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## Understanding the Discrimination and Quantification of Monoclonal Antibodies preparations using Raman Spectroscopy

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## **Abstract**

The use of Raman spectroscopy for analytical quality control of anticancer drug preparations in clinical pharmaceutical dispensing units is increasing in popularity, notably supported by commercially available, purpose designed instruments. Although not legislatively compulsory, analytical methods are frequently used post-preparation to verify the accuracy of a preparation in terms of identity and quantity of the drug formulation in solution. However, while the rapid, cost effective and label free analysis achieved with Raman spectroscopy is appealing, it is important to understand the molecular origin of the spectral contributions collected from the solution of actives and excipients, to evaluate the strength and limitation for the technique, which can be used to identify and quantify either the prescribed commercial formulation, and/or the active drug itself, in personalised solutions. In the current study, four commercial formulations, Erbitux®, Truxima®, Ontruzant® and Avastin® of monoclonal antibodies (mAbs), corresponding respectively to cetuximab, rituximab, trastuzumab and bevacizumab have been used to highlight the key role of excipients in discrimination and quantification of the formulations. It is demonstrated that protein based anticancer drugs such as mAbs have a relatively weak Raman response, while excipients such as glycine, trehalose or histidine contribute significantly to the spectra. Multivariate analysis (partial least square regression and partial least square discriminant analysis) further demonstrates that the signatures of the mAbs themselves are not prominent in mathematical models and that those of the excipients are solely responsible for the differentiation of formulation and accurate determination of concentrations. While Raman spectroscopy can successfully validate the conformity of mAbs intravenous infusion solutions, the basis for the analysis should be considered, and special caution should be given to excipient compositions in commercial formulations to ensure reliability and reproducibility of the analysis.

## 1. Introduction:

The analytical quality control (AQC) of anticancer drug solutions represents a significant daily workload for technicians and pharmacists in healthcare establishments. Commercial formulations of drugs are prescribed and may be diluted to a patient specific concentration in aqueous solutions, before delivery to the bedside. To ensure administration of the correct medication to patients, the injectable solutions are frequently analysed prior to being released from the preparation units. The two key parameters are (i) identification of the drug and (ii) validation of the concentration, to prevent either therapeutic failure or more importantly protect patients from adverse effects.

Monoclonal antibodies (mAbs) are biopharmaceutics produced by bioengineering technology; they are used as targeted therapy in cancer treatment and are associated with fewer and less severe side effects compared to other chemotherapeutics [1]. In addition to their valuable role in cancer therapy, they are used in preventing or modulating viral diseases [2], for multiple sclerosis [3] and some autoimmune diseases [4]. As a consequence, approaches for AQC need to be adapted. mAbs are glycoproteins of molecular weight ~150 kDa; their structure consists of two heavy chains, each of which contains 447 amino acids linked by 4 disulphide bonds, and two light chains, each containing 215 amino acids, linked by 2 disulphide bonds [5]. Typically, the different types of mAbs (chimeric, humanised and fully humanised types) [6], only differ in their sequences of amino acids, respectively 240 amino acids for chimeric mAbs and only 65 - 70 amino acids for the humanised mAbs [1]. Reversed phase high performance liquid chromatography (RP-HPLC) coupled to mass spectrometry is the main method for characterisation of proteins and intact mAbs molecules, although the extensive pre-column sample preparation and after-column ionisation steps for mass fragmentation and analysis make the technique costly and time consuming [7]. Furthermore, HPLC with different separation modes, including reversed phase, size exclusion chromatography, weak cation exchange chromatography and strong anion exchange chromatography, are used to identify and quantify mAbs with different precision profiles [8]. Capillary electrophoresis, coupled to electrospray ionisation-mass spectrometry, is used for the comprehensive study of mAbs structures as well as detection of dimer formation, despite the requirement for high separative affinity and mass detection sensitivity which involve cleaving mAbs into different levels of fragments [9]. The main drawback of capillary size electrophoresis is the adsorption of proteins in silica walls, resulting in a loss of precision in determining their migration time [10].

Reversed phase liquid chromatography with diode detectors is used for quantification of mAbs in their intact status, but long term stability of mAbs during the pre-column and column step at high temperature (60 – 90 °C) is not assured [11]. Immunoassay methods such as enzyme linked immunoassay (ELISA) can be used to check the stability of mAbs in infusion bags, as well as bioavailability in biological matrices (plasma, serum) [12].

Although a number of methods are available, their complexity, need for highly trained personnel and time-consuming protocols suggest that, in the context of daily AQC of large cohorts of samples, a simple, direct and rapid quantitative screening tool could greatly reduce the workflow and accelerate safe administration of mAbs to patients.

Vibrational spectroscopy, namely Raman and infrared spectroscopy, are label free and non-destructive methods. They have been demonstrated to be relevant tools to investigate the effect of stress conditions on the secondary and tertiary structure of mAbs aggregates and unfolded species during the manufacturing and formulation [13,14].

Vibrational spectroscopy have also been well described in literature for QC of injectable small molecule chemotherapeutic drugs such as taxanes [15] or anthracyclines [16]. Raman spectroscopy has been implemented for real time AQC of chemotherapeutic preparations in hospitals [17,18]. Recently, commercialised flow injection instrumentation QCPrep+® (Icônes Services, France), combining Raman with UV detection, has been reported for QC of chemotherapeutic preparations into healthcare establishment [19]. Moreover, miniaturisation of instruments and commercialisation of so-called handheld Raman open further perspectives for performing non-invasive measurements directly in plastic infusion bags, consequently reducing potentially hazardous exposure to solutions of staff members [20].

