TECHNICAL SPECIFICATION

# ISO/TS 21362

First edition
2018-06

# Nanotechnologies — Analysis of nano-objects using asymmetrical-flow and centrifugal field-flow fractionation

*Nanotechnologies — Analyse des nano-objets par fractionnement flux asymétrique et flux force centrifuge*



Reference number
ISO/TS 21362:2018(E)



ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Fax: +41 22 749 09 47
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

# Contents

Page

**Foreword** ..... **v**
**Introduction** ..... **vi**
**1 Scope** ..... **1**
**2 Normative references** ..... **1**
**3 Terms and definitions** ..... **1**
**4 Symbols and abbreviated terms** ..... **7**
**5 Principles of operation** ..... **8**
5.1 Field-flow fractionation (General) ..... 8
5.2 Specific applications by applied field ..... 10
5.2.1 Flow field ..... 10
5.2.2 Centrifugal field ..... 11
**6 Method development for AF4** ..... **13**
6.1 General ..... 13
6.2 Sample specifications ..... 14
6.3 Mobile phase specifications ..... 14
6.4 Fractionation ..... 15
6.4.1 Channel and membrane selection ..... 15
6.4.2 Injection and relaxation ..... 17
6.4.3 Optimizing flow conditions ..... 18
6.4.4 Elution programme ..... 18
6.4.5 Using FFF theory to select initial flow settings ..... 19
**7 Method development for CF3** ..... **19**
7.1 General ..... 19
7.2 Choice of mobile phase ..... 20
7.3 Field strength selection ..... 20
7.4 Field decay programme ..... 20
7.5 Channel flow rate selection ..... 21
7.6 Calculation of the relaxation time ..... 21
7.7 Calculation of sample injection delay ..... 21
**8 Analysis of nano-objects** ..... **21**
8.1 General ..... 21
8.2 Online size analysis ..... 22
8.3 Online concentration analysis ..... 23
8.3.1 General ..... 23
8.3.2 Mass-based methods ..... 23
8.3.3 Number-based methods ..... 24
8.4 Online material identification or composition ..... 25
8.5 Off-line analysis (Fraction collection) ..... 26
**9 Qualification, performance criteria and measurement uncertainty** ..... **26**
9.1 System qualification and quality control ..... 26
9.1.1 Basic system qualification ..... 26
9.1.2 Focusing performance ..... 27
9.1.3 Flow rate of the carrier liquid ..... 28
9.1.4 Separation field ..... 28
9.2 Method performance criteria ..... 28
9.2.1 Recovery ..... 28
9.2.2 Selectivity ..... 29
9.2.3 Retention ..... 29
9.2.4 Resolution ..... 29
9.3 Method precision and measurement uncertainty ..... 29

**10 General procedures for measurement of samples ..... 30**
10.1 General ..... 30
10.2 Calibration of retention time for online size analysis ..... 30
10.2.1 Calibration of the AF4 channel ..... 30
10.2.2 Calibration of AF4 retention time for online size measurements ..... 31
10.3 AF4 general measurement procedure ..... 31
10.4 CF3 general measurement procedure ..... 32
**11 Test report ..... 33**
11.1 General ..... 33
11.2 Apparatus and measurement parameters ..... 33
11.2.1 AF4 recording/reporting specifications ..... 33
11.2.2 CF3 recording/reporting specifications ..... 34
11.3 Reporting test results ..... 34
**Bibliography ..... 35**



# Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies*.

# Introduction

The capacity to isolate and analyse diverse populations of nano-objects and their agglomerates or aggregates, often suspended in, or extracted from, complex matrices, is critical for applications ranging from materials discovery and nanomanufacturing to regulatory oversight and environmental risk assessment. Furthermore, the ability to characterize these analytes with minimal perturbation of their natural or native state is highly desirable. The list of available techniques capable of achieving such objectives is relatively short, and while all techniques have advantages and disadvantages, and no single technique is solely adequate or appropriate for all possible applications and materials, a group of related separation techniques known collectively as field-flow fractionation (FFF), conceptually proposed by J. Calvin Giddings in 1966[1], offers many advantages for nanotechnology applications. In FFF, the analyte, suspended in a liquid medium, is fractionated by the application of a field (e.g. flow, centrifugal, electric, thermal-gradient, magnetic) perpendicular to the direction of flow of the analyte and mobile phase eluting through a thin defined channel. Separation occurs when the analyte responds to the applied field, such that populations with different response sensitivities reach equilibrium positions (i.e. in equilibrium with diffusional forces) higher or lower in the laminar flow streamlines perpendicular to channel flow, thus eluting differentially.

Among the FFF variants, asymmetrical flow FFF (variously abbreviated in the literature as AF4, A4F, AFFFF, AfFFF or AsFlFFF) and centrifugal FFF (abbreviated as CF3, also called sedimentation FFF and abbreviated as SdFFF), are available commercially and have been most widely adopted in the nanotechnology field (for convenience and simplicity, the abbreviations AF4 and CF3 are used throughout this document). AF4 is arguably the most versatile technique with respect to the wide range of applications, materials and particle sizes to which it has been applied. Symmetrical flow FFF (fFFF), the original "flow-based" technique as first described in 1976[2], has been supplanted commercially by AF4, introduced in 1987[3], due to several advantages, including a simpler channel design, the ability to visualize the sample through a transparent top channel wall, and reduced analyte band width. The theory and application of CF3 as it is presently applied was described by Giddings and coworkers in 1974[4], although a centrifugal field-based FFF system was first developed and tested independently by Berg and Purcell in 1967[5]. Other FFF field variants, such as thermal, electrical and magnetic, provide unique capabilities, but have been limited in the scope of their applications vis-à-vis nanotechnology or commercial availability.

Where FFF was once predominantly the domain of specialists, these instruments are now commonly and increasingly utilized in government, industry and academic laboratories as part of the nano-characterization toolbox. Two factors are driving this increase in nanotechnology utilization: maturation of commercial instrumentation and versatility with respect to coupling a wide range of detectors to FFF systems. In the latter case, recent developments have led to the use of highly sensitive elemental detectors (e.g. inductively coupled plasma mass spectrometer or ICP-MS), which offer enhanced characterization and quantification for many materials. Additionally, traditional concentration or sizing detectors, such as ultraviolet-visible (UV-Vis) absorbance, fluorescence, multi-angle light scattering (MALS) and dynamic light scattering (DLS), yield online data for eluting populations, and theoretically provide more accurate information than obtainable using off-line measurements of unfractionated polydisperse systems. The measured retention time of an eluting peak can also be used to determine the hydrodynamic size by AF4 based on theoretical relationships or calibration with a known size standard. CF3 has the unique capacity to rapidly separate species of the same size but differing in density.

Although FFF based techniques have the capacity to separate and characterize analytes over an extremely broad size range, from about 1 nm up to tens of micrometers, this document focuses primarily on materials in the nanoscale regime and their associative structures. The basic underlying principles, experimental approach, and hardware described here can be more broadly applied.

While this specification includes the most common online detection schemes for nano-object analysis, other less common forms of detection have been utilized or reported in the literature, including differential refractometry (primarily used for macromolecular analysis), particle tracking analysis, graphite furnace atomic absorption spectrometry, single particle ICP-MS, and small-angle X-ray



scattering. This number is likely to grow in the future, as new techniques emerge and existing ones are modified and evaluated for coupling to FFF.

In order to develop and validate methods for application of FFF to the analysis of nano-objects and their agglomerates or aggregates, and to properly report experimental results and conditions in order to enable reproducibility across laboratories, it is critical to specify key parameters to be controlled and reported. These parameters encompass all aspects of FFF methodology, including sample/analyte, instrumentation, fractionation, calibration, qualification, performance specifications, measurement uncertainty, and data analysis. This document identifies the key parameters and lays out a general approach to method development for AF4 and CF3.

General references and further reading on FFF theory and practise, as well as AF4 and CF3 applications to nanotechnology, can be found in the Bibliography[6]-[18].

# Nanotechnologies — Analysis of nano-objects using asymmetrical-flow and centrifugal field-flow fractionation

## 1 Scope

This document identifies parameters and conditions, as part of an integrated measurement system, necessary to develop and validate methods for the application of asymmetrical-flow and centrifugal field-flow fractionation to the analysis of nano-objects and their aggregates and agglomerates dispersed in aqueous media. In addition to constituent fractionation, analysis can include size, size distribution, concentration and material identification using one or more suitable detectors. General guidelines and procedures are provided for application, and minimal reporting requirements necessary to reproduce a method and to convey critical aspects are specified.

