the untreated material. **B)** Overlay of the Near-

UV CD spectra of the samples treated with the three concentrations of copper after 6 weeks at+25°C (one replicate per sample is shown). **C)** Zoomed view of Near-UV CD spectra of the samples treated with the three concentrations of copper after 6 weeks at+25°C (one replicate per sample is shown).

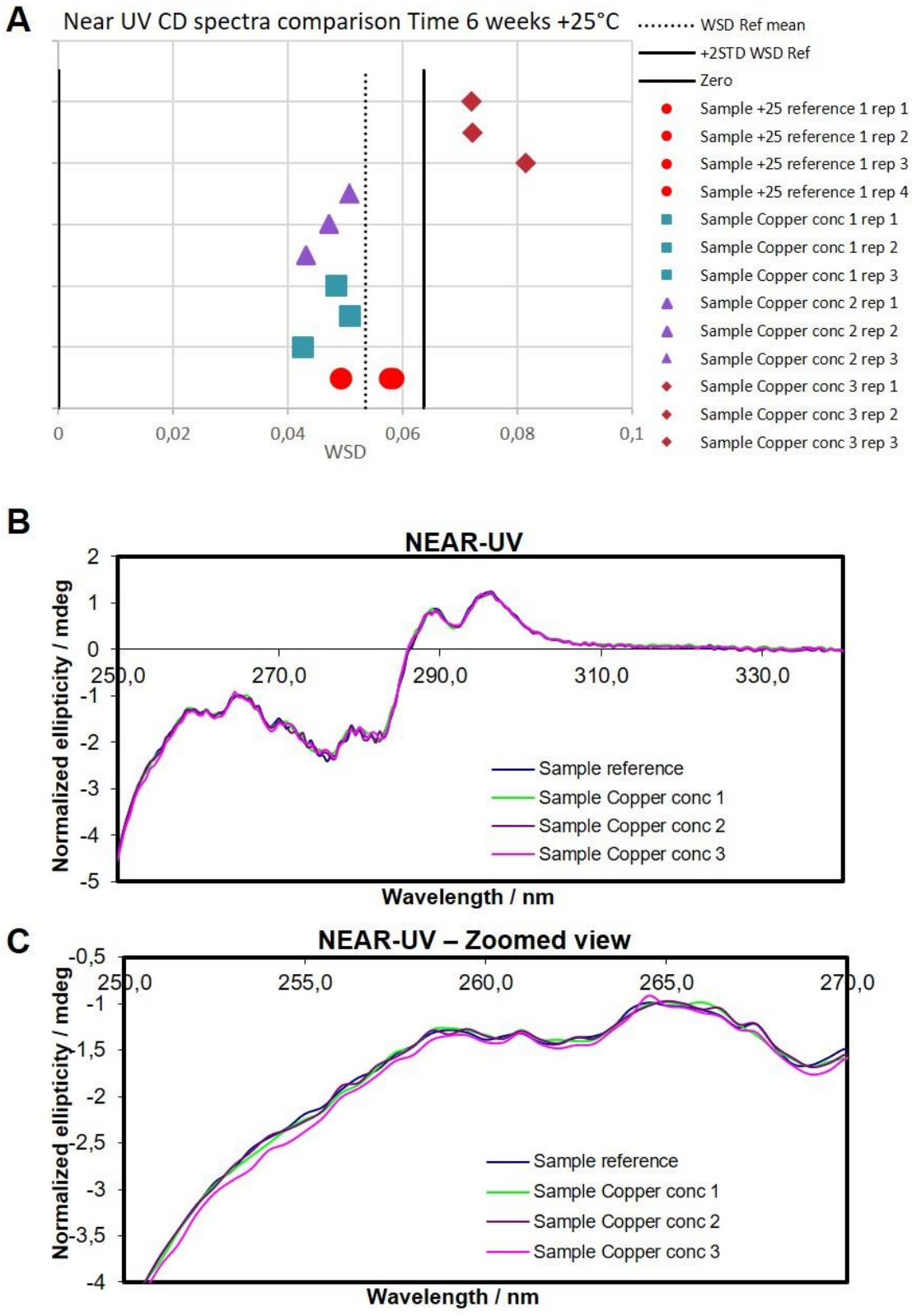


Table 10 – WSD values – Samples treated with copper after 6 weeks at +25°C.

Sample	WSD - Similarity
Sample +25 reference 1 rep 1	0.058331
Sample +25 reference 1 rep 2	0.049338
Sample +25 reference 1 rep 3	0.057612
Sample +25 reference 1 rep 4	0.049097
Sample Copper conc 1 rep 1	0.042606
Sample Copper conc 1 rep 2	0.050787
Sample Copper conc 1 rep 3	0.04849
Sample Copper conc 2 rep 1	0.043198
Sample Copper conc 2 rep 2	0.047124
Sample Copper conc 2 rep 3	0.050692
Sample Copper conc 3 rep 1	0.081516
Sample Copper conc 3 rep 2	0.072232
Sample Copper conc 3 rep 3	0.071983

Figure 9- Circular Dichroism of samples treated with copper after 6 weeks at +40°C. A) WSD comparison of the Near-UV CD spectra of the samples treated with copper after 6 weeks at +40°C. One replicate of the sample treated with concentration 3 fall within the range determined by the WSDs, although just below the upper limit while the other two replicates fall outside the range. Concentration 2 replicate 1 is not reported as determined as outlier (Box plot detection). B) Overlay of the Near-UV CD spectra of the samples treated with the three concentrations of copper after 6 weeks at+40°C. (one

replicate per sample is shown). C) Zoomed view of Near-UV CD spectra of the samples treated with the three concentrations of copper after 6 weeks at+40°C (one replicate per sample is shown).

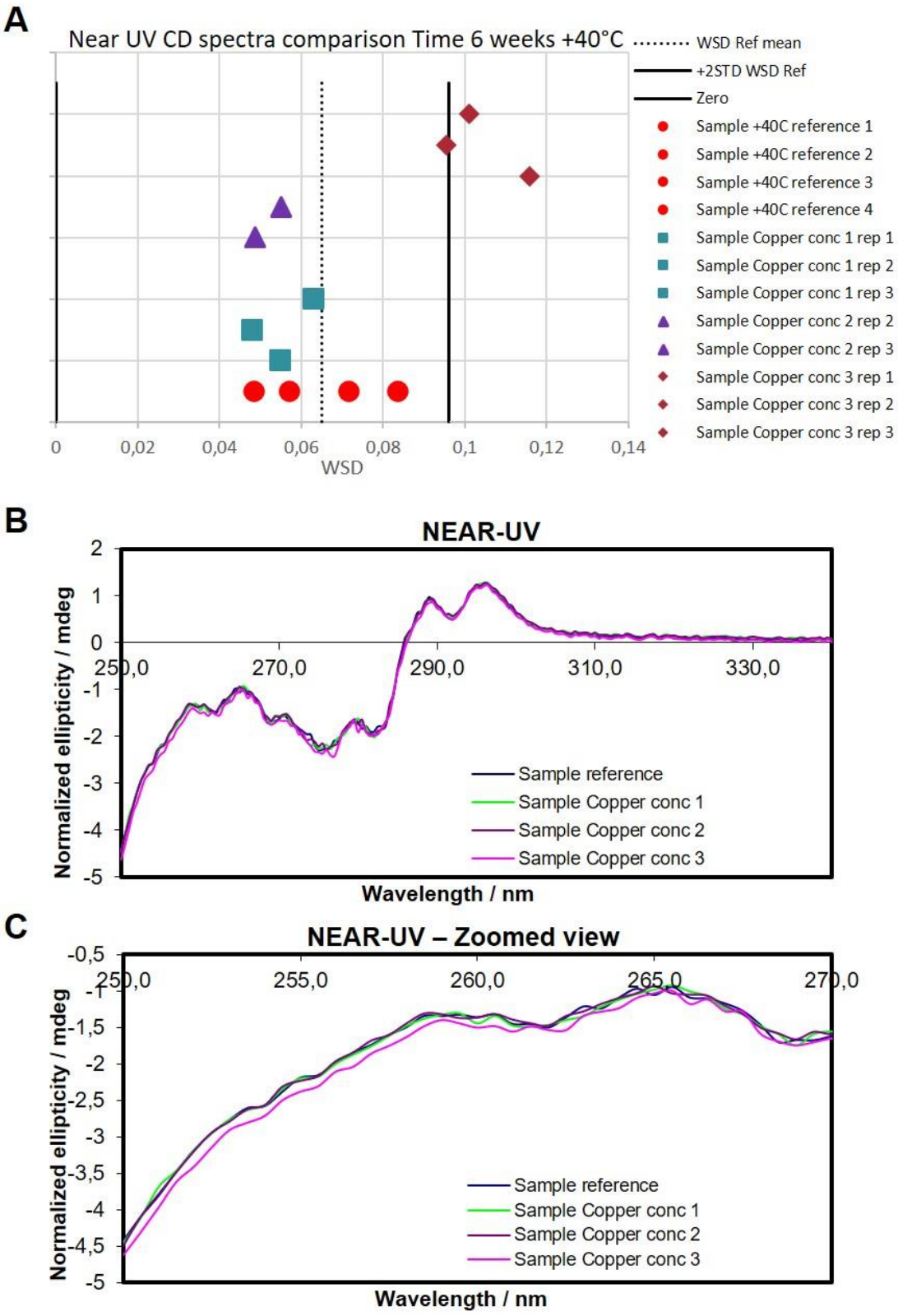


Table 11 – WSD values – Samples treated with copper after 6 weeks at +40°C.

Sample	WSD - Similarity
Sample +40C reference 1	0.048404
Sample +40C reference 2	0.071435
Sample +40C reference 3	0.083468
Sample +40C reference 4	0.057116
Sample Copper conc 1 rep 1	0.054899
Sample Copper conc 1 rep 2	0.04801
Sample Copper conc 1 rep 3	0.06304
Sample Copper conc 2 rep 1	0.65732
Sample Copper conc 2 rep 2	0.048623
Sample Copper conc 2 rep 3	0.054981
Sample Copper conc 3 rep 1	0.11574
Sample Copper conc 3 rep 2	0.095546
Sample Copper conc 3 rep 3	0.10098

3.2.1.3 Zinc and Nickel

Upon treatment with zinc, no differences in terms of tertiary structure were observed in any of the analysed conditions (**Figures 10, 11 and 12; Table 12, 13 and 14**).

Figure 10 - Circular Dichroism of samples treated with zinc at time zero. A) WSD comparison of the Near-UV CD spectra of samples treated with zinc at time zero. All samples fall within the range determined by the WSDs of the untreated material. B) Overlay of the Near-UV CD spectra of the samples treated with the three concentrations of zinc at time zero (one replicate per sample is shown).

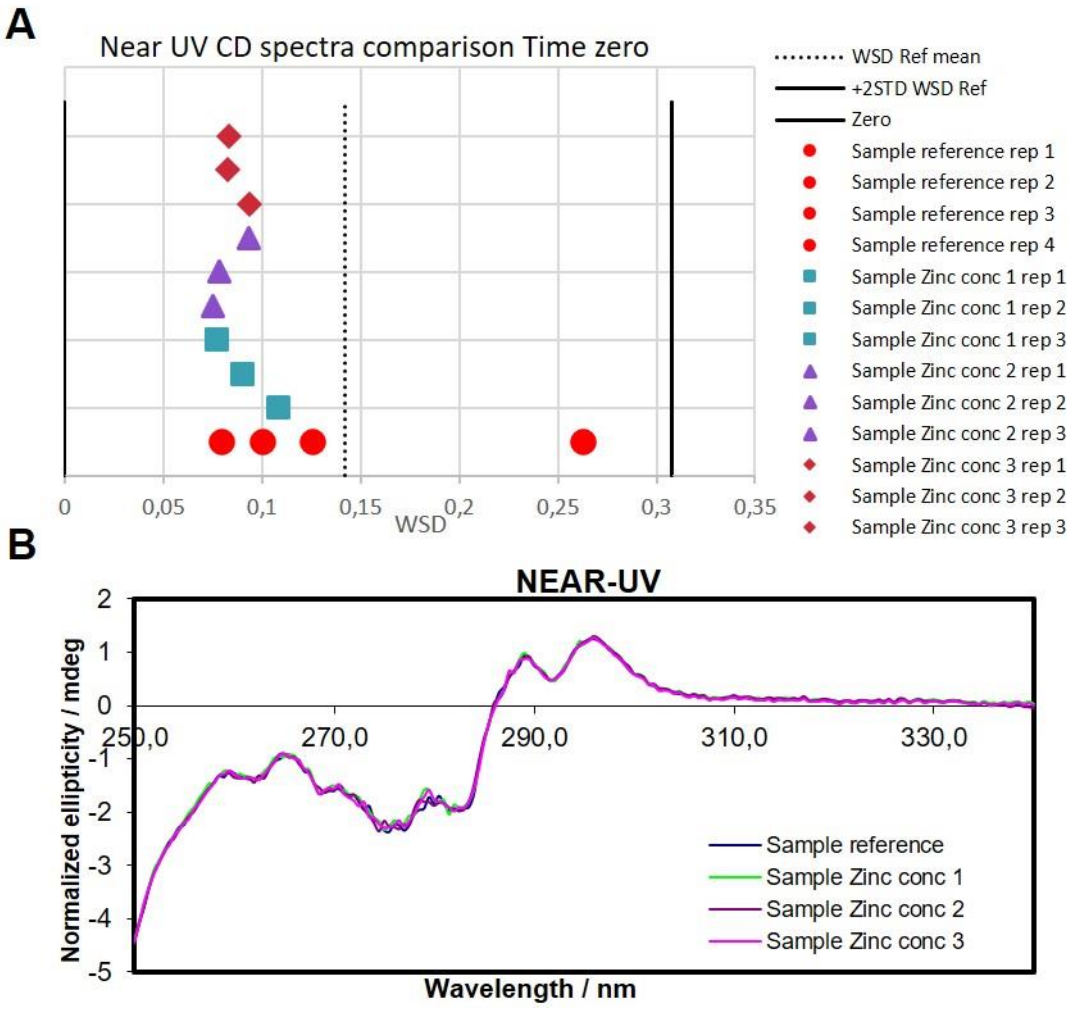


Table 12 – WSD values – Samples treated with zinc at time zero.

Sample	WSD - Similarity
Sample reference rep 1	0.12562
Sample reference rep 2	0.079401
Sample reference rep 3	0.099990
Sample reference rep 4	0.26283
Sample Zinc conc 1 rep 1	0.10862
Sample Zinc conc 1 rep 2	0.090156
Sample Zinc conc 1 rep 3	0.077167
Sample Zinc conc 2 rep 1	0.075212
Sample Zinc conc 2 rep 2	0.078368
Sample Zinc conc 2 rep 3	0.093474
Sample Zinc conc 3 rep 1	0.093474
Sample Zinc conc 3 rep 2	0.082368
Sample Zinc conc 3 rep 3	0.083100

Fig 11 - Circular Dichroism of samples treated with zinc copper after 6 weeks at +25°C. A) WSD comparison of the Near-UV CD spectra of samples treated with zinc after 6 weeks at+25°C. All samples fall within the range determined by the WSDs of the untreated material. Concentration 3 replicate 2 is not reported as determined as outliers (Box plot detection). B) Overlay of the Near-UV CD spectra of the samples treated with the three concentrations of zinc after 6 weeks at+25°C (one replicate per sample is shown).

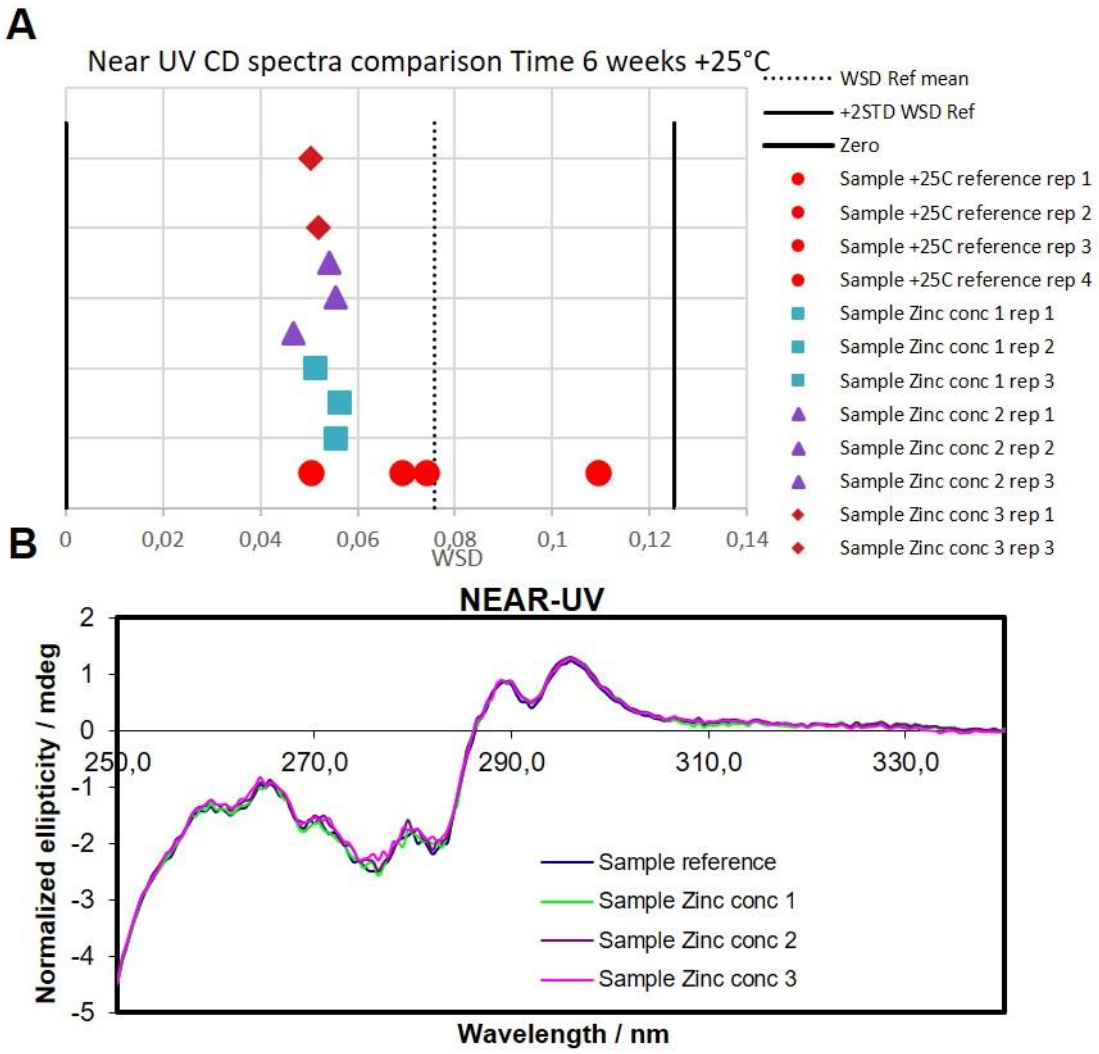


Table 13 – WSD values – Samples treated with zinc after 6 weeks at +25°C.

Sample	WSD - Similarity
Sample +25C reference rep 1	0.050364
Sample +25C reference rep 2	0.069091
Sample +25C reference rep 3	0.074089
Sample +25C reference rep 4	0.10939
Sample Zinc conc 1 rep 1	0.055452
Sample Zinc conc 1 rep 2	0.056401
Sample Zinc conc 1 rep 3	0.051237
Sample Zinc conc 2 rep 1	0.046761
Sample Zinc conc 2 rep 2	0.05544
Sample Zinc conc 2 rep 3	0.054176
Sample Zinc conc 3 rep 1	0.05195
Sample Zinc conc 3 rep 2	0.074697
Sample Zinc conc 3 rep 3	0.050397

Figure 12 - Circular Dichroism of samples treated with zinc copper after 6 weeks at +40°C. A) WSD comparison of the Near-UV CD spectra of samples treated with zinc after 6 weeks at+40°C. All samples fall within the range determined by the WSDs of the untreated material. B) Overlay of the Near-UV CD spectra of the samples treated with the three concentrations of zinc after 6 weeks at+40°C (one replicate per sample is shown).

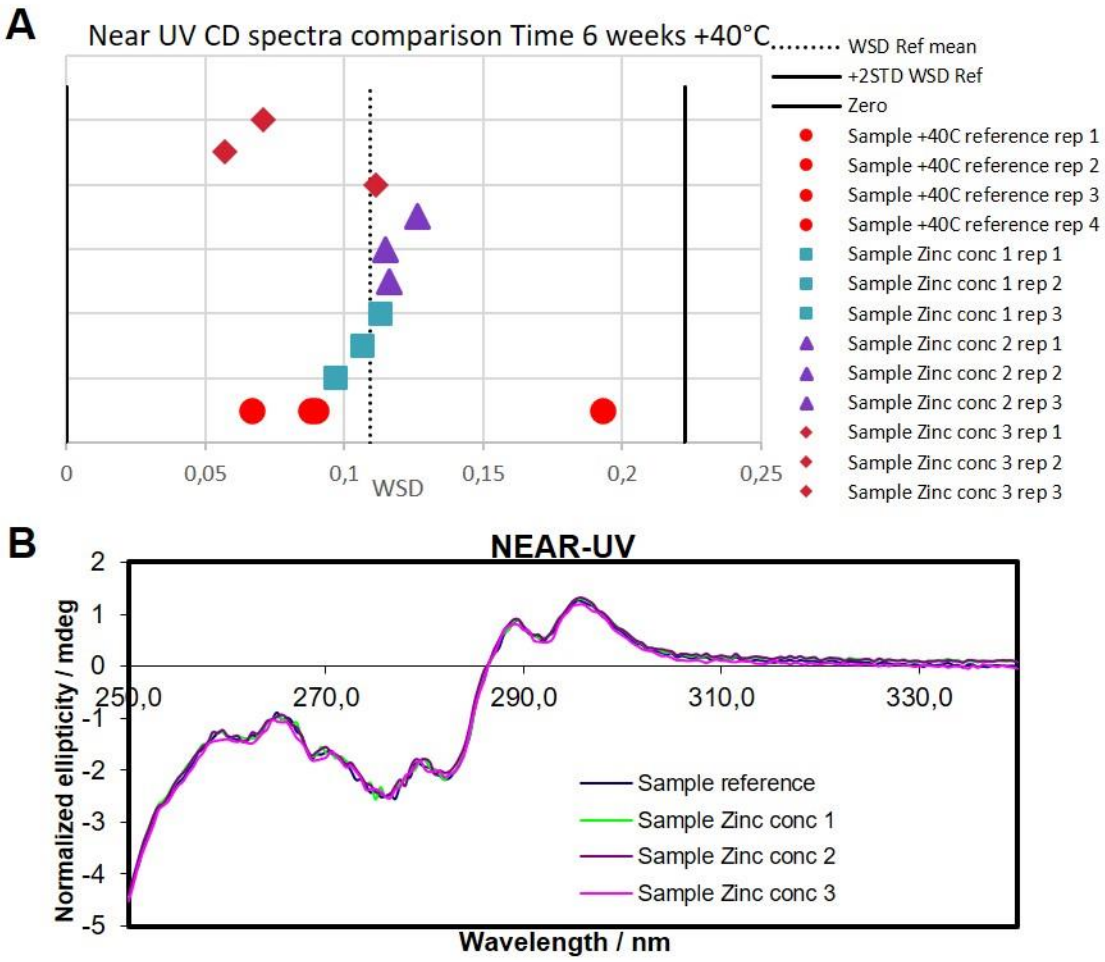


Table 14 – WSD values – Samples treated with zinc after 6 weeks at +40°C.

Sample	WSD - Similarity
Sample +40C reference rep 1	0.088099
Sample +40C reference rep 2	0.066771
Sample +40C reference rep 3	0.089704
Sample +40C reference rep 4	0.19296
Sample Zinc conc 1 rep 1	0.096651
Sample Zinc conc 1 rep 2	0.10660
Sample Zinc conc 1 rep 3	0.11281
Sample Zinc conc 2 rep 1	0.11619
Sample Zinc conc 2 rep 2	0.11480
Sample Zinc conc 2 rep 3	0.12656
Sample Zinc conc 3 rep 1	0.11137
Sample Zinc conc 3 rep 2	0.056967
Sample Zinc conc 3 rep 3	0.070657

In case of nickel as well, Near-UV CD spectra did not highlight any difference with respect to the reference material in any of the analysed conditions (**Figures 13, 14 and 15; Tables 15, 16 and 17**), except for one replicate per concentration, after incubation for 6 weeks at +25°C (**Fig 14**), that did not fall within the range determined by the WSDs of the untreated material. However as only one out of three replicates did not fall within the range determined by the WSDs, samples treated with nickel are considered not altered in their tertiary structure also after 6 weeks at +25°C.

Figure 13 - Circular Dichroism of samples treated with nickel at time zero. A) WSD comparison of the Near-UV CD spectra of samples treated with nickel at time zero. All samples fall within the range determined by the WSDs of the untreated material. B) Overlay of the Near-UV CD spectra of the samples treated with the three concentrations of nickel at time zero (one replicate per sample is shown).