However, reports of application of vibrational spectroscopy for QC of mAbs are few. Bazin *et al.*, [21] reported a study using a Multispec® analyser (Microdom, Taverny, France) which, coupling UV/vis to IR absorption, has been applied for QC of small molecule chemotherapeutic drugs and mAbs (cetuximab, rituximab, trastuzumab and bevacizumab). The true classification, respectively, was 97% and 99.95% for molecules and solvents, but there were difficulties in discriminating structurally related molecules such as anthracyclines (doxorubicin, epirubicin and daunorubicin) and oxazophosphorines (cyclophosphamide and ifosfamide) as well as mAbs. More recently, classification of selected mAbs (bevacizumab, rituximab, infliximab and ramucirumab) in their commercialised form by Raman micro-spectroscopy coupled to advanced machine learning data mining approaches has been documented [22]. Although the results are promising in the context of classification of formulations in terms of their characteristic spectra, the spectral origin of the analysis

outcome is not elucidated, while instrumental interferences or spectral artefacts can bias advanced supervised data mining methods [21,22]. Moreover, the sensitivity of Raman spectroscopy to detect and quantify features originating from the therapeutic antibodies within the complex, multicomponent injectable mixtures is not discussed. This highlights that, presently, the extent of information encompassed in Raman spectra collected from mAbs formulations is still not fully understood or explored in the context of applications to clinical AQC. Misconceptions about the true potential of the spectroscopic technique could lead to its misuse and misinterpretation of results. Therefore, the present study aims to provide further insights into the analysis of commercial mAbs formulations in the context of clinical administration AQC, by analysing 4 commonly administered formulations (Erbix®<sup>®</sup>, Truxima®<sup>®</sup>, Ontruzant®<sup>®</sup> and Avastin®<sup>®</sup>) of anticancer drugs (cetuximab, rituximab, trastuzumab and bevacizumab). The quantification and discrimination will be illustrated and discussed on the basis of the spectral features observed in the context of their respective chemical compositions.

## 2. Materials and Methods:

### 2.1 Monoclonal antibodies and sample preparation

The study has been conducted in collaboration with the Unité de Biopharmacie Clinique Oncologique (UBCO) of the University Hospital of Tours (CHU Tours, France). UBCO manages the daily preparation of systemic therapeutic solutions, and it provided samples of the 4 most frequently clinically prescribed mAbs formulations: ERBITUX®<sup>®</sup>, (Merck - cetuximab 5 gL<sup>-1</sup>), TRUXIMA®<sup>®</sup>, (Healthcare Celltrion - rituximab 10 gL<sup>-1</sup>), ONTRUZANT®<sup>®</sup>, (Samsung Bioepis - trastuzumab 150 mg powder for injection) and AVASTIN®<sup>®</sup> (Roche - bevacizumab 25 gL<sup>-1</sup>). Commercial formulations of mAbs contain several excipients, listed in Table 1. Throughout the paper for clarity, ERBITUX®<sup>®</sup>, TRUXIMA®<sup>®</sup>, ONTRUZANT®<sup>®</sup> and AVASTIN®<sup>®</sup> will refer to the commercial mAbs formulations while the respective antibodies themselves will be designated as CTX (cetuximab), RTX (rituximab), TRS (trastuzumab) and BEV (bevacizumab).

Based on recent administration records of solutions prepared at UBCO, relevant therapeutic ranges were defined. Ultimately 5 different concentrations were prepared for each commercial mAb as follows: AVASTIN®<sup>®</sup>: 2 gL<sup>-1</sup>, 4 gL<sup>-1</sup>, 8 gL<sup>-1</sup>, 12 gL<sup>-1</sup> and 15 gL<sup>-1</sup>; ERBITUX®<sup>®</sup>, TRUXIMA®<sup>®</sup> and ONTRUZANT®<sup>®</sup>: 0.5 gL<sup>-1</sup>, 1 gL<sup>-1</sup>, 2 gL<sup>-1</sup>, 3 gL<sup>-1</sup> and 4 gL<sup>-1</sup>.

The different concentrations were prepared from the stock formulations using serial dilutions in 0.9% normal saline (matrix used at the hospital for injection) and analysed directly with Raman spectroscopy. All samples were prepared freshly on the day of analysis.

Table 1: Excipients list present in the tested mAbs

<b>Brand</b>	<b>mAb</b>	<b>Excipients</b>
ERBITUX®	CTX	Sodium chloride, Glycine, Polysorbate 80, Citric acid monohydrate Sodium hydroxide and Water for injections.
TRUXIMA®	RTX	Sodium chloride, Tri-sodium citrate dihydrate, Polysorbate 80 and Water for injections.
ONTRUZANT®	TRS	L-histidine hydrochloride monohydrate, L-histidine, $\alpha,\alpha$ -trehalose dihydrate, polysorbate 20.
AVASTIN®	BEV	Trehalose dihydrate, Sodium phosphate, Polysorbate 20 and Water for injections.

## 2.2 Ultrafiltration of mAbs

Amicon® Ultra-0.5 mL (Merck, Germany) with cut-off points at 30K (Kilo-Daltons) were used for centrifugal dialysis of mAbs solutions. For the purpose of the study, 0.5 mL of ERBITUX® 4 g·L<sup>-1</sup>, TRUXIMA® 4 g·L<sup>-1</sup>, ONTRUZANT® 4 g·L<sup>-1</sup> and AVASTIN® 4 g·L<sup>-1</sup> were subjected to ultrafiltration. Filters were placed in a fixed angle centrifuge (Sigma Laboratory centrifuge 3-30 K) and spun at 14000 g at 4 °C for 10 minutes. At the end of a spinning cycle, solutions were separated in 2 fractions: the concentrate (retained in the filter) and the filtrate (passed through the membrane). Having high molecular weight, mAbs were retained in the filter, while excipients were found in the filtrate. Filters containing concentrated mAbs were topped up with 0.5 mL Milli Q water and subjected to another spinning cycle. To ensure efficient dialysis, 10 cycles were applied to each mAb solution. For Raman measurement, the washing procedure is important to avoid molecular contamination with glycerine residue on the membrane of filters [23]. Therefore, prior to filtering mAbs, 3 cycles of washings with 0.1 M NaOH solution followed by 3 cycles of rinsing with Milli Q water were undertaken (10 mins, 14,000g).