## 2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/TS 80004-1, *Nanotechnologies — Vocabulary — Part 1: Core terms*

ISO/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

ISO/TS 80004-6, *Nanotechnologies — Vocabulary — Part 6: Nano-object characterization*

## 3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/TS 80004-1, ISO/TS 80004-2, ISO/TS 80004-6 and the following, apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at https://www.iso.org/obp

**3.1**
**nano-object**
discrete piece of material with one, two or three external dimensions in the nanoscale (from approximately 1 nm to 100 nm)

Note 1 to entry: Generic term for all discrete nanoscale objects.

[SOURCE: ISO/TS 80004-2:2015, 2.2, modified — In the definition, "(from approximately 1 nm to 100 nm)" has been added. Note 1 to entry has been changed.]

**3.2**
**nanoparticle**
nano-object with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly

Note 1 to entry: If the dimensions differ significantly (typically by more than 3 times), terms such as nanofibre or nanoplate may be preferred to the term nanoparticle.

[SOURCE: ISO/TS 80004-2:2015, 4.4]

**3.3**
**field-flow fractionation**
**FFF**
separation technique where a field is applied to a liquid suspension passing along a narrow channel in order to induce separation of the particles present in the liquid, dependent on their differing mobility under the force exerted by the field

Note 1 to entry: The field can be, for example, gravitational, centrifugal, a liquid flow, electrical or magnetic.

Note 2 to entry: Using a suitable detector after or during separation allows determination of the size and size distribution of nano-objects.

[SOURCE: ISO/TS 80004-6:2013, 4.4, modified — The term "field flow" has been changed to "field-flow".]

**3.4**
**asymmetrical-flow field-flow fractionation**
separation technique that uses a cross flow field applied perpendicular to the channel flow to achieve separation based on analyte diffusion coefficient or size

Note 1 to entry: Cross flow occurs by means of a semipermeable (accumulation) wall in the channel, while cross flow is zero at an opposing nonpermeable (depletion) wall.

Note 2 to entry: By comparison, in symmetrical flow, the cross flow enters through a permeable wall (frit) and exits through an opposing semipermeable wall and is generated separately from the channel flow.

Note 3 to entry: Nano-objects generally fractionate by the "normal" mode, where diffusion dominates and the smallest species elute first. In the micrometre size range, the "steric-hyperlayer" mode of fractionation is generally dominant, with the largest species eluting first. The transition from normal to steric-hyperlayer mode can be affected by material properties or measurement parameters, and therefore is not definitively identified; however, the transition can be defined explicitly for a given experimental set of conditions; typically, the transition occurs over a particle size range from about 0,5 µm to 2 µm.

Note 4 to entry: Including both normal and steric-hyperlayer modes, the technique has the capacity to separate particles ranging in size from approximately 1 nm to about 50 µm.

**3.5**
**centrifugal field-flow fractionation**
separation technique that uses a centrifugal field applied perpendicular to a circular channel that spins around its axis to achieve size separation of particles from roughly 10 nm to roughly 50 µm.

Note 1 to entry: Separation is governed by a combination of size and effective particle density.

Note 2 to entry: Applicable size range is dependent on and limited by the effective particle density.

**3.6**
**channel**
<field-flow fractionation> thin ribbon-like chamber with a parabolic flow profile required for separation under the influence of a field applied perpendicular to the channel flow

Note 1 to entry: Channel thickness can vary and is defined by a spacer insert.

Note 2 to entry: In asymmetrical-flow field-flow fractionation, a trapezoidal channel is commonly used, typically with a maximum breadth of ca. 20 mm to 25 mm and length of ca. 100 mm to 300 mm.

Note 3 to entry: In asymmetrical-flow, one channel surface (depletion wall) is solid (impermeable) and the opposing surface (accumulation wall) consists of a semipermeable membrane on a porous frit.

Note 4 to entry: In centrifugal flow field-flow fractionation, both the inner and outer walls of the circular channel are solid (non-porous) and the channel is curved. A trapezoidal channel is commonly used, typically with a breadth of 10 mm to 20 mm and length of 300 mm to 550 mm.

**3.7**
**spacer**
<field-flow fractionation> thin plastic film with a cut-out that defines the thickness and lateral dimensions of the channel

Note 1 to entry: Trapezoidal or rectangular cut-outs are most commonly used in asymmetrical-flow field-flow fractionation.

Note 2 to entry: Typical spacer thickness used for separation of nano-objects ranges from 190 µm to 500 µm.

**3.8**
**channel thickness**
<field flow fractionation> nominal thickness as defined by the spacer

**3.9**
**effective channel thickness**
<field-flow fractionation> thickness due to compressibility or swelling of the semipermeable membrane at the accumulation wall, the effective value of which can differ from the nominal value for a given spacer and is determined using a well-defined analyte of known diffusivity under the test conditions

Note 1 to entry: The measured effective channel thickness depends on other factors, such as interactions between the analyte and the membrane and variability in spacer manufacturing.

**3.10**
**accumulation wall**
surface of a field-flow fractionation channel toward which sample components are forced by the applied field acting perpendicular to the channel flow

Note 1 to entry: In asymmetrical-flow field-flow fractionation, the accumulation wall is flat and consists of a semipermeable membrane on a porous frit substrate.

Note 2 to entry: In centrifugal field-flow fractionation, the accumulation wall is impermeable and curved, and is located farther from the axis of rotation relative to the depletion wall. In the rare case that the particles have a lower density than the aqueous medium, the depletion and accumulation walls are reversed.

**3.11**
**depletion wall**
surface of a field-flow fractionation channel opposite the accumulation wall, which is depleted in analyte due to the movement of analyte toward the accumulation wall in the applied field

Note 1 to entry: In asymmetrical-flow field-flow fractionation, the depletion wall is flat and impermeable.

Note 2 to entry: In centrifugal field-flow fractionation, the depletion wall is impermeable and curved, and located closer to the axis of rotation relative to the accumulation wall. When the effective particle density is lower than the density of the medium, the depletion and accumulation walls are reversed.

**3.12**
**carrier liquid**
**eluent**
**mobile phase**
liquid phase used to achieve separation and transport of analytes

Note 1 to entry: The eluent or mobile phase may contain salts, surfactants, and/or other chemical constituents that are required for optimized separation and recovery of an analyte.

Note 2 to entry: In this document, only aqueous phases are relevant, but organic solvents can also be used if equipment and channel are compatible.

**3.13**
**elution**
<field-flow fractionation> process by which analytes in the mobile phase, or eluent, are transported through, and exit from, the fractionation channel

**3.14**
**focusing**
**<**asymmetrical-flow field-flow fractionation**>** process by which, during and after sample injection a counter-balanced flow entering from opposite ends of the channel (inlet and outlet) is applied to focus the sample components into a thin band close to the inlet port and near the accumulation wall

Note 1 to entry: This step is necessary to minimize band broadening and to allow components to achieve an equilibrium localization (relaxation) within the channel.

Note 2 to entry: During focusing outward flow occurs only through the permeable membrane at the accumulation wall.

**3.15**
**relaxation**
**<**field-flow fractionation**>** process by which the sample components assume their equilibrium state with respect to the opposing forces of diffusion and the applied field before elution is initiated

Note 1 to entry: In flow field-flow fractionation there are two means to achieve relaxation: normal focusing relaxation and frit inlet or hydrodynamic relaxation.

Note 2 to entry: In centrifugal field-flow fractionation, stop-flow is used to achieve relaxation.

**3.16**
**injection flow**
<field-flow fractionation> flow that drives the sample out of the injection loop and into the fractionation channel

Note 1 to entry: Depending on instrument design, injection can occur via a separate injection port or through the channel inlet port.

**3.17**
**cross flow**
<flow field-flow fractionation> flow field applied perpendicular to the channel flow to achieve separation of analytes

Note 1 to entry: In asymmetrical-flow field-flow fractionation, cross flow is created by the pressure differential across a permeable membrane at the accumulation wall, which results in a downward force that decreases with increasing distance from the accumulation wall.

Note 2 to entry: Cross flow is generated by using a flow controller combined with a single pump or by use of a second dedicated pump.

**3.18**
**channel inlet flow**
<field-flow fractionation> eluent that enters the channel at the front end (upstream)

Note 1 to entry: In asymmetrical-flow field-flow fractionation, inlet flow is split between cross flow and channel flow during elution.

**3.19**
**channel flow**
<field-flow fractionation> eluent flow through the channel

Note 1 to entry: Channel flow is generally equivalent to the flow exiting the channel and entering the detectors under typical experimental conditions, but can differ if flow exiting the channel is split.

Note 2 to entry: In asymmetrical-flow field-flow fractionation, fluid loss through the permeable accumulation wall leads to a linearly decreasing channel-flow velocity. This gradient can be compensated using a trapezoidal channel design with decreasing channel breadth toward the outlet.

