CHALMERS
UNIVERSITY OF TECHNOLOGY

Investigation of ultra-high-pressure size exclusion columns and chromatographic parameters for polymer characterization

AMANDA VÄSTERNÄS

Master of Science Thesis

Department of Chemistry and Chemical Engineering
Chalmers University of Technology
AstraZeneca R&D, Mölndal
Gothenburg, Sweden 2015

MASTER'S THESIS

Investigation of ultra-high-pressure size exclusion columns and chromatographic parameters for polymer characterization

Master of Science Thesis

AMANDA VÄSTERNÄS

SUPERVISORS
Sara Richardson, Eivor Örnskov and Anna Maria Enlund
AstraZeneca R&D, Mölndal

EXAMINER
Gunnar Westman
Chalmers University of Technology

Department of Chemistry and Chemical Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenborg, Sweden 2015

Investigation of ultra-high-pressure size exclusion columns and chromatographic parameters for polymer characterization

Master of Science Thesis

AMANDA VÄSTERNÄS

Department of Chemistry and Chemical Engineering
Chalmers University of Technology
SE - 412 96 Göteborg
Sweden

In collaboration with AstraZeneca AB
SE-431 83 Mölndal
Sweden

Göteborg, Sweden 2015

Acknowledgements

First I would like to thank my supervisors Sara Richardson, Eivor Örnskov and Anna Maria Enlund for their enormous support and sharing of knowledge throughout the project. Many thanks to Magnus Fransson for his lesson in chemometrics and Anders Sparén for reading parts of my report. Also, many thanks to all colleagues at AstraZeneca, Mölndal for all help and making me feel very welcome. I am also grateful for the help from my examiner Gunnar Westman at Chalmers University of Technology.

Investigation of ultra-high-pressure size exclusion columns and chromatographic parameters for polymer characterization

AMANDA VÄSTERNÄS
Department of Chemistry and Chemical Engineering

This thesis project was performed at Pharmaceutical Development, AstraZeneca R&D Mölndal.

Abstract

Size exclusion chromatography (SEC) is a liquid chromatographic technique for separation of biomolecules and polymers, based on hydrodynamic volume or size. The growing demand of shorter analysis time and maintained resolution in SEC has developed ultra-high-pressure size exclusion chromatography (UHPSEC). The aim of this thesis work is to evaluate UHPSEC columns with a refractive index (RI) detector for development of methods for low amounts of polymer excipients with reduced analysis time. In addition, the aim is to investigate, in a screening design, what factors influence the chromatographic behavior of hydroxypropyl cellulose (HPC) in a selected UHPSEC column. Six different SEC columns were evaluated, one conventional SEC column and five UHPSEC columns. The conventional SEC column was TSKgel GMPWXL and the UHPSEC columns were Acquity APC AQ 200, Acquity APC XT 200, Acquity UPLC BEH 125 SEC, and Acquity UPLC BEH 200 SEC. Hydroxypropyl cellulose (HPC), Pluronic F108, polyethylene glycol (PEG) 6000 and PEG 20.000 were used as model polymers, whereas pullulan and polystyrene were used as standards.

Evaluation of Acquity APC AQ 200 showed that only pullulan standards eluted from the column, the other model polymers adsorbed to the column material. The Acquity APC XT 200 column with tetrahydrofuran (THF) as mobile phase and the conventional TSKgel GMPWXL column with phosphate buffer saline (PBS) as mobile phase had comparable limit of quantification (LOQ) and limit of detection (LOD) for Pluronic F108. However, for HPC LOQ was higher on the UHPSEC column. Of the tested columns, Acquity UPLC BEH 200 SEC had the lowest LOQ and LOD for HPC. Evaluation of the columns also showed that the analysis time was reduced with an UHPSEC column compared to the conventional column. The screening design on Acquity UPLC BEH 200 SEC for analysis of HPC revealed that column temperature and injection volume were predominant factors influencing peak height, peak area, the ratio of tailing and fronting, and back pressure.

Sammanfattning

SEC (size exclusion chromatography) är en vanlig kromatografisk teknik för separation av biomolekyler och polymerer, baserad på hydrodynamisk volym eller storlek. En ökande efterfråga på SEC metoder som har kortare analystid har lett till att UHPSEC (ultra high pressure SEC) har utvecklats. Syftet med detta examensarbete är att utvärdera UHPSEC kolonner med en brytningsindex (RI) detektor för utveckling av metoder för små mängder av polymera hjälpämnen. Dessutom är syftet att undersöka, med designade experiment, vilka faktorer som påverkar det kromatografiska beteendet av hydroxipropylcellulosa (HPC) i en UHPSEC kolonn. Sex olika SEC kolonner utvärderades, en konventionell SEC kolonn och fem UHPSEC kolonner. Den konventionella kolonnen var TSKgel GMPWXL och UHPSEC kolonnerna var Acquity APC AQ 200, Acquity APC XT 200, Acquity UPLC BEH 125 SEC, och Acquity UPLC BEH 200 SEC. Hydroxipropylcellulosa (HPC), Pluronic F108, polyetylenglykol (PEG) 6000 och PEG 20,000 användes som modellpolymerer, medan pullulan och polystyren användes som standarder.

Utvärdering av Acquity APC AQ 200 visade att endast pullulanstandarder eluerade från kolonnen, de andra modellpolymererna adsorberade till kolonnmaterialet. Acquity APC XT 200 kolonnen med tetrahydrofuran som mobilfas och den konventionella TSKgel GMPWXL kolonnen med fosfatbuffrad saltlösning som mobilfas hade jämförbar kvantifieringsgräns och detektionsgräns för Pluronic F108. För HPC däremot var kvantifieringsgränsen högre för UHPSEC kolonnen. Av de testade kolonnerna hade Acquity UPLC BEH 200 SEC den lägsta kvantifieringsgränsen och detektionsgränsen för HPC. Utvärdering av kolonnerna visade också att analystiden reducerades med UHPSEC kolonnerna jämfört med den konventionella SEC kolonnen. De designade experimenten på Acquity UPLC BEH 200 SEC för analys av HPC visade att kolonntemperatur och injektionsvolym var dominerande faktorer som påverkade topphöjd, topparea, toppform, och mottryck.

Table of contents

List of abbreviations

APC	Advanced polymer characterization
BEH	Ethylene bridged hybrid
Dn/dc	Refractive index increment
DOE	Design of experiment
FA	Formic acid
HPC	Hydroxylpropyl cellulose
HPLC	High performance liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
PBS	Phosphate buffer saline
PEG	Polyethylene glycol
RI	Refractive index
RSD	Relative standard deviation
SEC	Size exclusion chromatography
THF	Tetrahydrofuran
UHPSEC	Ultra-high-pressure size exclusion chromatography
UPLC	Ultra performance liquid chromatography

1 Introduction

Size exclusion chromatography (SEC) is the dominant liquid separation chromatographic technique for separation of biomolecules and polymers, based on hydrodynamic volume or size. During a SEC separation a solute is injected into a mobile phase that is pumped through a column where the separation occurs. The column is packed with non-adsorbing particles with pores. Depending on their size, the molecules will penetrate different fractions of the pores, thus smaller molecules are retained a longer time in the column (1). A conventional SEC method requires running times of typically half an hour up to several hours. There has been a growing demand, from chemists in polymer laboratories where many samples are being analyzed every day, for SEC methods that can provide faster analysis time but still maintain a high resolution (2). In addition, shorter analysis time is also beneficial as the quantities of mobile phase required for separation is reduced (2, 3). In conventional SEC high concentrations, typically one or several mg/ml of the molecule is needed for analysis. This is a consequence of less sensitive detectors used in SEC compared to detectors for HPLC of small molecules containing an UV light absorbing chromophore (2). It is also due to larger column dimensions of a SEC column and larger particle size of a SEC columns packing material compared to HPLC columns for small molecules.

Ultra-high-pressure size exclusion chromatography (UHPSEC) has been developed to meet said demands on conventional SEC (2, 3). Advances in column technology resulted 1990 in UHPSEC columns packed with smaller particles, sizes of sub 3 μm and 2 μm. In such columns the length is reduced, but the columns still have the same separation efficiency. The new type of column materials in UHPSEC columns are porous but stable during the higher pressures generated by the smaller particles (4). The advances in column technology opened up the possibility to operate at higher pressures and the analysis time can be up to ten times shorter (3). Furthermore, additional contribution to the shorter analysis time is that columns with small particles often have a higher optimum flow rate i.e. the flow rate where maximum efficiency is achieved, compared to columns packed with larger particles (2, 4).

In pharmaceutical, environmental, life science and food analysis, ultra-high performance liquid chromatography (UHPLC) for small molecules is a common technique. However, little is published about the potentials of UHPSEC (5). Future pharmaceutical formulations may include new or other types of polymer excipients at low concentrations. When developing these formulations new analytical separation methods are needed that are both faster and can be used for determining low concentrations. UHPSEC is therefore of interest as a potential solution for separation of polymer excipients aimed for pharmaceutical formulations.

1.1 Aim

The aim of this master thesis work is to investigate UHPSEC columns on a HPLC system with refractive index (RI) detector for development of methods for quantification and characterization of low amounts of polymer excipients. The UHPSEC columns that will be investigated in this study are two columns developed for advanced polymer characterization,

Acquity APC AQ and Acquity APC XT, as well as two Acquity UPLC BEH SEC columns developed for protein characterization. Three polymer excipients commonly used in pharmaceutical formulations; HPC, PEG, and Pluronic F108 will be used as model polymers. In addition, pullulan and polystyrene will be used as standard compounds. Limit of quantification (LOQ) and limit of detection (LOD) will be determined for the analytes on some of these UHPSEC columns as well as for a conventional SEC column.

The aim is also to identify, in a screening experimental design on one selected UHPSEC column, factors that have impact on the chromatography of HPC in terms of peak height, peak shape, and peak area as well as what factors influence back pressure.

2 Theoretical background

2.1 Size exclusion chromatography

Size exclusion chromatography (SEC) is a liquid chromatographic technique, predominantly used for separation of large biomolecules and polymers. The separation is entropy controlled and molecules are separated according to their hydrodynamic volume or size in a specific solvent and temperature (1). SEC is also referred to as gel permeation chromatography (GPC) and gel filtration chromatography (GFC) in the case of separation of lipophilic macromolecules and hydrophilic macromolecules respectively (2). A molecule dissolved in solvent is injected into a mobile phase that is pumped through a column where the separation takes place. The column has a stationary phase consisting of non-adsorbing particles with pores. Molecules will penetrate the pores and depending on the pore size, molecules of different sizes will penetrate different amount of pores (1). All molecules that are too large to penetrate the particle pores in the column will elute first in the volume between the pores called void or interstitial volume, V_o. A column's V_o is illustrated in Figure 1. Molecules capable of penetrate the pores will elute in decreasing size order as larger molecules penetrate a smaller fraction of pores compared to smaller molecules. Polymers small enough to enter all pores have access to the total permeation volume V_t of the column, which is V_o and total pore volume of the packing material V_i, see Equation i (6) and Figure 1.

$$V_t = V_o + V_i \qquad \text{(i)}$$

All SEC columns have a range of molecular weights that can be separated. The separation range of a column is defined as the molecular weight of the molecule that elute between V_o and V_t. It is in the V_i the separation occurs for polymers within the separation range. Furthermore, SEC columns have a total column volume, V_c defined in Equation ii, (6)

$$V_c = V_o + V_i + V_g \qquad \text{(ii)}$$

where V_g is the volume of the packing material.

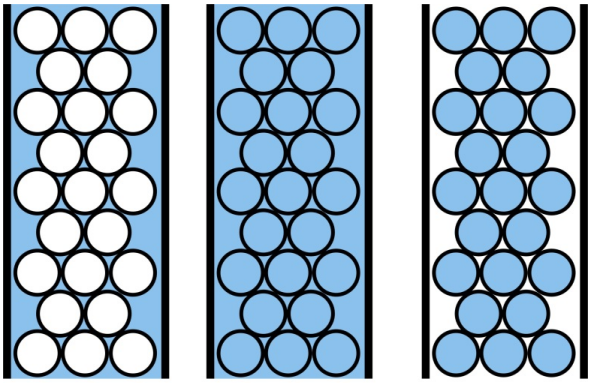

Figure 1. Schematic illustration of a SEC column's void or interstitial volume, V_o, (left), total permeation volume, V_t, (middle), and total pore volume of the packing, V_i, (right). (7)

There are different mechanisms used to explain the nature of SEC. The most accepted theory in SEC is the thermodynamic model (6). In the model, molecule retention within the column is based on the distribution of the molecule between V_o and V_i. (2) The retention volume, V_R of a molecule injected into the mobile phase can be described by Equation iii.

$$V_R = V_o + K_{SEC}V_i \quad \text{(iii)}$$

K_{SEC} is a thermodynamic parameter called the SEC distribution coefficient, which is defined as the proportion of molecule concentration in the V_i and the V_o (C_i/C_o). K_{SEC} can have a value between 0 and 1 and if K_{SEC} is equal to 0 or 1 the separation process will not occur. In the case $K_{SEC} = 0$, the molecule elutes in the V_o and in the case $K_{SEC}=1$ the molecule elutes in the V_t. In addition, if K_{SEC} is larger than 1, unwanted enthalpic interactions occur in the column. In equilibrium the distribution of a molecule in the V_o and the V_i can be described by Equation iv, (6)

$$K = exp^{-\frac{\Delta Ho}{RT}} exp^{\Delta So/R} \quad \text{(iv)}$$

where R is the gas constant, T is temperature, ΔH^o is enthalpy change and ΔS^o is conformational entropy changes when one mole of the molecule is transferred from V_o into the V_i during standard conditions. Ideally in SEC, only entropy controls the system and no repulsive or attractive interactions between the solute and the packing material exists. ΔH^o is negligible and K equals $K_{SEC,}$ see Equation v (6).

$$K_{SEC} = exp^{\Delta So/R} \quad \text{(v)}$$

When molecules reach the column, the concentration of the molecule within V_i is zero and the concentration of the molecule in V_o is larger than zero. A concentration gradient occurs, which “pulls” the molecule into the pores to equalize the chemical potential. The molecule will contract to fit the pore, thus loses entropy conformation. The shrinking of the molecule stops when the force pulling the molecule into the pores and the loss in entropy of the molecules are in equilibrium. The molecule concentration in V_o will be equal to zero as the sample zone moves through the column, which result in the molecules leaving the pores (2).

2.2 Non-exclusion retention effects

Ideally in SEC, as described above, there is no interaction between the stationary phase and the molecule, and the separation is depending only on the exclusion processes. However, non-exclusion retention mechanisms can occur in SEC, which affects the width and the position of the peak in the chromatogram. Enthalpy interactions, often attraction, but sometimes repulsion, between the molecule and mobile phase with the packing material is one common non-exclusion mechanism. An important property of a column packing material is therefore chemical inertness to the mobile phase and the molecule to minimize non-exclusion mechanisms (2). In case of unwanted interactions with the packing material, adjustments of the mobile phase can often solve this problem. Furthermore, high temperature can eliminate the adsorption to the packing material in some cases. Another unwanted effect

is degradation of high molecular weight polymers due to shearing, which can be solved by lowering the flow rate through the column (2).

2.3 Effect of column particle size

The current trend in SEC is reduction of the particle size of the packing material and reduction of column length (8). In liquid chromatography there are several advantages with columns packed with small particles compared to larger particles. By decreasing the particle size improvements in resolution of the column can be obtained or the same resolution can be maintained while the run time is decreased. Resolution is the difference in separation time of two peaks divided by the average width of the peaks. High resolution i.e. good separation is achieved when the difference in retention time between peaks is large and the peaks are narrow. Small particles can be packed with a higher density, which means V_o is decreased. As a result, the molecules diffusion distance between stationary phase and mobile phase is shorter. Diffusion can cause wider and asymmetrical peaks in a chromatogram and is a result of random movement from high concentration areas to areas with lower concentrations. When a solute moves through a column it spreads into a Gaussian shape and the longer retention time in the column the wider peak. Diffusion of polymers is slow which makes column particle size very important in SEC for polymers. Smaller particles within a column will reduce the diffusion and the chromatographic peak becomes narrower (4, 9).

2.4 Ultra-high-pressure size exclusion chromatography

The first UHPSEC columns were launched in 2010 developed for protein characterization in aqueous solution named Acquity UPLC BEH SEC. In 2013, UHPSEC columns were launched for advanced polymer characterization. Acquity APC columns became available for synthetic polymers both for organic and aqueous based systems (4). The UHPSEC columns are packed with small particles, sizes in ranges of sub 3 and sub 2 μm. In comparison, conventional SEC columns typically have particle size of 5-13 μm (2). Due to the smaller particle size, the column length of typically 25-30 cm for a conventional SEC column (10), can be reduced, but the UHPSEC column can still maintain the same efficiency (3).

Reduced particles sizes that are porous are mechanically unstable at very high pressures. To enable UHPSEC the packing materials in the columns have been improved and consist of porous inert ethylene bridged hybrid (BEH) particles, which is an inorganic silica-organic hybrid-based material (4). The material in conventional SEC is typically either semirigid polymer gels, rigid inorganic solids or soft hydrophilic polymer gels (6).

The BEH particles have different end-capping in different columns. The Acquity UPLC BEH SEC columns developed for characterization of proteins in aqueous mobile phases are diol end capped (11). The BEH particles in the Acquity APC XT column, suitable for operating in organic mobile phases, are end capped with tri-methyl silane, which masks silanols on the surface to make the BEH particles inert and non-retentive in organic mobile phases. In the corresponding column for aqueous mobile phases, Acquity APC AQ, the BEH particles are not end capped, hence they have free silanols (4). In addition, the BEH particles in

comparison to silica have lower silanol acidity and when the BEH particles are end capped the acidity is reduced even more (12).

2.5 Refractive index detector

The refractive index (RI) detector, also called differential refractive index (dRI) detector, measures the bulk property refractive index. It is a concentration sensitive detector where signals are generated based on differences in refractive index between the mobile phase and the analyte. Light from a light-emitting diode (LED) with typical wavelength between 660-880 nm passes a static flow cell containing the mobile phase and a cell containing the analyte flow (13). Light is detected by two photodiodes or by a photodiode array located behind each cell (13, 14). The overall refractive index changes in presence of an analyte, which will change the position of the light beam on the photodiodes. This shift in the relative intensity of light reaching the photodiodes is detected and the concentration of the analyte can be obtained (13). The difference in refractive index is proportional to the concentration of the analyte. All analytes that have a different refractive index compared to the mobile phase can be detected by the RI detector (6).

2.6 Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) are two parameters used in method validation. The definition of LOD and LOQ varies slightly between regulatory authorities. According to International Conference on Harmonization (ICH) and United States Pharmacopoeia (USP), LOD is the lowest amount of analyte in sample, which can be detected but not necessarily quantified under stated experimental conditions. LOQ is defined as the lowest amount of analyte in sample, which can be quantitatively determined with suitable precision and accuracy. The difference between LOD and LOQ is that it is not a requirement that the analyte can be quantified in LOD (15). Different approaches of how to determine LOD and LOQ exist. Among methods, LOD and LOQ can be determined based on calculations from the standard deviation of the blank, calculations from the linear regression curve at low concentrations and on signal-to-noise ratio. LOQ can also be calculated with a targeted relative standard deviation (RSD) based approach (15).

