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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

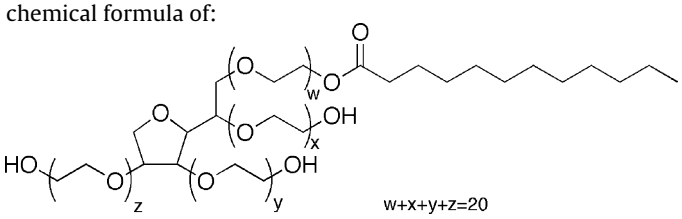
A novel fast and sensitive method has been developed for the specific simultaneous determination of polysorbate 20 (Tween 20) and unbound polyethylene-glycol (PEG) from liquid formulations in the presence of proteins and excipients. The quantitative determination is based on a fast liquid chromatographic (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).