International Journal of Pharmaceutics 649 (2024) 123643

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Prolonging the stability of cetuximab (Erbitux®) and panitumumab (Vectibix®): An orthogonal assessment of physicochemical, biological and microbiological properties of original opened glass vials and diluted saline preparations

Debora Carpanese [a,1], Valentina Rossi [a,1], Veronica Di Paolo [b], Luigi Quintieri [b], Alessandro Penna [c], Gaia Zuccolotto [a], Jessica Sebellin [d], Camilla Saran [d], Francesca Pipitone [d], Giorgia Miolo [b], Elisabetta De Diana [b], Nicola Realdon [b], Nicoletta Rigamonti [d], Francesca Di Sarra [d], Marina Coppola [d,2], Antonio Rosato [a,c,2,*]

[a] *Immunology and Molecular Oncology Unit, Veneto Institute of Oncology IOV-IRCCS, Padua, Italy*
[b] *Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Padua, Italy*
[c] *Department of Surgery, Oncology and Gastroenterology, University of Padua, Padua, Italy*
[d] *Pharmacy Unit, Veneto Institute of Oncology IOV-IRCCS, Padua, Italy*

A R T I C L E I N F O

Keywords:
Cetuximab
Panitumumab
Physicochemical stability
Biological stability
Microbiological stability
Diluted solution

A B S T R A C T

The two anti-epidermal growth factor receptor monoclonal antibodies (mAbs) cetuximab and panitumumab are the pillars for the treatment of EGFR-positive, *KRAS* wild-type metastatic colorectal cancers. However, stability data of these mAbs are generally missing or incomplete. Here, we report for the first time an orthogonal analysis of the stability of cetuximab (Erbitux®) and panitumumab (Vectibix®), either undiluted vial leftovers or saline dilutions in polyolefin/polyamide infusion bags. All samples were stored at 2–8 °C protected from light, according to their summary of product characteristics (SmPCs). Alternatively, opened vials and preparations were maintained at 25 °C for 15 h, and then stored again at 2–8 °C protected from light to mimic a temporary interruption of the cold chain. Vial leftovers proved stable up to 180 days when stored according to their SmPCs, while compounded preparations in infusion bags maintained their physiochemical, biological and microbiological stability up to 30 days. Additionally, no changes were detected up to 30 days for the same samples undergoing a thermal excursion. Our results provide additional rationale to the SmPCs, crucial especially in the case of reassignment and pre-preparation of bags. This information will allow hospitals to achieve significant cost savings, and better organization of the entire therapeutic process.

1. Introduction

Over the last years, monoclonal antibodies (mAbs) have rapidly escalated as biopharmaceuticals into cancer therapeutic settings, mainly due to their target specificity and stimulation of reliable anti-tumoral responses (Kaur, 2021). However, the high cost and limited stability, this latter being in the majority of cases 24 h in the refrigerated state as reported by their summary of product characteristics (SmPCs), negatively impact on the economic management of immunotherapies and immunotherapy trials. Nevertheless, the restrictions on mAbs shelf life principally rely on microbiological stability issues, which can be currently easily controlled as therapies are prepared in centralized compounding units under validated aseptic conditions (Lagarce, 2017; Vigneron et al., 2019; Rigamonti et al., 2023). Therefore, the validation of a prolonged stability is more related to a deeper investigation of mAbs physical and chemical properties and biological activity. The degradation pathways of mAbs can occur through complex modalities and throughout all stages of production, storage, and administration. In the

* Corresponding author at: Department of Surgery, Oncology and Gastroenterology, University of Padua, Padua, Italy, Via Giustiniani 2, 35128 Padua, Italy.
E-mail addresses: valentina.rossi@iov.veneto.it (V. Rossi), antonio.rosato@unipd.it (A. Rosato).

[1] co-first authorship.
[2] co-last authorship.

https://doi.org/10.1016/j.ijpharm.2023.123643
Received 19 September 2023; Received in revised form 24 November 2023; Accepted 27 November 2023
Available online 30 November 2023
0378-5173/© 2023 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

latter case, it is important to highlight that the physical and chemical modification of mAbs can lead not only to the loss of their bioactivity, but also to the enhancement of their immunogenicity with increasing risks of severe hypersensitivity reactions. Additionally, mAbs administered intravenously are often diluted in 0.9 % NaCl that add electrolytes potentially influencing the conformational stability, equilibrium solubility, intermolecular repulsion, and rate of formation of non-native aggregates (Le Basle et al., 2020). Overall, it is evident the necessity of maintaining rigorous safety standards, and data obtained by a single technique cannot be considered adequate to provide satisfactory information on mAbs alteration status (Kaur, 2021); (Farjami et al., 2019); (Thiagarajan et al., 2016). Thus, according to the international conference of consensus (ICH) Q5C (ICH, 1995), a comprehensive and representative description of mAbs stability requires complementary methodologies, such as physical for the assessment of aggregation and fragmentation phenomena, chemical to evaluate any degradation profile, and biological to estimate the conservation of mAbs functionality.

Cetuximab (Erbitux®) is a chimeric IgG1 mAb and epidermal growth factor receptor (EGFR) inhibitor, which is approved for wild-type *KRAS* metastatic colorectal (mCRC), non-small cell lung and head and neck cancers. Panitumumab (Vectibix®) is a fully humanized IgG2 mAb targeting EGFR with a higher affinity than cetuximab, which is authorized for the treatment of adult patients with wild-type *KRAS* mCRC. Both mAbs avoid the binding of endogenous ligands to EGFR, thus blocking receptor activation and downstream signalling pathways. According to the SmPCs, cetuximab is stable for 48 h at 25 °C after dilution and the opened vial for 24 h at 2–8 °C, while panitumumab for 24 h at 2–8 °C, but no indications regarding leftovers are available.

The main aim of this study was to provide information on the stability of cetuximab and panitumumab in terms of maintenance of their physiochemical, biological, and microbiological stability over time. Analysed samples were in-use vials and saline dilutions in polyolefin (POF)/polyamide infusion bags at selected concentrations, namely 4 and 1.5 mg/mL for cetuximab and 6 and 3 mg/mL for panitumumab, which were stored according to SmPCs at 2–8 °C, or experienced a temperature excursion of 15 h at 25 °C. Moreover, data from samples that were purposely chemically and thermally stressed served as references for the validation of stability evaluation methodologies. In this study, different types of analytical methods were employed to evaluate functional alterations, which were not previously appreciated from the application of sole binding assays. To the best of our knowledge, this is the first study that provides such comprehensive evaluation of the stability of either therapeutics (Farjami et al., 2019); (Paul et al., 2012; Le Guyader et al., 2020; Ikesue et al., 2010; Park et al., 2020; Le Guyader et al., 2021).

2. Materials and methods

2.1. mAbs and preparation of samples

Stability of cetuximab (Erbitux®, Merck KGaA, Darmstadt, Germany) and panitumumab (Vectibix®, Amgen Europe B.V., Breda, The Netherlands) was studied in original opened vials, presented as a ready-to-use solution of 5 mg/mL and 20 mg/mL, respectively. Leftovers were used without removing the Spike® CS-51 (Icu Medical, San Clemente, California, USA) from the vials. Both mAbs were diluted in 0.9 % sodium chloride solution in 50 mL POF/polyamide intravenous infusion bags (Baxter International, Deerfield, Illinois, USA), to obtain two concentrations covering the most common range used in clinical practices: 1.5 and 4 mg/mL for cetuximab, 3 and 6 mg/mL for panitumumab. The bags were prepared aseptically in a vertical laminar flow hood. The drug was withdrawn from each vial using a CSTD vial Spike®. At least three different batches of vials and bags were stored at each condition tested: 2–8 °C, and 25 °C for 15 h and then 2–8 °C (called ΔT° samples) to mimic an interruption of the cold chain. A reference sample was always obtained after the immediate opening of vials or drug compounding (day 0), and analyses were periodically performed at days 0, 14, and 30 for the bags, and additionally at days 60, 90, and 180 for vials stored at 2–8 °C, while for ΔT° samples at days 1, 14, and 30, unless otherwise indicated. As controls to evaluate the reliability of our methods, forced degradation studies were conducted. For thermal stress, cetuximab vials were heated for 15 h at 50 °C, or for 5 h at 60 °C or 70 °C, while panitumumab for 5 h at 50 °C. For chemical stress, both mAbs were also diluted 1:2 in trypsin (Gibco, Thermo Fisher Scientific, Waltham, USA).