In the method calculations from blank determination, the mean value and standard deviation of typically 10 replicates of blank samples are calculated. LOD and LOQ are thereafter determined by adding the mean value of the blank to 3 and 10 standard deviations respectively. One assumption is made in this method; when the analyte is present, a response signal larger than the background noise will appear. The method is fast, however there is no evidence that injections of low concentration produce a signal that can be distinguished from the blank injection. In this method, LOD is calculated with the following Equation:

$$LOD = y_0 + 3SD \quad \text{(vi)}$$

where y_0 is the concentration corresponding to the expected response of a blank (sample without analyte) and SD is the standard deviation of the blank. The equation is based on that

there are two risks of errors when samples are analyzed at zero or low concentrations; false positive risk that sample without analyte contains analyte and false negative that a sample containing analyte is a blank. For a given concentration, c the risk of making a false positive or false negative error has an acceptance limit of 5 %, i.e. 95 % confident that the sample can be distinguishable from blank. The concentration, c is described in Equation vii.

$$c = y_0 + 3.28SD \quad \text{(vii)}$$

However in this equation, 3.28 is rounded to 3, which means the risk of making a false negative and false positive error is 7 %. An estimation of Equation vii is to set y_0 equal to 0 or equal to the intercept of the linear regression of the method, hence the intercept will reflect the signal when the concentration of the analyte is zero (16). Equation viii describes the equation for LOQ.

$$LOQ = y_0 + 10SD \quad \text{(viii)}$$

LOD and LOQ can also be determined by linear regression. A prerequisite for this method is that the instrument response is linear related to the standard concentration. LOD and LOQ are calculated from Equation ix and x respectively,

$$LOD = \frac{3S}{b} \quad \text{(ix)}$$

$$LOQ = \frac{10S}{b} \quad \text{(x)}$$

where S is standard deviation of the response and b is the slope of the calibration curve. The standard deviation can be estimated by standard deviations of y-residuals or y-intercepts of the regression line (17, 18).

Signal to noise determination to determine LOD is an empirical approach where low concentrations of analyte are tested and compared to blank injections. Peak to peak noise is measured around the retention time of the analyte and is compared to the peak height. LOD and LOQ is the concentration that results in a response where the signal to noise ratio is equal to three and ten respectively. According to European Pharmacopeia, signal to noise ratio is calculated by Equation xi, (15)

$$\frac{S}{N} = \frac{2H}{h} \quad \text{(xi)}$$

where H is the peak height, h is peak to peak background noise observed over a distance equal to 20 times the width of the peak at half length.

In the target relative standard deviation (RSD) approach, LOQ is determined by precision studies. LOQ in this method is defined as the amount of the analyte that can be determined with a precision equal to a specific RSD value, typically 10 %. The difference between this method and previous described methods is that the precision of the measurement can be

considered sufficient at LOQ. With this method LOD can be determined as three tenths of LOQ. The RSD value is determined depending on the requirements of the method. Six different amounts of analyte are analyzed close to the target RSD value with six injections at each level. The RSD values are plotted against the amount analyte with Equation xii and LOQ is calculated from the obtained curve (17, 19).

$$RSD = level * p_1^{(1-p_2*(level))} \quad \text{(xii)}$$

2.7 Chemometrics

Chemometrics is a statistical and mathematical tool used to extract information from large amount of chemical data and to design experiments in a way where maximum amount of information is gained with few performed experiments (20).

2.7.1 Experimental design

Design of experiments (DOE) is used to plan and perform experiments that are representative for a given objective. Depending on the purpose of the experimental design different approaches may be relevant. Screening, optimization and robustness testing are three areas where DOE is applicable. In a screening design, the most influential factors are recognized as well as their appropriate range. In addition, if the experimental design is not reduced, information about interactions between factors can be observed. In this design, relatively few experiments are performed, and result in information about many factors. Optimization can be used after screening, when the important factors have been identified. The aim in such design is to identify combinations of important factors to achieve optimal operational conditions. Here, the question is how influential the important factors are. When performing optimization, more experiments are required per factor compared to the screening design. Lastly, robustness testing is used to discover how the response is influenced by small changes or fluctuations on factor levels, e.g. representing day-to-day variation of a system (21).

In experimental design, different factors are varied and the variations of factors are correlated to changes in response. For each factor, two or three levels are normally investigated, which will make up the experimental domain. The experiments are performed according to a predefined plan in a randomized order to minimize systemic errors. In many cases, experiments are formed around a standard reference, often a center point, and experimental points are thereafter built around the center point. Often two or three experiments are performed in the center point of the defined design domain to investigate the repeatability of the experiment and investigate possible curvature in the response (20, 21).

In a full factorial design, which forms the basis of DOE, all possible combinations of factor levels are performed. If K is the number of factors and two levels of each factor are to be investigated, the number of experiments will be 2^k. When several factors are of interest, a full factorial design is both time and resource consuming. In these cases, a fractional factorial design is possible, thus the number of experiments is reduced. When reducing experiments,

some factor effects are confounded, which means that the factor effects are not completely free from one another (20, 21).

The data obtained from the experiments is analyzed in a regression analysis that describes how the change of the factors will affect the response. Multiple linear regression (MLR) and partial least squares (PLS) are example of two linear regression models (21).

2.7.2 Multiple linear regression and partial least squares

Multiple linear regression (MLR) is a useful method to evaluate DOEs. MLR can be used to fit data to a model and the response can be described by a polynomial function seen in Equation xiii,

$$Y = \beta_0 + \sum \beta_i x_i \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j + \epsilon \qquad \text{(xiii)}$$

where $x_i ... x_k$ are the factors in the model and $\beta_i \ldots \beta_k$ are regression coefficients estimated by the MLR model. $\beta_{ij} x_i x_j$ is the interaction between factors, $\beta_{ii} x_i^2$ describes curvature in the model and ϵ is the random error (20). The regression coefficients are used to describe how influential the factors are. A drawback of MLR is that the model assumes that the variables are uncorrelated (22). In the MLR model a separate regression model is made for each response (20).

Partial least square analysis (PLS) is a multivariate linear regression method, which can be described by the same polynomial as the MLR in Equation xiii. PLS is useful when several responses in the data correlate, thus the model fit all responses simultaneously to variations of the factors in one single regression model. The PLS method, compared to the MLR method small amounts of missing data (<10 %) is accepted as long as it is in a random order. In a MLR, experiments that have missing values are discarded (22).

2.7.2.1 Predictive power and measure of fit

In MLR and PLS a predictive power, Q^2 can be estimated. Predictive power is a measurement of how well the model can predict the outcome in a new experiment, i.e. the variation in response that can be predicted by the model. A large Q^2 indicates that the model is good at predicting the response for a new experiment with small predictive errors. The Q^2 is correlated to the measure of fit, R^2, which is an estimation of the variation in response that can be explained by the model. In a good model both R^2 and Q^2 are close to 1. Insignificant terms in the model can be removed to obtain a model with a R^2 and Q^2 value closer to one (21).

3 Experimental

3.1 Chemicals

Tetrahydrofuran (THF, > 99.9 %, gradient grade for HPLC), methanol (MeOH, > 99.9 %, gradient grade for HPLC), sodium chloride (NaCl, ≥ 99.8 %, ACS reagent), sodium azide (NaN_3) were obtained from Sigma Aldrich (St. Louis, USA). Formic acid (FA, > 98 %, reagent grade) was supplied from Scharlau Chemie S.A (Barcelona, Spain). Sodium hydroxide solution (NaOH, 50-55 % in H_2O, for HPLC) was obtained from Fluka Analytical (Buchs, Switzerland). Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4*H_20$) was obtained from EMSURE Merck (Darmstadt, Germany). Di-sodium hydrogen phosphate dehydrate ($HNa_2O_4P*2H_2O$) was obtained from Applichem Panreac GmBh (Darmstadt, Germany). Purified water was prepared by a MilliQ water purification system from Merck Millipore (Billerica, USA).

Polymers used in this study were hydroxypropyl cellulose (HPC-SSL) obtained from Nisso Nippo Soda Co ltd. (Tokyo, Japan, No 39787555, batch NIH-2211) and Pluronic F108 prill from BASF Corporation (New Jersey, USA, No 583062). Further, two different polyethylene glycol (PEG) with different molecular weight were used, PEG 6000 obtained from ProSynth (Suffolk, England) and PEG 20.000 (standard for GPC) from Sigma Aldrich (St. Louis, USA, Lot BCBB1135 81298-1G). Five different polystyrene standards obtained from TOSOH Corporation (Tokyo, Japan) and five different pullulan standards from Showa Denko (Kawasaki, Japan) were used and their respective molecular weights can be seen in Table 1.

Table 1. Molecular weight for polystyrene and pullulan standards.

Name	Molecular weight [g/mole]
Polystyrene standards:	
- A-500 (TS-140)	5970
- F-1 (TS-508)	10400
- F-2 (TS-504)	18100
- F-10 (TS-144)	96400
- F-20 (TS-140)	190000
Pullulan standards:	
- P-5	5900
- P-10	11800
- P-50	47300
- P-100	112000
- P-200	212000

3.2Methods

In the following section, the methods and instruments used in this master thesis work are presented.

3.2.1 Size exclusion chromatography with refractive index detector

The experiments were performed on a HPLC system from Schimadzu Corporation (Kyoto, Japan) equipped with a LC-20AD pump and auto injector SIL-20A with a 500 µl sampling loop. A Millipore VM filter with pore size 0.05 µm was mounted in a filter holder between

the pump and the auto injector. The detector was an Opitilab T-rEX refractive index detector from Wyatt Technology Corporation, (Santa Barbara, USA). The temperature in the detector cell was 25 °C. The rinsing liquid for the auto sampler and needle wash consisted of 0.02 % (v/v) NaN_3 in MilliQ water for the water based system and THF for the organic based system. An external keystone hot pocket column heater from TermoFisher Scientific (San Jose CA, USA) was used for experiments conducted at temperatures higher than room temperature. The vials used were from Agilent Technologies (Santa Clara, USA), 2 ml clear vials for water based system and dark vials for organic based system. Astra 6.1.2.84 from Wyatt Technology Corporation was the software used for analysis.

3.2.1.1 Columns

In total, six different columns were evaluated, one conventional SEC column and five UHPSEC columns. The conventional SEC column was a TSKgel GMPWXL column with particle size 13 µm, dimensions 7.8 x 300 mm (inner diameter and length) and pore size 100 - 1000 Å obtained from TOSOH BIOSCIENCE GmbH (Darmstadt, Germany). The UHPSEC columns used were all obtained from Waters Corporation (Milford, USA). Acquity APC XT 200 for organic mobile phases and Acquity APC AQ 200 for water based mobile phases both with particle size 2.5 µm, pore size 200 Å, dimensions 4.6 x 150 mm were explored. In addition, two Acquity UPLC BEH SEC columns originally developed for analysis of peptides and proteins were tested, both with particle size 1.7 µm and dimensions 4.6 x 150 mm, Acquity UPLC BEH 125 SEC and Acquity UPLC BEH 200 SEC. These columns have pore size 125 Å and 200 Å respectively. The recommended operational parameters for each column are seen in Appendix 1.

3.2.1.2 Refractive index increment

The refractive index increment (dn/dc) used in PBS was 0.143 ml/g for HPC (23), 0.133ml/g for Pluronic F108 (24), 0.134ml/g for PEG 6000 (25), and 0.147 ml/g for pullulan (23). For polystyrene in THF dn/dc value was set to 0.1680 ml/g for A-500, 0.1828 for F-1 and F-2. For F-10 and F-20 the dn/dc value was 0.1845 ml/g (26). For HPC and Pluronic F108 in THF, the dn/dc was 0.0715 ml/g and 0.0603 ml/g respectively, which were determined experimentally.

3.2.1.3 Preparation of mobile phases and samples

All water based mobile phases were vacuum filtrated through a GSWP filter with pore size 0.22 µm followed by sonication in water bath for 20 minutes. The following water based mobile phases were prepared: 1) Phosphate buffer saline (PBS) consisted of a total concentration of 10 mM Na-phosphate (from 31.1 g $NaH_2PO_4*H_2O$ and 135.8 g $HNa_2O_4P*2H_2O$ in 25 liter H_2O), 135 mM NaCl and 0.12 mM NaOH pH 7.4 in MilliQ water, 2) 50 mM NaCl + 0.055 % (v/v) FA in MilliQ water pH 2.47, 3) 40 mM NaCl + 20 % (v/v) MeOH + 0.044 % (v/v) FA pH 2.82 in MilliQ water. THF was kept in a dark container and was used as mobile phase for the organic based system.

Stock solutions were prepared by weighing the samples on a microbalance and the samples were transferred into volumetric flasks. The volumetric flasks were filled to 2/3 of the total

volume with solution and left stirring one day before use. The sample was thereafter diluted to the total volume. If not noted, analytes were dissolved in the same solvent as the mobile phase. The volumetric flasks were not smaller than 25 ml and the minimum weight of the analyte was 5 mg. When dilutions were made, more than 0.5 ml was taken from stock solution and added to volumetric flasks with a minimum volume of 25 ml. Samples prepared with THF were protected from light.

3.2.2 Method development size exclusion chromatography

Below the experimental conditions for the evaluation of the six columns are described. In all experiments polystyrene F-2 for organic based system and pullulan P-50 for water based system were used at regular intervals as reference.

3.2.2.1 TSKgel GMPWXL column

Evaluation of the TSKgel GMPWXL column was performed using pullulan standards, HPC, Pluronic F108, and PEG 20.000, each 1 mg/ml with PBS as eluent. Flow rate was 0.5 ml/min, collection duration 30 minutes and injection volume 50 µl.

3.2.2.2 Acquity APC AQ 200 column

Evaluation of the Acquity APC AQ 200 column was performed with three different eluents: PBS, 50 mM NaCl + 0.055 % (v/v) FA, and 40 mM NaCl + 20 % (v/v) MeOH + 0.044 % (v/v) FA. The first and second mobile phases were used to elute pullulan standards P-5, P-10, P-100 and P-200, 1 mg/ml dissolved in PBS with flow rate 0.5 ml/min. HPC, Pluronic F108, PEG 20.000, 1 mg/ml were also analyzed in these two mobile phases. HPC, Pluronic F108 and PEG 20.000 were eluted with the second mobile phase also with elevated column temperatures 40 and 45 °C. In the third mobile phase, Pluronic F108, HPC and PEG 6000 each 1 mg/ml were analyzed with flow rate 0.3 ml/min. The collection duration on this column was 10 minutes and injection volume was 20 µl.

3.2.2.3 Acquity UPLC BEH 200 SEC and Acquity UPLC BEH 125 SEC columns

Evaluation of the Acquity UPLC BEH 200 SEC column was performed using pullulan standards, HPC, Pluronic F108, and PEG 6000, each 1 mg/ml with two mobile phases: PBS and 50 mM NaCl + 0.055 % (v/v) FA. Flow rate was 0.4 ml/min, collection duration 20 minutes, and injection volume 20 µl. Evaluation was also performed with column temperatures 40, 50 and 60 °C.

3.2.2.4 Acquity APC XT 200 column

Evaluation of the Acquity APC XT 200 was performed using polystyrene standards, HPC, and Pluronic F108, each 1 mg/ml with THF as eluent. Two different flow rates were evaluated; 0.5 ml/min and 1 ml/min. Collection duration was 10 minutes and injection volume 20 µl.

3.2.2.4.1 Determination of dn/dc values in THF

Dn/dc values for HPC and Pluronic F108 in room temperature with THF as eluent was determined by the mean value of three repeated runs. The column was disconnected from the HPLC system so that the mobile phase went directly from the pump to the RI-detector. The

auto injector injected 500 µl of six different concentrations between 1 mg/ml and 0.1 mg/ml of Pluronic F108 and HPC respectively dissolved in THF. For exact concentrations see Appendix 2. The flow rate was 0.5 ml/min. The Astra 6.1.2.84 software template "batch (determine dndc)" was used as method to determine the dn/dc value. When data had been collected, peaks were defined by the flat plateau region and associated with their respective concentration. From the defined peaks and known concentrations of HPC and Pluronic F108 the software calculated the dn/dc value.

3.2.3 Limit of quantification and limit of detection

LOQ was determined with the targeted RSD approach for both peak height and peak area with a target RSD of 10 %. Six injections of each quantity level were performed. Data was fitted to $RSD = level * p_1^{(1-p_2*(level))}$ using SigmaPlot 13.0 (San Jose, USA). LOD was reported as three tenths of the LOQ.

For the TSKgel GMPWXL column LOQ and LOD was determined with PBS as eluent for PEG 6000, HPC and Pluronic F108. The flow rate was 0.5 ml/min and the injection volume was 50 µl. The column was in room temperature and the collection duration was 30 minutes. LOQ and LOD were determined for HPC in PBS with the Acquity UPLC BEH 200 SEC column. The flow rate was 0.4 ml/min (maximum) and samples were injected with a volume of 20 µl. The collection duration for the auto injector was 15 minutes and the column temperature was 40 °C to reduce the back pressure generated by the column. For the Acquity APC XT column, LOQ with THF as mobile phase was determined with flow rate 0.5 ml/min, samples were injected with a volume of 20 µl and collection duration was 10 minutes.

3.2.4 Screening design

A randomized full factorial 2^4 experimental design was performed and evaluation of results was carried out with the software MODDE 10.0 and Simca 13.02.0 (Umetric, Umeå, Sweden). The experiments were performed on an Acquity UPLC BEH 200 SEC column with HPC. The response variables were HPC peak area, peak height, peak shape, and back pressure generated. To evaluate the peak shape, the ratio of the time between where the peak begins to the retention time and the time from the retention time to where the peak ends were obtained i.e. tailing divided by fronting, see Appendix 6. Factors were column temperature (22 °C - 40 °C), concentration of NaCl in the mobile phase (10 – 200 mM), pH in the mobile phase (2.4 -5.6), and injection volume (5 – 20 µl). pH was adjusted with FA. In addition, three experiments were performed in the center point (column temperature 30 °C, 50 mM NaCl, pH 3.8, and injection volume 10 µl) to evaluate reproducibility. HPC was diluted in the same concentration of NaCl as the mobile phase. The concentration of HPC was adjusted so the same mass was injected, 0.2 µg. The flow rate was 0.4 ml/min and collection time 20 minutes. The samples were injected twice and mean values of the response were calculated and used for analysis. The design can be seen in Appendix 3.

4 Results and Discussion

In the following section results and discussion from the evaluation of different UHPSEC columns (Acquity APC AQ, Acquity APC XT, and Acquity UPLC BEH SEC) are presented, using HPC, Pluronic F108, PEG 6000 and PEG 20.000 as model compounds. The results obtained were compared with the results obtained on a conventional TSKgel GMPWXL. Furthermore, LOQ and LOD for some of the model compounds on the different columns were determined. In addition, the results from the experimental design on the Acquity UPLC BEH 200 SEC column are presented.

