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Simultaneous determination of polysorbate 20 and unbound polyethylene-glycol in protein solutions using new core–shell reversed phase column and condensation nucleation light scattering detection

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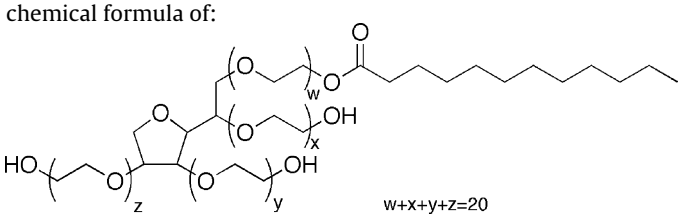
ABSTRACT

A novel fast and sensitive method has been developed for the specific simultaneous determination of polysorbate 20 (Tween 20) and unbound polyethylene-glycol (PEG) from liquid formulations in the presence of proteins and excipients. The quantitative determination is based on a fast liquid chromatographic (HPLC) separation and condensation nucleation light scattering detection (CNLSD or NQAD™). The method uses a Kinetex core–shell column (100 mm × 3 mm, 2.6 μm) and methanol–water–trifluoroacetic acid mobile phase. The rapid HPLC-CNLSD method presented here is suitable for quantifying polysorbate 20 in the range of 10–60 μg/ml and unbound PEG in the range of 2–40 μg/ml in protein solutions within good manufacturing practices (GMP) of the pharmaceutical industry.

1. Introduction

In liquid pharmaceutical formulations, surfactants are added to minimize protein absorption to surfaces (containers and syringes) and to reduce the air–liquid, solid–liquid interfacial surface tension in order to decrease the rate of protein denaturation that can lead to aggregation [1–3]. Proteins at the interface can unfold further, exposing more hydrophobic surface in order to enhance amphiphilicity. Surfactants fall into several categories based on their molecular charge. Nonionic surfactants include polyoxyethylene sorbitans, polyoxyethylene ethers and polyethylene-polypropylene glycols. They have heterogeneous molecular composition and non-chromophoric characteristic.

Polysorbate 20 (PS-20, polyoxyethylenesorbitan monolaurate) and polysorbate 80 (PS-80, polyoxyethylenesorbitan monooleate) are the most common polysorbates currently used in the formulation of protein biopharmaceuticals. Both types of polysorbates have a common backbone and only differ in the structures of the fatty acid side-chains. In solution the polysorbates occur as either monomers or in micelles depending on a number of factors including the polysorbate concentration, buffer composition, and temperature of the solution [4,5]. Polysorbate 20 (Tween 20) has a chemical formula of:



The number of possible molecular structures is greater than 1500, the average molar mass is approximately 1230 g/mol.

Regulatory agencies are increasingly asking for methods to quantify the amount of surfactants and other ingredients in the final product. Commonly used quantitative methods for polysorbates are quite time consuming (derivatization) and use hazardous solvents [6–9]. Tani et al. applied a method using size exclusion chromatography to determine polysorbates [10]. A kinetic spectrophotometic method for the determination of Tween 80 based on its interaction with 5(*p*-dimethylaminobenzylidene)rhodanine (PDR) in alkaline media was reported by Pourezza [11]. A fast and sensitive method for the specific determination of polysorbate 80 from liquid formulations in the presence of proteins and excipients based on charged aerosol detection (CAD) was reported in our previous study [12]. A HPLC assay with simple sample preparation for the measurement of polysorbate 20 was developed by Hewitt et

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al. [13]. The HPLC method was employed involving a mixed-mode stationary phase to quantify polysorbate 20 in protein solutions. Polysorbate 20 is retained, eluted with a step gradient and quantified as a single peak using an evaporative light-scattering detector.

PEGylation is a process in which one or more units of chemically activated polyethylene-glycol reacts with a biomolecule usually a protein or peptide, creating a putative new molecular entity possessing physicochemical and physiological characteristics that are distinct of its predecessor molecules. PEGylation is used as a drug modification technology to transform existing biopharmaceuticals to clinically more efficacious form. This process yield useful properties on the native molecule, resulting in improved pharmacokinetic and pharmacodynamic properties [14]. Most PEGs include molecules with a distribution of molecular weights, they are polydisperse. PEG has a chemical formula of:

$$HO–CH_2–(CH_2–O–CH_2–)_n–CH_2–OH$$

Determination of unbound or free PEG in final bio-products is required by authorities. Determination of trace PEG beside a large amount of protein and other excipients is quite challenging since PEGs have no UV activity. Typically, an evaporative light-scattering detector (ELSD) [15–17] is used to detect nonvolatile and UV-undetectable samples. Some investigations have been made on the concentration dependence of the ELSD [18–20]. Koropchak et al. [21] compared the analytical characteristics of commercial ELSD to those of an in-house constructed CNLSD and a commercial condensation particle counter for the measurement of PEG and polyethylene oxide with molecular masses ranging from 1000 to 45,000. The limits of detection for CNLSD were greatly improved compared to ELSD. Takahashi et al. applied CNLSD detection for supercritical fluid chromatography to detect synthetic polymers [22].

Condensation nucleation light-scattering detector is a new aerosol-based detector for HPLC separations. CNLSD uses condensation nucleation technology. The principle of the technique is based on nebulization and evaporation of the mobile phase at elevated temperature (35–100 °C) and consequent analyte condensation with supersaturated auxiliary water vapor. This leads to formation of relatively large droplets being detected by using scattered light with a laser photodetector system set-up at perpendicular arrangement [23,24]. Subsequently CNLSD detects the change in the aerosol size by measuring the increase in the number of particles counted using a Water-based Condensation Particle Counter (WCPC). The WCPC condenses water vapor onto particles and grows them to a size that can be detected individually using an optical sensor. The WCPC detector in the CNLSD will only condense vapor onto particles that are above a certain size, particles below this size are not counted. The increase in particle size tremendously increases the light-scattering signal and dramatically increases the sensitivity in comparison to ELSD [25]. Only particles above a critical size can act as condensation nucleation sites that increase the signal–noise ratio due to discrimination of small droplets from the mobile phase. The number of particles counted by the WCPC detector is then converted to an analog output signal.