**3.20**
**void volume**
<field flow fractionation> fluid volume defined by the channel dimensions plus the volume between the channel exit and the first detector

**3.21**
**void peak**
**<**field-flow fractionation**>** a peak appearing in the fractogram that corresponds to unretained, typically small sample components that are not in equilibrium with the separation field

Note 1 to entry: The void peak travels at the average carrier velocity and elutes before retained components.

Note 2 to entry: In this context, unretained means components that are not separated by the field and elute with the void peak. Unretained has a different meaning in traditional enthalpic-based chromatographic separations.

**3.22**
**void time**
time between initiation of elution and detection of the void peak defined at its maximum signal intensity

**3.23**
**retention time**
time between initiation of elution and detection of an analyte peak defined at its maximum signal intensity

Note 1 to entry: For a Gaussian peak, the maximum and peak centre are equivalent.

**3.24**
**retention parameter**
<field flow fractionation> dimensionless parameter equal to the ratio of the analyte zone centre-of-mass distance (from the accumulation wall) to the channel thickness

Note 1 to entry: A measure of the strength of interaction between the applied field and the analyte.

**3.25**
**retention ratio**
<field flow fractionation> ratio of the mean velocity of the analyte zone to the mean velocity of the mobile phase in the channel during elution

Note 1 to entry: This can be calculated theoretically or determined empirically from the ratio of the retention times associated with the void and analyte peaks, and is directly related to the retention parameter.

**3.26**
**selectivity**
<field-flow fractionation> measure of the ability of a method to separate analytes of different diffusion coefficient or size; empirically, the slope of a double logarithmic plot of diffusion coefficient versus retention ratio for analytes of known size, where a high value reflects a large change in retention time with a small variation in analyte size

Note 1 to entry: In centrifugal field-flow fractionation, selectivity is also dependent on effective mass, but the empirical relationship is defined in the same manner as asymmetrical-flow field-flow fractionation.

**3.27**
**resolution factor**
**fractionation power**
ratio of the difference in retention time to the average of the peak widths measured as the full width at half maximum for two adjacent eluting analytes

Note 1 to entry: Measure of the degree of separation between neighbouring or overlapping peaks.

**3.28**
**band broadening**
overall dispersion or widening of an analyte band as the analyte passes through a separation system

**3.29**
**zone broadening**
<field-flow fractionation> broadening of the width of the sample zone during separation in the channel

**3.30**
**normal mode (of elution)**
**Brownian mode**
<field-flow fractionation> mode of elution in which diffusion is the dominant opposing force to the applied orthogonal force (e.g., cross flow or centrifugal), resulting in relatively faster migration of smaller particles through the channel due to their higher location within the parabolic flow profile and an elution sequence where smaller particles elute before larger particles

Note 1 to entry: All nanoparticles are subject to normal or Brownian mode elution, which is dominant for particle diameters smaller than approximately 0,5 µm; nano-objects with at least one dimension greater than 0,5 µm might be subject to steric-hyperlayer mode elution. The upper limit for normal mode elution is not well defined and depends on both material and measurement factors.

Note 2 to entry: For centrifugal field-flow fractionation, the stated elution sequence assumes all particles have the same density; for particles that differ in both size and density, it is possible for the elution sequence to be reversed.

**3.31**
**steric-lift hyperlayer mode (of elution)**
<field-flow fractionation> elution in which diffusion forces are negligible, and motion of particles due to the applied orthogonal force (e.g., cross flow or centrifugal) is essentially impeded by resistance of the accumulation wall itself, resulting in an elution sequence that is reversed compared to normal mode

Note 1 to entry: Steric effects occur when larger particles form layers at the accumulation wall that, on average, project higher into the parabolic flow profile of the channel. As a result, larger particles will migrate faster than smaller particles. Hyperlayer or lift-hyperlayer occurs when the particles form thin layers above (extended from) the accumulation wall due to hydrodynamic effects, with larger particles more elevated than smaller particles resulting in their faster migration. Because steric and lift-hyperlayer are closely related, forming a continuum, and produce similar elution behaviour, they are commonly merged together.

Note 2 to entry: The lower limit for steric-hyperlayer mode elution is not well defined and can depend on both material and measurement factors such as the channel thickness and flow rate or the applied field strength. Generally, particles with an effective diameter greater than about 1 µm are subject to steric-hyperlayer elution, but the onset of steric-hyperlayer effects can occur over a range from about 0,5 µm to about 2 µm. The transition can be determined experimentally for a given set of conditions.

**3.32**
**fractogram**
<field-flow fractionation> two-dimensional graphic representation of data derived from an experiment, typically with one or more detector signals on the ordinate and retention time on the abscissa

Note 1 to entry: This is analogous to a chromatogram in traditional chromatography.

**3.33**
**recovery**
<field flow fractionation> ratio of the mass eluted during fractionation to the initial injected mass expressed as a percentage

Note 1 to entry: Determined experimentally using an appropriate mass sensitive detector, either off-line (directly measured in sample before injection and after collection of eluting peak) or online (by comparison of peak areas obtained with and without application of the force field).

Note 2 to entry: Recovery can be estimated based on the signal from non-mass-sensitive detectors, such as light scattering, subject to the influence of changes in detector response due to changes in the size distribution or other experimental conditions.

## 4 Symbols and abbreviated terms

| | |
|---|---|
| AF4 | asymmetrical-flow field-flow fractionation |
| CF3 | centrifugal field-flow fractionation |
| CTA | cellulose triacetate |
| CTAB | cetyltrimethylammonium bromide, $(C_{16}H_{33})N(CH_3)_3Br$ |
| DAD | diode array detector |
| DLS | dynamic light scattering |
| HF5 | hollow fibre flow field-flow fractionation |
| FFF | field-flow fractionation |
| F4 | flow field-flow fractionation |
| FWHM | full width at half maximum |
| ICP-MS | inductively coupled plasma mass spectrometry |
| OES | optical emission spectroscopy |
| MALS | multi-angle light scattering (static light scattering) |
| MWCO | molecular weight cut-off |
| NIR | near infrared |
| PES | polyethersulfone |
| PSL | polystyrene latex |
| PTA | particle tracking analysis |
| RC | regenerated cellulose |
| SDS | sodium dodecyl sulfate, $CH_3(CH_2)_{11}SO_4Na$ |
| SPP | sodium pyrophosphate |
| sp | single particle (inductively coupled mass spectrometry) |
| SPR | surface plasmon resonance |
| UV-Vis | ultraviolet-visible (wavelength range) |
| $\lambda$ | retention parameter |
| $R$ | retention ratio |
| $t_0$ | void time |
| $t_R$ | retention time (of analyte peak) |
| $\Delta t_R$ | retention time difference for two eluting peaks, centre-to-centre |
| $t_D$ | injection delay time |

| | |
|---|---|
| $w_{avg}$ | average width of two adjacent eluting peaks, typically defined as the full width at half maximum |
| $w$ | channel thickness (nominal, as defined by a spacer in AF4) |
| $w_{eff}$ | effective channel thickness (as determined empirically in AF4) |
| $l$ | centre of gravity distance from accumulation wall |
| $D$ | translational diffusion coefficient |
| $\dot{V}_c$ | volumetric cross flow rate in AF4 |
| $\dot{V}_{in}$ | volumetric channel inlet flow rate |
| $\dot{V}$ | volumetric channel outlet flow rate |
| $\dot{V}_i$ | volumetric injection flow rate |
| $v$ | migration velocity of the analyte zone in the channel |
| $\langle v \rangle$ | average longitudinal carrier (mobile phase) velocity in the channel |
| $V_0$ | void volume |
| $V_i$ | volume between the injection valve and the channel injection port |
| $V_l$ | sample loop volume |
| $S$ | selectivity |
| $R_s$ | resolution factor |
| $R$ (%) | recovery |
| $A_S$ | peak area of eluted sample |
| $A_D$ | peak area of sample injected directly into detector or eluted without cross-flow |
| $t_1$ | time over which $V_c$ or centrifugal field is held constant during an elution programme |
| $r_g$ | radius of gyration or root mean square radius |
| $d_H$ | hydrodynamic diameter (sphere-equivalent) |
| $d_V$ | volume-based equivalent spherical diameter |
| $RPS$ | rotational speed in revolutions per second |
| $RPS_0$ | rotational speed in revolutions per second at the beginning of the decay period |
| $G$ | centripedal acceleration (also referred to as centrifugal field strength) |

# 5 Principles of operation

## 5.1 Field-flow fractionation (General)

Field-flow fractionation is a flow-based separation methodology. Typical FFF operation predicates the release of a narrow sample band into a flowing mobile phase contained within a thin well-defined



channel. By comparison, traditional liquid chromatography utilizes a packed column containing a solid stationary phase that interacts with the analyte to achieve separation. In FFF, the mobile phase and analyte flow longitudinally through the channel. The channel is designed to separate the sample components along its length, resulting in the elution of constituents at different times. The channel and its large aspect ratio is designed to promote parabolic or near-parabolic laminar flow between two infinite planes under normal operational conditions. Though generally ignored, viscous drag at the edges of the channel can be accounted for using a correction factor[19]. Under parabolic flow, longitudinal fluid velocity at the walls of the channel approaches zero, due to frictional drag, and increases with distance from the walls. Maximum velocity occurs at the channel centre.