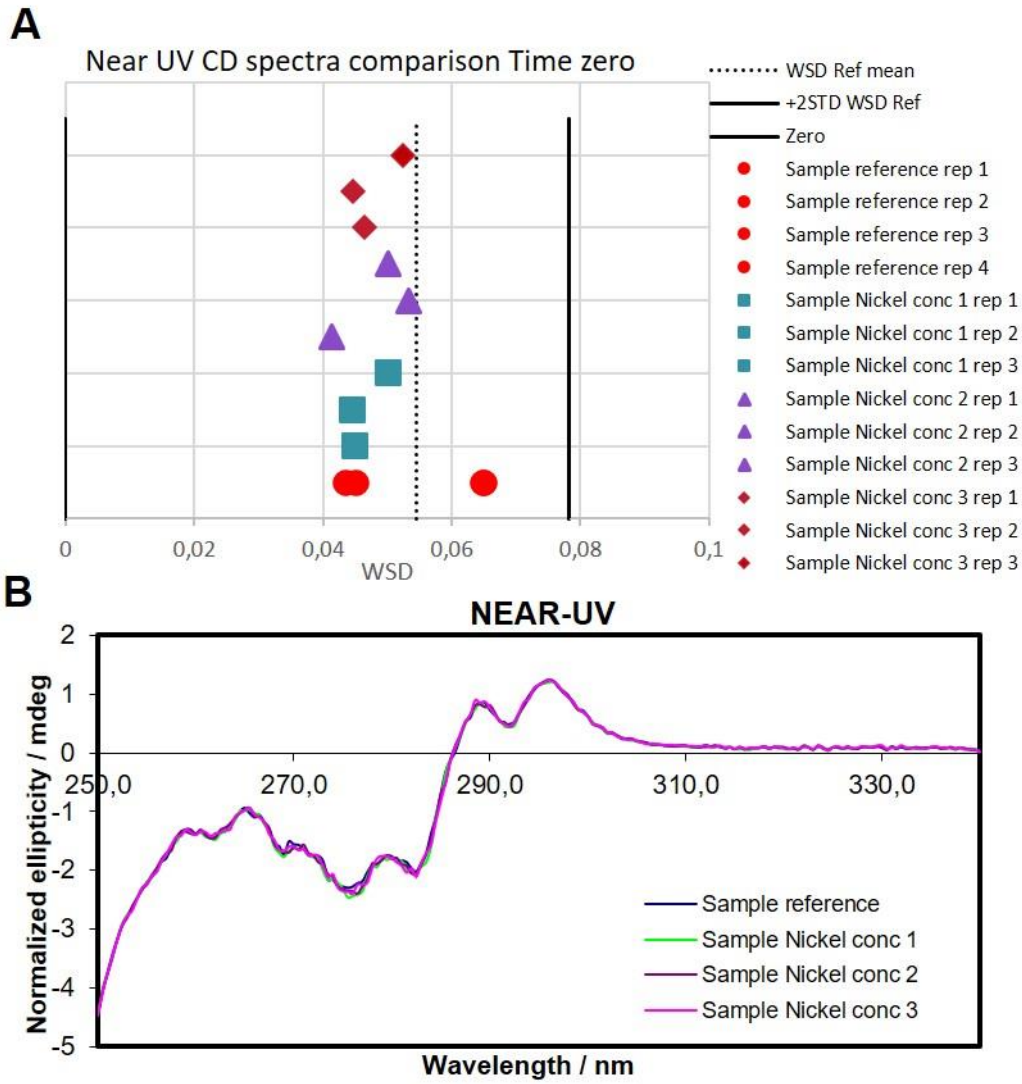


Table 15 – WSD values – Samples treated with nickel at time zero.

Sample	WSD - Similarity
Sample reference rep 1	0.064954
Sample reference rep 2	0.043505
Sample reference rep 3	0.045176
Sample reference rep 4	0.064755
Sample Nickel conc 1 rep 1	0.045037
Sample Nickel conc 1 rep 2	0.044504
Sample Nickel conc 1 rep 3	0.050128
Sample Nickel conc 2 rep 1	0.041324
Sample Nickel conc 2 rep 2	0.053404
Sample Nickel conc 2 rep 3	0.050208
Sample Nickel conc 3 rep 1	0.046388
Sample Nickel conc 3 rep 2	0.044641
Sample Nickel conc 3 rep 3	0.052506

Figure 14 - Circular Dichroism of samples treated with nickel after 6 weeks at +25°C. A) WSD comparison of the Near-UV CD spectra of samples treated with zinc after 6 weeks at+25°C. All the samples, except for one replicate per concentration, fall within the range determined by the WSDs of the untreated material incubated at the same conditions. B) Overlay of the Near-UV CD spectra of the samples treated with the three concentrations of nickel after 6 weeks at+25°C (one replicate per sample is shown).

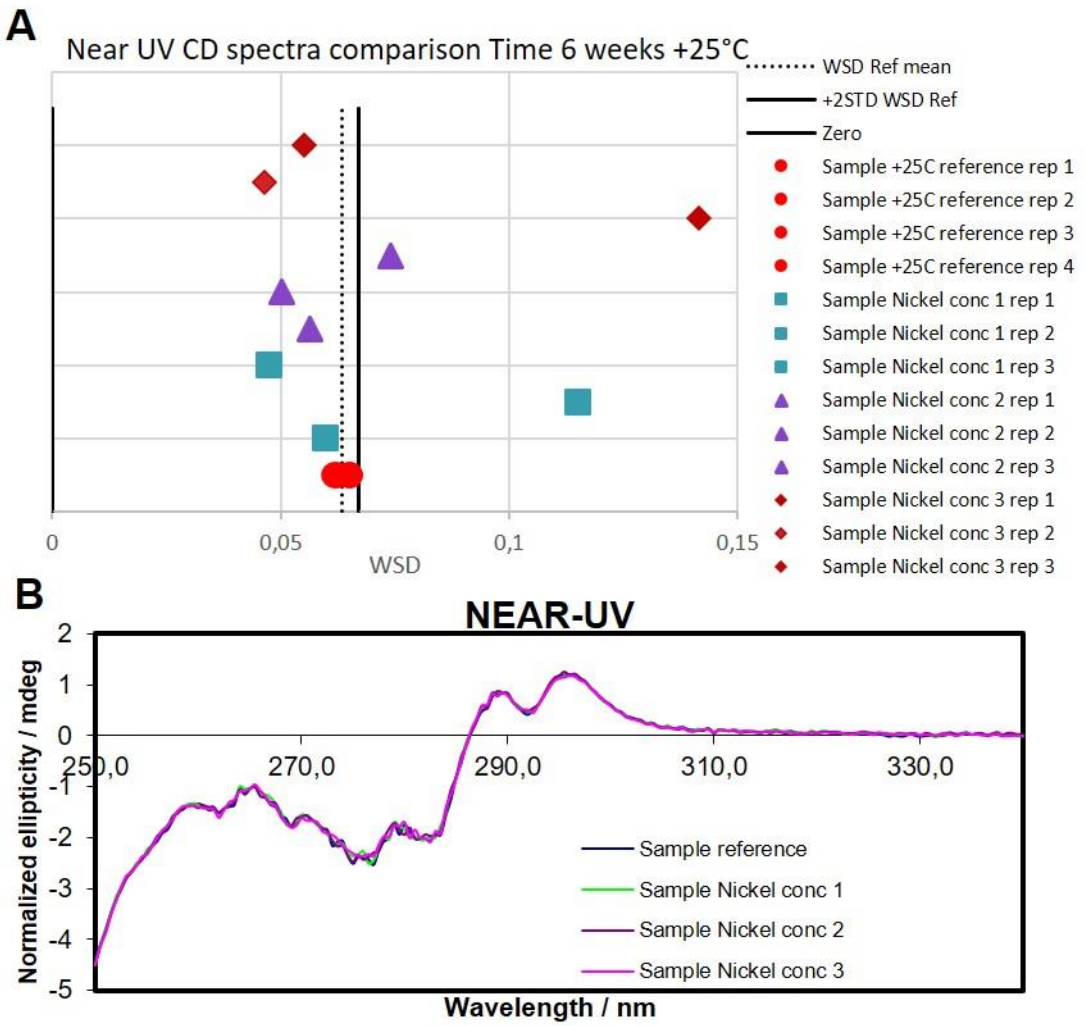


Table 16 – WSD values – Samples treated with nickel after 6 weeks at +25°C.

Sample	WSD - Similarity
Sample +25C reference rep 1	0.061644
Sample +25C reference rep 2	0.064889
Sample +25C reference rep 3	0.062078
Sample +25C reference rep 4	0.065106
Sample Nickel conc 1 rep 1	0.059884
Sample Nickel conc 1 rep 2	0.11491
Sample Nickel conc 1 rep 3	0.047373
Sample Nickel conc 2 rep 1	0.056395
Sample Nickel conc 2 rep 2	0.050204
Sample Nickel conc 2 rep 3	0.074024
Sample Nickel conc 3 rep 1	0.14147
Sample Nickel conc 3 rep 2	0.046403
Sample Nickel conc 3 rep 3	0.055224

Figure 15 - Circular Dichroism of samples treated with nickel after 6 weeks at +25°C. A) WSD comparison of the Near-UV CD spectra of samples treated with zinc after 6 weeks at+40°C. All samples fall within the range determined by the WSDs of the untreated material incubated at the same conditions. Concentration 3 replicate 3 and concentration 2 rep 3 are not reported as determined as outliers (Box plot detection). B) Overlay of the Near-UV CD spectra of the samples treated with the three concentrations of nickel after 6 weeks at+40°C (one replicate per sample is shown).

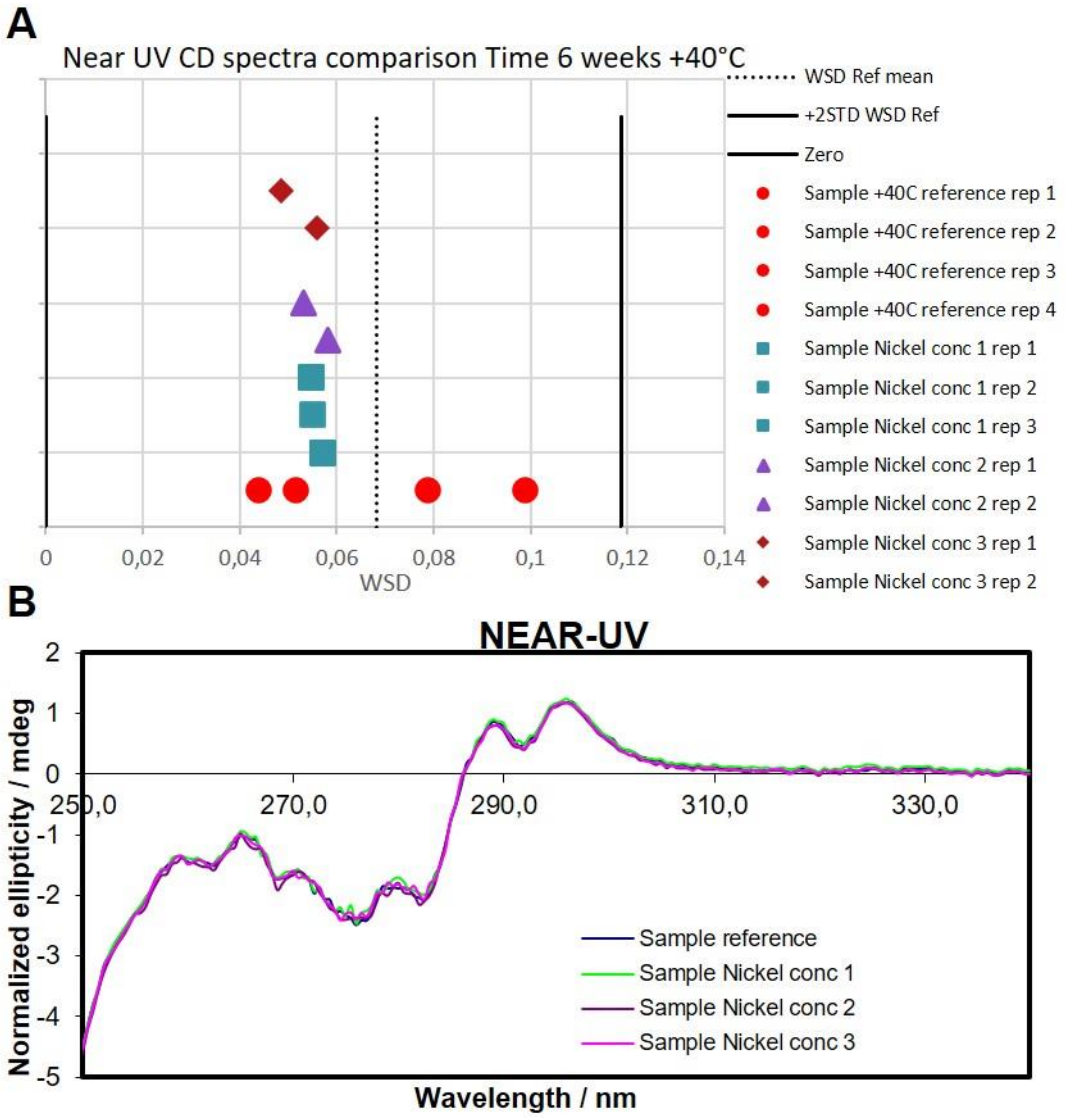


Table 17 – WSD values – Samples treated with nickel after 6 weeks at +40°C.

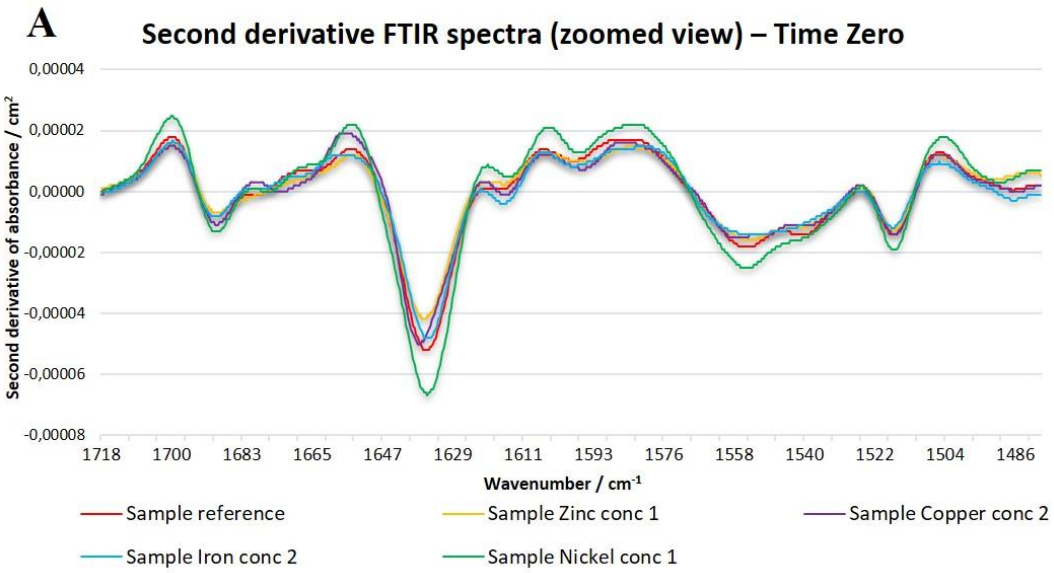
Sample	WSD - Similarity
Sample +40C reference rep 1	0.051375
Sample +40C reference rep 2	0.098793
Sample +40C reference rep 3	0.043784
Sample +40C reference rep 4	0.078694
Sample Nickel conc 1 rep 1	0.057082
Sample Nickel conc 1 rep 2	0.055037
Sample Nickel conc 1 rep 3	0.054742
Sample Nickel conc 2 rep 1	0.058112
Sample Nickel conc 2 rep 2	0.053112
Sample Nickel conc 2 rep 3	0.064773
Sample Nickel conc 3 rep 1	0.055797
Sample Nickel conc 3 rep 2	0.048430
Sample Nickel conc 3 rep 3	0.086485

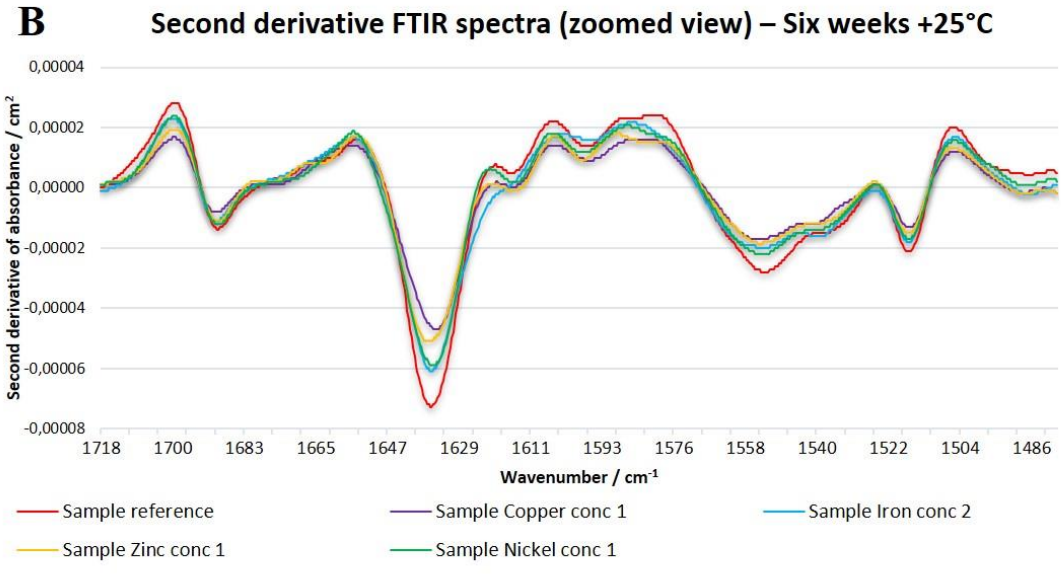
3.2.2 Conformation - Secondary structure

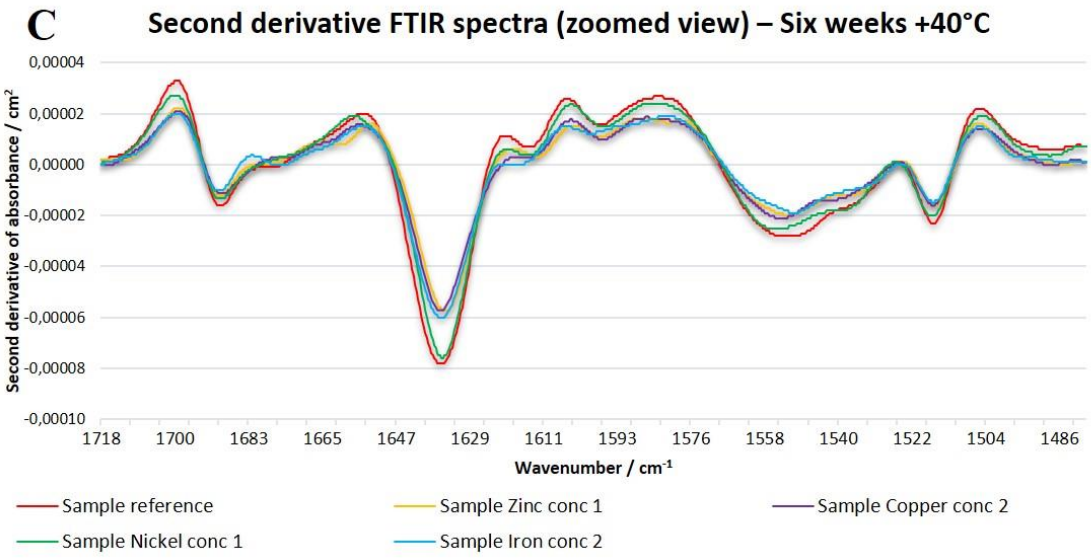
The secondary structure of the treated samples was evaluated by FT-IR spectroscopy. Far-UV Circular Dichroism was applied in the preliminary study (**Appendix 1**) but, since it was observed high variability and non-reproducibility of the spectra – due to a combination of the molecule's formulation excipients and the spiked elements - we decided to employ an orthogonal technique.

Figure 16 describes the modification to the FT-IR profiles upon treatment with the inorganic impurities. The overlay reports as example the FT-IR second derivative spectra, zoomed on the region 1720-1500 cm^{-1} (signals from the protein backbone) of the samples treated with the *Concentration Limit*. The effects of the four elements on the secondary structure are overall similar at all time points: major perturbations were observed for signals around 1554 cm-1 (α-helices/random coil), 1638 and 1690 cm-1 (β-sheet).

Figure 16 - Overlay of second derivative FT-IR spectra (All elements). Untreated material (red), samples treated with zinc (orange), nickel (green), iron (cyan) and copper (purple). One replicate of the Concentration Limit (30% PDE / daily dose of Fc-fusion protein) concentrations per sample is reported for time zero (A), after 6 weeks at +25°C (B), and after 6 weeks at +40°C (C). Major perturbations are observed for signals around 1554 cm-1 (α-helices/random coil), 1638 and 1690 cm-1 (β-sheet).


A
Second derivative FTIR spectra (zoomed view) – Time Zero
Second derivative of absorbance / cm²
0,00004
0,00002
0,00000
-0,00002
-0,00004
-0,00006
-0,00008
1718 1700 1683 1665 1647 1629 1611 1593 1576 1558 1540 1522 1504 1486
Wavenumber / cm⁻¹
Sample reference
Sample Zinc conc 1
Sample Copper conc 2
Sample Iron conc 2
Sample Nickel conc 1


B
Second derivative FTIR spectra (zoomed view) – Six weeks +25°C
Second derivative of absorbance / cm²
0,00004
0,00002
0,00000
-0,00002
-0,00004
-0,00006
-0,00008
1718 1700 1683 1665 1647 1629 1611 1593 1576 1558 1540 1522 1504 1486
Wavenumber / cm⁻¹
Sample reference
Sample Copper conc 1
Sample Iron conc 2
Sample Zinc conc 1
Sample Nickel conc 1


C
Second derivative FTIR spectra (zoomed view) – Six weeks +40°C
Second derivative of absorbance / cm²
0,00004
0,00002
0,00000
-0,00002
-0,00004
-0,00006
-0,00008
-0,00010
1718 1700 1683 1665 1647 1629 1611 1593 1576 1558 1540 1522 1504 1486
Wavenumber / cm⁻¹
Sample reference
Sample Zinc conc 1
Sample Copper conc 2
Sample Nickel conc 1
Sample Iron conc 2

This perturbation of the secondary structure becomes more evident when looking to the amide I and amide II band Wavenumber (cm^{-1}) calculated for the treated samples: modifications were observed at all time points, temperatures and concentrations.

These Wavenumber, sensitive to the backbone conformation, are reported in **Tables 18 (Iron), 19 (Copper), 20 (Zinc), 21 (Nickel)** where the values obtained for the treated samples are compared to the minimum and maximum values obtained for three replicates of the untreated material incubated at the same temperature (indicated in the table as Reference range). Non-comparable values are reported in bold.

Table 18. FT-IR: values of Amide I and Amide II – Iron. Amide I and II band Wavenumber (cm-1) at time zero at +5°C and after 6weeks at +25°C and +40°C. Results are compared to the minimum and maximum values obtained for the three replicates of reference material analysed in the same analytical session. Non-comparable values are reported in bold.

Samples treated with iron	**Wavenumber (cm^{-1})**					
	TIME ZERO		TIME 6 WEEKS +25°C		TIME 6 WEEKS +40°C	
	Amide I	**Amide II**	**Amide I**	**Amide II**	**Amide I**	**Amide II**
Conc 1 rep. 1	**1634.5**	**1547.9**	**1634.2**	**1547.4**	**1633.6**	**1546.4**
Conc 1 rep. 2	**1634.4**	**1548.5**	**1634.4**	**1547.6**	**1633.6**	**1547.1**
Conc 1 rep. 3	**1634.5**	**1548.7**	**1634.1**	**1547.5**	**1633.4**	**1546.4**
Conc 2 rep. 1	**1634.2**	**1548.4**	**1634.3**	**1548.9**	**1635.1**	**1548.1**
Conc 2 rep. 2	**1633.9**	**1549.1**	**1634.2**	**1548.4**	**1634.9**	**1548.4**
Conc 2 rep. 3	**1634.1**	**1549.2**	**1634.1**	**1548.5**	**1635.2**	**1548.7**
Conc 3 rep. 1	**1629.6**	**1547.7**	**1634.2**	**1547.6**	**1635.1**	**1547.7**
Conc 3 rep. 2	**1627.9**	**1547.7**	**1633.7**	**1547.2**	**1635.0**	**1547.7**
Conc 3 rep. 3	**1627.9**	**1545.8**	**1633.8**	**1547.4**	**1634.9**	**1547.7**
Reference range	1635.9-1636.0	1548.1-1548.2	1635.5-1635.7	1548.2-1548.3	1635.8-1635.9	1547.8-1547.9

Table 19. FT-IR: values of Amide I and Amide II – Copper. Amide I and II band Wavenumber (cm^{-1}) at time zero and after 6weeks at +25°C and +40°C. Results are compared to the minimum and maximum values obtained for the three replicates of reference material analysed in the same analytical session. Non-comparable values are reported in bold.

Samples treated with copper	**Wavenumber (cm^{-1})**					
	TIME ZERO		TIME 6 WEEKS +25°C		TIME 6 WEEKS +40°C	
	Amide I	**Amide II**	**Amide I**	**Amide II**	**Amide I**	**Amide II**
Conc 1 rep.1	**1633.6**	**1548.5**	**1635.3**	**1548.0**	**1635.2**	**1548.7**
Conc 1 rep.2	**1633.7**	**1548.4**	**1635.0**	**1550.0**	**1635.1**	**1549.0**
Conc 1 rep.3	**1633.8**	**1548.4**	1635.6	1548.3	**1635.1**	**1548.6**
Conc 2 rep.1	**1635.0**	**1547.9**	**1634.5**	**1549.4**	**1634.9**	**1548.2**
Conc 2 rep.2	**1634.8**	**1548.3**	**1634.5**	**1549.2**	**1634.8**	**1548.6**
Conc 2 rep.3	**1635.0**	**1548.0**	**1634.2**	**1549.0**	**1635.0**	**1548.8**
Conc 3 rep.1	**1635.1**	**1547.9**	**1634.9**	**1548.4**	**1634.5**	**1548.4**
Conc 3 rep.2	**1635.1**	**1548.4**	**1634.7**	1548.2	**1634.3**	**1548.7**
Conc 3 rep.3	**1634.8**	**1548.5**	**1634.7**	**1548.1**	**1634.7**	**1548.5**
Reference range	1635.6-1635.8	1546.8-1547.1	1635.5-1635.7	1548.2-1548.3	1635.8-1635.9	1547.8-1547.9

Table 20. FT-IR: values of Amide I and Amide II - Zinc. Amide I and II band Wavenumber (cm^{-1}) at time zero and after 6weeks at +25°C and +40°C. Results are compared to the minimum and maximum values obtained for the three replicates of reference material analysed in the same analytical session. Non-comparable values are reported in bold.