The speed of chromatographic separation can be increased with different approaches. The concept of core–shell stationary phases, was introduced by Horvath and coworkers [26,27]. Horvath applied 50 μm glass bead particles covered with styrene-divinylbenzene based ion exchange resin and became known as “pellicular” packing material. Later Kirkland presented, that 30–40 μm diameter core–shell packings (1 μm phase thickness, 100 Å pores) provided much faster separations, compared with the large porous particles used earlier in liquid chromatography [28]. Later on the core diameter was reduced and the thickness of active layer was cut to 0.5 μm and was used for fast separation of peptides and proteins [29]. Core–shell packing materials are commercially available in different diameters (2.7 and 5 μm). The 2.7 μm particles consist of a 1.7 μm nonporous core and a 0.5 μm porous silica layer, and the 5 μm particles consist of a 4.5 μm nonporous core and a 0.25 μm porous silica layer. Studies have proven [30] that the peak broadening is larger than it can be expected on the basis of the shorter diffusion path. It can be explained by the rough surface of particles in which the mass transfer rate is reduced through the outer stagnant liquid [31]. The latest core–shell stationary phase was released in the year of 2009. This core–shell technology performs particles, which consist of a 1.9 μm nonporous core and a 0.35 μm porous silica layer. This new technology is using sol–gel processing techniques that incorporate nano-structuring technology; a durable, homogeneous porous shell is grown on a solid silica core. Recent studies explain that this new core–shell (Kinetex™) stationary phase performs very efficient separations both for small and large molecules [32–36].

The aim of this study was to demonstrate the applicability of the new Kinetex™ column for fast and efficient separation of large and small molecules (protein, PEG and polysorbate). A simple LC/CNLSD method was developed and validated for polysorbate 20 and PEG determination in bio-formulation. The concept of coupling fast LC and CNLSD can be applied in routine analysis for various protein formulations. The selectivity of RP-LC based separation can be easily adjusted by varying the mobile phase composition (gradient program, organic modifier) and by changing the separation temperature. This present study shows an example of the direct fast determination of polysorbate 20 and unbound PEG from an injection solution containing a PEGylated protein, which belongs to the family of cytokines.

2. Experimental

2.1. Solvents and reference material

Acetonitrile, methanol (Gradient grade), trifluoroacetic acid (Uvasol), hydrogen peroxide 30% (Pro analysi) and hydrochloric acid 1N (Titripur), were purchased from Merck (Darmstadt, Germany). For measurements water was prepared freshly using a Milli-Q® equipment (Milli-Q gradient A10 by Millipore). The reference material as polysorbate 20 (preservative free, low peroxide, low carbonyls), L-methionine (Reagent grade) and 2,5-dihydroxybenzoic acid (Puriss, matrix substance for MALDI MS) were purchused from Sigma–Aldrich Ltd, Budapest. Dithiothreitol (PlusOne DTT) was obtained from BioRad Ltd., Budapest. The reference methoxypoly(ethylene-glycol) (Sunbright™) was purchased from NOF Corporation, Japan.

2.2. Equipment, column

Throughout the separations a Shimadzu LC-20 UFLC (Ultra Fast Liquid Chromatographic) system with Class VP software from Simkon Ltd. Budapest, Hungary, was employed. The system was equipped with a photodiode array detector in line with a CNLSD detector (Nano Quant Analyte Detector, QT-500, from Lab-Comp Ltd., Budapest). A CoreShell Kinetex C18 column (100 mm × 3 mm, 2.6 μm) column was used for the separation (Gen-Lab Ltd., Budapest).

The molar mass distribution of polysorbate 20 and PEG was measured with a Shimadzu Biotech Axima Confidence MALDI-TOF mass spectrometer (MS) using a Shimadzu AccuSpot NSM1, Nano Scale Spotter for LC-MALDI-TOF MS (Simkon Ltd., Budapest, Hungary).

3. Results and discussion

3.1. Optimization of the chromatographic conditions

For method development, the mobile phases were prepared by mixing different ratio of HPLC gradient grade acetonitrile, methanol, Milli-Q water and trifluoroacetic acid. The mixtures were degassed by sonication for 5 min. The composition of organic phase was varied between 100% acetonitrile and 100% methanol in 20% steps.

The stock solutions of reference standards (polysorbate 20 and PEG) were dissolved in water (1000 μg/ml), then it was diluted to 500 μg/ml for scouting gradient runs and 40 μg/ml as reference test solutions for polysorbate 20 and PEG content determination. Protein solutions containing 40 μg/ml polysorbate 20 and 40 μg/ml PEG was also applied for method development to find the suitable chromatographic conditions for a specific separation of polysorbate, PEG and protein origin peaks. The protein solution was injected directly without dilution. The protein in this example was a PEGylated-cytokine, which contains native disulfide bonds.

Linear gradients with 8, 16 and 32 min gradient span (from 35% B to 100% B) at different column temperatures (20, 40 and 60 °C) with different flow rates (0.40, 0.55, and 0.70 ml/min) were run for systematic method development. Gradient program, column temperature, mobile phase composition, injection volume, flow rate and detector (NQAD) parameters (such as evaporation temperature, gain, filter) were optimized applying a fractional factorial experimental design (Statistica 9 software was used to find the optimal condition).

Injecting 500 μg/ml polysorbate 20 showed that reverse phase separation and NQAD detection could distinguish many components of polysorbates (Fig. 1A). Two major peaks of polysorbate 20 can be detected and can be the target compounds of polysorbate determination from solutions containing low level of polysorbate 20. The first main peak is eluted at 14 min while the more hydrophobic major component peak pair is eluted at 15 min during the 32 min long scouting gradient (Fig. 1A). A polar peak group containing several polysorbate components is eluted between 3 and 6 min during the scouting gradient run. In the protein formulation the expected concentration of polysorbate 20 is 40 μg/ml.

The PEG components are eluted in one sharp peak when methanol was used as organic modifier (Fig. 1B). It was so surprising that adding acetonitrile to the mobile phase spoils the peak symmetry. When 100% acetonitrile was used as mobile phase organic modifier the tailing factor of PEG peak was higher than 3, while in the case of methanol it was around 1. Previously on other stationary phases we have not experienced this phenomenon. The PEG peak is less retained than the polysorbate main peaks. The selectivity with methanol as organic modifier was more advantageous than with acetonitrile. The expected quantity of unbound PEG in real samples is lower than 40 μg/ml.