## 2.3 Raman spectroscopic analysis

Raman spectra were collected using a Labram spectrometer (Horiba Jobin-Yvon, France) equipped with a 690 nm laser source delivering ~10 mW at the sample. Spectra were collected over the 150 and 3750 cm<sup>-1</sup> range with a 300 lines/mm grating, resulting in spectral

resolution of  $\sim 3 \text{ cm}^{-1}$ . 2 instrumentals set up were used. 1) *Macro set up*: A macro sampling cuvette holder (Horiba Jobin-Yvon, France) attached to the turret of the microscope has been used for recording using 500  $\mu\text{L}$  in quartz cuvette, thereby recording from a large sample volume and ensuring very high reproducibility in recording, without the need to refocus the laser each time, as well as reduced risk of photothermal damage to the sample by the laser. From each prepared concentration; 15 spectra have been recorded using 2 accumulations of 20 seconds, giving a total of 75 spectra recorded for each mAb in 0.9% NaCl. 2) *Microscope set up*: A x100 objective (Olympus, NA = 0.9) has been used to record Raman spectra from air dried drops on a  $\text{CaF}_2$  slide before and after the centrifugation cycle. The laser spot size was roughly  $1 \mu\text{m}$ . Spectra resulted from 2 accumulations of 60 seconds, recorded from 5 different sites within the drop.

#### 2.4 Data handling:

Raman data sets have been pre-processed and analysed using MATLAB (Mathworks, USA).

##### a) Quantitative analysis:

Firstly, data were EMSC corrected (Extended Multiplicative Scattering Correction) in the range  $300 - 3750 \text{ cm}^{-1}$  [24]. Secondly, Partial Least Squares Regression (PLSR) analysis has been applied to the fingerprint region of corrected spectra ( $600 - 1800 \text{ cm}^{-1}$ ). A 100-fold Leave K Out Cross Validation (LKOCV) has been applied, in which 2/3 of the data set were selected randomly for calibration and the remaining 1/3 for validation. The process was run automatically in a random way, with no repetition of data in each set. The output from PLSR was evaluated using the linearity of the regression model ( $R^2$ ), the Root Mean Square Error of Cross Validation (RMSECV) as well as the regression coefficients, highlighting the variables (spectral features) used to construct the regression models.

##### b) Discriminant analysis:

Partial Least Squares Discriminant Analysis (PLSDA) is a well-established supervised classification method and was employed in this study to demonstrate the discriminative potential of the analytical techniques. Prior to analysis, data were subjected to pre-processing with EMSC in the range  $300 - 3750 \text{ cm}^{-1}$  followed by baseline correction (Lieber correction) and vector normalisation in order to remove the background interference. To perform the analysis, Raman spectra were cut to the fingerprint region ( $600 - 1800 \text{ cm}^{-1}$ ), in which the most relevant spectral features are observed. 2/3 of the data was used for calibration and the



remaining 1/3 for classification. The output of PLSDA is presented under confusion matrix allowing calculation of specificity and sensitivity of the discrimination [25].

### 3. Results and Discussion:

#### 3.1 Spectral characterisation of commercial mAbs solutions studied

##### a) Air dried stock formulations

To elucidate the spectral features and aid interpretation of the quantitative analysis, the following section uses Raman spectra collected from air dried drops of stock commercial mAbs formulations, the spectra of which are presented in [Figure 1](#). mAbs are proteins and therefore the main features are observed at  $\sim 640\text{ cm}^{-1}$  (tyrosine),  $755\text{ cm}^{-1}$  (tryptophan),  $954\text{ cm}^{-1}$  (C-H stretching of  $\alpha$ -helix structure),  $1001\text{ cm}^{-1}$  (phenylalanine),  $1237\text{ cm}^{-1}$  (amide III),  $1335\text{ cm}^{-1}$  and  $1445\text{ cm}^{-1}$  ( $\text{CH}_2$  deformation),  $1551\text{ cm}^{-1}$  (amide II) and  $1668\text{ cm}^{-1}$  (amide I) [26,27]. Notably, however, while Raman spectra can reflect modifications in chemical compositions of samples, it is not expected that modifications in small sequences in amino acids compared to the overall size of mAbs can lead to the large spectral variations observed in [Figure 1](#) (highlighted regions). [Table 1](#) provides a list of excipients found in commercialised mAbs solutions that can possibly contribute significantly to the spectra collected. Excipients are, however, relatively small molecular entities compared to antibodies ( $\approx 150\text{ kD}$ ), and thus they can be separated for independent spectral characterisation using ultrafiltration.