2.2. Physicochemical stability

Physical stability was assessed by visual examination of both vials and bags: colour changes and particulate matter were evaluated after preparation (day 0), and every week up to days 30 or 180. pH measurements were performed using a Mettler Toledo FiveEasy™ pH-meter (Merck KGaA), while the total protein concentration was assessed by measuring the absorbance at 279 nm on a NanoDrop One spectrophotometer (Thermo Scientific). Values were acceptable if they did not vary by more than 1 pH unit or 1 mg/mL from the initial measurement, respectively. Turbidity was evaluated by measuring the absorbance of sample solutions at 280 and 350 nm using the Ultraviolet (UV)-Vis spectrophotometer Cary 60 (Agilent Technologies, California, USA). The aggregation index (AI) was calculated as reported elsewhere (Hawe et al., 2009). AI% $<$ 10 were considered lacking of sub/visible soluble aggregates. The tertiary structure changes were analysed by UV between 250 and 320 nm in second derivative mode, analysing samples diluted to 0.5 mg/mL in 0.9 % sodium chloride solution. The representative peaks specific for aromatic amino acids were studied at 252, 259, and 265 nm for phenylalanine (Phe), 275 for tyrosine (Tyr), 285 for Tyr and tryptophan (Trp), 289, 292, and 295 for Trp (Paul et al., 2012); (Lahlou et al., 2009); (Bardo-Brouard et al., 2016). Sodium Dodecyl Sulphate (SDS) PAGE analysis was carried out under both reducing and non-reducing conditions in pre-cast polyacrylamide NuPAGE 4 to 12 % (v/v) Bis-Tris, 1.0 mm, Midi Protein Gel (Thermo Scientific). Antibody samples mixed with NuPAGE LDS Sample Buffer and, in reducing condition, NuPAGE Sample Reducing Agent (both from Thermo Scientific) were further added. All samples were loaded onto the gels at a concentration of 10 μg/μL together with reference SHARPMASS™ VI Protein molecular weight marker (5–245 kDa, Euroclone, Milan, Italy). Following electrophoresis, gels were stained for 1 h with GelCode TM Blue Stain (Thermo Scientific). The presence of sub-molecular size fragments and/or aggregates such as oligomers was evaluated by size exclusion high-performance liquid chromatography (SEC-HPLC). The separation was performed with a TSK G3000-SWXL column (7.8 mm x 30 cm x 5 μm, TOSOH Bioscience, Rivoli, Turin, Italy) maintained at a constant temperature of 25 °C. The mobile phase consisted of 0.1 M disodium hydrogen phosphate and 0.1 M sodium sulphate at pH 6.8, and the flow rate was 0.6 mL/min. The detection wavelength was set up at 280 nm.

2.3. Pharmacological stability

The $EGFR^+$ and *KRAS* and *BRAF* wild-type (Pozzi, 2016) human colorectal adenocarcinoma Caco-2 cell line (HTB-37, ATCC, Manassas, VA, USA) was cultured in High Glucose w Sodium Pyruvate w/o L-Glutamine Dulbecco's Modified Eagle Medium (DMEM, EuroClone), supplemented with 1 % of 2 mM Ultra-Glutamine, 10 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 100 U/mL Penicillin, 100 μg/mL Streptomycin (all from Lonza, Verviers, Belgium) and 10 % (v/v) heat-inactivated Foetal Bovine Serum (Gibco), hereafter referred as to complete medium. Cells were incubated at 37 °C in a humidified 5 % CO_2 atmosphere. Samples were analysed for their ability to bind EGFR by flow cytometry. Caco-2 cells ($3x10^5$/sample) were labelled for 30 min in ice with each mAb sample at a final concentration of 50 μg/mL, and then stained with secondary antibodies: for cetuximab, FITC rat anti-human IgG (clone M1310G05, BioLegend, London, UK) for

30 min in ice; for panitumumab, FITC mouse anti-human IgG (clone G18-145, Becton Dickinson, Franklin Lakes, NJ, USA) for 1 h at room temperature (RT). Control cells were represented by cells labelled with the secondary antibody only. Samples were analysed using FACSCalibur flow cytometry (Becton Dickinson) and FlowJo software (TreeStar Inc., Olten, Switzerland), and the difference between the mean fluorescence intensity (ΔMFI) was calculated by subtracting from the value obtained the MFI of the cells labelled with the secondary antibody only. The maintenance of the effector functions was also assessed with two different methods. The capability of mAbs to interfere with cell proliferation was monitored by the ATPlite luminescence Adenosine triphosphate (ATP) detection assay system (PerkinElmer Life Sciences, Boston, USA) as reported elsewhere (Montagner, 2015), testing in triplicate two different dilutions for each sample: 0.5 and 0.05 mg/mL for cetuximab, 0.17 and 0.017 mg/mL for panitumumab. For testing the induction of apoptosis, Caco-2 cells ($2x10^6$/well) were let adhere on a 6-well flat-bottom plate overnight, and then were incubated with cetuximab and panitumumab at a final concentration of 0.06 and 0.05 mg/mL in complete medium, respectively. Cells incubated with medium only were used as control of proliferation. After 72 h of incubation, necrosis and apoptosis were assessed by cytofluorimetric analysis using eBioscience Annexin V Apoptosis Detection Kit APC (Thermo Fisher Scientific) according to the manufacturer's instructions. Samples were analysed using FACSCalibur flow cytometry and FlowJo software, and the percentages of early (propidium iodide (PI)-negative and Annexin V-positive) and late apoptotic (PI-positive and Annexin V-positive) cells were calculated.

2.4. Microbiological stability

Sterility tests were performed on days 30 or 180 to detect the presence of microbial contamination using a pool of three batches for each condition. Samples were incubated in trypticase soy broth (TSB) and fluid thioglycolate medium (FTM, both from Merck KGaA), and incubated at 25 and 37 °C for 14 days. As positive controls, an aerobic bacterial culture of *Cupriavidus necator* was used for the TSB test, while a community of anaerobic bacteria composed of *Lentimicrobium* sp, *Fermentimonas caenicola,* and *Methanomassilii coccaceae* sp was used for the FTM test (kindly provided by the Department of Biology, University of Padua). Broths opalescence indicated the presence of microbial growth. The same sample pools were also investigated for the presence of pyrogens using the Limulus Amebocyte Lysate (LAL) PYROGENT™ Plus Gel Clot test kit with 0.125 endotoxin unit (EU)/mL sensitivity (Lonza, Basel, Switzerland), according to the manufacturer's instructions.

2.5. Statistical analysis

Results are presented as mean ± standard deviations (SDs) from at least three separate samples. Parametric tests with Welch's correction test were used, when required, to compare results with the reference sample (day 0) using GraphPad Prism 7.0 software. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Physicochemical stability

For each mAb, the opened vials and bags at the different dilutions and storage conditions maintained their physicochemical stability throughout the entire time of analysis (30 days; 180 days for the vials stored at 2–8 °C). Indeed, solutions conserved their transparency and no colour changes were visible (data not shown), while the measured pH and concentration of each sample remained unchanged (Fig. 1a and 1b). Cetuximab vials heated at 50 °C for 15 h showed no modification of these parameters; conversely, after 5 h at 60 °C or 70 °C samples become opalescent in the first case and evidenced a visible and not resuspendable white precipitate in the latter (data not shown). Consequently, the apparent total protein concentration lowered and dramatically increased, respectively, due to changes in the refraction index of the solutions. As for panitumumab, 5 h at 50 °C worsened imperceptibly the limpidity of the solution (data not shown), but this was sufficient to increase the detected protein concentration. All samples treated with trypsin changed in both the pH values and total protein concentration. Accordingly, the AI% remained within the predefined limits (<10) for all sampling times, storage conditions, and concentrations, demonstrating the lack of aggregate formation. On the other hand, heating over 50 °C increased turbidity in all samples, this being particularly evident for cetuximab that evidenced an AI of 47.195 ± 10.024 % at 60 °C. Interestingly, trypsin treatment of cetuximab slightly increased the AI% that however remained within acceptable values (Fig. 1c).

Antibody fragmentation or aggregation was also assessed by SDS-PAGE. Gels under non-reducing conditions revealed a single band at ∼ 150 kDa, corresponding to the expected molecular weight (MW) of intact cetuximab (MW = 152 kDa, Fig. 2a and 2b) or panitumumab (MW = 147 kDa, Fig. 2c and 2d) monomers. This was observed for both opened glass vials or diluted bag solutions and either storage conditions, and also for the cetuximab vial heated at 50 °C for 1 h. Under reducing conditions, the same samples revealed two major bands representing the heavy (MW ∼ 50 kDa) and light (MW ∼ 25 kDa) chains. Panitumumab heated at 50 °C for 5 h displayed similar results and no satellite bands were detected, even though bands appeared less intense for some batches. In contrast, cetuximab samples heated at 60 °C and 70 °C proved to contain aggregates, since in non-reducing conditions the vial heated at 60 °C displayed different satellite bands at MW > 150, while at 70 °C the sample was unable or poorly able to migrate. Only for this latter case, reducing conditions led to appreciate the presence of different bands ranging from 52 to 15 kDa. Cetuximab treated with trypsin revealed collateral bands, indicating that mAb fragmentation occurred: in non-reducing conditions, the most represented bands appeared slightly lower 50 kDa and at ∼ 10–11 kDa. The latter was also detectable in reducing conditions, along with a band in the proximity of the light chain (Fig. 2a and 2b). Panitumumab treated with trypsin showed both fragmentation and aggregation processes, as in non-reducing conditions different bands were detectable, especially at MW > 240 KDa, while in reducing conditions just two bands were observed with MW resembling those of the single heavy (H) and light (L) chains of the mAb (Fig. 2c and 2d).