For further method development only the diluted polysorbate 20, PEG and protein solutions were used. The results obtained during the method development showed that resolution and selectivity between the peaks of polysorbate, PEG and protein origin peaks can be improved applying low column temperature such as 20–40 °C (data not shown). Other beneficial parameter is the mobile phase composition. Adding methanol as organic modifier beside or instead of acetonitrile enhances the efficiency (sharper peaks for PEG and for the protein) and thus the resolution can be increased.

3.2. Optimized HPLC-CNLSD conditions

The design of experiments was evaluated in terms of separation speed, selectivity and resolution. The shortest possible separation is about 7–10 min with sufficient selectivity and resolution. The data

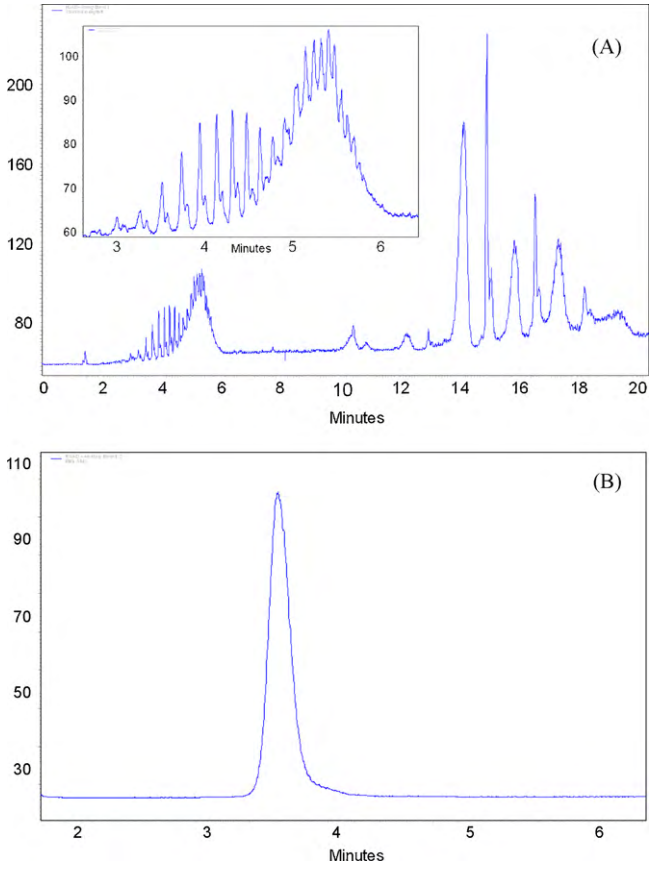

Fig. 1. Scouting–condensation nucleation light scattering detection (CNLSD or NQAD™) chromatograms of 500 μg/ml polysorbate 20 (A) and 100 μg/ml PEG (B). In (A), the retention range of less retained polysorbate compounds is zoomed. Chromatographic conditions: Kinetex C18 column packed with 2.6 (m shell particles (100 mm × 3 mm); mobile phase: methanol–water–triflouroacetic acid gradient elution (35–100% "B", in 32 min); flow: 0.5 ml/min; column temperature: 30 °C; injection volume: 5 μl; detection: CNLSD; gain: 10×; evaporating temperature: 70 °C; filter: 0.6 s; gas (nitrogen) pressure: 28–30 psi.

obtained during the method optimization, full factorial experimental designs were used to find the optimum condition for separation. The main effect of three factors such as (1) gradient span, (2) column temperature and (3) flow rate on the selectivity and resolution were estimated with a 2^3 standard factorial design on the basis of nine chromatographic runs (including center point). The calculated effects were plotted on Pareto charts (data not shown). Temperature and gradient time showed significant effect on resolution between PEG and polysorbate peaks while the effect of flow rate was negligible. Contour plots of resolution versus column temperature and gradient span were calculated. According to these plots, 20–30 °C as column temperature provides the highest peak resolution. Another experimental design was conducted to find the optimum in mobile phase composition. A 2^2 standard factorial design (including center point) was used to estimate the effect of acetonitrile–methanol ratio and separation temperature. On the basis of five chromatographic runs, Pareto charts and contour/surface plots were calculated. The results obviously showed that methanol is a much better organic modifier than acetonitrile for this purpose. Significantly higher resolutions and better selectivity were obtained if methanol was used as organic modifier instead of acetonitrile.

The optimized method uses a Kinetex C18 column packed with 2.6 (m shell particles (100 mm × 3 mm) and methanol–water–triflouroacetic acid mobile phase gradient at a flow rate of 0.6 ml/min. Mobile phase "A" consists of