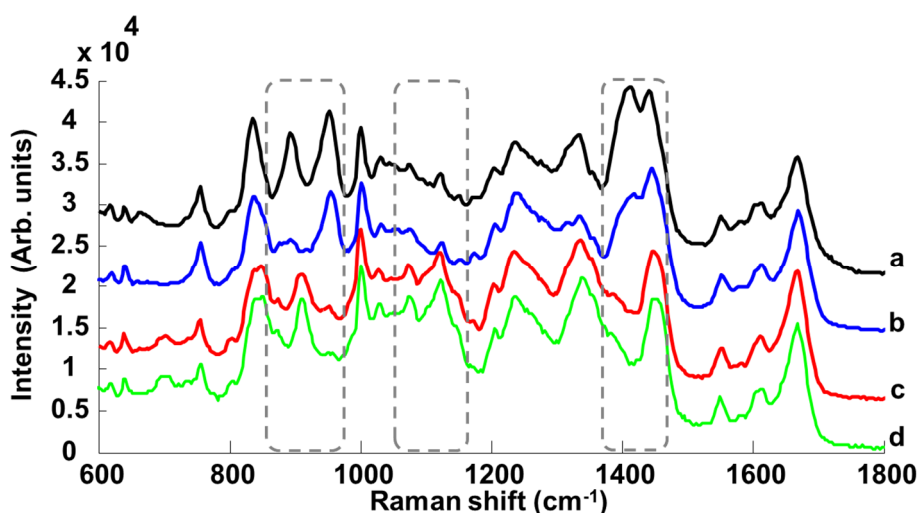


Figure 1: Mean Raman spectra recorded from air dried drops of the commercial stock solutions: ERBITUX® 5 gL<sup>-1</sup> (a), TRUXIMA® 10 gL<sup>-1</sup> (b), ONTRUZANT® 21 gL<sup>-1</sup> (c) and AVASTIN® 25 gL<sup>-1</sup> (d). Spectra are offset for clarity.

#### b) Air dried filtrates: excipients

Although the excipients list is provided for each product and they can be purchased individually, the concentration of each one of these ingredients in the formula is not disclosed. Therefore, for the purpose of this study, it was deemed preferable to consider the fraction of product corresponding to excipients as a whole to clearly differentiate the spectral contributions of mAbs and the combination of all other ingredients. Using a centrifugal filter with a 30K (Kilo-Daltons) cut-off, the excipients can be separated from the mAbs. The filtrate, fraction passing through the membrane, can be analysed following air drying. Figure 2 presents the spectra of the filtrates collected from the 4 mAbs commercial formulations. Clearly, the fractions collected significantly differ between mAbs.

The spectrum collected from ERBITUX® filtrate (Figure 2a) is dominated by glycine [28]. Characteristic bands of the amino acid can be observed at 895 cm<sup>-1</sup> (COOH deformation), 1330 cm<sup>-1</sup> (CH<sub>2</sub> wagging), 1411 cm<sup>-1</sup> (CH<sub>2</sub> and CH<sub>3</sub> stretching) and 1444 cm<sup>-1</sup> (CH<sub>2</sub> deformation).

For TRUXIMA® (Figure 2b), strong features can be observed at 838 cm<sup>-1</sup> (CH<sub>2</sub> rocking), 957 cm<sup>-1</sup> (CH<sub>2</sub> rocking) and 1417 cm<sup>-1</sup> (C-H bending) correspond to specific features of polysorbate 80 [29].

ONTRUZANT® and AVASTIN® (Figure 2c and 2d) both exhibit strong contributions from trehalose and histidine [30]. The features observed at 841 cm<sup>-1</sup> (C-O-C skeletal structure and C-C stretching), 911 cm<sup>-1</sup> and 1122 cm<sup>-1</sup> C-C stretching of saccharides (trehalose), while band

at  $1076\text{ cm}^{-1}$  (=C-N deformation),  $1349\text{ cm}^{-1}$  (ring (C-N) deformation) and  $1457\text{ cm}^{-1}$  (N-H bending) are specific peaks of histidine added to mAbs formulations [31].

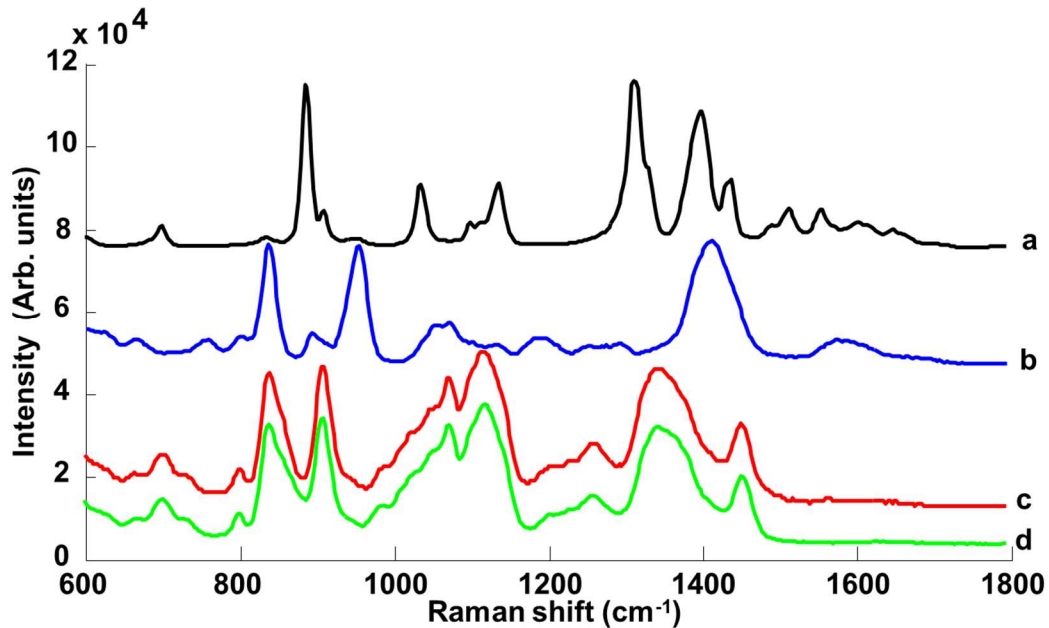


Figure 2: Raman spectra from filtrates ERBITUX® (a), TRUXIMA® (b), ONTRUZANT® (c) and AVASTIN® (d). Spectra are offset for clarity.

c) Air dried concentrates: mAbs

An advantage of performing dialysis of excipients using centrifugal ultrafiltration is the isolation of the mAbs, hence enabling unambiguous identification of its characteristic Raman spectroscopic signature. Unlike the filtrates, the spectra of the concentrates of the ultrafiltration process, the pure mAbs, exhibit a much higher degree of similarity (Figure 3). It is therefore clearly demonstrated that the excipients strongly contribute to the spectral signatures collected from stock formulations.