Second-derivative spectroscopy of the UV spectra showed no evident shifts in the aromatic amino acids region (250–300 nm), indicating that cetuximab and panitumumab vials and bags retained their tertiary structure as compared with day 0. This can be observed in samples stored at 2–8 °C for up to 180 and 30 days, respectively, or when stored for 15 h at 25 °C before being returned to 2–8 °C for 30 days (blue and red lines, respectively, Fig. 3a and 3b), as well as in cetuximab vials heated at 50 °C (grey line, Fig. 3a). In contrast, both mAbs stressed samples displayed multiple shifts, and the characteristic peaks of the aromatic amino acids Phe, Tyr and Trp were no longer identifiable. Notably, for cetuximab heated at 60 °C the tertiary structure was completely altered.

SEC-HPLC profiles of cetuximab and panitumumab showed the peak corresponding to their monomeric forms (hereafter referred as "main SEC peak") with a retention time of 11.4 and 13.3 min, respectively. Both mAbs solutions tested at day 0 (i.e., immediately after vials opening), after storage at 2–8 °C for 180 days, and at 30 days after thermal excursion, yielded a chromatogram showing a main SEC peak with a relative total area under the curve (AUC) ≥ 98 %. No variation of percent area of the main SEC peak was observed after dilution of both antibody formulations with 0.9 % sodium chloride. Conversely, thermally stressed samples (i.e., after heating at 60 °C or 50 °C for cetuximab and panitumumab, respectively) produced chromatograms presenting minor peaks with shorter retention time and suggestive of oligomers formation (Fig. 4a). Moreover, chemical stress induced by trypsin led to

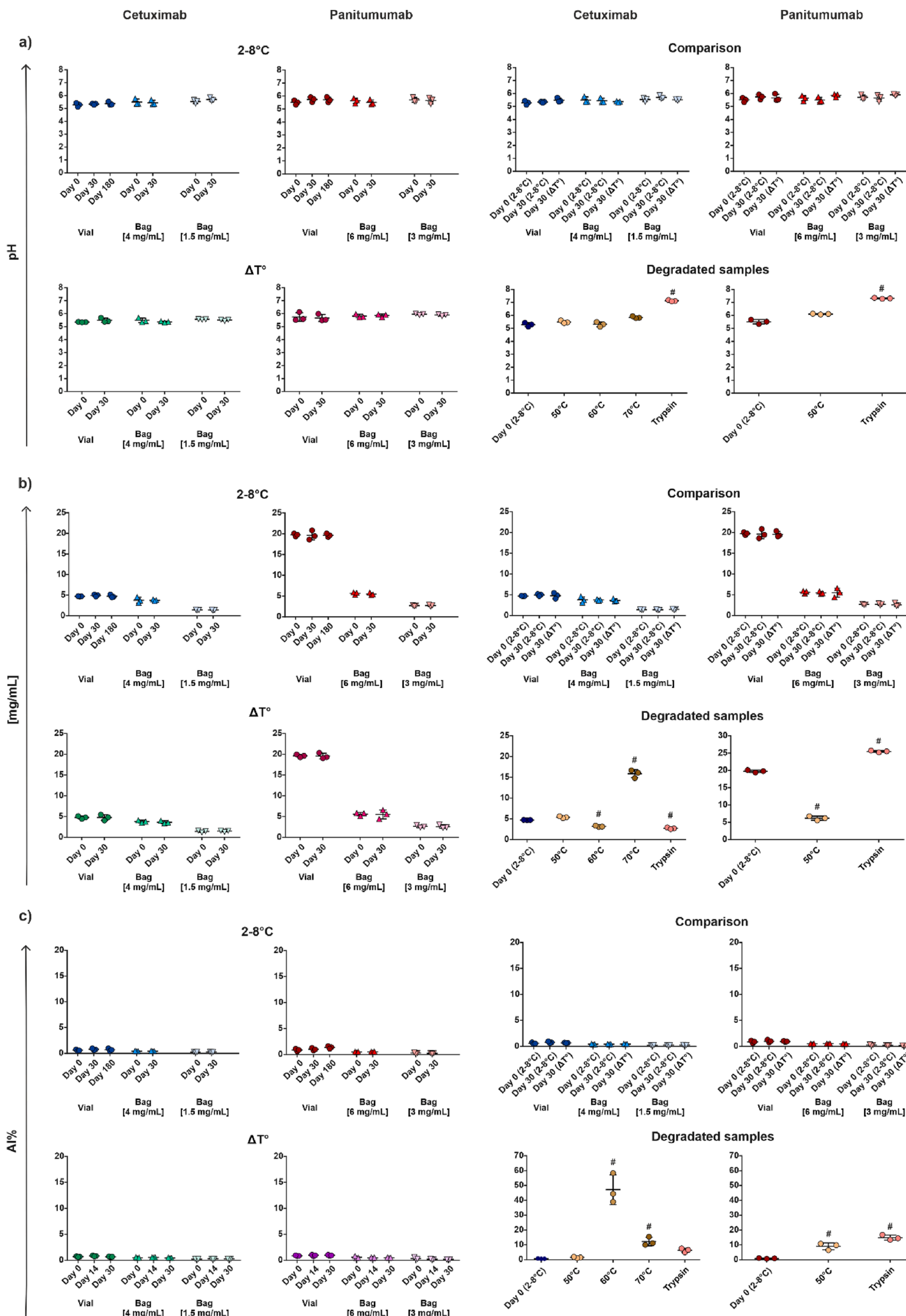

Fig. 1. Physiochemical stability of cetuximab and panitumumab samples. Samples (three different batches for each condition and experiment) were stored at 2–8 °C or experienced a temporary interruption of the cold chain (ΔT°), to be tested thereafter for up to 30 or 180 days. Stress studies (thermal and chemical) were performed: cetuximab vials were heated for 15 h at 50 °C, or for 5 h at 60 °C or 70 °C, while panitumumab for 5 h at 50 °C; for chemical stress, both mAbs were also diluted 1:2 in trypsin. Then, changes in pH values (a), total protein concentration (b), and presence of aggregates (c) were assessed. Values were considered to be acceptable if they did not vary by more than 1 pH unit (a) or 1 mg/mL (b) from the initial measurement, while values of AI% < 10 were considered as the absence of sub/visible soluble aggregates (c). Differences in these values are evidenced with # symbols.

