# MOLAR MASS DETERMINATION OF COLLAGEN PEPTIDES

## WHITE PAPER

Establishing a cost-effective, easy-to-use QC method for the accurate, reliable and precise determination of collagen peptides molar mass distributions

# WHITE PAPER

## SCOPE OF WORK

Collagen peptides (also referred to as hydrolysed collagen or collagen hydrolysate) are produced from natural proteins by hydrolysis. They are soluble in cold water, are highly digestible and used in dietary supplements and functional foods.

Collagen peptides are relatively small proteins with typical molar masses of less than 10.000 g/mol comprising at least two and at most 100 amino acids. Common to nearly all macromolecules, collagen peptides do not exhibit only one specific molar mass but a molar mass distribution, MMD. This MMD influences their properties.

GPC/SEC is an established technique to measure MMDs of macromolecules, such as collagen peptides, but suffers from 2 limitations:

- It is a relative technique which allows determination of close to true molar masses only
    - if reference materials chemically matching the analyte structure are available
    - or if advanced detectors such as online viscometers or online light scattering detectors are applied
- It is a liquid chromatographic technique which requires highly qualified and trained users, especially when advanced detectors such as online viscometers or online light scattering detectors are employed.

The purpose of the present work was to develop a simple, cost-effective and robust high precision GPC/SEC method for QC, allowing to provide close to true molar masses for collagen peptides. The method should be applicable in any laboratory allowing for reliable inter laboratory comparison. Therefore, the use of molar mass sensitive detectors should be avoided.

The single steps in the study were

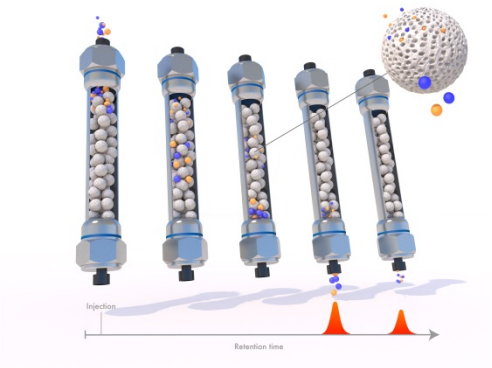
- identification of collagen peptides suitable as reference materials and determination of their characteristics
- establishing a suitable calibration procedure
- round robin test of the final approach

## SCOPE OF WORK

Establish a cost-effective, easy-to-use QC method for the accurate, reliable and precise determination of collagen peptides molar mass distributions, allowing for reliable inter laboratory comparison.

# INTRODUCTION TO GPC/SEC

GPC/SEC is a chromatographic technique in which a sample is separated according to the size of the molecules. The separation takes place in columns filled with porous particles. Molecules which are larger than the pores of the particles are excluded from the pores and remain in the flowing eluent stream, eluting first from the column. Molecules smaller than the pores diffuse in and out of the pores, resulting in later elution with decreasing size.[1]




## Calibration in GPC/SEC

To determine the required molar mass information by GPC/SEC a *calibration curve* can be applied to relate the elution volume to the molar mass of the eluting macromolecules. The most common approach to construct the calibration curve is injecting a series of chemically identical, narrowly distributed standards of known molar masses. The elution volumes at peak maxima are plotted versus the molar masses of the standards resulting in the desired calibration curve.

However, GPC/SEC separates by the *size of the molecules in solution*. Because at a given molar mass the sizes of macromolecules of different chemical structures differ from each other, the molar mass determined by GPC/SEC depends on the chemical structure of the standards applied for calibration. In order to derive the true molar masses, the chemical structure of the analyte and the calibration standards must therefore be identical. If materials with narrow molar mass distributions are not available, broadly distributed reference materials of known molar mass having the same structure as the analyte can also be applied. Alternatively, molar mass sensitive detectors such as light scattering detectors or viscometers can be used.[1,2]

## Broad standard calibration in GPC/SEC

Benoit and Grubisic empirically showed that polymers eluting at the same GPC/SEC elution volume have identical sizes (hydrodynamic volumes).[2] The relation between chemically different macromolecules eluting at the same elution volume can be expressed as:

$$M_2 = A \times M_1^B \qquad \text{Equation 1}$$

, where A and B are parameters, which depend on the Mark-Houwink parameters of the two polymers in the eluent. Usually A and B which relate the molar mass of the narrowly distributed standards ($M_1$) to the molar mass of the analyte at the same elution volume ($M_2$) are unknown and need to be determined.

