

# Revision of the AF4 calibration experiment

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Abstract:

Asymmetrical flow field-flow fractionation is a versatile chromatographic fractionation method. In combination with at least one detection technique it is used for size-based separation of colloids, biomolecules and polymers. Although often used 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 to obtain the channel width  $w$ . 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  $D$ . We show that the best way to conduct is to consider the area of the channel and  $D$  of the calibrant while neglecting the small peak which occurs in the void peak region.

Highlights:

- An AF4 data evaluation software is presented
- The comparison of AF4 channel width calibration procedures shows inconsistent results
- A deviation analysis identifies the measured void peak as an error-prone parameter

Keywords:

Asymmetrical flow field-flow fractionation, [channel width](#) ~~void peak~~ determination, size determination, calibration

## 1. Introduction

AF4 (asymmetrical flow field-flow fractionation) is a chromatographic technique that can be used to separate samples due to their diffusion coefficient[1]. It is a member of the FFF (field-flow fractionation) family invented by J. Calvin Giddings [2]. Compared to more commonly applied separation methods like SEC and HPLC[3], FFF techniques are based on the interaction of the analyte with a physical field which separates the sample to a corresponding physical size[4]. In principle, the method is applicable to a huge variety of samples, including small biomolecules[5,6], nanoparticles and polymers [3,4] up to big agglomerates like protein aggregates[7], virus-like particles [8] and drug carrier systems [9]. Nowadays, AF4 is the most commonly used flow FFF method, where the separation channel is formed of a solid wall and a frit covered by a membrane.

The development of dedicated measurement protocols can be complicated due to the high number of adjustable parameters[10]. This includes instrumental specifications like the channel shape, its related parameters length  $L$ , the channel width  $b_0$  and  $b_L$  and the choice of the membrane material. The three typical variable experimental conditions are elution flow  $V_e$ , applied cross-flow  $V_c$  and the focusing flow and period. AF4 has to be combined with at least one detection technique, typically MALLS (multi-angle laser light scattering), UV/Vis and/or RI (refractive index) detection. Also, on-line NMR [11], mass spectrometry [12] and SAXS (Small-angle X-ray scattering) [13] have been applied as detection techniques.

In addition to the parameters listed above, the channel thickness  $w$  and the separation volume  $V$  have to be known precisely in order to allow a correct determination of the diffusion coefficient, and, thereby, the size of a measured sample [1,10]. However, due to swelling of the applied membrane in the channel, these magnitudes are not directly accessible with current devices, which reasons the necessity of suitable calibration methods.

Although AF4 theory has been elaborated and well documented in literature, the transfer to its application quantitative evaluation software ~~lacks~~ still lags behind compared to methods like AUC (analytical ultracentrifugation). Here, several software solutions and a couple of evaluation methods are already available and can be used even without in-depth knowledge of the underlying algorithmic considerations [14,15]. This may be also a reason, why the practical handling of AF4 data evaluation is handled very inconsistently by different authors. While some authors suggest a direct conversion from measurement data is directly possible from a measurement [16], others suggest an external measurement using a calibrant with a known diffusion coefficient [10,17] or an external size measurement via coupled MALLS [18].

Therefore, we fill this gap with an implementation of the known procedures and compare them to each other.

In this work, we want to compare some of the different approaches reported up to now in literature. In the past, the validity of the no-field method in AF4 has already been disproved successfully [19]. A reported approach, which aims to measure the thickness of the membrane directly by a micrometer [5,10,17] has to be questioned as the measurement cannot be not conducted *in situ*.

## 2. Theory

The sample is injected into a flat channel with a solid upper wall and a lower wall that allows the streaming solvent to pass partially (Fig. 1). In current devices this wall is made of a frit covered by an ultrafiltration membrane. The inlet flow  $V_{in}$  is, thereby, split to a crossflow  $V_c$  (which is distributed uniformly over the horizontal section of the channel) and an elution flow  $V_e$  forming parabolic flow profile typical for all FFF variants:

$$V_{in} = V_e + V_c \quad (1)$$

The “broadness” of the parabole representing the velocity gradient depends on the plate distance  $w$ , also designated as channel width.  $L_1$ ,  $L_2$ ,  $L_3$ ,  $b_{01}$  and  $b_{21}$  describe the channel dimensions as shown in Fig. 1.