4.1 Evaluation of the conventional column TSKgel GMPWXL

The results obtained from the analysis of pullulan standards, HPC, Pluronic F108 and PEG 20.000 on the conventional TSKgel GMPWXL column are presented below. PBS was used as mobile phase with flow rate 0.5 ml/min and injection volume of 50 µl.

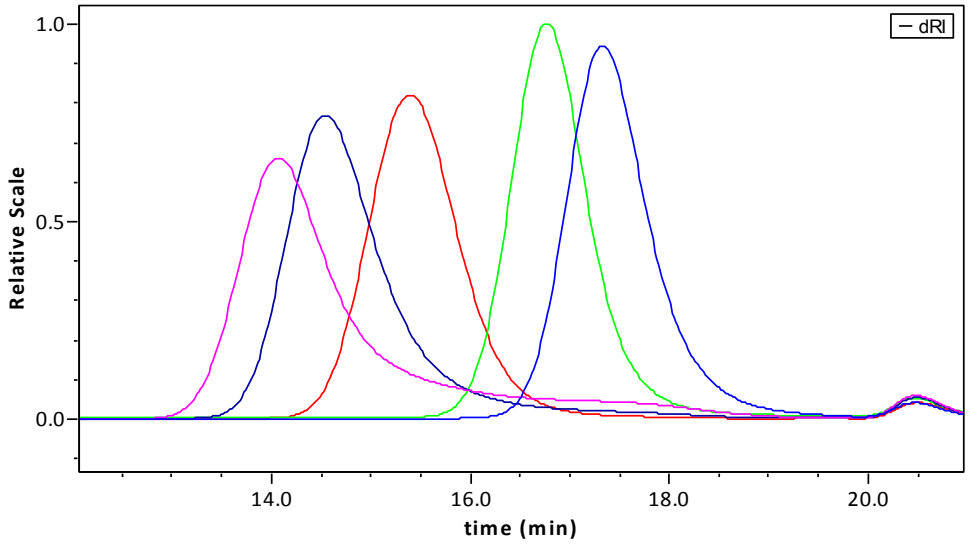

Figure 2. Overlaid RI chromatograms of five pullulan standards from left; P-200, P-100, P-50, P-10 and P-5. System peak at 20.5 minutes. Mobile phase was PBS with flow rate 0.5 ml/min and the column was TSKgel GMPWXL. Injection volume was 50 µl.

Overlaid RI chromatograms of pullulan standards, P-5, P-10, P-50, P-100 and P-200 are presented in Figure 2. The pullulan standards, with molecular weights between 5900 - 212000 g/mol, had retention times between 14.1 and 17.3 minutes. Pullulans with the lowest molecular weights (P-5, P-10 and P-50) resulted in symmetrical Gaussian shaped peaks, however the two larger molecular weight pullulans (P-100 and P-200) had light tailing.

In Figure 3, overlaid RI chromatograms of HPC, PEG 20.000 and Pluronic F108 separated on the TSKgel GMPWXL column are visualized. The retention time for HPC was 15.9 minutes. HPC is more polydisperse, hence generated broader peak. PEG 20.000 and Pluronic F108 generated narrower peaks with retention times 15.5 and 16.2 minutes, respectively.

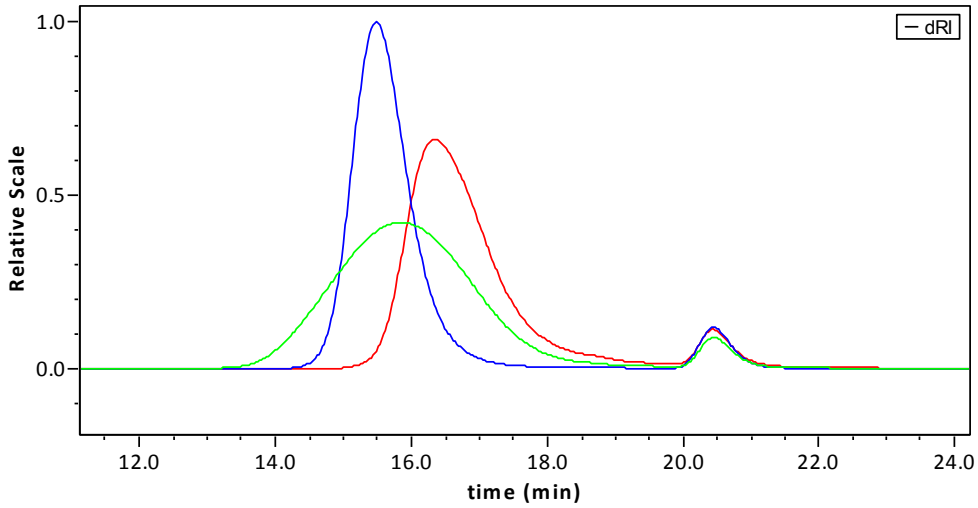

Figure 3. Overlaid RI chromatograms of HPC (green), PEG 20.000 (blue) and Pluronic F108 (red). System peak at 20.5 minutes. Mobile phase was PBS with flow rate 0.5 ml/min and the column was TSKgel GMPWXL. Injection volume was 50 µl.

For the lower molecular weight pullulans, PEG 20.000, HPC and Pluronic F108 respectively, PBS can be used as mobile phase on the TSKgel GMPWXL column. For pullulan standards with molecular weights 112000 and 212000 g/mol with, the use of PBS as mobile phase, resulted in peak tailing on this column. The polymers tested were well separated from the system peak.

4.2 Evaluation of the UHPSEC column Acquity APC AQ 200

The results obtained from the analysis of pullulan standards, HPC, Pluronic F108 and PEG 20.000 on the UHPSEC column Acquity APC AQ are presented below. Injection volume was 20 µl.

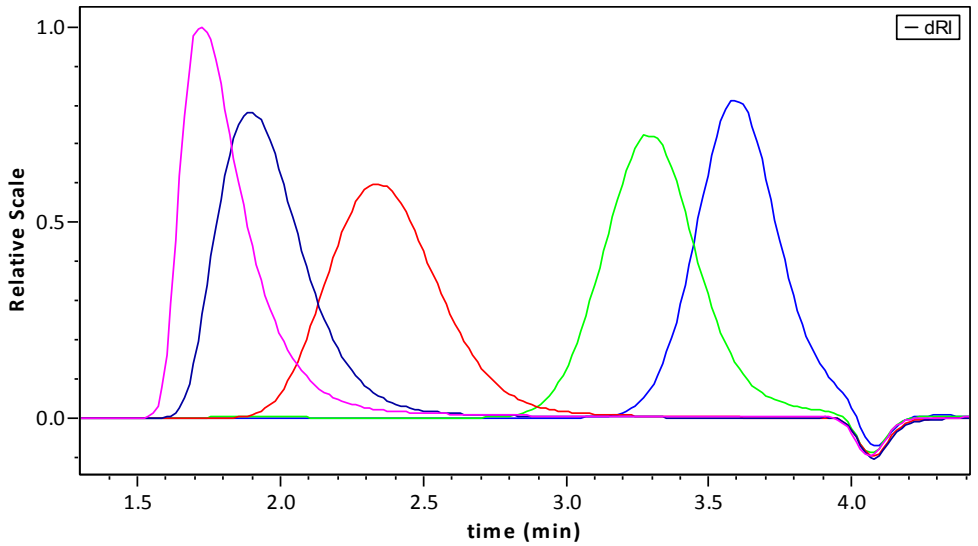

Figure 4. Overlaid RI chromatograms of five pullulan standards from left; P-200, P-100, P-50, P-10 and P-5. System peak at 4.1 minutes. Mobile phase was PBS with flow rate 0.5 ml/min and the column was Acquity APC AQ 200. Injection volume was 20 µl.

In Figure 4, overlaid RI chromatograms of pullulan standards; P-5, P-10, P-50, P-100 and P-200 separated on an Acquity APC AQ 200 column are presented and mean mass recoveries

of two replicates of each standard can be seen in Table 2. The mobile phase was PBS with flow rate 0.5 ml/min. Pullulan standards eluted from the column with retention times between 1.7 and 3.6 minutes. Pullulan A-500 with molecular weight 5970 g/mol interfered with the system peak thus is not optimal for analysis on this column with PBS as mobile phase.

Table 2. Mean mass recoveries of five pullulan standards separated on Acquity APC AQ 200 column with PBS as mobile phase and flow rate 0.5 ml/min (n=2).

	P-5	P-10	P-50	P-100	P-200
Mass recovery %	90 %	91 %	91 %	90 %	89 %

Under the same conditions Pluronic F108 (2, 1, 0.5, 0.25 mg/ml), HPC (2, 1, 0.5, 0.25 mg/ml), and PEG 20.000 dissolved in PBS did not elute from the Acquity APC AQ column, see Figure 5. Pullulan P-50 eluted from the column and is presented as a peak in the chromatogram. In an attempt to elute these model compounds from the Acquity APC AQ 200 a number of chromatographic conditions were tested. The flow rate was reduced to 0.2 ml/min from 0.5 ml/min still, HPC, Pluronic F108, PEG 20.000 did not elute from the Acquity APC AQ 200 column (chromatograms not shown). A more acidic mobile phase was tested, 50 mM NaCl + 0.055 % (v/v) FA, pH 2.47, but the polymers did not elute from the column (chromatograms not shown). With this mobile phase pullulan standards dissolved in PBS resulted in peaks in the RI chromatogram, see Appendix 4.

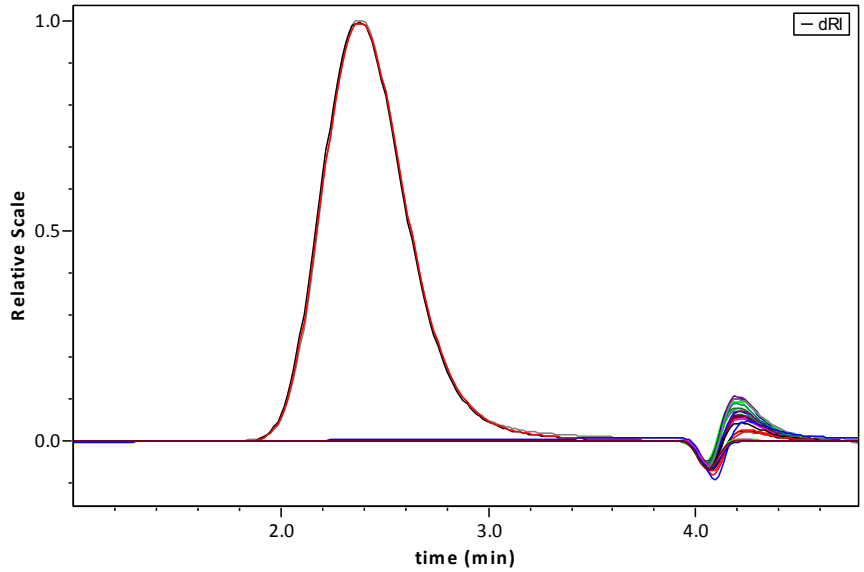

Figure 5. Overlaid RI chromatograms of Pluronic F108 (2, 1, 0.25 and 0.05 mg/ml), HPC (2, 1, 0.25 and 0.05 mg/ml), PEG 20.000 (1 mg/ml) and Pullulan P-50 (1 mg/ml). Mobile phase was PBS with flow rate 0.5 ml/min and the column was Acquity APC AQ 200. Injection volume was 20 µl.

Higher column temperatures, 40 and 45 °C (maximum temperature for the column) were generated with a column oven in an attempt to elute Pluronic F108, PEG 20.000 and HPC with mobile phase 50 mM NaCl + 0.055 % (v/v) FA and flow rate 0.5 ml/min, but without success. Addition of 20 % MeOH to the mobile phase, i.e. a mobile phase consisting of 40 mM NaCl + 20 % (v/v) MeOH + 0.044 % (v/v) FA did not elute the polymers. The flow rate was 0.3 ml/min due to the higher back pressure generated, 14.3 MPa at flow rate 0.3 ml/min. For all the experimental conditions described above pullulan P-50 eluted from the column.

Pullulan standards can be separated with the Acquity APC AQ 200 column with mass recoveries between 89-91 %. However, under the tested conditions, the Acquity APC AQ column was not suitable for separation of Pluronic F108, PEG 20.000 and HPC as the polymers did not elute from the column. Most likely secondary interactions like adsorption of these polymers to the packing material occurred. The packing material in the column is uncapped, which means free silanol groups are disposable on the surface. During analysis the back pressure increased successively, which also indicates adsorption of the polymers to the packing material in the column. Researchers at Waters Corporation have also verified the adsorption of polymers to the column packing material.

4.3 Evaluation of the UHPSEC columns Acquity UPLC BEH SEC

The results obtained from the analysis of pullulan standards, HPC, Pluronic F108 and PEG 6000 on the UHPSEC column Acquity UPLC BEH 200 SEC is presented below. Acquity UPLC BEH 125 SEC was also tested for analysis of PEG 6000.

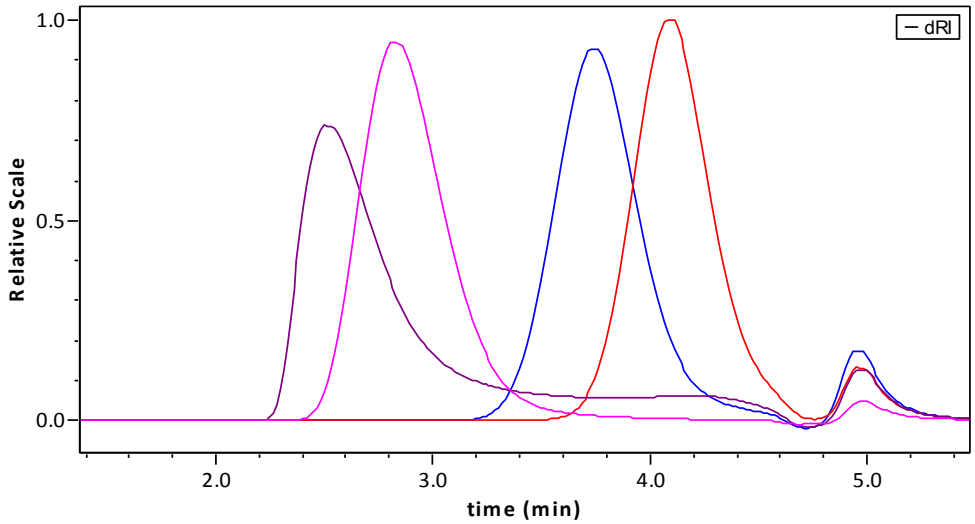

Figure 6. Overlaid RI chromatograms of four pullulan standards from left, P-100, P-50, P-10 and P-5. System peak at 5 minutes. Mobile phase was PBS with flow rate 0.4 ml/min and the column was Acquity UPLC BEH 200 SEC. Injection volume was 20 µl.

The evaluation of the Acquity UPLC BEH 200 SEC was performed at 0.4 ml/min (maximum flow rate for the column) and injection volume was 20 µl. Overlaid RI chromatograms of pullulan standards, P-5, P-10, P-50, P-100 are presented in Figure 6. The pullulan standards, with molecular weights between 5900 - 112000 g/mol, had retention times between 2.5 and 4.2 minutes. Pullulans with the lowest and highest molecular weights (P-100 and P-5) interfered with the system peak and P-10 and P-50 resulted in symmetrical Gaussian shaped peaks. HPC generated a broad peak with tailing on this column with PBS as mobile phase, chromatogram is visualized in Figure 7.

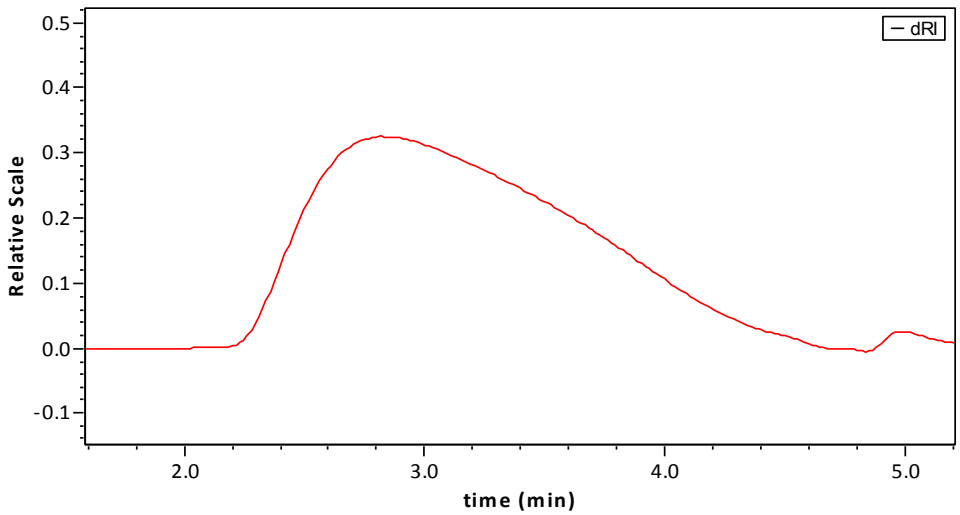

Figure 7. RI chromatogram of HPC. System peak at 5 minutes. Mobile phase was PBS with flow rate 0.4 ml/min and the column was Acquity UPLC BEH 200 SEC. Injection volume was 20 µl.

The UPLC BEH 200 SEC column is packed with smaller particles, 1.4 µm compared to the Acquity APC (2.5 µm) and TSKgel GMPWXL (13 µm). To reduce the back pressure generated by the smaller particles, separation were performed with column temperature 40 °C, which generated a back pressure of 15.1 MPa. When PBS was used as mobile phase, PEG 6000 eluted close to the system peak, see Figure 8.

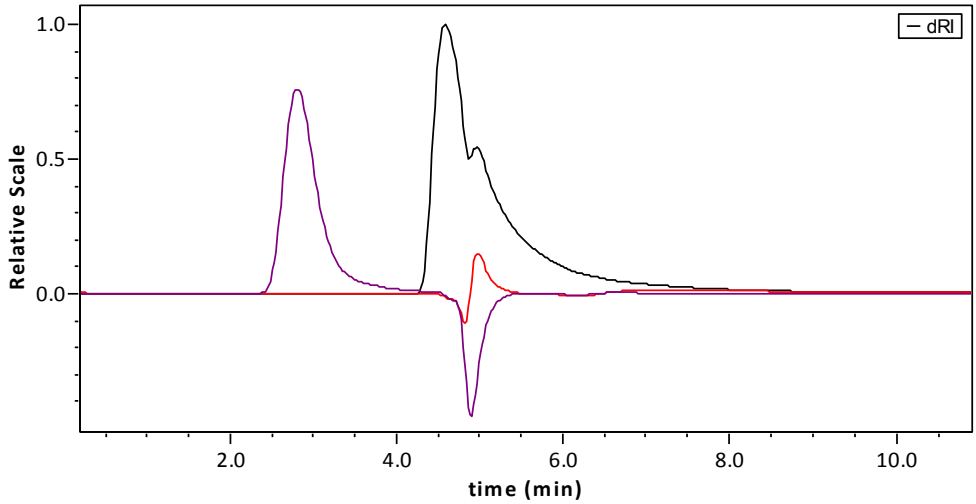

Figure 8. Overlaid RI chromatograms of pullulan P-50 (purple), Pluronic F108 (red) and PEG 6000 (black). Mobile phase was PBS with flow rate 0.4 ml/min and the column was Acquity BEH UPLC 200 SEC. Injection volume was 20 µl and the column temperature was 40 °C.