For this purpose, a calibration curve is established first using standards which might differ in chemical structure from the analyte (base calibration). Afterwards one or more broadly distributed reference samples, for which one or more molar mass averages are known ($M_n$ or $M_w$, target values) and which are of the same chemical structure as the material to be analyzed, are run on the column

set to be calibrated. For each chromatogram of the reference samples the molar mass averages are calculated using a test set of A and B to convert the previously established base calibration. The relations between the test parameters A and B, the chromatogram (S(V)) and the calculated average molar masses are given by:

$$M_{n,i}(A,B) = \frac{\sum S_i(V)}{\sum S_i(V)/M_2} = \frac{\sum S_i(V)}{\sum S_i(V)/(A \times M_1^B)} \qquad \text{Equation 2}$$

$$M_{w,i}(A,B) = \frac{\sum S_i(V) \times M_2}{\sum S_i(V)} = \frac{\sum S_i(V) \times A \times M_1^B}{\sum S_i(V)} \qquad \text{Equation 3}$$

, here $M_{n,i}(A,B)$ and $M_{w,i}(A,B)$ are the number and weight average molar masses for chromatogram i, when applying the parameters A and B. $M_1(V)$ is the molar mass of the base calibration at elution volume V. The so calculated molar masses averages depend on the selection of the parameters A and B. Agreement between the calculated molar mass averages and the target values will result only if A and B are chosen correctly. Applying suitable optimization algorithms allows varying A and B until the best agreement between the calculated molar mass averages for the chromatograms of the broadly distributed reference samples with the target values is achieved. The so identified optimal set of A and B can now be applied to convert the base calibration into a calibration curve representing the chemical structure of the analyte.

## Molar mass determination by GPC/SEC with advanced detection

The broad standard calibration approach, as described above requires a set of broadly distributed reference samples of known true molar masses. These molar masses could be determined using GPC/SEC with either online viscometry or online light scattering detection.

### *GPC/SEC with Viscosity detection*

Benoit and Grubisic empirically showed that polymers eluting at the same GPC/SEC elution volume have identical products of intrinsic viscosity, [n], and molar mass ([n]xM).[3]

An online viscometry detector attached to a GPC/SEC instrument provides, in combination with a concentration detector, information about the intrinsic viscosity at each point of the elution curve.

By running a series of narrowly distributed standards of known molar masses and determining their intrinsic viscosities using the viscosity detector, a universal calibration curve can be established by plotting the product [n]xM as a function of elution volume. The chemical structure of the standards applied for setting up the universal calibration curve is allowed to differ from the analyte's chemical structure.

For determining the analyte's molar mass, its intrinsic viscosity at each elution volume is derived using the viscosity detector. By taking at each elution volume the ratio of [n]xM from the calibration curve and the experimentally derived intrinsic viscosity of the eluting sample, the true molar mass of the eluting macromolecule is calculated, allowing determining the true molar mass averages of the sample.

### *GPC/SEC with light scattering detection*

Besides GPC/SEC with viscosity detection static light scattering is one of the few absolute techniques to measure true molar masses.[4] Attached to a GPC/SEC instrument an online light scattering detector provides, in combination with a concentration detector, information about the sample molar mass at each point of the elution curve. Calibration with reference materials is thus not required, only the determination of system related constants and parameters.

However, light scattering is a technique generally more applicable to molecules of higher molar mass. If molecules of lower molar mass are to be analyzed, concentration needs to be enhanced to achieve suitable signal intensity. The enhanced concentrations might result in column overloading which in turn will reduce chromatographic resolution. The reduced resolution affects determination of the molar mass distribution, finally resulting in incorrect determination of molar mass averages.

In addition, molar mass determination by light scattering requires the accurate knowledge of the specific refractive index increment, dn/dc, which depends on the composition. The applied dn/dc value needs to be constant over the eluting peak and accurately known. Otherwise severe errors might result for the molar masses determined.[5]

## Suitable options for collagen peptides

For the characterization of the future collagen peptides reference materials on-line viscometry was preferred over light scattering detection for 2 reasons:

- Collagen peptides have relatively low molar masses and while both detectors are better with higher molar masses, viscometers often have a better signal quality at low molar masses, specifically when working at typical low GPC/SEC concentrations.
- Collagen peptides are not uniform with respect to amino acid composition, thus dn/dc might not only vary between different collagen peptide samples, but also across the peak of the sample. While the accuracy of GPC/SEC-light scattering results is highly influenced by the accuracy of the dn/dc this is not the case for GPC/SEC-viscometry results.