To induce separation between different constituents of a sample, a field is applied in a direction perpendicular to the flow axis (see Figure 1). The force of the applied field moves constituents toward the accumulation wall (depicted at bottom in Figure 1), and is opposed by the diffusional force inherent to the affected species. Constituents that differ in size (AF4) or mass (size)/density (CF3) become localized at different positions within the channel's parabolic flow profile, causing each population to be displaced downstream at a velocity equal to the mean parabolic flow at that position (where position refers to distance from the accumulation wall at a right angle to the direction of channel flow, and is a mean value for the analyte zone or "cloud", *l*). Smaller constituents elute before larger ones; this is termed normal mode or Brownian elution and applies to most nano-objects. Fractionation of large species (micrometre size) occurs by steric-hyperlayer interactions (steric-hyperlayer mode, frequently referred to simply as steric mode), in which smaller species elute after larger ones (i.e. reverse elution order compared to normal mode). Steric-hyperlayer mode might be relevant, for example, when samples contain large agglomerates of nano-objects or constituents are highly polydisperse. The transition from normal to steric-hyperlayer mode is not well defined, and can be influenced by factors other than size (e.g. cross flow rate)[20]; for a given sample and set of experimental conditions, the transition range can be determined empirically.

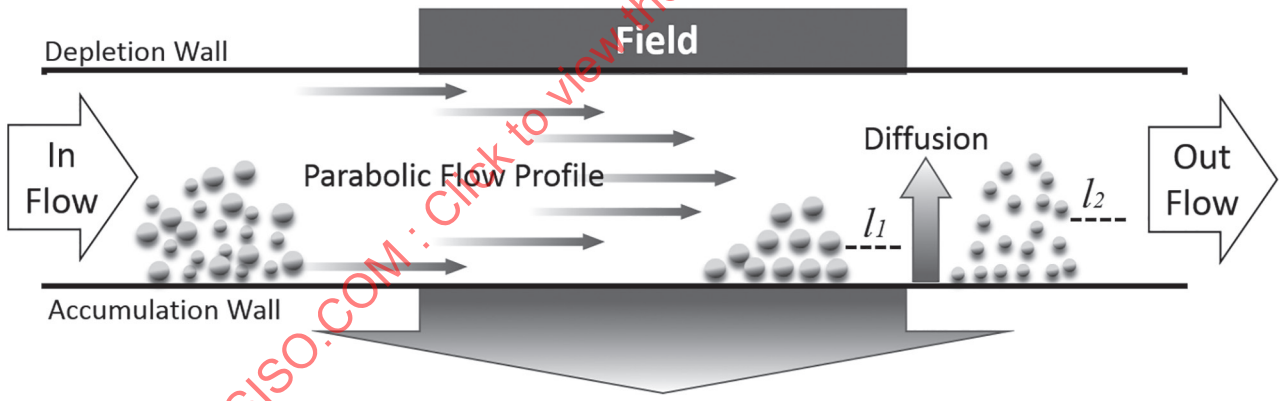



**Figure 1 — Schematic illustration of field-flow fractionation, showing channel flow velocity profile, applied field, diffusive motion, size separation, centre of gravity distance ($l_1$, $l_2$) for each population (in normal mode elution) and direction of channel flow in and out to detector**



Fractionation is achieved during passage through the channel based on the velocity flow profile, after which the mobile phase containing separated constituents exits to online detectors and/or a fraction collector for off-line analysis. Common detectors used for analysis of nano-objects and their aggregates or agglomerates (NOAA) include ultraviolet-visible (UV-Vis) absorbance, fluorescence, multi-angle light scattering (MALS), dynamic light scattering (DLS) and element detectors such as the inductively coupled plasma mass spectrometer (ICP-MS). Other detectors are used more commonly for polymer or macromolecular analytes (e.g. viscometer, differential refractometer) or less commonly for NOAAs (refer to Clause 8 for more detail).

Band broadening results from analyte zone broadening during sample migration in the FFF channel and from experimental (also called secondary) band broadening. Zone broadening is described by FFF theory and is a function of channel height, diffusion coefficient, retention parameter and channel flow[21][22]. Zone broadening can be calculated for a given diffusion coefficient and known flow rates. Secondary band broadening is due to dead volume and mixing effects in the fluid path of the analyte

zone exiting the FFF channel and passing through the detector chain. Secondary band broadening can be determined only experimentally, and care should be taken to minimize it by minimizing the volume in the fluid path downstream of the separation channel and between detectors.

## 5.2 Specific applications by applied field

### 5.2.1 Flow field

Flow field-flow fractionation (F4) is an elution technique whereby constituents are separated by flow control in a fluid medium. In F4 the separation force is generated by a cross flow field that is acting perpendicular to the channel flow. The cross flow in F4 is generated by a second pump or flow controller. In symmetrical F4, the cross flow enters the channel via a porous frit at the depletion wall and exits the channel via a semipermeable membrane on a porous frit the opposing accumulation wall. The membrane permeability is chosen such that it allows the mobile phase to pass through, while retaining the analyte. The sample fractions elute from the channel and enter the detectors by the main channel flow. In F4, the retention time, $t_R$, of constituents can be predicted by a modified version of the Giddings formula [23]:

$$t_R = \frac{\pi \eta d_H w^2}{2kT} \frac{\dot{V}_c}{\dot{V}} \quad (1)$$

where

$kT$ is the thermal energy (Boltzmann's constant × absolute temperature);

$\eta$ is the dynamic (or absolute) viscosity of the carrier liquid;

$d_H$ is the sphere-equivalent hydrodynamic diameter;

$w$ is the channel thickness;

$\dot{V}$ is the volumetric flow rate through the channel (outlet flow);

$\dot{V}_c$ is the volumetric cross flow rate.

Thus, when the cross flow and channel flow rates are held constant in the F4 system, the retention time is proportional to the sphere-equivalent hydrodynamic diameter of the separated constituents or inversely proportional to their diffusion coefficient, $D$, via the Stokes-Einstein relationship ($D = kT/3\pi\eta d_H$).

Whereas the analyte zone centre-of-mass distance from the accumulation wall, $l$, determines the rate at which the constituent population elutes in the parabolic flow profile, it can be expressed in a non-dimensional form as the retention parameter, $\lambda = l/w$, a measure of the strength of interaction between the applied field and the analyte. For flow FFF, the retention parameter can be expressed as Formulae [2] and [3]:

$$\lambda = \frac{V_0 D}{\dot{V}_c w^2} \quad (2)$$

where $V_0 = t_0 \dot{V}$ is the void volume and $t_0$ is the void time associated with the void peak that comprises unretained constituents and elutes before all retained or fractionated species. The retention parameter is related to a key empirically determined value known as the retention ratio, $R = t_0/t_R$. $R$ is defined explicitly as Formula [3]:

$$R = \frac{v}{\langle v \rangle} \quad (3)$$



where

$v$ is the migration velocity of the analyte zone through the channel;

$\langle v \rangle$ is the average channel (longitudinal) velocity of the mobile phase.

For R < < 1, the following expression is valid:

$$R = 6\lambda \tag{4}$$

Formula (4) is accurate to within 5 % when $\lambda < 0{,}02$[11]. Additionally, when $R$ is in the range 0,03 to 0,17, an approximation for λ in Formula (2) can be applied to account for particle shape[24]:

$$D = \frac{\dot{V}_c w^2}{A V_0} R \tag{5}$$

where the constant $A$ corresponds to a value of 6, 12 or 18, for spheres, thin rods or thin disks, respectively.

Asymmetrical-flow field-flow fractionation (AF4) has replaced symmetrical flow in commercial instrumentation. In AF4, the cross flow is generated by a second pump or a flow controller, by which fluid is passed through the semipermeable membrane at the accumulation wall due to a pressure differential across the membrane surface; in this case, the opposing depletion wall is solid and nonpermeable (though typically transparent). Accounting for this asymmetry, the relationship between analyte diffusion coefficient, retention time, flow conditions and the channel thickness in normal mode elution under constant cross flow is described by[3]:

$$t_R = \frac{w^2}{6D} \ln\left(1 + \frac{\dot{V}_c}{\dot{V}}\right) \tag{6}$$

In this case, $t_R = C \times d_H$, where $C$ is a constant for the experimental conditions used and the type of analyte present. By calibrating retention time using spherical particles of known size (or known diffusion coefficient), the hydrodynamic size can be directly estimated from measured retention time for the unknown sample. The relationship in Formula (6) holds true for the linear range defined by the calibration. The accuracy of the estimate will also depend on how closely the calibrant's physical and chemical properties match those of the analyte.

