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**Revision of the AF4 calibration experiment, reply to the reviewers’ comments**

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**First reviewer:**

**Comments on supporting information:**

We are very grateful for the detailed comments of the first reviewer. Besides the minor incidents

we identified 3 main issues to be improved:

1. It had to be ensured that the designated volumes should represent the complete physical volume for consistency of the channel and don’t refer to the *effective separation volume*. For this reason, we had to change the used surface from the effective separation Az to the complete surface AL in method 3 (wgeo), 4(whyd),5(wapp). However, this deviation only affects the results partially and the drawn conclusions from the data could be kept.
2. We integrated the extrapolations for channel breadths according to the approximated shape model in method 2. Also here, only minor influences on the overall result could be observed, which do not imply a necessary change in the interpretation of the data. To become consistent, the index of b now clearly indicates its position on x axis.
3. The position of 2CF in method 5 could be identified to be correct, but inconsistent due to a typo error (now corrected) in the first equation, where CF occurred.

* The keywords include "void peak determination" It might be better to replace this with :channel thickness determination" or "channel width determination"

Indeed, the proposal fits the topic better, therefore, we adopted the change.

* The position of the sample focusing is represented by z% which is equal to the ratio of the distance z0 from the inlet and the channel length L. It is not a percentage. It should be noted that z% must be a function of assumed channel geometry and focusing flow rates. Therefore, for given focusing flow rates, one would expect z% for an assumed classical rectangular channel without triangular endpieces to be a little different to z% for a trapezoidal channel with triangular endpieces. All were assumed to be 12%, or 0.12. The same value of 12% was assumed for the experimental results taken from the literature. It is not explained in the manuscript how the z% is calculated. This should be included. It should also be confirmed that the literature results were also obtained for a z% of 12%.

For our measurements z% was simply set in the measurement software of our devices and taken. Indeed, the Wyatt Eclipse control software designates the focus position as a percentage of the total channel length, which already has been passed. Here, also the differences in channel shapes are considered for the focus position.

Unfortunately, this value was not given explicitly together with the applied literature data. For this reason, our value 12 % had to be taken as an assumption. Also, we did not find enough information to reconstruct the exact focus position from the literature data. This information was already given in our first submission, therefore, no changes had to be made. In the revised version, the information is given in line xxxxxx.

We stated in our revision that this might be an additional error source. However, in the revised version, we included an additional analysis for the respective literature data at deviating values at 9% and 15 % in order to show that a possible deviation does not the impair our findings. As we stated, we could expand the functionality of our software in the future; still, then the respective flow parameters have to be given order to reconstruct the exact focus position according to Wang et al, 2018.

* The first two methods are similar. The measured void time yields the channel volume (with different assumed channel geometries). The different geometries should have a minor effect on the obtained channel volume. The volume could be divided by the membrane area (which is known accurately) to obtain channel thickness. But rather than doing this, the elution time of a standard is used to obtain R, and hence <lambda>. From <lambda>, the ratio of channel volume to the square of channel thickness is obtained, and since it is assumed that channel volume has been obtained from the void time, then channel thickness is obtained.

This description of the used algorithm is correct. We would like to add here that the suggestion to divide the obtained volume by the known channel area is actually equivalent to the approach 4 described below besides the more accurate description of the volume.

* The third method takes the measured void time and the measured elution time of the standard to obtain R, solves for <lambda>, and from this the ratio of channel volume to the square of channel thickness is obtained (given the symbol S). S is therefore equal to the ratio of the area of the membrane AL to the channel thickness w. At this point the accurately known area of the membrane is taken into account to obtain channel thickness and from this the channel volume. The authors incorrectly use the area Az from z0 to outlet, rather than the total area AL.

We have checked the respective derivation of the volume equation. Indeed the reviewer is right and we are grateful that this mistake could be detected. We have integrated the correction in the reevaluation of all data sets. However, the correction did not have significant impact on the overall interpretation and only slightly reduced the observed discrepancies regarding this algorithm. This is only little wonder as *AL* only differs ~14 %, from *Az* while the observed discrepancy was much larger.

* The fourth method uses only the measured void time to obtain channel thickness and channel volume with knowledge of AL.