# EXPERIMENTAL

## Samples and mobile phase

**Dextran/Pullulan**

Dextran and Pullulan standards (PSS, Mainz, Germany), with a molar mass at the peak maximum of $M_p$ = 180 g/mol up to $M_p$ = 106.000 g/mol were applied to establish the universal calibration curve.

**Collagen fragments**

Collagen fragments (CNBr-Peptides) for establishing the base calibration for the broad calibration approach were purchased from FILK (Research Institute of Leather and Plastic Sheeting, Freiberg, Germany). The standard mixture contains molar masses between 3500 and 38.000 g/mol certified by the manufacturer. These materials exhibit chromophores for UV/VIS detection and are generally suitable for GPC/SEC calibration. However, the lack of knowledge on the exact amounts of each component prevents preparing accurately known concentrations as is required for determining intrinsic viscosities to establish a universal calibration curve.

**Collagen peptide samples**

Five different samples, 13R078, 13R079, 833593, 891600 and 890435, were obtained from different manufacturers and were used as reference materials after molar mass determination. The true molar masses of these samples were determined by GPC/SEC with online viscosity detection.

Six additional samples (sample A - sample F) were used in a round robin experiment to identify the suitability of the developed calibration approach.

**Mobile phase**

Phosphate buffer pH 5.30 with 0.2 M NaCl. (13.27 g $KH_2PO_4$, 0.44 g $Na_2HPO_4$, 11.69 g NaCl in 1.0 L deionized water) was applied as GPC/SEC eluent. All salts were obtained from Sigma Aldrich in p.A. purity. Water was deionized in-house using an ion exchanger (Behr, Germany).

## Instrumentation and software

The GPC/SEC equipment comprised a PSS SECcurity GPC/SEC system (PSS, Mainz, Germany), equipped with an isocratic pump, a four channel on-line degassing device, a column thermostat and an autosampler. The detector system in this study was a combination of a SECcurity UV/VIS (λ=214 nm), a viscometer detector Viscotek H502 (Viscotek, Houston, USA) and a SECcurity Refractive Index Detector, RI. Data acquisition and analysis was performed using PSS WinGPC UniChrom Version 8.2 (PSS, Mainz, Germany).

Only the viscosity and the RI detector were applied to determine the properties and characteristics of the collagen peptide reference materials, as the pullulan and dextran samples, required for universal calibration, are not UV/VIS active.

For broad standard calibration with the collagen peptide reference materials and the collagen fragments only the UV/VIS detector was employed.

All measurements were carried out at a temperature of 40° C and a flow rate of 0.5mL/min. A small amount of benzoic acid (internal flow marker) was added to the solvent to prepare the sample solution. At least two concentrations were prepared for each sample. After dissolving overnight, each

sample solution was analyzed in duplicate without further pretreatment.

GPC/SEC-Viscometry: Typical sample concentrations were 3-7 g/L, an injection volume of 100 µL was applied.

Broad standard calibration: Typical sample concentration was 2 g/L, an injection volume of 20 µL was applied.

## Separation columns

Two column types were used which were equivalent in separation performance

a) PSS PROTEEMA columns (5µm particle size, 100Å porosity, 8.0 mm ID x 300 mm); with PSS PROTEEMA precolumn (8.0 mm ID x 50 mm) (PSS, Mainz, Germany)