Samples treated with Zinc	**Wavenumber (cm^{-1})**					
	TIME ZERO		TIME 6 WEEKS +25°C		TIME 6 WEEKS +40°C	
	Amide I	**Amide II**	**Amide I**	**Amide II**	**Amide I**	**Amide II**
Conc 1 rep.1	**1635.5**	**1546.1**	**1634.8**	**1548.8**	**1634.4**	**1548.1**
Conc 1 rep.2	**1635.2**	**1547.6**	**1634.9**	**1549.0**	**1634.4**	**1548.9**
Conc 1 rep.3	**1635.4**	**1547.5**	**1634.8**	**1549.0**	**1634.6**	**1548.6**
Conc 2 rep.1	**1635.2**	1548.1	**1635.2**	1548.2	**1634.7**	**1548.9**
Conc 2 rep.2	**1634.9**	**1548.6**	**1635.1**	**1548.9**	**1634.6**	**1549.3**
Conc 2 rep.3	**1635.0**	**1548.4**	**1635.1**	**1549.1**	**1634.4**	**1549.8**
Conc 3 rep.1	**1634.7**	**1545.8**	**1634.9**	**1548.7**	**1634.8**	**1548.7**
Conc 3 rep.2	**1634.7**	**1544.0**	**1634.9**	**1549.0**	**1635.1**	**1548.8**
Conc 3 rep.3	**1634.7**	**1542.2**	**1635.0**	**1548.9**	**1635.4**	**1548.0**
Reference range	1635.9-1636.0	1548.1-1548.2	1635.5-1635.7	1548.2-1548.3	1635.8-1635.9	1547.8-1547.9

Table 21. FT-IR: values of Amide I and Amide II – Nickel. Amide I and II band Wavenumber (cm^{-1}) at time zero and after 6 weeks at +25°C and +40°C. Results are compared to the minimum and maximum values obtained for the three replicates of the reference material analysed in the same analytical session. Non-comparable values are reported in bold.

Samples treated with nickel	**Wavenumber (cm^{-1})**					
	TIME ZERO		TIME 6 WEEKS +25°C		TIME 6 WEEKS +40°C	
	Amide I	**Amide II**	**Amide I**	**Amide II**	**Amide I**	**Amide II**
Conc 1 rep.1	**1635.1**	1548.2	**1635.3**	**1548.4**	**1634.4**	**1548.6**
Conc 1 rep.2	**1635.1**	**1547.7**	**1635.4**	**1548.5**	**1635.1**	**1547.6**
Conc 1 rep.3	**1635.2**	**1548.3**	**1635.4**	**1548.4**	**1635.1**	**1547.6**
Conc 2 rep.1	**1634.8**	**1547.8**	**1634.9**	**1548.5**	**1634.5**	**1548.9**
Conc 2 rep.2	**1635.0**	**1548.0**	**1635.0**	**1548.5**	**1634.9**	**1548.5**
Conc 2 rep.3	**1634.8**	1548.1	**1634.9**	**1548.5**	**1635.1**	1547.9
Conc 3 rep.1	**1634.4**	**1548.3**	**1635.4**	**1548.4**	**1635.0**	**1549.8**
Conc 3 rep.2	**1634.8**	**1548.0**	**1635.1**	**1548.6**	**1634.5**	**1552.8**
Conc 3 rep.3	**1634.7**	**1547.9**	**1635.1**	**1548.5**	**1634.5**	**1552.7**
Reference range	1635.9-1636.0	1548.1-1548.2	1635.5-1635.7	1548.2-1548.3	1635.8-1635.9	1547.8-1547.9

3.2.3 Aggregation and fragmentation

In order to evaluate if the presence of the four inorganic leachables could modify the propensity to aggregation or fragmentation of the molecule, treated samples were analysed by SEC-UPLC and compared to the untreated material incubated at the same temperature.

3.2.3.1 Iron

Figures 17 and **18** and **Table 22** report the results obtained upon treatment with iron. In this case at time zero no differences were observed between treated and untreated samples, regardless of the concentration employed. However, after 6 weeks of storage at +25°C, the addition of iron induced a major increase of the Low Molecular Weight species (LMW) and almost complete loss of the monomer with respect to the untreated material incubated at the same conditions, most likely due to fragmentation. This effect was confirmed also after incubation at +40°C (**Fig 18 B**).

Figure 17. Summary of SEC results - Iron. %HMW (yellow), %LMW (green) and %Monomer (blue) are reported for each sample as average of three independent replicates. Results are reported for treated samples at time zero and after 6 weeks at +25°C and +40°C.

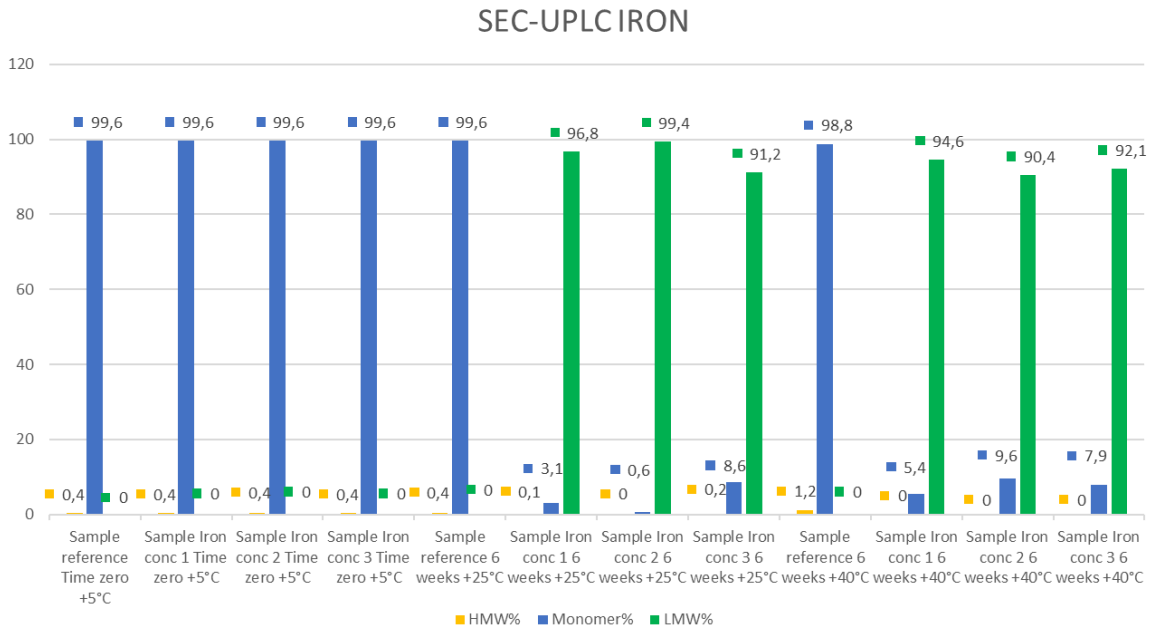
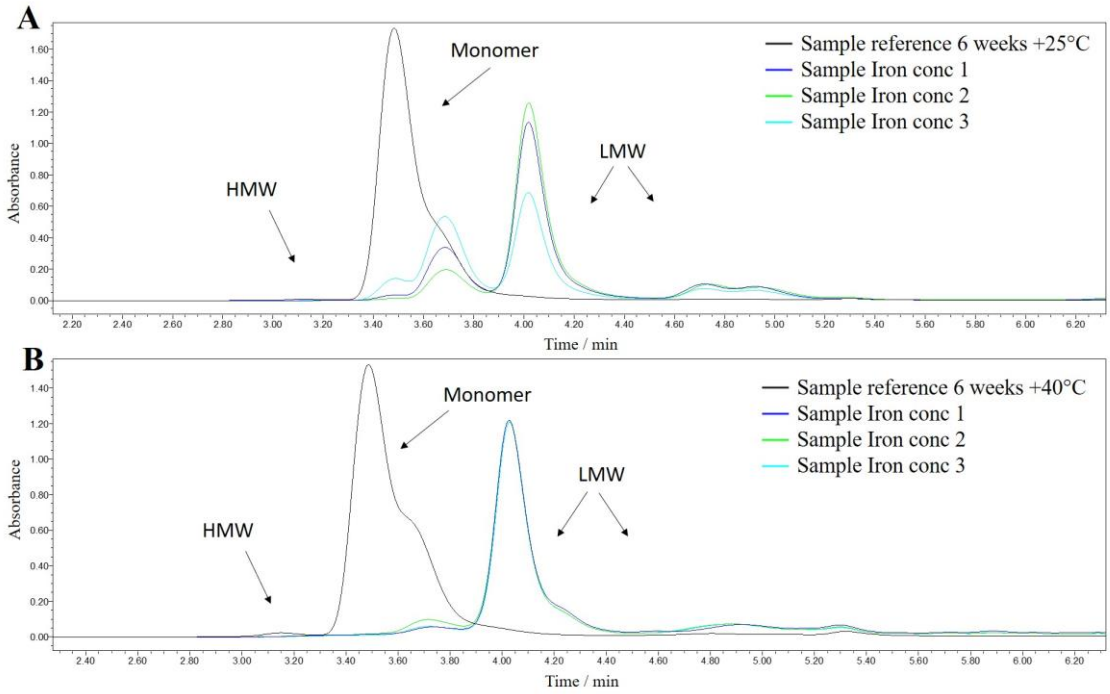


Table 22. Summary of SEC-UPLC results on aggregation - Iron. Area (%) of HMW, monomer and Low Molecular Weight species in samples at time zero and after 6 weeks at +25°C and +40°C. Results for the treated samples are expressed as average of three independent replicates and are compared to the minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature. Non-comparable values are reported in bold.

Aggregation - IRON					
Condition	**Parameter**	**Reference range**	**Sample Iron conc 1**	**Sample Iron conc 2**	**Sample Iron conc 3**
Time zero +5°C	% HMW	0.4 - 0.5	0.4	0.4	0.4
	% Monomer	99.6 - 99.5	99.6	99.6	99.6
	% LMW	0	0	0	0
Time 6 week +25°C	% HMW	0.4 - 0.5	**0.1**	**0**	**0.1**
	% Monomer	99.6 - 99.5	**3.1**	**0.6**	**8.7**
	% LMW	0	**96.8**	**99.4**	**91.2**
Time 6 week +40°C	% HMW	1.1 - 1.4	**0.0**	**0.0**	**0.0**
	% Monomer	98.8 - 98.7	**5.4**	**9.6**	**7.9**
	% LMW	0	**94.6**	**90.1**	**92.1**

Figure 18. SEC results – Iron Overlay of the chromatograms of untreated material (black) and samples treated with iron (blue, green and cyan) after 6 weeks at +25°C **(A)** and at +40°C **(B).** One replicate per sample is reported.



Legend: HMW High Molecular Weight species
LMW Low Molecular Weight species

As shown in **Figure 18**, one LMW species above all others contributed to the high percentage of fragmentation reported in **Figure 17** for samples incubated at +25°C and +40°C.

To characterize the nature of this LMW species, samples incubated for 6 weeks at +40°C were analysed by SEC-MALS in order to determine its molar mass. **Figure 19** reports the overlay of the SEC-MALS chromatograms of samples treated with iron and incubated for 6 weeks at +40°C while **Table 23** reports the molecular weights calculated for the detected species: in case of the LMW one the molecular weight was determined as 134.1 KDa, almost 48 KDa less than the monomer.

Considering that the Fc-fusion protein used for this study is comprised of an IgG1 with an extracellular domain (ECD) protein fused to the C-terminus via a linker,

this result suggested this species corresponded to the molecule deprived of the fused extracellular domain (ECD) protein.

Figure 19. SEC-MALS on samples treated with Iron –Overlay (**A**) and zoomed view (**B**) of the UV chromatograms of untreated material at time zero (black) and samples treated with iron (blue, green and violet) after 6 weeks at +40°C.

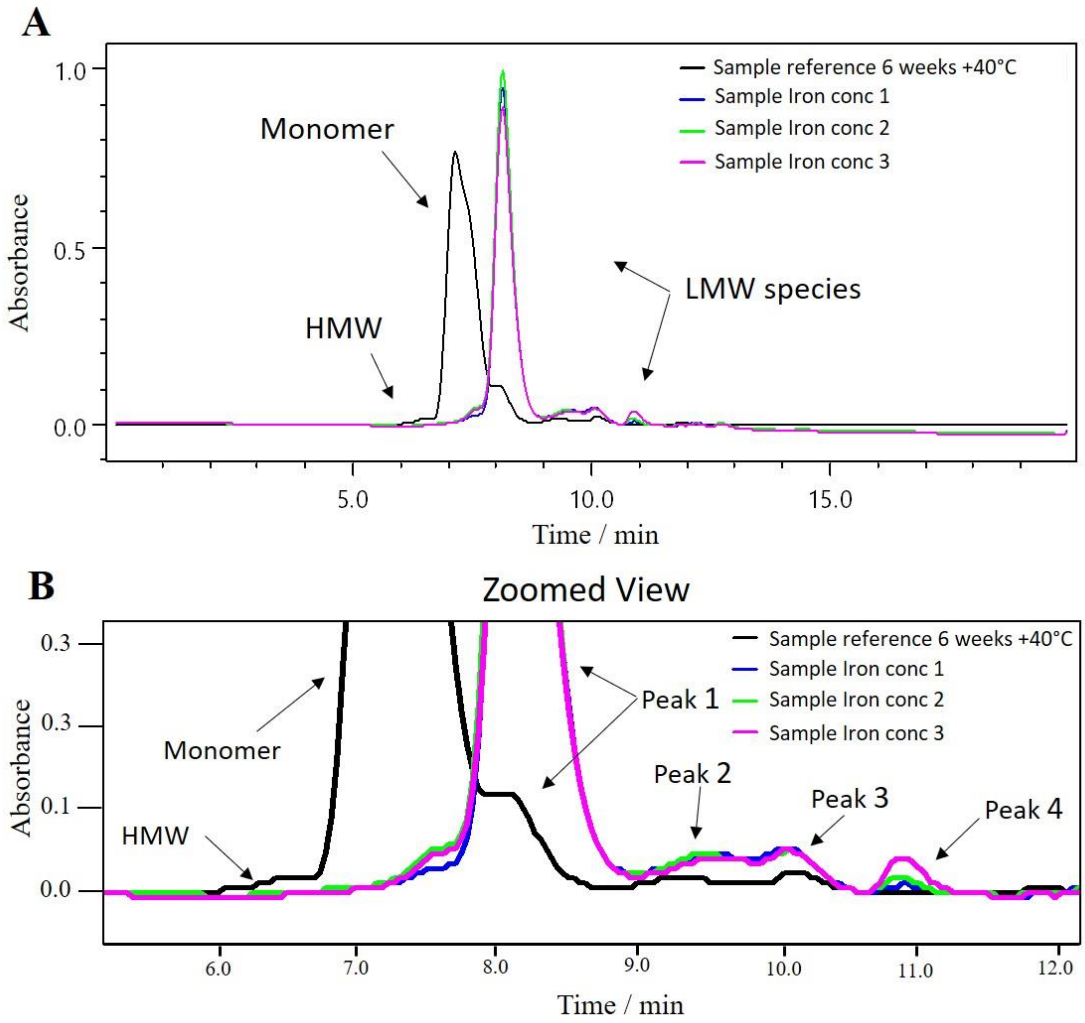


Table 23 – SEC-MALS Results (molar masses). Molar masses are reported only for species > 2% (LOQ of the method).

Molecular Weight (kDa)						
Sample	**Monomer**	**High Molecular Weight**	**Low Molecular Weight**			
			Peak 1	**Peak 2**	**Peak 3**	**Peak 4**
Sample Time zero	191.0	n.a.	n.a.	n.a.	n.a.	n.a.
Sample reference 6 weeks +40°C	182.1	n.a.	144.1	n.a.	n.a.	n.a.
Sample Iron conc 1 6 weeks +40°C	n.a.	n.a.	134.1	65.7	45.5	n.a.
Sample Iron conc 2 6 weeks +40°C	198.8	n.a.	134.1	66.5	47.2	n.a.
Sample Iron conc 3 6 weeks +40°C	192.5	n.a.	134.1	65.4	45.5	32.5

n.a.: not applicable (< LOQ)

This LMW species was further investigated collecting it from Sample Iron conc 3 incubated for 6 weeks at +40°C with an UPLC-fraction collector (**Figure 20**) and by analysing it by LC-MS (**Table 24**, **Figure 21).**

The LC-MS analysis showed that the Light Chain (LC) of the Fc-fusion protein remained intact while the Heavy Chain (HC) presented a lower molecular weight, 50894.45 Da (the molecular weight of the intact HC calculated for the reference sample- aligned to the theoretical value - is 65841.18 Da). Such lower weight was due to a cleavage between an Histidine and a Valine, five residues downstream the linker connecting the IgG1 domain and the ECD one, confirming the hypothesis based on SEC-MALS data.

Figure 20. UPLC – fraction collection.

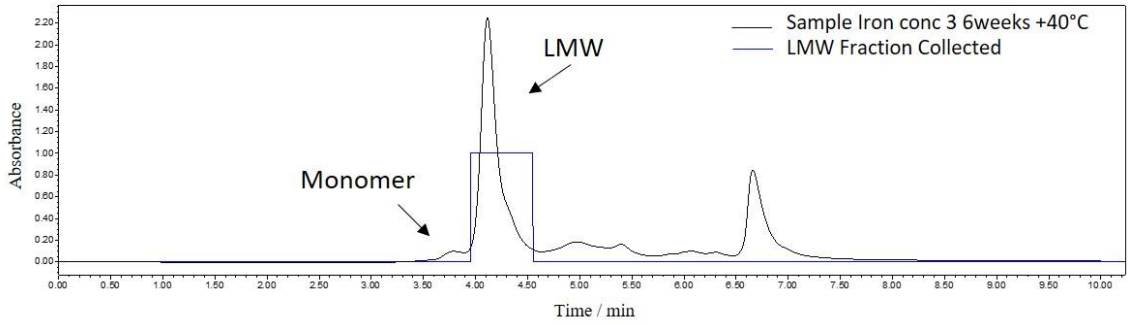
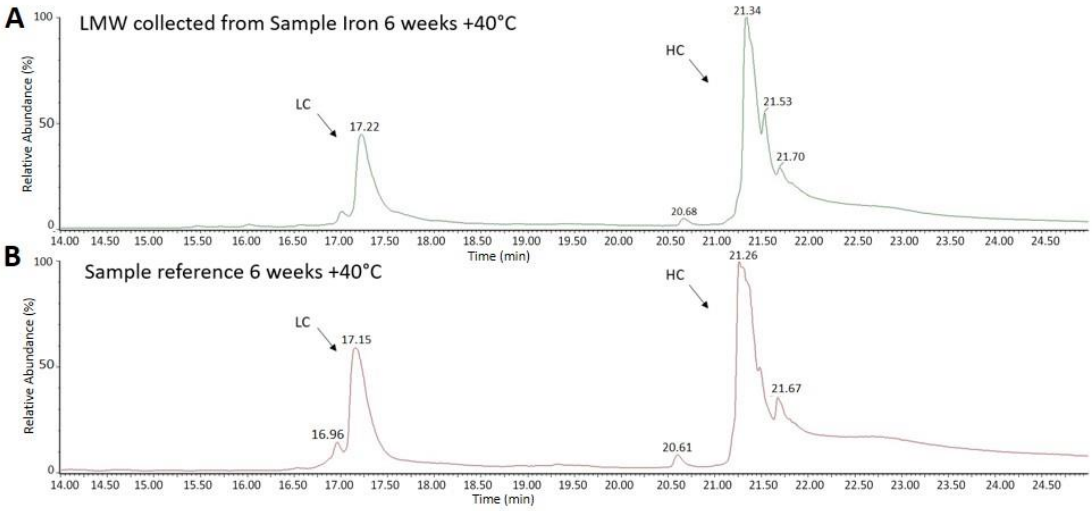


Table 24: ESI-MS: LMW fragment of Fc-fusion protein treated with iron conc 3 6weeks +40°C collected from SEC

Sample	Peak Residues	Expected mass (Da)	Observed mass (Da)	Cleavage site
LMW collected from Sample Iron conc 3 6 weeks +40°C	HC:1–607	65841.29	65841.18	-
Sample reference 6 weeks +40°C	HC:1–475	50891.36	50894.45	His–Val

Figure 21. ESI-MS: total ion current (TIC) of A) LMW (fragment of Fc-fusion protein observed in sample iron conc 3 6 weeks +40°C by LC/ESI-MS; **B)** Sample reference time zero by LC/ESI-MS



3.2.3.2 Copper, Zinc and Nickel

Figure 22 and Table 25 report the results obtained upon treatment with copper, zinc and nickel. In terms of monomer purity no differences were observed by SEC-UPLC at time zero and after incubation for 6 weeks at +25°C. Indeed, all samples show a %HMW (%High Molecular Weight) around 0.4-0.5% that is consistent with the percentage obtained for the reference material incubated at the same storage conditions. Only after 6 weeks at +40°C we observed a slight increase of the %HMW species ascribable, however, to the effect of the storage temperature rather than to the interaction of the molecule with the metal ions, as the same effects were observed also in the untreated material stored at +40°C (**Table 25, Figure 23**).

Figure 22. SEC results – Zinc, Copper and Nickel. Overlay of the chromatograms of untreated material (black) and samples treated with copper, zinc and nickel at time zero (**A**), after 6 weeks at +25°C (**B**) and at +40°C (**C**)**.** One replicate per sample is reported.

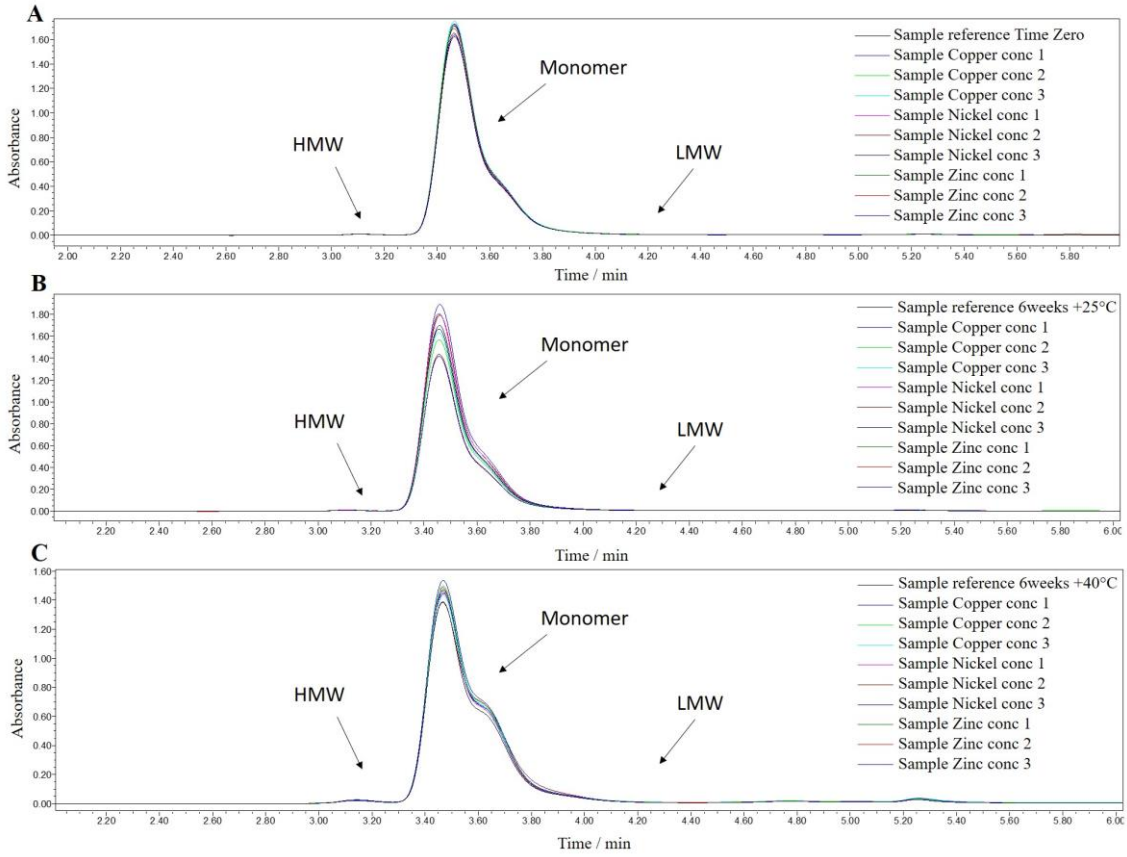
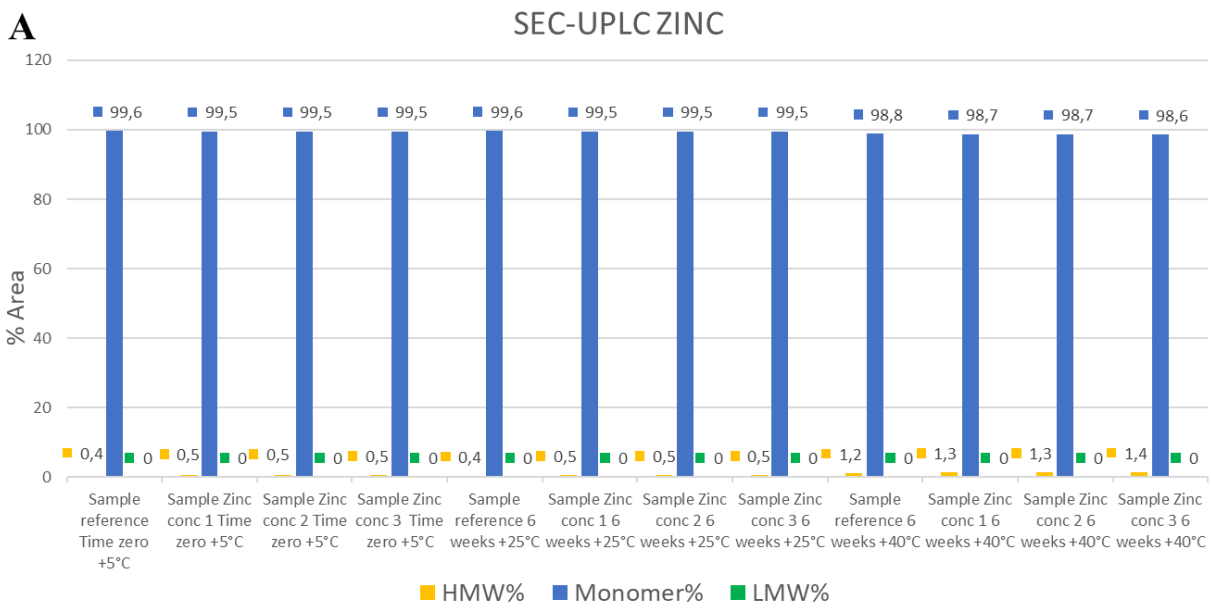


Table 25 - Summary of SEC-UPLC results on aggregation – Copper, Nickel and Zinc. Area (%) of monomer, HMW and LMW species in samples at time zero and after 6 weeks at +25°C and +40°C. Results for the treated samples are expressed as average of three independent replicates and are compared to the minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature. Non-comparable values are reported in bold.