$V_c$  transports the particles to the membrane. As a consequence, the opposed translational diffusion  $J_z$  determines the average velocity zone and hence the time of elution. Mathematical descriptions of AF4 experiments and derivations have been described in literature [1,10,20,21]. They describe phenomena by physical and geometric approaches. As shown in the supporting information, rigorously applying a coordinate system to the channel shape allows to express these formulas for random channel shapes as well.

Thereby, we only state a short description of those formulas that are used in our evaluation approach which is essentially built up on existing theory. While the physical relationships are widely known and well documented, this is not always the case for their translations into an evaluation procedure. This might seem to be a trivial step as the physical content is well elaborated. However, having a closer look to the physical formalism, it turns out that the overdetermined nature of the system allows several possible evaluation procedures with different input parameters.

Considering the number of different approaches which exist for calibration [10,18] and their variations in detail, the implementation affects not only the evaluation but also the required measurement setup and, of course, the final measurement result. The lack of such standardized evaluation procedures impairs the reproducibility of measurements and may be one of the reasons why the analytical characterization potential is not exhausted to its potential up to now [3].

In total, 5 different calibration ways are described here briefly. The explicit derivation of the underlying formalisms and algorithmic considerations are stated in the supporting information.

The retention ratio  $R$ , defined as

$$R = \frac{t_{void}}{t_e} \quad (2)$$

with the time of the void peak  $t_{void}$  (the time which is required for a particle to travel if no retention occurs) and any possible point of time during the evaluation) and the time of sample elution  $t_e$ .

This is connected to the relative mean layer distance  $\lambda$  by the classical FFF retention equation:

$$R = 6\lambda \cdot \left( \coth\left(\frac{1}{2\lambda}\right) - 2\lambda \right) \quad , \quad (3)$$

which is often simplified to

$$R_{approx} = 6\lambda \quad (4)$$

The primary separation size of AF4 is the translational diffusion coefficient  $D$ . For AF4, the relevant correlation of  $\lambda$  and  $D$  has been elaborated [1] as

$$\lambda = \frac{D \cdot V^0}{V_c \cdot w^2} \quad (5)$$

For a typical AF4 measurement the channel volume  $V^0$  and the channel width  $w$  are critical sizes for the evaluation.

Recently, we used a calibration method [22] that makes use of the volume calculation as reported by Wahlund and Giddings [1] and then adjusts  $w$  by a simple bisection accordingly to Eq. (3) and (4). A similar method was reported independently before [20,2,20]. This formalism does not include the steric effect which has to be considered for larger particles. This can be quantified by a more refined version of Eq. (3). [23] An overview over possible “levels of sophistication” of variants of Eq. (3) has been given by Håkansson et al. [20]

Fig. 2 shows that bisection is easily applicable due to the strict ~~monotonicity~~ ~~monotony~~ of the retention equation within the relevant scope and sufficient for being used on modern CPUs. It can be replaced by an even more efficient conversion if required [243]. Here, ~~t~~ The separation volume  $V^0$  is estimated according to [1]:

$$\lambda = \frac{V^0}{V_c \cdot w^2} \quad (6)$$

~~In the following, we refer to this method as “classical” calibration method with the calculated separation volume  $V^{cla}$  and channel height  $w^{cla}$ .~~

$$t_0 = \frac{V^{cla}}{V_c} \ln \left( \frac{z_0 - (V_e + V_c)/V_c}{1 - (V_e + V_c)/V_c} \right) \quad (67)$$

~~In the following, we refer to this method as “classical” calibration method with the calculated separation volume  $V^{cla}$  and channel height  $w^{cla}$ .  $z_0$  designates the relative focus position of the sample at the beginning of a measurement. It is given as ratio of the start position (i.e. the skipped channel length) and the total channel length  $L$ . This description neglects the tapering of the ends and assumes a simple rectangular shape of the channel.~~

The formalism for the relevant method has been adjusted for narrowing trapezoidal channel shapes as follows [245]. ~~and It is used as a such for the second method similar to the first one.~~  $\gamma$  is a correction term for approximating the correct channel surface. ~~In order to distinguish the calculated channel widths and volumes, we refer to the results here as  $V^{appGeo}$  and  $w^{appGeo}$ .~~

$$t_0 = \frac{V^{app}}{V_c} \ln \left( 1 + \frac{V_c}{V_e} \left( 1 - \frac{b_0 z - \frac{(z^2)(b_0 - b_L)}{2L} - \gamma}{A_L} \right) \right) \quad (87)$$

The separation volume of the channel can also be a product of the surface and height of the channel. For this reason, we introduce a third calibration method which yields the separation volume  $V^{geo}$  and its corresponding size  $w^{geo}$ . ~~Making~~ ~~We make here~~ use of the simple geometric relationship passed channel area  $A_{L\pm}$  and the channel height:

$$V^{geo} = A_{L,z} \cdot w^{geo} \quad (98)$$

Thereby, it leads to the intuitive observation that variations of any parameters don't affect the calculated volume and the channel width linearly-equally.