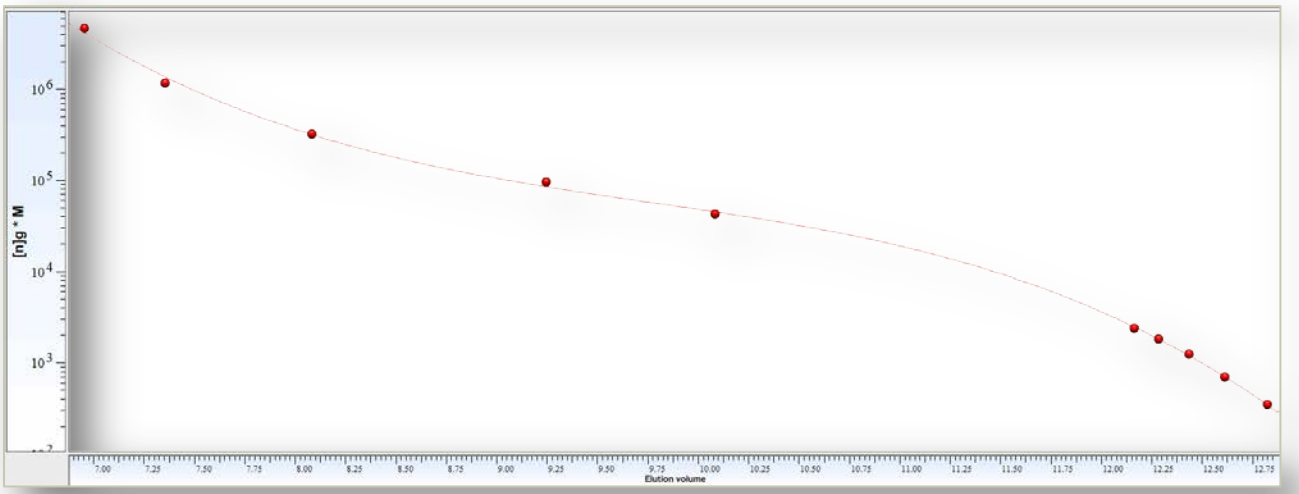
b) TSK SWXL G2000 columns (5µm particle size, 100Å porosity, 7.8 mm ID x 300 mm); with TSK precolumn (7.8 mm ID x 50 mm) (Tosoh, Tokyo, Japan)

## RESULTS AND DISCUSSION

### Characterization of collagen peptides using GPC/SEC-Viscometry-RI

Monodisperse monosaccharides and narrowly distributed pullulans and dextrans with given $M_p$ values were used to establish the universal calibration curve. The intrinsic viscosity of each standard was determined online. The universal calibration curve was obtained by plotting the product log([n]xM) vs. V as shown in Figure 1. The resulting data points were fitted by a 3rd order polynomial, resulting in residuals of less than <5%.

Figure 1: Universal Calibration Curve on PSS PROTEEMA columns constructed using monosaccharides and narrowly distributed pullulan and dextran standards




To obtain true molar masses, the broadly distributed collagen peptides were analyzed using the same conditions as applied for establishing the universal calibration curve.
Table 1 lists the average weight average molar masses, Mw, and the average intrinsic viscosities, IV, of the collagen peptides including their standard deviations.

Table 1: Weight average molar masses, $M_w$, and intrinsic viscosity, IV, of the collagen peptides reference samples (average values and standard deviations from duplicate injections of four individual sample preparations)

| Sample | $M_w$ [g/mol] | IV [mL/g] |
|---|---|---|
| 13R078 | 1960 ± 130 | 4.57 ± 0.07 |
| 833593 | 2820 ± 270 | 5.56 ± 0.27 |
| 13R079 | 3920 ± 200 | 6.60 ± 0.20 |
| 891600 | 4480 ± 380 | 7.49 ± 0.12 |
| 890435 | 9020 ± 680 | 9.58 ± 0.80 |

For the further study the weight average molar masses, $M_w$, were preferred over the number average molar masses, $M_n$, which have a higher experimental error.[6] With the $M_w$ provided in Table 1 the collagen peptides can be used as broadly distributed reference materials to convert a base calibration to a collagen calibration using the broad standard calibration approach.

## Application of collagen peptides for broad calibration using GPC/SEC-UV/VIS

While the pullulan and monosaccharide/dextran standards cover a suitable molar mass separation range and had the required purity to establish accurate concentrations as required for the viscometry measurements, and thus and for constructing a universal calibration curve, they lack chromophores for UV detection. Because a UV based method was requested, the dextrans and pullulans had to be replaced by UV-active calibrants suitable for establishing the base calibration.

A sample of collagen fragments (CNBr peptides) was identified as suitable candidate for the base calibration when using GPC/SEC systems with only UV/VIS detection.

A typical chromatogram of the CNBr peptides with indication of the molar masses at the peak maximum as provided by the supplier is shown in Figure 2.