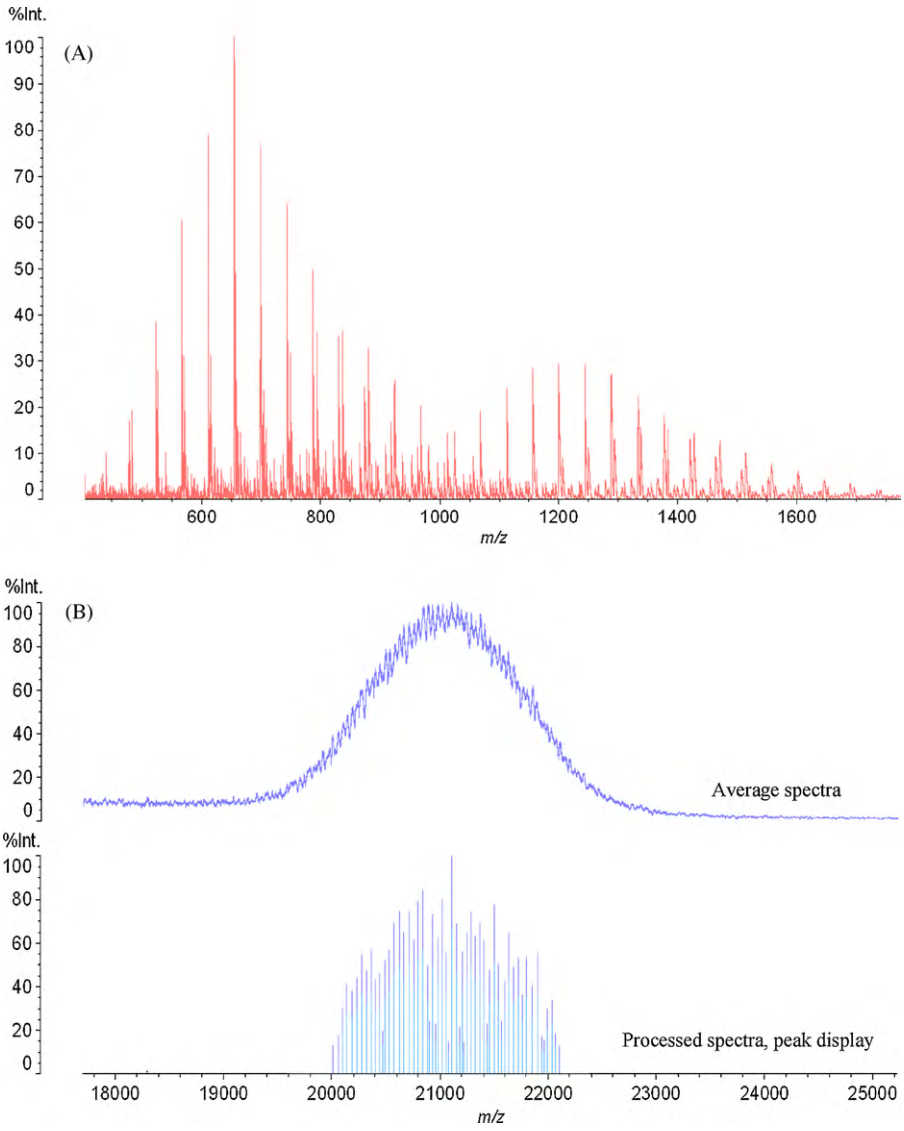

Fig. 2. MALDI-TOF MS spectra of (A) polysorbate 20 and (B) PEG (poly(ethylene-glycol), Sunbright™). Matrix substance: 2,5-dihydroxybenzoic acid (10 mg/ml) in acetonitrile–water–trifluoroacetic acid (50:50:0.1), sample preparation: 0.5 μl matrix solution and 0.5 μl 500 μg/ml polysorbate 20 and PEG solution. The spectra were collected in linear positive mode.

methanol–water–triflouroacetic acid in 100:900:1 (v/v/v) ratio, and mobile phase "B" with 900:100:1 (v/v/v) ratio. The gradient program starts with 83% "B" eluent, and a 4.5 min gradient duration is applied up to 100% "B" and it is kept till 6.5 min. After this gradient elution program, the column is re-equilibrated (post-run or pre-run) with the starting mobile phase composition for 3 min. The injection volume was set as 5 μl. The detector parameters are set as follows: gain, 10×; evaporating temperature, 70 °C; filter, 0.6 s; gas (nitrogen) pressure, 28–30 psi. The sample compartment is thermostated at 4 °C to avoid protein degradation or aggregation.

3.3. Mass spectrometric measurements

During the method development stage mass spectrometric (MS) detection was also used in addition to CNLSD. The aim of MS measurements was to determine which substances eluted in the polysorbate main (target) peak. Preliminary measurements were performed with MALDI-TOF (matrix-assisted laser desorption/ionization – time of flight) MS to determine the mass distribution of polysorbate 20. The matrix substance (2,5-dihydroxybenzoic acid) was dissolved (10 mg/ml) in acetonitrile–water–triflouroacetic acid (50:50:0.1). Then the matrix solution was mixed with the 500 μg/ml polysorbate 20 solution on the target plate (0.5 + 0.5 μl). The spectra were collected in linear positive mode in the range of 400–3000 Da (Fig. 2A). The mass of polysorbate 20 substances shows bimodal distribution. Similar type mass distribution was observed previously for polysorbate 80 [12]. The separation of all polysorbate isomers is not possible with reverse phase chromatography thus MS detection was applied to conclude which polysorbate components are coeluted in the target main chromatographic peak, which was chosen as the test compound of polysorbate determination (t_r = 3.2 min in Fig. 4 and Fig. 6). Applying a splitter built in after the column, the mobile phase flow rate was splitted (in 1:99 ratio) toward the CNLSD detector (99% flow) and to the AccuSpot (1% flow) to collect a fraction of the target polysorbate peak on a MALDI plate. The major target peak contained at least 11 different polysorbate 20 compounds in the mass range of 1000–1500 Da (data not shown).

MALDI-TOF MS measurements were also achieved to determine the average mass of PEG. The sample preparation of PEG on the MALDI plate was similar as it was applied in the case of polysorbate. 2,5-dihydroxybenzoic acid as matrix solution (10 mg/ml)

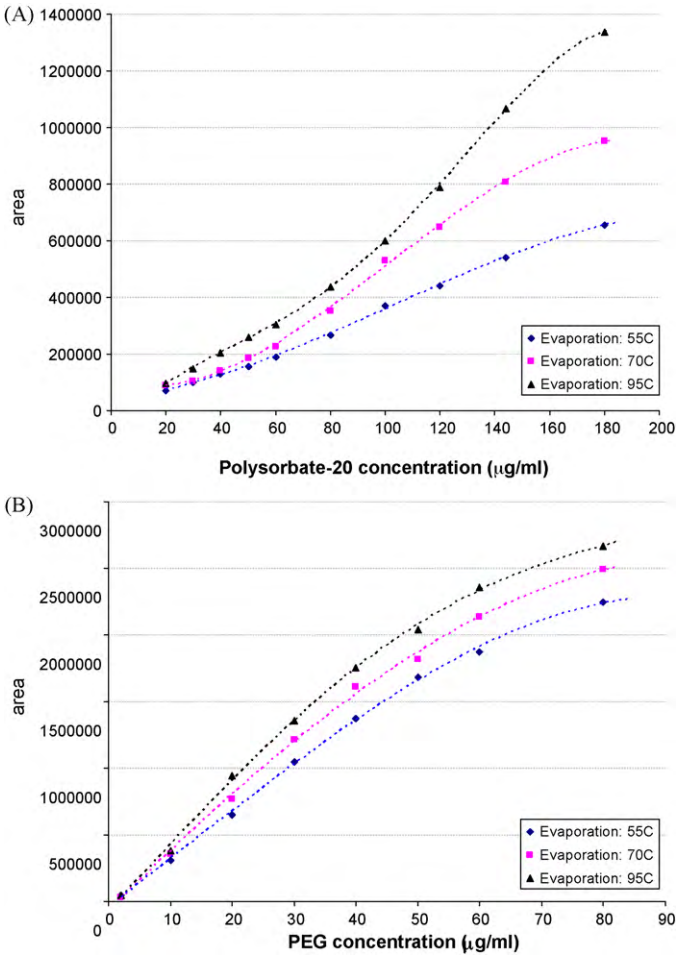

Fig. 3. CNLSD detector response of polysorbate 20 (A) and PEG (B) when different evaporation temperature (55 °C, 70 °C and 95 °C) is applied.

was mixed with the 500 μg/ml PEG solution on the target plate (0.5 + 0.5 μl). The spectra were collected in linear positive mode in the range of 5000–30000 Da (Fig. 2B). The mass distribution of PEG components showed Gaussian distribution in the mass range of 20–22 kDa. The average mass of the applied PEG is approximately 21 kDa. The processed peak spectra of PEG shows a 44 Da repeating unit.