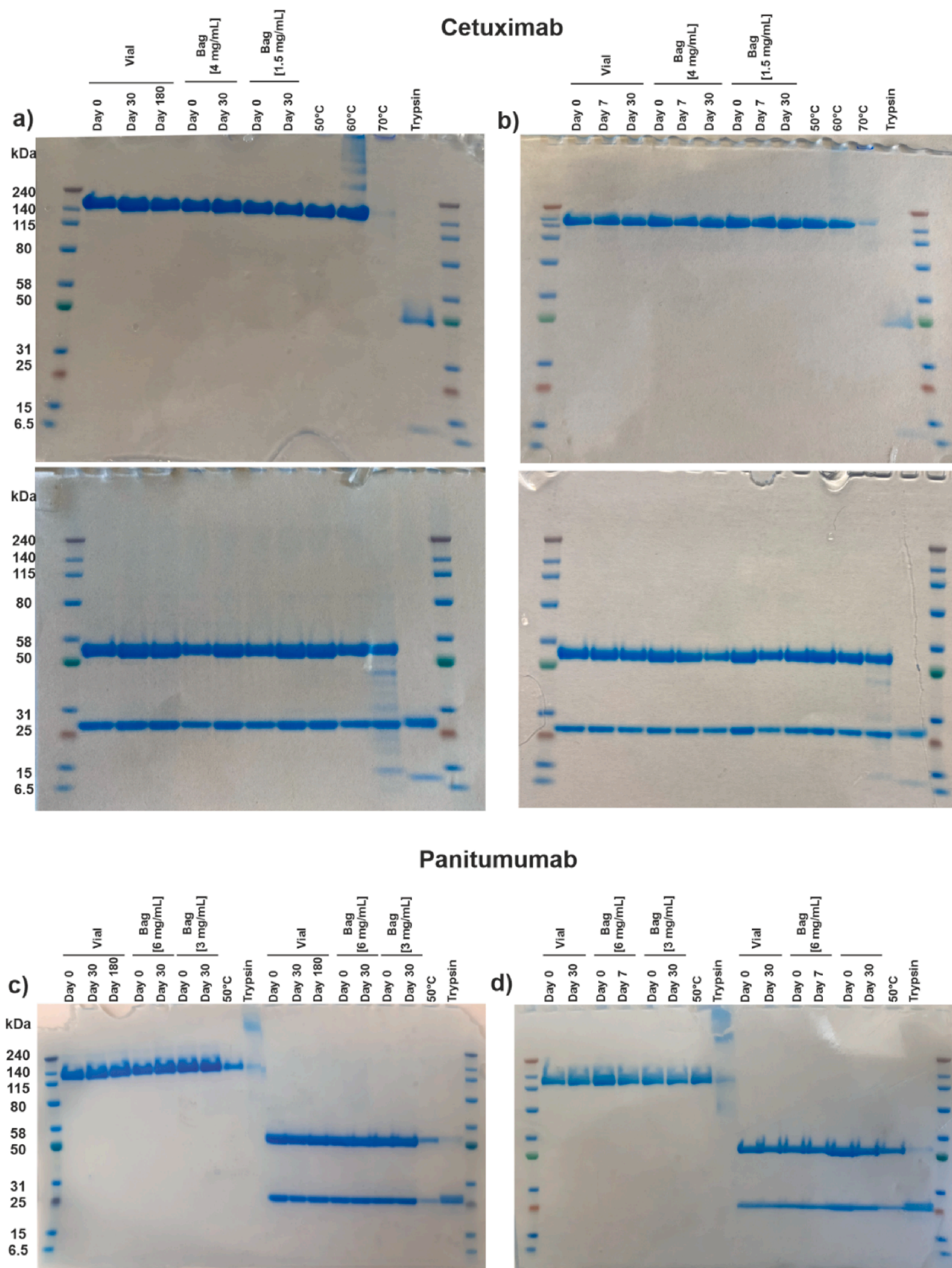

Fig. 2. Representative SDS-PAGE analysis conducted on cetuximab and panitumumab samples. Non-reduced (up) and reduced (down) SDS-PAGE of cetuximab samples stored at 2–8 °C (a) or samples that underwent thermal excursion (b), together with vials heated at 50 °C, 60 °C, and 70 °C, or incubated with trypsin. Non-reduced (left) and reduced (right) SDS-PAGE of panitumumab samples stored at 2–8 °C (c) or that underwent thermal excursion (d), together with vial samples heated at 50 °C or incubated with trypsin.

chromatographic profiles indicating a sharp decrease of the monomeric forms, and the formation of several fragments (Fig. 4b).

3.2. Pharmacological stability

To ascertain the pharmacological stability of protein-based therapeutics with specific targeting activity such as mAbs, it is essential to

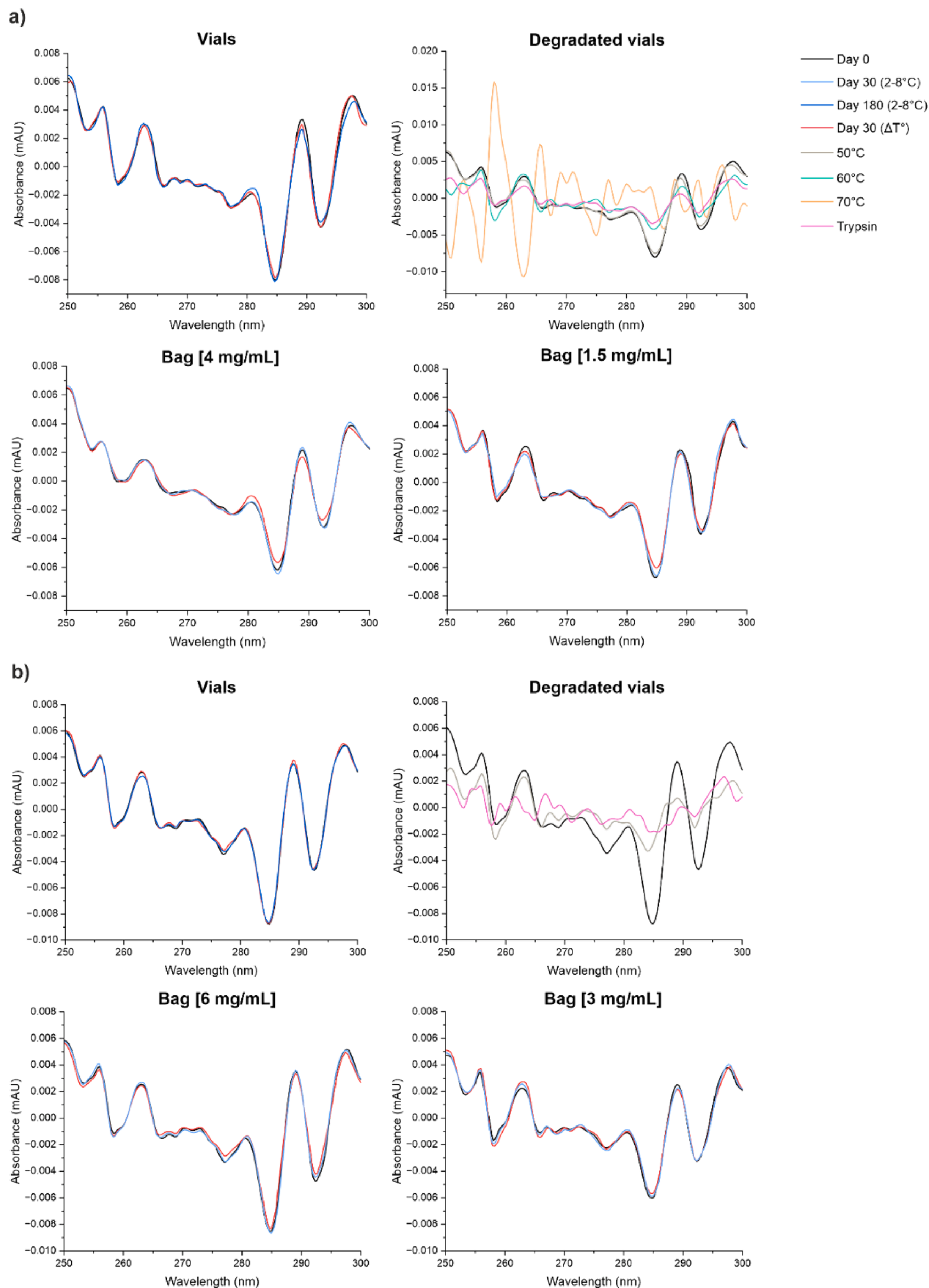

Fig. 3. Example of second-derivative UV spectra of cetuximab (a) and panitumumab (b). Spectra obtained after opening vials or diluting mAbs in bags at selected concentrations (day 0, black lines) were compared to the same samples stored at 2–8 °C for 30 (light blue lines) or 180 days (blue lines), or that experimented a temporary interruption of the cold chain (ΔT°) and tested at 30 days (red lines), together with vials that underwent thermal or chemical stress: heat at 50 °C, 60 °C or 70 °C (grey, green, and orange lines, respectively), incubation with trypsin (pink lines). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