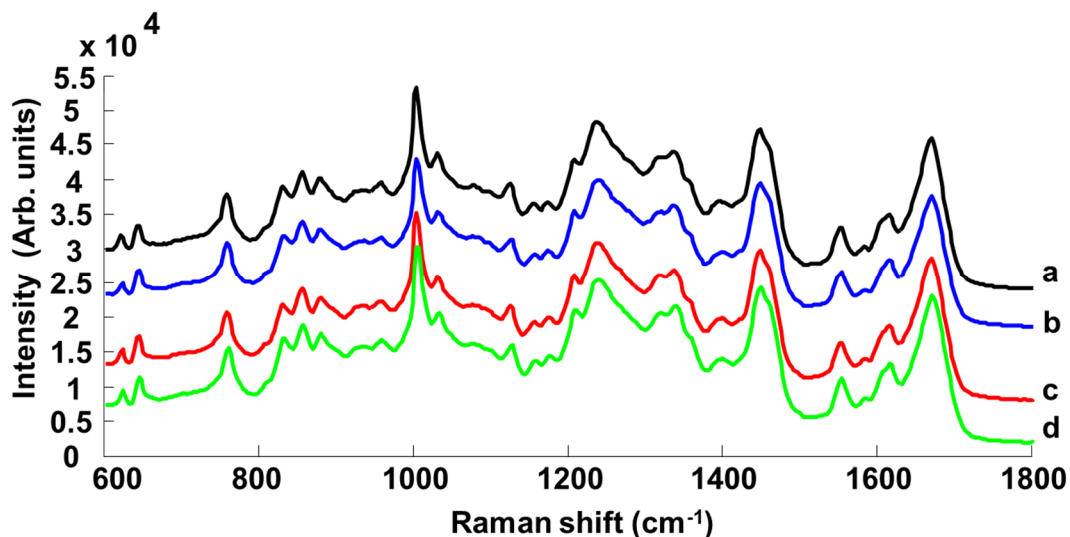


Figure 3: Raman spectra concentrates after cycle 10 of ultrafiltration ERBITUX® (a), TRUXIMA® (b), ONTRUZANT® (c) and AVASTIN® (d). Spectra are offset for clarity.

### 3.2 Quantitative analysis by Raman spectroscopy:

Quantitative analysis is better conducted in the native aqueous environment. Notably, however, although water is commonly described as a weak scatterer, enabling Raman analysis of aqueous liquid samples [16], within the range of concentrations, the relatively intense OH bending vibration at  $1638\text{ cm}^{-1}$  compared to the mAbs signal results in a strong underlying background (Figure 4).

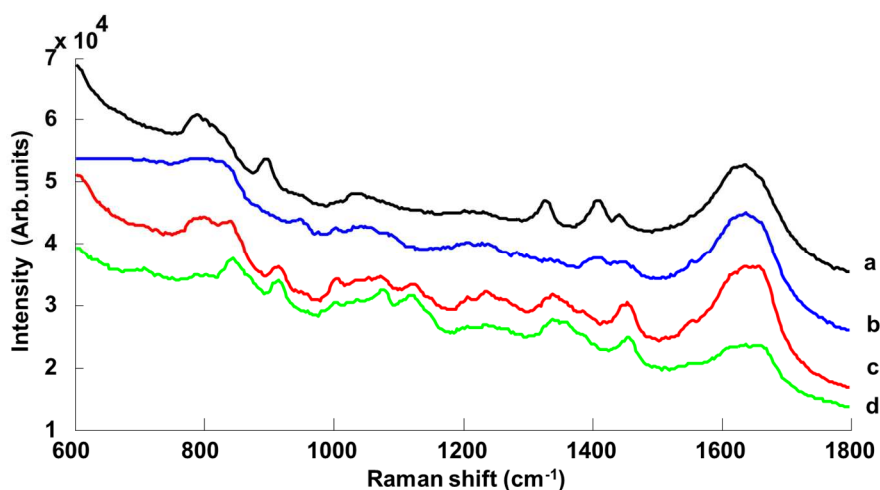


Figure 4: Raman spectra recorded from commercial stock solutions: a) ERBITUX®  $5\text{ gL}^{-1}$ , b) TRUXIMA®  $10\text{ gL}^{-1}$ , c) ONTRUZANT®  $21\text{ gL}^{-1}$  and d) AVASTIN®  $25\text{ gL}^{-1}$ . Spectra are offset for clarity.

Figure 5 presents an example of PLSR analysis using ERBITUX® in 0.9% NaCl solutions. It can be observed in Figure 5a that the RMSECV decreases as a function of the number of latent variables (LVs) included in the regression models. Errors bars represent the standard deviation and they suggest that, above 5 LVs, no significant improvement is achieved. Moreover, overfitting of data should be avoided by limiting as much as possible the number of LVs, thus results presented correspond to  $n = 5$ . Figure 5b presents the regression plot with an  $R^2$  value equal to 0.9996 and RMSECV of  $0.0253 \text{ g}\cdot\text{L}^{-1}$ . These criteria support the quality of the fitting achieved and the performance of Raman spectroscopy to deliver quantitative analysis for ERBITUX®.