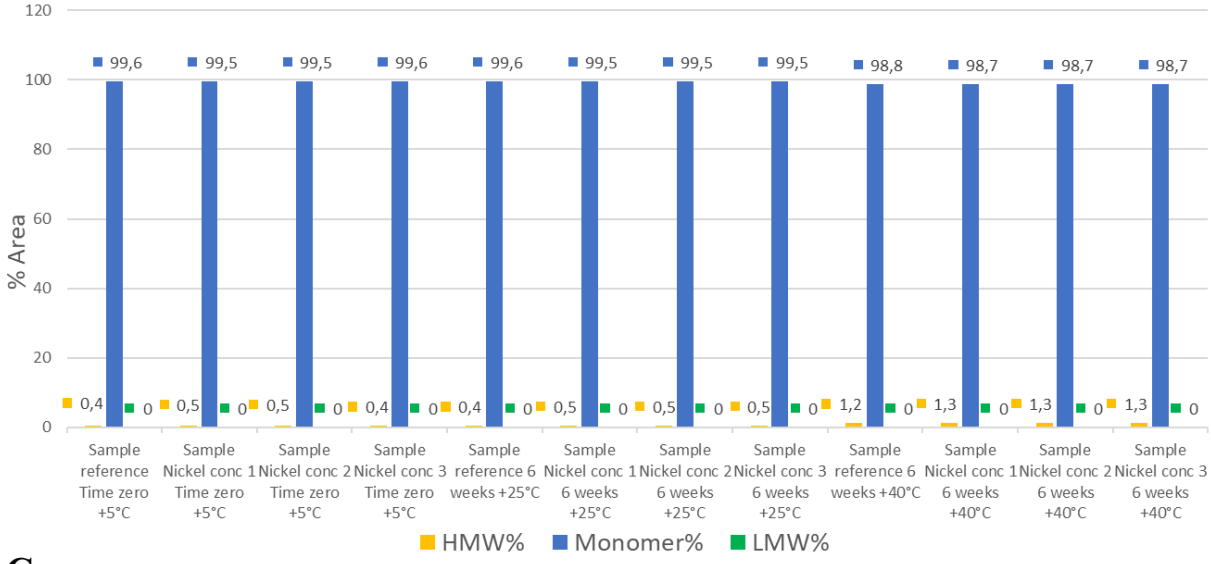
Aggregation - COPPER					
Condition	**Parameter**	**Reference range**	**Sample Copper conc 1**	**Sample Copper conc 2**	**Sample Copper conc 3**
Time zero +5°C	% HMW	0.4 - 0.5	0.4	0.4	0.4
	% Monomer	99.6 - 99.5	99.6	99.6	99.6
	% LMW	0.0	0	0	0
6 week +25°C	% HMW	0.4 - 0.5	0.4	0.4	0.4
	% Monomer	99.6 - 99.5	99.6	99.6	99.6
	% LMW	0.0	0	0	0
6 week +40°C	% HMW	1.2 – 1.3	1.2	1.2	1.2
	% Monomer	98.8 - 98.7	98.8	98.8	98.8
	% LMW	0.0	0	0	0
Aggregation - NICKEL					
Condition	**Parameter**	**Reference range**	**Sample Nickel conc 1**	**Sample Nickel conc 2**	**Sample Nickel conc 3**
Time zero +5°C	% HMW	0.4 - 0.5	0.5	0.5	0.4
	% Monomer	99.6 - 99.5	99.5	99.5	99.6
	% LMW	0.0	0	0	0
6 week +25°C	% HMW	0.4 - 0.5	0.5	0.5	0.5
	% Monomer	99.6 - 99.5	99.5	99.5	99.5
	% LMW	0.0	0	0	0
6 week +40°C	% HMW	1.2 – 1.3	1.3	1.3	1.3
	% Monomer	98.8 - 98.7	98.7	98.7	98.7
	% LMW	0.0	0	0	0
Aggregation - ZINC					
Condition	**Parameter**	**Reference range**	**Sample Zinc conc 1**	**Sample Zinc conc 2**	**Sample Zinc conc 3**
Time zero +5°C	% HMW	0.4 - 0.5	0.5	0.5	0.5
	% Monomer	99.6 - 99.5	99.5	99.5	99.5
	% LMW	0.0	0	0	0
6 week +25°C	% HMW	0.4 - 0.5	0.5	0.5	0.5
	% Monomer	99.6 - 99.5	99.5	99.5	99.5
	% LMW	0.0	0	0	0
6 week +40°C	% HMW	1.2 – 1.3	1.3	1.3	**1.4**
	% Monomer	98.8 - 98.7	98.7	98.7	**98.6**
	% LMW	0.0	0	0	**0**

Figure 23. Summary of SEC results – Copper, Zinc and Nickel. %HMW (yellow), %LMW (green) and %Monomer (blue) are reported for each sample as average of three independent replicates. Results are reported for treated samples at time zero and after 6 weeks at +25°C and +40°C.

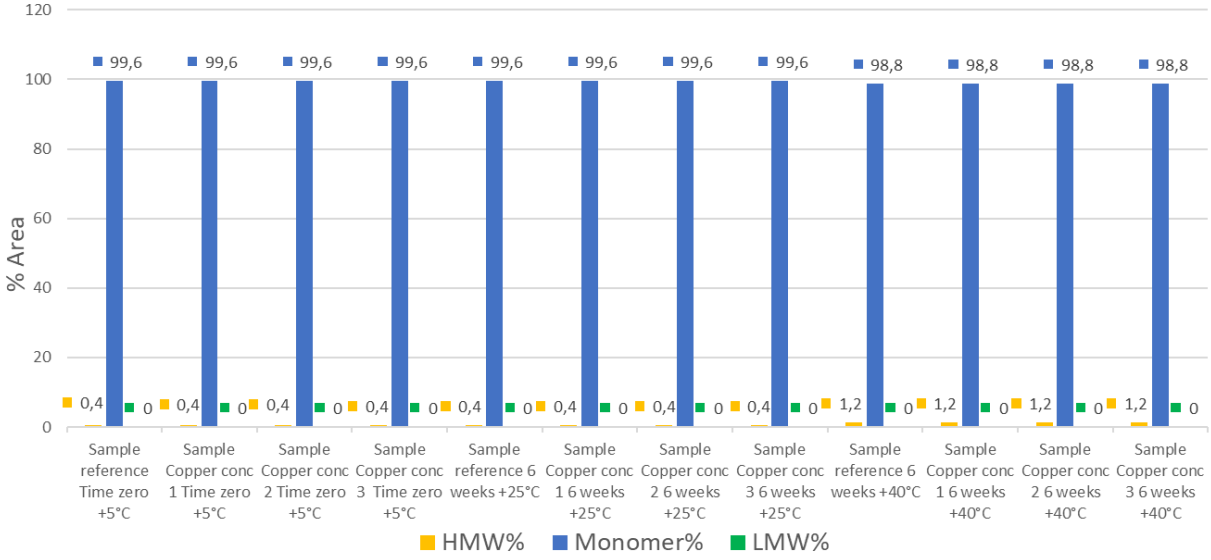
A


SEC-UPLC ZINC
% Area
120
100
80
60
40
20
0
99,6 99,5 99,5 99,5 99,6 99,5 99,5 99,5 98,8 98,7 98,7 98,6
0,4 0 0,5 0 0,5 0 0,5 0 0,4 0 0,5 0 0,5 0 0,5 0 1,2 0 1,3 0 1,3 0 1,4 0
Sample reference Time zero +5°C
Sample Zinc conc 1 Time zero +5°C
Sample Zinc conc 2 Time zero +5°C
Sample Zinc conc 3 Time zero +5°C
Sample reference 6 weeks +25°C
Sample Zinc conc 1 6 weeks +25°C
Sample Zinc conc 2 6 weeks +25°C
Sample Zinc conc 3 6 weeks +25°C
Sample reference 6 weeks +40°C
Sample Zinc conc 1 6 weeks +40°C
Sample Zinc conc 2 6 weeks +40°C
Sample Zinc conc 3 6 weeks +40°C
HMW% Monomer% LMW%

B


SEC-UPLC NICKEL
% Area
120
100
80
60
40
20
0
99,6 99,5 99,5 99,6 99,6 99,5 99,5 99,5 98,8 98,7 98,7 98,7
0,4 0 0,5 0 0,5 0 0,4 0 0,4 0 0,5 0 0,5 0 0,5 0 1,2 0 1,3 0 1,3 0 1,3 0
Sample reference Time zero +5°C
Sample Nickel conc 1 Time zero +5°C
Sample Nickel conc 2 Time zero +5°C
Sample Nickel conc 3 Time zero +5°C
Sample reference 6 weeks +25°C
Sample Nickel conc 1 6 weeks +25°C
Sample Nickel conc 2 6 weeks +25°C
Sample Nickel conc 3 6 weeks +25°C
Sample reference 6 weeks +40°C
Sample Nickel conc 1 6 weeks +40°C
Sample Nickel conc 2 6 weeks +40°C
Sample Nickel conc 3 6 weeks +40°C
HMW% Monomer% LMW%

C


SEC-UPLC COPPER
% Area
120
100
80
60
40
20
0
99,6 99,6 99,6 99,6 99,6 99,6 99,6 99,6 98,8 98,8 98,8 98,8
0,4 0 0,4 0 0,4 0 0,4 0 0,4 0 0,4 0 0,4 0 0,4 0 1,2 0 1,2 0 1,2 0 1,2 0
Sample reference Time zero +5°C
Sample Copper conc 1 Time zero +5°C
Sample Copper conc 2 Time zero +5°C
Sample Copper conc 3 Time zero +5°C
Sample reference 6 weeks +25°C
Sample Copper conc 1 6 weeks +25°C
Sample Copper conc 2 6 weeks +25°C
Sample Copper conc 3 6 weeks +25°C
Sample reference 6 weeks +40°C
Sample Copper conc 1 6 weeks +40°C
Sample Copper conc 2 6 weeks +40°C
Sample Copper conc 3 6 weeks +40°C
HMW% Monomer% LMW%

3.2.4 Oxidation

RP-UPLC was employed to monitor the oxidation levels of the most relevant oxidation site of the molecule upon interaction of the Fc-fusion protein with the four elements: Met-ECD1, a methionine located on the heavy chain of the Fc-fusion protein, in the ECD domain.

3.2.4.1 Iron

In case of iron, no increase of oxidation was observed at time zero and after 6 weeks of incubation at +25°C: indeed, also in case of concentrations 2 and 3 variations are within method variability. After 6 weeks at +40°C, instead, a concentration-dependent increase was observed, with respect to the untreated material incubated at the same condition as reported in **Table 26**.

Table 26. **Summary of RP-UPLC results on oxidation – Iron.** Area (%) of the oxidized Met-ECD1 in samples incubated at time zero and for 6 weeks at +25°C and +40°C. Results for the treated samples are expressed as average of three independent replicates and compared to minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature. Non-comparable values are reported in bold.

Conditions	Samples	% Area Met-ECD1 oxidized
Time zero +5°C	Sample reference range	3.1 – 3.6
	Sample Iron conc 1	3.3
	Sample Iron conc 2	3.2
	Sample Iron conc 3	3.4
6 weeks +25°C	Sample reference range	3.1 – 3.6
	Sample Iron conc 1	3.5
	Sample Iron conc 2	**4.2**
	Sample Iron conc 3	**4.7**
6 weeks +40°C	Sample reference range	4.4 – 5.0
	Sample Iron conc 1	**6.1**
	Sample Iron conc 2	**8.5**
	Sample Iron conc 3	**9.2**

Eventually, peptide mapping by LC-MS was performed on samples incubated for 6 weeks at +40°C to better characterize the extent of the oxidation observed by RP-UPLC. This analysis showed that all the iron concentrations were able to induce oxidation in a concentration-dependent manner not only on Met-ECD1, but also on other two methionines: Met-HC (situated on the HC) and, especially, on Met-ECD2 situated on the ECD region (**Table 27**). No oxidation on other residues was observed.

Table 27. LC-MS Peptide Mapping - Oxidation (%) of methionine residues in samples incubated with iron for 6 weeks at +40°C. Non-comparable values are reported in bold.

Peptide Mapping Results			
Samples 6 weeks +40°C	**Met-HC Ox%**	**Met-ECD1 Ox%**	**Met-ECD2 Ox%**
Sample reference	3.1	4.5	3.5
Sample Iron conc 1	**3.9**	**5.6**	**10.1**
Sample Iron conc 2	**4.6**	**6.9**	**12.8**
Sample Iron conc 3	**4.6**	**7.1**	**15.3**

3.2.4.2 Copper

For copper as well, we detected by RP-UPLC a concentration-dependent increase of the oxidation levels of Met-ECD1 only after incubation at +40°C for 6 weeks (**Table 28**). This increase can be, however, considered significant only in case of concentrations 2 and 3 (taking into account also results obtained after incubation for 44 weeks, see section 3.3.2).

As in case of iron, an additional investigation by peptide mapping of these samples revealed that all tested concentrations were able to induce almost the same degree of oxidation on the three methionine residues monitored by the applied method (**Table 29**). No oxidation on other residues was observed.

Table 28. **Summary of RP-UPLC results on oxidation – Copper.** Area (%) of the oxidized Met-ECD in samples incubated at time zero and for 6 weeks at +25°C and +40°C. Results for the treated samples are expressed as average of three independent replicates and compared to minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature. Non-comparable values are reported in bold.

Conditions	**Samples**	**% Area Met-ECD1 oxidized**
Time zero +5°C	Sample reference range	3.1 – 3.6
	Sample Copper conc 1	3.3
	Sample Copper conc 2	3.3
	Sample Copper conc 3	3.4
6 weeks +25°C	Sample reference range	3.1 – 3.6
	Sample Copper conc 1	3.2
	Sample Copper conc 2	3.1
	Sample Copper conc 3	3.3
6 weeks +40°C	Sample reference range	4.4 – 5.0
	Sample Copper conc 1	**5.8**
	Sample Copper conc 2	**7.1**
	Sample Copper conc 3	**8.5**

Table 29. LC-MS Peptide Mapping - Oxidation (%) of HC methionine residues in samples incubated with copper for 6 weeks at +40°C. Non-comparable values are reported in bold.

Peptide Mapping Results			
Samples 6 weeks +40C	**Met-HC Ox%**	**Met-ECD1 Ox%**	**Met-ECD2 Ox%**
Sample reference	3.1	4.5	3.5
Sample Copper conc 1	**3.3**	**5.2**	**3.9**
Sample Copper conc 2	**3.8**	**6.3**	**4.7**
Sample Copper conc 3	**4.4**	**7.4**	**5.6**

3.2.4.3 Zinc and Nickel

In case of zinc (**Table 30**) and nickel (**Table 31**) at time zero and after 6 weeks at +25°C and +40°C no differences in terms of oxidation were observed.

Table 30. **Summary of RP-UPLC results on oxidation – Zinc.** Area (%) of the oxidized Met-ECD1 in samples incubated at time zero and for 6 weeks at +25°C and +40°C. Results for the treated samples are expressed as average of three independent replicates and compared to minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature. Non-comparable values are reported in bold.

Conditions	Samples	% Area Met-ECD1 oxidized
Time zero +5°C	Sample reference range	4.7 – 5.8
	Sample Zinc conc 1	4.8
	Sample Zinc conc 2	4.8
	Sample Zinc conc 3	4.7
6 weeks +25°C	Sample reference range	3.1 – 3.6
	Sample Zinc conc 1	3.6
	Sample Zinc conc 2	3.2
	Sample Zinc conc 3	3.4
6 weeks +40°C	Sample reference range	4.4 – 5.0
	Sample Zinc conc 1	4.7
	Sample Zinc conc 2	4.8
	Sample Zinc conc 3	4.6

Table 31. **Summary of RP-UPLC results on oxidation – Nickel.** Area (%) of the oxidized Met-ECD1 in samples incubated at time zero and for 6 weeks at +25°C and +40°C. Results for the treated samples are expressed as average of three independent replicates and compared to minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature. Non-comparable values are reported in bold.

Conditions	Samples	% Area Met-ECD1 oxidized
Time zero +5°C	Sample reference range	4.7 – 5.8
	Sample Nickel conc 1	5.1
	Sample Nickel conc 2	5.0
	Sample Nickel conc 3	4.7
6 weeks +25°C	Sample reference range	3.1 – 3.6
	Sample Nickel conc 1	3.4
	Sample Nickel conc 2	3.5
	Sample Nickel conc 3	3.3
6 weeks +40°C	Sample reference range	4.4 – 5.0
	Sample Nickel conc 1	4.5
	Sample Nickel conc 2	4.7
	Sample Nickel conc 3	4.9

3.2.4.4 Methionine content by Reverse-Phase UPLC (RP-UPLC)

In the DP formulation placebo Methionine is included as antioxidant excipient. In order to better clarify if the observed protein oxidation was happening after complete oxidation of the antioxidant an additional RP-UPLC analysis was performed to quantify this free amino acid. Only samples treated with copper and iron, stored for 6 weeks at +25°C and +40°C were analysed.

In samples treated with iron (**Table 32**) after 6 weeks, regardless of the temperature of incubation, a similar decrease in the concentration of the free methionine (of about 13-15%) was observed in samples treated with iron in comparison to the

reference material incubated under the same storage conditions (**Figure 24**). In case of the placebo incubated at +25°C the same decrease was observed while placebo incubated at +40°C presented a slightly higher decrease (17-18%). These data confirmed that methionine residues' oxidation was not due to the incapacity of the antioxidant to protect the Fc-fusion protein.

As demonstrated for the samples treated with iron, also in samples treated with copper (**Table 33**) the oxidation of methionines (Met-HC, Met-ECD1 and Met-ECD2) was not due to the incapacity of the placebo's methionine to protect the Fc-fusion protein (**Figure 24**). Indeed, in comparison to the reference material incubated under the same storage conditions, after 6 weeks at +25°C treated samples only showed a 13-14% decrease of the excipient while after 6 weeks at +40°C an 8-10% decrease. In case of placebos similar decreases were observed: 13-14% after incubation at +25°C and 11-13% after incubation at +40°C.

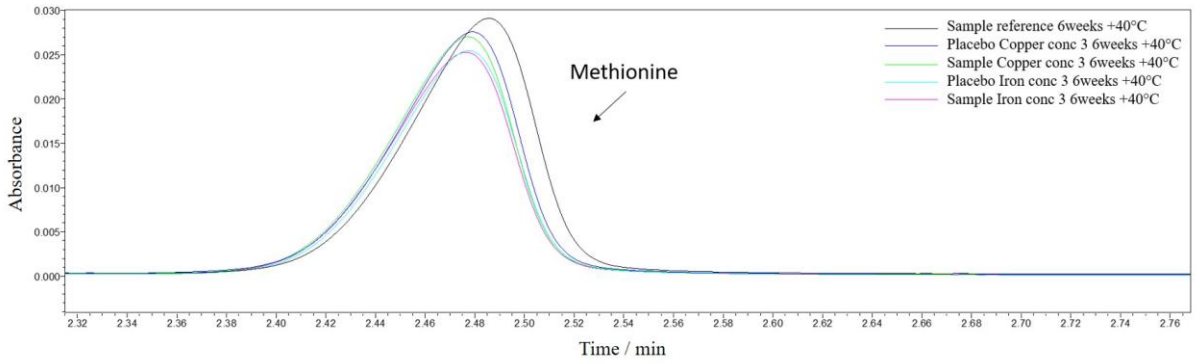
Table 32. Methionine quantification by RP-UPLC. Samples treated with iron and incubated for 6 weeks at +25°C and at +40°C.

Samples	**Met (mM)**	
	6 weeks +25°C	**6 weeks +40°C**
Sample reference rep 1	5.196	5.030
Sample reference rep 2	5.166	5.037
Sample reference rep 3	5.160	5.028
Placebo reference rep 1	5.256	5.317
Placebo reference rep 2	5.247	5.336
Sample Iron conc 1 rep 1	4.372	4.223
Sample Iron conc 1 rep 2	4.357	4.469
Sample Iron conc 1 rep 3	4.399	4.434
Placebo Iron conc 1	4.502	4.400
Samples Iron conc 2 rep 1	4.547	4.345
Samples Iron conc 2 rep 2	4.342	4.408
Samples Iron conc 2 rep 3	4.485	4.417
Placebo Iron conc 2	4.525	4.359
Samples Iron conc 3 rep 1	4.433	4.323
Samples Iron conc 3 rep 2	4.345	4.301
Samples Iron conc 3 rep 3	4.391	4.361
Placebo Iron conc 3	4.496	4.345

Table 33. Methionine quantification by RP-UPLC. Samples treated with copper and incubated for 6 weeks at +25°C and at +40°C.

Samples	**Met (mM)**	
	6 weeks +25°C	**6 weeks +40°C**
Sample reference rep 1	5.196	5.030
Sample reference rep 2	5.166	5.037
Sample reference rep 3	5.160	5.028
Placebo reference rep 1	5.256	5.317
Placebo reference rep 2	5.247	5.336
Sample Copper conc 1 rep 1	4.461	4.627
Sample Copper conc 1 rep 2	4.518	4.569
Sample Copper conc 1 rep 3	4.556	4.603
Placebo Copper conc 1	4.542	4.624
Samples Copper conc 2 rep 1	4.412	4.604
Samples Copper conc 2 rep 2	4.567	4.575
Samples Copper conc 2 rep 3	4.403	4.654
Placebo Copper conc 2	4.568	4.614
Samples Copper conc 3 rep 1	4.494	4.608
Samples Copper conc 3 rep 2	4.543	4.691
Samples Copper conc 3 rep 3	4.503	4.590
Placebo Copper conc 3	4.577	4.725

Figure 24 – Methionine Quantification – RP-UPLC - Zoomed view of the chromatograms of samples incubated at +40°C for 6 weeks, treated with concentration 3 of copper (green) or concentration 3 of iron (pink). Placebos treated with concentration 3 of copper (violet) or concentration 3 of iron (cyan), both stored for 6 weeks at +40°C are reported as well. The profile of the untreated sample incubated at +40°C for 6 weeks is reported in black.



3.2.5 Biological characterization

To analyse potential changes in the biological activity of the Fc-fusion protein after the interaction with the four metal ions, binding affinities and kinetic rate constants were studied by Surface Plasmon Resonance (SPR).

The following section reports the functional characterization of the interaction between the Fc-fusion protein and two of its ligands:

- the binding between the Fab portion of the molecule and the Fab ligand;
- the binding between the ECD fused protein portion of the molecule and the ECD ligand.

These analyses were performed on samples reported in **Table 5**, chosen as representative concentration: more in details for iron and copper, where significant alterations were detected in this time point, the lowest concentrations were chosen, while for zinc and nickel, where no significant alteration were seen in this time point, the highest concentrations were chosen.

The interaction was observed in terms of Absolute KD and Relative KD. The Absolute KD is the equilibrium dissociation constant between the Fc-fusion protein and its ligands and is expressed in molarity. Absolute KD and affinity are inversely related as the lower the KD value, the higher the molecule's affinity.

The Relative KD constant relates the Absolute KD obtained for the reference material (sample reference time zero) and the sample under examination, allowing to relate data obtained in different sessions as well.

3.2.5.1 Fab portion binding activity

In this section are reported the results of the characterization of the interaction Fc-fusion protein/Fab ligand.

As reported in **Table 34,** untreated samples stored for 6 weeks at +25°C and +40°C showed comparable binding affinities, 94% and 91% of Relative KD, respectively; comparability was observed as well with respect to the untreated sample at time zero, indicating that the temperature of incubation in "normal" conditions does not modify the functional activity of the Fab portion.

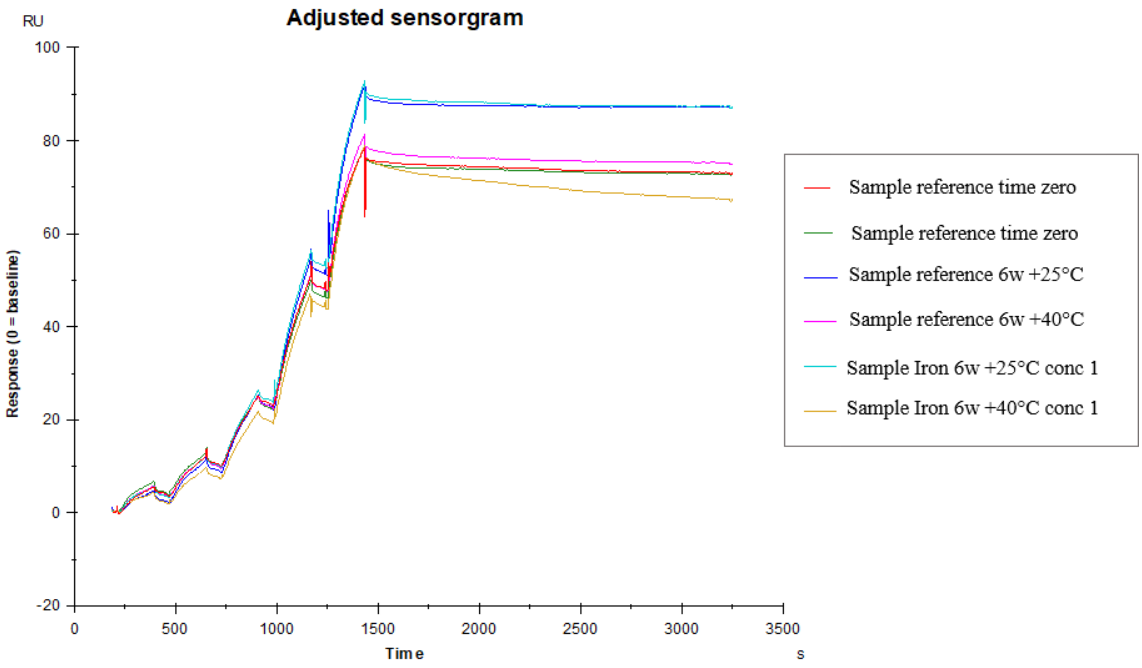
In case of samples treated with zinc, nickel and copper no differences were observed upon incubation with the inorganic elements.

Most interesting results were instead observed in case of Iron. Samples treated with iron for 6 weeks at +25°C showed a comparable binding behaviour with respect to the untreated material incubated at the same conditions of temperature and storage time. In contrast, samples stored for 6 weeks at +40°C showed a decreased binding affinity toward the Fab-ligand. Such decreased binding affinity is ascribable to a difference in both association and dissociation profiles (more evident in this latter case), as shown in **Figure 25**.

Table 34. Fab portion binding activity - Results are reported in terms of absolute KD (lower the KD value, higher the affinity binding) and relative KD (affinity value related to the reference material at time zero). Treated samples were considered not comparable to the reference material if material if their Relative KD experienced a decrease > 25% in comparison to the Relative KD of the untreated material incubated in the same storage conditions (temperature and weeks). Non-comparable values are reported in bold.