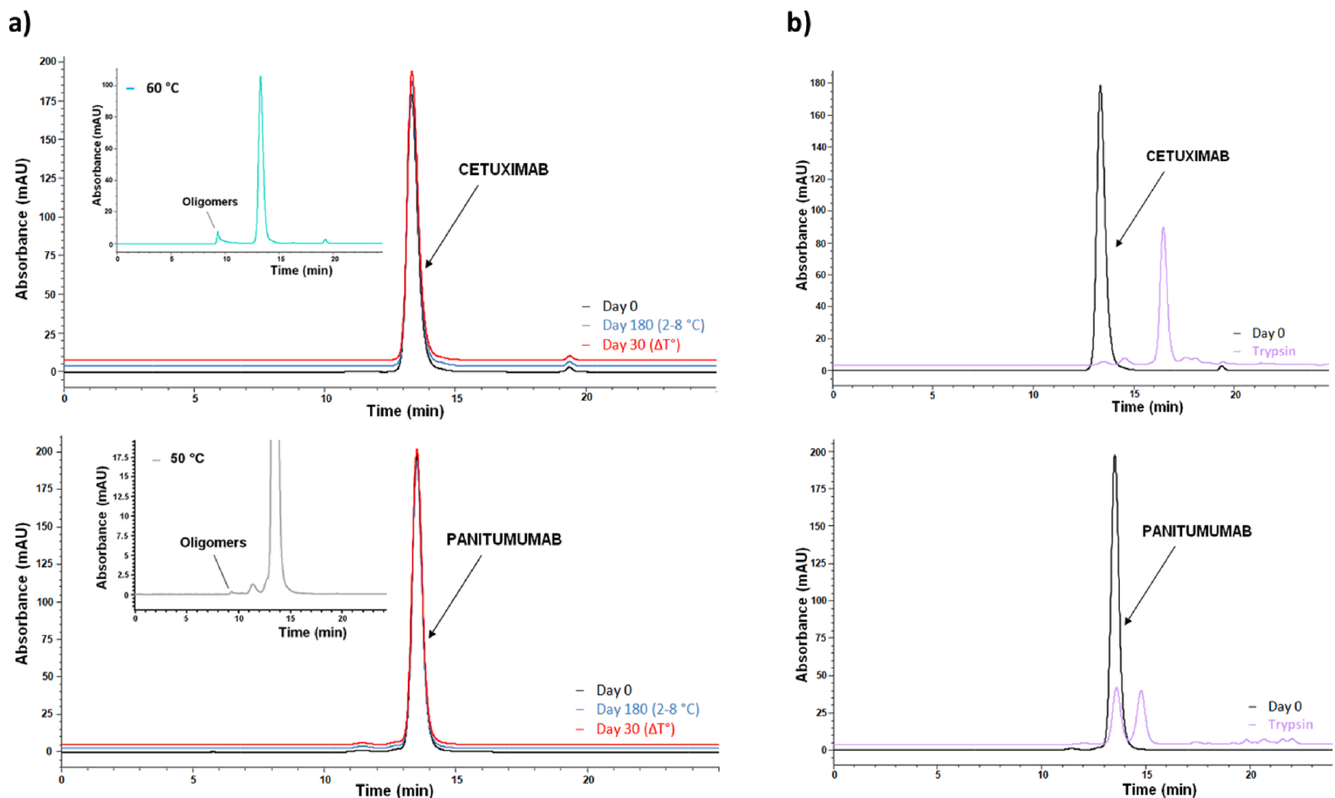

Fig. 4. Example of SEC-HPLC profiles of cetuximab and panitumumab opened vials. Chromatograms of cetuximab and panitumumab opened vials at day 0 (black line) were analysed together with samples stored at 2–8 °C for 180 days (light blue line) or that experimented a temporary interruption of the cold chain (ΔT°) and tested at 30 days (red lines) (a). Chromatograms of selected vials samples that underwent thermal stress, respectively cetuximab heat at 60 °C, and panitumumab at 50 °C (green and grey lines) were produced (a). SEC-HPLC profiles of cetuximab and panitumumab opened vials at day 0 (black line) were compared with same sample underwent chemical stress due to trypsin (pink lines) (b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

first assess the maintenance of target binding and recognition. To this aim, EGFR-expressing Caco-2 cells were stained with cetuximab and panitumumab samples and FITC-labelled secondary antibodies, and analysed by flow cytometry. Results evidenced that vials and bags of either mAbs stored at 2–8 °C or after an interruption of the cold chain, as well as cetuximab vial heated for 15 h at 50 °C, preserved their ability to recognize and bind the EGFR up to 30 or 180 days (Fig. 5a and 5b). On the other hand, the remaining heat and chemical-stressed samples decreased their binding activity, except for cetuximab vials heated at 60 °C that showed a 3-fold increase in binding capability compared to the reference sample (day 0; $p < 0.0001$).

Cetuximab and panitumumab samples were also analysed for their antiproliferative and pro-apoptotic activities by the ATPlite proliferation assay and apoptotic cells quantification by flow cytometry, respectively. Cell growth was affected in the same manner by all cetuximab and panitumumab samples analysed, with a decrease ranging between 10 and 20 % as compared to untreated control cells. In contrast, all stressed samples lost the antiproliferative ability with the exception of the 50 °C-heated cetuximab vials (Fig. 6).

Similar results were obtained by flow cytometry monitoring of cell apoptosis, and confirmed that cetuximab and panitumumab opened vials and selected dilutions in POF/polyamide bags preserved their pharmacological activity when stored at 2–8 °C up to 180 and 30 days, respectively, and up to 30 days after experiencing a temporary interruption of the cold chain for 15 h. Again, unlike panitumumab, cetuximab vials proved to be less affected by heat stress, and to preserve their pharmacological stability after heating at 50 °C for 15 h (Fig. 7).

3.3. Microbiological stability

Sterility studies on pooled samples of opened vials and diluted mAbs in bags stored at 2–8 °C for 180 and 30 days, respectively, or stored for 30 days at 2–8 °C after 15 h at 25 °C, showed no microbial growth after 14 days of incubation in TSB and FTM mediums. LAL test resulted negative for the same samples, indicating the absence of pyrogens (<0.125 EU/mL, data not shown). These results indicate that cetuximab and panitumumab vial leftovers, as well as their preparations, can be maintained under sterile conditions for extended periods if vial handling and drug compounding occurred under aseptic conditions.

4. Discussion

The long-term stability of mAbs and their derived preparations is a key issue for daily hospital practice as well as for researchers and manufacturers (Rigamonti et al., 2023); (Ma et al., 2020). For the majority of these biologicals, the common shelf-life reported by their SmPCs is 24 h, with diffuse cases of lack in stability data of opened vials or their dilutions in specific media and/or storage materials. Moreover, evaluation of mAbs stability turns out extremely challenging due to the requirement of several techniques and diversified expertise (Le Basle et al., 2020).

Here, different orthogonal methods were employed to assess the stability of cetuximab (Erbitux®, 5 mg/mL) and panitumumab (Vectibix®, 20 mg/mL) glass vial leftovers and diluted preparations in 0.9 % NaCl in POF/polyamide infusion bags. Dilutions were prepared according to the clinical practice, corresponding to the lowest and highest average concentrations usually employed: 1.5 and 4 mg/mL for cetuximab, 3 and 6 mg/mL for panitumumab. Samples were stored according