3.4. Investigation of CNLSD condition and detector response

In the case of CNLSD detection, three parameters are available for the user to set the optimal condition such as gain, filter and evaporation temperature. Gain is a simple signal amplifying option, while the filtering opportunity does a signal smoothing process as a function of time. The only one valid parameter to adjust the signal to noise ratio and/or the signal–mass correlation is the evaporation temperature. Both for PEG and polysorbate 20 the evaporation temperature was set for three different values such as 55, 70 and 95 °C, and the signal (peak area) versus mass (concentration) functions were obtained (Fig. 3).

In the case of polysorbate 20 (Fig. 3A) the fitted plots contain two inflexion points in the range of 20–200 μg/ml (one order). A convex curve can be fitted between 20 and 140 μg/ml to the measured points, while in higher concentration region the curves shift into concave form. The lower the evaporation temperature is the more linear the fitted curve. The signal to noise ratio is obviously higher as the evaporation temperature is increased. Over 110–120 μg/ml the 95 °C evaporation performs approximately 2-times higher sensitivity as the 55 °C evaporation provides. In the range of 20–60 μg/ml polysorbate 20 (the other points are ignored) the linear correlation coefficient (R^2) is larger than 0.99 on each three evaporation temperature thus the method can be considered as linear in this narrow range, the CNLSD can perform as a good mass concentration detector.

For PEG (Fig. 3B) the response of CNLSD was slightly saturated for the higher concentrations studied (above 50 μg/ml). The obtained slopes are significantly different. The peak area is considerably higher as the evaporation temperature is increased. In the range of 2–50 μg/ml CNLSD works as a linear detector ($R^2 > 0.99$). It is worthy to mention that the obtained peak area was much higher for PEG than for polysorbate if the same concentration is compared. Probably the sensitivity difference between PEG and polysorbate 20 are related to their property of interaction with condensing water.

3.5. Method validation

A fast, gradient reversed-phase HPLC method with condensation nucleation light scattering detection was developed to separate and determine polysorbate 20 and PEG components, and can be applied for in process control (IPC) analysis for routine work. The selectivity between polysorbate 20, PEG and protein origin peaks can be changed significantly with varying the column temperature or changing the ratio of methanol and acetonitril in the mobile phase. It is very useful when polysorbate 20 or PEG is measured from different matrixes (different protein solutions).

The HPLC-CNLSD method was validated. The measurement of polysorbate 20 concentration can be based on the first main peak (most polar) of polysorbate 20 while PEG elutes in a sharp single peak. The method validation was performed in accordance with the recent guidelines [37–39]. The data obtained during the method validation are summarized in Table 1.

3.5.1. Specificity

To prove that the determination of polysorbate 20 and PEG is selective and free from any disturbing effects, reference solutions, protein containing solutions and stressed protein (oxidized, reduced and deamidated) solutions were tested.

Deamidation of asparagine and glutamine in proteins results a free carboxylic acid group. This process is accompanied by changes in polarity and hydrophobicity, mass and charge. Changes in polarity and hydrophobicity can be followed using RP-HPLC. In this example the RP-HPLC chromatogram showed a small amount of a less polar product after deamidation. Fig. 4 chromatogram B shows a small shouldered-peak after the major protein peak. Deamidation was achieved by adding 100 μl hydrochloric acid (1 N) to 900 μl protein solution. Then the mix was incubated at 30 °C for 3 h.

Reduction of the protein can also affect the polysorbate and PEG determination. The model protein has intramolecular disulfide bonds. The disulfide bonds in this protein were reduced to sulfhydryls with dithiothreitol (DTT). 0.5 mg of DTT was measured to 1 ml protein solution then it was incubated at 30 °C for 60 min. The protein reduction yielded a more hydrophobic compound than the native form (peak 5 in Fig. 4C).

The protein used in this study contains methionines in its sequence. Hydrogen peroxide oxidation of two methionines is relatively fast in the case of this protein. Oxidation of both methionine results in alterations of protein structure that affect the apparent molecular size and polarity, thus can be separated from the native molecule with reversed-phase liquid chromatography. Therefore it was necessary to verify that oxidized protein forms do not disturb the polysorbate or PEG determination, and baseline separation can be achieved between the polysorbate main peak and oxidized protein peaks (Fig. 4D). The oxidation was performed by adding 1% (v/v) hydrogen peroxide (30%) into the protein solution, and after

Table 1
Summary of method validation data.

Parameter	Polysorbate	PEG
Specificity[a]	Complies	Complies
Linearity		
Correlation	$R^2 > 0.99$	$R^2 > 0.99$
Intercept	Confidence interval of "Y" intercept ($P = 95\%$) contains the origin	Confidence interval of "Y" intercept ($P = 95\%$) does not contain the origin
Residuals	The residuals plotted uniformly and randomly around the regression line	The residuals plotted uniformly and randomly around the regression line
Accuracy[b]		
Average recovery	98.2%	102.3%
Correlation	$R^2 > 0.99$	$R^2 > 0.99$
Residuals	The residuals plotted uniformly and randomly around the regression line	The residuals plotted uniformly and randomly around the regression line
Precision[c]		
RSD% (n = 6)	5.5%	4.4%
Intermediate precision		
F-test	$F = 1.90$ ($F_{critical} = 5.05$)	$F = 2.35$ ($F_{critical} = 5.05$)
t-test	$t = 2.09$ ($t_{critical} = 2.23$)	$t = 1.88$ ($t_{critical} = 2.23$)
Limit of quantitation[d]	10 μg/ml (RSD = 7.9%, $n = 5$)	2 μg/ml (RSD = 8.3%, $n = 5$)

[a] To prove specificity, reference solutions, protein containing solutions and stressed protein (oxidized, reduced and deamidated) solutions were injected.
[b] Mean value of the recovery in the range of LOQ – 150% of nominal concentration.
[c] Relative standard deviation (RSD) of recovery data.
[d] Concentration, where RSD% of repeated peak areas ($n = 5$) not exceed 10%.

a 2 h long incubating (30 °C) the oxidation was stopped by adding 0.5 mg methionine to the solution.