Sample	Absolute KD (nM)		Relative KD (%)	
	Average	CV% (Coefficient of variation)	Average	CV% (Coefficient of variation)
Sample reference Time zero	718	3.6	100	-
Sample reference 6 weeks +25°C	761	1.4	94	4.4
Sample reference 6 weeks +40°C	789	1.3	91	1.8
Sample Iron conc 1 6 weeks +25°C	821	8.8	88	5.8
Sample Iron conc 1 6 weeks +40°C	1468	4.0	**49**	0.9
Sample Copper conc 1 6 weeks +25°C	669	10.6	108	13.7
Sample Copper conc 1 6 weeks +40°C	870	9.3	83	12.4
Sample Nickel conc 3 6 weeks +25°C	707	5.2	102	2.1
Sample Nickel conc 3 6 weeks +40°C	752	3.9	96	0.8
Sample Zinc conc 3 6 weeks +25°C	689	1.2	104	4.3
Sample Zinc conc 3 6 weeks +40°C	774	1.3	93	4.4

Figure 25. Surface Plasmon Resonance - Biacore sensogram of the binding Fc-fusion protein/ Fab-ligand of untreated samples and samples treated with iron (conc 1 stored for 6 weeks at +25°C and at +40°C). One replicate per sample is reported. Sample iron conc 1 6 weeks +40°C (orange) showed a significant decrease of the binding affinity with respect to the reference material 6 weeks +40°C (pink). One replicate per sample is reported.



3.2.5.2 ECD portion binding affinity

In this section are reported the results of the characterization of the interaction Fc-fusion protein/ECD ligand. Analyses were performed in two analytical sessions due to the instrument's limitations in terms of allowed samples per run.

In **Table 35** (Session I) **and Table 36 (**Session II) are reported the Absolute KD (nM) and the Relative KD (%) calculated for samples incubated at +25°C and +40°C for 6 weeks (treated ones and untreated material). In each analytical session the untreated material at time zero was included as bridge sample.

Table 35. ECD portion binding activity - Session I. - Results are reported in terms of Absolute KD (lower the KD value, higher the affinity binding) and Relative KD (affinity value related to the reference material at time zero. Treated samples were considered not comparable to the reference material if material if their Relative KD experienced a decrease > 25% in comparison to the Relative KD of the untreated material incubated in the same storage conditions (temperature and weeks). Non-comparable values are reported in bold.

Samples	Absolute KD (nM)		Relative KD (%)	
	Average	CV% (Coefficient of variation)	Average	CV% (Coefficient of variation)
Sample reference Time zero	76	3.3	100	-
Sample reference 6weeks +25°C	184	10.7	**41**	15.0
Sample reference 6weeks +40°C	83	3.3	91	7.6
Sample Iron conc 1 6weeks +25°C	211	9.8	**36**	5.5
Sample Iron conc 1 6weeks +40°C	474	10.0	**16**	5.7
Sample Copper conc 2 6weeks +25°C	78	2.2	97	6.5

Table 36. ECD portion binding activity - Session II. - Results are reported in terms of Absolute KD (lower the KD value, higher the affinity binding) and Relative KD (affinity value related to the reference material at time zero). Treated samples were considered not comparable to the reference material if material if their Relative KD experienced a decrease > 25% in comparison to the Relative KD of the untreated material incubated in the same storage conditions (temperature and weeks). Non-comparable values are reported in bold.

Sample	Absolute KD (nM)		Relative KD (%)	
	Average	CV% (Coefficient of variation)	Average	CV% (Coefficient of variation)
Sample reference Time zero	79	0.3	100	-
Sample reference 6 weeks +25°C	201	5.3	**39**	5.7
Sample Copper conc 2 6 weeks +40°C	84	0.9	94	1.3
Sample Nickel conc 3 6 weeks +25°C	79	1.0	100	1.4
Sample Nickel conc 3 6 weeks +40°C	84	0.6	94	1.0
Sample Zinc conc 3 6 weeks +25°C	79	2.2	100	2.6
Sample Zinc conc 3 6 weeks +40°C	84	0.5	94	0.9

Untreated samples stored for 6 weeks at +25°C and +40°C (Session I) showed unusual results: while no differences were observed between the sample at time zero and the sample incubated at +40°C for 6 weeks, for the reference stored at +25°C for 6 weeks a very low Relative KD was observed (41%). This uncommon result was confirmed in Session II, where the sample was retested.

Samples treated with zinc, nickel and copper and stored for 6 weeks at +25°C were instead characterized by a relative KD aligned to that of the untreated time zero sample. Only samples treated with iron showed a very low Relative KD (36%), similar to that of the untreated material incubated at the same storage conditions.

Overall, results obtained for samples incubated at +25°C for 6 weeks are most likely ascribable to sample contamination and/or degradation (very plausible in case of the reference stored at +25°C for 6 weeks, in which the binding should not be abolished, based on the manufacturer's experience). Based on these considerations biological data obtained for samples at +25°C will not be taken into consideration and further discussed.

The untreated material stored for 6 weeks at +40°C showed comparable binding affinities with respect to the untreated material at time zero. Only a slight, non-significant decrease in the binding with the ECD ligand was observed that could be appreciated only by looking to the sensorgram (**Figure 26**), indicating that the temperature of incubation in "normal" conditions does not modify the functional activity of the ECD portion**.**

In case of samples treated with zinc, nickel and copper no differences were observed upon incubation for 6 weeks at +40°C (**Figure 27**). Only in case of samples treated with iron a significant decrease in the Relative KD (16%) was observed.

Figure 26– Surface Plasmon Resonance - Session 1. Biacore sensogram of the binding Fc-fusion protein/ ECD ligand for samples incubated for 6 weeks at +40°C (iron-treated and untreated ones). The untreated material at time zero is reported as well reported.

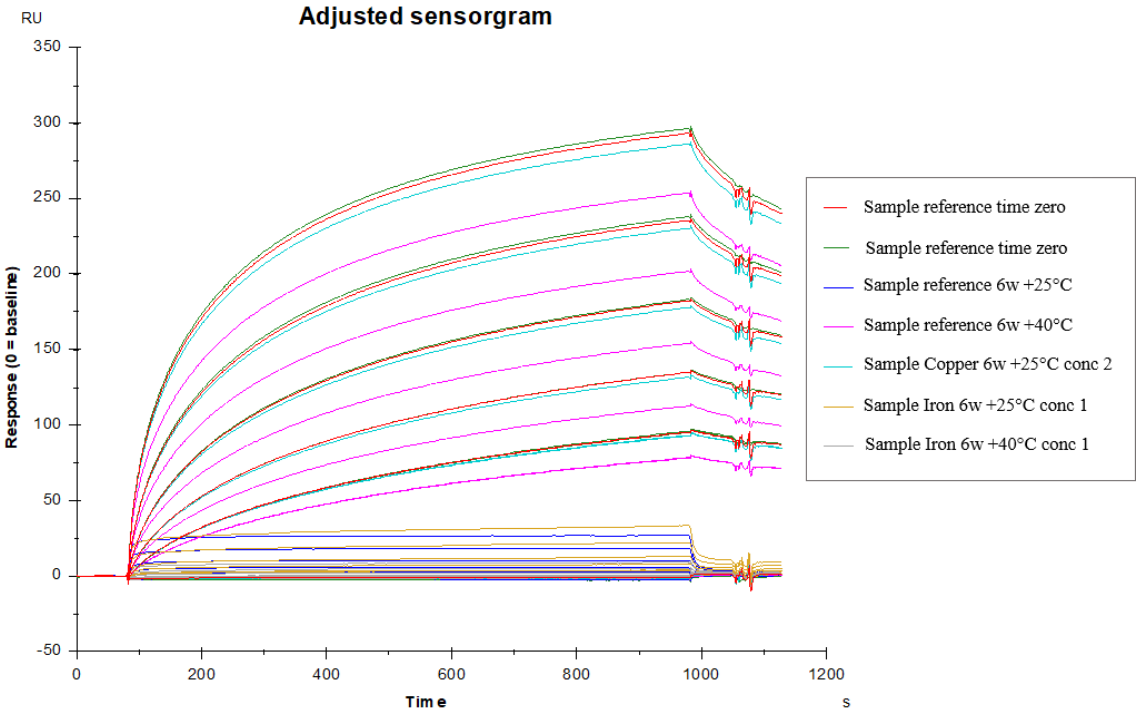
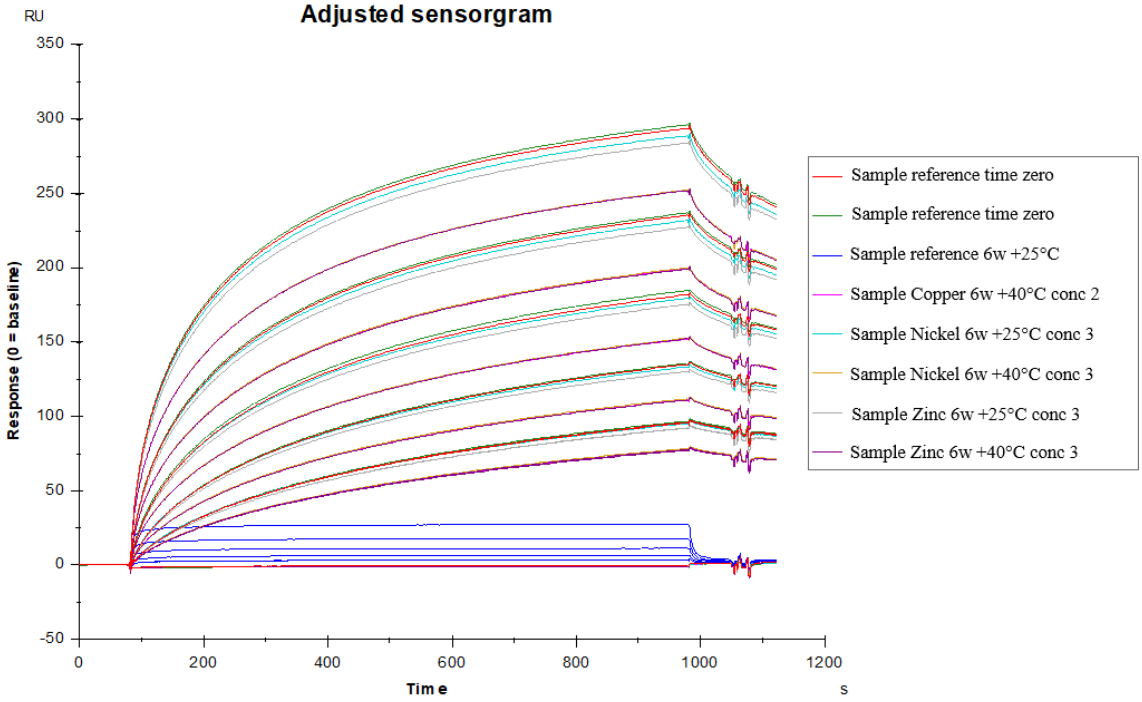


Figure 27 – Surface Plasmon Resonance - Session 2. Biacore sensogram of the binding Fc-fusion protein/ ECD ligand for samples incubated for 6 weeks at +40°C (zinc, nickel and copper-treated and untreated ones). The untreated material at time zero is reported as well reported.



3.3 Stability studies – 44 weeks

The second part of our study was focused on the characterization of treated samples after incubation at +5°C, +25°C and +40°C for 44 weeks. Samples were analysed with a reduced analytical panel (**Table 2**), revised upon the evaluation of the results obtained in the previous time point and based on the visual inspection of vials opened after 44 weeks of incubation (**Appendix 2**).

More in detail, all 44 weeks samples (treated and untreated) did not satisfy the required basic characteristics of a parenteral solution: absence of visible particulate and clarity and colourless solution [28]. Indeed, all samples showed the presence of visible particles and alterations in terms of clarity and colour, suggesting samples had undergone strong degradation (**Figure 39, Appendix 2**). Based on these considerations, the analytical panel was modified: visual inspection and pH measurements were added (**Appendix 2**); oxidation, aggregation and fragmentation were monitored as in the previous time point.

The secondary structure was not monitored as its alterations were already detected after 6 weeks. The tertiary structure as well was not monitored because of the high number of particles observed that, beside complicating CD analyses, suggest strong degradation of samples.

3.3.1 Aggregation and fragmentation

3.3.1.1 Iron

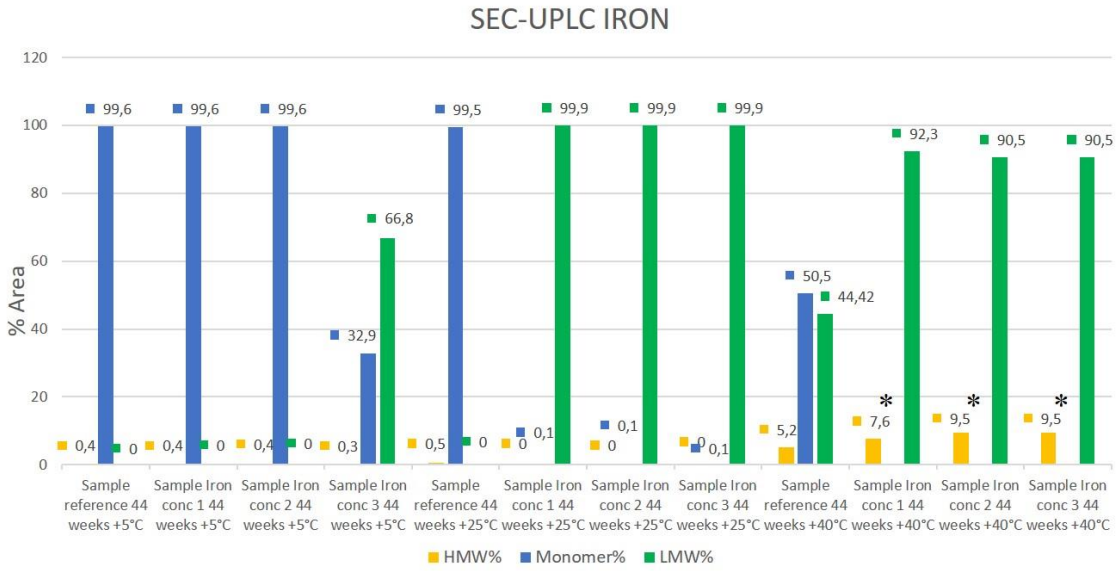
Results of the SE-UPLC analysis on samples treated with Iron and incubated for 44 weeks at +5°C, +25°C and +40°C are reported in **Table 37** and **Figure 28**.

Table 37. Summary of SEC-UPLC results on aggregation – Iron. Area (%) of HMW, monomer and Low Molecular Weight species in treated samples after 44 weeks at +5°C, +25°C and +40°C. Results for the treated samples are expressed as average of three independent replicates and are compared to the minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature. Non-comparable values are reported in bold.

Aggregation - IRON					
Condition	**Parameter**	**Reference range**	**Sample Iron conc 1**	**Sample Iron conc 2**	**Sample Iron conc 3**
44 week +5°C	% HMW	0.3 – 0.4	0.4	0.4	0.3
	% Monomer	99.7 – 99.6	99.6	99.6	**32.9**
	% LMW	0	0	0	**66.8**
44 week +25°C	% HMW	0.5 – 0.6	**0**	**0**	**0**
	% Monomer	99.5 – 99.4	**0.1**	**0.1**	**0.1**
	% LMW	0	**99.9**	**99.9**	**99.9**
44 week +40°C	% HMW	4.6 – 5.8	**N.I.***	**N.I.***	**N.I.***
	% Monomer	50.0- 51.8			
	% LMW	44.2 – 43.5	**92.3**	**90.5**	**90.5**

N.I.: Not integrable as HMW and monomer peak not enough separated (see Figure 29).

Figure 28. Summary of SEC results – Iron. %HMW (yellow), %LMW (green) and %Monomer (blue) are reported for each sample as average of three independent replicates. Results are reported for treated and untreated samples after 44 weeks at +5°C, +25°C and +40°C.



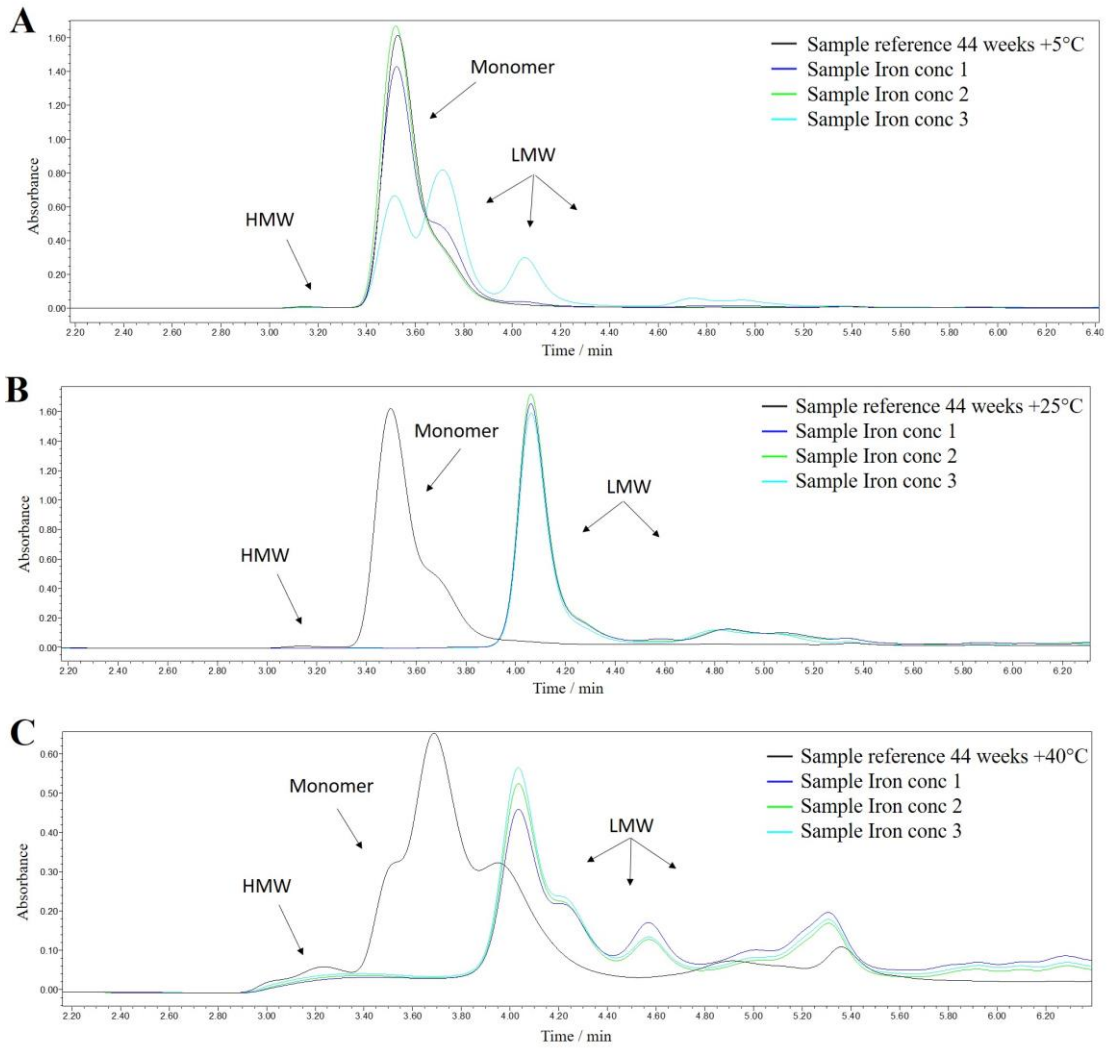
* These HMWs values includes both HMW and monomer species, as these peaks were not enough separated (see Figure 29)

After storage for 44 weeks at +5°C only samples treated with iron concentration 3 showed differences with respect to the reference material incubated at the same storage conditions: a decrease of the monomeric species (from 99% to 32.9%) and a concomitant increase of LMW ones. As can be observed in **Figure 29A** it is important to note that in case of samples incubated at +5°C this LMW increase only partially depends on the fragment characterized in section 3.2.3.1 (molecule deprived of the ECD): a discrete increase of another LMW species is indeed observed.

In case of samples treated with iron and stored for 44 weeks at +25°C and +40°C results were similar to those observed for these samples after 6 weeks, but more pronounced: complete loss of the monomer and increase of different LMW species. In this case the main LMW species was the molecule deprived of its ECD.

Note that after 44 weeks at +40°C also the untreated material presented a decrease of the monomer and an increase of LMW species. Its chromatographic profile is however different with respect to those of the treated samples incubated at the same temperature, indicating that the degradation effect attributable to the storage temperature is different than the degradation effect attributable to iron.

Fig 29. SEC results – Iron Overlay of the chromatograms of untreated material (black) and samples treated with iron (blue, green and cyan) after 44 weeks at +5°C **(A)**, at +25°C **(B)**, at +40°C **(C)**. One replicate per sample is reported.



Legend: HMW High Molecular Weight species
LMW Low Molecular Weight species

3.3.1.2 Copper

In case of samples treated with copper (**Figure 30, Table 38**) incubated for 44 weeks at +5°C no changes were observed with respect to the untreated material stored under the same conditions.

After 44 weeks at +25°C and +40°C no differences were observed between untreated and treated samples in case of concentrations 1 and 3. The same profile and pattern of species was indeed observed in all samples, regardless of the presence of copper, confirming the observations made at the previous time point: copper does not modify the propension to aggregation/fragmentation of the molecule. Note that at +40°C a noticeable increase of LMW species and concomitant loss of monomer (about 50%) was observed in all samples due to the temperature of storage.

Unexpectedly, in case of concentration 2 at both +25°C and +40°C it was observed an increase of LMW species (the main one being the same described for Iron, Fc-fusion protein deprived of the ECD portion) not previously observed after 6 weeks. A possible explanation is that these alterations seen were due to sample contamination and/or degradation likely occurred after opening and using samples for other analyses.

Table 38 - Summary of SEC-UPLC results on aggregation – Copper.

Area (%) of HMW, monomer and Low Molecular Weight species in treated samples after 44 weeks at +5°C, +25°C and +40°C. Results for the treated samples are expressed as average of three independent replicates and are compared to the minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature. Non-comparable values are reported in bold.

Aggregation - COPPER					
Condition	**Parameter**	**Reference range**	**Sample Copper conc 1**	**Sample Copper conc 2**	**Sample Copper conc 3**
44 week +5°C	% HMW	0.3 – 0.4	0.4	0.4	0.4
	% Monomer	99.7 – 99.6	99.6	99.6	99.6
	% LMW	0	0	0	0
44 week +25°C	% HMW	0.5 – 0.6	0.5	**0.2**	0.5
	% Monomer	99.5 – 99.4	99.5	**1**	99.5
	% LMW	0	0	**99.8**	0
44 week +40°C	% HMW	4.6 – 5.8	4.7	**1**	**3.4**
	% Monomer	50.0- 51.8	52.7	**7.6**	**53.5**
	% LMW	44.2 – 43.5	42.6	**91.4**	**43.1**

Fig 30. Summary of SEC results – Copper. %HMW (yellow), %LMW (green) and %Monomer (blue) are reported for each sample as average of three independent replicates. Results are reported for treated samples after 44 weeks at +5°C, +25°C and +40°C.

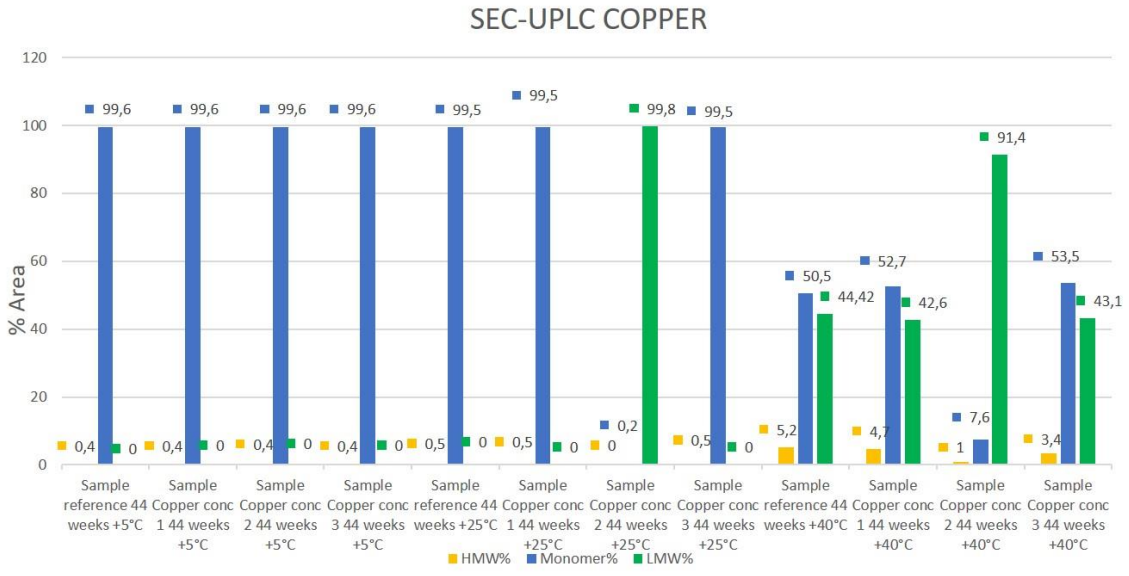
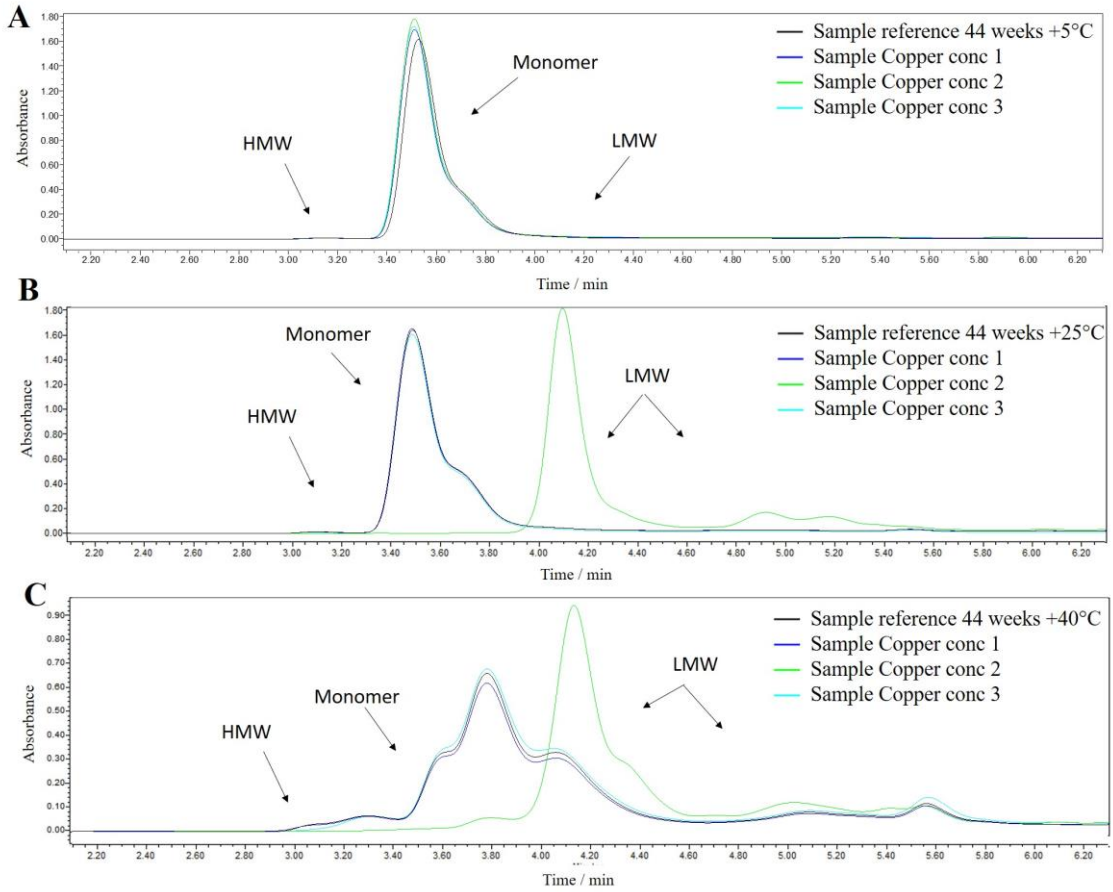


Figure 31. SEC results – Copper Overlay of the chromatograms of untreated material (black) and samples treated with copper (blue, green, cyan and pink) after 44 weeks at +5°C **(A)**, at +25°C **(B)**, at +40°C **(C)**. One replicate per sample is reported.