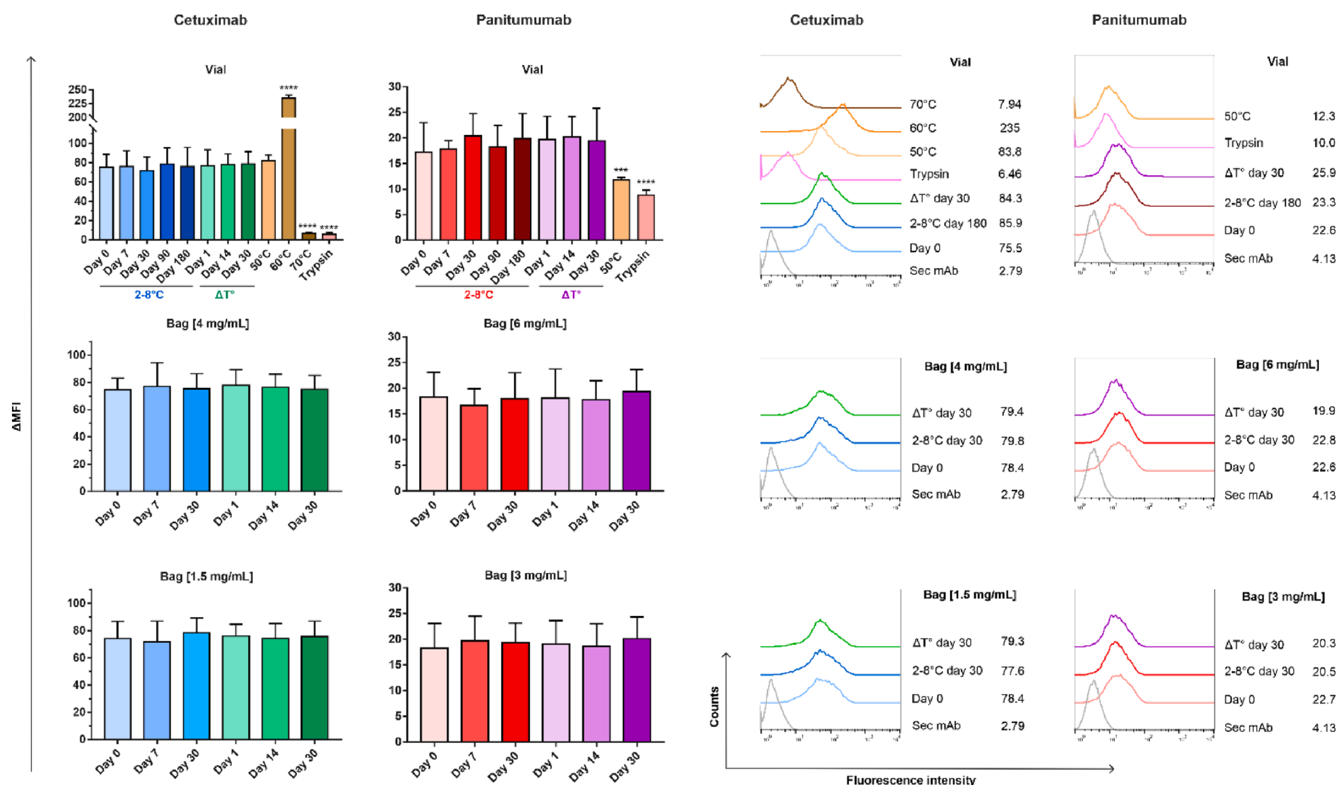

Fig. 5. Binding of cetuximab and panitumumab samples to EGFR-expressing Caco-2 cells by flow cytometry. Caco-2 cells were labelled with each mAb sample at a final concentration of 50 µg/mL, and subsequently with the proper FITC-labelled secondary mAb. Analyses were performed confronting the binding obtained at day 0 (opened vials or diluted samples in bags), with that of the same samples stored at 2–8 °C up to 30 or 180 days, or after temporary interruption of the cold chain (ΔT°) up to 30 days, together with vials under stress conditions: 15 h at 50 °C, 5 h at 60 °C or 70 °C for cetuximab, 5 h at 50 °C for panitumumab; and for both mAbs incubation in trypsin. a) Data are reported as mean of the ΔMFI values measured for 3 to 5 independent experiments and batches. ΔMFI was obtained by calculating the difference between the MFI emitted by the sample and the MFI emitted by the secondary antibody (sec mAb) alone. Student's *t*-test was applied to compare each sample with the reference, i.e. the drug (in vial or bag) stored at 2–8 °C in the dark on day 0 (***$p = 0.001$, ****$p < 0.0001$, $p > 0.05$ if not indicated). b) Example of a flow-cytometry analysis performed for a single batch of cetuximab and panitumumab and for each condition.

to their SmCPs, or experienced a thermal excursion. Physiochemical, biological and microbiological stability were periodically verified up to 30 days, and up to 180 days for opened vials stored at 2°-8°C, performing studies using three to five different batches for each mAb and condition. In all cases, samples proved to be stable, as no physical or chemical deterioration signs were detected, and the biological activity was fully preserved. Indeed, no variation in appearance, pH, or total protein content of more than 1 pH unit or 1 mg/mL, respectively, was measured. No visible aggregation was detected by turbidimetry assessment, and both SDS-PAGE and SEC-HPLC analyses mutually confirmed the absence of aggregation or fragmentation. In fact, second-derivative UV spectroscopy demonstrated that the tertiary structures of the mAbs were not modified, as also confirmed by SDS-PAGE performed both in reducing and non-reducing conditions. Notably, in non-reducing conditions only the characteristic band of an IgG1 (cetuximab) or IgG2 (panitumumab) was detectable, while in reducing conditions two bands belonging to the single H and L chains were visible. For SEC-HPLC, no difference in the percentage of the main and minor peaks was detected as respect to control samples (day 0). In this regard, potential aggregation phenomena were also evaluated with techniques that are more sensitive than SDS-PAGE (SEC-HPLC and second-derivative UV spectra assessment) and do not require denaturation processes, thus leading to assess both covalent and non-covalent aggregates. Cetuximab and panitumumab specifically bind to the extracellular domain of the EGFR overexpressed by tumour cells, thereby preventing ligand binding and blocking the receptor in its autoinhibitory monomeric conformation (García-Foncillas, 2019). Therefore, to evaluate the biological stability and the ability to successfully recognize and bind EGFR, both mAbs samples were analysed by flow cytometry for staining the $EGFR^+$ and *KRAS* and *BRAF* wild-type human colorectal adenocarcinoma Caco-2 cell line. However, since binding assessment only is inadequate to exhaustively evaluate the maintenance of mAbs functional activity, effector mechanisms were also evaluated. Cetuximab and panitumumab possess multiple effector functions mainly based on the competition with the endogenous EGFR ligands, and thus result in receptor functions inhibition. Since EGFR downstream signalling pathways are involved in the control of cell survival, cell cycle progression, angiogenesis, cell migration, and invasion/metastasis (Scaltriti and Baselga, 2006), the antiproliferative and proapoptotic activities were evaluated on Caco-2 cells as well. Results clearly demonstrated that all tested samples retained their effector functions and therefore their pharmacological activity. These data confirm the physiochemical findings, and provide essential complementary data to depict the overall stability of the mAbs and their dilutions. Furthermore, the microbiological stability of all samples was also successfully ascertained by testing the absence of bacterial growth and endotoxins contamination.

As suggested by ICH guidelines (ICH, 1995), purposely chemically and thermally stressed samples were used in this work as a stability validating reference for the different methodologies. Interestingly, cetuximab demonstrated to be less affected by heat stress, as all results from 50 °C-heated vials were comparable to reference sample (vial at day 0). On the other hand, instability was clearly observed for vials heated for 5 h at 60 °C or 70 °C, whose solutions were modified in appearance suggesting the occurrence of aggregation phenomena (Li et al., 2016). This was confirmed by variation in total protein concentration and AI%, as well as SDS-PAGE. Nonetheless, while aggregates

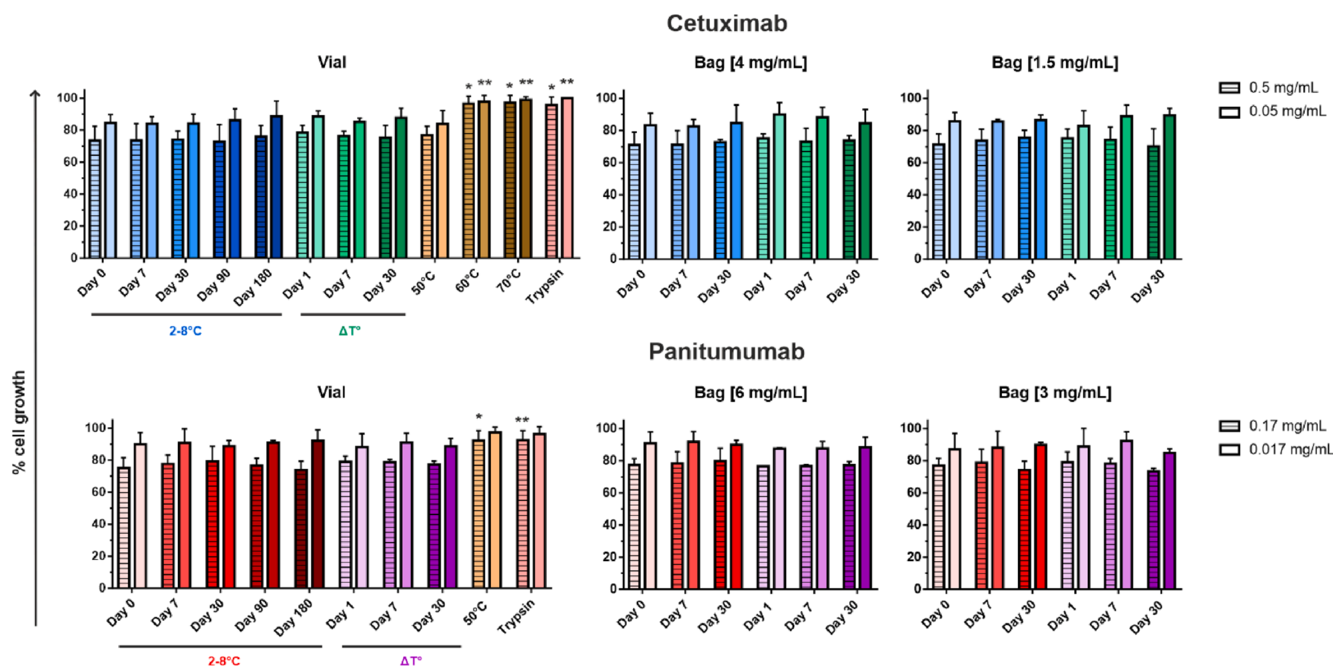

Fig. 6. Evaluation of the antiproliferative activity of cetuximab and panitumumab samples Caco-2 cells by ATPlite test. Caco-2 cells were incubated with each mAb sample for 72 h at two different final concentrations: 0.5 mg/mL (stripped bars) and 0.05 mg/mL (plain coloured bars) for cetuximab and 0.17 mg/mL (stripped bars) and 0.017 mg/mL (plain coloured bars) for panitumumab. As positive control of proliferation, cells were incubated with complete medium only (untreated control). The percentage of cell growth was calculated by determining the counts per second (cps) values according to the formula: [(cps tested – cps blank) / (cps untreated control – cps blank)] x 100, with cps blank referring to the cps of wells that contained only medium and ATPlite solution. Each bar reports the mean % of cell growth calculated for 3 to 4 independent experiments and batches ± SD. Student's *t*-test was applied to compare each sample (vial or infusion bag) at a specific final concentration with the reference at day 0 (*$p < 0.05$, **$p < 0.01$, $p > 0.05$ if not indicated).

