Revision of the AF4 calibration experiment (Supplementary information)

Benedikt Häusele, Maxim Benjamin Gindele, Helmut Cölfen

Abstract

Asymmetrical field-flow fractionation is a versatile chromatographic fractionation method. In combination it is used for size-based separation of colloids, biomolecules and polymers. Although used often as pure separation method, a well-elaborated theory is available that allows precise quantification of the translational diffusion coefficient D. Still, current literature suggests different ways to transform this theory into applicable experimental procedures and no "gold standard" for correct data processing exists. While some sources report a direct way to extract diffusion information from the fractogram, others suggest the necessity of an external calibration measurement. In this work, we compare the different approaches and calibration algorithms based on original and literature data using our own open-source AF4 evaluation software. Based on the results, we conclude that available AF4 setups do not fulfill the requirements for absolute measurements of D yet and direct meaurements of w.

Determination of geometrical channel Volume V^{geo}

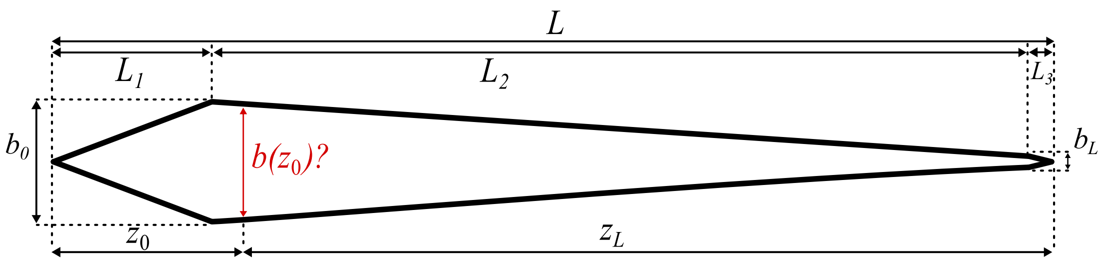


Fig. S.1: Channel dimensions. The width $b(z_L)$ depends on the focus position and is not accessible via bare channel data.

AF4 channel have a trapezoidal shape with measures indicated in fig. S.1. For all further considerations, the channel plane is split into three sections (1,2,3) with their correspoding lengths L_1, L_2, L_3. To simplify the further calculations, they are subsumed as in the following:

$$L = L_1 + L_2 + L_3 = L_{12} + L_3 \tag{S.1}$$

As the sample is focussed at a certain channel position on the beginning, this has to be considered. The relative focus position $z_{\%}$is related to the other focus-related magnitudes by

$$z_0 = z_{\%} L = L - z_L \tag{S.2}$$

The channel height difference $b_{\triangle}$on the section 2 is

$$b_{\triangle} = b_0 - b_L \geqq 0 \tag{S.3}$$

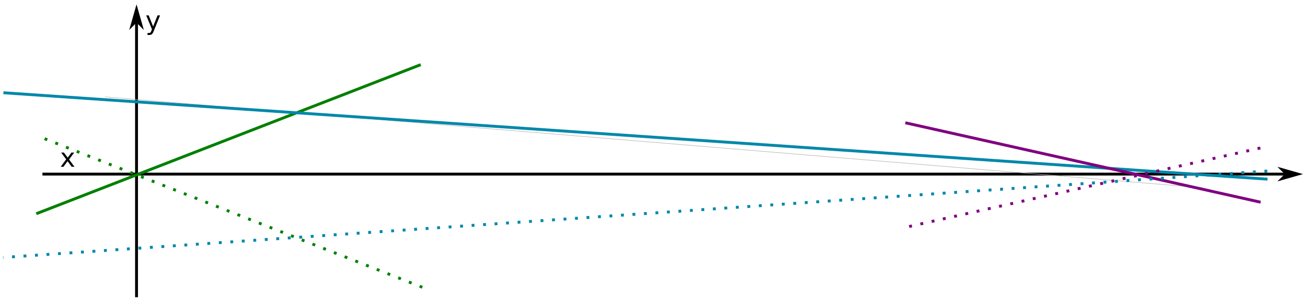


Fig. S.2: Channel dimensions as a set of 3 pairs of straight lines

Volume calculation may be conducted for the trapezoidal by simple decomposition of the channel plane into elementary geometrical objects. However, a concise analytical approach is more appropriate as the result can be displayed as a function of $z_{\%}$. In addition the corresponding $b(z_{\%})$ is not known initially. Similar derivations have already been conducted with the approximation of dividing the shape into two sections.[1–4] The approach may be useful for further hydrodynamic considerations as for example, the elution flow $V_e(x)$ in AF4 is a position-dependent size. For the trapezoidal plane shape, the channel is described by the enclosure of three pairs of straight line S.2. All expressions here are not optimized for mathematical elegance, but rather for being translated into an understandable and well-maintainable calculation routine. This is achieved by extensive subsitution of the known variables. Subsuming of these magnitudes helps to simplify the later expressions and the transformation into an Due to the reason of symmetry, only three borders have to described exactly:

$$\frac{1}{2}b(x) = E(x) \begin{cases} \boxed{e_1(x) = m_1 x = \frac{b_0}{2L_1} \cdot x} & \forall \quad 0 \leqq \quad x \quad \leqq L_1 \\ \boxed{e_2(x) = m_2 x + t_2 = -\frac{b_\Delta}{2L_2} \cdot x + \frac{1}{2}\left(b_0 + \frac{L_1}{L_2} b_\Delta\right)} & \forall \quad L_1 < \quad x \quad \leqq L_{12} \\ \boxed{e_3(x) = m_3 x + t_3 = -\frac{b_L}{2L_3} \cdot x + \frac{L b_L}{2L_3}} & \forall \quad L_{12} < \quad x \quad \leqq L \end{cases} \tag{S.4}$$

As all dimensions here are known, the slopes and offsets of the lines can be calculated directly and don't have to be resubsituted after the following substitutions. The calcuation of geometrical volume of the trapezoidal channel has to be adapted according to whether the focus position z_0 is located left or right to the position of maximal channel extent (i.e. if $z_0 < L_1$ or $z_0 \geqq L_1$). In the algorithm later, rather the plane is used explicitly, which is obtained easily

V^{geo}: Distal focussing with $z_0 \geqq L_1$

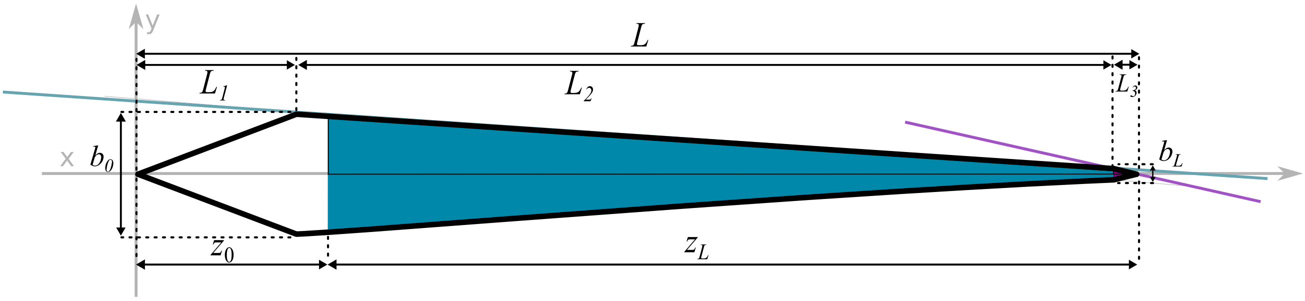


Fig. S.3: Area Section passed by the sample during the measurement marked with the color of the corresponding line in the case of distal focussing

In this case, the channel volume V^{geo} is the product of the channel width w and the colored area the x, y-plane of Fig. S.3. It is described by:

$$
\begin{aligned}
V^{\text{geo}} &= \left(\boxed{A_2} + \boxed{A_3}\right) \cdot w \\
&= 2 \cdot \left(\boxed{\int_{z_0}^{L_{12}} e_2(x)\,\mathrm{d}x} + \boxed{\int_{L-L_3}^{L} e_3(x)\,\mathrm{d}x}\right) \cdot w \\
&= \left(\boxed{(L_{12} - z_0)\,(m_2\,(L_{12} + z_0) + 2t_2)} + \boxed{\tfrac{1}{2} \cdot L_3 \cdot b_L}\right) \cdot w
\end{aligned}
\tag{S.5}
$$

V^{geo}: Proximal focussing with $z_0 < L_1$

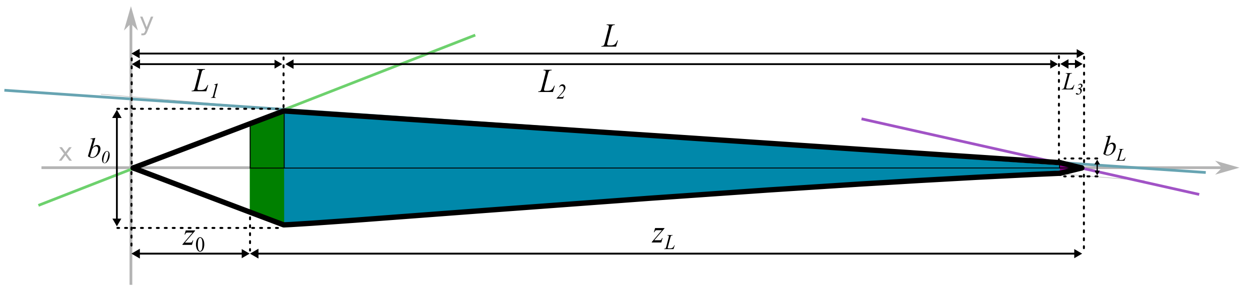


Fig. S.4: Area Section passed by the sample during the measurement marked with the color of the corresponding line in the case of proximal focussing

Outgoing from the previous result, the full area section of section 2 has to be considered, i.e. first z_0 is replaced by L_1, then the area part of section 1 is added:

$$
\begin{aligned}
V^{\text{geo}} &= \left(\boxed{A_1} + \boxed{A_2} + \boxed{A_3}\right) \cdot w \\
&= 2 \cdot \left(\boxed{\int_{z_0}^{L_1} e_1(x)\,\mathrm{d}x} + \boxed{\int_{L_1}^{L_{12}} e_2(x)\,\mathrm{d}x} + \boxed{\int_{L-L_3}^{L} e_3(x)\,\mathrm{d}x}\right) \cdot w \\
&= \left(\boxed{m_1 \cdot (L_1^2 - z_0^2)} + \boxed{m_2 L_{12} L_2 + m_2 L_1 L_2 + 2t_2 L_2} + \boxed{\tfrac{1}{2} \cdot L_3 \cdot b_L}\right) \cdot w \\
&= \left(\boxed{m_1 \cdot (L_1^2 - z_0^2)} + \boxed{\tfrac{1}{2}(b_0 + b_L) L_2} + \boxed{\tfrac{1}{2} \cdot L_3 \cdot b_L}\right) \cdot w
\end{aligned}
\tag{S.6}
$$

Determination of “hydrodynamic” channel height w^{hyd} and Volume V^{hyd}

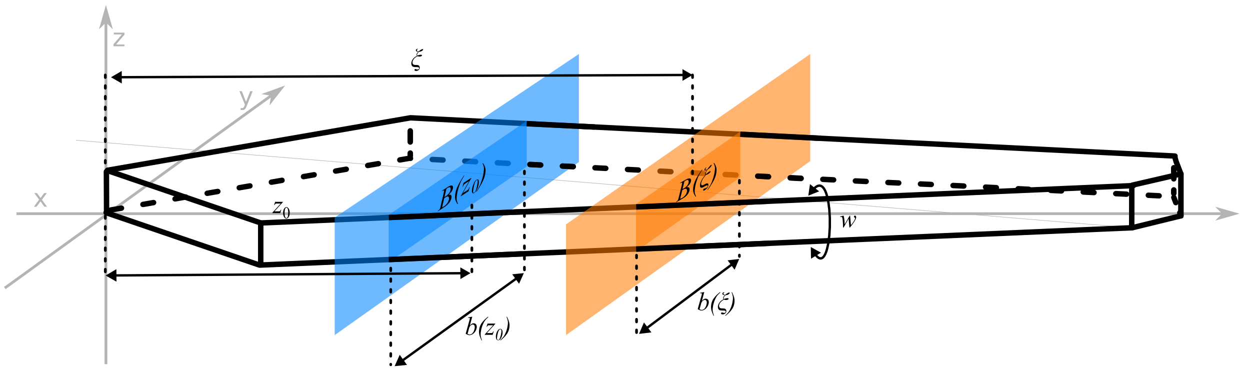


Fig. S.5: Cross sections $B(\xi)$ of the channel at different positions ξ

Here, the derivation is analogously conducted as described in literature[1, 5, 6], but using the straight equations S.4 for description of the channel above. This takes into consideration that $b(z)$ is variable over the whole channel length. In addition, also focussing into the first channel section is considered, for this reason, the the surface cannot just be corrected by a constant term as reported[6]. $t_{\text{void}}$is the void time of an unretained species which can be obtained by integration over the channel positions ξ. Although this derivation leads to rather laborious expressions, it has the advantage that no additional assumptions are necessary.

$$t_{\text{void}} = \int_0^{t_{\text{void}}} \mathrm{d}t = \int_{z_0}^{L} \frac{1}{v_{\text{m}}(\xi)} \,\mathrm{d}\xi \tag{S.7}$$