As PEG 6000 eluted close to the system peak (Figure 8), a column with lower molecular separation range may be suitable for analysis of PEG 6000. The same type of column with pore size 125 was tested, Acquity UPLC BEH 125 SEC. However, due to the small particles (1.7 µm) and small pore size (125 Å) the column generated too high back pressures for the HPLC system when PBS was used as mobile phase. When flow rate was 0.3 ml/min and the column temperature was 40 °C the back pressure was over 20 MPa, which resulted in leakage of the mobile phase at the inlet of the column and at the filter holder. This limitation in the

HPLC system resulted in that no further experiments were performed on this column. Furthermore, as seen in Figure 8, Pluronic F108 was not visible in the chromatogram, i.e. did not elute from the column. When 50 mM NaCl + 0.055 % (v/v) FA was used as mobile phase, Pluronic F108 eluted from the column, see Figure 10. Chromatograms with this mobile phase with HPC and PEG 20.000 are also presented in Figure 9 and Figure 11 respectively.

PEG 6000 eluted in the permeation volume on the Acquity UPLC BEH 200 SEC column and the Acquity UPLC BEH 125 SEC was not suitable for a HPLC instrument as higher back pressures were generated. PBS as mobile phase for separation of Pluronic F108 on the Acquity UPLC BEH 200 SEC column should be avoided, however 50 mM NaCl + 0.055 % (v/v) FA as eluent was suitable. Analysis of HPC was possible in both PBS and 0.055 % (v/v) FA in 50 mM NaCl as mobile phase.

4.3.1 Analysis of HPC, Pluronic F108 and PEG 20.000 at different temperatures on Acquity UPLC BEH 200 SEC

In the section below, analysis of HPC, Pluronic F108 and PEG 20.000 on the UHPSEC column Acquity UPLC BEH 200 SEC at three different column temperatures, 40, 50 and 60 °C is presented. The mobile phase was 50 mM NaCl + 0.055 % (v/v) FA and the flow rate was 0.4 ml/min (maximum flow rate for the column). These chromatograms are shown in Figure 9 (HPC), Figure 10 (Pluronic F108) and Figure 11 (PEG 20.000).

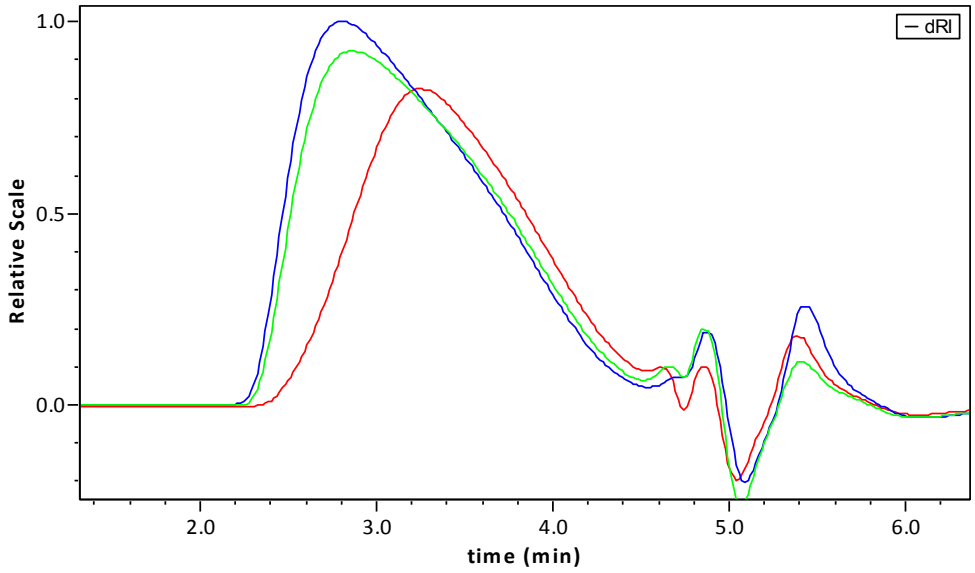

Figure 9. Overlaid RI chromatograms of HPC at three different column temperatures. The blue peak represents 40 °C, green 50 °C and red 60 °C. Mobile phase was 50 mM NaCl + 0.055 % (v/v) FA with flow rate 0.4 ml/min and the column was Acquity UPLC BEH 200 SEC. Injection volume was 20 µl.

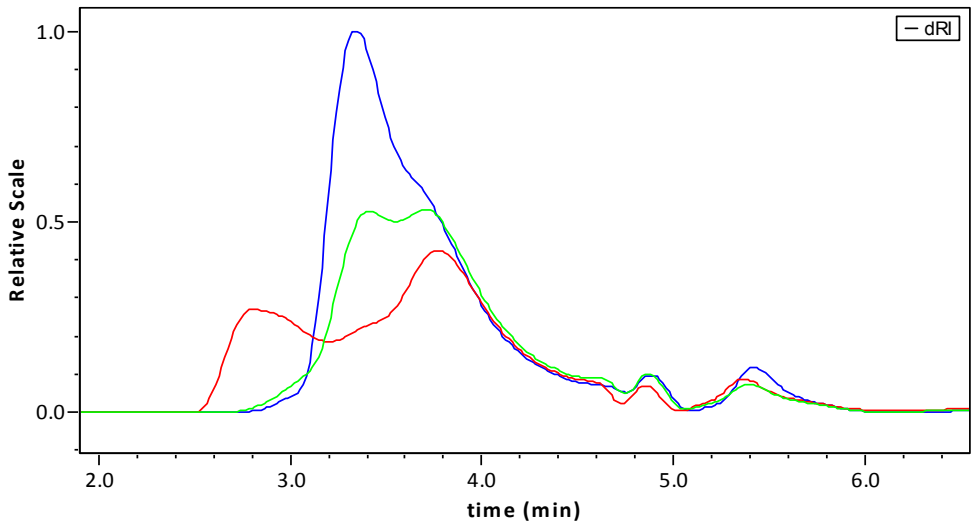

Figure 10. Overlaid RI chromatograms of Pluronic F108 at three different column temperatures. The blue peak represents 40 °C, green 50 °C and red 60 °C. Mobile phase was 50 mM NaCl + 0.055 % (v/v) FA with flow rate 0.4 ml/min and the column was Acquity UPLC BEH 200 SEC. Injection volume was 20 µl.

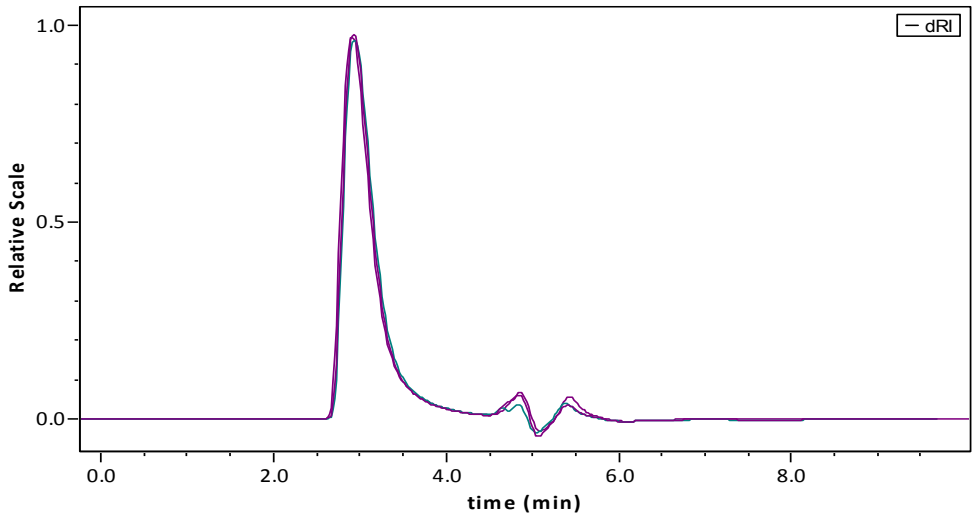

Figure 11. Overlaid RI chromatograms of PEG 20.000 at three different column temperatures; 40, 50 and 60 °C. Mobile phase was 50 mM NaCl + 0.055 % (v/v) FA with flow rate 0.4 ml/min and the column was Acquity UPLC BEH 200 SEC. Injection volume was 20 µl.

Column temperature was affecting the shapes of the peaks for HPC and Pluronic F108. For HPC the peak obtained a Gaussian shape when separation took place at 60 °C, however the height was lower compared to separations at 40 and 50 °C (Figure 9). For Pluronic F108 both peak shape and peak height differed with temperature (Figure 10) whereas the peak shape for PEG 20.000 did not change with temperature (Figure 11).

4.4 Evaluation of the UHPSEC column Acquity APC XT 200

The results obtained from the analysis of polystyrene standards, HPC and Pluronic F108 on the UHPSEC column Acquity APC XT 200 are presented below. THF was used as mobile phase and injection volume was 20 µl.

Overlaid RI chromatograms of five polystyrene standards with molecular weights between 5970 and 190000 g/mol separated on this column with flow rate 0.5 ml/min and 1 ml/min are depicted in Figure 12 and Figure 13. The retention times of polystyrene standards with a flow rate of 0.5 ml/min and 1 ml/min were between 1.9-3.5 minutes and 0.8-1.75 minutes respectively. All polystyrene standards for both flow rates resulted in Gaussian shaped peaks.

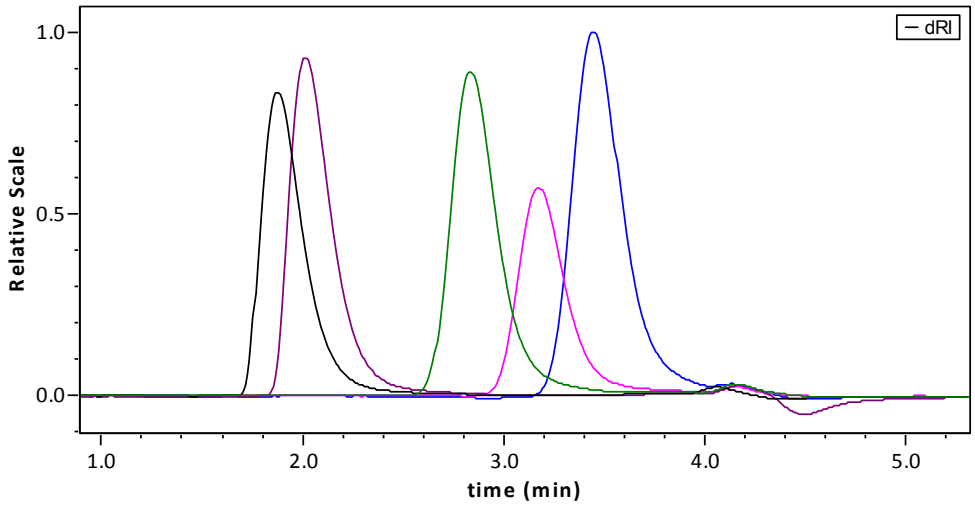

Figure 12. Overlaid RI chromatograms of five polystyrene standards from left F-20, F-10, F-2, F-1 and A-500. System peak at 4.2 minutes. Mobile phase was THF, flow rate 0.5 ml/min and the column was Acquity APC XT 200. Injection volume was 20 µl.

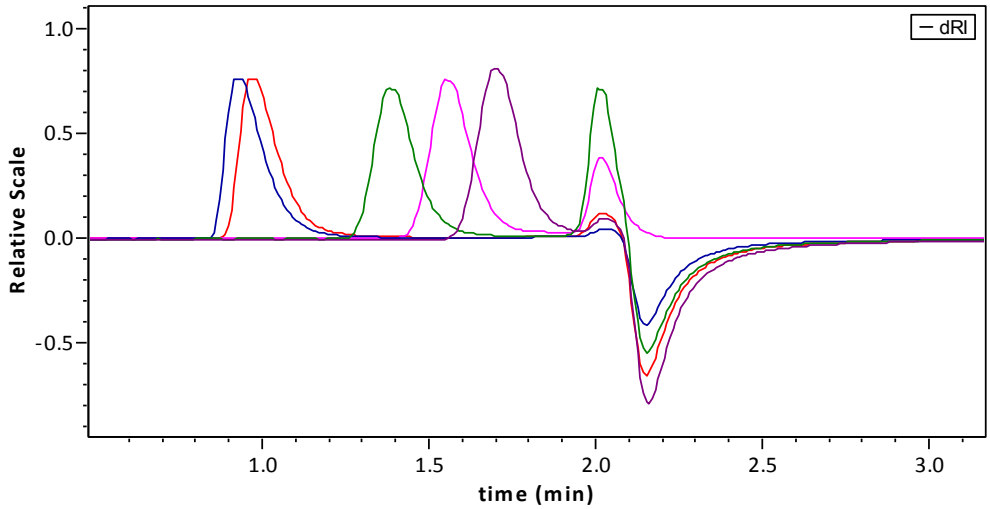

Figure 13. Overlaid RI chromatograms of five polystyrene standards from left F-20, F-10, F-2, F-1 and A-500. System peak at 2 minutes. Mobile phase was THF, flow rate 1 ml/min and the column was Acquity APC XT 200. Injection volume was 20 µl.

Pluronic F108 and HPC were analyzed at flow rates 0.5 and 1 ml/min and are illustrated in Figure 14. Pluronic F108 generated a bimodal peak and HPC generated a broad peak with tailing. The retention times for HPC and Pluronic F108 were 2.1 and 2.6 minutes respectively with flow rate 0.5 ml/min. The retention times were 1.1 and 1.3 minutes respectively with flow rate 1 ml/min. The use of an UHPLC system instead of a HPLC system could result in further decrease in retention times. Back pressure is a limitation in the HPLC system and

therefore the analysis was not performed at the columns maximum flow rate, hence the column can operate at flow rates up to 2 ml/min with THF as mobile phase.

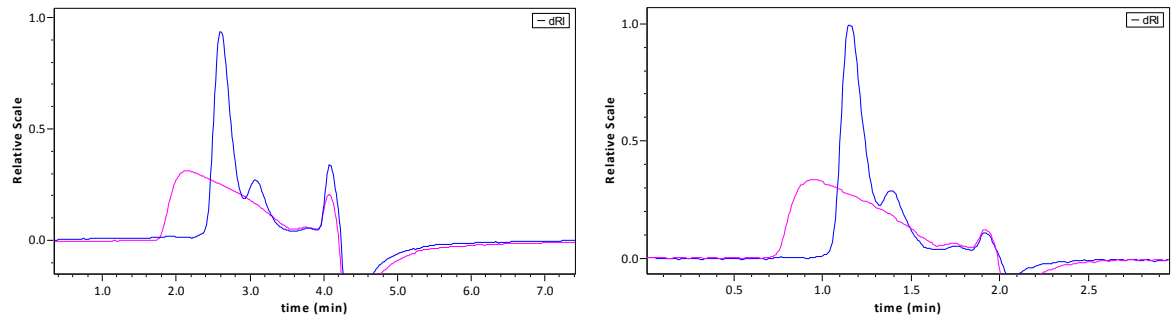

Figure 14. Overlaid RI chromatograms of HPC (purple) and Pluronic F108 (blue). Mobile phase was THF with flow rate 0.5 ml/min (left) and 1 ml/min (right). The column was Acquity APC XT 200. Injection volume was 20 µl.

Mass recoveries for polystyrene standards, HPC, and Pluronic F108 can be seen in Table 3. Mass recoveries were identical for HPC and Pluronic F108 at the two flow rates. The fact that the recovery was over 100 percent indicates that it was hard to determine the best way to integrate the peak. To calculate mass recovery dn/dc values are required. Dn/dc values for HPC and Pluronic F108 in THF could not be found in the literature, thus these values had to be determined experimentally. Dn/dc values for Pluronic F108 and HPC with THF as mobile phase in room temperature was determined to 0.0603 ml/g and 0.0715 ml/g respectively. The obtained chromatograms for the determination of dn/dc can be seen in Appendix 2. For polystyrene standards mass recoveries were lower with the higher flow rate.

Table 3. Mass recoveries of polystyrene standards, Pluronic F108 and HPC with flow rates 0.5 and 1 ml/min.

Sample name	Mass recovery (%) 0.5 ml/min	Mass recovery (%) 1 ml/min
A 500	96	58
F-1	80	74
F-2	107	61
F-10	101	81
F-20	100	90
Pluronic F108	105	105
HPC	100	100

4.5 Limit of quantification and limit of detection

In the following section, the results from the determination of LOQ and LOD based on the targeted RSD approach for the model compounds on the different columns are presented. This method was used for determination of LOQ and LOD as variations in peak height and peak area between repeatable injections with the same mass was observed.

4.5.1 Limit of quantification on the conventional column TSKgel GMPWXL

In Figure 15, the RSD variation in peak height as a function of the quantity of HPC injected onto TSKgel GMPWXL is illustrated. In Appendix 5, corresponding graphs of LOQ determined for PEG 6000 and Pluronic F108, both for peak height and peak area as well as for HPC for peak area, are presented. From these data, LOQ for HPC was 0.24 µg based on

peak height and 0.29 µg based on peak area. For Pluronic F108, the obtained LOQ was 0.16 µg based on peak height and 0.19 µg based on peak area. PEG 6000 obtained the lowest LOQ, 0.06 µg for both peak area and peak height. All obtained LOQ values are summarized in Table 4.

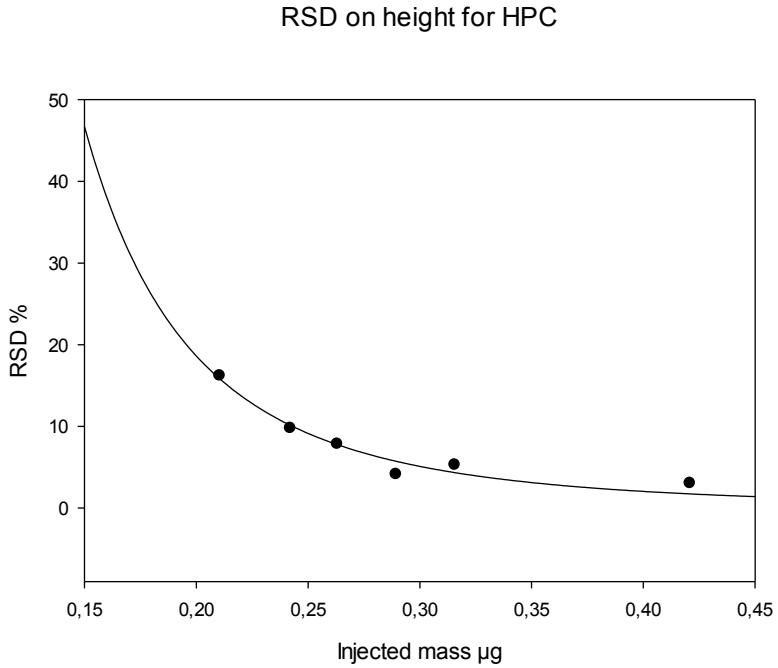

Figure 15. RSD variation in peak height as a function of the quantity of HPC injected onto the TSKgel GMPWXL column.