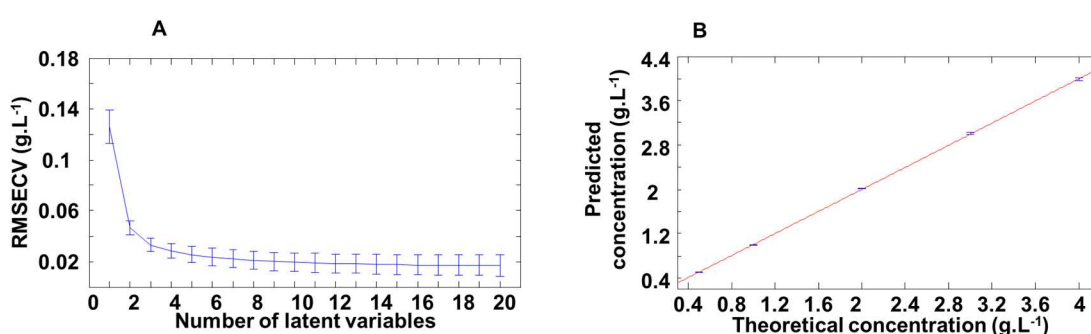


Figure 5: PLSR analysis performed on Raman spectra collected from ERBITUX® in 0.9% NaCl. A) RMSECV and B) Regression plot with 5 LVs.

Table 2 summarises the PLSR results obtained for the 4 mAbs formulations studied.  $R^2$  is consistently greater than 0.99, indicating acceptable regression can be achieved for all. AVASTIN® displays, however, a significantly higher RMSECV compared to other mAbs, with a value of  $0.3599 \text{ g}\cdot\text{L}^{-1}$ . This can be explained by the clinical concentration range extending from  $2 \text{ g}\cdot\text{L}^{-1}$  to  $15 \text{ g}\cdot\text{L}^{-1}$ , while for others the highest concentration does not exceed  $4 \text{ g}\cdot\text{L}^{-1}$ . For the purpose of comparison, the RMSECV can be expressed as a percentage of the mean concentration of the range tested, in which case, a value of 4.4% is found for AVASTIN®, while for ERBITUX®, TRUXIMA® and ONTRUZANT®, respective values of 1.2%, 2.4% and 4% are obtained. Thus, depending on the mAb formulation considered, the outcome of the analysis differs, but PLSR of AVASTIN® and ONTRUZANT® deliver a similar outcome once the range used is considered. While dialysis of excipients by means of ultrafiltration enables separation and collection of the mAbs from commercial products, the process also modifies the concentrations of the constituent fractions. The mAbs fraction is retained, but significantly concentrated within the filter of the centrifugal device, and

therefore its concentration does not reflect the composition of the stock solution. As a consequence, no attempt to perform PLSR on the concentrate has been performed, due to poor correlation with the concentration in the commercial product.

Table 2: PLSR results obtained for the 4 mAbs.

<b>Brand</b>	<b>mAb</b>	<b><math>R^2</math></b>	<b>RMSECV (<math>\text{g}\cdot\text{L}^{-1}</math>)</b>	<b>LVs</b>
ERBITUX®	CTX	0.9995	0.0275	5
TRUXIMA®	RTX	0.9984	0.0516	5
ONTRUZANT®	TRS	0.9951	0.0843	5
AVASTIN®	BEV	0.9950	0.3599	6

Regression coefficients in Figure 6 provide information about the spectral features used during PLSR. For ERBITUX® (Figure 6a), dominant peaks at  $895\text{ cm}^{-1}$ ,  $1330\text{ cm}^{-1}$ ,  $1411\text{ cm}^{-1}$  and  $1444\text{ cm}^{-1}$  correspond to features previously observed in the filtrate and are thus associated with the formulation excipients (Figure 2). Similarly, for AVASTIN® (Figure 6d) the main bands are found at  $846\text{ cm}^{-1}$ ,  $911\text{ cm}^{-1}$ ,  $1001\text{ cm}^{-1}$ ,  $1076\text{ cm}^{-1}$ ,  $1117\text{ cm}^{-1}$ ,  $1341\text{ cm}^{-1}$  and  $1457\text{ cm}^{-1}$ , suggesting contributions predominantly from excipients. Although a small feature is observable at  $1668\text{ cm}^{-1}$  that can be attributed to the amide I band of proteins, the model remains dominated by other constituents of the formulation. For TRUXIMA® (Figure 6b) and ONTRUZANT® (Figure 6c), the regression coefficients exhibit lower signal to noise ratio. The spectrum collected from the stock solution of TRUXIMA®  $10\text{ g}\cdot\text{L}^{-1}$  (Figure 4b) already displays weak features while the range tested is between  $0.5\text{ g}\cdot\text{L}^{-1}$  and  $4\text{ g}\cdot\text{L}^{-1}$ . While analysis of the filtrate for ONTRUZANT® and AVASTIN® demonstrated that trehalose was the main excipient contributing to the spectra, the 2 mAbs are not prescribed in the same range of concentrations. For instance, AVASTIN® was tested between  $2\text{ g}\cdot\text{L}^{-1}$  and  $15\text{ g}\cdot\text{L}^{-1}$  compared to  $0.5\text{ g}\cdot\text{L}^{-1}$  to  $4\text{ g}\cdot\text{L}^{-1}$  for ONTRUZANT®. As a result, all constituents are more diluted in solution. Although all PLSR deliver relatively good RMSECV, it appears the analysis strongly depends on the ability to detect spectral variations not specifically attributed to the active mAbs.