$v_{\text{m}}(\xi)$ is the migration velocity of the eluent at a channel position ξ. It dependends on the flow velocity $\dot{V}(\xi)$ at the position and the y, z cross-sectional area $B(\xi)$ at (Fig S.5)

$$v_{\text{m}}(\xi) = \frac{\dot{V}(\xi)}{B(\xi)} = \frac{\dot{V}(\xi)}{b(\xi) \cdot w} \tag{S.8}$$

The term $b(\xi)$ is described with the aid of eq. S.4 and will require a case-by-case approach. The change of the flow velocity $\dot{V}(\xi)$ is exactly the total loss in the applied crossflow. It has its maximum at the inlet position with

$$\dot{V}(0) = \dot{V}_{\text{in}} = \dot{V}_{\text{e}} + \dot{V}_{\text{c}} \tag{S.9}$$

and its minimum with

$$\dot{V}(L) = \dot{V}_{\text{e}} \tag{S.10}$$

As this is distributed uniformly over the membrane surface, the decay is proportional to the area the eluent has already passed. This leads to the expression

$$\dot{V}(\xi) = \dot{V}_{\text{in}} - V_c \cdot \frac{A(\xi)}{A_L} = \dot{V}_{\text{in}} - V_c \cdot \frac{\int_0^{\xi} b(x)\,\mathrm{d}x}{\int_0^{L} b(x)\,\mathrm{d}x} = \dot{V}_{\text{in}} - V_c \cdot \frac{2 \cdot \int_0^{\xi} E(x)\,\mathrm{d}x}{A_L} \tag{S.11}$$

The total area $A_L$can be easily derived by letting of eq. S.6 with letting $z_0 = 0$:

$$A_L = \boxed{A_1} + \boxed{A_2} + \boxed{A_3} \quad = \boxed{\tfrac{1}{2} b_0 L_1} + \boxed{\tfrac{1}{2}(b_0 + b_L) L_2} + \boxed{\tfrac{1}{2} L_3 b_L} \tag{S.12}$$

To evaluate $A(\xi)$ correctly, the integrals have to be split according to the conditions in eq. S.4. This is required which corresponds to the cases needed for $b(\xi)$. Merging eq. S.7 and S.11 gives the expression

$$v_{\mathrm{m}}(\xi) = \frac{\dot{V}_{\mathrm{in}} - V_c \cdot \frac{2 \cdot \int_0^{\xi} E(x)\,\mathrm{d}x}{A_L}}{2 \cdot E(\xi) \cdot w} = \frac{1}{2 \cdot w} \cdot \frac{\dot{V}_{\mathrm{in}} - V_c \cdot \frac{2 \cdot \int_0^{\xi} E(x)\,\mathrm{d}x}{A_L}}{E(\xi)} \tag{S.13}$$

Inserting into eq. S.7 gives:

$$t_{\mathrm{void}} = 2 \cdot w \cdot \int_{z_0}^{L} \frac{E(\xi)}{\dot{V}_{\mathrm{in}} - V_c \cdot \frac{2 \cdot \int_0^{\xi} E(x)\,\mathrm{d}x}{A_L}}\,\mathrm{d}\xi \tag{S.14}$$

This expression quantifies a linear conversion factor C_{F} for the relationship of t_{void} and w. This promises a simple relationship between those two basic magnitudes with

$$t_{\mathrm{void}} = 2 \cdot C_{\mathrm{F}} \cdot w \tag{S.15}$$

and

$$C_{\mathrm{F}} = \int_{z_0}^{L} \frac{E(\xi)}{\dot{V}_{\mathrm{in}} - V_c \cdot \frac{2 \cdot \int_0^{\xi} E(x)\,\mathrm{d}x}{A_L}}\,\mathrm{d}\xi \tag{S.16}$$

Similar to the calculation of V^{geo}above, a case-by case analysis is required depending on z_0. Due to the section-wise definition of the integrand, the integrals then have to be split accordingly to the partial domain of $E(\xi)$.

V^{hyd}: Distal focussing with $z_0 \geqq L_1$

Here, the outer integral of eq. S.14 is split into the sections with $L_1 < \xi \leqq L_{12}$ and $L_{12} < \xi$:

$$C_{\mathrm{F}} = \int_{z_0}^{L_{12}} \frac{E(\xi)}{\dot{V}_{\mathrm{in}} - \frac{2V_c}{A_L}\int_0^{\xi} E(x)\,\mathrm{d}x}\,\mathrm{d}\xi + \int_{L_{12}}^{L} \frac{E(\xi)}{\dot{V}_{\mathrm{in}} - \frac{2V_c}{A_L}\int_0^{\xi} E(x)\,\mathrm{d}x}\,\mathrm{d}\xi \tag{S.17}$$

As ξ is now located only on one of the section within each summand, the inner integrals can be split for the different domains of $E(x)$. Integrals independent from ξ are directly substituted with their corresponding area section from eq. S.12, only the last integral is solved.

$$\begin{aligned}
C_{\mathrm{F}} &= \int_{z_0}^{L_{12}} \frac{e_2(\xi)}{\dot{V}_{\mathrm{in}} - \frac{2V_c}{A_L}\left(\int_0^{L_1} e_1(x)\,\mathrm{d}x + \int_{L_1}^{\xi} e_2(x)\,\mathrm{d}x\right)}\,\mathrm{d}\xi \\
&\quad + \int_{L_{12}}^{L} \frac{e_3(\xi)}{\dot{V}_{\mathrm{in}} - \frac{2V_c}{A_L}\left(\int_0^{L_1} e_1(x)\,\mathrm{d}x + \int_{L_1}^{L_{12}} e_2(x)\,\mathrm{d}x + \int_{L_{12}}^{\xi} e_3(x)\,\mathrm{d}x\right)}\,\mathrm{d}\xi \\
&= \int_{z_0}^{L_{12}} \frac{m_2 \cdot \xi + t_2}{\dot{V}_{\mathrm{in}} - \frac{2V_c}{A_L}\left(\frac{1}{2}A_1 + \frac{1}{2}m_2(\xi^2 - L_1^2) + t_2(\xi - L_1)\right)}\,\mathrm{d}\xi \\
&\quad + \int_{L_{12}}^{L} \frac{m_3 \cdot \xi + t_3}{\dot{V}_{\mathrm{in}} - \frac{2V_c}{A_L}\left(\frac{1}{2}A_1 + \frac{1}{2}A_2 + \frac{1}{2}m_3(\xi^2 - L_{12}^2) + t_3(\xi - L_{12})\right)}\,\mathrm{d}\xi
\end{aligned} \tag{S.18}$$

In order to transform the integrand terms into ordinary rational functions and simplify the analytical solutions This can be rearranged by using substitutions for the occurring prefactors $\alpha_i, \beta_i, \gamma_i, \delta_i$, the quadratic polynomials $P(\xi)$ and its discriminants Δ_i:

$$\begin{aligned}
&\alpha_2 = \tfrac{t_2}{m_2} \quad \beta_2 = -\tfrac{\dot{V}_c m_2}{A_L} \quad \gamma_2 = -\tfrac{2\dot{V}_c t_2}{A_L} \\
&\delta_2 = \dot{V}_{\mathrm{in}} - \tfrac{\dot{V}_c}{A_L}\left(A_1 - m_2 L_1^2 - 2t_2 L_1\right) \qquad \Delta_2 = 4\beta_2\delta_2 - \gamma_2^2 \\
&\alpha_3 = \tfrac{t_3}{m_3} \quad \beta_3 = -\tfrac{\dot{V}_c m_3}{A_L} \quad \gamma_3 = -\tfrac{2\dot{V}_c t_3}{A_L} \\
&\delta_3 = \dot{V}_{\mathrm{in}} - \tfrac{\dot{V}_c}{A_L}\left(A_1 + A_2 - m_3 L_{12}^2 - 2t_3 L_{12}\right) \quad \Delta_3 = 4\beta_3\delta_3 - \gamma_3^2
\end{aligned} \tag{S.19}$$

$$\begin{aligned}
P_2(\xi) &= \beta_2\xi^2 + \gamma_2\xi + \delta_2 \\
P_3(\xi) &= \beta_3\xi^2 + \gamma_3\xi + \delta_3
\end{aligned} \tag{S.20}$$

For solving the integral it is important to know the sign of Δ_2 and Δ_3. Inserting S.9, it can be shown (see below) that it is not possible to determine the scope of Δ_2 exactly for the general case and the case-by-case analysis has to be conducted "at runtime". To simplify the display of this expression, it is split and each summand treated separately:

$$C_{\mathrm{F}} = C_{\mathrm{F2}} + C_{\mathrm{F3}} \tag{S.21}$$

$$
\begin{aligned}
C_{\mathrm{F2}} &= m_2 \cdot \int_{z_0}^{L_{12}} \frac{\xi + \alpha_2}{\beta_2 \xi^2 + \gamma_2 \xi + \delta_2} \,\mathrm{d}\xi \\
&= m_2 \cdot \left(\int_{z_0}^{L_{12}} \frac{\xi}{P_2(\xi)} \,\mathrm{d}\xi + \int_{z_0}^{L_{12}} \frac{\alpha_2}{P_2(\xi)} \,\mathrm{d}\xi \right) \\
&= m_2 \cdot \left(\left[\tfrac{\ln P_2(\xi)}{2\beta_2} \right]_{z_0}^{L_{12}} + \left(\alpha_2 - \tfrac{\gamma_2}{2\beta_2} \right) \int_{z_0}^{L_{12}} \frac{\mathrm{d}\xi}{P_2(\xi)} \right) & [7] \\
&= \begin{cases} m_2 \cdot \left(\left[\tfrac{\ln P_2(\xi)}{2\beta_2} \right]_{z_0}^{L_{12}} + \left(\alpha_2 - \tfrac{\gamma_2}{2\beta_2} \right) \left[\frac{2}{\sqrt{\Delta_2}} \cdot \arctan\left(\frac{2\beta_2 \xi + \gamma_2}{\sqrt{\Delta_2}} \right) \right]_{z_0}^{L_{12}} \right) & \forall \Delta_2 > 0 \\ m_2 \cdot \left(\left[\tfrac{\ln P_2(\xi)}{2\beta_2} \right]_{z_0}^{L_{12}} + \left(\alpha_2 - \tfrac{\gamma_2}{2\beta_2} \right) \left[-\frac{2}{\sqrt{-\Delta_2}} \cdot \operatorname{artanh}\left(\frac{2\beta_2 \xi + \gamma_2}{\sqrt{-\Delta_2}} \right) \right]_{z_0}^{L_{12}} \right) & \forall \Delta_2 < 0 \end{cases} & [8] \\
&= \begin{cases} m_2 \cdot \left(\tfrac{1}{2\beta_2} \left(\ln P_2(L_{12}) - \ln P_2(z_0) \right) + \left(\tfrac{2}{\sqrt{\Delta_2}} \right) \left(\alpha_2 - \tfrac{\gamma_2}{2\beta_2} \right) \left(\arctan \frac{2\beta_2 L_{12} + \gamma_2}{\sqrt{\Delta_2}} - \arctan \frac{2\beta_2 z_0 + \gamma_2}{\sqrt{\Delta_2}} \right) \right) & \forall \Delta_2 > 0 \\ m_2 \cdot \left(\tfrac{1}{2\beta_2} \left(\ln P_2(L_{12}) - \ln P_2(z_0) \right) - \left(\tfrac{2}{\sqrt{-\Delta_2}} \right) \left(\alpha_2 - \tfrac{\gamma_2}{2\beta_2} \right) \left(\operatorname{artanh} \frac{2\beta_2 L_{12} + \gamma_2}{\sqrt{-\Delta_2}} - \operatorname{artanh} \frac{2\beta_2 z_0 + \gamma_2}{\sqrt{-\Delta_2}} \right) \right) & \forall \Delta_2 < 0 \end{cases} \\
&= \begin{cases} m_2 \cdot \left(\tfrac{1}{2\beta_2} \ln \tfrac{P_2(L_{12})}{P_2(z_0)} + \left(\tfrac{2}{\sqrt{\Delta_2}} \right) \left(\alpha_2 - \tfrac{\gamma_2}{2\beta_2} \right) \left(\arctan \frac{2\beta_2 L_{12} + \gamma_2}{\sqrt{\Delta_2}} - \arctan \frac{2\beta_2 z_0 + \gamma_2}{\sqrt{\Delta_2}} \right) \right) & \forall \Delta_2 > 0 \\ m_2 \cdot \left(\tfrac{1}{2\beta_2} \ln \tfrac{P_2(L_{12})}{P_2(z_0)} - \left(\tfrac{2}{\sqrt{-\Delta_2}} \right) \left(\alpha_2 - \tfrac{\gamma_2}{2\beta_2} \right) \left(\operatorname{artanh} \frac{2\beta_2 L_{12} + \gamma_2 - 2\beta_2 z_0 - \gamma_2}{\sqrt{-\Delta_2} \cdot \left(1 - \frac{2\beta_2 L_{12} + \gamma_2}{\sqrt{-\Delta_2}} \cdot \frac{2\beta_2 z_0 + \gamma_2}{\sqrt{-\Delta_2}} \right)} \right) \right) & \forall \Delta_2 < 0 \end{cases} \\
&= \begin{cases} m_2 \cdot \left(\tfrac{1}{2\beta_2} \ln \tfrac{P_2(L_{12})}{P_2(z_0)} + \left(\tfrac{2}{\sqrt{\Delta_2}} \right) \left(\alpha_2 - \tfrac{\gamma_2}{2\beta_2} \right) \left(\arctan \frac{2\beta_2 L_{12} + \gamma_2}{\sqrt{\Delta_2}} - \arctan \frac{2\beta_2 z_0 + \gamma_2}{\sqrt{\Delta_2}} \right) \right) & \forall \Delta_2 > 0 \\ m_2 \cdot \left(\tfrac{1}{2\beta_2} \ln \tfrac{P_2(L_{12})}{P_2(z_0)} - \left(\tfrac{2}{\sqrt{-\Delta_2}} \right) \left(\alpha_2 - \tfrac{\gamma_2}{2\beta_2} \right) \left(\operatorname{artanh} \frac{2\beta_2 (L_{12} - z_0)}{\left(\sqrt{-\Delta_2} - \frac{(2\beta_2 L_{12} + \gamma_2)(2\beta_2 z_0 + \gamma_2)}{\sqrt{-\Delta_2}} \right)} \right) \right) & \forall \Delta_2 < 0 \end{cases}
\end{aligned} \tag{S.22}
$$