4.5.2 Limit of quantification on the UHPSEC column Acquity UPLC BEH 200 SEC

In Figure 16, RSD variation in peak height as a function of the quantity of HPC injected onto the Acquity UPLC BEH 200 SEC is illustrated. From the graph, LOQ for peak height was determined to 0.09 µg for HPC. LOQ for HPC based on peak area was determined to 0.08 µg. In Appendix 5, the RSD variation in peak area against the quantity plot is presented and the obtained LOQ values are summarized in Table 4.

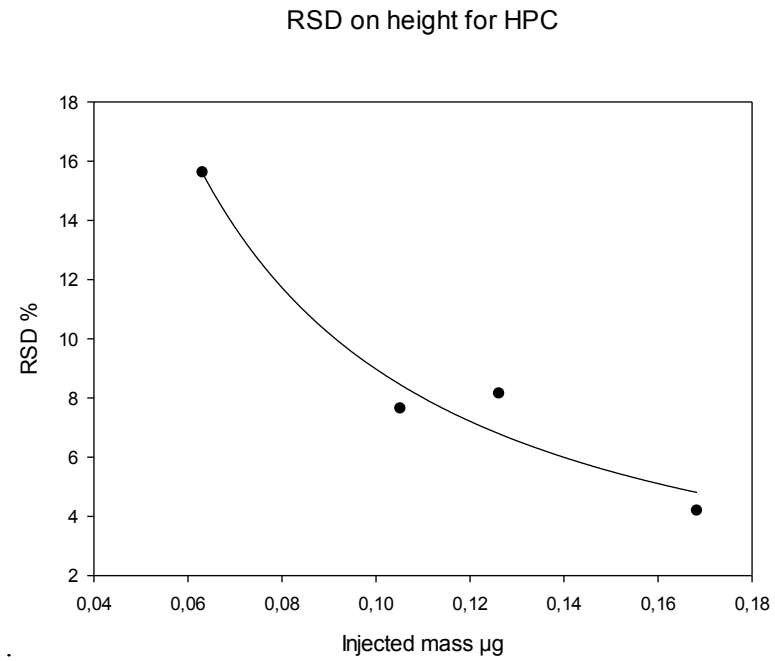

Figure 16. RSD variation in peak height as a function of the quantity of HPC injected onto the Acquity UPLC BEH 200 SEC column.

4.5.3 Limit of quantification on the UHPSEC column Acquity APC XT 200

In Figure 17, the RSD variation in peak height as a function of the quantity of HPC injected onto the Acquity APC XT 200 column is illustrated. From the graph, LOQ based on peak height was 1.22 µg for HPC. LOQ for peak area was 1.54 µg. For Pluronic F108, the obtained LOQ was 0.15 µg based on peak height and 0.26 µg based on peak area. The obtained LOQ values are found graphically in Appendix 5 and in Table 4.

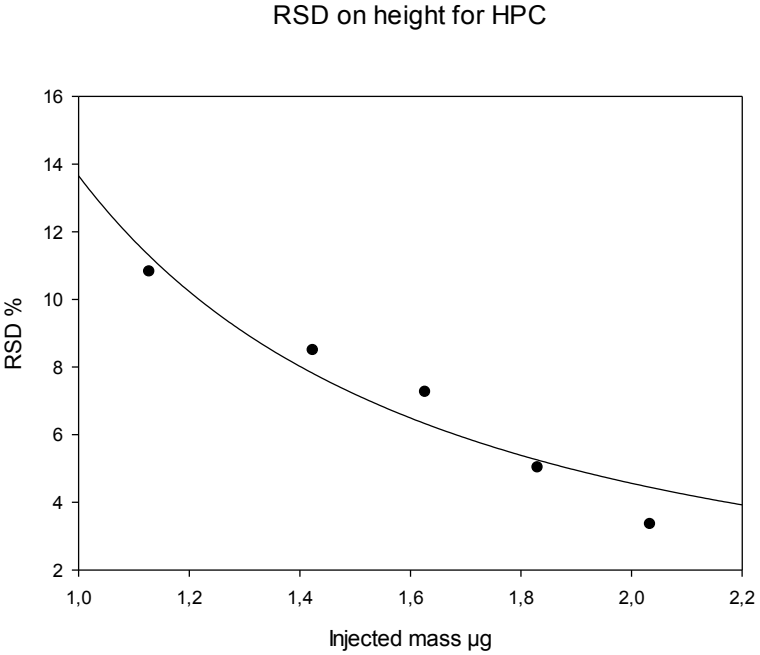

Figure 17. RSD variation in peak height as a function of the quantity of HPC injected onto the Acquity APC XT 200 column.

4.5.4 Summary of limit of quantification

In Table 4, the determined LOQ values for the model compounds on the different columns are summarized. Lowest LOQ for HPC were obtained with the UHPSEC column, Acquity UPLC BEH 200 SEC, based on both peak height and peak area. Lowest LOQ based on peak height for Pluronic F108 was obtained with the UHPSEC column for organic based mobile phases, Acquity APC XT 200. When LOQ was based on peak area however, Pluronic F108 had lowest LOQ with the conventional TSKgel GMPWXL. TSKgel GMPWXL column was preferred for analysis of PEG 6000. PEG 6000 is not soluble in THF and was therefore not tested with the Acquity APC XT 200 column and the Acquity UPLC BEH 200 SEC column has not the right separation range for PEG 6000.

Table 4. Summary of LOQ for peak height and peak area.

Column	Polymer	Mobile phase	LOQ based on height [µg]	LOQ based on area [µg]
TSKgel GMPWXL	HPC	PBS	0.24	0.29
	Pluronic F108	PBS	0.16	0.18
	PEG 6000	PBS	0.06	0.06
Acquity UPLC BEH 200 SEC	HPC	PBS	0.09	0.08
Acquity APC XT 200	HPC	THF	1.22	1.54
	Pluronic F108	THF	0.15	0.26

4.5.5 Limit of detection on the TSKgel GMPWXL, Acquity UPLC BEH 200 SEC, and Acquity APC XT 200

Limit of detection was obtained with the targeted RSD approach and is presented in Table 5. In this method, LOD is reported as LOQ times 0.3.

Table 5. LOD with the targeted RSD approach for respective column and polymer.

Column	Polymer	Mobile phase	LOD based on height [µg]	LOD based on area [µg]
TSKgel GMPWXL	HPC	PBS	0.07	0.09
	Pluronic F108	PBS	0.05	0.05
	PEG 6000	PBS	0.02	0.02
Acquity UPLC BEH 200 SEC	HPC	PBS	0.02	0.02
Acquity APC XT 200	HPC	THF	0.37	0.46
	Pluronic F108	THF	0.05	0.08

4.6 Screening design

A screening design was performed to evaluate factors influencing the following response variables; peak height, peak shape in terms of ratio between tailing and fronting, peak area, and back pressure respectively. The factors tested were column temperature, injection volume, pH and concentration of NaCl in the mobile phase. The effect of run order and day on the performed experiments was also obtained. Both an MLR and PLS model were generated and in these models experiment number 13 was considered as an outlier and therefore discarded from the evaluation. In the MLR model for peak area, experiment number six was also an outlier and discarded.

4.6.1 Multiple Linear Regression model with peak height as response variable

The screening design revealed that temperature was the prominent factor affecting peak height of HPC, followed by injection volume and the concentration of NaCl in the mobile phase. The coefficient plot and contour plot of the MLR model for peak height are visualized in Figure 18. In the ranges tested, low temperature, low injection volume and low concentration of NaCl in the mobile phase resulted in high peak heights. An interaction term between column temperature and injection volume was included in the model, but not of statistical significance (Figure 18). The Q^2 and R^2 were 0.99 and 0.90 respectively, which indicates a good model.

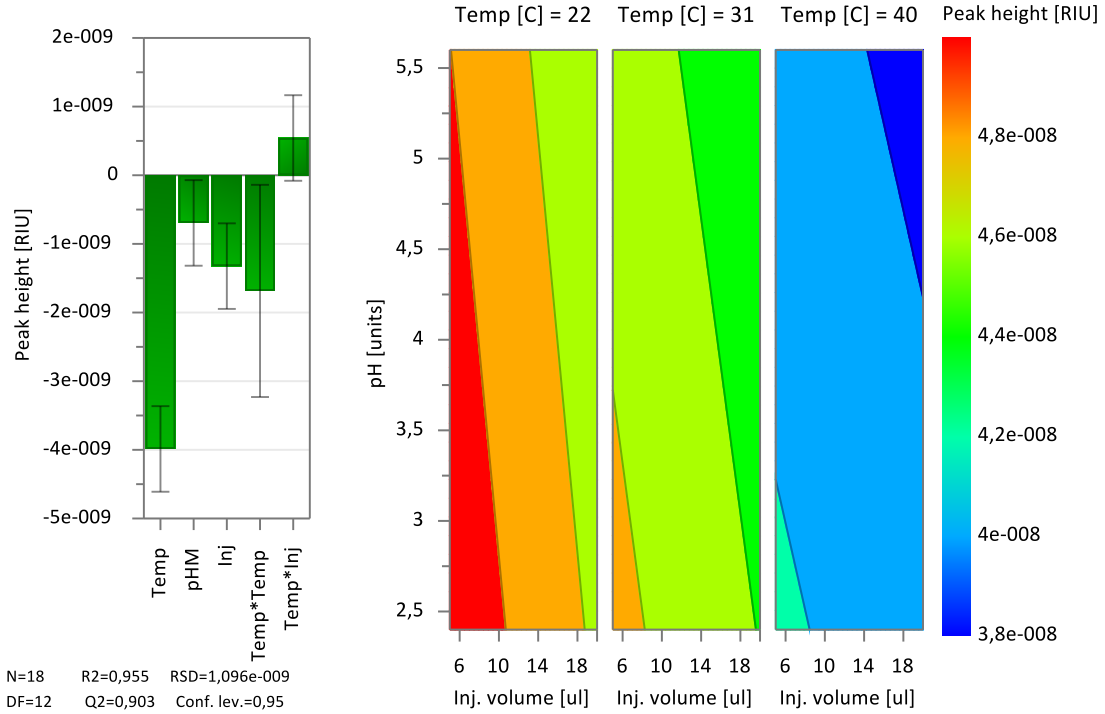

Figure 18. Coefficient plot (left) and contour plot (right) for the MLR model with peak height as response variable. The error bars represent 95 % confidence interval.

4.6.2 Multiple Linear Regression model with peak shape as response variable

To evaluate peak shape of HPC, the time between where the peak begins to the retention time and the time from the retention time to where the peak ends were obtained i.e. fronting and tailing (An example is illustrated in Appendix 6). The ratio of tailing and fronting was calculated and used as a response variable (The retention time is defined as the time at maximum peak height). In the case of a symmetrically distributed peak shape the ratio is expected to be close to 1. In the case of fronting the ratio is below 1 and in the case of tailing above 1. The obtained Q^2 and R^2 values for the MLR model were 0.75 and 0.92.

From the coefficient and contour plot visualized in Figure 19, it is clearly seen that temperature was the most influential factor affecting the ratio between tailing and fronting. Higher temperatures in the selected range tended to have a shape closer to a Gaussian form, but no experimental conditions made the peaks totally symmetrically distributed. Two interaction terms of statistical significance with temperature were also included in the MLR model, namely pH and concentration of NaCl in the mobile phase.

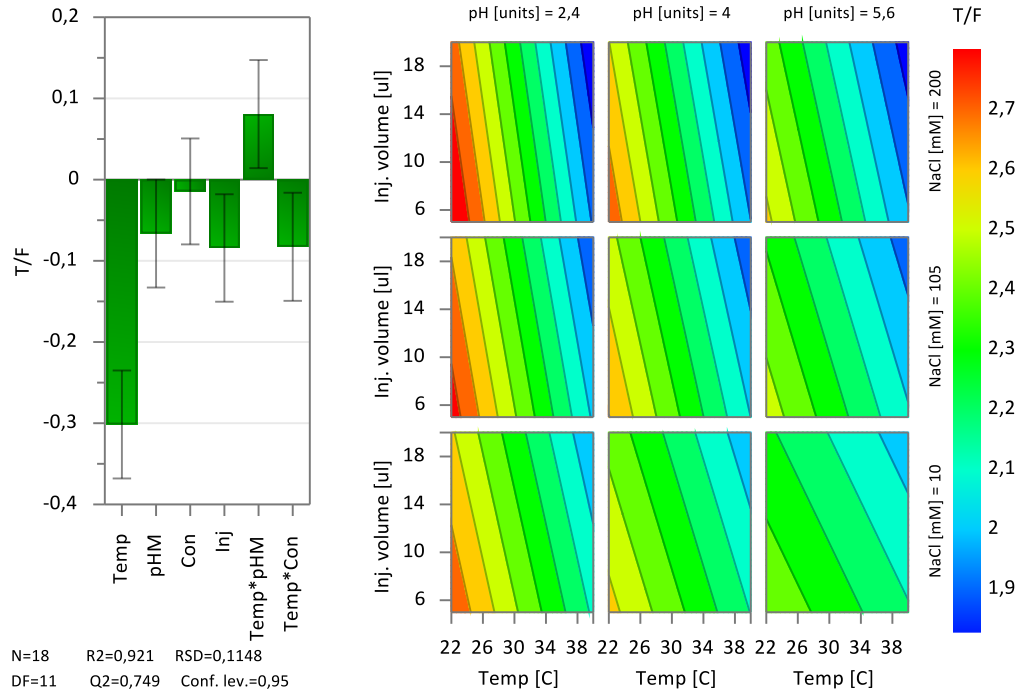

Figure 19. A coefficient plot and contour plot for the MLR model of the ratio between tailing and fronting. The error bars represent 95 % confidence interval.

The interaction plots presented in Figure 20, revealed that the ratio between tailing and fronting in the lower temperature range will decrease with decreasing concentration of NaCl. In opposite, in the higher temperature range, the ratio between tailing and fronting will decrease with increasing concentration of NaCl in the mobile phase. Further, the factor pH in the mobile phase seemed to affect the shape of the peak at the lower temperature range tested, and less effect of the factor pH was observed at the higher temperature range. Last, data showed that injection volume influenced the response, seen in the coefficient and contour plot in Figure 19. Volumes closer to 20 µl compared to 5 µl resulted in more symmetrically distributed shapes of the HPC peak.

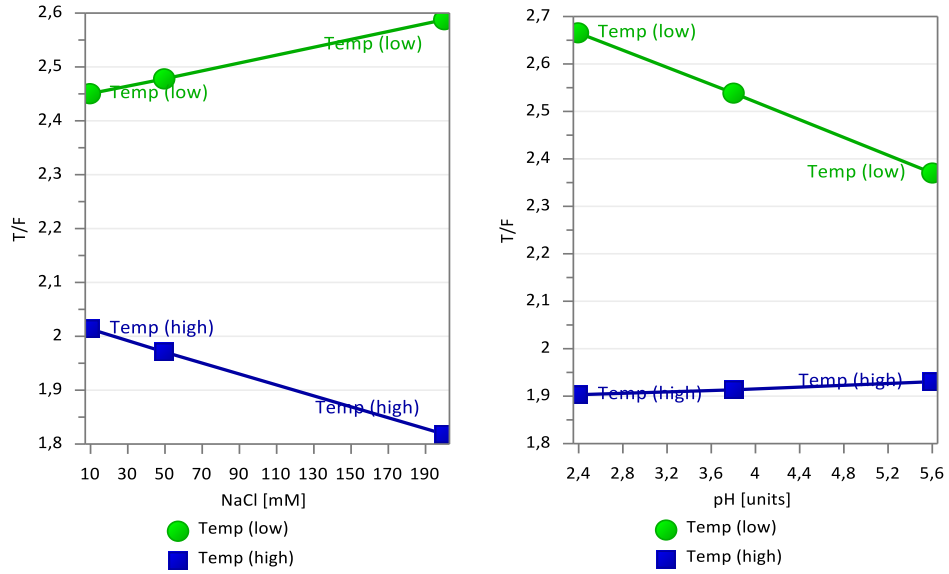

Figure 20. Interaction plot for ratio between tailing and fronting between column temperature and concentration of NaCl in the mobile phase (left) and column temperature and pH in the mobile phase (right).

4.6.3 Multiple Linear Regression model with peak area as response variable

Factors influencing peak area were also investigated and resulted in a MLR model with a Q^2 value of 0.96 and R^2 value of 0.99. The results in form of a coefficient, contour and interaction plot are shown in Figure 21 and Figure 22.

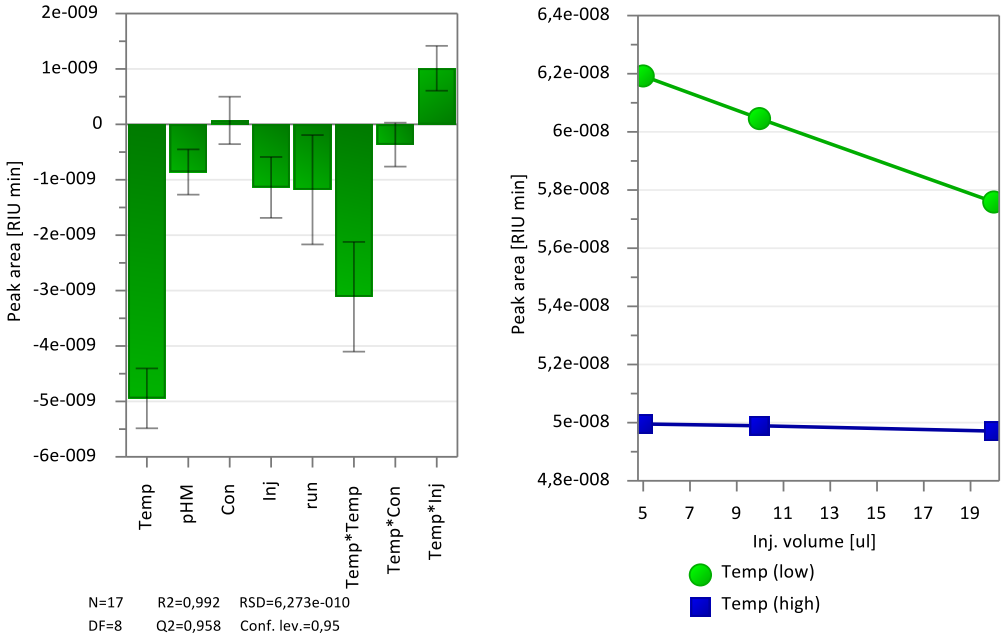

Figure 21. Coefficient plot (left) and interaction plot (right) for the MLR model with peak area as response. Error bars represent 95 % confidence interval. The error bars represent a 95 % confidence interval.