The calibration is conducted by rearranging and substituting the terms

$$\frac{\lambda V_C}{D} = \frac{V^{geo} V^{geo}}{w^2} := S \quad (102)$$

in Eq. (4) and determine  $S$  analogously to  $w$  as in the classical approach via bisection. In a second step, inserting the geometrical definition of  $V^{geo}$  as indicated in Eq. (8)

that was used for the separation gives a leads to a simple solution for  $w$  with

$$w = \frac{A_{L,z}}{S} \quad (110)$$

$A_L$  can be obtained by simple geometric considerations as described in the supporting information. Using Eq. (8), now explicitly,  $V^{geo}$  the volume can be calculated as well. Dedicated derivations of the channel plane calculation is given in the supplementary information. As already stated, in this approach, all the hydrodynamic information is already used to calculate  $S$  in this approach. By this approach it is ensured, that the calculated volume, width and the channel area fit together geometrically by including the term into the calculation.

A fourth way of calculating the width and the volume is based on considerations concerning the flow velocities and hydrodynamic processes in the channel. The same rigorous equations for the description of the channel shape were used as for the calculation of  $V^{geo}$ . This leads to a direct linear relationship of  $t_{void}$  and  $w$ :

$$w t_{void} = 2 \cdot w^2 \cdot t_g \cdot C_F \quad (121)$$

The "conversion factor"  $C_F$  is determined via the hydrodynamic and geometric properties of the measurement. It can be obtained by solving the integral:

$$C_F = \int_{z_0}^L \frac{E(\xi)}{V_{in} - V_C \cdot \frac{2 \cdot \int_0^\xi E(x) dx}{A_L}} d\xi \quad (132)$$

This expression is derived based on a known approach [254], but independent from the shape and more suited to relate  $w$  and  $t_{void}$  directly. The function  $E(x)$  describes the shape of the channel in dependence of its longitudinal position  $x$ . In eq. 13,  $x$  marks the positions in the channel to the current position  $\xi$  of a hypothetical non-retained species, which moves with the mean velocity.  $A_L$  is the complete surface of the channel including the non-separating part, i.e.

$$A_L = 2 \int_0^L E(x) dx \quad (143)$$

A detailed derivation and an attempt for an analytical solution of Eq. 11-12-143 is given in the supporting information. Using Eq. 5,  $V^{hyd}$  can be calculated. The values obtained by this method are denoted as  $V^{hyd}$  in the following. A detailed derivation for the factor  $C_F$  and  $V^{hyd}$  is given in the supporting information. The obvious advantage of this method is the independence from an external diffusion coefficient i.e. no calibration measurement is involved in this procedure. This is a formalized method which is equivalent to calibration free conversion approaches [16,256,267].

The fifth algorithm also makes use of this conversion factor. Here, it is used to substitute the void time  $t_0$  ~~to~~  $t_{\text{void}}$ . As no experimental void time is used in this approach, we designated the calculated results with  $w^{\text{noT}}$  and  $V^{\text{noT}}$ . This way, Eq. 2 can be written as

$$R = \frac{2C_{FW}w^{\text{noT}}}{t_e} \quad (154)$$

By reformulating Eq. 5 with the substituted volume according to Eq. 108,  $\lambda$  can be calculated ~~written~~ as:

$$\lambda(w) = \frac{DA_{\text{ZL}}}{V_c w} \quad (165)$$

Now Eq. 35 can be merged with Eq. (144):-

$$\frac{2C_{FW}}{t_e} = 6\lambda(w) \cdot \left( \coth\left(\frac{1}{2\lambda(w)}\right) - 2\lambda(w) \right) \quad (167)$$

$w^{\text{noT}}$  can now be easily determined numerically from Eq. 156 and Eq. 176. This calibration calculation procedure is advantageous as  $t_{\text{void}}$  ~~to~~ does not have to be determined experimentally and be read from the fractogram. However, it requires a calibration measurement for the determination of  $t_e$  and a known  $D$ .