Legend: HMW High Molecular Weight species
LMW Low Molecular Weight species

3.3.1.3 Zinc and Nickel

Table 39 and **Figure 32** report the results obtained upon treatment with zinc and nickel and incubation for 44 weeks at +5°C, +25°C and +40°C. In terms of aggregation and fragmentation no differences were observed between treated and untreated samples incubated under the same storage conditions. Only after 44 weeks at +40°C we observed – as in case of samples treated with copper - a noticeable increase of LMW species and concomitant loss of monomer (about 50%): such alteration is, however, ascribable also in this case to the storage temperature rather than to the interaction of the

molecule with the metal ions, as the same effects were observed also in the untreated material stored at +40°C (**Figure 33**).

Table 39 - Summary of SEC-UPLC results on aggregation – Nickel and Zinc. Area (%) of HMW, monomer and Low Molecular Weight species in treated samples after 44 weeks at +5°C, +25°C and +40°C. Results for the treated samples are expressed as average of three independent replicates and are compared to the minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature**.** Non-comparable values are reported in bold.

Aggregation - NICKEL					
Condition	**Parameter**	**Reference**	**Sample Nickel conc 1**	**Sample Nickel conc 2**	**Sample Nickel conc 3**
44 week +5°C	% HMW	0.3 – 0.4	0.4	0.4	0.3
	% Monomer	99.6 – 99.7	99.6	99.6	99.7
	% LMW	0	0	0	0
44 week +25°C	% HMW	0.5 – 0.6	0.5	0.5	0.5
	% Monomer	99.4 – 99.5	99.5	99.5	99.5
	% LMW	0	0	0	0
44 week +40°C	% HMW	4.6 – 5.8	**6.1**	**6**	**6**
	% Monomer	50.0- 51.8	**52.3**	**52**	**52**
	% LMW	44.2 – 43.5	**41.6**	**42**	**42**
Aggregation - ZINC					
Condition	**Parameter**	**Reference**	**Sample Zinc conc 1**	**Sample Zinc conc 2**	**Sample Zinc conc 3**
44 week +5°C	% HMW	0.3 – 0.4	0.3	0.4	0.3
	% Monomer	99.6 – 99.7	99.7	99.6	99.7
	% LMW	0	0	0	0
44 week +25°C	% HMW	0.5 – 0.6	0.5	0.5	0.5
	% Monomer	99.4 – 99.5	99.5	99.5	99.5
	% LMW	0	0	0	0
44 week +40°C	% HMW	4.6 – 5.8	**6.3**	**6.3**	**6.6**
	% Monomer	50.0- 51.8	51.7	**52.0**	51.6
	% LMW	44.2 – 43.5	**42**	**41.5**	**41.8**

Figure 32. Summary of SEC results – Zinc and Nickel. %HMW (yellow), %LMW (green) and %Monomer (blue) are reported for each sample as average of three independent replicates. Results are reported for treated samples after 44 weeks at +5°C, +25°C and +40°C.

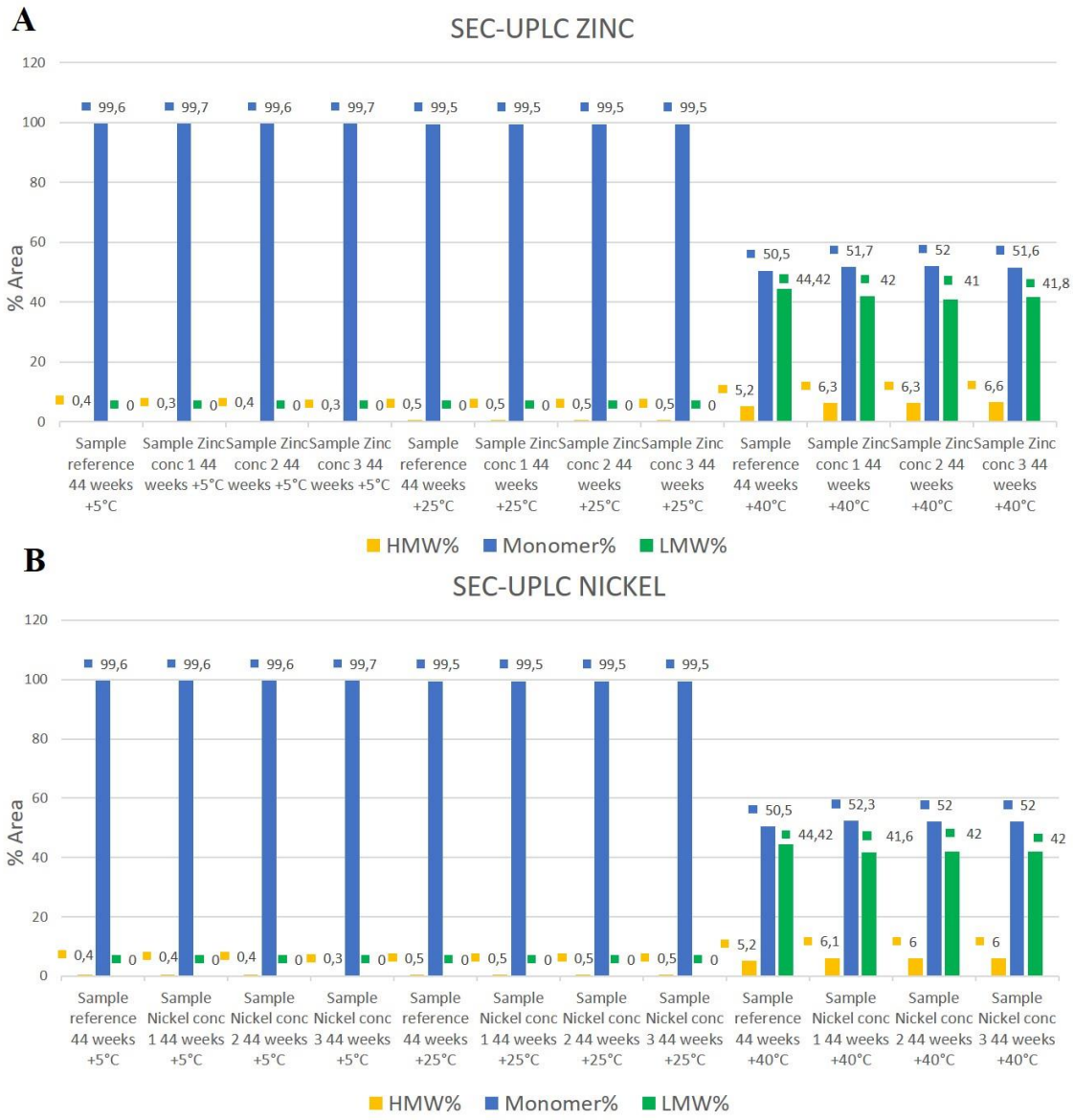
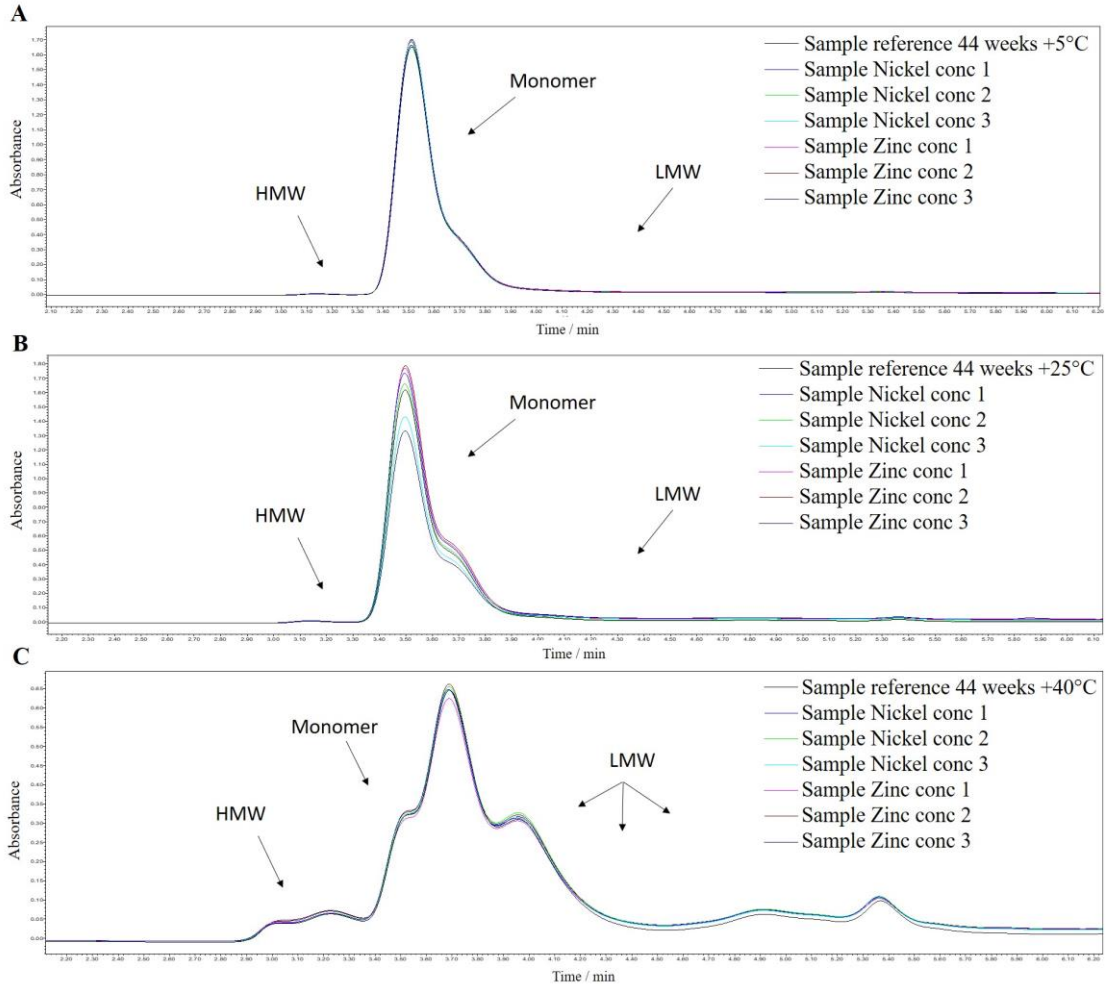


Figure 33. SEC results – Nickel and Zinc Overlay of the chromatograms of untreated material (black) and samples treated with zinc and nickel after 44 weeks at +5°C (**A**), at +25°C (**B**) and at +40°C (**C**). One replicate per sample is reported.



3.3.2 Oxidation

Following the interesting results obtained after 6 weeks, the oxidation of Met-ECD1 was monitored by RP-UPLC also on treated samples incubated for 44 weeks at +5°C, +25°C and +40°C.

3.3.2.1 Iron

In case of iron after 44 weeks of incubation at +5°C and at +25°C no increase of oxidation was observed: indeed, also in case of concentration 3 variations are within method variability. After 44 weeks at +40°C, instead, a strong increase was observed for all the tested concentrations, with respect to the untreated material incubated at the same conditions (**Table 40).** Concentration 1 and 2 lead to almost comparable values - being the difference between the two concentrations very small (0.5 ppm, see **Table 1**) – while in case of concentration 3 (2.8 ppm more than concentration 2) as observed after 6 weeks a concentration-dependent effect can be more appreciated.

Table 40. **Summary of RP-UPLC results on oxidation – Iron.** Area (%) of the oxidized Met-ECD1 in samples incubated for 44 weeks at +5°C, +25°C and +40°C. Results for the treated samples are expressed as average of three independent replicates and compared to minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature. Non-comparable values are reported in bold.

Oxidation - IRON		
Conditions	**Samples**	**% Area Met-ECD1 oxidized**
44 weeks +5°C	Sample reference range	2.7 – 3.3
	Sample Iron conc 1	3.2
	Sample Iron conc 2	3.2
	Sample Iron conc 3	3.3
44 weeks +25°C	Sample reference range	2.3 – 3.7
	Sample Iron conc 1	2.7
	Sample Iron conc 2	3.3
	Sample Iron conc 3	**4.3**
44 weeks +40°C	Sample reference range	13.2 –13.7
	Sample Iron conc 1	**31.7**
	Sample Iron conc 2	**30.9**
	Sample Iron conc 3	**38.1**

3.3.2.2 Copper

In case of copper after 44 weeks oxidation was observed as significantly increased only in case of concentration 2 and 3 upon incubation at +40°C (**Table 41)**, confirming the results obtained after 6 weeks. Indeed, variations observed at +25°C are within method variability.

Table 41. **Summary of RP-UPLC results on oxidation – Copper.** Area (%) of the oxidized Met-ECD1 in samples incubated for 44 weeks at +5°C, +25°C and +40°C. Results for the treated samples are expressed as average of three independent replicates and compared to minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature. Non-comparable values are reported in bold.

Oxidation - Copper		
Conditions	**Samples**	**% Area Met-ECD1 oxidized**
44 weeks +5°C	Sample reference range	2.7 – 3.1
	Sample Copper conc 1	2.7
	Sample Copper conc 2	2.8
	Sample Copper conc 3	2.8
44 weeks +25°C	Sample reference range	2.8 – 3.7
	Sample Copper conc 1	3.2
	Sample Copper conc 2	**5.0**
	Sample Copper conc 3	**3.8**
44 weeks +40°C	Sample reference range	13.9 – 14.3
	Sample Copper conc 1	13.5
	Sample Copper conc 2	**16.9**
	Sample Copper conc 3	**21.7**

3.3.2.3 Zinc and Nickel

In case of treatment with zinc (**Table 42**) and nickel (**Table 43**) also after 44 weeks of incubation no differences were observed in terms of oxidation. Only in case of nickel a non-significant increase, ascribable to method variability, was observed in treated samples incubated at +40°C.

Table 42. **Summary of RP-UPLC results on oxidation – Zinc.** Area (%) of the oxidized Met-ECD1 in samples incubated for 44 weeks at +5°C, +25°C and +40°C. Results for the treated samples are expressed as average of three independent replicates and compared to minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature. Non-comparable values are reported in bold.

Oxidation - Zinc		
Conditions	**Samples**	**% Area Met-ECD1 oxidized**
44 weeks +5°C	Sample reference range	2.7 – 3.3
	Sample Zinc conc 1	2.7
	Sample Zinc conc 2	2.9
	Sample Zinc conc 3	2.9
44 weeks +25°C	Sample reference range	2.3 – 3.7
	Sample Zinc conc 1	3.4
	Sample Zinc conc 2	3.5
	Sample Zinc conc 3	3.7
44 weeks +40°C	Sample reference range	13.2 –13.7
	Sample Zinc conc 1	13.2
	Sample Zinc conc 2	13.7
	Sample Zinc conc 3	13.6

Table 43. **Summary of RP-UPLC results on oxidation – Nickel.** Area (%) of the oxidized Met-ECD1 in samples incubated for 44 weeks at +5°C, +25°C and +40°C. Results for the treated samples are expressed as average of three independent replicates and compared to minimum and maximum values obtained for the six replicates of untreated material incubated at the same temperature. Non-comparable values are reported in bold.

Oxidation – Nickel		
Conditions	**Samples**	**% Area Met-ECD1 oxidized**
44 weeks +5°C	Sample reference range	2.7 – 3.1
	Sample Nickel conc 1	2.7
	Sample Nickel conc 2	2.5
	Sample Nickel conc 3	2.5
44 weeks +25°C	Sample reference range	2.8 – 3.7
	Sample Nickel conc 1	2.9
	Sample Nickel conc 2	3.4
	Sample Nickel conc 3	3.5
44 weeks +40°C	Sample reference range	13.9 – 14.3
	Sample Nickel conc 1	**15.2**
	Sample Nickel conc 2	**15.2**
	Sample Nickel conc 3	**15.4**

4. Discussion:

This Ph.D. project provides an overview of how inorganic impurities can affect the physicochemical stability of a Fc-fusion protein, depending on the nature of the inorganic ion and on the stability conditions.

The inorganic leachables investigated in this study - nickel, zinc, copper and iron - are classified in diverse ways according to ICH Q3D [17], the international guideline employed in the risk assessment of the elemental impurities during the development of biopharmaceuticals.

Copper is assigned to Class 3, which contains elements with relatively low toxicity when the administration of a drug is by oral route but higher toxicity in case of inhalation or parenteral administration (the case of our Fc-fusion protein). Indeed, based on this guideline ("*for parenteral and inhalation products, the potential for inclusion of these elemental impurities should be evaluated during the risk assessment*") since Copper is intentionally added in the cell culture media and in the feed during the manufacturing process, it must be monitored.

Nickel, a component of stainless steel and therefore commonly found in final drug products, is of major concern: being a class 2A element it is regarded as most dangerous since it is toxic for humans independently from the route of administration. Indeed, in ICH Q3D [17] is expressly declared that the elements belonging to class 2A "*have relatively high probability of occurrence in the drug product and thus require risk assessment across all potential sources of elemental impurities and routes of administration (as indicated).*"

Zinc and iron are not sorted in the classes foreseen by the ICH Q3D (indicated in the guideline as *Other Elements*), due to their low inherent toxicity. While for nickel and copper a PDE is established by the guideline, for zinc and iron it is not: for these

elements PDEs were therefore calculated according to ICH Q3D's Appendix 1 (Method for establishing exposure limits) employing suitable documents, the "Assessment report Ferric, Ferric citrate coordination complex" [29] written by EMA and the "Toxicological Review of Zinc and Compounds" [30] written by U.S. EPA, as described in section 1.1.

Our study monitored how the interaction of the four metal ions with a Fc-fusion protein could affect its quality attributes. In **Table 44** are summarized the alterations observed.

Table 44. Summary of the observations upon treatment of the Fc-fusion protein with the four metal ions.

Element	Tertiary Structure by CD	Secondary Structure by FTIR	Aggregation and Fragmentation by SE-UPLC	Oxidation by RP-UPLC and Peptide Mapping	Particles, color degree and pH (after 44 weeks of incubation)	Biological characterization
Ni^{+2}	No alterations observed.	Alteration of the secondary structure for all concentrations at all conditions.	No alterations observed.	No alterations observed.	Presence of visible particles; stable pH over time; solutions' color altered.	No alterations observed.
Cu^{+2}	Alteration of the tertiary structure for samples treated with concentration 3 (1 ppm) at all conditions.	Alteration of the secondary structure for all concentrations at all conditions.	No alterations observed.	Oxidation observed for all the samples incubated at +40°C for 6 and 44 weeks treated with the three concentrations.	Presence of visible particles; stable pH over time; solutions' color altered.	No alterations observed.
Zn^{+2}	No alterations observed.	Alteration of the secondary structure for all concentrations at all conditions.	No alterations observed.	No alterations observed.	Presence of visible particles; stable pH over time; solutions' color altered.	No alterations observed.

Fe^{+3}	Major alterations of the tertiary structure for all samples incubated with the three concentrations for 6 weeks both at +25°C and +40°C.	Alteration of the secondary structure for all concentrations, at all conditions.	Increase of LMW content in all samples incubated for 6 weeks and 44 weeks at +25°C and +40°C (the highest conc even after 44 weeks at +5°C). The main fragment is ascribable to the molecule deprived of the fused extracellular domain (ECD) protein.	Oxidation observed for all samples incubated at +40°C for 6 and 44 weeks treated with the three concentrations tested.	Presence of visible particles; alterations of pH over time for samples stored at +25°C and +40°C. Significant color alterations for all samples incubated for 44 weeks, particularly for those stored at +40°C.	Fab portion's binding abolished in case of samples incubated for 6 weeks at +40°C and not at +25°C. ECD portion's binding abolished for samples treated with iron and stored for 6 weeks at +40°C (data of samples stored at +25 were not taken into consideration).

Aggregation [31] and fragmentation [32] are important quality attributes monitored during pharmaceutical development since aggregates and fragments are of particular concern given their potential effect on efficacy and immunogenicity. In case of samples treated with zinc, nickel and copper no modifications due to the treatment with the three elements were detected. Only after 44 weeks at +40°C a significant decrease of the monomer purity (about 50%) was observed but it was ascribable to the incubation time and temperature.

Most relevant results were instead observed in case of iron. Indeed, all tested concentrations were characterized by an important loss of monomer upon incubation at +25°C and +40°C after 6 weeks. Such loss of monomer was coupled to a concomitant increase of a LMW species (134.1 KDa) corresponding to the molecule deprived of the fused extracellular domain (ECD) protein.

This result was confirmed also after 44 weeks. In addition, at this latter time point we observed a decrease of the monomer purity in case of concentration 3 (5.5 ppm) incubated at +5°C: the SEC profile is however different with respect to those of the above-cited samples at +25°C and +40°C, suggesting that at this temperature a

different degradation event may occur, triggered by very high concentrations of the element.

It is well known that most of the backbone fragmentation events in therapeutic proteins occur at one of the following residues: Asp, Gly, Ser, Thr, Cys or Asn [32]. Furthermore, fragmentation upon interaction of a mAb with iron at +40°C was previously described: Ouellette et al. [33] demonstrated that iron, in presence of histidine in the formulation placebo, catalyses the fragmentation of IgG antibodies containing a lambda Light Chain: the cleavage is localized between residues present in the vicinity of the disulfide bond holding LC and HC.

Our results, interestingly, describes that in conditions similar to those of Ouellette et al. (presence of histidine in formulation placebo and incubation of samples treated with 2.5-50 ppm of iron for 1 month at +40°C) an alternative cleavage may occur: in our case it was localized between a Histidine and a Valine located on the ECD fused protein, five residues downstream the linker that binds ECD and IgG1 domains, as detected by intact molecule analysis by LC-MS.

Oxidation was chosen to be monitored as metal catalyzed oxidation (MCO) [9] typically occurs on a protein in the presence of metal ions. This chemical modification is particularly critical as it may induce structural changes that can potentially impact biological efficacy, clearance, safety, and immunogenicity [2] of a biologic. Although oxidation can occur on different amino acids such as cysteines, tryptophans, lysines and methionines, the latter are the most susceptible ones: their oxidation generates methionine sulfoxide, a product which is more polar and less hydrophobic [34-35].

In this study we focused on the most relevant oxidation site of our molecule, a methionine located in the ECD portion of the Fc-fusion protein (Met-ECD1) and we observed an increase of its oxidation level only after treatment with iron and

copper (concentration-dependent) upon incubation at + 40°C, both after 6 and 44 weeks. This increase was induced by all the tested concentrations in case of iron while only by concentrations 2 and 3 in case of copper. Notably, such increased oxidation -particularly dramatic in case of iron (in terms of magnitude) - was observed in spite of the presence of an antioxidant excipient in the formulation buffer (Methionine 5mM) that was consumed only to a small extent in treated samples (about 15%).

Interestingly, reducing peptide mapping was performed as additional test on samples treated with iron and copper revealing that oxidation also occurred - in a concentration-dependent manner - on other two methionine residues: one localized on the Heavy Chain (Met-HC) and the other on the ECD fused protein (Met-ECD2). This latter residue, in particular, together with Met-ECD1 was indicated by this technique as experiencing the highest increment. Considering that both amino acids are localized in the ECD of the Fc-fusion protein, nearby the cleavage site identified by intact molecule analysis, it is plausible that oxidation is one of the key factors contributing to fragmentation upon treatment with iron.

No differences, in terms of Met-ECD1 oxidation, were instead observed for nickel and zinc, under all stability study conditions, very likely due to their low standard reduction potential (SRP). Copper and iron have in fact a positive SRP value (Fe^{3+}=+0.77V and Cu^{2+}=+0.34V) while zinc and nickel have a negative SRP value (Zn^{2+}=-0.76V and Ni^{2+}=-0.25V): the more positive the value is, the more likely the substance is to be reduced.

The higher order structure (HOS) of a protein - intended as secondary, tertiary and quaternary structure - is a fingerprint covering structural quality attributes potentially linked to the function of a Fc-fusion protein that therefore needs to be monitored during the development of a biologic. Modifications and perturbations of the

folding may lead, for example, to loss of function and immunogenicity [36]. For instance, the interaction between inorganic leachables and biologics could lower disulphide bonds' stability, ultimately leading to non-native structures, because of the higher propensity to unfold and to form aggregates [37].