$$
\begin{aligned}
C_{\mathrm{F3}} &= m_3 \cdot \int_{L_{12}}^{L} \frac{\xi + \alpha_3}{\beta_3 \xi^2 + \gamma_3 \xi + \delta_3} \,\mathrm{d}\xi \\
&= m_3 \cdot \left(\int_{L_{12}}^{L} \frac{\xi}{P_3(\xi)} \,\mathrm{d}\xi + \int_{L_{12}}^{L} \frac{\alpha_3}{P_3(\xi)} \,\mathrm{d}\xi \right) \\
& \cdots \text{ analogously to eq. S.22 } \cdots \\
&= \begin{cases} m_3 \cdot \left(\tfrac{1}{2\beta_3} \ln \tfrac{P_3(L)}{P_3(L_{12})} + \left(\tfrac{2}{\sqrt{\Delta_3}} \right) \left(\alpha_3 - \tfrac{\gamma_3}{2\beta_3} \right) \left(\arctan \frac{2\beta_3 L + \gamma_3}{\sqrt{\Delta_3}} - \arctan \frac{2\beta_3 L_{12} + \gamma_3}{\sqrt{\Delta_3}} \right) \right) & \forall \Delta_3 > 0 \\ m_3 \cdot \left(\tfrac{1}{2\beta_3} \ln \tfrac{P_3(L)}{P_3(L_{12})} - \left(\tfrac{2}{\sqrt{-\Delta_3}} \right) \left(\alpha_3 - \tfrac{\gamma_3}{2\beta_3} \right) \left(\operatorname{artanh} \frac{2\beta_3 (L - L_{12})}{\left(\sqrt{-\Delta_3} - \frac{(2\beta_3 L + \gamma_3)(2\beta_3 L_{12} + \gamma_3)}{\sqrt{-\Delta_3}} \right)} \right) \right) & \forall \Delta_3 < 0 \end{cases} \\
&= \begin{cases} m_3 \cdot \left(\tfrac{1}{2\beta_3} \ln \tfrac{P_3(L)}{P_3(L_{12})} + \left(\tfrac{2}{\sqrt{\Delta_3}} \right) \left(\alpha_3 - \tfrac{\gamma_3}{2\beta_3} \right) \left(\arctan \frac{2\beta_3 L + \gamma_3}{\sqrt{\Delta_3}} - \arctan \frac{2\beta_3 L_{12} + \gamma_3}{\sqrt{\Delta_3}} \right) \right) & \forall \Delta_3 > 0 \\ m_3 \cdot \left(\tfrac{1}{2\beta_3} \ln \tfrac{P_3(L)}{P_3(L_{12})} - \left(\tfrac{2}{\sqrt{-\Delta_3}} \right) \left(\alpha_3 - \tfrac{\gamma_3}{2\beta_3} \right) \left(\operatorname{artanh} \frac{2\beta_3 \left(L - L_{12} \right)}{\left(\sqrt{-\Delta_3} - \frac{(2\beta_3 L + \gamma_3)(2\beta_3 L_{12} + \gamma_3)}{\sqrt{-\Delta_3}} \right)} \right) \right) & \forall \Delta_3 < 0 \end{cases}
\end{aligned} \tag{S.23}
$$

V^{hyd}: Proximal focusing with $z_0 < L_1$

If the sample was focused to a point with $z_0 < L_1$, the in addition to the solution above, also the eluent migration through the first sections has to be considered. The evaluation of the expression can be conducted analogously for the second and third summand as shown above with adaption of the lower limit of integration for the second:

$$
\begin{aligned}
C_{\mathrm{F}} \quad = \quad & \int_{z_0}^{L_1} \frac{e_1(\xi)}{\dot{V}_{\mathrm{in}} - \frac{2V_c}{A_L}\left(\int_0^{\xi} e_1(x)\,\mathrm{d}x\right)}\,\mathrm{d}\xi \\
+ \quad & \int_{L_1}^{L_{12}} \frac{e_2(\xi)}{\dot{V}_{\mathrm{in}} - \frac{2V_c}{A_L}\left(\int_0^{L_1} e_1(x)\,\mathrm{d}x + \int_{L_1}^{\xi} e_2(x)\,\mathrm{d}x\right)}\,\mathrm{d}\xi \\
+ \quad & \int_{L_{12}}^{L} \frac{e_3(\xi)}{\dot{V}_{\mathrm{in}} - \frac{2V_c}{A_L}\left(\int_0^{L_1} e_1(x)\,\mathrm{d}x + \int_{L_1}^{L_{12}} e_2(x)\,\mathrm{d}x + \int_{L_{12}}^{\xi} e_3(x)\,\mathrm{d}x\right)}\,\mathrm{d}\xi \\
= \quad & \int_{z_0}^{L_1} \frac{m_1 \cdot \xi}{\dot{V}_{\mathrm{in}} - \frac{2V_c}{A_L}\left(\frac{1}{2}m_1\xi^2\right)}\,\mathrm{d}\xi \\
+ \quad & \int_{L_1}^{L_{12}} \frac{m_2 \cdot \xi + t_2}{\dot{V}_{\mathrm{in}} - \frac{2V_c}{A_L}\left(\frac{1}{2}A_1 + \frac{1}{2}m_2(\xi^2 - L_1^2) + t_2(\xi - L_1)\right)}\,\mathrm{d}\xi \\
+ \quad & \int_{L_{12}}^{L} \frac{m_3 \cdot \xi + t_3}{\dot{V}_{\mathrm{in}} - \frac{2V_c}{A_L}\left(\frac{1}{2}A_1 + \frac{1}{2}A_2 + \frac{1}{2}m_3(\xi^2 - L_{12}^2) + t_3(\xi - L_{12})\right)}\,\mathrm{d}\xi
\end{aligned}
\tag{S.24}
$$

Substitution is done similarly as above:

$$
\begin{aligned}
& \beta_1 = -\tfrac{\dot{V}_c m_1}{A_L} \quad \delta_1 = \dot{V}_{\mathrm{in}} \\
& \alpha_2 = \tfrac{t_2}{m_2} \quad \beta_2 = -\tfrac{\dot{V}_c m_2}{A_L} \quad \gamma_2 = -\tfrac{2\dot{V}_c t_2}{A_L} \\
& \delta_2 = \dot{V}_{\mathrm{in}} - \tfrac{\dot{V}_c}{A_L}\left(A_1 - m_2 L_1^2 - 2t_2 L_1\right) \qquad \Delta_2 = 4\beta_2\delta_2 - \gamma_2^2 \\
& \alpha_3 = \tfrac{t_3}{m_3} \quad \beta_3 = -\tfrac{\dot{V}_c m_3}{A_L} \quad \gamma_3 = -\tfrac{2\dot{V}_c t_3}{A_L} \\
& \delta_3 = \dot{V}_{\mathrm{in}} - \tfrac{\dot{V}_c}{A_L}\left(A_1 + A_2 - m_3 L_{12}^2 - 2t_3 L_{12}\right) \quad \Delta_3 = 4\beta_3\delta_3 - \gamma_3^2
\end{aligned}
\tag{S.25}
$$

$$
\begin{aligned}
P_2(\xi) &= \beta_2\xi^2 + \gamma_2\xi + \delta_2 \\
P_3(\xi) &= \beta_3\xi^2 + \gamma_3\xi + \delta_3
\end{aligned}
\tag{S.26}
$$

Then, in analogy, to the case $z_0 \geqq L_1$, C_F can be expressed as

$$
\begin{aligned}
C_\mathrm{F} &= C_\mathrm{F1} + C_\mathrm{F2} + C_\mathrm{F3} \\
&= m_1 \cdot \int_{z_0}^{L_1} \left(\frac{\xi}{\beta_1 \cdot \xi^2 + \delta_1} \right) \mathrm{d}\xi \\
&+ m_2 \cdot \int_{L_1}^{L_{12}} \left(\frac{\xi + \alpha_2}{\beta_2 \xi^2 + \gamma_2 \xi + \delta_2} \right) \mathrm{d}\xi \\
&+ m_3 \cdot \int_{L_{12}}^{L} \left(\frac{\xi + \alpha_3}{\beta_3 \xi^2 + \gamma_3 \xi + \delta_3} \right) \mathrm{d}\xi
\end{aligned}
\tag{S.27}
$$

with

$$
\begin{aligned}
C_\mathrm{F1} &= m_1 \cdot \int_{z_0}^{L_1} \left(\frac{\xi}{\beta_1 \cdot \xi^2 + \delta_1} \right) \mathrm{d}\xi \\
&= \frac{m_1}{\beta_1} \cdot \int_{z_0}^{L_1} \left(\frac{\xi}{\frac{\delta_1}{\beta_1} + \xi^2 W} \right) \mathrm{d}\xi \\
&= \frac{m_1}{\beta_1} \cdot \tfrac{1}{2} \left[\ln\!\left(\left| \frac{\delta_1}{\beta_1} + \xi^2 \right| \right) \right]_{z_0}^{L_1} \\
&= \frac{m_1}{2\beta_1} \cdot \left(\ln \left| \frac{\delta_1}{\beta_1} + L_1^2 \right| - \ln \left| \frac{\delta_1}{\beta_1} + z_0^2 \right| \right)
\end{aligned}
\tag{S.28}
$$

$$
C_\mathrm{F2} = \begin{cases}
m_2 \cdot \left(\frac{1}{2\beta_2} \ln \frac{P_2(L_{12})}{P_2(L_1)} + \left(\frac{2}{\sqrt{\Delta_2}} \right) \left(\alpha_2 - \frac{\gamma_2}{2\beta_2} \right) \left(\arctan \dfrac{2\beta_2 L_{12} + \gamma_2}{\sqrt{\Delta_2}} - \arctan \dfrac{2\beta_2 L_1 + \gamma_2}{\sqrt{\Delta_2}} \right) \right) & \forall \Delta_2 > 0 \\
m_2 \cdot \left(\frac{1}{2\beta_2} \ln \frac{P_2(L_{12})}{P_2(L_1)} - \left(\frac{2}{\sqrt{-\Delta_2}} \right) \left(\alpha_2 - \frac{\gamma_2}{2\beta_2} \right) \left(\operatorname{artanh} \dfrac{2\beta_2 (L_{12} - L_1)}{\left(\sqrt{-\Delta_2} - \frac{(2\beta_2 L_{12} + \gamma_2)(2\beta_2 z_0 + \gamma_2)}{\sqrt{-\Delta_2}} \right)} \right) \right) & \forall \Delta_2 < 0
\end{cases}
\tag{S.29}
$$

$$
C_\mathrm{F3} = \begin{cases}
m_3 \cdot \left(\frac{1}{2\beta_3} \ln \frac{P_3(L)}{P_3(L_{12})} + \left(\frac{2}{\sqrt{\Delta_3}} \right) \left(\alpha_3 - \frac{\gamma_3}{2\beta_3} \right) \left(\arctan \dfrac{2\beta_3 L + \gamma_3}{\sqrt{\Delta_3}} - \arctan \dfrac{2\beta_3 L_{12} + \gamma_3}{\sqrt{\Delta_3}} \right) \right) & \forall \Delta_3 > 0 \\
m_3 \cdot \left(\frac{1}{2\beta_3} \ln \frac{P_3(L)}{P_3(L_{12})} - \left(\frac{2}{\sqrt{-\Delta_3}} \right) \left(\alpha_3 - \frac{\gamma_3}{2\beta_3} \right) \left(\operatorname{artanh} \dfrac{2\beta_3 (L - L_{12})}{\left(\sqrt{-\Delta_3} - \frac{(2\beta_3 L + \gamma_3)(2\beta_3 L_{12} + \gamma_3)}{\sqrt{-\Delta_3}} \right)} \right) \right) & \forall \Delta_3 < 0
\end{cases}
\tag{S.30}
$$