### 5.2.2 Centrifugal field

In centrifugal field-flow fractionation (CF3), the separation force is established by a centrifugal field applied perpendicular to the main channel flow (see Figure 2). The centrifugal field is generated by spinning a circular channel, which creates a centrifugal force across the channel and perpendicular to the axial channel flow. This force causes the constituents to migrate toward the accumulation wall (typically the outer wall, unless the particle density is lower than the medium). In CF3 separation, the centrifugal field increases with the buoyant mass, which depends on the mass of the particle, the effective density of the particle and the density of the fluid. As a result, larger and denser constituents are retained more strongly relative to smaller less dense species, and therefore elute more slowly from the channel. This means for constituents with a uniform density, separation in a CF3 is accomplished based on the species size; smaller size components elute before larger ones. According to theory, the retention time for nano-objects in CF3 can be predicted according to the following Formula[4]:

$$t_R = \frac{\pi \Delta\rho \, w G d_v^3 V_0}{36 k T \dot{V}} \tag{7}$$

where

$\Delta\rho$ is the density difference between analyte and eluent;

$d_v$ is the volume-based equivalent spherical diameter;

$\dot{V}$ is the volumetric channel flow rate;

$V_0$ is the void volume;

$G$ is the radial acceleration due to centrifugation [see Formula (10)]; and other parameters are identical to Formula (1).

Due to the combined effects of size and density, separation of constituents with similar size but different densities are possible. Similarly, a significant increase in peak-to-peak resolution is possible using CF3 compared with AF4, though the range of analyte size and type is more limited (e.g. materials with density sufficiently different from the suspending media).

The measurement capabilities of CF3 are based on a theoretical relationship between retention volume, $V_r$, and particle size:

$$V_r = \frac{V_0}{6\lambda\left[coth(1/2\lambda) - 2\lambda\right]} \approx \frac{V_0}{6\lambda} \tag{8}$$

For CF3, the retention parameter can be expressed as[2][3]:

$$\lambda = \frac{6kT}{\pi\Delta\rho G d_v^3} \tag{9}$$

where other terms are defined as before and $G$ is given by:

$$G = \left(2\pi \times RPS\right)^2 r \tag{10}$$

where

$RPS$ is the angular speed expressed in revolutions per second; and

$r$ is the radius of rotation of the circular channel.

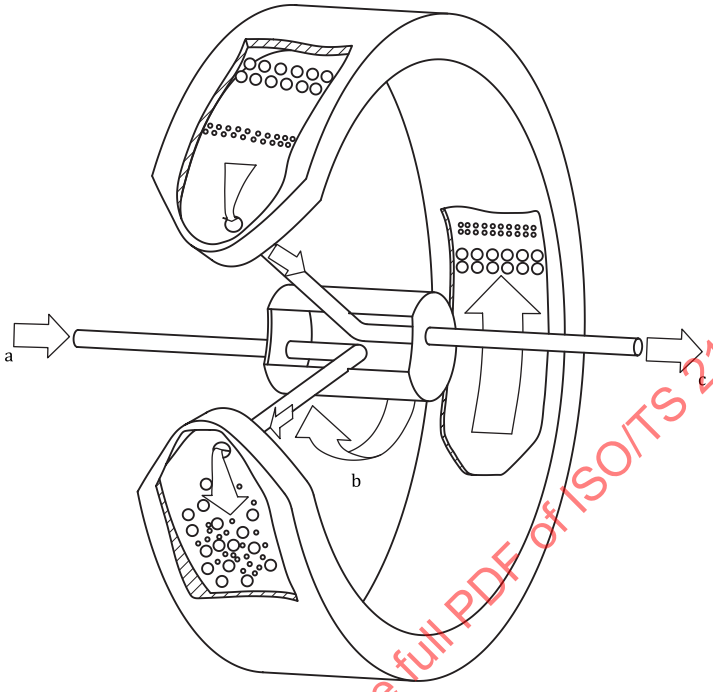
As with AF4, the retention parameter is a key empirically determined value related to the retention ratio, and the same relationship in Formula (3) applies. Evaluating retention times of retained constituents provides a continuum of quantitative values of $d_H$, yielding a distribution of constituent sizes. Since the larger retention times increase resolution, while lower retention times decrease resolution, the RPS should be adjusted appropriately.

CF3 requires a relaxation time as well as the focus and relaxation steps of AF4. During the relaxation time, a stop flow procedure is necessary so that each sample component forms a steady-state distribution near the accumulation wall. The centrifugal field causes constituents to migrate toward the accumulation wall and this migration is opposed by dispersive flux according to the Stokes-Einstein relationship. At steady-state, diffusion of constituents and centrifugal effects are balanced. The achievement of steady-state from initial distributions across the channel thickness is termed relaxation in CF3. The theoretical relaxation time is predicted by:

$$t_{eq} = \frac{18\eta w}{\Delta\rho G d_H^2} \tag{11}$$

where the parameters are defined as before[25].

According to Formula (11), relaxation time is also related to the difference in density between the constituents and the carrier liquid. The effect of this is that a long relaxation time is, in principle, necessary to obtain appropriate relaxation for small constituents with a low density difference relative to the liquid medium.




a Flow in
b Spin
c Flow out

**Figure 2 — Schematic of CF3, showing separation field and size fractionation**



# 6 Method development for AF4

## 6.1 General

The application of AF4 to a specific sample is dependent on the sample properties, the instrumentation utilized, and the objective(s) of the measurement; method development and optimization are typically required for each sample type or set of conditions. Due to the complexity and material-dependent nature of AF4, it is not possible to specify a single universal method that is applicable to all NOAA-containing samples, experimental conditions and purposes. Occasionally, the objective is simply to fractionate and collect analytes for further investigation or application; method fine-tuning might not be required or necessary in this case. AF4 based methods are also used to screen unknown samples for quality, to determine, for example, if a sample has changed over time or varies from batch to batch, or contains components other than the primary analyte; in this case, a single method may be used repeatedly once it has been validated for its purpose with respect to, for example, repeatability, intermediate precision and sensitivity. In most cases the objective is to fractionate and characterize the NOAAs with respect to size, shape, concentration, state of agglomeration, etc., often for samples that are unknown, modified, or complex in nature. The guidelines in 6.2 to 6.4 are intended to summarize the principal components of method development and to specify the parameters and conditions necessary to develop, optimize and validate a method.

## 6.2 Sample specifications

The following factors can significantly influence the quality of fractionation and the accuracy of results:

— concentration or quantity (mass) of injected analyte;

— surface charge;

— surface coating;

— core material;

— size and shape.

Sufficient material should be present to yield a reasonable detector signal-to-noise ratio (as specified by the manufacturer), while avoiding sample overloading that can lead to abnormal (depressed or coeluting) retention behaviour and poor recovery. For spherical nano-objects, a range from roughly $10^7$ to $10^{11}$ total injected particles is recommended[8], but validation of the sample quantity should be performed as part of any optimization process and is channel dependent. The objective is to identify a concentration range and injection volume that yields consistent retention and recovery results, where consistent can be user-defined based on a valid statistical approach (e.g. 95 % confidence interval, within $x \cdot \sigma$ where $\sigma$ is the standard deviation of the mean).

As a rule of thumb, the analyte concentration should not exceed about 1 g/l; this value provides only a "ball park" upper limit to initiate the optimization process and actual optimal concentrations can be far lower. Sample volume is established by the sample loop used on the injector; different size loops (i.e. with different volumes) can be interchanged to control injection volume, or injection quantities smaller than the loop volume can be injected manually or using an autosampler if the injected quantity is known with reasonable accuracy. It might also be necessary to dilute the sample into a compatible medium that does not alter the analyte form or stability. Similarly, it might be necessary to concentrate the analyte(s) prior to analysis (e.g. by stirred cell ultrafiltration or centrifugation). One of the first steps towards optimization should be the injection of a range of analyte quantities to evaluate detector response and recovery/loss. This can be achieved with "default" conditions or using mid-range typical settings for flow rates and a simple mobile phase that is selected to be compatible with known properties of the analyte (see 6.3).

Surface charge can substantially influence analyte-membrane interactions and affect retention behaviour. Common commercially available membranes (polyethersulfone, regenerated cellulose) carry a residual negative surface charge in aqueous media. Nano-objects with a positive surface charge (based on measurement of the zeta potential, for example), will adhere to the membrane, resulting in loss of analyte. Conversely, negatively charged analytes will exhibit repulsive interactions that increase recovery, but might also lead to inadequate retention and consequently poor fractionation if not properly balanced. The optimization and validation process should take these factors into consideration; options include selecting an appropriate mobile phase composition (see 6.3), selecting an appropriate membrane material or modifying the membrane surface, and altering the flow conditions to obtain an acceptable balance between recovery, retention and selectivity.