The comparison of the CD spectra of samples treated with iron highlighted major changes upon incubation for 6 weeks at +25°C and +40°C. Interestingly, they suggest a different level of modification of the tertiary structure depending on the incubation temperature (**Figures 5B and 6B**): at +25°C spectra perturbations were observed only around 255 nm, 275 nm and 285 nm (absorption of phenylalanine, tyrosine and tryptophan) while at +40°C modifications were observed also in the region where disulphide-bridges absorb (310-330 nm).

The tertiary structure of samples treated with copper appeared perturbed only in case of concentration 3 (1 ppm) at time zero, +25°C and +40°C: alterations were registered around 250-265 nm and around 275 nm, indicating different absorption of phenylalanine and tyrosine, respectively.

In case of samples treated with nickel or zinc, no alterations of the tertiary structure were detected by Circular Dichroism in any of the analysed conditions.

In terms of secondary structure, FTIR spectroscopy demonstrated alterations in all the treated samples, not only in case of iron and copper, but also in case of zinc and nickel. These alterations were seen not only in samples treated with the *Concentration Limit*, but also when treated with the lower ones (tested only for copper and iron), indicating that the stability of the secondary structure is the most sensitive to the presence of metal ions.

Alterations in the quality attributes described above (aggregation, oxidation and conformation) should always be considered in a broader scenario, evaluating if and how

they affect the biological activity of a biotherapeutic. To this aim the biological activity of the Fc-fusion protein after the interaction with the four metal ions (after 6 weeks) was evaluated in terms of binding affinities and kinetic rate constants of the interactions between the Fc-fusion protein and its ligands: Fab ligand and ECD ligand.

Significant decreased binding affinities with respect to both ligands were detected only in case of iron upon incubation at +40°C. Apparently, upon incubation at +25°C no differences were highlighted. It is important to note, however, that in case of samples incubated at +25°C data are not available in terms of interaction Fc-fusion protein/ECD ligand.

Obtained data are coherent with what observed in terms of fragmentation as only iron-treated samples are characterized by a significant increase of the fragment corresponding to the protein without the ECD region. Such fragmentation lead to an abolished capacity to bind the ECD ligand (detected at +40°C but plausible at +25°C as well) and, in case of incubation at +40°C, to a reduced ability to bind the Fab ligand as well.

5. Conclusions

Manufacturing issues linked to chemical impurities are not uncommon: our data will be helpful to explain effects of process-related impurities and to help process troubleshooting.

Most interesting results were obtained for iron and copper. Not only the *Concentration Limit* (iron conc 2 = 2.8 ppm; copper conc 2= 0.4 ppm), but also lower ones (iron conc 1 = 2.2 ppm; copper conc 1= 0.05 ppm) are in fact capable to trigger oxidation under accelerated conditions (+40°C) and, in case of iron, fragmentation (that is linked to a loss of the biological activity). Furthermore, our data suggest that, depending on the incubation temperature (+25°C or +40°C), iron induces different alterations in terms of HOS. Nickel, despite being classified as most potentially dangerous in the ICH Q3D is the least capable (together with zinc, that is not included in the classification) to induce alterations of the QAs of the investigated Fc-fusion protein.

In conclusion, our data suggest that keeping the levels of the four inorganic impurities under the safety threshold limits does not necessarily guarantee the product quality, for which thresholds seems to be less permissive in case of iron and copper. While nickel and zinc slightly altered the physicochemical properties of the Fc-fusion protein, iron and copper appeared to be more harmful for the Quality Attributes' stability therefore may cause significant alterations of the product quality such as to potentially alter its efficacy

6. Appendix 1 – Preliminary Study

The rationale behind the choice of the elements' concentrations employed was based not only on PDEs values (see section 1.1.) but also on a preliminary study carried out to determine, for each element, which were the minimum concentrations that could cause a measurable effect on Higher Order Structure (secondary and tertiary structure), oxidation and aggregation. Far- and Near-UV Circular Dichroism, RP-UPLC and SEC-UPLC were employed for this purpose.

The Fc-fusion protein was treated with decreasing concentrations of each element (**Table 45 – Range of concentration tested**) and samples were tested with the above-cited techniques.

RP-UPLC (for Met-ECD1 oxidation) and SEC-UPLC (for aggregation) did not identify any alteration even at the highest concentrations tested. Only by Circular Dichroism were observed modifications to the protein's conformation in case of iron and copper.

Based on these results, the minimum concentration of copper and iron capable to cause an alteration of the Fc-fusion protein (concentration 3) was selected in accordance to CD data (**Table 45 - Minimum concentration that gives an alteration**) – 5.5 ppm for iron and 1 ppm for copper. Results obtained by Circular Dichroism are described in the following section.

In case of Nickel and Zinc, also by CD no alterations were observed even employing very high concentrations: since further increasing the element quantities would have not been significant and representative of real cases, concentration 3 for these elements was set to 5.5 ppm for zinc (100 times the PDE value) and 1.5 ppm (60 times the PDE value) for nickel.

Table 45. Preliminary study overview.

Element	Analytical Technique	Range of concentration tested	Minimum concentration that gives an alteration
Iron	CD Near-UV	from 1 to 5.5 ppm	5.5 ppm
	CD Far-UV	from 1 to 30 ppm	Non-consistent data
	RP-UPLC	from 5 to 40 ppm	No alterations observed
	SE-UPLC	from 1 to 40 ppm	No alterations observed
Copper	CD Near-UV	from 1 to 7.5 ppm	1 ppm
	CD Far-UV	from 1 to 7.5 ppm	Non-consistent data
	RP-UPLC	from 1 to 30 ppm	No alterations observed
	SE-UPLC	from 1 to 40 ppm	No alterations observed
Zinc	CD Near-UV	from 1 to 5.5 ppm	No alterations observed
	CD Far-UV	from 1 to 5.5 ppm	No alterations observed
	RP-UPLC	from 5 to 30 ppm	No alterations observed
	SE-UPLC	from 1 to 40 ppm	No alterations observed
Nickel	CD Near-UV	from 1 to 1.5 ppm	No alterations observed
	CD Far-UV	from 1 to 1.5 ppm	No alterations observed
	RP-UPLC	from 1.5 to 35 ppm	No alterations observed
	SE-UPLC	from 1 to 40 ppm	No alterations observed

6.1 Far- and Near-UV Circular Dichroism (CD)- Preliminary study

6.1.1 Circular Dichroism – Materials and methods

CD spectra were collected on a Chirascan V100 Spectropolarimeter at room temperature using cuvettes with a path length of 10 mm for Near-UV measurements (250-340 nm) and 1 mm for Far-UV ones (190-260 nm). Spectra have been collected at

a step size of 0.5 nm, a bandwidth of 1 nm and a response time of 2 s with a 3-scan average in both wavelength ranges.

For Near-UV analyses samples were diluted to 1 mg/ml in water MilliQ, for Far-UV ones to 0.1 mg/mL in water MilliQ. After subtraction of the baseline (DP placebo diluted in water MilliQ with the same samples' dilution factor) the spectra were normalized for the value of absorbance at 280 nm (Near-UV) or at 215 nm (Far-UV).

The normalized CD spectra have been compared by means of the "Chirascan qBiC Comparison Suite" software, provided by the instrument supplier, that allows to calculate a numeric value expressing similarity among spectra: the Weighted Spectral Difference (WSD) [20,21]. This parameter accounts for the absolute differences in signals between spectra, taking into account both intensity and profile variations. Treated samples were considered comparable to the reference material if their WSD was between 0 (identity) and WSDmean+2STD, where WSDmean is the average of the WSDs calculated for three replicates of the untreated samples incubated at the same temperature.

6.1.2 Circular Dichroism - Results

Inorganic salts were dissolved into H_2O MilliQ and then added to the Fc-fusion protein to obtain the desired concentrations:

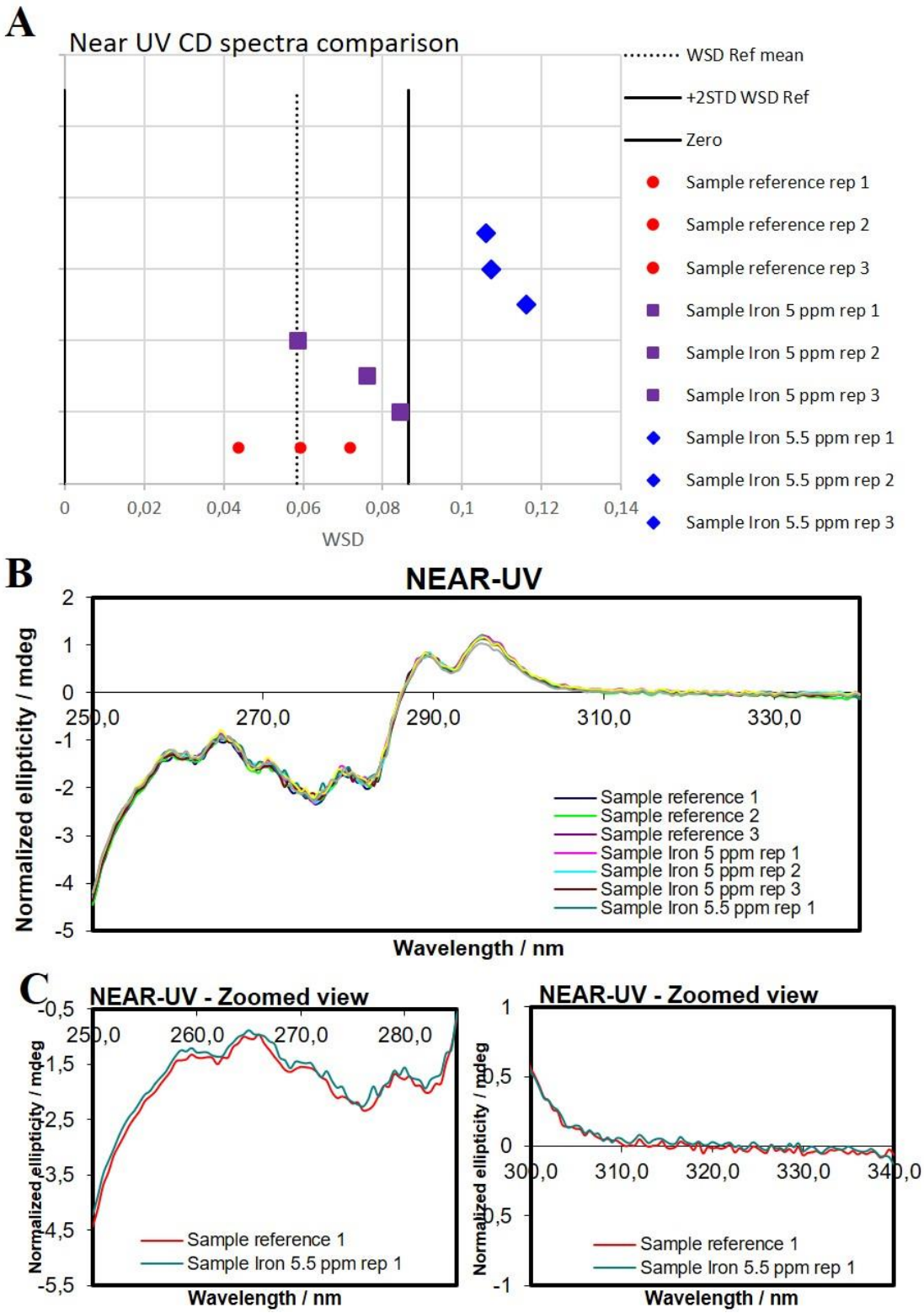
- Fe^{3+}: from 1 to 30 ppm (Far-UV); from 1 to 10 ppm (Near-UV CD)
- Cu2+: from 1 to 7.5 ppm (both Far- and Near-UV)
- Ni^{2+}: from 1 to 1.5 ppm (both Far- and Near-UV)
- Zn^{2+}: from 1 to 5.5 ppm (both Far- and Near-UV)

Iron

In case of Iron it was observed a perturbation of Near-UV spectra (**Figure 34**) only upon addition of 5.5 ppm of iron: perturbations were registered in the region 260-280 nm, mostly in terms of intensity.

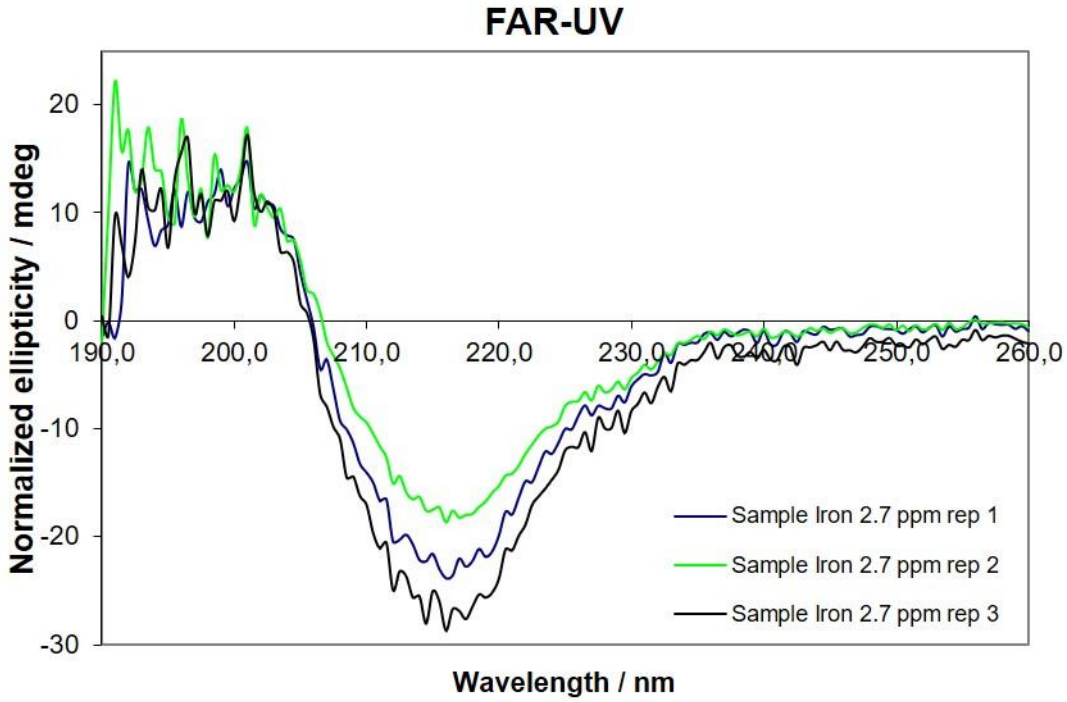
Figure 34 – CD Near-UV of samples treated with iron. A) WSD comparison of the Near-UV CD spectra of samples treated with 5 and 5.5 ppm of iron. Only starting from 5.5 ppm, samples' WSDs do not fall within the range determined by the WSDs of the

untreated material, indicating different tertiary structure **B)** Overlay of the Near-UV CD spectra of samples treated with 5 and 5.5 ppm of iron (Full view). **C)** Zoomed views of the Near-UV CD spectra of untreated samples and samples treated with 5.5 ppm of iron.



In the Far-UV region it was observed extremely high variability - due to a combination of the molecule's formulation excipients and the spiked element - between replicates of the same sample (**Figure 35**), therefore the selection of concentration 3 was based only on Near-UV data.

Figure 35- Far-UV CD spectra upon treatment with Iron. The three replicates of the sample treated with 2.7 ppm of Iron are reported as example of the variability observed in case of Far-UV spectra.



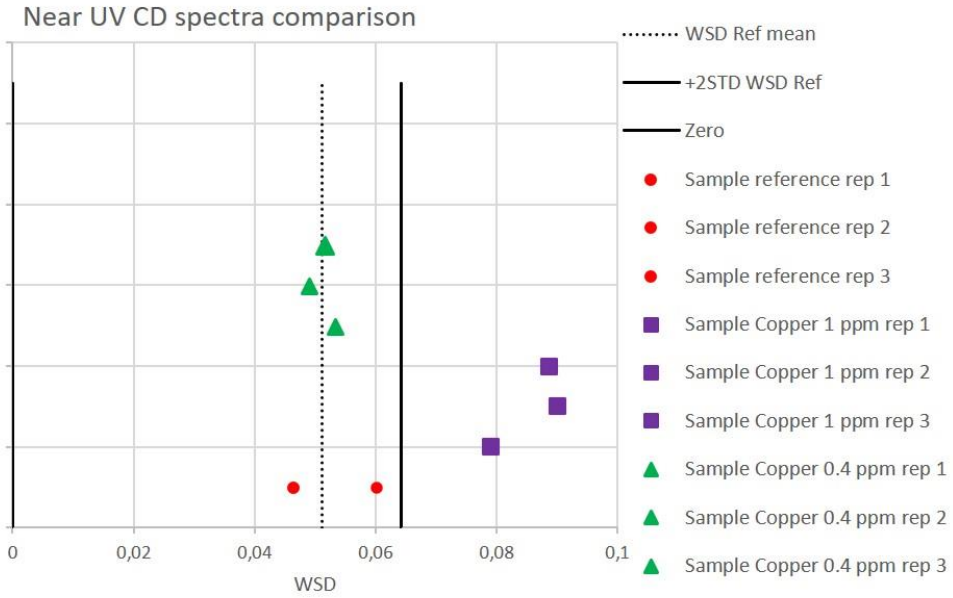
Copper

Upon treatment with copper, Near-UV analysis could detect perturbation of the tertiary structure starting from 1 ppm. As shown in **Figure 36** major perturbation were detected in the regions around 250-260 nm where phenylalanine residues typically absorb.

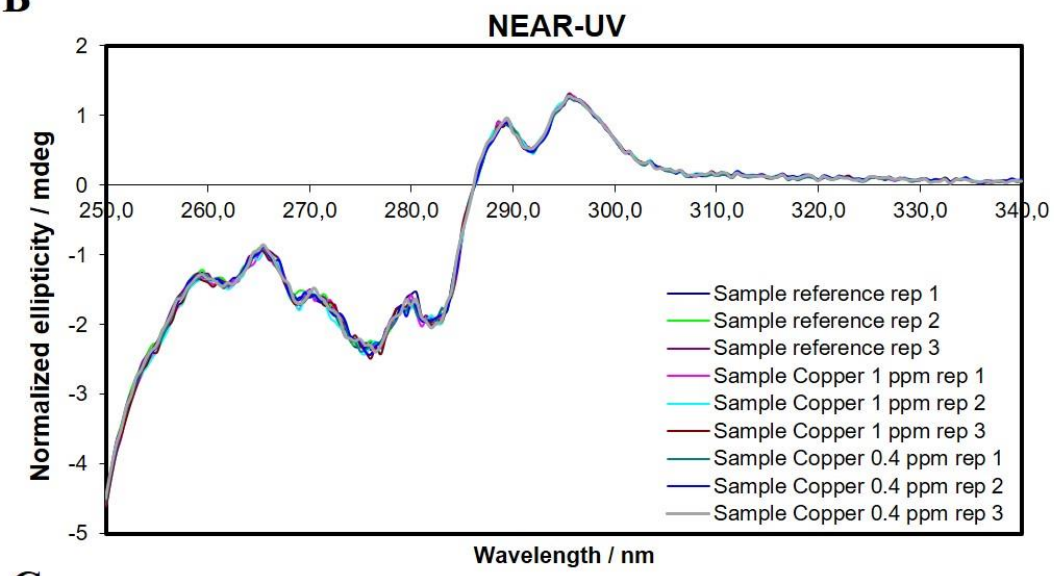
Figure 36 – CD Near-UV of samples treated with copper. A) WSD comparison of the Near-UV CD spectra of samples treated with 1 ppm and 0.4 ppm of copper. Only starting from 1 ppm, samples' WSDs do not fall within the range determined by the

WSDs of the untreated material, indicating different tertiary structure B) Overlay of the Near-UV CD spectra of samples treated with 1 and 0.4 ppm of copper (Full view). C) Zoomed views of the Near-UV CD spectra of untreated sample and sample treated with 1 ppm of copper.

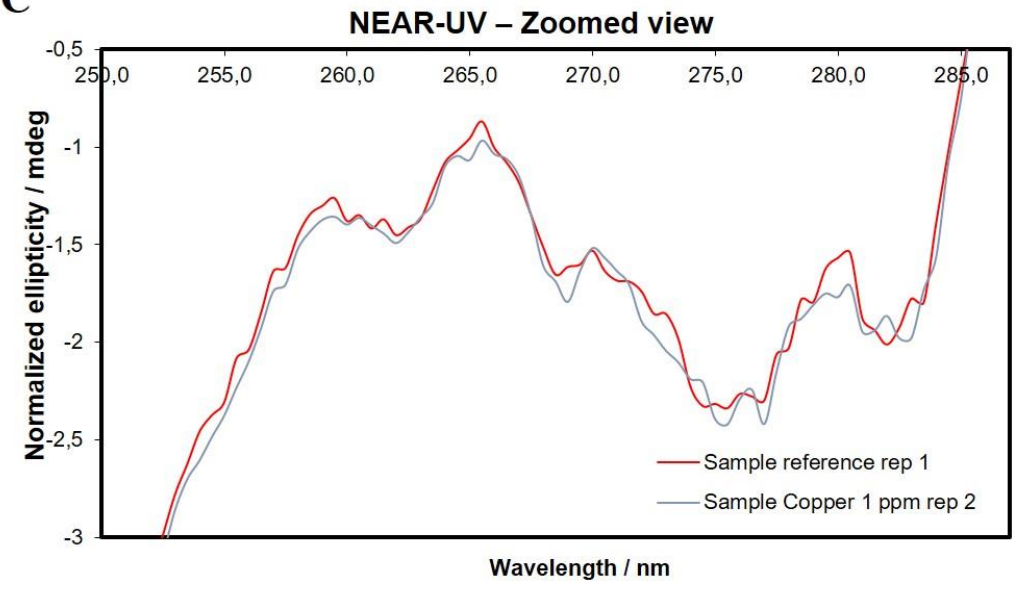
A


Near UV CD spectra comparison
WSD Ref mean
+2STD WSD Ref
Zero
Sample reference rep 1
Sample reference rep 2
Sample reference rep 3
Sample Copper 1 ppm rep 1
Sample Copper 1 ppm rep 2
Sample Copper 1 ppm rep 3
Sample Copper 0.4 ppm rep 1
Sample Copper 0.4 ppm rep 2
Sample Copper 0.4 ppm rep 3
0
0,02
0,04
0,06
0,08
0,1
WSD

B

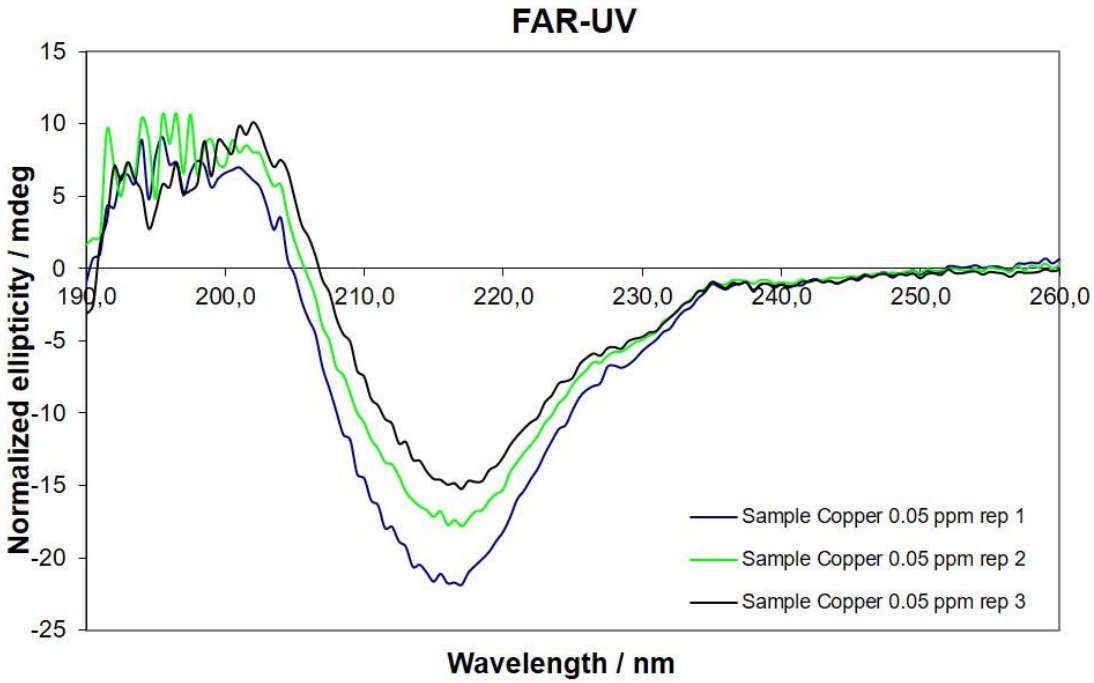

NEAR-UV
Normalized ellipticity / mdeg
Wavelength / nm
Sample reference rep 1
Sample reference rep 2
Sample reference rep 3
Sample Copper 1 ppm rep 1
Sample Copper 1 ppm rep 2
Sample Copper 1 ppm rep 3
Sample Copper 0.4 ppm rep 1
Sample Copper 0.4 ppm rep 2
Sample Copper 0.4 ppm rep 3

C


NEAR-UV – Zoomed view
Normalized ellipticity / mdeg
Wavelength / nm
Sample reference rep 1
Sample Copper 1 ppm rep 2

As in case of Iron, in the Far-UV region it was observed also for copper extremely high variability between replicates of the same sample (**Figure 37**), therefore the selection of concentration 3 was based only on Near-UV data.

Figure 37 – Far-UV CD spectra upon treatment with Copper. The three replicates of the sample treated with 0.05 ppm of Coper are reported as example of the variability observed in case of Far-UV spectra.



7. Appendix 2 – Visual inspection and pH measurement

When boxes containing samples incubated for 44 weeks were opened, visible changes of solutions were observed. Therefore, to evaluate the extent of presence of visible particles, the color-changing and the pH variation, Visible Inspection and pH measurement were performed as described in the following section.

Both the presence of metal ions and the conditions of incubation appeared to be involved in color-changing phenomenon, indeed not only treated samples but also reference materials underwent a color variation. Furthermore, the phenomenon of yellowing does not seems to be linked to the observed pH variations (only samples treated with iron showed a pH variation), in fact even in samples with a stable pH over time, a clearly visible color variation was observed (i.e. samples with a pH of 5.5, such as samples treated with copper, nickel and zinc showed a significant yellowing).

The presence of particles was seen in almost all samples and placebo, particularly critical for samples stored at +40°C, while it should be noted that in all samples stored at +25°C an increase in opacity was observed, particularly higher in the placebo samples.