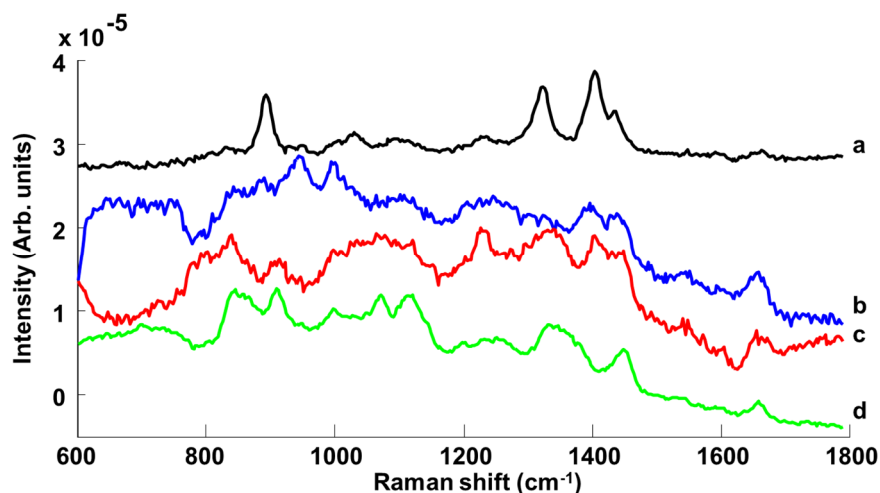


Figure 6: Regression coefficients of the PLSR. a) ERBITUX®, b) TRUXIMA®, c) ONTRUZANT® and d) AVASTIN®. Spectra are offset for clarity

### 3.3 Discriminant analysis using PLSDA

PLSDA has been used to discriminate between 0.9% NaCl mAbs solutions prepared within the clinical range using the 4 mAbs commercial formulations (Table 3). Specificity and sensitivity have been calculated from the confusion matrix using 6 LVs. All mAbs delivered 100% sensitivity and specificity, except for solutions prepared from TRUXIMA®, for which the values were determined to be 98.9% and 96.7%, respectively. Misclassification observed between mAbs solutions has been attributed to their weak Raman signal (Figure 3) For instance, misclassifications observed are TRUXIMA® solutions recognised as ONTRUZANT® due to some shared bands between them. One can see that the spectral signature for the stock solution of TRUXIMA® at 10 g·L<sup>-1</sup> presents broad and weak bands (Figure 4). Therefore, at lower concentrations, it can be expected that the specificity of the analysis is lost, leading to false identifications.

Table 3: Specificity and sensitivity % of PLSDA of Raman using 6 LVs, data pre-processed with EMSC, Lieber correction and vector normalisation.

Brand	mAb	Specificity %	Sensitivity %
ERBITUX®	CTX	100	100
TRUXIMA®	RTX	96.7	98.9
ONTRUZANT®	TRS	100	100
AVASTIN®	BEV	100	100

Figure 7a and 7b displays first and second regression coefficients from PLSDA. Regression coefficient enables to highlight the wavenumbers, hence the chemical species, used for the discrimination of the 4 mAbs solutions (Table 3). It is clearly demonstrated that there is a strong correspondence between the regression coefficient and the spectral features observed in filtrates. Negative features of the first regression coefficient (Figure 7a) at  $841\text{ cm}^{-1}$ ,  $911\text{ cm}^{-1}$ ,  $1076\text{ cm}^{-1}$ ,  $1120\text{ cm}^{-1}$ ,  $1349\text{ cm}^{-1}$  and  $1454\text{ cm}^{-1}$  match the main spectral features found in spectra collected from filtrates of AVASTIN® (Figure 2d) and ONTRUZANT® (Figure 2c). Bands at  $895\text{ cm}^{-1}$ ,  $1330\text{ cm}^{-1}$  and  $1411\text{ cm}^{-1}$  correspond, however, to peaks found in ERBITUX® (Figure 2a). Unambiguously, analysis of the PLSDA regression coefficients demonstrates that the identification of mAbs solutions cannot be achieved based on the spectral signatures of the antibodies themselves but rather on the spectral signatures of the unique combination of excipients of the commercial mAbs.

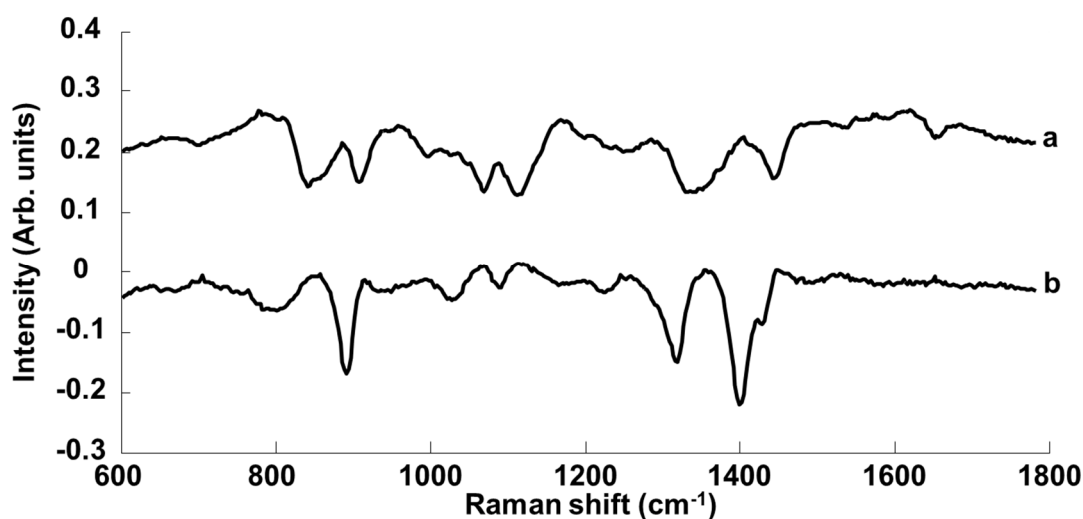


Figure 7: First (a) and second (b) regression coefficients of the PLSDA. Spectra are offset for clarity