Coatings on the surface of nano-objects might influence interactions with the membrane and retention, as they can impart a surface charge, decrease or increase membrane adhesion, or provide stability toward agglomeration during injection, focusing and relaxation.

Additionally, the core material of the nano-object, its size and its shape all influence retention. Size and shape separation are often primary objectives of AF4, whereas material-dependent retention behaviour can be a benefit or a complication. Nano-objects generally elute at a retention time inversely proportional to their diffusion coefficient, irrespective of their shape.

## 6.3 Mobile phase specifications

Mobile phase composition is a key variable in AF4, and should be chosen both to ensure compatibility with the analyte and to optimize operational performance. The properties of the mobile phase



(composition, ionic strength, and pH) should be specified as part of the optimization and validation process.

Ideally, for nano-objects that carry a net electrostatic charge, the addition of a small quantity (e.g. 0,5 to 5 mmol/L level) of electrolyte to the mobile phase is recommended to modulate analyte-membrane interactions. Inert electrolytes such as ammonium nitrate or sodium chloride are commonly used for this purpose and typically yield similar results. Deionized water, without added electrolyte, has also been used successfully for fractionation of some analytes. Sodium azide, a biocide, has also served a dual purpose as an electrolyte and an antimicrobial agent in AF4 systems. However, use of azide might present a hazard and a waste disposal issue that should be evaluated for long-term use.

In general, the use of highly concentrated electrolyte solutions, such as isotonic saline or phosphate buffered saline, should be avoided. If required for specific applications, the analyst should evaluate the stability of the analytes in those solutions prior to performing fractionation. Additionally, the refractive index and/or viscosity of these solutions can differ significantly from a dilute aqueous mobile phase, and this can affect online measurements (e.g. size analysis by DLS).

Strong attractive electrostatic interactions between the analyte and the membrane surface can be mitigated by the addition of appropriately charged surfactants (e.g. positively charged CTAB or negatively charged SDS) to the mobile phase. To avoid formation of micelles that can interfere with the separation and analysis of the target analyte, surfactants should be used at levels well below their critical micelle concentration. Surfactants should also be used only when the interactions of the surfactant and analyte are known. Combining an inert electrolyte with an appropriate surfactant has proven to be an efficient and successful approach for optimizing the mobile phase when oppositely charged analytes and membranes are present.

In addition to facilitating acceptable fractionation of a sample, the mobile phase can be further adjusted to improve separation performance with respect to $R$, $R_S$, $S$, and $R(\%)$[8]. Generally, the total ionic strength (including contributions from the surfactant, if present) should be kept below approximately 2 mmol/L in order to minimize analyte loss due to "salting out" of charged species. One can first vary the ionic strength and composition to obtain an optimal mobile phase, using fixed mid-range flow rate values (e.g. $\dot{V}$ = 0,5 ml/min and $\dot{V}_c$ = 0,3 ml/min to 1 ml/min). The pH of the mobile phase might also be an important parameter, especially for nano-objects that naturally contain, or are coated with, protolyzable sites. Any prior knowledge of the analyte and its suspending medium will expedite substantially the selection and optimization of the mobile phase.

NOTE 1 Sodium azide absorbs light at 254 nm and can cause interference during UV absorbance measurements.

NOTE 2 The use of multivalent ions (e.g. $Ca^{2+}$, $Mg^{2+}$) and certain buffers can enhance the formation of agglomerates.

NOTE 3 Surfactants and additives can contain a significant number of solid particles and/or can be contaminated by ambient dust particles. Therefore, passing the prepared mobile phase through a membrane with nominal pore size of 0,1 µm is recommended to avoid forming obstructions in the FFF tubing or valves.

NOTE 4 Follow the FFF and detector instrument manufacturer recommendations for pH compatibility with the mobile phase.

## 6.4 Fractionation

### 6.4.1 Channel and membrane selection

Channels currently used for AF4 are predominantly trapezoidal in shape, with the width narrowing from the inlet to the exit (see Figure 3). Relative to a simple rectangular channel (constant breadth along z-axis), this geometry compensates for the loss of channel flow velocity along the length of the channel due to the asymmetric design and loss of flow through the membrane, it provides more consistent cross flow along the length of the channel, it minimizes analyte dilution and it reduces band broadening due to differential axial zone velocities in the parabolic streamlines[26].

The dimensions of the trapezoidal channel are defined by the tip-to-tip length ($L$), thickness ($w$), inlet channel breadth $b_0$ and outlet channel breadth $b_L$, as shown in Figure 3; $b_{z'}$ is the breadth at the focusing point along the z-axis of the channel.

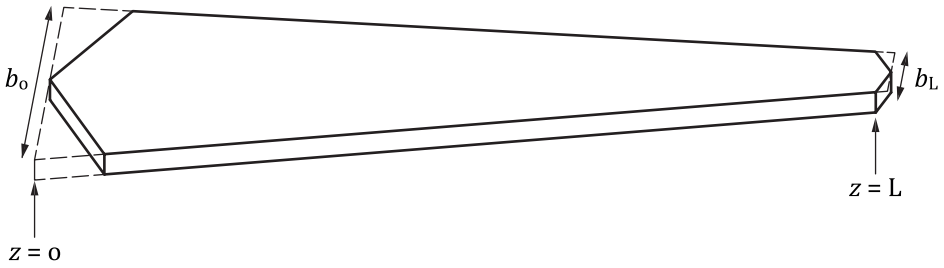



**Figure 3 — Depiction of a trapezoidal channel geometry. Adapted from Litzen and Wahlund 1991[26]**

Channel selection (i.e. nominal thickness, breadth and the channel length, as defined by a plastic spacer insert) is a necessary consideration in the optimization process when optimal selectivity is required. For nano-objects with maximum dimensions in the nanosize range, a relatively large channel thickness (e.g. 350 µm) works well under most experimental conditions. However, the properties of the sample itself might dictate a larger or, more likely, a smaller channel thickness. The following guidelines apply:

Increasing the channel thickness

— results in greater dilution of sample, due to increased channel volume;

— results in greater broadening of analyte bands due to axial distribution across flow streamlines that vary in velocity;

— yields better fractionation with higher resolution or selectivity, because the laminar flow profile is expanded over a greater distance; this allows analytes more time to separate into different size populations; and

— yields increasing retention times.

Decreasing the channel thickness

— yields narrower analyte bands with less broadening;

— results in less dilution of sample; and

— yields reduced fractionation quality and selectivity.

For example, all things being equal, increasing $w$ positions the analyte at a lower point in the parabolic velocity profile, thereby increasing residence time in the channel. This in turn influences fractionation, recovery and retention time. These are general guidelines. In practice, adjustment of flow conditions also influences fractionation quality and longitudinal band broadening, as does the properties of the analyte itself. Therefore, the channel thickness and flow rates should be intermittently varied to meet acceptable performance criteria.

Channel length is typically in the range between about 100 mm and 300 mm. Shorter channels offer faster elution with less dilution and band broadening, while longer channels might provide better fractionation, particularly with polydisperse samples. Channels with a reduced length (e.g. 70 mm) are now commercially available; these shorter channels significantly reduce sample dilution and analysis time, while improving the limit of detection, but at the expense of resolving power[26] (this compromise may be appropriate in specific applications).

Membrane selection can influence fractionation quality and recovery. Membranes are commonly classified based on a “molecular weight cut-off” (MWCO) value, with values ranging from 5 kDa to

30 kDa for AF4 applications[1]. A lower MWCO value retains smaller analytes in the channel; higher MWCO values allow larger species to permeate the membrane and produce less resistance to cross flow. As the MWCO decreases, the channel pressure increases faster with flow rate. A MWCO value of 10 kDa works for most nano-object applications, but the choice of pore size should always be validated for a specific sample (e.g. by determining recovery). Regenerated cellulose (RC) and polyether sulfone (PES) membranes are used for the vast majority of applications due to their availability as pre-cut inserts for specific channel geometries from instrument vendors and the wide range of available MWCO values. However, other membrane materials are available (e.g. polyvinylidene difluoride (PVDF), cellulose triacetate (CTA), either pre-cut from instrument vendors or in sheets from other commercial sources. Commercially sourced sheets should be carefully cut to size by the user to fit the specific channel geometry, using a procedure provided by the instrument manufacturer if possible. The quality of the membrane is critical for good performance, and vendor supplied pre-cut membranes are generally evaluated for quality. Both RC and PES are hydrophilic and exhibit low protein binding, while PES has relatively higher flow performance. Typically, RC and PES membranes carry a residual negative surface charge that should be considered with respect to the analyte during method development and mobile phase formulation.