Evaluation of $\boldsymbol{\Delta}_2$

To avoid an additional case-by-case analysis for the integration, the discriminants the polynomials $P_2(\xi)$ and $P_3(\xi)$ each were resubsituted to derive that only one of the cases

$$
\int \frac{\mathrm{d}\xi}{\beta_i \xi^2 + \gamma_i \xi + \delta_i} \begin{cases}
= \dfrac{2}{\sqrt{\Delta_i}} \cdot \arctan \left(\dfrac{2\beta_i \xi_i + \gamma_i}{\sqrt{\Delta_i}} \right) & \forall \quad \Delta_i > 0 \\
= -\dfrac{2}{\sqrt{-\Delta_i}} \cdot \operatorname{artanh} \left(\dfrac{2\beta_i \xi_i + \gamma_i}{\sqrt{-\Delta_i}} \right) & \forall \quad \Delta_i < 0
\end{cases}
\quad [8]
$$

has to be applied for the evaluation of C_F:

$$
\begin{aligned}
\Delta_2 &= 4\beta_2\delta_2 - \gamma_2^2 \\
&= 4 \cdot \left(-\tfrac{\dot{V}_c m_2}{A_L}\right) \cdot \left(\dot{V}_{\text{in}} - \tfrac{\dot{V}_c}{A_L}\left(A_1 - m_2 L_1^2 - 2t_2 L_1\right)\right) - \left(-\tfrac{2\dot{V}_c t_2}{A_L}\right)^2 \\
&= -4 \cdot \tfrac{\dot{V}_c m_2 \dot{V}_{\text{in}}}{A_L} + 4 \cdot \tfrac{\dot{V}_c^2 m_2 A_1}{A_L^2} - 4 \cdot \tfrac{\dot{V}_c^2 m_2^2 L_1^2}{A_L^2} - 4 \cdot \tfrac{2\dot{V}_c^2 m_2 t_2 L_1}{A_L^2} - 4 \cdot \tfrac{\dot{V}_c^2 t_2^2}{A_L^2} \\
&= \left(4 \cdot \tfrac{\dot{V}_c^2}{A_L^2}\right) \cdot \left(-m_2 A_1 + m_2^2 L_1^2 - 2m_2 t_2 L_1 - t_2^2\right) - 4 \cdot \tfrac{\dot{V}_c m_2 \dot{V}_{\text{in}}}{A_L} \\
&= \left(\tfrac{\dot{V}_c^2}{A_L^2}\right) \cdot \left(-\tfrac{L_1}{L_2} b_0 b_\Delta - \tfrac{L_1^2}{L_2^2} b_\Delta^2 + 2\tfrac{L_1}{L_2} b_0 b_\Delta + 2\tfrac{L_1^2}{L_2^2} b_\Delta^2 - b_0^2 - 2\tfrac{L_1}{L_2} b_0 b_\Delta - \tfrac{L_1^2}{L_2^2} b_\Delta^2\right) + 2 \cdot \tfrac{b_\Delta \dot{V}_c \dot{V}_{\text{in}}}{L_2 A_L} \\
&= \left(\tfrac{\dot{V}_c^2}{A_L^2}\right) \cdot \left(-\tfrac{L_1}{L_2} b_0 b_\Delta - b_0^2\right) + 2 \cdot \tfrac{b_\Delta \dot{V}_c \dot{V}_{\text{in}}}{L_2 A_L} \\
&= \left(\tfrac{\dot{V}_c}{A_L}\right) \cdot \left(-\tfrac{\dot{V}_c}{A_L}\tfrac{L_1}{L_2} b_0 b_\Delta - \tfrac{\dot{V}_c}{A_L} b_0^2 + 2\tfrac{b_\Delta}{L_2}\dot{V}_{\text{in}}\right) \\
&= \left(\tfrac{\dot{V}_c}{A_L^2}\right)\left(\dot{V}_{\text{in}} \cdot \left(2\tfrac{b_\Delta}{L_2} A_L\right) - \dot{V}_c \cdot \left(\tfrac{L_1}{L_2} b_0 b_\Delta + b_0^2\right)\right) \\
&= \left(\tfrac{\dot{V}_c}{A_L^2}\right)\left(\dot{V}_{\text{in}} \cdot \left(2\tfrac{b_\Delta}{L_2}\left(\tfrac{1}{2} b_0 L_1 + \tfrac{1}{2}(b_0 + b_L) L_2 + \tfrac{1}{2} L_3 b_L\right)\right) - \dot{V}_c \cdot \left(\tfrac{L_1}{L_2} b_0 b_\Delta + b_0^2\right)\right) \\
&= \left(\tfrac{\dot{V}_c}{A_L^2}\right)\left(\dot{V}_{\text{in}} \cdot \left(\tfrac{L_1}{L_2} b_\Delta b_0 + b_\Delta b_0 + b_\Delta b_L + \tfrac{L_3}{L_2} b_L b_\Delta\right) - \dot{V}_c \cdot \left(\tfrac{L_1}{L_2} b_0 b_\Delta + b_0^2\right)\right)
\end{aligned}
\tag{S.31}
$$

It turns out that the sign of the discriminant cannot be determined exactly without prior knowledge about the parameters. and the sign of the discriminants have to be determined “at runtime”.

Simplified formulation w^{hyd} and Volume V^{hyd}

A much shorter version has already been derived with the assumptions $L_{23} = L_2 + L_3$ and $b(L) \approx b(L_{12}) = b_L$.[6]
Here,

$$\begin{aligned} t_{\text{void}} &= \frac{V^{\approx\text{geo}}}{\dot{V}_{\text{c}}} \ln\left(1 + \frac{\dot{V}_{\text{c}}}{\dot{V}_{\text{e}}}\left(1 - \frac{w\left(b_0 z_0 - \frac{z_0^2 b_\Delta}{2L} - Y\right)}{V^{\text{geo}}}\right)\right) \\ &= \frac{V^{\approx\text{geo}}}{\dot{V}_{\text{c}}} \ln\left(1 + \frac{\dot{V}_{\text{c}}}{\dot{V}_{\text{e}}}\left(1 - \frac{b_0 z_0 - \frac{z_0^2 b_\Delta}{2L} - Y}{\int_0^L b(z)\,\mathrm{d}z}\right)\right) \end{aligned} \tag{S.32}$$

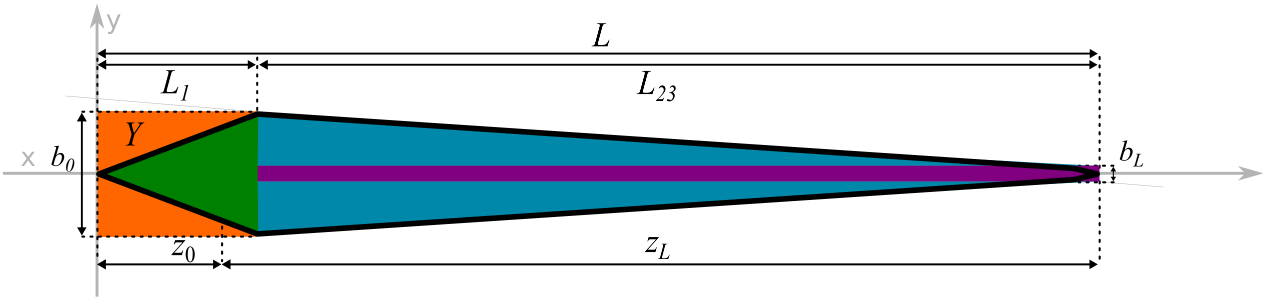


Fig. S.6: Total area of an AF4 channel in the simplified model.

With these assumptions, the channel surface A_L is computed as

$$A_L = \int_0^L b(z)\,\mathrm{d}z = \boxed{\tfrac{1}{2} b_0 L_1} + \boxed{b_L L_{23}} + \boxed{\tfrac{1}{2} b_\Delta L_{23}} \tag{S.33}$$

Y is the enclosed area of the elongation from, $e_2(x)$ y-axis and $e_1(x)$ and its symmetrical counterpart (Fig. S.7 and S.8). It can be calculated by simple geometrical considerations (Fig.)as

$$\boxed{Y} = \boxed{2 \cdot \tfrac{1}{2} e_2(0) L_1} = \boxed{\tfrac{1}{2}\left(b_0 + \tfrac{L_1}{L_{23}} b_\Delta\right) L_1} \tag{S.34}$$

The area, which is relevant for separation, could also be calculated according to the geometrical considerations from above. In this simplified version, the the proximal and distal focusing cases have to be distinguished:

Distal focusing with $z_0 \geqq L_1$

In this case, only the relevant part on the elongated surface is considered (Fig. S.7).

$$\frac{V^{\approx\text{geo}}}{w} = \int_{z_0}^{z_L} b(z)\,\mathrm{d}z = \frac{1}{2} b_\Delta \left(L_{23} - z_0\right) \tag{S.35}$$

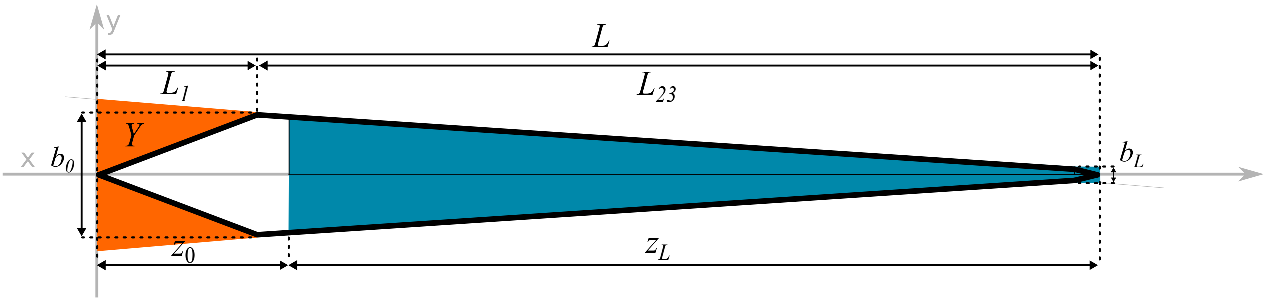


Fig. S.7: Simplified model of relevant passed area sections acccording to literature[6] in case of a distal focusing point.

Proximal focusing with $z_0 < L_1$

In this case, the additional space on the left part has to be considered as well (Fig. S.8).

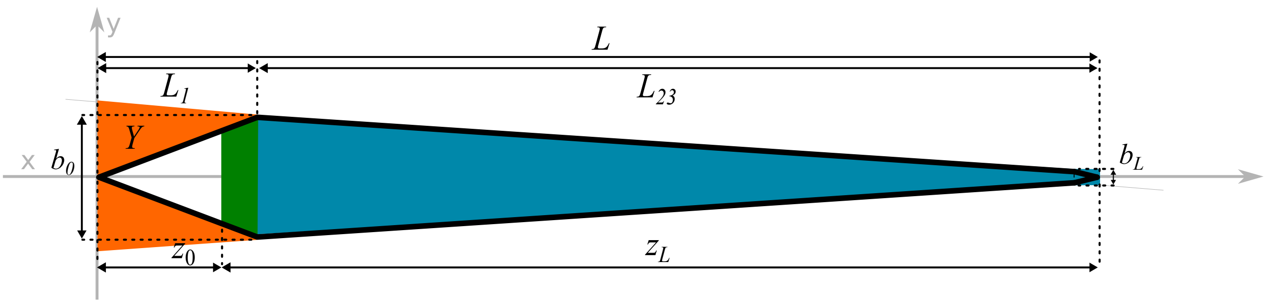


Fig. S.8: Simplified model of relevant passed area sections acccording to literature[6] in case of a proximal focusing point.

$$\frac{V^{\approx\text{geo}}}{w} = \int_{z_0}^{z_L} b(z)\,\mathrm{d}z = \boxed{\frac{b_0}{2L_1}(L_1^2 - z_0^2)} + \boxed{\frac{1}{2} b_\Delta L_{23}} \tag{S.36}$$

Determination of without experimental t_{void}

If a calibration measurement with known diffusion coefficient is used. Using the relationship of R can be formulated as used. R_D stands for the dependency of R the calibration

$$R_D(w) = 6\lambda\left(\coth\left(\frac{1}{2\lambda}\right) - 2\lambda\right) \tag{S.37}$$

$$\lambda = \frac{DV}{\dot{V}_\text{c} w^2} = \frac{DA_z}{\dot{V}_\text{c} w} \tag{S.38}$$

In order to eliminate t_{void} as experimental input, eq. S.15 is used as a substitution for the retention ratio, resulting in an expression solely dependent on t_e and w:

$$R_{t_\text{e}}(w) = \frac{2C_\text{F} w}{t_\text{e}} \tag{S.39}$$

By adjusting w such that

$$(R_{t_\text{e}} - R_D)^2 \rightarrow \min$$

, w can be calculated. This corresponds (as an exact solution) to the literature to the "calibration method 5", presented by Wahlund[1] and remains as the only recommended calibration method according to the results presented in the main paper.