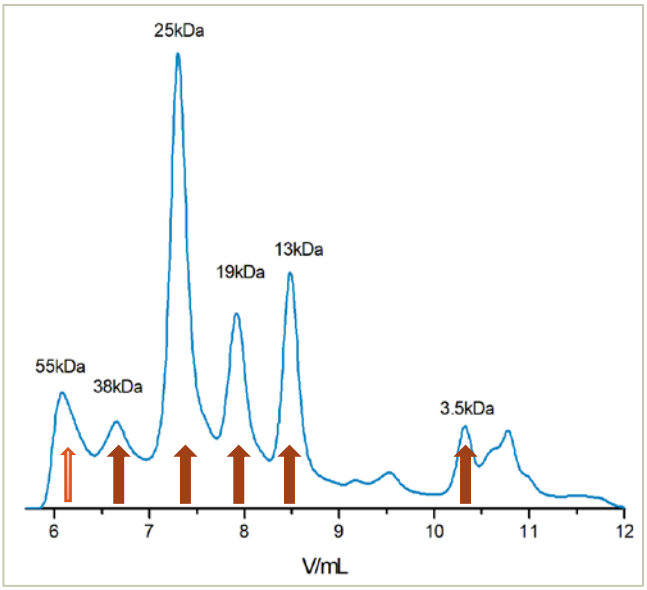



Figure 2: Chromatogram of the CNBr peptides sample on the PSS PROTEEMA column. The peaks with given molar mass information and dark red arrows were used to construct the base calibration. Please note that the peak with 55kDa molar mass is not as nicely separated on all columns as shown in this example. Since the peak elutes close to the exclusion limit of the columns recommended for this application, a loss in column resolution can result in only 5 peaks being visible.

The molar masses and elution volumes of the 5 peaks below 38kDa were used to construct the base calibration. To convert the calibration curve constructed using the CNBr peptides into a calibration curve for collagene peptides, the five collagen peptide reference materials were run using the same experimental conditions as applied to run the CNBr peptide sample (Figure 3).

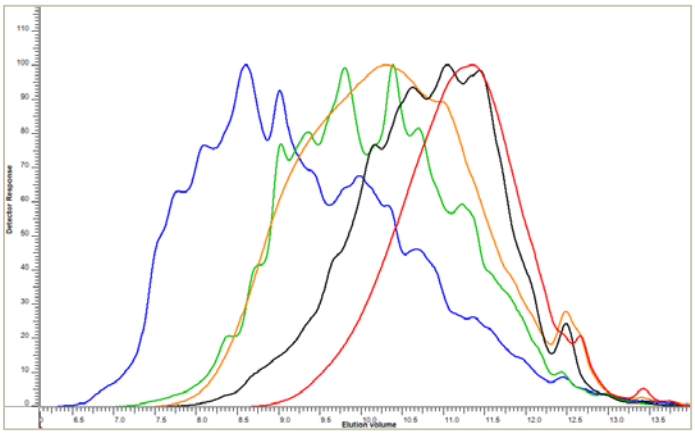
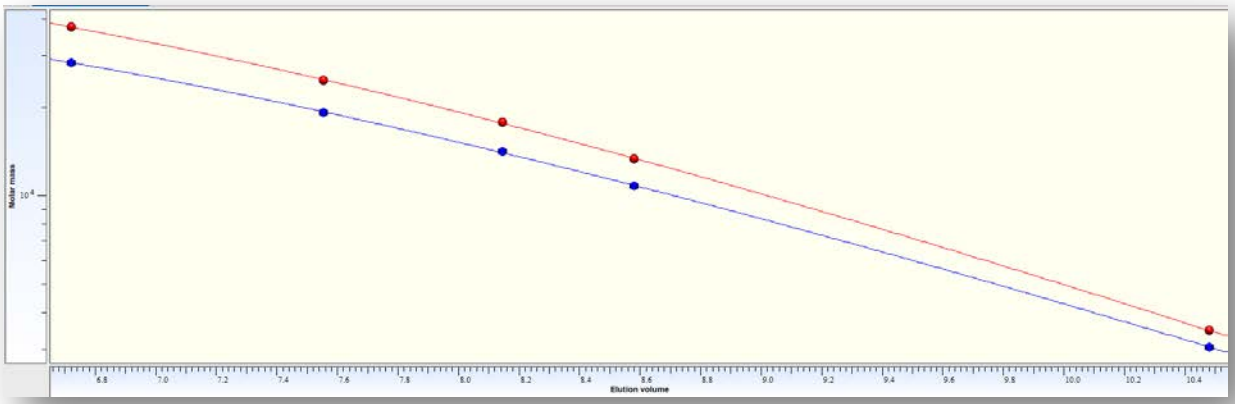


Figure 3: Overlay of the chromatograms of the collagen peptide reference materials
13R078
833593
13R079
891600
890435
on PSS PROTEEMA column.