An alternative configuration or mode of operation involves the so-called split flow or slot outlet on an otherwise typical trapezoidal channel. In this case, the fluid flow exiting the channel is divided into two streams. An analyte-free stream exists near the depletion wall (located at the top of the channel), and is removed via an additional outlet port installed on the channel's top block near the main channel outlet. Simultaneously, the analyte-containing flow stream located further from the depletion wall, exits the channel via a port located at the end of the channel (i.e. the primary channel outlet port). This latter flow is transferred directly to the connected detectors, while the split flow is directed to waste. Split or slot operation can significantly improve the sensitivity of detection by yielding a more concentrated analyte flow (less dilution). It can also be used in place of a standard flow splitter (post-channel) to reduce the flow rate reaching the detector, if necessary, without the disadvantage of sample loss. Care should be taken to avoid sample loss through the slot outlet, and distortion of the peak shape, which could compromise resolution.

Hollow fibre flow FFF (HF5) is used primarily for the analysis of proteins and other bioanalytical applications, where small sample quantity is a limiting factor. HF5 incorporates a cylindrical channel with a semipermeable hollow fibre shell and uses a radially applied cross flow. This has the benefits of reduced channel volume (less sample dilution) and high cross flow density (fast separations). It is easily adapted for collection and analysis of dissolved species by prudent selection of the membrane pore size and collection of the filtrate during focusing. HF5 therefore has the capacity to separate nano-objects from dissolved species with minimal sample dilution. For instance, its use has been demonstrated for samples containing Ag nano-objects in equilibrium with a range of dissolved Ag species[27]. However, at this time HF5 is not routinely used for nano-object analysis and is beyond the scope of this document.

Other channel geometries and designs (e.g. flat channel with exponentially decreasing width) have been described in the literature or are available for specific instrument platforms, but their use is not routine and is beyond the scope of this document.

### 6.4.2 Injection and relaxation

An AF4 analysis is initiated when the sample is injected into the channel. Depending on the channel and instrument design, injection can occur through a separate injection port or via the main inlet port at the front end of the channel. Initially, the sample analyte cloud is distributed across the depth of the channel. Before fractionation can begin, a relaxation process is applied. This process occurs during the focusing step, during which the analyte equilibrates in the mobile phase and is focused into a narrow band near the accumulation wall of the channel. The duration of the focusing step depends on whether injection and inlet flow occur through separate ports (requiring two relaxation steps) or through a shared port (requiring a single relaxation step), and on the cross flow rate generated during focusing.

1) The term "molecular weight" is obsolete, but still widely used by industry to define membrane pore size. The correct term, relative molecular mass, is dimensionless but given in units of Dalton (Da), which is the symbol for the unified atomic mass unit numerically equivalent to 1 g/mol. The International Committee for Weights and Measures (CIPM) has categorized Da as a non-SI unit accepted for use with the SI.

A simple relationship can be used to estimate the minimum relaxation (focusing) time, $\tau$, required in minutes[28]:

$$\tau = 1{,}2\frac{V_0}{\dot{V}_c} \tag{12}$$

During focusing, mobile phase flows from opposite ends of the channel and exits through the permeable accumulation wall. The focusing point can be adjusted by balancing the opposing flow rates, and can be visualized using a retained species that is coloured (e.g. blue dextran or gold nanoparticles). The desired result of focusing/relaxation is a narrow symmetrical band containing the analyte(s) that is equilibrated throughout the thickness of the channel.

Focusing can cause agglomeration of the analyte and loss of sample due to membrane interactions, if the loading is too high and there is insufficient repulsive force to sustain stability of the nano-objects in the mobile phase. In this case the analyst can consider lowering the sample quantity injected, modifying the mobile phase in accordance with 6.2 (e.g. using surfactants) or modifying the sample prior to injection (e.g. using a stabilizing surface coating). Another option is to use a frit-inlet channel, which reduces the concentration stress on the sample[29]. In this case, hydrodynamic relaxation of the sample is achieved by the compressive action of the stop-less frit flow and the need for focusing is bypassed, thereby mitigating undesired analyte interactions with the membrane. As a consequence of the stop-less frit-inlet flow, relaxational band broadening is increased relative to conventional AF4 with focusing[30].

### 6.4.3 Optimizing flow conditions

Once an initial mobile phase and analyte concentration (nano-object quantity) have been selected or determined empirically, the channel flow rate $\dot{V}$ should be fixed within the range (0,5 to 1,0) ml/min. Below 0,5 ml/min, very slow elution velocity can lead to substantial loss of analyte (poor recovery), while above 1,0 ml/min the channel pressure increases rapidly such that a high $\dot{V}_c$ is required to fractionate very small analytes, thereby limiting the available experimental range. A mid-range $_c$ should then be selected to evaluate if fractionation and recovery are reasonable (constituents at least partially resolved and $R$(%) ≥ 70 %). Additionally, the absence of unretained analytes (collocated within the void peak) should be confirmed. Once these conditions are met, starting with a low to intermediate $\dot{V}_c$ in the range (0,5 to 1,0) ml/min, $\dot{V}$ should be varied to optimize $\dot{V}_c / \dot{V}$ such that *R(%)* is maximized, 0,03 ≤ $R$ ≤ 0,2 and $S$ approximately equal to 1. If these performance parameters cannot be optimized to acceptable levels, then the channel thickness might need to be changed in accordance with 6.4.1. Alternatively, FFF theory can be consulted directly to select an appropriate initial set of flow conditions. Commercial software might be available to facilitate this process. Theoretically derived flow conditions should still be validated experimentally.

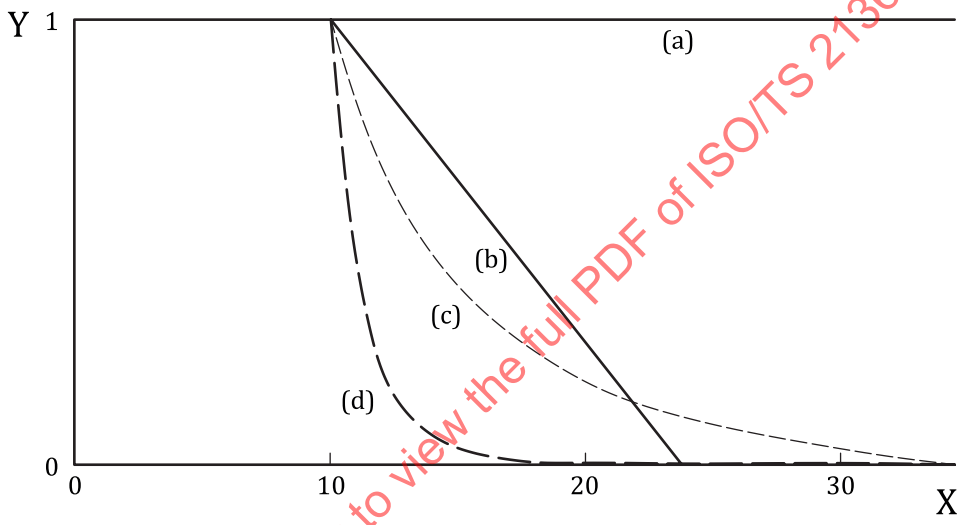
### 6.4.4 Elution programme

Although the decision to use a constant cross flow (fixed $\dot{V}_c$) versus cross flow programming ($\dot{V}_c$ reduction gradient) is not defined by the need to determine retention time-based particle size, a programmed cross flow decay introduces an additional complication (i.e. a discretization algorithm is necessary in the case of a variable cross flow[31][32]). If the dynamic range of constituent sizes to be separated is small (e.g. monomer-dimer separation), a constant cross flow should be applied. For many applications, a large size range exists, and therefore a decay of the cross flow during elution is favourable to facilitate rapid elution with an acceptable $R_S$ (i.e. where $R_S$ > 1). Elution programming is particularly useful for highly polydisperse or multimodal samples, where a wide range of constituent sizes are present; in this case, a single value for $\dot{V}_c$ might not be practical, resulting in an excessive and unnecessarily long elution time.

Different elution programmes can be utilized depending on the specific circumstances and available options for a specific commercial device, and a trial and error approach may be necessary to select the optimum approach. Programmes typically follow one of four types (see Figure 4): (a) constant mode (no

change in $\dot{V}_c$), (b) time-delayed linear programme ($\dot{V}_c$ initially held constant for a period of time, $t_1$, followed by a linear decrease to zero), (c) time-delayed power-law programme ($\dot{V}_c$ initially held constant, followed by a power-law decay to zero), and (d) time-delayed exponential programme ($\dot{V}_c$ initially held constant, followed by an exponential decrease to zero). The initial cross flow rate in Figure 4 is constant up to 10 min for all decay programmes. In decay programmes, the eluting peaks are typically collected for off-line use and/or analysed by online detectors capable of determining particle size (e.g. DLS or MALS) or concentration (e.g. UV-Vis or ICP-MS) of nano-objects.