Detailed description of applied algorithms

Classical Calibration

Inputs:

- void peak t_{void}
- elution flow $\dot{V}_{\mathrm{e}}$
- elution time t_{e}
- cross flow $\dot{V}_{\mathrm{c}}$
- diffusion coefficient D
- relative focus $z_{\%}$;

Outputs:

- channel width w
- channel volume V^0

Constants:

- $w_{\min} \leftarrow 10^{-4}$
- $w_{\max} \leftarrow 10$

Temporary variables:

- measured retention R_{meas}
- variation δ_w
- λ

Calculations:

1 Calculate volume:

$$V^0 \leftarrow \frac{V_c \cdot t_{\mathrm{void}}}{\ln\left(\frac{z_{\%} - {(V_e+V_c)}/{V_c}}{1 - {(V_E+V_c)}/{V_c}}\right)}$$

2 Calculate R_{meas}:

$$R_{\mathrm{meas}} \leftarrow \frac{t_{\mathrm{void}}}{t_{\mathrm{e}}}$$

3 Initialize w and δ:

$$w \leftarrow \frac{w_{\max} + w_{\min}}{2}$$

$$\delta_w \leftarrow \frac{w_{\max} - w_{\min}}{4}$$

4 Find w such that $|R_{\text{meas}} - R_{\text{calc}}| \stackrel{!}{=} \min$ by bisection:

for $i \leftarrow 0$ to 50 **do**
  $\lambda \leftarrow \frac{D \cdot V^0}{V_C \cdot w^2}$
  $R_{\text{calc}} \leftarrow 6\lambda \left(\frac{1}{\tanh(1/2\lambda)} - 2\lambda \right)$ # ${}^1\!/_{\tanh(x)} = \coth(x)$
  if $R_{\text{calc}} > R_{\text{meas}}$ **then**
    $w \leftarrow w + \delta_w$
  else
    $w \leftarrow w - \delta_w$
  end if
  $\delta_w \leftarrow \delta_w / 2$
end for

Classical Calibration under consideration of the simpified trapezoidal shape model

Inputs:

- void peak t_{void}
- elution flow $\dot{V}_{\mathrm{e}}$
- elution time t_{e}
- cross flow $\dot{V}_{\mathrm{c}}$
- diffusion coefficient D
- relative focus $z_{\%}$;
- L_1, L_2, L_3
- b_0, b_L

Outputs:

- channel width w
- channel volume $V^{\approx\mathrm{geo}}$

Constants:

- $w_{\min} \leftarrow 10^{-4}$
- $w_{\max} \leftarrow 10$

Temporary variables:

- measured retention R_{meas}
- Channel surface area A_L
- λ
- T_1

Calculations:

1 Calculate volume:

$$L_{23} \leftarrow L_2 + L_3, \qquad b_\Delta \leftarrow b_0 - b_L$$
$$A_L \leftarrow \tfrac{1}{2} b_0 L_1 + b_L L_{23} + \tfrac{1}{2} b_\Delta L_{23}$$
$$Y \leftarrow \tfrac{1}{2}\left(b_0 + \tfrac{L_1}{L_{23}}\right) L_1$$
$$\mathrm{T}_1 \leftarrow b_0 z_0 - \tfrac{z_0^2 b_\Delta}{2L} - Y$$
$$\mathrm{T}_1 \leftarrow 1 - \tfrac{\mathrm{T}_1}{A_L}$$
$$\mathrm{T}_1 \leftarrow \ln\left(1 + \tfrac{\dot{V}_{\mathrm{c}}}{\dot{V}_{\mathrm{e}}} \mathrm{T}_1\right)$$
$$V^{\approx\mathrm{geo}} \leftarrow \tfrac{\dot{V}_{\mathrm{c}} t_{\mathrm{void}}}{\mathrm{T}_1}$$

2 Calculate R_{meas}:

$$R_{\mathrm{meas}} \leftarrow \frac{t_{\mathrm{void}}}{t_{\mathrm{e}}}$$

3 Initialize w and δ:

$$w \leftarrow \frac{w_{\max} + w_{\min}}{2}$$
$$\delta_w \leftarrow \frac{w_{\max} - w_{\min}}{4}$$

4 Find w such that $|R_{\text{meas}} - R_{\text{calc}}| \stackrel{!}{=} \min$ by bisection:

for $i \leftarrow 0$ to 50 **do**
  $\lambda \leftarrow \frac{D \cdot V^{\approx \text{geo}}}{V_C \cdot w^2}$
  $R_{\text{calc}} \leftarrow 6\lambda \left(\frac{1}{\tanh(1/2\lambda)} - 2\lambda \right)$ # $1/\tanh(x) = \coth(x)$
  if $R_{\text{calc}} > R_{\text{meas}}$ **then**
    $w \leftarrow w + \delta_w$
  else
    $w \leftarrow w - \delta_w$
  end if
  $\delta_w \leftarrow \delta_w / 2$
end for

Calibration of channel height by V^{geo}

Inputs:

- void peak t_{void}
- elution time t_{e}
- cross flow $\dot{V}_{\text{c}}$
- diffusion coefficient D
- relative focus $z_{\%}$;
- L_1, L_2, L_3
- b_0, b_L

Outputs:

- channel height w
- channel volume V^{geo}

Temporary variables:

- measured retention R_{meas}
- calculated retention R_{calc}
- variation δ_λ
- λ
- S
- L_{12}, L
- z_0
- m_1, m_2
- t_2
- A_z
- A_3

Constants:

- $\lambda_{\text{min}} \leftarrow 10^{-5}$
- $\lambda_{\text{max}} \leftarrow 100$

Calculations:

1 Calculate R_{meas}:

$$R_{\text{meas}} \leftarrow \frac{t_{\text{void}}}{t_{\text{e}}}$$

2 Initialize λ and δ_λ:

$$\lambda \leftarrow \frac{\lambda_{\text{min}} + \lambda_{\text{max}}}{2}$$

$$\delta_\lambda \leftarrow \frac{\lambda_{\text{max}} - \lambda_{\text{min}}}{4}$$

3 Find λ such that $|R_{\text{meas}} - R_{\text{calc}}| \overset{!}{=} \text{min}$ by bisection:

for $i \leftarrow 0$ to 50 **do**
  $R_{\text{calc}} \leftarrow 6\lambda\left(\frac{1}{\tanh(1/2\lambda)} - 2\lambda\right)$ # $1/\tanh(x) = \coth(x)$
  if $R_{\text{calc}} > R_{\text{meas}}$ **then**
    $\lambda \leftarrow \lambda + \delta_\lambda$
  else
    $\lambda \leftarrow \lambda - \delta_\lambda$
  end if
  $\delta_\lambda \leftarrow \delta_\lambda / 2$
end for

4 Calculate substitution term S:

$$S \leftarrow \frac{\lambda \cdot \dot{V}_{\text{c}}}{D}$$

5 Calculate passed channel area A_z:

$A_3 \leftarrow \dfrac{1}{2} \cdot b_L L_3 \qquad L_{12} \leftarrow L_1 + L_2 \qquad L \leftarrow L_{12} + L_3 \qquad z_0 \leftarrow z_{\%} \cdot L$

if $z_0 \geqq L_1$ **then**

$\quad m_2 \leftarrow \dfrac{b_0 - b_L}{2 \cdot L_2} \qquad b_\Delta \leftarrow b_0 - b_L \qquad t_2 \leftarrow \dfrac{1}{2}\left(b_0 + \dfrac{L_1}{L_2} b_\Delta\right)$

$\quad A_z \leftarrow (L_{12} - z_0) \cdot (m_2(L_{12} + z_0) + t_2) + A_3$

else

$\quad m_1 \leftarrow \frac{b_0}{2L_1}$

$\quad A_z \leftarrow m_1 \cdot (L_1^2 - z_0^2) + \frac{1}{2}(b_0 + b_L)L_2 + A_3$

end if

6 Calculate w: $\qquad w \leftarrow \dfrac{A_z}{S}$

7 Calculate V^{geo}: $\qquad V^{\mathrm{geo}} \leftarrow A_z \cdot w$

Calibration of channel height by V^{hyd}

Inputs:

- void peak t_{void}
- elution flow $\dot{V}_{\text{e}}$
- cross flow $\dot{V}_{\text{c}}$
- relative focus $z_{\%}$;
- L_1, L_2, L_3
- b_0, b_L

Outputs:

- channel width w
- channel volume V^{hyd}

Temporary variables:

- z_0
- $\dot{V}_{\text{in}}$
- L_{12}, L
- b_Δ
- m_1, m_2, m_3
- t_2, t_3
- α_2, α_3
- $\beta_1, \beta_2, \beta_3$
- γ_2, γ_3
- $\delta_1, \delta_2, \delta_3$
- Δ_2, Δ_3
- $C_{\text{F1}}, C_{\text{F2}}, C_{\text{F3}}, C_{\text{F}}$
- T_1, T_2

Calculations:

1 Calculate ”derived” parameters:

$L_{12} \leftarrow L_1 + L_2 \qquad L \leftarrow L_{12} + L_3 \qquad z_0 \leftarrow z_{\%} \cdot L \qquad b_\Delta \leftarrow b_0 - b_L \qquad \dot{V}_{\text{in}} \leftarrow \dot{V}_{\text{e}} + \dot{V}_{\text{c}}$

2 Calculate slopes and offsets of the border lines of the channel plain:

$m_1 \leftarrow \frac{b_0}{2L_1} \qquad m_2 \leftarrow -\frac{b_\Delta}{2L_2} \qquad m_3 \leftarrow -\frac{b_L}{2L_3}$

$t_2 \leftarrow \frac{1}{2}\left(b_0 + \frac{L_1}{L_2} b_\Delta\right) \qquad t_3 \leftarrow \frac{L b_L}{2L_3}$

3 Calculate area sections of the channel plain:

$A_1 \leftarrow \frac{1}{2} b_0 L_1 \qquad A_2 \leftarrow \frac{1}{2}(b_0 + b_L) L_2 \qquad A_3 \leftarrow \frac{1}{2} L_3 b_L$

$A_L \leftarrow A_1 + A_2 + A_3$

4 Simple numerical integration via Riemann sum (see subroutines):

$C_{\text{F}} \leftarrow \text{calcCF}(.....)$

5 Calculate w:

$w \leftarrow \frac{t_{\text{void}}}{2 \cdot C_{\text{F}}}$

6 Calculate V^{hyd} with passed area:

$V^{\text{hyd}} \leftarrow A_\xi \cdot w$

S subroutines

S1 calcCF

```
# initialize ξnear z_0 on the ξ-grid
```
$\Delta\xi \leftarrow \frac{L}{n}$
$\xi < z_0$
while $\xi < z_0$ **do**
  $\xi \leftarrow \xi + \Delta\xi$
end while
# Calculate $C_{\mathrm{F}1}$
while $\xi < z_0$ **do**
  $A_\xi \leftarrow m_1\xi^2$
  $\dot{V}_\xi \leftarrow \dot{V}_{\mathrm{in}} - \dot{V}_{\mathrm{c}}\frac{A_\xi}{A_L}$
  $E_{\dot{V}\xi} \leftarrow \frac{m_1\xi}{\dot{V}_\xi}$
  $C_{\mathrm{F}}1 \leftarrow C_{\mathrm{F}}1 + E_{\dot{V}\xi}\Delta\xi$
  $\xi \leftarrow \xi + \Delta\xi$
end while
#
while $\xi < L_{12}$ **do**
  $A_\xi \leftarrow A_1 + m_2\left(\xi^2 - L_1^2\right) + 2t_2\left(\xi - L_1\right)$
  $\dot{V}_\xi \leftarrow \dot{V}_{\mathrm{in}} - \dot{V}_{\mathrm{c}}\frac{A_\xi}{A_L}$
  $E_{\dot{V}\xi} \leftarrow \frac{m_2\xi+t_2}{\dot{V}_\xi}$
  $C_{\mathrm{F}}2 \leftarrow C_{\mathrm{F}}2 + E_{\dot{V}\xi}\Delta\xi$
  $\xi \leftarrow \xi + \Delta\xi$
end while
#
while $\xi < L$ **do**
  $A_\xi \leftarrow A_1 + A_2 + m_3\left(\xi^2 - L_{12}^2\right) + 2t_3\left(\xi - L_{12}\right)$
  $\dot{V}_\xi \leftarrow \dot{V}_{\mathrm{in}} - \dot{V}_{\mathrm{c}}\frac{A_\xi}{A_L}$
  $E_{\dot{V}\xi} \leftarrow \frac{m_3\xi+t_3}{\dot{V}_\xi}$
  $C_{\mathrm{F}}3 \leftarrow C_{\mathrm{F}}3 + E_{\dot{V}\xi}\Delta\xi$
  $\xi \leftarrow \xi + \Delta\xi$
end while

General formulation of numerical C_{F} integration for arbitrary channel shapes

The simple integration process above for three channel sections can also be generalized by the following when the channel shape is provided as a set of functions $e_s(x)$:

for $s \leftarrow 1$ to S **do**
  while $\xi < L$ **do**
    $A_\xi \leftarrow \sum_{\sigma=1}^{s} A_\sigma + \int_{L1s}^{\xi} e_s(x)\,\mathrm{d}x$
    $\dot{V}_\xi \leftarrow \dot{V}_{\mathrm{in}} - \dot{V}_{\mathrm{c}}\frac{A_\xi}{A_L}$
    $E_{\dot{V}\xi} \leftarrow \frac{e_s(\xi)}{\dot{V}_\xi}$
    $C_{\mathrm{F}} \leftarrow C_{\mathrm{F}} + E_{\dot{V}\xi}\Delta\xi$
    $\xi \leftarrow \xi + \Delta\xi$
  end while
end for

Calibration of channel height by V^{hyd}

Inputs:

- void peak t_{void}
- elution flow $\dot{V}_{\mathrm{e}}$
- cross flow $\dot{V}_{\mathrm{c}}$
- relative focus $z_{\%}$;
- L_1, L_2, L_3
- b_0, b_L

Outputs:

- channel width w
- channel volume V^{hyd}

Temporary variables:

- z_0
- $\dot{V}_{\mathrm{in}}$
- L_{12}, L
- b_Δ
- m_1, m_2, m_3
- t_2, t_3
- α_2, α_3
- $\beta_1, \beta_2, \beta_3$
- γ_2, γ_3
- $\delta_1, \delta_2, \delta_3$
- Δ_2, Δ_3
- $C_{\mathrm{F1}}, C_{\mathrm{F2}}, C_{\mathrm{F3}}, C_{\mathrm{F}}$
- $\mathrm{T}_1, \mathrm{T}_2$

Calculations:

1 Calculate "derived" parameters:

$L_{12} \leftarrow L_1 + L_2 \qquad L \leftarrow L_{12} + L_3 \qquad z_0 \leftarrow z_{\%} \cdot L \qquad b_\Delta \leftarrow b_0 - b_L \qquad \dot{V}_{\mathrm{in}} \leftarrow \dot{V}_{\mathrm{e}} + \dot{V}_{\mathrm{c}}$