may be mainly caused by the instauration of intermolecular bonds between intact portions of the mAb in the 60 °C-heated samples, their detection in the 70 °C-heated samples was coupled with other fragments with variable MW. Indeed, the tertiary structure of either samples proved to be altered, as additionally confirmed by SEC-HPLC analyses. Furthermore, for the 60 °C-heated vial samples several blueshifts were observed in the tertiary structure of the mAbs by second-derivative UV spectra, specifically for phenylalanine (252, 259, and 265 nm) and tryptophan (292 nm), while it was profoundly altered for the 70 °C-heated sample. As a consequence, the pharmacological activity of these samples was impaired, with compromised ability of binding EGFR and therefore interfering with cell proliferation and apoptosis induction. Interestingly, for cetuximab vials heated at 60 °C a 3-fold increase in the fluorescent signal was detected in the flow-cytometric binding assay. This might be related to the presence of aggregates established through intermolecular bonds, which lead to the exposure of multiple intact Fc portions highly recognizable by the FITC-labelled secondary mAb (Rombach-Riegraf, 2014; Nishi et al., 2014; Rasmussen et al., 2021).

Panitumumab resulted more prone to heat-induced fragmentation and aggregation, already showing signs of molecular alteration after 50 °C thermal stress condition of incubation. Indeed, while no visible changes in the solution occurred, the calculated total protein concentration sensibly decreased in comparison to the reference sample. Moreover, physical instability data were collected by turbidimetry measurement, with a 10-fold increase of the AI%, and by both SEC-HPLC and second-derivative UV spectra methodologies, with blueshifts and redshifts (285 and 292 nm, and 295 nm, respectively) observed for phenylalanine, tyrosine and tryptophan. The SDS-PAGE analyses showed the characteristic bands of the intact mAb and no satellite bands, although the detected bands were less intense in some batches. This may be the consequence of the formation of a small amount of low MW fragments not detectable in these experimental conditions. Indeed, the vial heated at 50 °C demonstrated a loss in the mAbs pharmacological activity in terms of EGFR-binding, inhibition of cell proliferation, and induction of apoptosis. Aggregation tends to occur when protein solutions are exposed to temperatures above their temperature of melting (Tm), *i.e.* the temperature at which 50 % of the protein loses its native structure. Although belonging to different IgG subclasses, both mAbs exhibit similar thermal stability profiles with a Tm of approximately 60 °C, with slight differences mainly due to different intramolecular conformations in the Fab region (Tang et al., 2021). Nonetheless, also mAb formulations can affect thermal stability of compounds. In this regard, data collected revealed a substantial difference between panitumumab and cetuximab when exposed to 50 °C for 15 h. Reasonably, we advance that this phenomenon can be likely attributed to differences in the formulations of the therapeutics. Indeed, only Erbitux contains glycine and polysorbate 80, as reported in its SmPCs. Glycine is employed as stabilizer or buffering agent, and affects thermal stability due to its zwitterionic nature and small size (Platts and Falconer, 2015). Concurrently, polysorbate 80 is an effective surfactant in preventing aggregation, and plays a crucial role as chemical chaperone by accelerating protein refolding rates and thereby mitigating the tendency for aggregation between the Fab and Fc regions (Gervasi et al., 2018). Ultimately, for both cetuximab and panitumumab vials, the chemical stress induced by co-incubation with trypsin, an enzyme that cleaves proteins on the C-terminal side of lysine and arginine residues (García-Foncillas, 2019), changed their physio-chemical and biological properties. Particularly, both mAbs were affected in pH, concentration, and AI %, albeit this last value still lay in an acceptable range for cetuximab. Chemical stress led to the formation of several fragments, as confirmed by SEC-HPLC analyses and SDS PAGE performed in both reducing and non-reducing conditions, where panitumumab showed aggregation processes as well. Furthermore, trypsin treatment and consequently chemical stress-induced fragments dramatically affected the biological function of the mAbs.

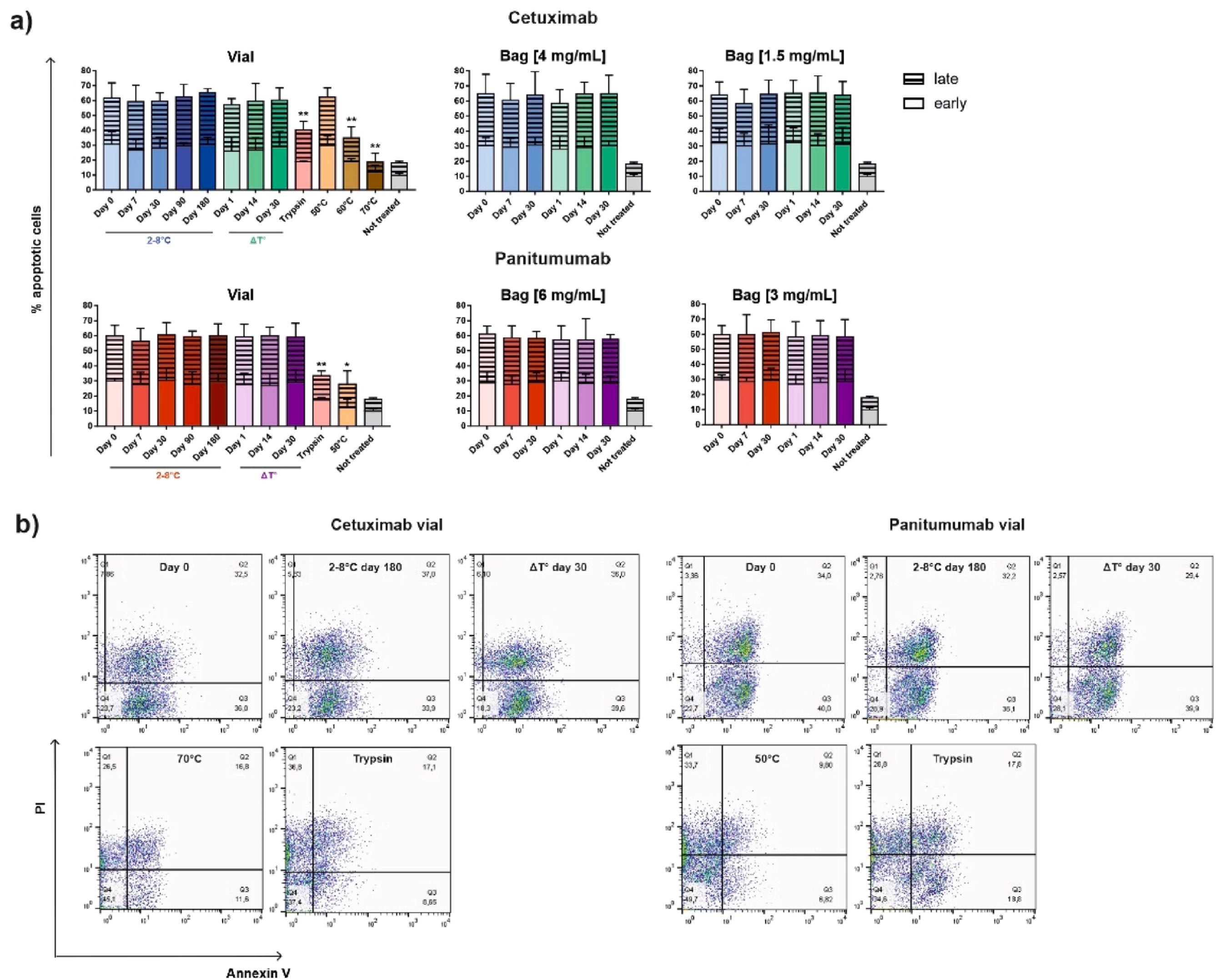

Fig. 7. Evaluation of the proapoptotic activity of mAb samples against Caco-2 cells by flow cytometry. Caco-2 cells were incubated with each mAb sample for 72 h at a final concentration of 0.06 for cetuximab and 0.05 mg/mL for panitumumab. Cells incubated with medium only were used as control. Cell death and apoptosis were assessed by cytofluorimetric analysis staining cells with APC-labeled Annexin V and PI, and the percentages of early (PI-negative and Annexin V-positive) and late apoptotic (PI-positive and Annexin V-positive) cells were calculated. a) Data reported as mean % of early apoptotic (plain coloured bars) or late apoptotic (striped bars) cells, obtained from 3 to 5 independent experiments and batches. Student's *t*-test was applied to compare each sample with the pertinent reference sample (day 0 sample) (*$p < 0.05$, **$p < 0.01$, $p > 0.05$ if not indicated). b) Example of a dot-plot flow-cytometric analysis performed for a single batch of cetuximab and panitumumab opened vials maintained for 180 days at 2–8 °C, at day 30 after undergoing a 15 h interruption of the cold chain, and under thermal and chemical stress conditions.