As already stated above, this method corresponds to the suggestion the reviewer stated above - besides the fact that our formalism encapsulates the shape-dependence a bit more clearly and allows a better adaption to other possible shapes by sampling adjusting the expressions for *AL* and *E*(*x*). Indeed, we were initially interested especially this method, as it would have provided a very elegant way to avoid the calibration experiment entirely.

* The fifth method uses the measured elution time of a standard, and effectively finds a channel thickness that is consistent with this elution time (taking into account that membrane area AL is known).

We would like to add that this is the only method which is consistent with elution time and the respective membrane area.

* If all measurements are accurate, then results would be expected to be in agreement. There is a great deal of disagreement between the different approaches, however. Methods 1 and 2 do not make use of the knowledge of AL, and the results for w and V0 are inconsistent with AL. This should disqualify these approaches.

The void time is certainly the most uncertain measurement. The authors show the estimation of void time in each case (Fig. 5), and it is often not an easy decision on where the peak lies. The fourth method uses only a measurement of void time and the results for w and V0 are much higher than for methods 3 and 5. This suggests that void time is overestimated. If this is true, then method 3 would overestimate R and <lambda>, and this would reduce the obtained w and V0. This is consistent with the results. This observation is consistent with their Fig. 9 which suggests convergence of results for lowered void times. One might expect that the fifth method which does not rely on a void time measurement should be least open to measurement error. The authors come to this same conclusion. In fact, the calibration of channel thickness and volume with the measurement of elution time of a submicron standard was recommended by Wahlund (ref 10 in this manuscript), Litzen (ref 17), and Hakansson et al (ref 20). The approach was used previously for other FFF techniques.

We added the citations 17 and 20 to the references which are equivalent to our fifth algorithm.

* Line 62: software still lags behind

The typo was corrected.

* Line 87: The dimensions of the channel: L1, L2, L3, b0, bL are better illustrated in Fig. S.1 of the supplementary information than in Fig. 1. The lower section of Fig. 1 also perpetuates an incorrect model of submicron particle distribution across channel thickness. All particles sizes have exponential distributions next to the membrane. The smaller particles are not driven away from the membrane.

zxzzzzzz

* Line 111: *t*e is not defined. It is the elution time for some particle size.

A definition was added.

* Line 113: The equation is not strictly correct for AF4, and this equation does not include a steric correction. The steric correction may be insignificant for the samples considered in the manuscript, however.

We added a short passage on the steric equation

* Line 117: *D* is not defined.

A definition was added.

* Line 123: strict monotonicity of the retention equation

The incorrect word “monotony” (a typical germanism) was replaced.

* Line 126: This equation 6 is incorrect (compare with eq 5). It is not clear what this equation is supposed to illustrate.

Equation 6 was supposed to illustrate the alternate expression to access the substitution term *S*. However, we admit that from the given unclear text, this definition was impossible for the reader to understand and misplaced in the document. For this reason, we removed the formula completely, adapted the numeration and integrated the formula into the new Eq. 9, which also covers the alternate of nature *S*.

* Line 129: z% is not defined. From the original publication (Wahlund & Giddings) it is clear that z% is supposed to represent the ratio of z0/L. It should also be stated that this equation was derived for a channel of constant breadth having no triangular endpieces.

The respective variable was defined and the comment on the simplified channel shape was added.

* Line 134: The equation should include z0 rather than z, and the b0 in this equation is not the b0 as defined earlier. It should represent the channel breadth extrapolated back to x = 0, not the breadth at L1. Also, AL is not defined (should be equal to the membrane area). The area Y is incorrectly defined in Section 3 Fig. S.1 of the supplementary information. It is correctly defined by eq S.3 where it corresponds to the area enclosed by the extrapolated channel walls from L1 back to x = 0, excluding the channel inlet endpiece. The bL should also correspond to an extrapolated breadth, although this difference is less significant.

The variables were adapted according to the suggestions of the reviewers. Consequently, we renamed the used channel breadths at b(x=L1) and b(x=L2) as b1 and b2 througout the manuscript and used b0 and bL correctly for their extrapolated counterparts. Indeed, as the reviewer already indicated, the numerical differences of b0 and b1 (b2 and Bl respectively) did only differ on a minor scale, therefore, these corrections, which only affect did not affect our overall result.

Indeed, one might see b0≈b1 and b2≈bl as a tolerable approximation.