2 Calculate slopes and offsets of the border lines of the channel plain:

$m_1 \leftarrow \frac{b_0}{2L_1} \qquad m_2 \leftarrow -\frac{b_\Delta}{2L_2} \qquad m_3 \leftarrow -\frac{b_L}{2L_3}$

$t_2 \leftarrow \frac{1}{2}\left(b_0 + \frac{L_1}{L_2} b_\Delta\right) \qquad t_3 \leftarrow \frac{L b_L}{2L_3}$

3 Calculate area sections of the channel plain:

$A_1 \leftarrow \frac{1}{2} b_0 L_1 \qquad A_2 \leftarrow \frac{1}{2}(b_0 + b_L)L_2 \qquad A_3 \leftarrow \frac{1}{2} L_3 b_L$

$A_L \leftarrow A_1 + A_2 + A_3$

4 Numerical integration for C_{F} via Riemann sum (see subroutine **S1** of V^{hyd} above):

$C_{\mathrm{F}} \leftarrow \mathrm{calcCF}(.....)$

6 Calculate w by minimizing $\Delta^2 = (R_{t_{\mathrm{e}}} - R_D)^2$:

\# Initialize:

$w_{\mathrm{L}} \leftarrow 1, w_{\mathrm{R}} \leftarrow 1000, w_{\mathrm{M}} \leftarrow \frac{w_{\mathrm{L}} + w_{\mathrm{R}}}{2}$

$\Delta_{w_{\mathrm{L}}} \leftarrow \mathrm{RDiff}(\Delta_{w_{\mathrm{L}}}), \Delta_{w_{\mathrm{R}}} \leftarrow \mathrm{RDiff}(\Delta_{w_{\mathrm{R}}}), \Delta_{w_{\mathrm{M}}} \leftarrow \mathrm{RDiff}(\Delta_{w_{\mathrm{M}}})$

while conv $< 10^{-8}$ **do**

  if $\Delta_{w_{\mathrm{L}}} > \Delta_{w_{\mathrm{M}}}$ and $\Delta_{w_{\mathrm{M}}} > \Delta_{w_{\mathrm{R}}}$ **then** # "Leap" right along gradient descent

    $w_{\mathrm{L}} \leftarrow w_{\mathrm{M}}, \Delta_{w_{\mathrm{L}}} \leftarrow \Delta_{w_{\mathrm{M}}}$

    $w_{\mathrm{M}} \leftarrow w_{\mathrm{R}}, \Delta_{w_{\mathrm{M}}} \leftarrow \Delta_{w_{\mathrm{R}}}$

    $w_{\mathrm{R}} \leftarrow w_{\mathrm{R}} + |w_{\mathrm{L}} - w_{\mathrm{M}}|$

    $\Delta_{w_{\mathrm{R}}} \leftarrow \mathrm{RDiff}(w_{\mathrm{R}})$

  else if $\Delta_{w_{\mathrm{L}}} < \Delta_{w_{\mathrm{M}}}$ and $\Delta_{w_{\mathrm{M}}} < \Delta_{w_{\mathrm{R}}}$ **then** #"Leap" left along gradient descent

    $w_{\mathrm{R}} \leftarrow w_{\mathrm{M}}, \Delta_{w_{\mathrm{R}}} \leftarrow \Delta_{w_{\mathrm{M}}}$

$w_{\mathrm{M}} \leftarrow w_{\mathrm{L}}, \Delta_{w_{\mathrm{M}}} \leftarrow \Delta_{w_{\mathrm{L}}}$
$w_{\mathrm{L}} \leftarrow w_{\mathrm{L}} - |w_{\mathrm{R}} - w_{\mathrm{M}}|$
$\Delta_{w_{\mathrm{L}}} \leftarrow \mathrm{RDiff}(w_{\mathrm{L}})$
else if $\Delta_{w_{\mathrm{L}}} > \Delta_{w_{\mathrm{M}}}$ and $\Delta_{w_{\mathrm{M}}} < \Delta_{w_{\mathrm{R}}}$ **then** #Shrink both distances about half
$w_{\mathrm{L}} \leftarrow \frac{w_{\mathrm{L}}+w_{\mathrm{M}}}{2}, \Delta_{w_{\mathrm{L}}} \leftarrow \mathrm{RDiff}(w_{\mathrm{L}})$
$w_{\mathrm{R}} \leftarrow \frac{w_{\mathrm{R}}+w_{\mathrm{M}}}{2}, \Delta_{w_{\mathrm{R}}} \leftarrow \mathrm{RDiff}(w_{\mathrm{R}})$
end if
end while

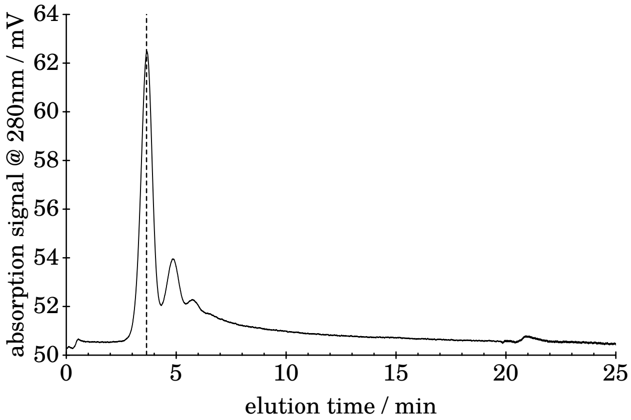
S subroutines

S2 RDiff

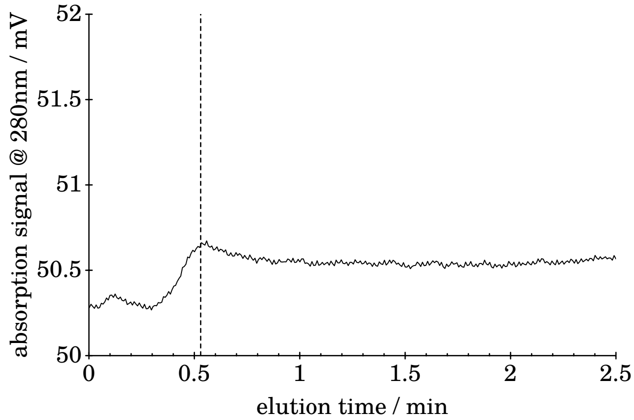
calculate $\Delta = (R_{t_{\mathrm{e}}} - R_D)^2$:
$R_{t_{\mathrm{e}}} \leftarrow \frac{2C_{\mathrm{F}}w}{t_e}$
$\lambda \leftarrow \frac{DA_z}{\dot{V}_{\mathrm{c}}w}$
$R_D \leftarrow 6\lambda\left(\coth\left(\frac{1}{2\lambda}\right) - 2\lambda\right)$
return $(R_{t_{\mathrm{e}}} - R_D)^2$

Complete data sets

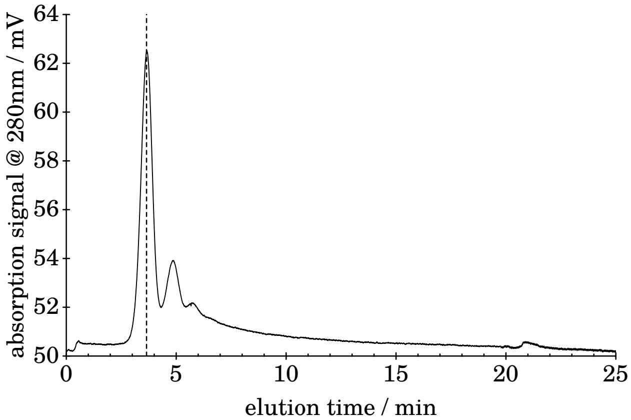
Measured raw data



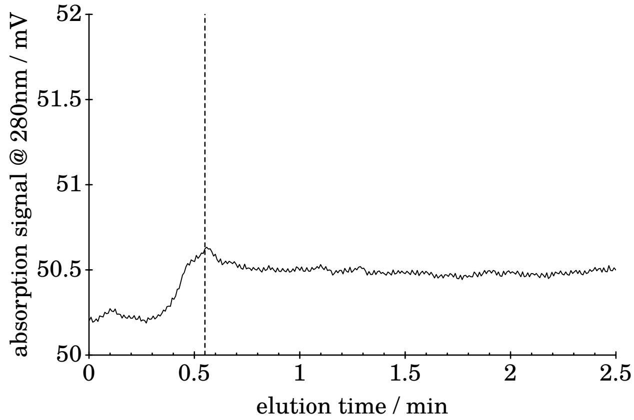
(a) Position of t_e, replicate 1.



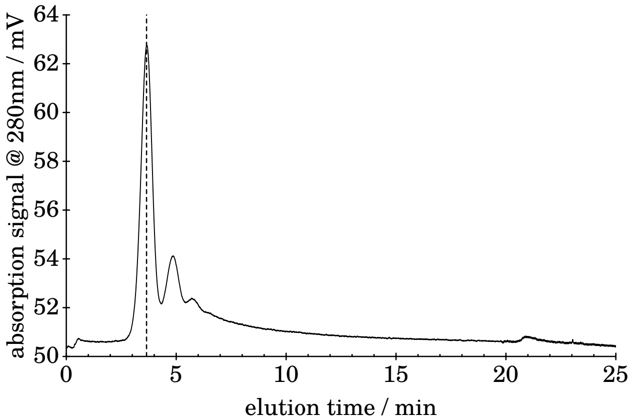
(b) Detailed starting section of fractogram S.9a; Position of t_{void}, replicate 1.



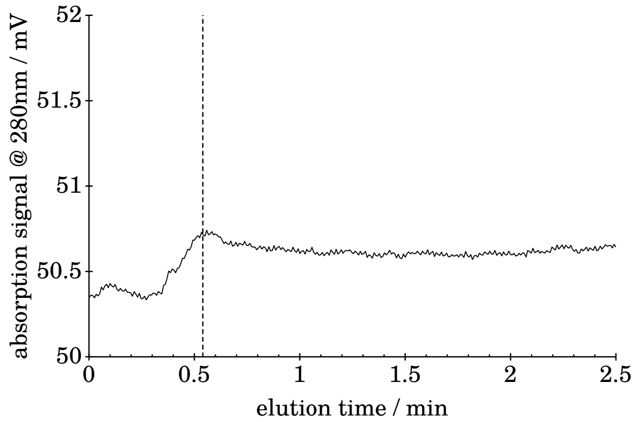
(c) Position of t_e, replicate 2.



(d) Detailed starting section of fractogram S.9c; Position of t_{void}, replicate 2.

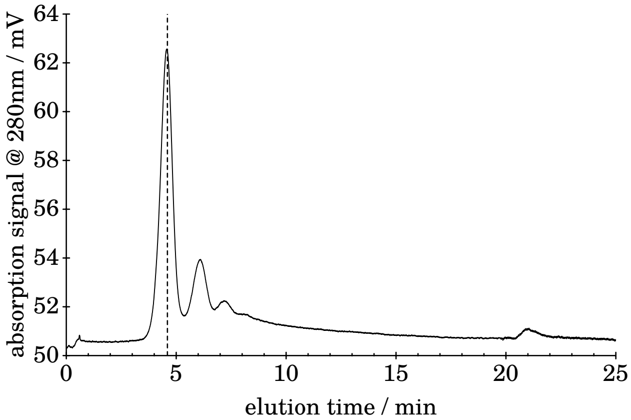


(e) Position of t_e, replicate 3.

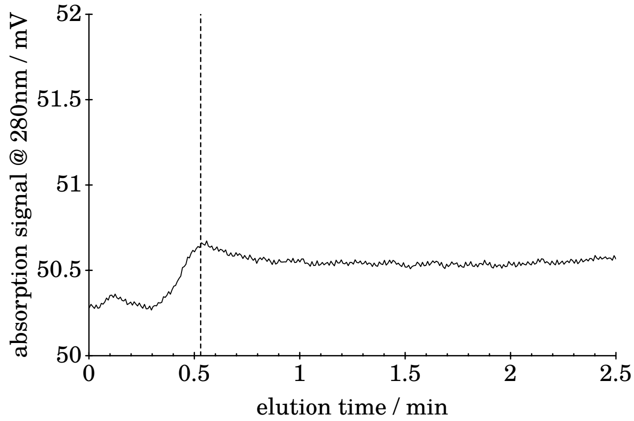


(f) Detailed starting section of fractogram S.9e; Position of t_{void}, replicate 3.

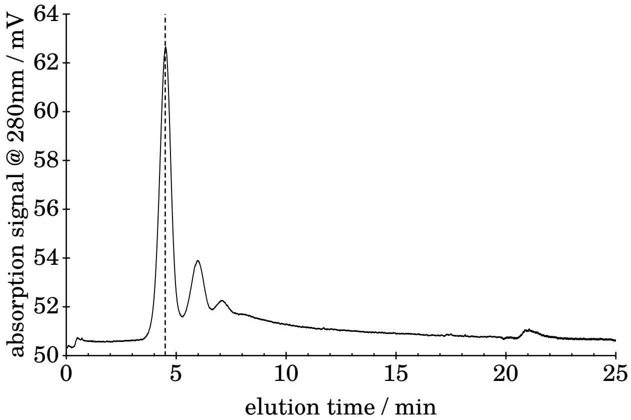
Fig. S.9: Raw fractograms of BSA measurements at $\dot{V}_c = 2.5 ^{\mathrm{ml}}/_{\mathrm{min}}$



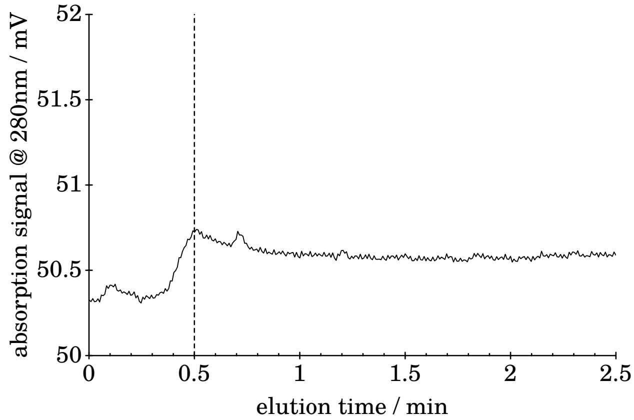
(a) Position of t_e, replicate 1.