Resolution of $R_s \gg 1.5$ was achieved between the PEG, the polysorbate main peak (target peak, $t_r = 3.2$ min in Fig. 4) and the protein or protein origin impurity or degradants peaks, therefore the method can be considered as a specific method for unbound PEG and polysorbate 20 determination. Fig. 4 shows the chromatograms obtained through the specificity study.

Placebo samples were also tested in term of specificity. The placebo components (sugar and buffer) give signals at column dead

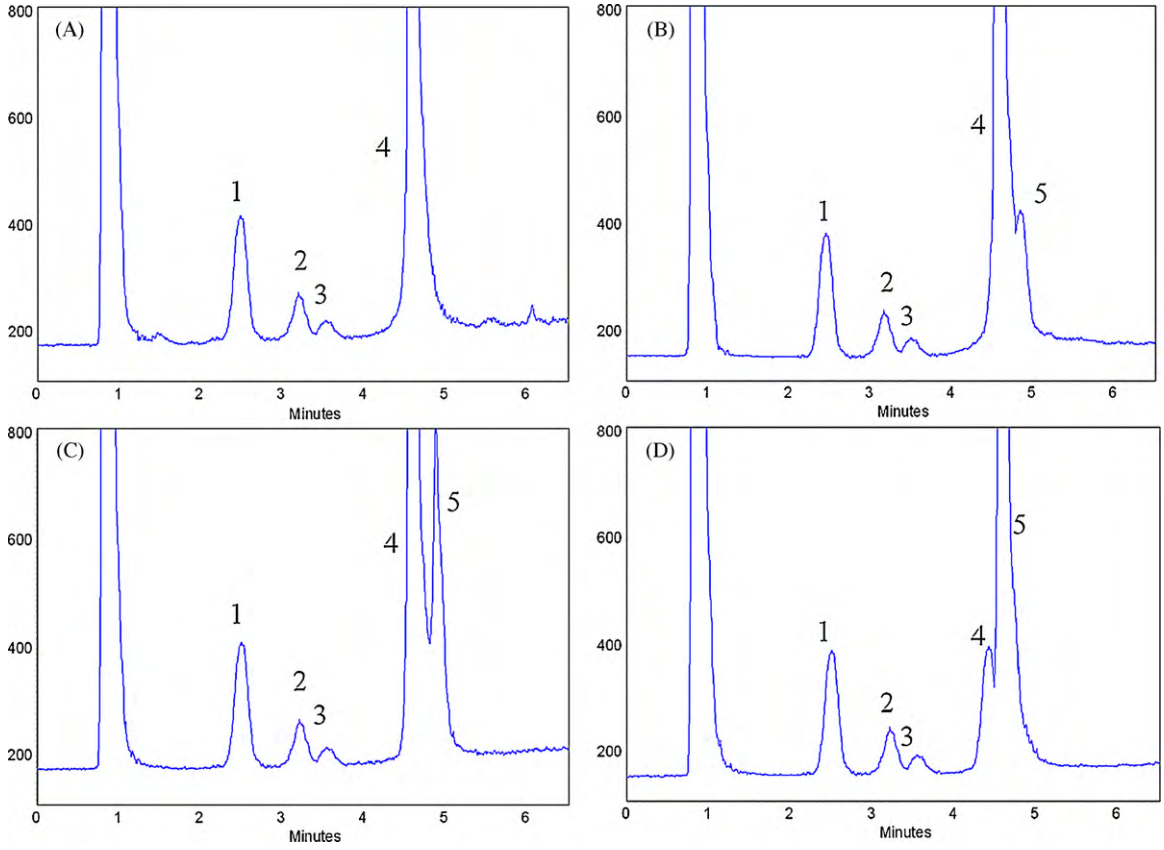

Fig. 4. Chromatograms of stressed protein solutions containing 10 mg/ml protein, 40 μg/ml polysorbate 20 and 40 μg/ml PEG. Chromatograms: protein solution (A), protein solution containing deamidated protein (B), protein solution containing reduced form (C), and protein solution containing oxidized protein form (D). Peaks: 1: PEG; 2 and 3: major peaks of polysorbate 20 origin; 4 and 5: peaks of protein origin. Chromatographic conditions: Kinetex C18 column packed with 2.6 (m shell particles (100 mm × 3 mm) Mobile phase "A" consists of methanol–water–triflouroacetic acid in 100:900:1 (v/v/v) ratio, and mobile phase "B" with 900:100:1 (v/v/v) ratio. The gradient program starts with 83% "B" eluent, and a 4.5 min gradient duration is applied up to 100% "B" and it is kept till 6.5 min. Flow rate: 0.6 ml/min; column temperature: 30 °C; injection volume: 5 μl. Detector parameters: gain, 10×; evaporating temperature, 70 °C; filter, 0.6 s; gas (nitrogen) pressure, 28–30 psi.