## Materials and methods

AF4Eval is our current version of hydrodynamic evaluation software for AF4 data. The user can create profiles for channel shapes and corresponding calibrations for a measurement set. Data are provided in a standardized csv-file format. Further input formats such as AniML[27,28,29,30] are planned to be added. As ~~to~~  $t_{\text{void}}$  and  $t_e$  have to be determined manually for some calibration methods, we integrated a simple graphical element (Fig. 4) with movable bars to the user interface to enable the user to pick the peak position manually without transferring the chosen value as it would be required in a spreadsheet application.

AF4Eval is written in C++14. Its source code obtainable via github and can be used without charge for academic purposes: <https://github.com/biocystal777/AF4Eval>. Data shown in this report where obtained with a version compiled with g++ 6.3 under Debian Gnu/Linux 9.5, using the framework Qt 5.7 and the plotting library Qwt 6.1.2[301].

Table 1: Required input parameters for the described calibration algorithms

	Classical	Approximated Volume	Geometrical	Hydrodynamic	Without $t_{\text{void}}$
	$D_{\text{calib}}$ $t_{\text{e}}$	$D_{\text{calib}}$ $t_{\text{e}}$	$D_{\text{calib}}$ $t_{\text{e}}$	$t_{\text{void}}$	$D_{\text{calib}}$ $t_{\text{e}}$
Inputs	$V_{\text{e}}$ $V_{\text{c}}$ $z\%$	$V_{\text{e}}$ $V_{\text{c}}$ $z\%$ $L_1, L_2, L_3$ $b_0, b_L$	$V_{\text{c}}$ $L_1, L_2, L_3$ $b_0, b_L$	$V_{\text{e}}$ $V_{\text{c}}$ $z\%$ $L_1, L_2, L_3$ $b_0, b_L$	$V_{\text{e}}$ $V_{\text{c}}$ $z\%$ $L_1, L_2, L_3$ $b_0, b_L$
Side condition	-	-	Eq. 89	Eq. 98	Eq. 98
Outputs	$w$ $I^0$	$w$ ( $w^{\text{appgeo}}$ ) $I^0$ ( $I^{\text{appgeo}}$ )	$w$ ( $w^{\text{geo}}$ ) $I^0$ ( $I^{\text{geo}}$ )	$w$ ( $w^{\text{hyd}}$ ) $I^0$ ( $I^{\text{hyd}}$ )	$w$ ( $w^{\text{noT}}$ ) $I^0$ ( $I^{\text{noT}}$ )

Based on the theory above we implemented 5 calibration algorithms. The calibration-dependent three methods enable an estimation of the void peak time from the geometrical properties of the calibrated channel. Thus, manual readout of the void peak is avoided entirely and the methods can be integrated in a fully automated procedure. The direct conversion turns out to be useful if no appropriate standard is available. These algorithms vary in their specific required input magnitudes (Tab. 1). A pseudocode of the applied calculations representation is given in the supporting information.

In addition, an error analysis function was implemented that allows to estimate the uncertainties of the methods. The error analysis allows to define a range  $R$  of the estimated uncertainty  $\delta X$  from -100% to 100% for the input quantity  $X_i$  and a grid resolution parameter. The method then iterates over  $X+\delta X$  of  $R$  while conducting the assigned algorithm and gives the deviation of the output quantity  $Y_j$ . The method seems trivial from a mathematical perspective, however, it gives a good overview, how the deviation of one quantity affects the result while the other quantities are kept constant. This is especially useful if the impact cannot be foreseen due to the involvement in several numerical procedures. This way, the individual impact of the uncertainty of each variable can be easily quantified for each experimental condition. Other functionalities, such as the evaluation of size distributions based on the calibration, are not discussed in this publication.

### 3. Experiments

The experiments were conducted with a Wyatt Eclipse DualTec Separation system. The setup was coupled with a degassing unit (G1322A), an isocratic pump (G1310B) and an autosampler (G1328C), all from the Agilent 1260 series. Signals were recorded using the software Astra 6.1.7.17 with a sample rate of 0.5 Hz. A detector chain, consisting of a Dawn Heleos 8<sup>+</sup> MALLS detector (wavelength = 663 nm) a UV/Vis-DA (diode array) detector (G1315C, Agilent series 1100) and an RI detector (G7162A, Agilent



series 1260). Before usage, an interdetector distance correction was conducted according to the elution peak of a BSA measurement using the respective function in Wyatt. Only signals with a decent signal-to-noise ratio were considered.