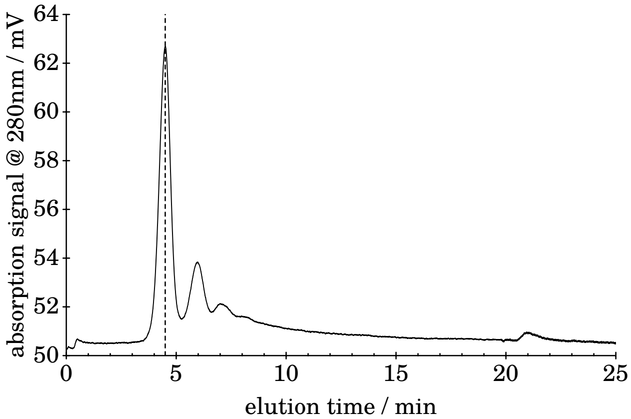
(b) Detailed starting section of fractogram S.10a; Position of t_{void}, replicate 1.



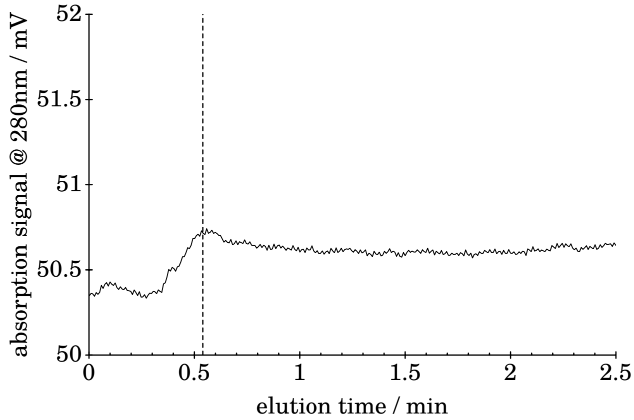
(c) Position of t_e, replicate 2.



(d) Detailed starting section of fractogram S.10c; Position of t_{void}, replicate 2.

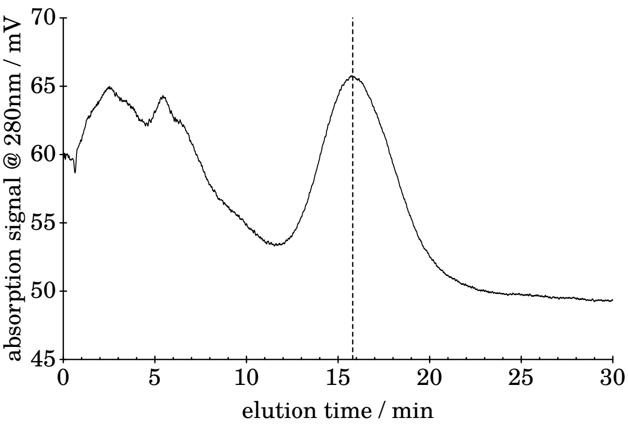


(e) Position of t_e, replicate 3.

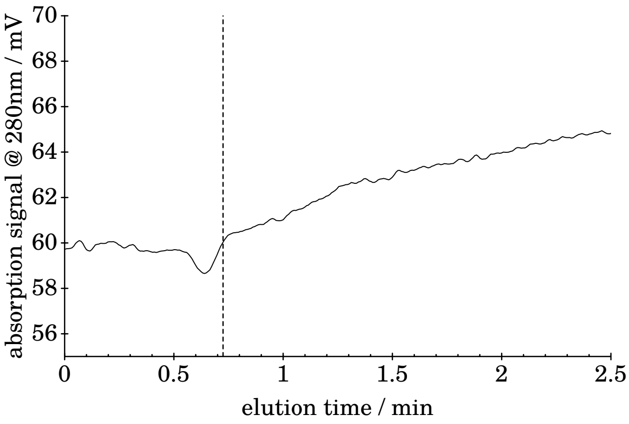


(f) Detailed starting section of fractogram S.10e; Position of t_{void}, replicate 3.

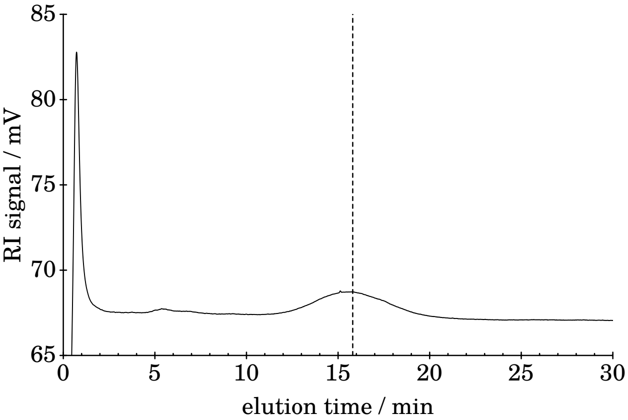
Fig. S.10: Raw fractograms of BSA measurements at $\dot{V}_c = 3.5 \, ^{ml}/_{min}$



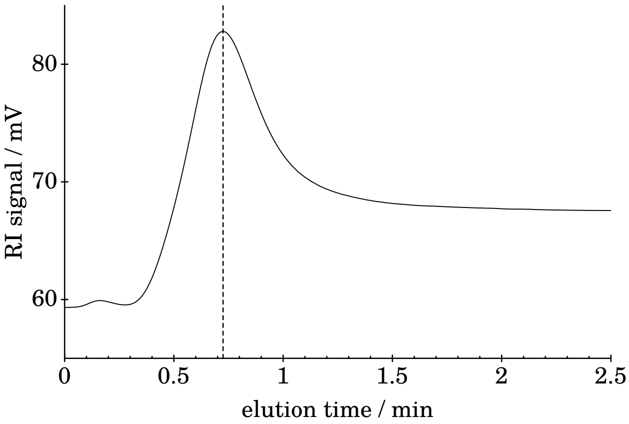
(a) Position of t_e with UV detection signal



(b) Detailed starting section of fractogram S.11a with UV detection signal and position of t_void.

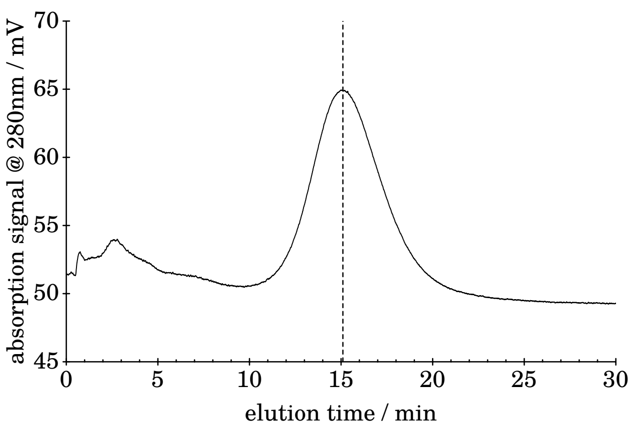


(c) Position of t_e with RI detection signal

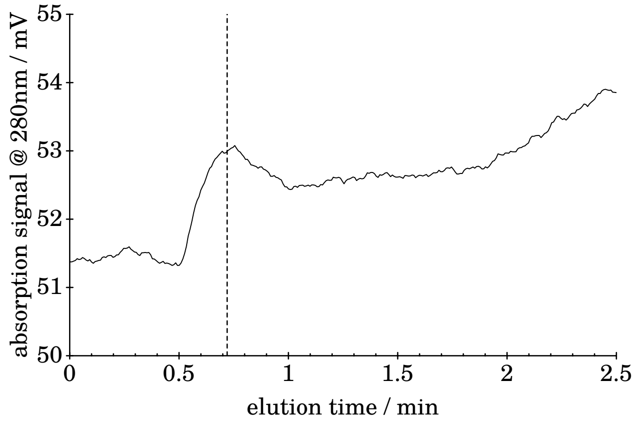


(d) Detailed starting section of fractogram S.11c with RI detection signal and position of t_void.

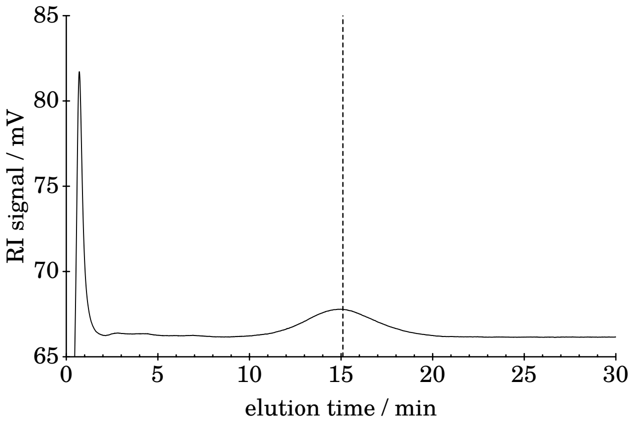
Fig. S.11: Raw fractograms of PS measurements at $\dot{V}_\mathrm{c} = 0.5 ^{\mathrm{ml}}/_{\mathrm{min}}$, replicate 1.



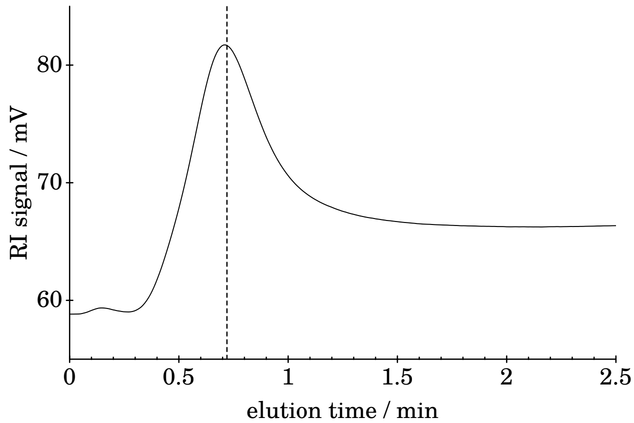
(a) Position of t_e with UV detection signal



(b) Detailed starting section of fractogram S.12a with UV detection signal and position of t_void.

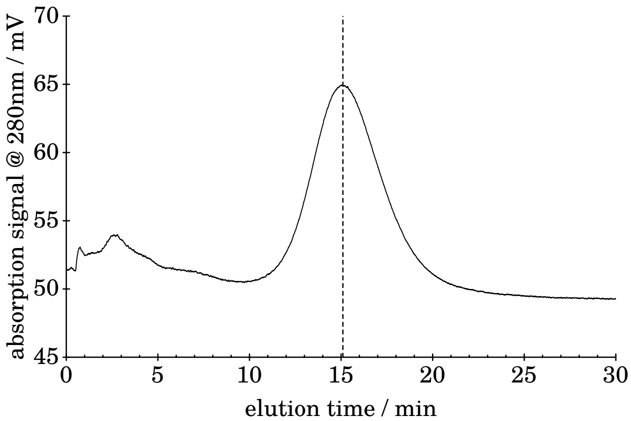


(c) Position of t_e with RI detection signal

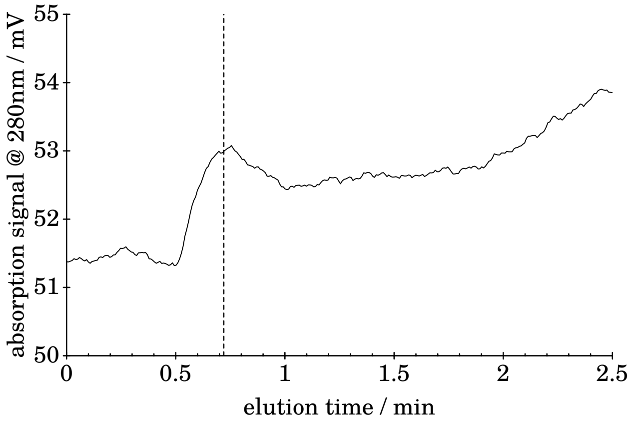


(d) Detailed starting section of fractogram S.12c with RI detection signal and position of t_void.

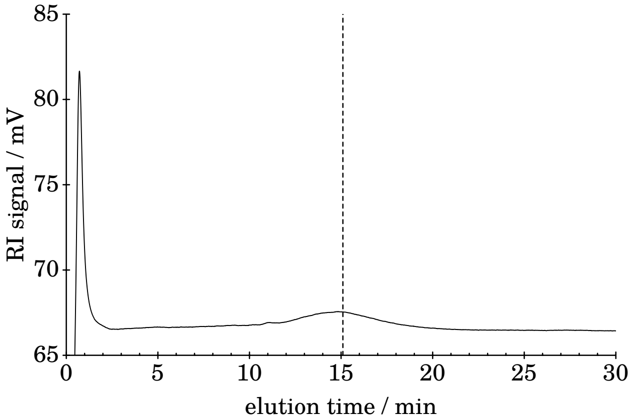
Fig. S.12: Raw fractograms of PS measurements at $\dot{V}_\mathrm{c} = 0.5 ^{\mathrm{ml}}/_{\mathrm{min}}$, replicate 2.