The resulting chromatograms of the five collagen peptides (compare Figure 3) and their $M_w$ as shown in Table 1 were then applied to adjust the parameters of the broad standard approach and thereby establishing a calibration curve for collagene peptides.

Figure 4 shows a comparison of the base calibration corresponding to the CNBr peptides and the collagen peptide calibration curve. A significant shift between both calibration curves is observed, indicating the necessity to apply the broad calibration correction to the base calibration in order to achieve true molar masses. The application of the CNBr-peptide calibration only would result in an overestimation of the molar masses for collagen fragments.

Figure 4: Overlay of calibration curves constructed using CNBr peptides (base calibration, red) and collagen peptide calibration curve obtained by broad standard calibration (blue) on PSS PROTEEMA column



Once the calibration curve is established it can be used for the molar mass determination of collagen peptides of unknown molar mass.

Table 2 compares the target values as obtained from universal calibration with the weight average molar masses resulting after adjusting the parameters A and B of the broad calibration approach.

Table 2: Comparison of weight average *molar masses from universal calibration (target values) with weight average molar masses derived based on converted CNBr peptide calibration*

| | $M_w$ [g/mol] | |
|---|---|---|
| **Sample** | Values recalculated adjusted calibration curve | Target values (Universal Calibration) |
| **13R078** | 1929 | 1960 |
| **833593** | 2776 | 2820 |
| **13R079** | 4025 | 3920 |
| **891600** | 4596 | 4480 |
| **890435** | 8806 | 9020 |

## ROUND ROBIN TEST

To test the validity of the calibration approach, a round robin test was conducted involving four different laboratories. The task was determination of weight average molar masses for 6 collagen peptides, which were not applied during calibration.

The eluent, the concentration and injection volume were specified as described in the experimental section. A flow rate of 0.5mLmin$^{-1}$ was requested. The laboratories were, however, free in selecting one of the two column types described in the experimental section.

For the calibration the above developed procedure with the CNBr peptides and the 5 broad reference materials was demanded. The CNBr peptides sample was applied to establish the base calibration, which was converted into a calibration for collagen peptides using the five samples as shown above.

Afterwards 8 additional collagen peptide samples A – F were evaluated using the so established calibration curve. The results are summarized in Table 5 and shown in Figure 5.

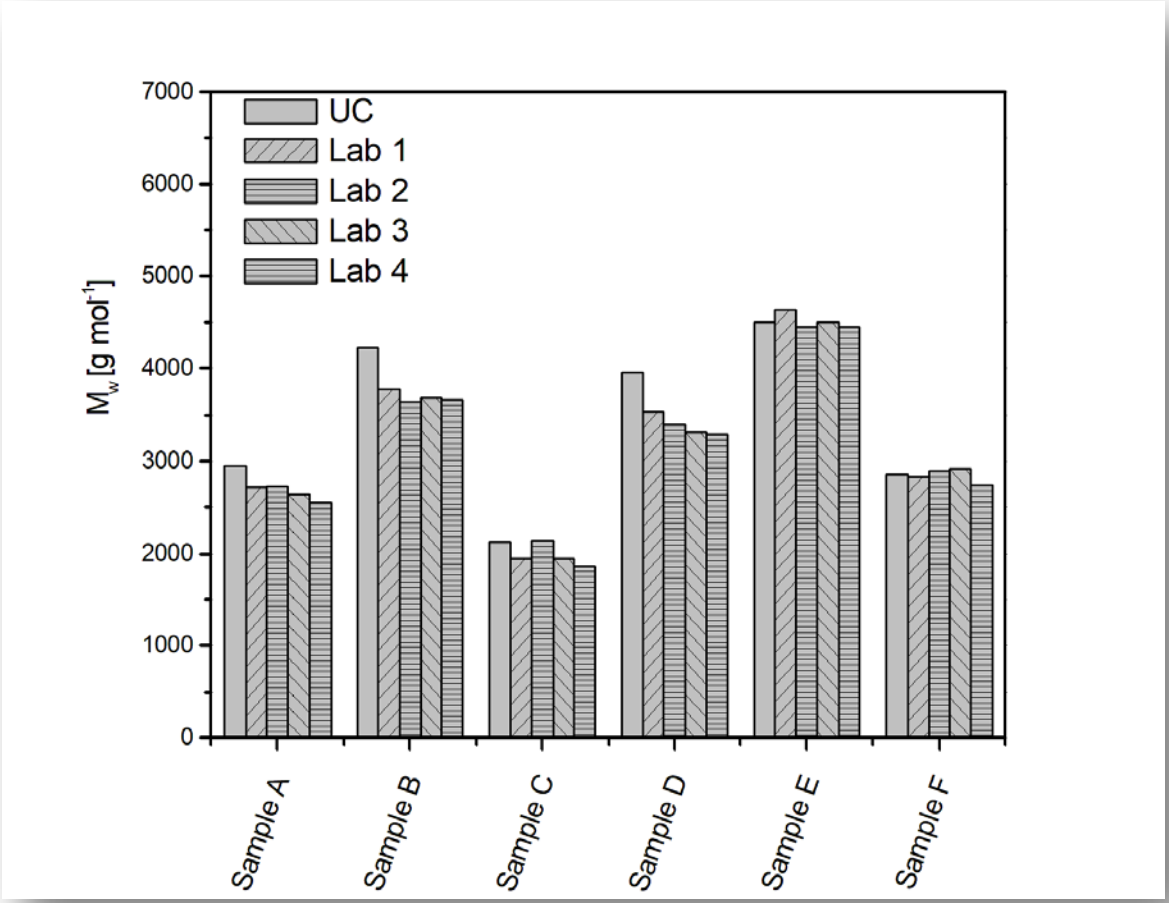
On average the value derived for the molar mass of a sample at a laboratory differs by less than 4% from the mean of the sample.