The model contained both a temperature and temperature as a square factor with statistical significance, which reveals that temperature had great influence on peak area. Low temperature correlated with larger peak area. Acidic pH in the mobile phase and low injection volumes in the range tested also seemed to be factors in favour of large peak areas. Run order was also a factor with statistical significance. The performance of the column with respect to peak area seemed to be worse towards the end of experiments performed.

The interaction term between temperature and injection volume had statistical significance in the MLR model. In Figure 21 (right), the interaction plot between these factors is presented. In this figure as well as in Figure 22, it is observed that at the lower temperatures the volume injected influence the peak area more compared to at the higher temperatures where the effect of injection volume seemed to be less.

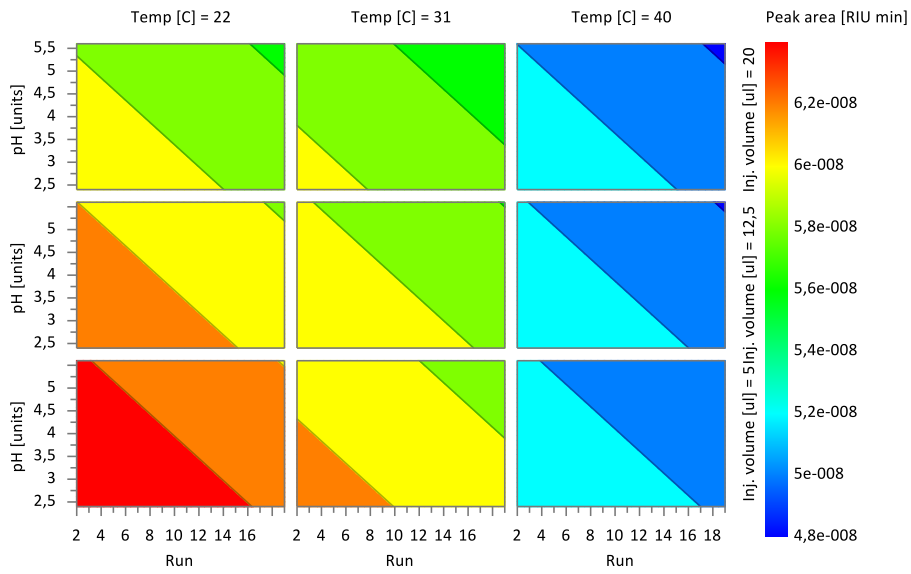

Figure 22. Contour plot for the MLR model with peak area as response.

4.6.4 Multiple Linear Regression model with back pressure as response variable

The last response investigated was back pressure under each experimental condition. The performed designed experiments generated a MLR model with Q^2 and R^2 equal to 0.99 and 0.99 respectively. As illustrated in the coefficient plot in Figure 23, temperature was the only significant factor influencing the pressure. It is clearly seen that higher temperatures resulted in lower back pressures. pH in mobile phase, concentration of NaCl, and injection volume were included in the MLR model but did not influence the pressure significantly.

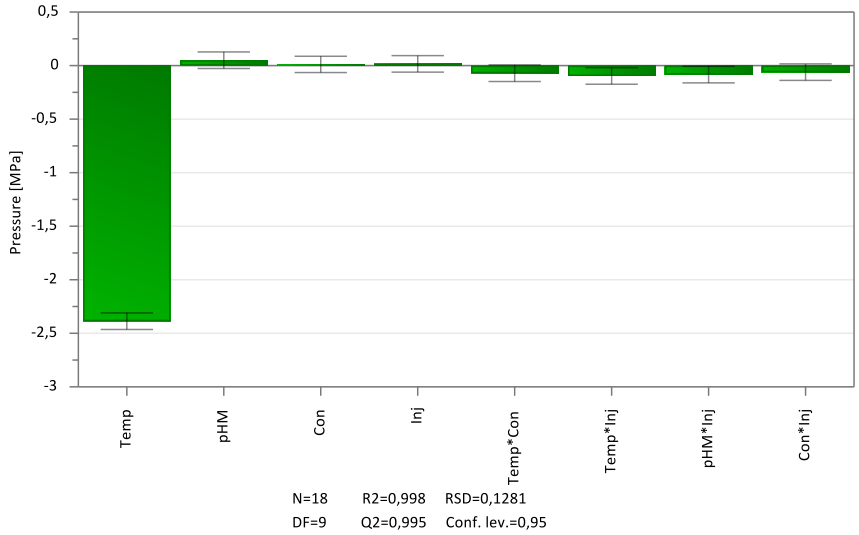

Figure 23. Coefficient plot presenting impact and directionality of factors in the MLR model for back pressure generated in the experiments. The error bars represent 95 % confidence interval.

4.6.5 Partial Least Squares model

In the PLS model, all responses are fitted to variations of the factors in one single regression model. The model generated two components and the Q^2 value was 0.74 and R^2 values were 0.61 and 0.88. In Figure 24, a score plot is presented. The trend in the score plot is that experiments above the x-axis, t(2), had injection volumes of 5 µl and below the x-axis the

injection volume was 20 µl. Left of the y-axis, t(1) experiments was performed at 22 °C and right of the y-axis at 40 °C. The separation of injection volume and column temperature indicates that these factors had large impact on the model.

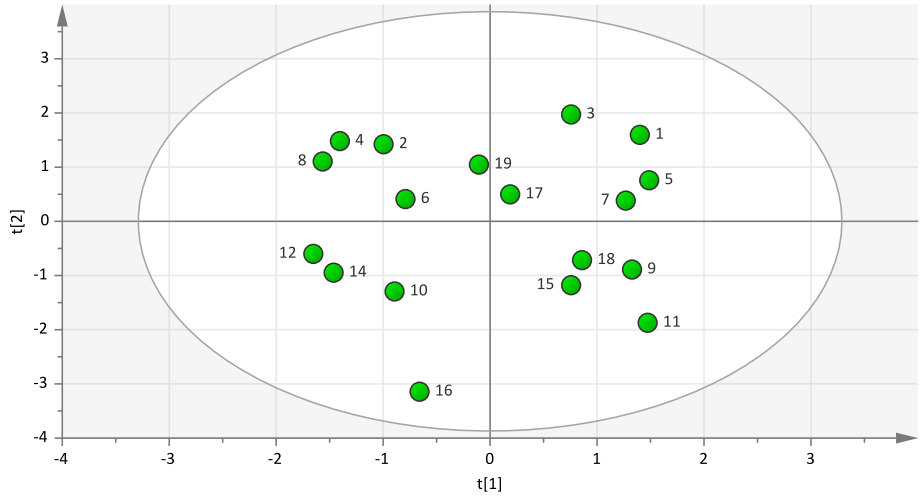

Figure 24. Score plot of experiment 1-19 in the screening design. Experiments 17-19 were center points.

Experiments 17, 18 and 19 were center points and as can be seen in the score plot in Figure 24, two of the center points (experiment 17 and 18) are adjacent to each other, however one is further apart (experiment 18). Ideally, the center points should give the same results, thus this result indicates that variation between runs performed with the same conditions occurred. This can be a result of that unknown factors not included in the model influenced the response variables.

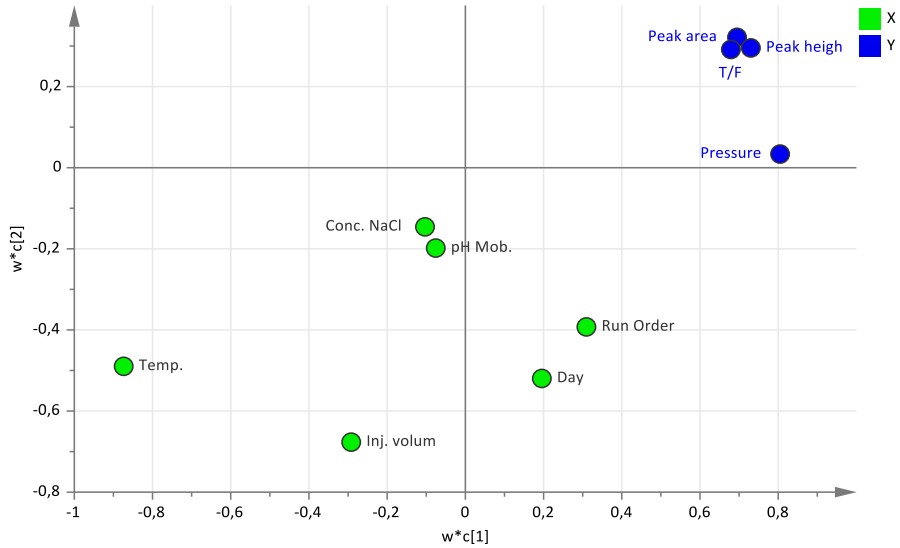

Figure 25. Loading plot from the screening design.

From the PLS model a loading plot was obtained, which is illustrated in Figure 25. The loading plot shows that peak area, peak height, and the ratio between peak fronting and peak tailing are correlated. Temperature and injection volume are on the opposite side, which indicates negative correlation between these response variables. The model suggests that pH

and the concentration of NaCl in the mobile phase were factors less influential on the response variables. It is also clear that day and run order influenced all of the response variables. The same result was obtained in the MLR model where run order was a factor with statistical significance for the area of HPC. A possible explanation might be that SEC columns need to be conditioned between changes in the mobile phase. The variation in day and run order might be a result of the column not being conditioned long enough between runs. An additional explanation might be that the column performance decreased during the performed experiments.

5 Conclusion

In conclusion, the UHPSEC columns evaluated in this master thesis work enabled shorter analysis time compared to the conventional SEC column. The total analysis time could be reduced by two thirds from typically 30 minutes to ten minutes by changing from a conventional column to an UHPSEC column. For Pluronic F108 and HPC the retention times were reduced approximately six fold with an UHPSEC column compared to a conventional SEC column.

No universal column and mobile phase composition for the studied polymers could be found with low LOQ and LOD, hence the UHPSEC columns seem to be more specific to analyte and composition of mobile phase compared to the conventional TSKgel GMPWXL column.

With the UHPSEC column developed for advanced polymer characterization in aqueous mobile phases, Acquity APC AQ 200, was not usable for analysis of HPC, Pluronic F108 and PEG 20.000, which are common polymers in pharmaceutical formulations. Most likely, adsorption of the polymers to the un-capped silanol column material occurred. Of the tested polymers, only pullulan standards eluted from this column.

The UHPSEC column for organic based mobile phases, Acquity APC XT 200, the lowest LOQ and LOD were obtained for Pluronic F108 based on peak height. However, a higher LOQ was obtained based on peak area compared to the conventional TSKgel GMPWXL. Furthermore, this UHPSEC column did not improve the ability to detect HPC at a lower concentration, thus a higher LOQ and LOD for HPC were obtained compared to the conventional TSKgel GMPWXL.

With the UHPSEC column developed for protein and peptide characterization in aqueous mobile phases, Acquity UPLC BEH 200 SEC, the lowest amount of HPC could be analyzed, thus the column had lowest LOQ and LOD based on both peak area and peak height. A drawback found with the Acquity UPLC BEH 200 SEC column was that Pluronic F108 did not elute with PBS as mobile phase. Change of mobile phase however, enabled separation of Pluronic F108 on this column.

The screening design revealed main factors that can be used for further method optimization to enable determination of low concentration of HPC at reduced analysis time. Temperature had significant impact on all responses. At the lower temperature tested, peak height was high and peak area was large. This data indicates that HPC should be analyzed in the lower temperature range. At the tested conditions, all peaks were broad and had tailing i.e. were not Gaussian distributed. However, the shape of the peak appeared more Gaussian at higher temperatures and at low temperatures the peak shape had more tailing. In addition, temperature was the only factor that significantly influenced the back pressure. Furthermore, from the screening design, injection volume was a significant factor influencing peak height, peak shape as well as the peak area for HPC. The results indicate that injection volume should be kept in the lower range to obtain high peak height and large peak area. This is

however compromising peak shape as lower injection volumes indicated that the ratio between tailing and fronting increased. The concentration of NaCl and pH in the mobile phase was less significant factors. Moreover, run order and day were factors influencing the response variables, which could be a result of decreasing performance of the Acquity UPLC BEH 200 SEC column with time, or that the column needed to be conditioned with a larger volume between changes in mobile phase composition.

One of the tested UHPSEC columns on a HPLC system with RI detector can be used for quantification and characterization of low amounts of HPC. The method can be optimized further by using a low injection volume and a low column temperature. For future studies, other detectors can be tested that might enable quantification and characterization at even lower amounts. One of the main advantages of using an UHPSEC column compared to a conventional column is however that the analysis time was reduced.

References

1. Malawer EGS, L. Handbook of size exclusion chromatography and related techniques: New York Marcel dekker inc; 1995. Available from: https://books.google.se/books?id=syubUqw-kf4C&pg=PP10&lpg=PP10&dq=Chi-san,W.+(1995)+Handbook+of+size+exclusion+chromatography+and+related+techniques.+2nd+edition.+New+York+Marcel+dekker+inc.&source=bl&ots=x4zvwW1KEL&sig=PrEaSRojpP1Z6j0XAdwuBXvT1ew&hl=en&sa=X&ei=86QeVcayNIWPsAGms4K4DA&redir_esc=y#v=onepage&q=Chisan%2CW.%20(1995)%20Handbook%20of%20size%20exclusion%20chromatography%20and%20related%20techniques.%202nd%20edition.%20New%20York%20Marcel%20dekker%20inc.&f=false. [150301].

2. Berek D. Size exclusion chromatography--a blessing and a curse of science and technology of synthetic polymers. Journal of separation science. 2010;33(3):315-35.

3. Bouvier E, Summers M, O'Leary M. Recent Advancements in Polymer Characterization. The R&D Daily. 2013.

4. Uliyanchenko E. Size-exclusion chromatography-from high-performance to ultra-performance. Analytical and bioanalytical chemistry. 2014;406(25):6087-94.

5. Uliyanchenko E, Schoenmakers PJ, van der Wal S. Fast and efficient size-based separations of polymers using ultra-high-pressure liquid chromatography. Journal of chromatography A. 2011;1218(11):1509-18.

6. Mori S, Barth HG. Size Exclusion Chromatography: Springer-Verlag Berlin Heidelberg; 1999.. Available from: https://books.google.se/books?hl=en&lr=&id=wKbvCAAAQBAJ&oi=fnd&pg=PA1&dq=Size+Exclusion+Chromatography:+SpringerVerlag+Berlin+Heidelberg&ots=0VJ9iFnCKy&sig=tO2e6ahbKTz8pubGd2Afy9RlJrk#v=onepage&q=Size%20Exclusion%20Chromatography%3A%20Springer-Verlag%20Berlin%20Heidelberg&f=false. [150301].

7. György H, Mihály K. Chromatographic methods . Available from: http://elte.prompt.hu/sites/default/files/tananyagok/IntroductionToPracticalBiochemistry/ch06.html. [20150515].

8. Guillarme D. Reversed phase, size exclusion and ion exchange chromatography for biomolecules characterization. 2015.

9. Hofe T, Reinhold G. The Challenge of Using Small Particle Packing Materials in SEC/GPC risk and possibilities. Chromatography Today. 2011:18-23.

10. Trathnigg B. Size-exclusion chromatography of Polymers. Enzyclopedia of analytical Chemistry. 2000:8008-34.

11. Waters Corporation. Care and use manual ACQUITY UPLC protein BEH SEC columns and standards Milford, MA, USA 2015. Available from: http://www.waters.com/webassets/cms/support/docs/720003385en.pdf. [150301].

12. Bouvier ESP, Koza SM. Advances in size-exclusion separations of proteins and polymers by UHPLC. TrAC Trends in Analytical Chemistry. 2014;63:85-94.

13. Dolan JW. Avoiding refractive index detector problems. LCGC NORTH AMERICA. 2012;30(2):1032-7.

14. Wyatt Technology Corporation. Optilab® T-rEX™ Differential refractometer with extended range. 2010. Available from: http://fmch2012.umcs.lublin.pl/sites/default/files/Optilab%20T-rEX.pdf. [150413].

15. Shrivastava A, Gupta VB. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. 2011. 2(1):21-5.

16. Morsing T, Ekman C, Bengtsson O. Grundläggande statistik för analytisk kemi.Astra Hässle AB. 1996.

17. Vial JL, Mapihan K, Jardy A. What is the best means of estimating the detection and quantification limits of a chromatographic method? Chromatographia Supplement. 2003;57:303-6.

18. ICH Harmonised tripartite guideline. Validation of Analytical Procedures: Text and Methodology. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf. [150318].

19. Mani DR, Abbatiello SE, Carr SA. Statistical characterization of multiple-reaction monitoring mass spectrometry (MRM-MS) assays for quantitative proteomics. BMC bioinformatics. 2012;13 Suppl 16:S9.

20. Harang V. Aspects of optimisation of separation of drugs by chemometrics: Uppsala University; 2003.

21. Eriksson L, Johansson E, Kettaneh-Wold N, Wikström C, Wold S. Design of Experiments Principle and Application. Umeå UMETRICS AB; 2008. Available from: http://books.google.se/books?id=mgoHFzEpg5oC&printsec=frontcover&hl=sv#v=onepage&q=doi&f=false. [150305].

22. Wold S, Sjöström M, Eriksson B. PLS-regression: a basic tool of chemometrics. Chemometrics and Intelligent Laboratory Systems. 2001(58):109–30.

23. Wyatt Technology Europe GmbH. dn/dc Database 2014. Available from: http://www.wyatt.eu/index.php?id=wyatt-dndc-database.[150201].

24. Erlandsson B, Wittgren B, Brinkmalm G. Development of a size exclusion chromatography method for the determination of molar mass for poloxamers. J Pharm Biomed Anal. 2003;31(5):845-58.

25. Tumolo T, Angnes L, Baptista MS. Determination of the refractive index increment (dn/dc) of molecule and macromolecule solutions by surface plasmon resonance. Analytical biochemistry. 2004;333(2):273-9.

26. Corporation APS. Light Scattering dn/dc Values Mentor OH, USA: American Polymer standard corporation; 2015 [250316]. Available from: http://www.ampolymer.com/dn-dcValues.html.

27. Waters Corporation. Product solution ACQUITY APC COLUMNS. Milford, MA, USA 2015. Available from: http://www.waters.com/webassets/cms/library/docs/720004580en.pdf. [250316].

28. Waters Corporation. Care and use manual ACQUITY APC Columns. Milford, MA, USA 2014. [250316] Available from: https://www.waters.com/webassets/cms/support/docs/720004667en.pdf.

29. TOSOH BIOSCIENCE. SEC COLUMNS SEC SIZE EXCLUSION CHROMATOGRAPHY. Stuttgart, Germany. Available from: http://www.separations.eu.tosohbioscience.com/NR/rdonlyres/50628A87-A204-4053-8B02-4BF793DD579D/0/B11L02A_TSKgelSECBrochure.pdf. [250310]

Appendices

Appendix 1 - Recommended column parameters

In Table 6, the evaluated columns in this master thesis work are listed with their respective recommended column parameters.