In the channel, an SC W350 spacer from Wyatt was used. The detailed dimensions are given in Table 3, together with the measurement conditions and related input parameters for the algorithms. Two samples were used: BSA and PS (Polystyrene) nanospheres. 3 replicates for each condition were measured. The eluent was 50 mM NaNO<sub>3</sub> for measurements with BSA. The injected sample amount was 20 µl. For the measurement with PS (Polystyrene) nanospheres, a 0.1 % w/v of SDS (sodium dodecyl sulfate) was used. The nanosphere standard particles had a nominal diameter of 60 nm. For our calculations, we assumed a hydrodynamic radius of 32 nm, which included the estimated influence of the surface stabilizer.

Measurements were conducted at 293 K. For each spacer a new 5 kDa Millipore regenerated Cellulose membrane was used. Detailed measurement program and sequence setup is given in the supplementary information.

## 4. Results

### 4.1 Comparison of algorithms with test measurements

First, we tested the algorithms with data acquired from our own field-flow fractionation. Standard protocols with a constant crossflow were used. Before elution, the sample was focused at  $z\% = 12\%$  of the total channel length. The details of the measurement protocols are given in the supporting information (Table S.1-S.3). BSA was measured at  $V_c = 2.5 \text{ mlmin}^{-1}$  and  $V_c = 3.5 \text{ mlmin}^{-1}$ . To provide an example for a lower crossflow, additional measurements with PS nanospheres were conducted at  $V_c = 0.5 \text{ mlmin}^{-1}$ . For BSA, the signals of the UV-Vis detector were chosen, for the PS measurements, UV and RI were considered.

Table 2: Parameters used for calibration experiments with test measurements

Parameter	unit	BSA $V_c 2.5$			BSA $V_c 3.5$			PS $V_c 0.5$		
		r1	r2	r3	r1	r2	r3	r1	r2	r3
$t_{\text{void}} - t_0$	min	0.53	0.55	0.54	0.6	0.5	0.51	0.73	0.72	0.72
$t_e$	min	3.65	3.65	3.35	4.6	4.5	4.5	15.8	15	15.1
$V_c$	mlmin <sup>-1</sup>		2.5			3.5			0.5	
$V_c$	mlmin <sup>-1</sup>		1.0			1.0			1.0	
$D_{\text{calib}}$	cm <sup>2</sup> s <sup>-1</sup>		6.1*10 <sup>-7</sup>			6.1*10 <sup>-7</sup>			6.74*10 <sup>-8</sup>	
$z\%$	%		12			12			12	
$b_0$	mm		22			22			22	
$b_L$	mm		3			3			3	
$L_1$	mm		20			20			20	
$L_2$	mm		150			150			150	
$L_3$	mm		3			3			3	

The plotted signals of the fractograms were used to pick positions of  $t_{\text{void}} - t_0$  and  $t_e$  for the BSA monomer peak, as shown exemplarily in Fig. 5. The fractograms have been corrected in time by its offset due to the focusing-related steps. I.e. “0” on the time axis displays the actual time of elution from the focusing position  $z\%$ .



The complete fractograms and extracted peak positions of all 9 measurements are given in the supporting information (Fig. S.6.1 9-S.6.513). All information was collected (Table 2) and processed by the 5 calibration algorithms of AF4Eval. The calculated channel widths and volumes are displayed in Fig. 6. The acquired retention times were typical for our AF4 setup and comparable to a lot of other measurements with similar conditions. In all three cases,  $V^{\text{appGeo}}$  is smaller than the calculated-  $V^{\text{cla}}$ . This is simply reasoned by the smaller surface, when the trapezoidal tapering is considered in the calculation.

Consequently, the same observation is made for the relation of  $w^{\text{appGeo}}$  and  $w^{\text{cla}}$ . As each of our calibration calculations was deduced from essentially the same established AF4 theory, we would have expected each of the calculations to lead to a very similar result. Instead, introducing the geometrical side condition of Eq. 89 ( $w^{\text{geo}}$  and  $V^{\text{geo}}$ ) made the calculated channel volume and related width shrink dramatically compared to the other calibration results.