7.1 Visual Inspection – Materials and methods

Visual inspection (samples incubated for 44 weeks) was performed following the indication reported on *European Pharmacopoeia (Ph. Eur.), 2.2.2 Degree of Coloration of Liquids.* For our purposes the brown-yellow (BY) color scale was considered and the BY certiPUR color reference solutions (Merck) were used to determine the color of the solutions. A white and black background (**Figure 38**) lighted with non-glaring light was used for visual inspection of product containers. The white background aids in the detection of dark-colored particles, while light or refractile

particles will be visible against the black background. A LED light source, positioned above, was used. Each vial was held by its top and was carefully swirled by rotating the wrist or inverting the vial to start contents of the vial moving in a circular motion.

Figure 38 – Visual Inspection Background

7.2 pH measurement – Materials and methods

pH measurements (samples incubated for 44 weeks) were performed on a calibrated Mettler Toledo Seven Multi pH meter, equipped with a microelectrode.

7.3 Visual Inspection – Results

A visual inspection was performed to determine the degree of coloration, the degree of clarity and the possible presence of visible particles in samples incubated for 44 weeks with the four metal ions. One replicate per sample was tested, together with the corresponding treated placebo.

The reference scale used to determine the degree of coloration (brown-yellow, BY), includes a range that goes from BY7 (colorless) to BY1 (more colored). Based on the DP specifications this value, for the Fc-fusion protein solution, should have a BY7 coloration.

As reported in **Table 46**, after 44 weeks of incubation at +5°C and at +25°C there was only a slight change in coloration for untreated samples and placebos (BY6, except for untreated sample reference 44 weeks at +5°C), while more appreciable yellowing was seen upon incubation at +40°C (BY4 Fc-fusion protein, BY5 placebo).

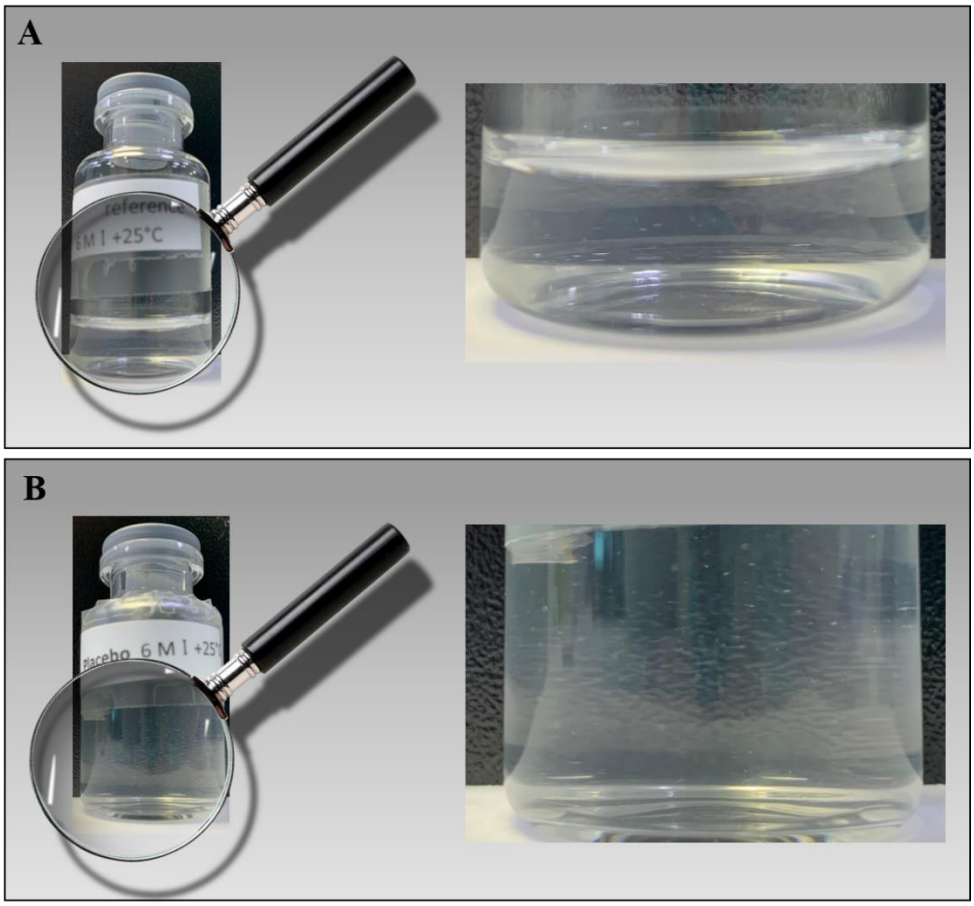
In terms of visible particles and clarity, deviations from the specification ranges were observed mainly for untreated samples and placebos incubated at +25°C, as can be seen in **Figure 39**.

Table 46 – Degree of coloration (BY scale). Samples should be comparable to the reference color BY7. Non-comparable values are reported in bold

Color Degree			
Sample - 44 weeks	+5°C	+25°C	+40°C
Untreated sample	BY7	**BY6**	**BY4**
Placebo reference	**BY6**	**BY6**	**BY5**
Sample Iron conc 1	**BY6**	**BY6**	**BY2***
Sample Iron conc 2	**BY6**	**BY6**	**BY2***
Sample Iron conc 3	**BY6**	**BY5**	**BY2**
Placebo Iron conc 1	**BY6**	**BY6**	**BY4**
Placebo Iron conc 2	**BY6**	**BY6**	**BY5**
Placebo Iron conc 3	**BY6**	**BY6**	**BY2**
Sample Copper conc 1	**BY6**	**BY6**	**BY5**
Sample Copper conc 2	**BY6**	**BY6**	**BY5**
Sample Copper conc 3	**BY6**	**BY6**	**BY4**
Placebo Copper conc 1	**BY6**	**BY6**	**BY6**
Placebo Copper conc 2	BY7	**BY6**	**BY5**
Placebo Copper conc 3	**BY6**	**BY6**	**BY5**
Sample Zinc conc 1	BY7	BY7	**BY5**
Sample Zinc conc 2	BY7	BY7	**BY5**
Sample Zinc conc 3	BY7	**BY6**	**BY5**
Placebo Zinc conc 1	BY7	BY7	**BY6**
Placebo Zinc conc 2	BY7	BY7	**BY6**
Placebo Zinc conc 3	BY7	**BY6**	**BY6**
Sample Nickel conc 1	BY7	**BY6**	**BY5**
Sample Nickel conc 2	BY7	**BY6**	**BY5**
Sample Nickel conc 3	BY7	**BY6**	**BY5**
Placebo Nickel conc 1	BY7	**BY6**	**BY6**
Placebo Nickel conc 2	BY7	**BY6**	**BY6**
Placebo Nickel conc 3	BY7	**BY6**	**BY6**

* One replicate BY1

Fig 39 – Visual Inspection – Reference. Presence of visible particles in untreated sample (**A**) and untreated placebos (**B**) incubated for 44 weeks at +25°C.



Samples and placebos treated with iron showed only a slight change of color when stored at +5°C (**Figure 40 A**) and at +25°C (**Figure 40 B**), while a more significant color change was observed for samples stored at +40°C (from BY4 to BY1), as can be seen in **Figure 40 C**.

In terms of visible particles and opacity deviations from the specification ranges were observed for all samples and placebos, but mainly for material incubated at +40°C, especially in case of concentration 3 (**Figure 41).**

Figure 40 – Visual Inspection – Iron. Samples treated with iron conc 2 and stored for 44 weeks at +5°C (**A**); at +25°C (**B)**; at +40°C (**C**) compared to BY certiPUR color reference solutions.

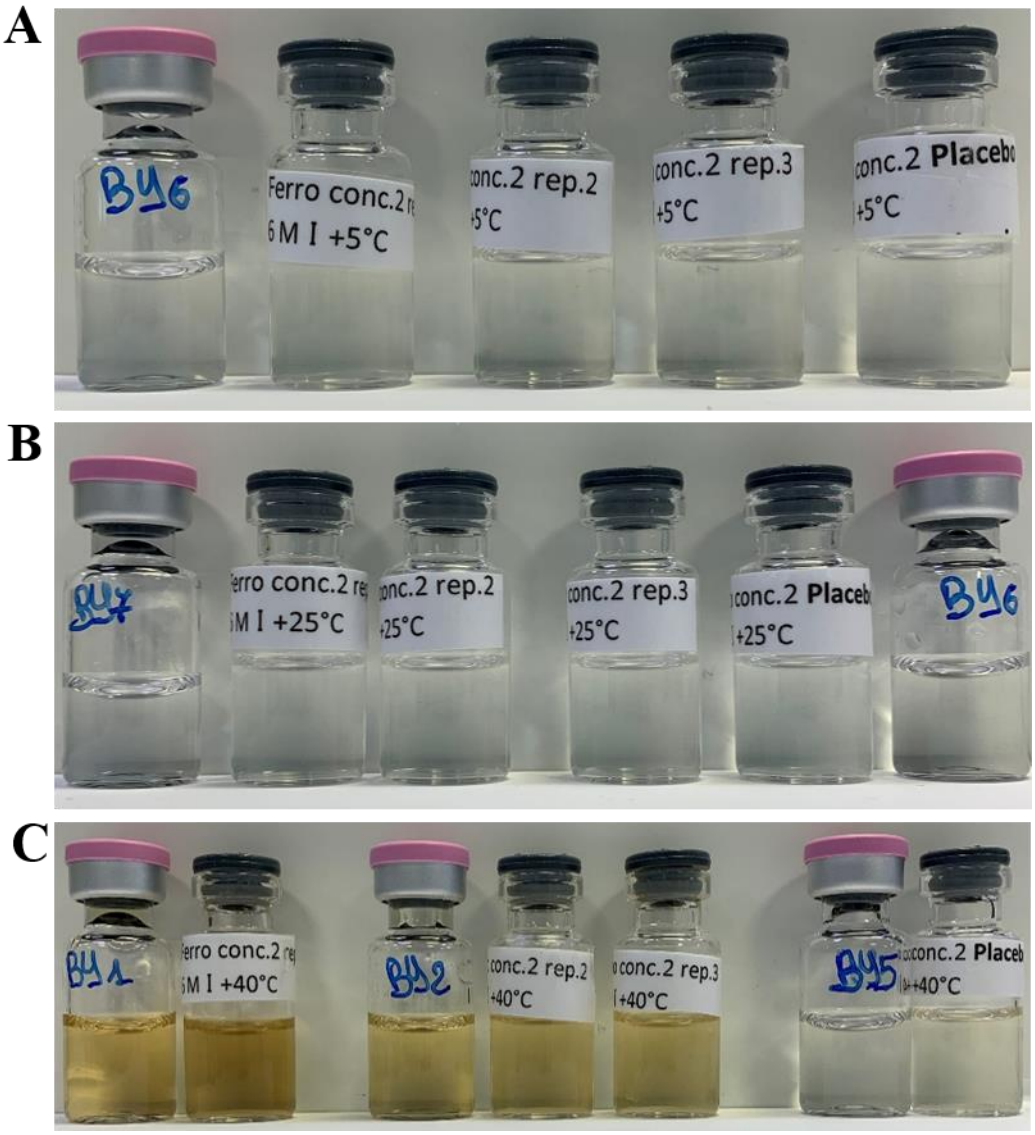
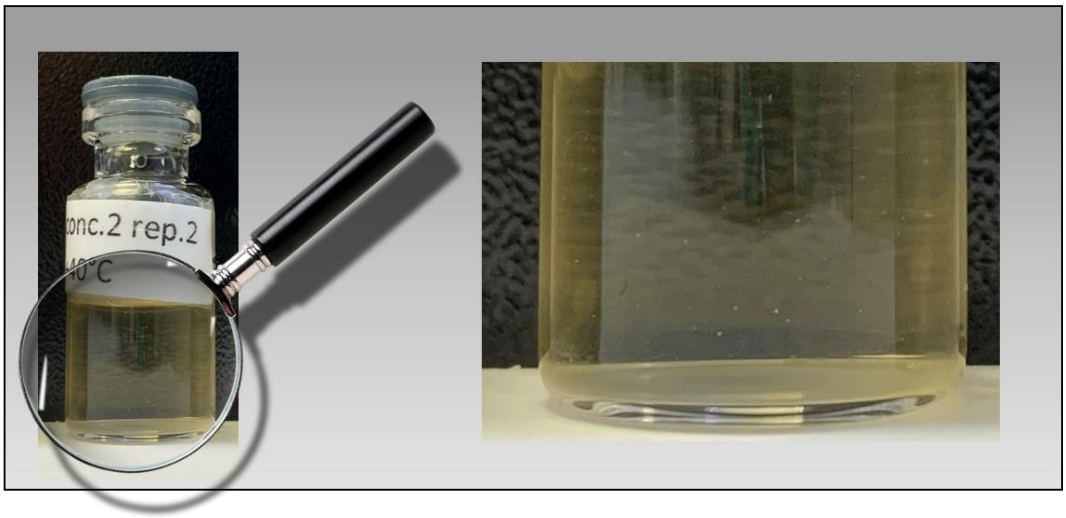


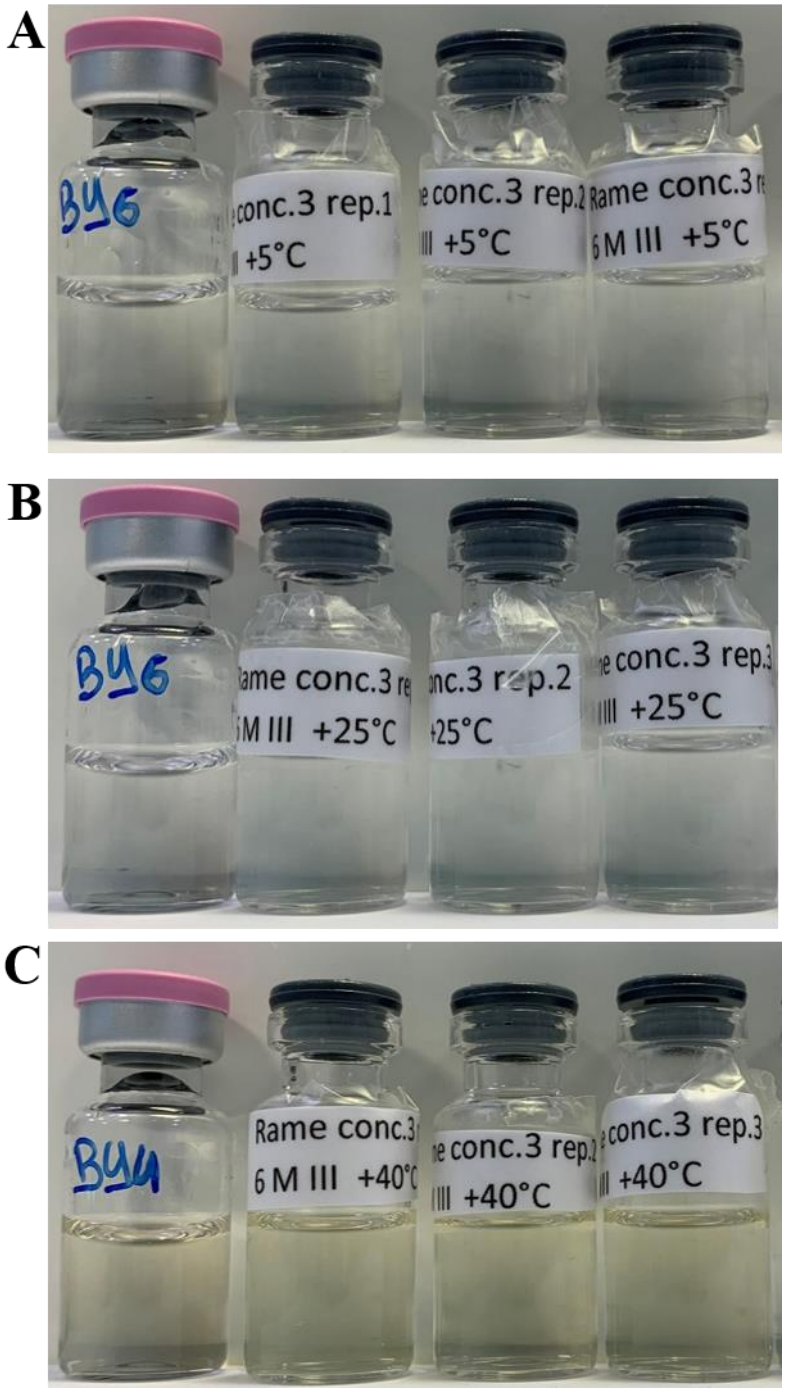
Fig 41 – Visual Inspection – Iron. Presence of visible particles in samples treated with iron conc 2 and stored for 44 weeks at +40°.



Samples and placebos treated with copper showed a slight change in coloration when stored at +5°C (**Figure 42 A**) and at +25°C (**Figure 42 B**). As in case of iron a more significant color change was observed upon incubation at +40°C (from BY4 to BY5), as can be seen in **Figure 42 C**.

In terms of visible particles a low number was observed in treated samples while a grater one in treated placebos (especially for those stored at +40°C). In terms of clarity all samples and placebos appeared clear, with the exception of those stored at + 25°C that appeared more opalescent.

Figure 42 – Visual Inspection – Copper. Samples treated with copper conc 3 and stored for 44 weeks at +5°C (**A**); at +25°C (**B**); at +40°C (**C**) compared to BY certiPUR color reference solutions.

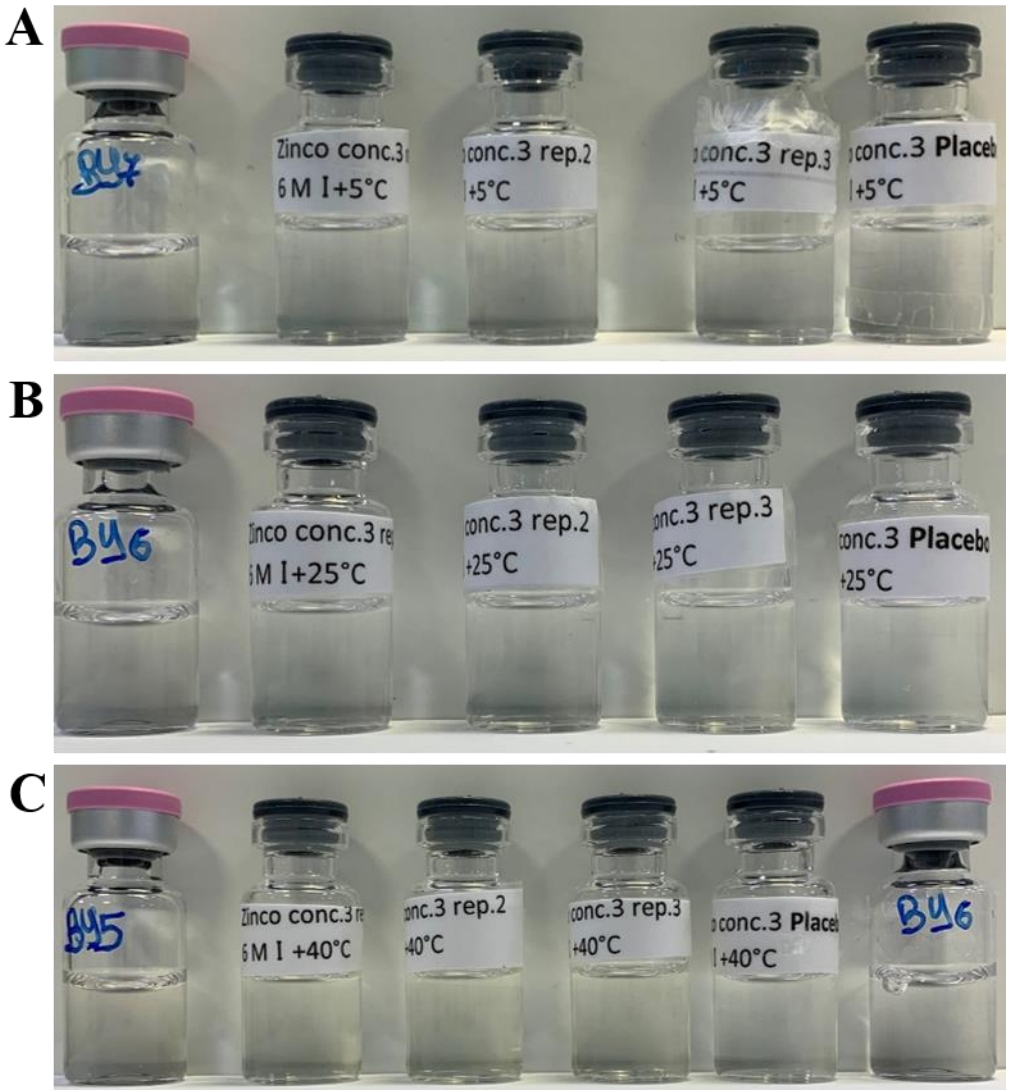


Samples and placebos treated with zinc (**Figure 43**) and stored at +5°C and +25°C did not show any alteration of coloration (except a change to BY6 for samples

and placebos treated with conc 3, stored for 44weeks at +25°C). A more significant colour change was instead observed for all concentrations upon incubation at +40°C: BY5 in case of samples and BY6 in case of placebos.

In case of visible particles only a modest increase was observed in all samples, while opacity was found increased only in samples stored at +25°C.

Figure 43 – Visual Inspection – Zinc. Samples treated with zinc conc 3 and stored for 44 weeks at +5°C (**A**); at +25°C (**B**); at +40°C (**C**) compared to BY certiPUR color reference solutions.



In case of samples and placebos treated with nickel (**Figure 44**) a behaviour similar to that observed for zinc was observed. Indeed, after incubation at +25°C only a slight change in coloration for both protein and placebo samples (BY6) was observed.

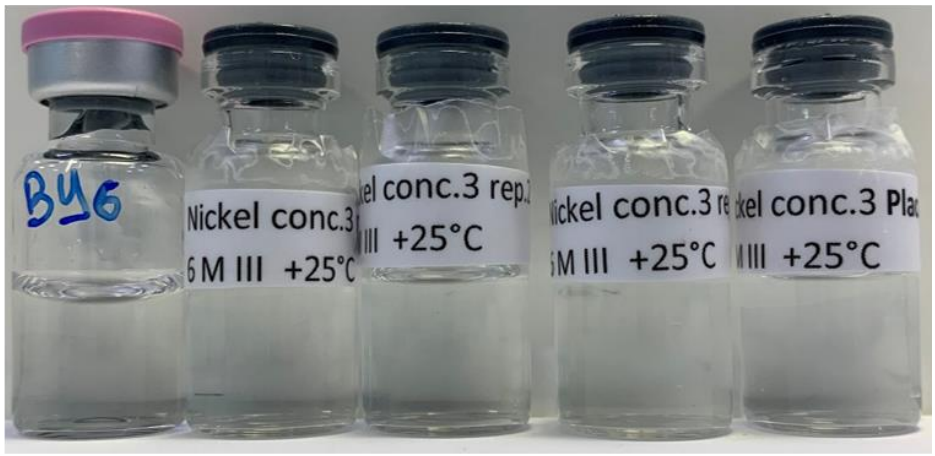
Upon incubation at +40°C a more significant colour change was observed for all concentrations: BY5 in case of samples and BY6 in case of placebos.

In case of visible particles, a low presence was seen in all samples and placebo, but a significant increase was observed in treated placebos incubated at +40°C, while opacity was found slightly increased in all samples and placebos stored at +25°C

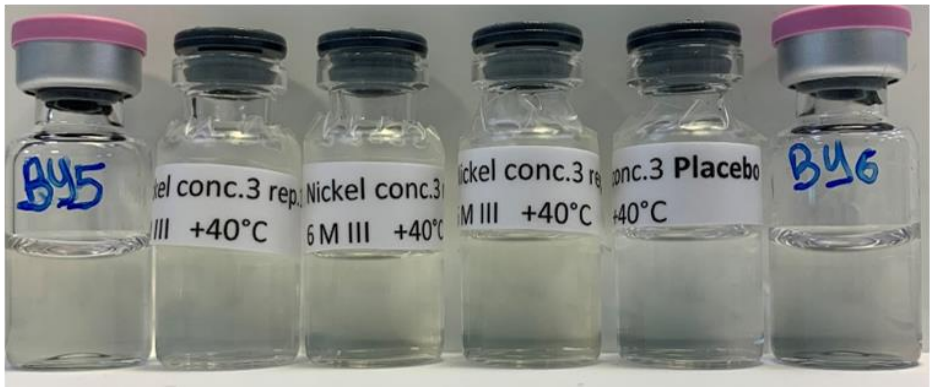
Figure 44 – Visual Inspection – Nickel. Samples treated with nickel conc 3 and stored for 44 weeks at +5°C (**A**); at +25°C (**B**); at +40°C (**C**) compared to BY certiPUR color reference solutions.

A

B



C



7.4 pH measurement – Results

The pH values of all treated samples upon storage for 44 weeks at +5°C, +25°C and +40°C were measured and are reported in **Table 47**.

Based on the DP specifications the pH of the Fc-fusion protein solution should be comprised between 5.4 and 5.6: in case of the untreated material, regardless of the temperature of incubation, after 44 weeks no difference were observed with respect to the acceptable limits.

In case of samples treated with iron, pH changes towards more acidic pHs were registered only upon incubation at +25°C and +40°C for all concentrations.

In case of samples treated with zinc, nickel and copper no modifications to the pH was observed.

Table 47. pH values. pH measurement of samples incubated for 44 weeks at +5°C, +25°C, +40°C. Results are expressed as average of three independent replicates. The pH reference range is 5.4 -5.6. Non-comparable values are reported in bold.

Samples 44 weeks	**pH**		
	+5°C	**+25°C**	**+40°C**
Untreated sample	5.6	5.5	5.4
Sample Iron conc 1	5.5	**4.6**	**4.3**
Sample Iron conc 2	5.5	**4.7**	**4.4**
Sample Iron conc 3	5.5	**4.9***	**5.0**
Sample Copper conc 1	5.5	5.6	5.5
Sample Copper conc 2	5.5	5.5	5.4
Sample Copper conc 3	5.5	5.5	5.4
Sample Zinc conc 1	5.5	5.5	5.4
Sample Zinc conc 2	5.5	5.5	5.4
Sample Zinc conc 3	5.5	5.5	5.4
Sample Nickel conc 1	5.5	5.5	5.5
Sample Nickel conc 2	5.5	5.5	5.4
Sample Nickel conc 3	5.5	5.5	5.4

* This value was calculated on a single replicate

8. References

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