Table 6 Record of evaluated columns and their recommended column parameters.

Column	Application focus	Molecular weight range	Recommended mobile phase composition	Recommended flow rate range mL/min	Maximum back pressure	Recommended temperature
TSKgel GMPWXL[b] 13 µm, 7.8 x 300 mm and pore size 100-1000 Å	Polymers	0.5-8000 kg/mole	Aqueous based < 50 % polar organic mobile phases	0.3-0.6	2 MPa	< 80 °C
Acquity APC XT 200 2.5 µm 4.6 x150 mm[a]	Polymers	3-70 kg/mole	Organic based	In THF ≤ 2	-	< 90°C
Acquity APC AQ 200 2.5 µm 4.6 x150 mm[a]	Polymers	3-70 kg/mole	Aqueous based	In water ≤1.4	-	< 45 °C
Acquity UPLC BEH 200 SEC 1.7 µm 4.6 x150 mm[c]	Peptides and protein	10-450 kg/mole	Aqueous based < 20 % organic solvent as mobile phase (acetonitrile)	0.1-0.4	7000 psi ~ 50 Mpa	4-60°C
Acquity UPLC BEH 125 SEC 1.7 µm 4.6 x150 mm[c]	Proteins	1-80 kg/mole	Aqueous based < 20 % organic solvent as mobile phase (acetonitrile)	0.1-0.4	1000 psi ~ 65 Mpa	4-60 °C

a (27, 28), b (27, 29) c (11)

Appendix 2 – Refractive index increment for Pluronic F108 and HPC in THF

Solutions of 1.0092 mg/ml Pluronic F108 and 1.0084 mg/ml of HPC in THF were prepared for the determination of dn/dc values. These solutions were diluted into five concentrations (Pluronic F108: 0.10092, 0.30276, 0.5046, 0.60552 and 0.80736 mg/ml, HPC: 0.10084, 0.30252, 0.5042, 0.60504 and 0.89672 mg/ml). Samples were injected with a volume of 500 µl.

The dn/dc value for Pluronic F108 was 0.0603 mL/g in THF at 25 °C. The value was obtained from three repeated measurements. Dn/dc of the first measurement was 0.0579 ± 0.0014 mL/g with a R^2 of 0.99. The chromatogram with defined peaks and differential refractive index versus concentration of Pluronic F108 is presented in Figure 26.

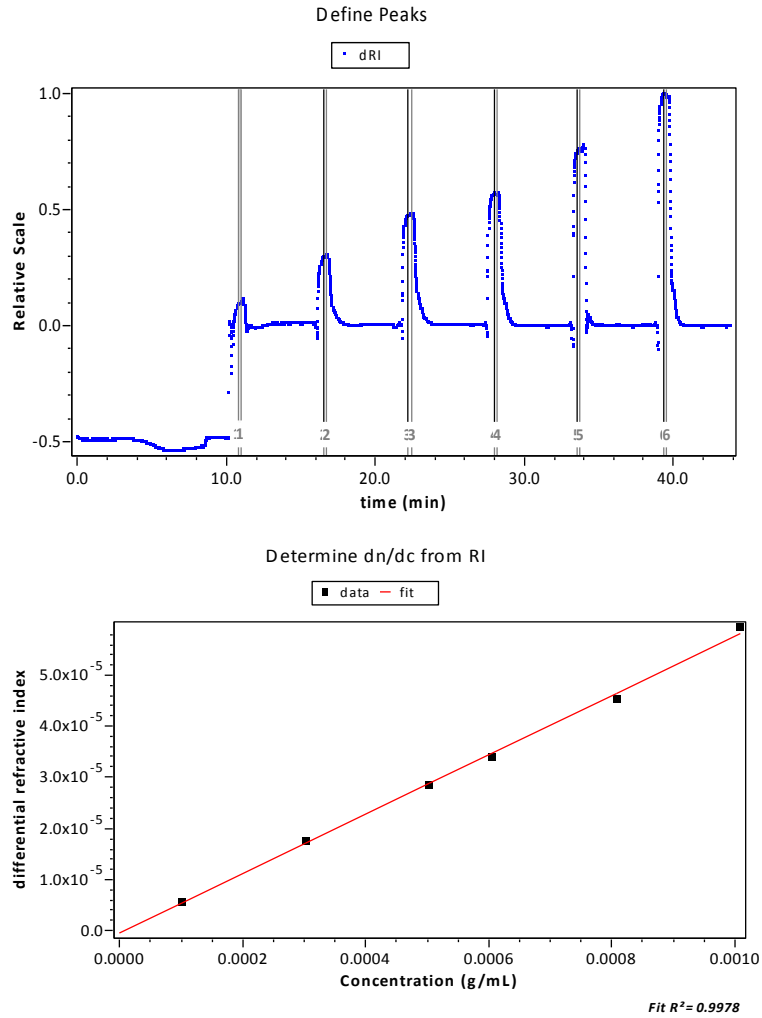

Figure 26. Chromatogram and defined peaks of Pluronic F108 at six concentrations injected and differential refractive index as function of concentration Pluronic F108. Sample volume was 500 µl.

In the second measurement, dn/dc was 0.0608 ± 0.0018 mL/g with a R^2 of 0.99. The chromatogram with defined peaks and differential refractive index versus concentration of Pluronic F108 is presented in Figure 27.

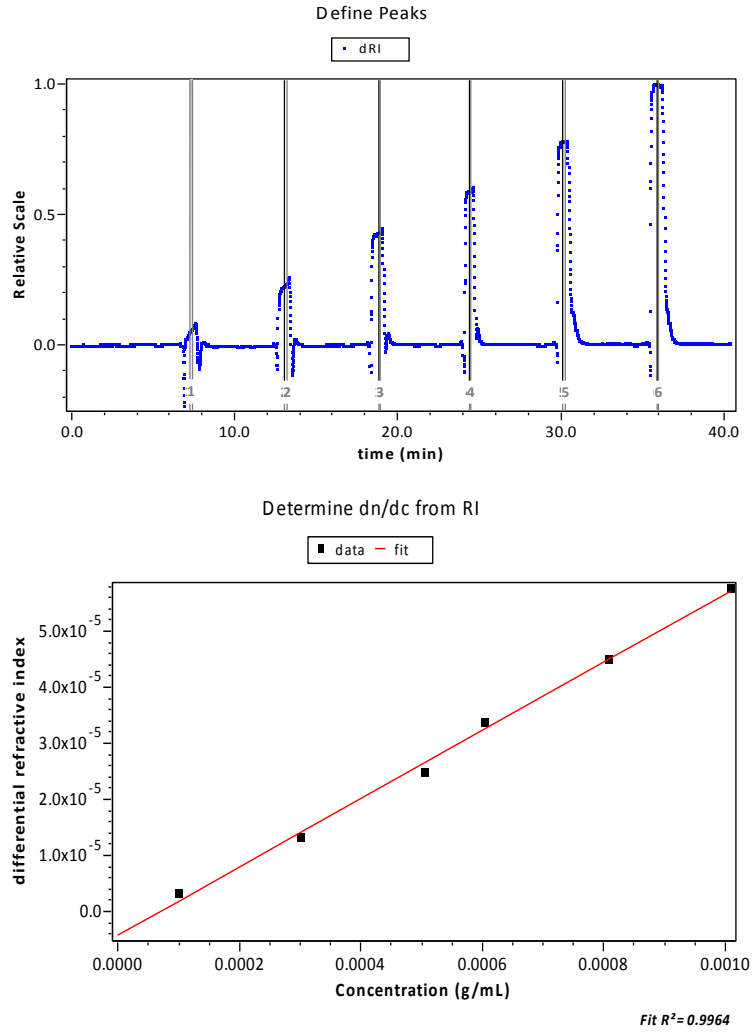

Figure 27. a) Chromatogram and defined peaks of Pluronic F108 at six concentrations injected and differential refractive index as function of concentration Pluronic F108. Sample volume was 500 µl.

The third measurement, dn/dc was 0.0621 ± 0.0026 mL/g with a R^2 value of 0.99. The chromatogram with defined peaks and differential refractive index versus concentration of Pluronic F108 is presented in Figure 28.

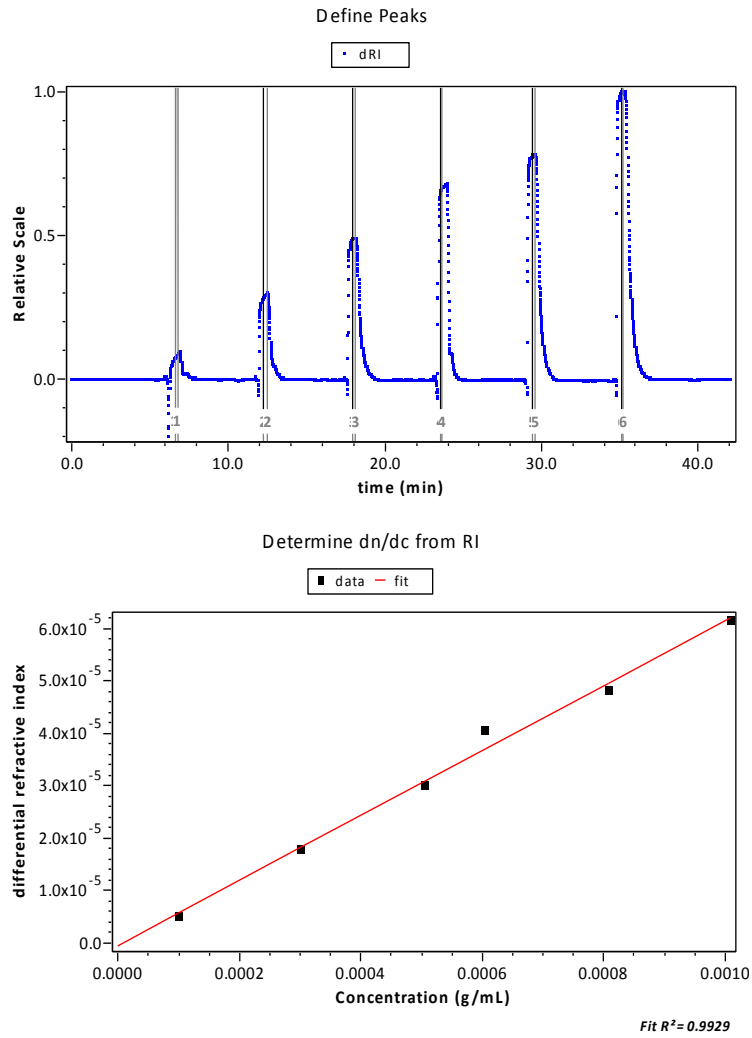

Figure 28. Chromatogram and defined peaks of Pluronic F108 at six concentrations injected and differential refractive index as function of concentration Pluronic F108. Sample volume was 500 µl.

The dn/dc value for HPC was 0.0715mL/g in THF at 25 °C. The value was obtained from three repeated measurements. Dn/dc of the first measurement was 0.0739 ± 0.0025 mL/g, with a R^2 of 0.99. The chromatogram with defined peaks and differential refractive index versus concentration of HPC is presented in Figure 29. The peak corresponding to the concentration 0.60504 mg/ml was considered as an outlier and was excluded to obtain a higher R^2 score.

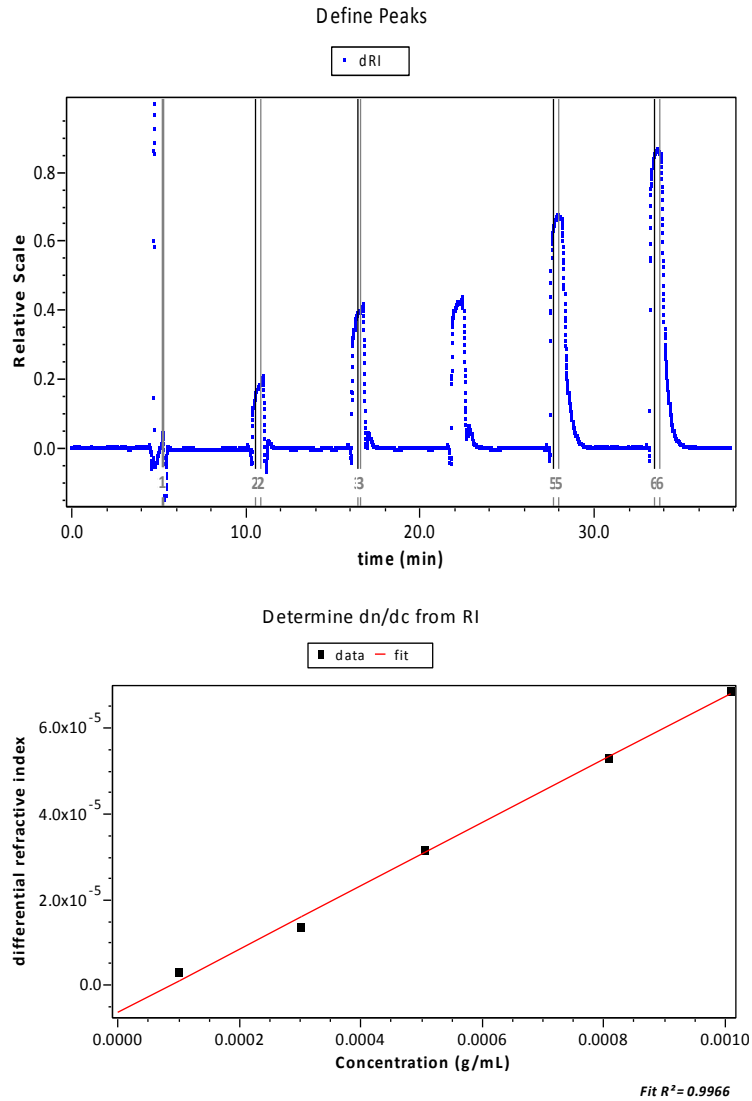

Figure 29. Chromatogram and defined peaks of Pluronic F108 at six concentrations injected and differential refractive index as function of concentration HPC. Sample volume was 500 µl.

In the second measurement, dn/dc was 0.0704 ± 0.0021 mL/g with a R^2 of 0.99. The chromatogram with defined peaks and differential refractive index versus concentration of HPC is presented in Figure 30.

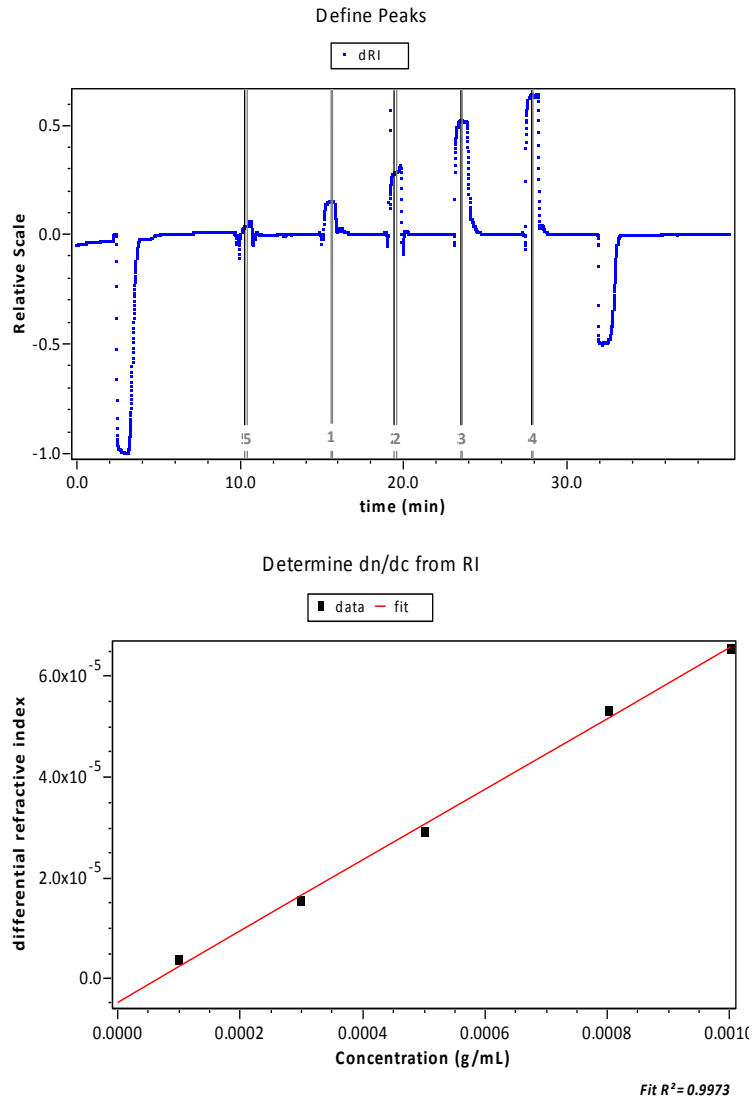

Figure 30. Chromatogram and defined peaks of Pluronic F108 at five concentrations injected and differential refractive index as function of concentration HPC. Sample volume was 500 µl.

The third measurement, dn/dc was 0.0702 ± 0.0020 mL/g with a R^2 value of 0.99. The chromatogram with defined peaks and differential refractive index versus concentration of HPC is presented in Figure 31.

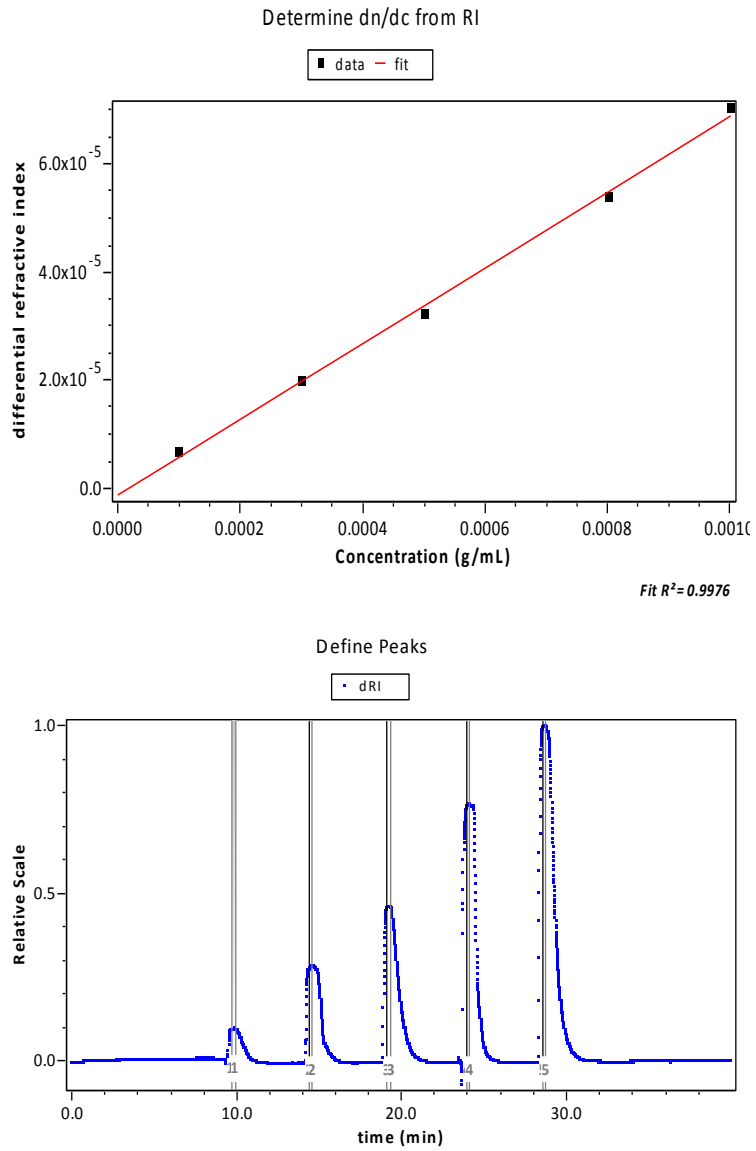

Figure 31. Chromatogram and defined peaks of Pluronic F108 at five concentrations injected and differential refractive index as function of concentration HPC. Sample volume was 500 µl.