In addition, the data are compared to true weight average molar masses which were independently derived by GPC/SEC-Viscometry (column UC). The average deviation between the molar mass derived at a laboratory and the true value is less than 9%, which confirms that the above established calibration approach does not only allow for reliable inter laboratory comparison but also result in close to true molar masses.

Table 3: Average molar masses of collagen peptide samples derived in different laboratories using the suggested approach with broad standard calibration.

| Sample | $M_w$ [g/mol] UC | $M_w$ [g/mol] Lab1 | $M_w$ [g/mol] Lab2 | $M_w$ [g/mol] Lab3 | $M_w$ [g/mol] Lab4 |
|---|---|---|---|---|---|
| Sample A | 2950 | 2720 | 2730 | 2650 | 2550 |
| Sample B | 4230 | 3780 | 3640 | 3680 | 3660 |
| Sample C | 2120 | 1950 | 2140 | 1950 | 1870 |
| Sample D | 3960 | 3530 | 3390 | 3300 | 3280 |
| Sample E | 4500 | 4640 | 4450 | 4495 | 4450 |
| Sample F | 2860 | 2830 | 2890 | 2920 | 2740 |

Figure 5: Comparison of weight average molar masses of collagen peptide samples derived in different laboratories using the suggested approach with broad standard calibration.



# PROCEDURE FOR BROAD CALIBRATION WITH COLLAGEN PEPTIDE REFERENCE MATERIALS USING PSS WINGPC WITH GUIDED BROAD CALIBRATION

The CNBr-Peptides and the five collagen peptide references are commercially available for the construction of GPC/SEC calibration curves for collagen peptides using only a UV/VIS detector. The details are described in a Collagen Peptides Monograph.[7]

The applied broad standard calibration approach is often implemented in commercial GPC/SEC software packages. A user-friendly software wizard guides the user through the task in PSS WinGPC UniChrom.

The procedure includes the following steps:

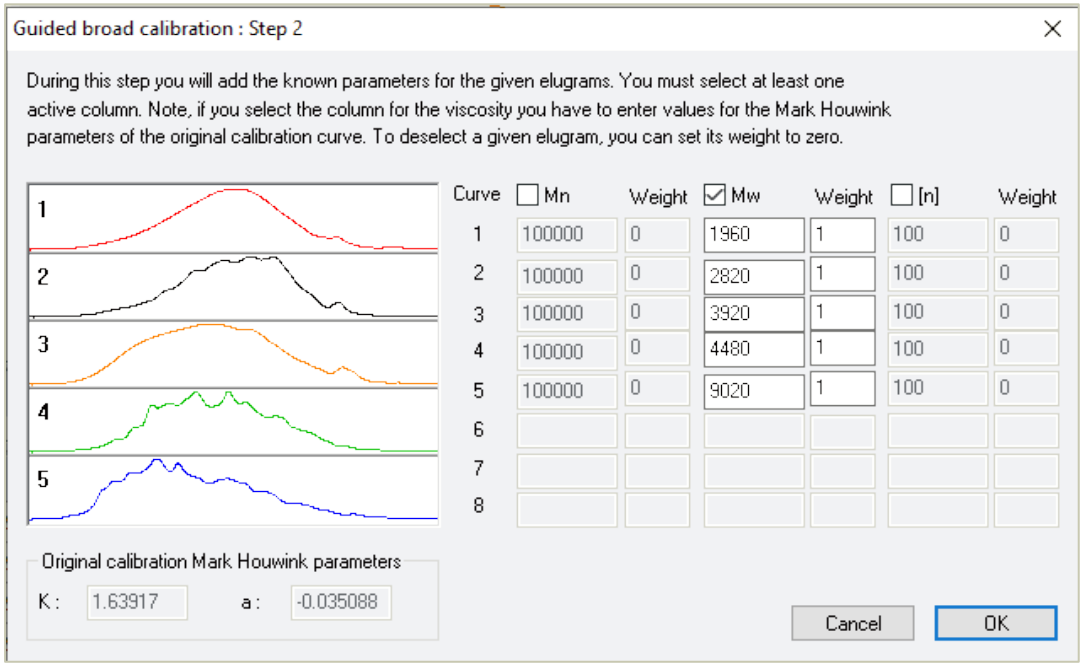
1) Run the CNBr-Peptides sample and construct a five point base calibration based on the measured elution volume and the certified molar masses.

2) Run the broadly distributed Collagen Peptide reference materials and add the resulting chromatograms to an overlay.

3) Start the Guided broad calibration wizard.

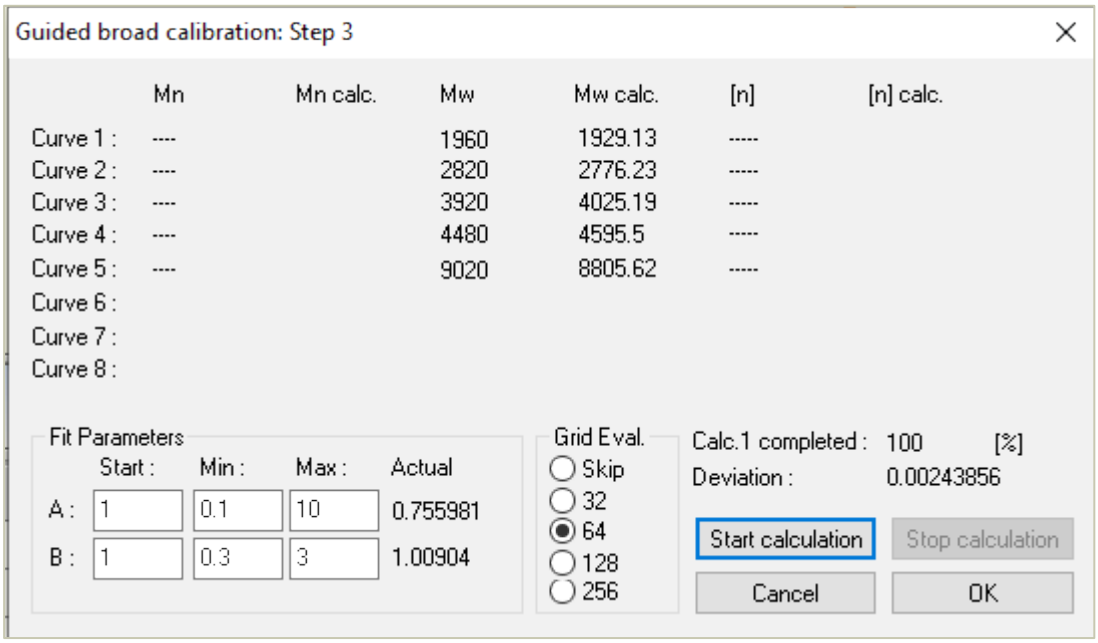
4) Use the overlay and assign the weight average molar masses as provided for the reference materials to each of the chromatograms.

Figure 6: Step 2 of the guided setup porcessing the overlay of chromatograms of the five collagen peptide reference samples with assigned weight average molar masses



5) Allow PSS WinGPC to automatically adjust the base calibration curve such that best agreement between target values and calculated molar masses is achieved for the chromatograms.

Figure 7: Step 3 of the guided setup showing the comparison of target values and derived values after adjusting the Fit Parameters




6) Save the calibration curve for future analysis of unknown collagen peptides.

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