However, our strict analytical approach was suited to use *E*(*x*) also to be used for the extrapolation easily. The respective equations were added to the supporting information and given in the main paper.

* Line 139: The Vcla of eq 7 and the Vapp of eq 8 are supposed to represent the complete channel volume. (Vapp is also written as Vappgeo and V≈geo in some places.) The Vgeo of eq 9 is inconsistent with these. Az should be defined clearly as shown by Fig. 3 - the area of the membrane from z0 to the outlet. But for consistency, Vgeo should be equal to the product of AL and wgeo.

As stated below,

* Lines 142 to 150: This description of the third method is confusing. It is better explained in the supplementary information. In this approach the definition of S makes sense only if Vgeo represents the total channel volume, in which case eq 11 should be written w = AL / S.

We have updated the explanation and adopted the usage of AL for all geometrical volume calculations in order to be ensure a consistent meaning (= the entire physical volume of the channel) for all channels.

* Lines 151 to 170: This fourth method should yield results for V0 almost identical to the second method. This method does not consider elution time of a standard. The only difference between this method and the second method with regard to determination of V0 is the treatment of the outlet endpiece, provided b0, bL and Y are correctly calculated for eq 8 used in the second method.

Again, we have to thank the reviewer for his comments. After applying the changes for extrapolating to b0 and bL in order to calculate the correction *Y* and exchanging the separation area for the complete area, indeed the resulting volumes became closer. The methods are indeed equivalent as the derivation of relation the t0 =2 w CF

* Lines 171 to 180: The fifth method does not consider measurement of void time, only elution time of a standard. wnoT is not defined. It is supposed to represent the result for w when a void time t0 is not considered.

In the revised version, we defined

* Line 173: The numerator should be written wnoT / (2 CF) (see eq 12)

Indeed, in the old document, there was a discrepancy between the eq. 12 and it application in eq. 14. We thank the reviewer for detecting this mismatch. However, we reviewed the equation and found the position of 2CF to be correct.

Also, we double-checked the correct application of this equation in the given pseudo-code and, of course, our coded implementation. In the numerator. The putative error arose due to a mix-uo in eq. 11, where *t*0 was swapped erroneously with *w*. This was verified as eq. 12 has to be the same as eq. S.2.9in the supporting information, where its derivation is explained in detail. Thereby, we corrected eq. to be alike S.2.9.

* Line 175: This equation should include AL and not Az (compare with eq 5)

The correction was applied in the main manuscript as well as for the supporting information. The data were reevaluated using the correct membrane.

Results changed?

* Line 177: The numerator of the left-hand side should be written wnoT / (2 CF)

Here the same correction applies as has been described for the comment of line 173.

* Table 1 has wappgeo and Vappgeo as outputs for method 2. The text referred to Vapp.

*V*appGeo and *w*appGeohave now been defined explicitly in the text and are used consistently.

* Lines 221 to 227: The size of the PS nanospheres is not given.

The information (60 nm diameter) was added in the experiemental section.

* Line 244: Reference to Fig. S.9-S.13 is incorrect

We adapated the numbering to as well and added the chapter number and now refer to S.6.1-S.6.5

**Comments on supporting information:**

* The void time is represented by tvoid in the supplementary information while t0 is used in the manuscript.

We changed the variable to tvoid in the manuscript as well. While t0 was used in literature before, tvoid seems more

* Section 3

It has already been stated that the b0 and bL for eq S.1 are not the b0 and bL at x = z0 and x = L1+L2. (In the FFF literature, the distance along the channel is usually represented by z and the distance across the channel thickness by x. Hence the focusing position zʹ, or z0 as in this manuscript.) It has been stated that Fig. S.1 incorrectly represents the area Y. It has also been stated that the V≈geo in eq S.1 should represent the total channel volume, not the volume defined by eq S.4. Incidentally, the final result on the right-hand side of eq S.4 is incorrect for the incorrect assumption. It should be the equivalent of (b(z0)+bL)(L-z0)/2

Likewise, the final result on the right-hand side of eq S.5 is incorrect for this incorrect assumption. The b<DELTA> should have been replace by (b0+bL)

As the reviewers points of critics concerning the extrapolation and the channel shapes was valid, we reformulated the entire section.