Whereas removing the dependence on the known  $D_{\text{calib}}$  (for determination of  $w^{\text{hyd}}$ ) led to dramatically increased calculated channel widths, which are highly unrealistic as the spacer thickness of 350  $\mu\text{m}$  represents the maximal possible distance between membrane and upper wall. Interestingly, the related separation volume  $V^{\text{hyd}}$  was nearly the same as for the established first two variants as they use a very similar assumption for the derivation of the respective volume. This indicates that the formalism is indeed correct. Finally, removing  $t_{\text{void}}$  from the list input parameters by making use of Eq. 112 led to a result, which preserves the correct geometric relation, and resulted in a channel thickness within the expected range.

Also, it could be observed that the ratio the calculated  $V^{\text{cla}}$ ,  $V^{\text{appGeo}}$  their respective calculated  $w$  were increased when a higher crossflow was applied although the membrane area should be independent from  $V_c$ . For  $w^{\text{hyd}}$  and  $V^{\text{hyd}}$  also an increase with constant ratio was observed. The difference was too big to be explained by the higher pressure on the membrane. The discrepancy was not observed for the other algorithms.

In order to exclude potential errors in our own experimental setups, we applied the algorithms on published fractogram data.

#### 4.2 Comparison of algorithms using published data in literature

For the reevaluation we screened the respective AF4-related literature for data sets which provided fractograms and required information on the applied measurement conditions if possible. Especially the focus position  $z_0$  was not always indicated. For our investigations, we decided, to assume a value which is typically used for channels without frit-inlet. The implication of possible deviations of this real value are discussed below separately. We chose to work with measurements made with spherical gold nanoparticles [16,324], Silica particles[16] and silver nanospheres[32,33,34]. Measurements in the used literature had been conducted using trapezoidal channel geometries with different channel dimensions and AF4 devices from different vendors. The respective diffusion coefficient was calculated according to the specifications made in the respective publication and the expected shell thickness for polymer coatings[354,356]. All used parameters are displayed in Table 3 and Table 4. We applied the algorithms in the same way as we did for our own measured data.

Table 3: Parameters used for calibration calculation with literature data

Parameter	unit	Lit_Au1[302]	Lit_Au2[16]		LitAu3[16]		Lit_Sil[16]
			Vc1_0	Vc0_75	Vc1_0	Vc0_75	
$t_{\text{void}} \neq t_0$	min	0.593	0.89	0.89	0.992	0.992	0.96
$t_e$	min	4.42	4.55	0.992	9.45	9.45	13.3
$V_c$	mlmin <sup>-1</sup>	0.8	1.0	0.75	1.0	0.75	0.2
$V_e$	mlmin <sup>-1</sup>	0.5	1.0		1.0		0.2
$D_{\text{calib}}$	cm <sup>2</sup> s <sup>-1</sup>	6.1*10 <sup>-7</sup>	6.1*10 <sup>-7</sup>		6.74*10 <sup>-8</sup>		1.13*10 <sup>-7</sup>
$z\%$	%	12	12		12		12
$b_0$	mm	22	22		22		22
$b_L$	mm	3	3		3		5
$L_1$	mm	20	20		20		22
$L_2$	mm	150	150		150		213
$L_3$	mm	3	3		3		5

Table 4: Parameters used for calibration calculation with literature data

Parameter	unit	LitAG[343]						Lit_Ag_42nm[324]
		10 nm	20nm	30nm	50nm	70nm	90nm	
$t_{\text{void}} \neq t_0$	min	0.75	0.75	0.75	0.75	0.75	0.75	0.75
$t_e$	min	1.81	4.32	7.85	10.11	12.02	14.32	10.39
$V_c$	mlmin <sup>-1</sup>				0.4			1
$V_e$	mlmin <sup>-1</sup>				1.0			0.4
$D_{\text{calib}}$	cm <sup>2</sup> s <sup>-1</sup>	4.29*10 <sup>-7</sup>	2.14*10 <sup>-7</sup>	1.43*10 <sup>-7</sup>	8.58*10 <sup>-8</sup>	6.13*10 <sup>-8</sup>	4.76*10 <sup>-8</sup>	1.02*10 <sup>-7</sup>
$z\%$	%				12			12
$b_0$	mm				20			24
$b_L$	mm				5			3
$L_1$	mm				20			22
$L_2$	mm				250			152
$L_3$	mm				5			3

The results of our evaluations are displayed in Fig.7. For the data set Lit\_Au1, we could confirm all effects of previous findings:  $w^{\text{geo}}$  was usually significantly smaller than the other calculated channel distances, while the calculated value for  $w^{\text{hyd}}$  increased. Only in two cases we found the opposite result, but also here, the presence of differences as such was remarkable.