Appendix 3 – Worksheet used for chemometrics

Table 7. The complete worksheet used for chemometrics.

Exp No	Run order	Column temperature [C°]	pH in mobile phase	% (v/v) FA in mobile phase	Conc. of NaCl [mM]	Sample volume injected [µl]	Day	Peak area [RIU min]	Back pressure [MPa]	Peak height [RIU]	Tailing/ fronting
13	1	22	2.4	0.084	200	20	1	5,302E-08	18,1	4,209E-08	2.11018
4	2	40	5.6	0	10	5	1	5,061E-08	15,4	3,963E-08	2,12147
8	3	40	5.6	0	200	5	1	4,984E-08	15,6	3,751E-08	1,73329
2	4	40	2.4	0.084	10	5	2	5,191E-08	15,3	3,98E-08	1,9697
3	5	22	5.6	0	10	5	2	6,088E-08	20	4,876E-08	2,47328
12	6	40	5.6	0	10	20	2	5,03E-08	15,4	3,921E-08	2,09696
19	7	30	3.8	0.0005	50	10	2	5,886E-08	17,9	4,544E-08	2,33567
14	8	40	2.4	0.084	200	20	3	5,128E-08	15,2	3,895E-08	1,79511
6	9	40	2.4	0.084	200	5	3	5,627E-08	15,2	4,172E-08	1,98888
17	10	30	3.8	0.0005	50	10	3	5,875E-08	18	4,538E-08	2,34522
1	11	22	2.4	0.084	10	5	3	6,241E-08	19,6	4,944E-08	2,67363
10	12	40	2.4	0.084	10	20	4	5,018E-08	15,3	3,901E-08	1,82237
7	13	22	5.6	0	200	5	4	6,182E-08	20,2	4,805E-08	2,61025
5	14	22	2.4	0.084	200	5	4	6,238E-08	20	4,865E-08	2,7623
15	15	22	5.6	0	200	20	4	5,629E-08	20,2	4,42E-08	2,3115
9	16	22	2.4	0.084	10	20	5	5,748E-08	20,2	4,527E-08	2,54101
18	17	30	3.8	0.0005	50	10	5	5,874E-08	18,2	4,576E-08	2,30981
16	18	40	5.6	0	200	20	6	4,693E-08	16,1	3,524E-08	1,7451
11	19	22	5.6	0	10	20	6	5,532E-08	20,1	4,481E-08	2,06281

Appendix 4 –Chromatograms of pullulan on Acquity APC AQ 200

Overlaid RI chromatograms of pullulan standards; P-5, P-10, P-50, P-100 and P-200 dissolved in PBS separated on an Acquity APC AQ 200 column is presented in Figure 32. The mobile phase was 50 mM NaCl + 0.055 % (v/v) FA with flow rate 0.5 ml/min.

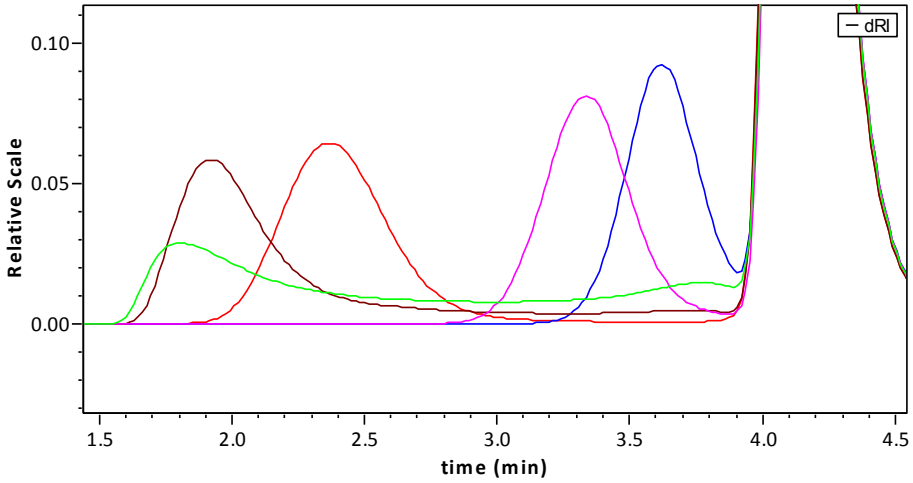

Figure 32. Overlaid RI chromatograms of five pullulan standards from left; P-200, P-100, P-50, P-10 and P-5. A system peak is seen at 4.2 minutes. Mobile phase was 50 mM NaCl + 0.055 % (v/v) FA with flow rate 0.5 ml/min and the column was Acquity APC AQ 200. Injection volume was 20 µl.

Appendix 5 – Limit of quantification

Below the variation in RSD for peak height and peak area as a function of the quantity of some model compounds injected onto TSKgel GMPWXL, Acquity UPLC BEH 200 SEC and Acquity APC XT 200 is presented.

In Figure 33 to Figure 37, LOQ determination on the conventional TSKgel GMPWXL is presented. In Figure 33, RSD of peak area as a function of the quantity of HPC is presented. In Figure 34 and in Figure 35, RSD of peak height and peak area respectively, as a function of the quantity of PEG 6000 is presented. In Figure 36 and Figure 37, RSD of peak height and peak area respectively, as a function of the quantity of Pluronic F108 is presented.

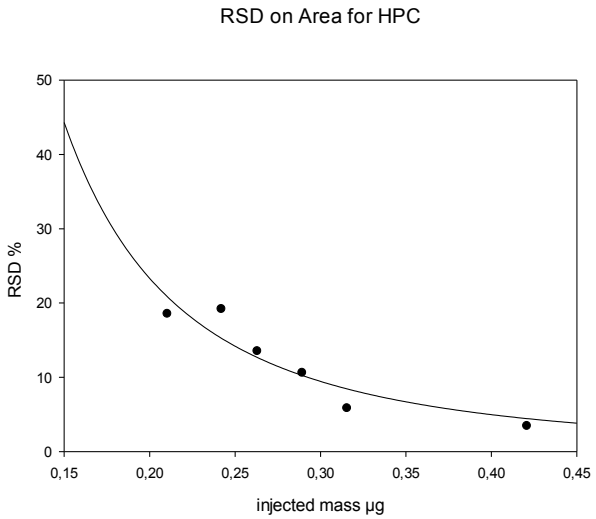

Figure 33. RSD variation in peak area as a function of the quantity HPC injected. The column was TSKgel GMPWXL SEC column.

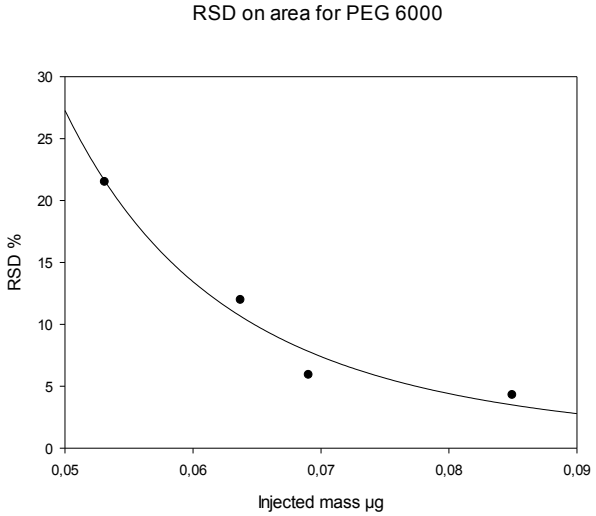

Figure 34. RSD variation in peak area as a function of the quantity PEG 6000 injected. The column was TSKgel GMPWXL SEC column.

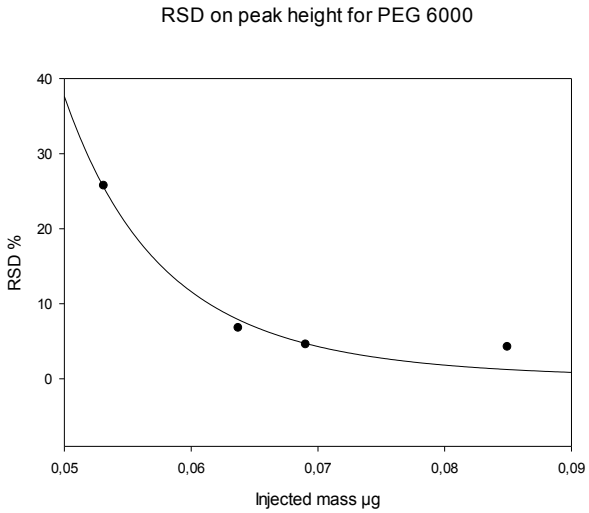

Figure 35. RSD variation in peak height as a function of the quantity PEG 6000 injected. The column was TSKgel GMPWXL SEC column.

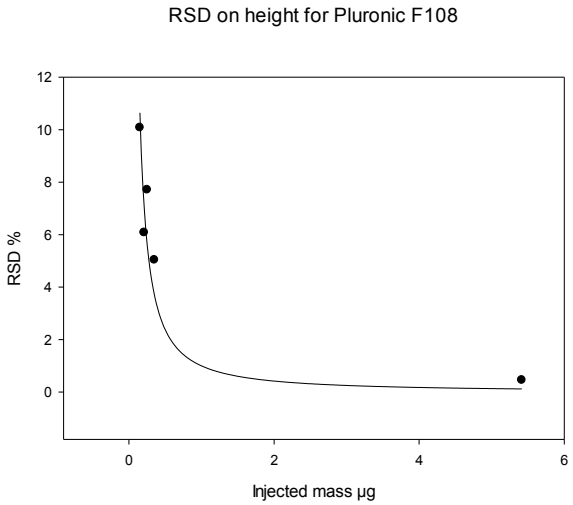

Figure 36. RSD variation in peak height as a function of the quantity Pluronic F108 injected. The column was TSKgel GMPWXL SEC column.

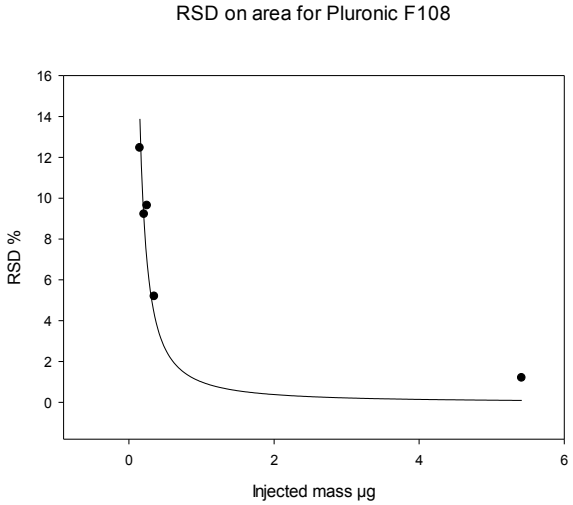

Figure 37. RSD variation in peak area as a function of the quantity Pluronic F108 injected. The column was TSKgel GMPWXL SEC column.

In Figure 38, LOQ determination on the UHPSEC column Acquity UPLC BEH 200 SEC is presented. In Figure 33, RSD of peak area as a function of the quantity of HPC is presented.

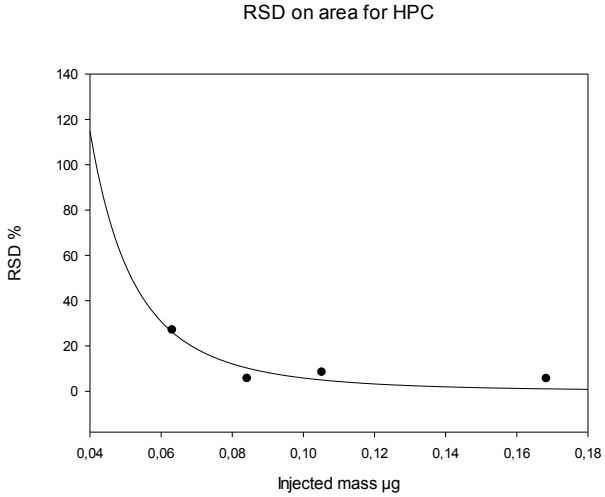

Figure 38. RSD variation in peak area as a function of the quantity HPC injected. The column was Acquity UPLC BEH 200 SEC.

In Figure 39 to Figure 41, LOQ determination on the UHPSEC column Acquity APC XT 200 is presented. In Figure 39, RSD of peak area as a function of the quantity of HPC is presented. In Figure 40 and Figure 41, RSD of peak height area and peak height as a function of the quantity of Pluronic F108 is presented.

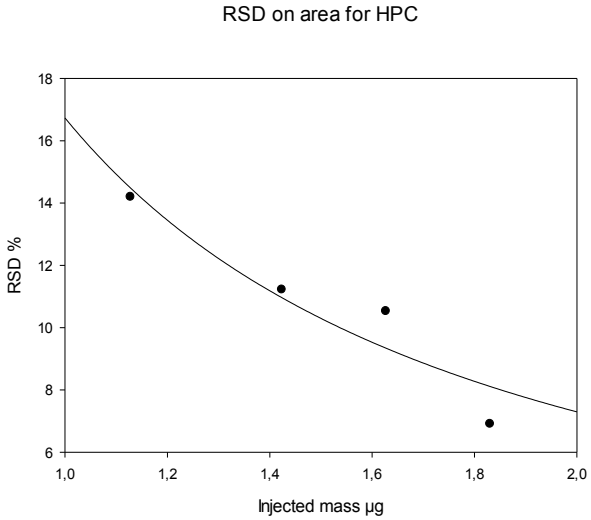

Figure 39. RSD variation in area height as a function of the quantity HPC injected. The column was Acquity APC XT 200.

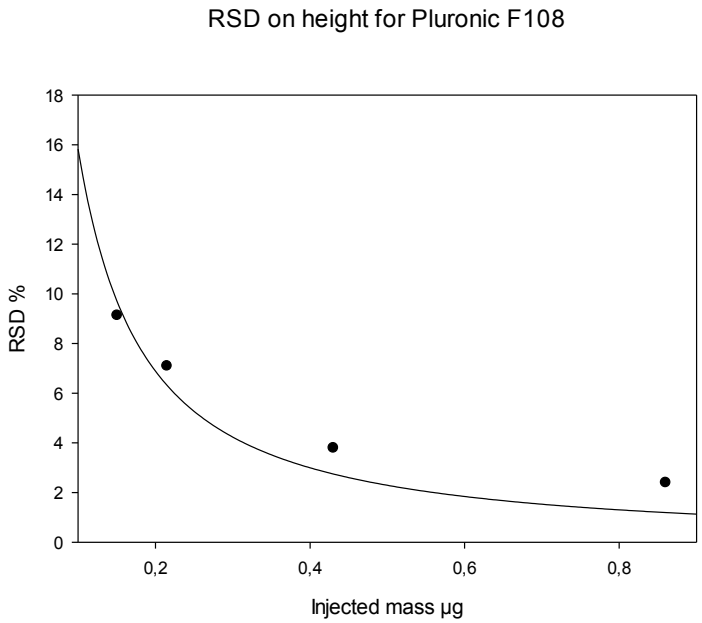

Figure 40. RSD variation in peak height as a function of the quantity HPC injected. The column was Acquity APC XT 200.

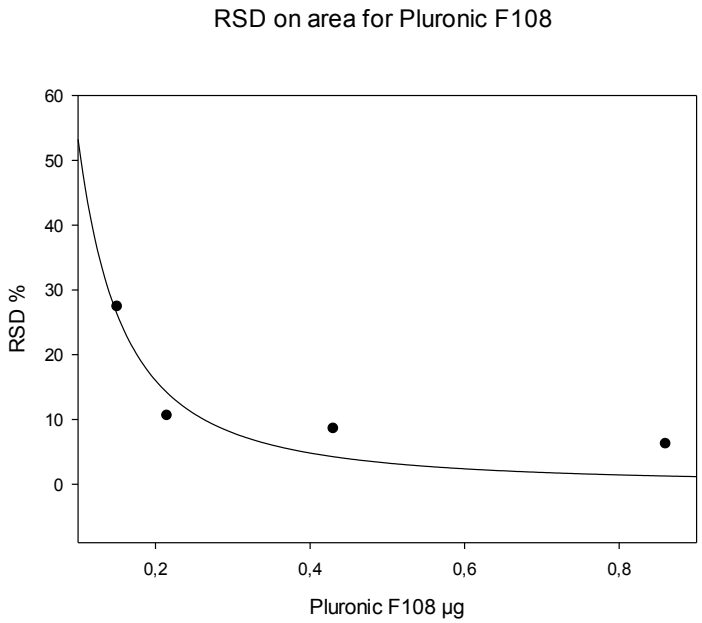

Figure 41. RSD variation in peak area as a function of the quantity Pluronic F108 injected. The column was Acquity APC XT 200.

Appendix 6 – Ratio between tailing and fronting

In Figure 42, the ratio between tailing and fronting is illustrated.

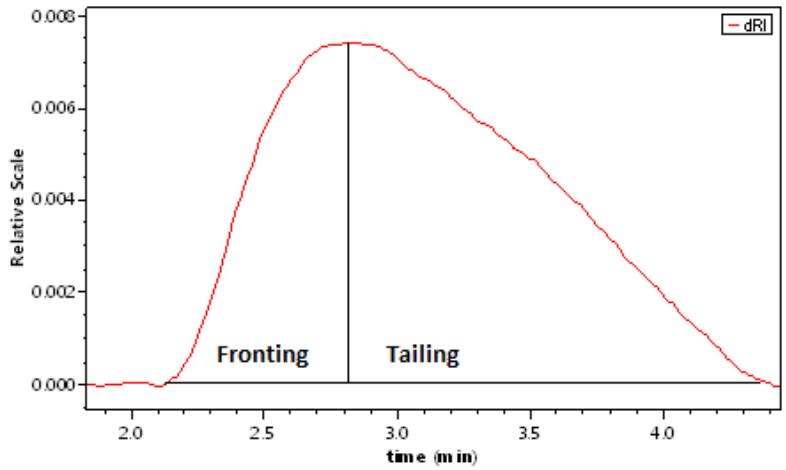

Figure 42. Fronting and tailing.