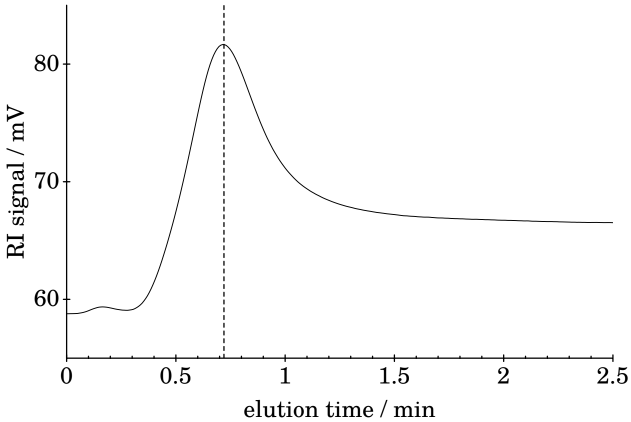
(a) Position of t_e with UV detection signal



(b) Detailed starting section of fractogram S.13a with UV detection signal and position of t_{void}.



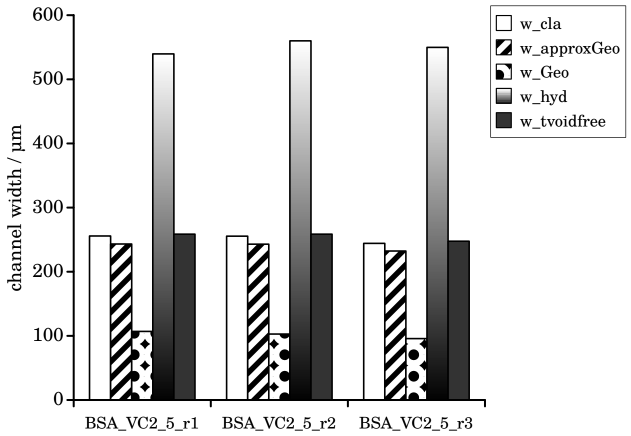
(c) Position of t_e with RI detection signal



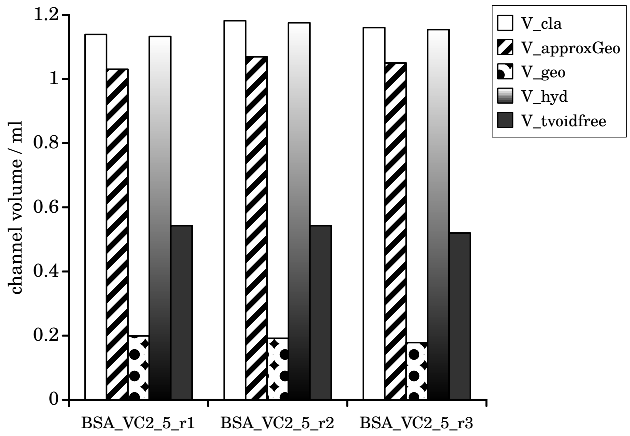
(d) Detailed starting section of fractogram S.13c with RI detection signal and position of t_{void}.

Fig. S.13: Raw fractograms of PS measurements at $\dot{V}_c = 0.5 \,{}^{ml}\!/_{min}$, replicate 3.

Evaluated results of calibration experiments with BSA and PS

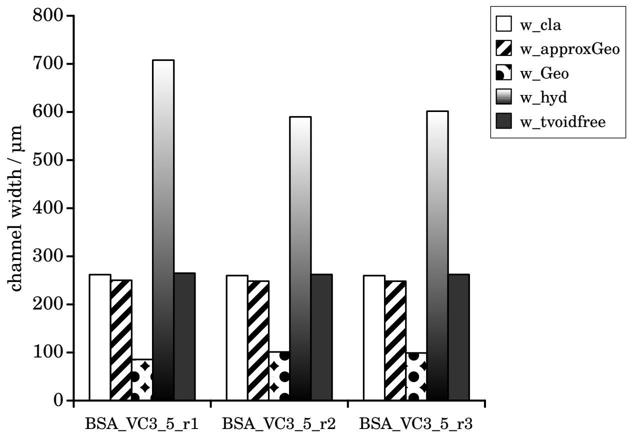


(a) Calibration results for w

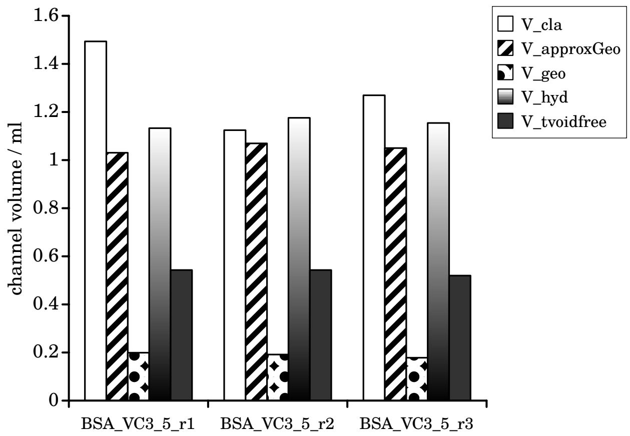


(b) Calibration results for V

Fig. S.14: Results of w and V from all 5 calibration algorithms for BSA measurements at $\dot{V}_\mathrm{c} = 2.5^{\mathrm{ml}}/_{\mathrm{min}}$

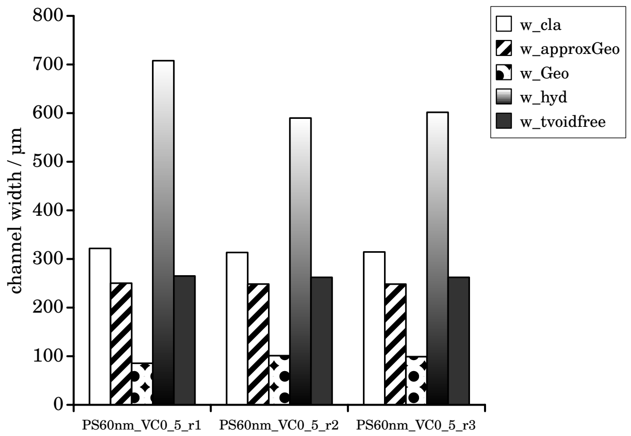


(a) Calibration results for w

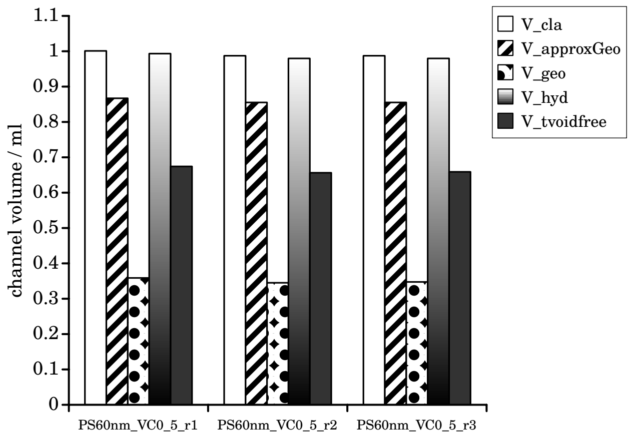


(b) Calibration results for V

Fig. S.15: Results of w and V from all 5 calibration algorithms for BSA measurements at $\dot{V}_\mathrm{c} = 3.5^{\mathrm{ml}}/_{\mathrm{min}}$



(a) Calibration results for w



(b) Calibration results for V

Fig. S.16: Results of w and V from all 5 calibration algorithms for PS measurements at $\dot{V}_\mathrm{c} = 0.5^{\mathrm{ml}}/_{\mathrm{min}}$

Deviation influences

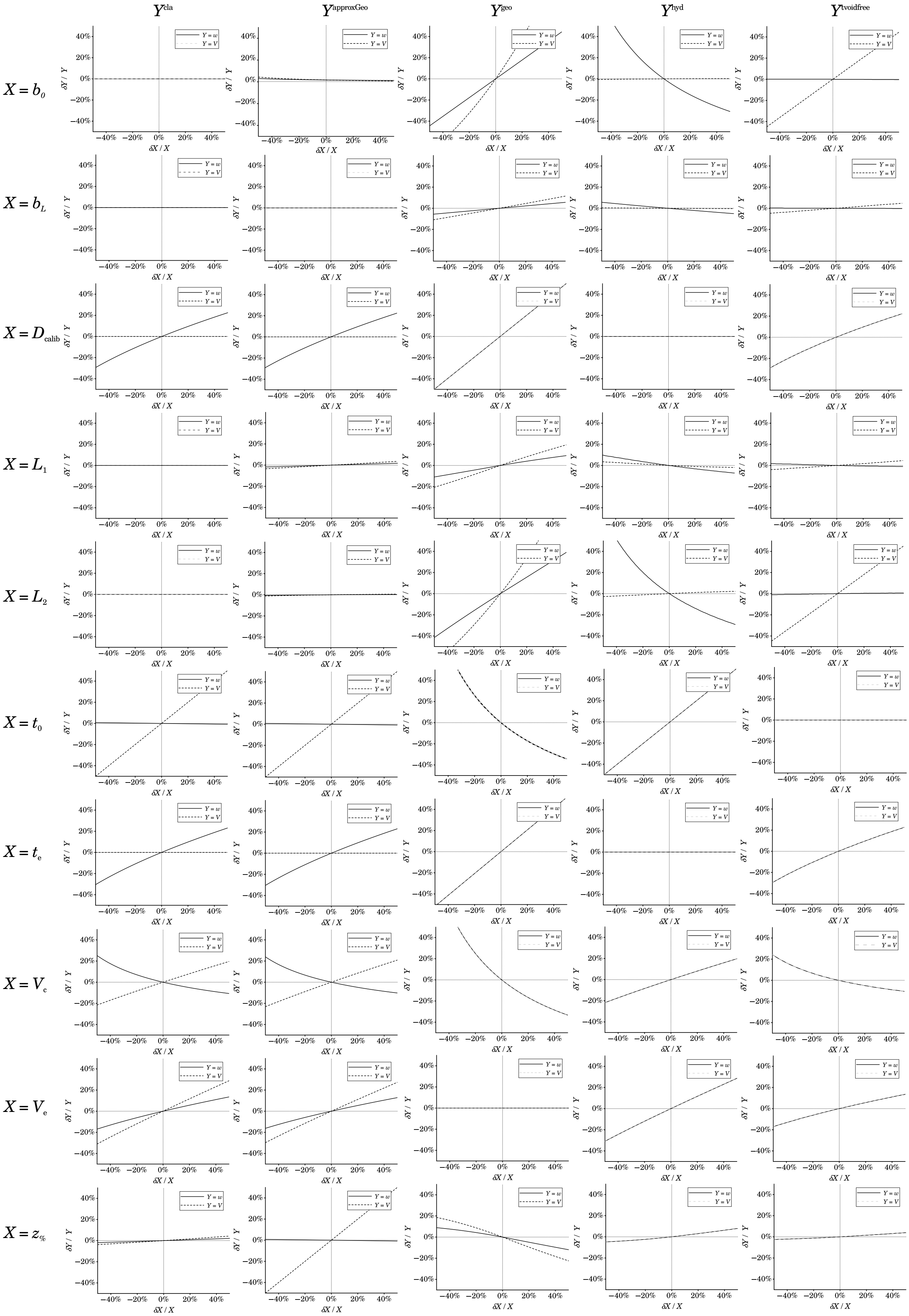


Fig. S.17: asdf

Detailed measurement program

Table S.1: Steps of an A4F measurement, the time of the Separation step was varied

Step	start time	mode	crossflow	focus flow
	[min]	E = Elution, F = Focus, I = Injection	+ = on, - = off	
Focussing	0	E	-	-
	1	E	+	-
	3	F	-	+
	4	F&I	-	+
Separation	8	E	+	-
	43	E&I	-	-
	48	E	-	-

References

[1] K.-G. Wahlund, *Journal of chromatography. A* **2013**, *1287*, 97–112.

[2] E. Magnusson, A. Håkansson, J. Janiak, B. Bergenståhl, L. Nilsson, *Journal of chromatography. A* **2012**, *1253*, 127–133.

[3] H. Bolinsson, Y. Lu, S. Hall, L. Nilsson, A. Hakansson, *Journal of chromatography. A* **2018**, *1533*, 155–163.

[4] A. Håkansson, E. Magnusson, B. Bergenståhl, L. Nilsson, *Journal of chromatography. A* **2012**, *1253*, 120–126.

[5] K. G. Wahlund, J. C. Giddings, *Anal Chem* **1987**, *59*, 1332–1339.

[6] A. Litzén, K.-G. Wahlund, *Anal. Chem.* **1991**, *63*, (Ed.: P. A. Jansson), 1001–1007.

[7] I. Bronstein, K. Semendjajew, G. Musiol, H. Mühlig in *Taschenbuch der Mathematik*, Harri Deutsch, **2008**, Chapter 21.7.1.2, p. 1077.

[8] I. Bronstein, K. Semendjajew, G. Musiol, H. Mühlig in *Taschenbuch der Mathematik*, Harri Deutsch, **2008**, Chapter 21.7.1.2, p. 1076.

[9] I. Bronstein, K. Semendjajew, G. Musiol, H. Mühlig in *Taschenbuch der Mathematik*, Harri Deutsch, **2008**, Chapter 21.7.1.3, p. 1078.