Due to our lack of knowledge of  $z\%$  we repeated the analysis for our own data as well for the literature data also for the alternative values 8% and 16%. The respective results do not vary significantly from the observations made for 12% and are displayed in the supporting information (S.6.19).

It can be summarized that applying AF4 theory blindly leads to conspicuously inconsistent results. As we counterchecked the derivation of our formulas and implementation into the software multiple times, we speculated this behavior to be reasoned by a systematic measurement error. For this reason, we continued and investigated the influence of measurement uncertainties on the resulting sizes.

#### *Influence of measurement uncertainties on the calibration result*

In the following, we implemented an additional feature which displays the effect of deviations to the final results. The task was implemented by a generic preprocessor macro, which keeps all but one parameter constant and iterates over a grid of the remaining parameters to be investigated. Thereby, we plot the impact of a deviation  $\delta X$  of a parameter  $X$  to the relative deviations of the resulting volume and channel widths ( $\delta Y$ ). This analysis is useful as due to the included numeric procedures and the relationship between the two output variables and its inputs is not obvious, especially when an input variable is used multiple times. In addition, this analysis can be used as a base for an estimation of the total propagation of uncertainty. Here, we show the result of this analysis, where the central parameters  $\delta X=0$  are taken from the parameter set BSA\_Vc2\_5\_r1.

Fig. 8 shows the influence of deviations to 4 of the parameters, we expected to exhibit the highest possible uncertainty.  $t_{0, \text{void}}$  and  $t_e$  have to be considered as they are the primary raw measured results. The physical dimensions of the channel area and the applied flow volumes are assumed to be measured with high accuracy, thereby, we don't discuss them further here in detail. Also, we skip the discussion on the first algorithm due to its similarity to the second one. However, the resulting data of full analysis of all 5 algorithms and 10 parameter sets is shown in the supporting information. The diffusion coefficient externally acquired has to be considered here, as for many samples, different numerical values have been published [36,37,38,39,40,41]. Also, side conditions like temperature and the temperature-dependent viscosity can influence the effective diffusion coefficient[10], where the latter one is often not known exactly in practice. The analysis shows that all procedures but the fourth (where  $D$  is not used) scale with  $D$ . However, the dependence was significantly stronger for the third procedure, while the volume is completely neglected for the second. The same relationships are shown for the input variable  $t_e$ . Interestingly, a deviation of the focus position seems only to contribute significantly if the actual position is strongly displaced from its designated position. The relation of the deviations of  $t_{0, \text{void}}$  were remarkable. While the linear relationship of the calculated volume was obvious, there was almost no impact on  $w^{\text{appGeo}}$ . For the third and fourth procedures, we found a proportional and a nearly inverse proportional relationship. As logical consequence a deviation of  $t_{0, \text{void}}$  leads to a divergence of the results in these procedures as it could be seen in the experimental results. This consideration lead to a closer consideration of an impact of the void peak time.

#### *4.3 Convergence of the calibration calculations via the adjustment of $t_{0, \text{void}}$*

Similarly, to the analysis above,  $t_{0, \text{void}}$  was varied for all 5 algorithms and the resulting channel widths. The analysis was conducted for one measurement per triplicate. The results, shown in Fig. 9, indicate a convergence of all algorithms for  $w$  and  $V$  for a value of  $t_{0, \text{void}}$  which is significantly dislocated from the respective measured value. We concluded that the position of the intersection point rather designates the position which should represent the actual position of  $t_{\text{void}}$  by its definition (the time used of passing the channel with the average migration velocity, starting from the focus position). -This confirms prior observations that the measured void peak might yield wrong results [1,10].