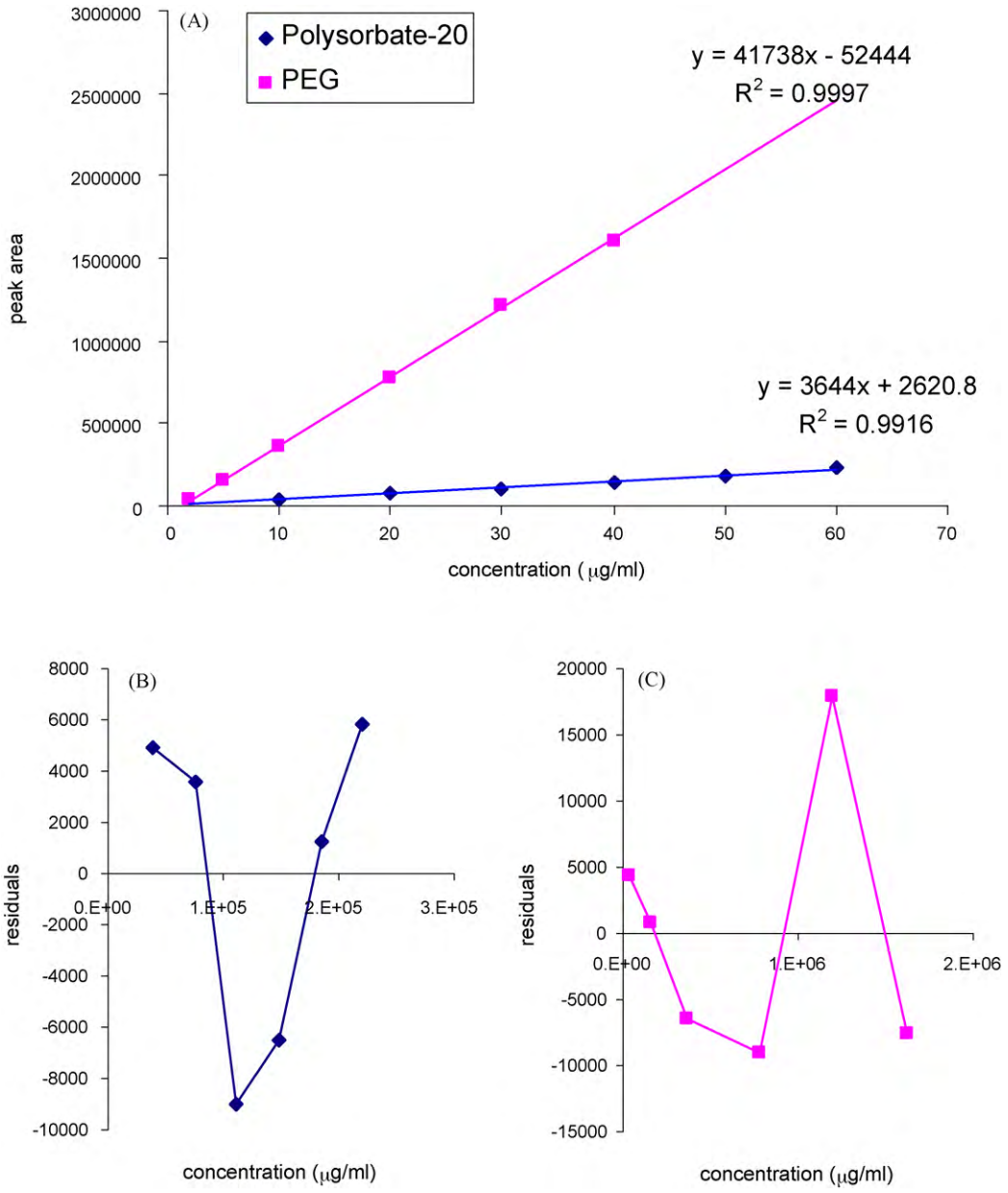

Fig. 5. Linear regression. (A) Fitted linear curves, correlation coefficients and equations, (B) residuals of polysorbate 20 regression and (C) residuals of PEG regression.

time (they are not retained), thus excipients have no importance regarding method specificity.

3.5.2. Linearity of response

In Section 3.4 it can be seen that the detector response is not linear when a broad concentration range is investigated. In the range of our interest (in possible concentration range of real samples) linear curve fitting can be applied and linear calibration can be valid for determination of polysorbate 20 and unbound PEG with CNLSD detection.

The linearity of response was assessed by injecting reference standards prepared in sample solvent. The concentration range of compounds was investigated from the quantitation limit (LOQ) up to the 150% of the theoretical (expected) polysorbate content (range: 10–60 μg/ml polysorbate 20) and to the acceptance limit of unbound PEG or PEG residue (range: 2–40 μg/ml PEG). Six independent results were obtained. The results were analyzed by linear regression (Fig. 5). The correlation coefficients, r^2, were found $r^2 > 0.99$ ($r^2 = 0.9916$ for polysorbate 20 and $r^2 = 0.9997$ for PEG), confidence interval of "Y" intercept ($P = 95\%$) for polysorbate 20 peak contained the origin (−15480 to 20722) but for the PEG peak the fitted curve did not contain the origin, it had negative intercept (confidence interval: −73733 to −31156). The residuals plotted uniformly and randomly around the regression line for both analyte (Fig. 5B and C). It can be seen that CNLSD detection can be applied as a linear detector in a relatively narrow concentration range, however in broad range it works as non-linear detector.

3.5.3. Accuracy

Samples for recovery test were prepared as follows: reference material (polysorbate 20 and PEG) in the range of LOQ – 150% of theoretical value was spiked to protein solutions, which did not contain polysorbate 20, and PEG. polysorbate and PEG were pipetted from a stock solution (dissolved in water) then the solutions were homogenized (1 min shaking).