The respective erroneous area formula could be dropped entirely as only the surface AL was needed in the adopted calculation.

Instead, we integrated now the existing approach of Litzén and Wahlund (ref 6 in supporting information), who defined and subtracted the *skipped* part of membrane area in eq. S.3.5 in order to include focusing correction.

For z0 >L1, this area was already given by

(b0z0 – ( z0²(b0 - bl ) ) / 2L - Y) .

The area of Y was corrected in terms of the new extrapolated b0 simply as

0.5\*b0\*L1

For z0 < L1, the skipped area becomes a simple triangle with

0.5 \* (b1/L1) \* z0² .

This term was applied analogously in eq. S.3.6. As stated above the prediction of the reviewer 151-179 was confirmed by the reevaluation of our data.

* Section 4

Eq S.2 should have AL in the numerator, not Az.

The formula was corrected; (in addition, we chose to include the chapter numbers in all formula names.)

* Section 5

For the classical calibration with simplified trapezoidal channel, the pseudo code for T1 should use the extrapolated b at x = 0 rather than b0

As stated, the old b0 was reassigned as b1.

We included a formula based on e2(x) in order to extrapolate for the new b0.

Indeed, this is simply achieved by b0=e2(0) and bL = e2(L). This only affects method 2(wappGeo). The corresponding illustrations were adapted and data reevaluated

* For the calibration of channel height by Vgeo, the pseudocode for m2 is missing a negative sign. Also, Az should not be calculated. w should be estimated from AL / S, and Vgeo should be estimated from AL w. With the incorrect use of Az, one would expect w to be underestimated by around 12% and V0 by around 25%.

We exchanged the erroneous usage of Az by AL. Indeed, an increase of ~14% for w could be observed. However, still w is significantly underestimated by this method, thereby, none of our interpretations were affected by removing this error. As there was no need for the calculation Az anymore, the step was completely removed. Thereby, also the calculation of m2 in this step could be dropped entirely (Indeed, the negative sign was present, but merged with numerator).

* For the calibration of channel height by Vhyd, the final calculation of Vhyd should be given by the product of AL and w.
* Section 7

The flow conditions during sample introduction and focusing are not clear from the tables given in Section 7 of the supplementary information. In Table S.1, for example, the channel outlet flow rate Ve is given as 1 mL/min. This must be during elution. The first minute is designated E = Elution yet there is no cross flow. Does this mean that channel inlet flow rate is also 1 mL/min? From 1 to 3 minutes is also designated E with cross flow rate of 2.5 mL/min. Does this mean that channel inlet flow rate was 3.5 mL/min during this time with Ve = 1 mL/min? From 3 to 4 minutes is designate F = Focus with a focus flow rate of 1.5 mL/min. Is this flow split at a certain ratio to enter the channel at the inlet and outlet? If so, the ratio should be given as this determines z%. This focus flow would result in a cross flow rate of 1.5 mL/min also, but no cross flow rate is listed. The same conditions apply to the period from 4 to 8 minutes, designated F+I = Focus+Injection.

* Does the injection flow contribute to cross flow, or is focus flow reduced to keep a constant cross flow? From 8 to 43 minutes is designated E = Elution for which cross flow rate is given as 2.5 mL/min and Ve is supposedly 1 mL/min so that channel inlet flow rate must be 3.5 mL/min.

**Second reviewer:**

* “Th[e] authors claimed that the AF4 system setup used in the manuscript was inadequate to provide the requirements for measuring D absolutely. The authors concluded that the only possible option is to calibrate the retention time using size standards and channel volume.”

xxxxx

* “The authors should explain clearly the discrepancy between the measurands in figure 6. What is the reason of having different channel thickness and volume for BSA at different cross flow rates (3.5 mL/min and 2.5 mL/min)? The methods have the same %z values (12%) so both methods have the same effective channel area. It is hard to believe that the channel is getting bigger by 20% at higher cross flow rate. Channel dimensions such as thickness and volume (channel length times channel cross section area) should be constant regardless of run conditions and sample type.”

xxx

* “The experimental data are not conclusive and obtained only from one of the commercial systems available in the market. There are three vendors who manufacture the AF4 system. Wyatt technology is one of them. The authors should reach out to other two vendors to get the information they need to test the analysis software using the literature data published by other AF4 users. “