### 3.4 Perspectives and caution

Raman spectroscopy holds numerous promises for applications in the clinical environment. The technology is evolving at great pace, incorporating automation, miniaturisation and hyphenated instruments such as UV coupled to Raman spectroscopy. In addition, chemometrics helps to push the limits of the technique [32], facilitating implementation of translational research. In principle, Raman spectroscopy is a powerful tool delivering a specific molecular fingerprint of a sample for characterisation or even quantification of



compounds. However, one should bear in mind that all molecules do not have equal Raman response. It has been well documented that most of chemotherapeutic agents such as small molecules like doxorubicin, daunorubicin and epirubicin exhibit strong and sharp features within the clinical range [16]. Numerous drugs have conjugated rings in their chemical structures which are particularly active in Raman spectroscopy. Therefore, their detection, identification and quantification can even be easily achieved [33]. Furthermore, the strength of the signal for those drugs have open encouraging perspective for direct analysis into containers (infusion bags, syringe) [15,20]. In contrast, mAbs do not deliver particularly intense Raman signals in solutions, especially diluted. As a consequence, other constituents of the formulation can dominate the spectra collected.

In the analysis of the aqueous formulations, firstly, it can be observed that, although the water is a relatively weak scatterer, as the primary constituent of the formulation, its contribution to the spectra collected is quite noticeable (Figure 4), further highlighting the limited Raman response from proteins bands. Secondly, other compounds used as excipients can also be observed. Unambiguously, when it comes to quantification and discrimination, excipients play a major role in the analysis outcome.

There is no specific legislation governing analytical quality control of chemotherapeutic solutions. However, for drugs with narrow therapeutic windows, centralized units for preparation of chemotherapy inside hospital have established systematic prevention strategies such as double visual check coupled to an additional method to detect preparation errors. Gravimetric [34] or video assisted control protocols are currently employed in process production methods [35]. In addition, numerous units also implement analytical methods to control and validate the final product [35]. There is, however, no general consensus on optimum protocols, and techniques currently used include conventional high-performance liquid chromatography, thin-layer chromatographic, high-performance liquid chromatography, flow injection analysis, but also increasingly more popular and accepted spectroscopy techniques (near infrared, mid-infrared and Raman spectroscopy). While separation techniques have the advantage of specificity, spectroscopy approaches remain attractive for low cost and speed of analysis. There are no international guidelines which specifically address ACQ of personalised chemotherapeutic solutions. For instance, the French Good Preparation Practices controls of chemotherapeutics solutions, established by the ANSM (National Agency for Drug Safety and Products for Health), are part of good practice to guarantee that necessary and appropriate analyses have been applied to ensure the quality of raw material, product packaging and therapeutic preparations before release. In the

context of AQC, results of analysis of anticancer drugs in solutions are acceptable, as long as the method employed is experimentally validated and appropriate to ensure the conformity of the solutions prepared for administration to the prescribed therapy. Clearly, for the case of intravenous solutions prepared from commercial formulations of mAbs, the excipients are the majority components and dominate the Raman spectra of the solutions. This observation raises a number of concerns, in the absence of clear guidelines or legislation available for this particular situation. On one hand, the sensitivity of Raman analysis to identify intravenous solutions based on their specific spectral signatures can be used to verify the current commercial mAbs formulations and the concentrations used in the preparation of the solution. Raman has indeed a strong potential for AQC for rapid control of solutions for release. On the other hand, the discrimination and quantification of solutions are based on the excipients not the spectral contribution from mAbs themselves. Experimentally, some of the ingredients found in commercial products act as internal standard, enabling indirect analysis of mAbs in solutions. While from the analytical point of view this is not an aberrant application of the technique, there is nevertheless a higher risk of errors in the identification process. The approach relies only on the specificity of the composition of excipients in commercial products. However, it can be seen in Table 3 that polysorbate 80, polysorbate 20 or trehalose are found in multiple mAbs formulations. Raman analysis is presently based on signal originating from a mixture of molecules rather than a single ingredient specifically attributed to a specific mAb. Although the number of commercially available mAbs is currently increasing, the selection of excipients used by the pharmaceutical industry is well established and tends to remain consistent. As a result, there is a high degree of duplication of ingredients found in mAbs formulations which could significantly compromise the robustness of the models established for AQC with Raman spectroscopy. If two or more commercial products have the same, or similar, excipient compositions, they will display Raman signatures with a high degree of similarity of feature positions and intensities, which could compromise the identification of mAbs in solutions. Indeed, the study included only four examples of mAbs used in clinics and further investigation is required to cover a broader range of commercial products and fully address the issue of the role of excipients in the Raman based AQC protocols.

## **5. Conclusion**

Raman spectroscopy is a powerful tool for AQC. It presents a number of advantages, such as label free, rapid and cost-effective molecular characterisation of samples. There is no doubt

that the technique can address needs for clinical or industrial applications, for instance for therapeutic solutions analysis. It is, however, crucial to recognise that Raman spectroscopy delivers a global analysis of a sample. On one hand, quantitative analysis can be performed with a high degree of accuracy and discrimination of solutions can be achieved with a high classification rate. On the other hand, all the outcomes of the multivariate analysis are based on excipients not the mAbs itself. It is crucial to raise awareness among the clinical community that the origin of the signature collected needs to be understood to avoid misuse of the technique. Presently, it is clearly demonstrated that, in the case of mAbs, the response to Raman spectroscopy for proteins in solutions of clinically relevant concentrations is limited, and therefore other compounds dominate the analysis. It is crucial to proceed carefully as all models presently validated on instruments used in research or dedicated to QC of anticancer solutions can become obsolete if the formula is modified by manufacturers. Indeed, the present study remains a preliminary demonstration and further investigation including additional mAbs and more importantly formula sharing excipients in their composition could bring further insight in the real potential of Raman spectroscopy for this specific application.

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