5. Conclusions

In conclusion, this work provides a clear-cut evidence that physicochemical stability, biological activity and microbiological safety of both cetuximab and panitumumab can be a dramatically prolonged with respect to the indications reported in the SmPC. Moreover, data from thermal and chemical stress-treated mAbs may represent a valuable support information for a wider understanding of pharmaceuticals formulations influence on stability, and possibly can contribute to its improvement throughout production and delivery steps.

Together, these results support the use of pooling residues in pharmacy practice, which can lead to time-saving strategies for pharmaceuticals preparation, reassignment and waste reduction, with a positive economic outcome for hospitals and clinical research funding, as well an improved patient management.

CRediT authorship contribution statement

Debora Carpanese: . **Valentina Rossi:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Veronica Di Paolo:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Luigi Quintieri:** . **Alessandro Penna:** . **Gaia Zuccolotto:** Data curation, Formal analysis, Writing – review & editing. **Jessica Sebellin:** Methodology, Resources, Validation, Writing – original draft, Writing – review & editing. **Camilla Saran:** . **Francesca Pipitone:** Resources, Writing – review & editing. **Giorgia Miolo:** . **Elisabetta De Diana:** Formal analysis, Writing – review & editing. **Nicola Realdon:** . **Nicoletta Rigamonti:** . **Francesca Di Sarra:** Resources, Writing – review & editing. **Marina Coppola:** Conceptualization, Resources, Supervision, Visualization, Writing – review & editing. **Antonio Rosato:** Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – review &

editing, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors acknowledge grants from Fondazione AIRC under 5 per mille - ID.22759 program to AR, and from Istituto Oncologico Veneto (IOV) BANDO IOV 2023 FREEDA to DC.

The authors would like to thank Elena Sabatini (IOV-IRCCS, Padua, Italy) for providing support in the set-up of the materials.

References

Bardo-Brouard, P., Vieillard, V., Shekarian, T., Marabelle, A., Astier, A., Paul, M., 2016. Stability of ipilimumab in its original vial after opening allows its use for at least 4 weeks and facilitates pooling of residues. Eur. J. Cancer 58, 8–16.

Farjami, A., Siahi-Shadbad, M., Akbarzadehlaleh, P., Roshanzamir, K., Molavi, O., 2019. Evaluation of the physicochemical and biological stability of cetuximab under various stress condition. J. Pharm. Pharm. Sci. 22 (1), 171–190.

García-Foncillas, J., et al., 2019. Distinguishing Features of Cetuximab and Panitumumab in Colorectal Cancer and Other Solid Tumors. Front. Oncol. 9 (September), 1–16.

Gervasi, V., Dall Agnol, R., Cullen, S., McCoy, T., Vucen, S., Crean, A., 2018. Parenteral protein formulations: An overview of approved products within the European Union. Eur. J. Pharm. Biopharm. 131 (July), 8–24.

Hawe, A., Kasper, J.C., Friess, W., Jiskoot, W., 2009. Structural properties of monoclonal antibody aggregates induced by freeze-thawing and thermal stress. Eur. J. Pharm. Sci. 38 (2), 79–87.

"ICH: Topic Q 5 C: Stability Testing of Biotechnological/Biological Products - Step 5," *Eur. Med. Agency*, no. December 1995, 1996.

Ikesue, H., Vermeulen, L.C., Hoke, R., Kolesar, J.M., 2010. Stability of cetuximab and panitumumab in glass vials and polyvinyl chloride bags. Am. J. Heal. Pharm. 67 (3), 223–226.

Kaur, H., 2021. Stability testing in monoclonal antibodies. Crit. Rev. Biotechnol. 41 (5), 692–714.

Lagarce, F., 2017. Centrally Prepared Cytotoxic Drugs: What Is the Purpose of Their Quality Control? Pharm. Technol. Hosp. Pharm. 2 (1), 29–33.

Lahlou, A., Blanchet, B., Carvalho, M., Paul, M., Astier, A., 2009. Mechanically-induced aggregation of the monoclonal antibody cetuximab. Ann. Pharm. Fr. 67 (5), 340–352.

Le Basle, Y., Chennell, P., Tokhadze, N., Astier, A., Sautou, V., 2020. Physicochemical Stability of Monoclonal Antibodies: A Review. J. Pharm. Sci. 109 (1), 169–190.

Le Guyader, G., Vieillard, V., Mouraud, S., Do, B., Marabelle, A., Paul, M., 2020. Stability of nivolumab in its original vials after opening and handing in normal saline bag for intravenous infusion. Eur. J. Cancer 135, 192–202.

Le Guyader, G., Vieillard, V., Paul, M., 2021. Physicochemical stability study of MYL-1401O, a biosimilar of trastuzumab, following a transient temperature excursion. J. Oncol. Pharm. Pract. 27 (4), 847–856.

Li, W., Prabakaran, P., Chen, W., Zhu, Z., Feng, Y., Dimitrov, D.S., 2016. Antibody aggregation: Insights from sequence and structure. Antibodies 5 (3), 1–23.

Ma, H., Ó'Fágáin, C., O'Kennedy, R., 2020. Antibody stability: A key to performance - Analysis, influences and improvement. Biochimie 177, 213–225.

Montagner, I.M., et al., 2015. Drug conjugation to hyaluronan widens therapeutic indications for ovarian cancer. Oncoscience 2 (4), 373–381.

Nishi, H., Mathäs, R., Fürst, R., Winter, G., 2014. Label-free flow cytometry analysis of subvisible aggregates in liquid IgG1 antibody formulations. J. Pharm. Sci. 103 (1), 90–99.

Park, D., Kim, J., Yun, J., Park, S.J., 2020. Evaluation of the Physico-Chemical and Biological Stability of SB8 (Aybintio), a Proposed Biosimilar to Bevacizumab, Under Ambient and In-Use Conditions. Adv. Ther. 37 (10), 4308–4324.

Paul, M., Vieillard, V., Jaccoulet, E., Astier, A., 2012. Long-term stability of diluted solutions of the monoclonal antibody rituximab. Int. J. Pharm. 436 (1–2), 282–290.

Platts, L., Falconer, R.J., 2015. Controlling protein stability: Mechanisms revealed using formulations of arginine, glycine and guanidinium HCl with three globular proteins. Int. J. Pharm. 486 (1–2), 131–135.

Pozzi, C., et al., 2016. The EGFR-specific antibody cetuximab combined with chemotherapy triggers immunogenic cell death. Nat. Med. 22 (6), 624–631.

Rasmussen, R.W., Botha, J., Prip, F., Sanden, M., Nielsen, M.H., Handberg, A., 2021. Zoom in on antibody aggregates: A potential pitfall in the search of rare ev populations. Biomedicines 9 (2), 1–17.

Rigamonti, N., Sebellin, J., Pipitone, F., Realdon, N., Carpanese, D., Coppola, M., 2023. A Method for Risk Assessment Evaluating the Safety, Stability and Efficacy in Clinical Practice of Anticancer Drug Preparations in the Centralized Compounding Unit of the Veneto Institute of Oncology-IRCCS. Pharmaceutics 15 (5), pp.

Rombach-Riegraf, V., et al., 2014. Aggregation of human recombinant monoclonal antibodies influences the capacity of dendritic cells to stimulate adaptive T-cell responses in vitro. PLoS One 9 (1), pp.

Scaltriti, M., Baselga, J., 2006. The epidermal growth factor receptor pathway: A model for targeted therapy. Clin. Cancer Res. 12 (18), 5268–5272.

Tang, Y., Cain, P., Anguiano, V., Shih, J.J., Chai, Q., Feng, Y., 2021. Impact of IgG subclass on molecular properties of monoclonal antibodies. Mabs 13 (1), pp.

Thiagarajan, G., Semple, A., James, J.K., Cheung, J.K., Shameem, M., 2016. A comparison of biophysical characterization techniques in predicting monoclonal antibody stability. Mabs 8 (6), 1088–1097.

Vigneron, J., D'Huart, E., Demoré, B., 2019. Stability studies in oncology: A marketing tool for pharmaceutical companies, a scientific mission for hospital pharmacists. J. Oncol. Pharm. 2 (2), 1–5.