The results of an analysis for this intersectional  $t_{\text{void}}$  are shown in Fig. 10. Besides the balanced results for the single measurements we can also show that the discrepancies of the results between the different crossflows now completely vanish. ~~Similar results could be obtained for the evaluation of the given~~

~~literature data (Supporting information). Consequently, only a much smaller value can be considered as the “true” void peak position by its definition (the time used of passing the channel with the average migration velocity, starting from the focus position).~~

## 5. Discussion

### 5.1 Invalidity of the measured void peak

Our results show several implications towards working out best practices in AF4 calibration. As it could be demonstrated, the measured “void peaks” are not suited to reproduce calibration results which are consistent with the elaborated AF4 theory. An obvious potential error might be a bad correction of the offset volume in our measurement setup. However, the error has been shown to occur abundantly in literature data as well. As a consequence,  $t_{0, \text{void}}$  would be a very unreliable source of information just because of this pitfall. Moreover, we even believe the observed small peak in AF4 should not be related to this magnitude at all.

### 5.2 Alternate hypothesis for the occurrence of the “void peak”

Finally, we want to give another possible explanation for the reason behind the occurrence of the little observable peak, which has been considered as the void peak. To the best of our knowledge this explanation has not been reported elsewhere up to now. FFF is fundamentally based on the friction between the solvent and the channel wall. This friction is responsible for forming the parabolic flow profile. However, the friction to the side walls is usually not considered at all. Still, it should be assumed that a similar velocity gradient is present immediately at these side channel walls. While we assume the cross-flow to be uniformly distributed over the channel area and the migration velocity to be dependent solely on x- and z position referring to Fig. 3, this is probably not true at these border regions. A more sophisticated hypothetical flow distribution is shown in Fig. 110.

Because of the friction of solvent and channel side wall, 4 regions with different flow are present in this model. Due to diffusion, a sample particle can easily migrate from one of these zones into another. By this effect, a small amount of the sample partially eludes the retention effect of the crossflow. This portion is then detected much earlier than the retained sample. However, it does not accompany a “solvent front”, a term used for the description other chromatography techniques, which has no actual correspondent in field-flow-fractionation: Due to the parabolic flow distribution, the single laminar layers of the solvent flow are permanently displaced against each other. As a result, no common elution speed for all layers should exist. It has been reported that the observed peak intensity increases with decreasing  $V_c$  [254,412]. This might be reasoned by an increase of the permeable (i.e. unaffected from crossflow) area in the presented model.

To give experimental evidence, dedicated mathematical models will have to be developed in the future. Therefore, the physical nature of  $t_{0, \text{void}}$  has to be considered as a theoretical auxiliary construct (i.e. “the time needed to flush the separation volume if there was no flow gradient along the diameter” as it was the case for very big wall distance). One should not rely on an experimental value for this parameter consequently. Approaches already exist, which substitute its value by the information on the channel shape and the external calibration measurement using a calibrant with known diffusion coefficient. Our fifth algorithm belongs to this group as well as some simplified versions in literature [10,17,20,432,434] or the replacement of the known  $D$  with a related property of the calibrant [18]. According to our study, this groups remains the only valid approach to conduct the calibration properly.

## 6. Conclusions

We have shown that the measured void peak is not a reliable value for the evaluation of field-flow-fractionation data. Based on this observation, we could confirm this hypothesis by Wahlund [10] and give strong evidence how a gold standard calibration for the evaluation of AF4 data with current instruments can be performed. Our dedicated software for the evaluation has the potential to greatly improve the practical handling of AF4 data. As already known, the information about channel volume and channel width are the critical quantities for contemporary machines. Avoiding  $t_{void}$  as an error-prone calibration input is likely to improve accuracy of AF4 measurements. The development of our software will be continued, considering a list of features for numerous possible extensions:

- Alternative calibration methods as investigated recently [18]. Also, distance measurement has improved continuously [44], therefore we think that further calibration methods based on combination of the channel with such a device might be an additional orthogonal tool for the calibration.
- Crossflow gradients [5,46,47], steric effects and decays [10,20,21]. Our final goal will be to provide an open and extensible reference implementation, which gathers all these state-of-the-art evaluation methods.
- Advanced handling of light scattering data.
- AF4 related deconvolution techniques [22,48].
- Improved focus point determination as recently presented [43].
- An API for scripting languages.

As distance measurement by optical methods has improved continuously over the last decades up to submicrometer precision a direct measurement of  $w$  will be an additional improvement. If the width could be provided directly, the presented formalism could be applied easily to determine  $D$  without prior calibration. It was stated by Wahlund[10] that, unfortunately, hydrodynamic calculations were not available in commercial software. Our platform-independent and vendor-agnostic implementation is designed to fill this gap, relying on a state-of-the-art technology stack. We encourage users of AF4 get in contact to discuss possible extensions for their specific needs.

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