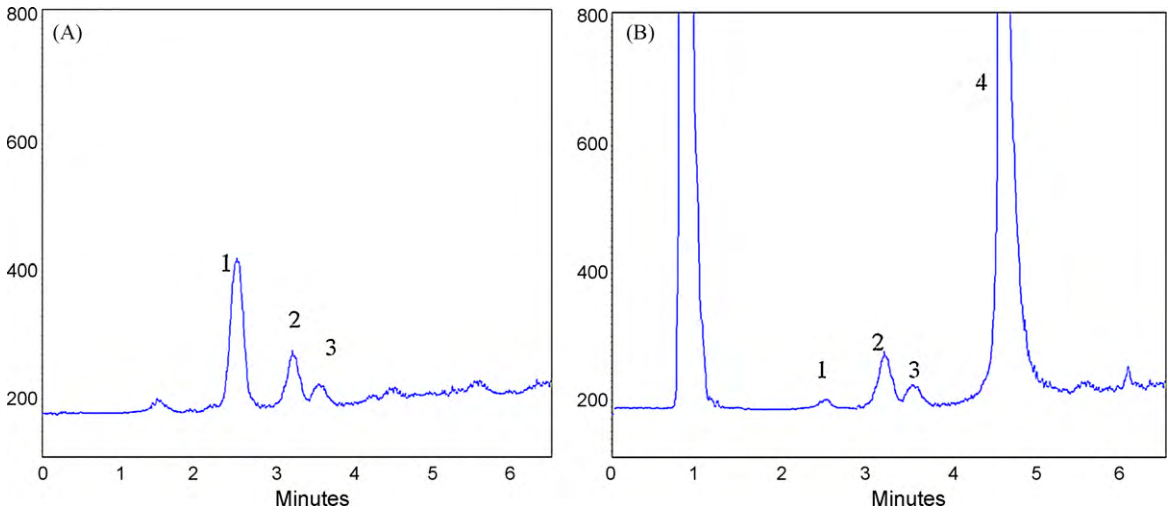

Fig. 6. Chromatograms of reference (A) and test solution (B). Peaks: 1: PEG; 2 and 3: major peaks of polysorbate 20 origin; 4: peak of protein origin. Chromatographic conditions: Kinetex C18 column packed with 2.6 (m shell particles (100 mm × 3 mm) Mobile phase "A" consists of methanol–water–triflouroacetic acid in 100:900:1 (v/v/v) ratio, and mobile phase "B" with 900:100:1 (v/v/v) ratio. The gradient program starts with 83% "B" eluent, and a 4.5 min gradient duration is applied up to 100% "B" and it is kept till 6.5 min. Flow rate: 0.6 ml/min; column temperature: 30 °C; injection volume: 5 μl. Detector parameters: gain, 10×; evaporating temperature, 70 °C; filter, 0.6 s; gas (nitrogen) pressure, 28–30 psi.

Sample concentrations were determined by reference to a calibration line constructed from standards containing the respective analyte in LOQ – 150% around the theoretical polysorbate 20 and PEG concentration. Six independent results were obtained. The results were analyzed by linear regression. The correlation coefficients (r^2) were found $r^2 > 0.99$ and the residuals plotted uniformly and randomly around the regression line. The obtained average recovery of polysorbate 20 was 98.2% and it was 102.3% for PEG.

3.5.4. Precision, intermediate precision

Precision was examined by the relative standard deviation (RSD) of simultaneously prepared samples ($n = 6$). The test samples contained 40 μg/ml polysorbate 20 and 20 μg/ml PEG. The RSD% of six analyses should be lower than 10% criteria was applied. For evaluating the intermediate precision, the analysis was repeated ($n = 6$) at another day and the obtained results were compared by *t*-test and *F*-test. No significant differences were observed between the two repeated analyses. The RSD% obtained for precision study were RSD = 5.5% (first day) and 4.2% (second day) for polysorbate and RSD = 4.4% (first day) and 4.1% (second day) for PEG. The obtained *F*-value for intermediate precision test was found as $F = 1.90$ and $F = 2.35$ ($F_{critical} = 5.05$), while *t*-test resulted a value of $t = 2.09$ and $t = 1.88$ ($t_{critical} = 2.23$).

3.5.5. Limit of quantitation

Quantitation limit were determined by the RSD of peak areas obtained by five repeated injections (of standard solutions). The RSD < 10% for LOQ concentration criteria were considered for this purpose. The obtained RSD of peak areas was 7.9% in the case of 10 μg/ml polysorbate 20 solution while the 2 μg/ml PEG solution yielded a 8.3% RSD of five repeated injections. As a result 10 μg/ml polysorbate 20 and 2 μg/ml PEG were considered as LOQ.

3.5.6. Stability of sample and stock solutions

The stability of sample solution was studied on standard as well as on test-sample (protein containing) solution at the concentration of nominal value (40 μg/ml polysorbate 20 and 10 μg/ml PEG). The solutions were stored in a sample compartment and were chromatographed 12 times within a 48-h period. RSD of peak areas was obtained, and linear regression was calculated for the fitted curve. The confidence interval ($P = 95\%$) of slopes contained the value of zero and the residuals plotted uniformly and randomly around the regression line for both compounds. The obtained RSD during the 48-h test was smaller than 5% (4.8% for polysorbate and 4.2% for PEG). The standard and test solutions were proved to be stable for 48 h. There were no detectable degradants on the chromatograms during the stability study.

For the establishment of the stability of standard stock solution, 3 standard stock solutions were prepared and stored in refrigerator for one week. For the measurements freshly diluted solutions were used and injected every day, and the differences between the peak areas injected at the beginning and actual one were calculated. No more than 5% difference was observed each day (the highest deviation was 4.6%). The stock solutions of the reference substances were considered to be stable, for at least 7 days.

3.6. Determination of polysorbate 20 and unbound PEG from real samples

For routine measurement of polysorbate 20, the determination is based on the peak area of the first polysorbate 20 origin main peak (target polysorbate 20 compound). This peak elutes at $t_r = 3.2$ min (peak 2 in Fig. 4 and in Fig. 6). The calculation is based on external standard method. The average peak area obtained from three parallel weighing of standard polysorbate 20 solutions is used as reference peak area. It is necessary to mention, that the peak are ratio of polysorbate compounds are variable depending on the vendor and batch, therefore the same polysorbate should be used as reference material as was applied in the injection solution to be measured. The determination of unbound PEG requires using a calibration curve for quantifying PEG since the fitted curve of PEG calibration does not contain the origin (Section 3.5.2).

The stock solutions of reference standards (both polysorbate 20 and PEG) were dissolved in water (1000 μg/ml), and then polysorbate 20 was diluted to 40 μg/ml as reference solutions and PEG solutions were diluted as 2, 20 and 40 μg/ml to acquire the linear calibration. The two compounds can be combined in one diluted reference solution. The test samples were injected without dilution. Two repeated injections of both samples and reference solutions were performed. Fig. 6 shows the chromatogram of a reference solution and a real sample.

4. Conclusion

On the basis of this study, the use of fast chromatographic technique (applying partially porous packings) coupled with condensation nucleation light scattering detection for the quantification of polysorbate 20 and unbound PEG from protein solutions is practical. Adequate separation of these compounds was achieved within 7 min. The time reducing and solvent saving characteristics of the fast separation is exceptionally beneficial, compared to the most widely used conventional HPLC technique. Generally the separation of polysorbate and PEG compounds from protein origin peaks can be achieved within 10 min depending on the characteristic of protein and other excipients (matrix components). Separation can be optimized by varying the mobile phase composition (gradient program, organic modifier) and by changing the separation temperature.

Applying CNLSD detection does not necessitate the use of a mass spectrometry detector, which is more expensive. The response of CNLSD is generally not linear but in an adequate narrow concentration range (inside 1 or 2 concentration order, depending on the analyte) can be used as a linear detector. Moreover, the detector was shown to be fully compatible with HPLC in gradient mode. This technique can be constructive for the determination of other non-chromophoric excipients. The concept of applying a fast RP-HPLC separation in combination with CNLSD detection can be useful in routine analysis for many protein formulations.

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