To be utilized, a desired flow programme shall be compatible with the capabilities and limitations of the instrument (e.g. mass flow controllers, syringe pumps). For example, small changes over a large $\dot{V}_c$ range may not be possible at the lowest $\dot{V}_c$ values. The instrument vendor should be consulted with regard to these limitations.




**Key**

X elution time (min)

Y cross flow (arb. units)

**Figure 4 — Graphical illustration of common elution programmes**



### 6.4.5 Using FFF theory to select initial flow settings

FFF theory can, in principle, be used as a tool in planning experiments and selecting initial conditions and parameters as part of the method development and optimization process, including programmed cross flow decay. Use of theoretical relationships to predict optimal settings for an experiment can minimize the need for trial and error. Commercial software might be available to facilitate this process, though settings should be validated by the user since non-ideal and material-specific effects can influence the fractionation process.

## 7 Method development for CF3

### 7.1 General

As with AF4, the application of CF3 to a specific sample is dependent on the sample properties and the objective(s) of the measurement. Thus, it is not possible to specify a single universal method that is applicable to all nano-object containing samples. To obtain good separation, choice of mobile phase, field strength, field decay and relaxation time are important. The guidelines set out in 7.2 to 7.7 are

intended to summarize the principal components of method development and to specify the parameters and conditions necessary to develop, optimize and validate a method.

## 7.2 Choice of mobile phase

The absence of a stationary phase in CF3 enables the use of a variety of aqueous mobile phases with a range of ionic strength and pH values. However, proper adjustment of the pH and ionic strength of the mobile phase is necessary to avoid sample-wall interactions and sample agglomeration. As mentioned before, carriers with very low ionic strength can result in repulsion between the sample species and the accumulation wall, which in turn results in early elution of the sample. Use of carriers with high ionic strengths could result in low analyte recovery owing to reduction in electrostatic repulsion ultimately leading to attraction between the sample and the accumulation wall. The pH of the mobile phase should also be adjusted to minimize particle-wall interactions. The rule of thumb is to adjust the pH of the mobile phase to a value above the isoelectric points of both the sample and the accumulation wall to avoid attraction of the sample to the wall or other particles. For nanoparticles and environmental colloids, which are often negatively charged, use of anionic surfactant solutions, such as SDS, sodium pyrophosphate (SPP), or FL-70 (a commercial formulation containing nonionic and ionic surfactants) is recommended. For positively charged colloids, cationic surfactants such as CTAB can be used.

## 7.3 Field strength selection

The optimum field strength should result in high-resolution separation without compromising analyte recovery[33]. To achieve a high-resolution separation, a ratio of the analyte retention time to the void time ($t_r/t_0$) of 5 has been typically recommended. However, in practice this ratio can be as low as 2, which reduces the sample resolving power while still separating the sample peak from the void peak. Therefore, the required field strength for high-resolution CF3 can be derived from the corresponding retention formulae. Ultimately, resolution implies the capacity to fractionate particles of different sizes and/or effective densities.

Extremely high field strengths can result in strong analyte-wall interactions, and thus low analyte recovery or Brownian to lift-hyperlayer mode transition. Peak broadening can also occur if retention times are excessive, which would reduce the separation quality. As a rule of thumb, $t_r/t_0$ should be less than 20.

## 7.4 Field decay programme

For samples with a broad size distribution or which contain particles widely separated in size, the retention time for the particles eluting last can be excessively long when a constant field is applied, particularly for Brownian mode separations using CF3. This cannot be solved by simply reducing the field strength, as the smallest particles can merge with the void peak. To reduce the run time, the applied field is programmed to decay with time during the run. This field decay programming allows the smallest particles to be resolved from the void peak and larger particles to be eluted faster from the channel, resulting in a reduced run time. To ensure a uniform resolving power, the field strength should be reduced using a power programme[34]:

$$RPS(t) = RPS_0 \left( \frac{t_1 - t_a}{t - t_a} \right)^4 \tag{13}$$

where

$RPS(t)$ is the rotational speed at time $t$;

$RPS_0$ is the rotational speed during application of the constant field;

$t_1$ is the period of the constant field preceding the field decay; and

$t_a$ $= -8 \times t_1$.

Equations for Brownian mode FFF are not valid when programming is used during the fractionation. In this case conversion of the retention time to size is achieved either by elaborate calculations incorporated into the sample processing software or by direct calibration using materials of known size and density.

### 7.5 Channel flow rate selection

The channel flow rate affects both the overall run time and the analyte resolution. The resolution can be improved by lowering the channel flow rate, thus minimizing nonequilibrium peak broadening. However, this inevitably increases run time. Typically, in Brownian mode, channel flow rates in the range 0,3 ml/min to 2 ml/min are used. For samples with a broad size distribution, high channel flow rates should be avoided in order to minimize lift-hyperlayer perturbations on the larger particles.

Channel and detector flow rates are not necessarily equivalent (e.g. flow could be split post-channel), and therefore should not be used interchangeably.

### 7.6 Calculation of the relaxation time

As explained earlier, a pre-elution time period is needed to establish the equilibrium position of the analyte cloud across the height of the channel where the field and diffusion induced migrations are balanced. For a polydisperse analyte, the relaxation time is calculated for the smallest size component of the distribution, and can be approximated as the time for these particles to traverse the channel thickness under the applied field strength. For CF3 the relaxation time is calculated from Formula (10) [35]. To guarantee a fully relaxed sample in CF3, a relaxation time 1,5 × longer than the minimum calculated value is recommended.

### 7.7 Calculation of sample injection delay

Before beginning relaxation, the analyst should ensure that the sample plug is introduced into the channel completely. Therefore, the volume between the injection valve and the channel injection port, $V_i$, should be known precisely. This volume can be measured with sufficient accuracy by measuring the length of the connecting tubing and knowing its internal diameter. The injection delay, $t_D$, can then be calculated by means of a simple relationship:

$$t_D = \frac{\left(V_i + V_l\right)}{\dot{V}_i} \tag{14}$$

where

$V_l$ is the sample loop volume; and

$\dot{V}_i$ is the volumetric injection flow rate.

## 8 Analysis of nano-objects

### 8.1 General

The analysis of nano-objects subjected to AF4 or CF3 is dependent on the objective of the experiment, the availability of appropriate detectors (online or off-line) and the properties of the sample itself. Typically, nano-object “size” and “concentration” are the most common measurands. Additionally, composition or material identification may be desired.

## 8.2 Online size analysis

An appropriate online detector and associated method should be selected to measure the size of fractionated nano-objects. The most common and widely available methods include determination of hydrodynamic diameter based on retention time (after calibrating with a material consisting of spherical particles of known diameter), determination of hydrodynamic diameter based on DLS (typically the z-average diameter[36])[2], and determination of radius of gyration $r_g$ based on MALS data (applicable for $r_g$ > 10 nm using a red laser). To avoid a band-broadening effect on sizing, the volume of the flow cell should be as small as possible for online size analysis methods; otherwise, correction of band broadening should be performed. Direct measurement of size by DLS or MALS, when appropriate, is preferable to retention based size measurement, because calibration is not required for the former.

Particle size analysis based on retention behaviour requires calibration of the AF4 retention time, preferably using spherical particles of known size or known diffusion coefficient that overlap the size range of the analyte(s). From the calibration curve so constructed, the spherical-equivalent hydrodynamic diameter can be assigned for any measured value of $t_R$ for an unknown analyte. Polystyrene latex (PSL) spheres are frequently used for this purpose. However, non-ideal material-specific retention behaviour can significantly bias the calibration procedure[37][38]. For instance, the use of PSL spheres might not be appropriate if this value is then used to calculate the diffusion coefficient or hydrodynamic size of nano-objects having a substantially different composition or surface nature (e.g. gold nanoparticles). For this reason, spherical particles of known size with composition and surface chemistry that match the analyte as closely as possible should be used as calibrants[3].

Alternatively, using a single known monodisperse spherical particle it is possible to obtain the effective channel thickness, $w_{\text{eff}}$, where:

$$w_{\text{eff}} = \sqrt{\frac{6 D_{t_R}}{ln\left(1 + {}^{V_c}\!/\!_{\dot{V}}\right)}} \tag{15}$$

Once established under the conditions of measurement, $w_{\text{eff}}$ can then be used in Formula (6) to determine $D$ for an unknown analyte; size is obtained by application of the Stokes-Einstein equation (see 5.2.1) with the assumption of spherical geometry. The nominal value of the channel thickness, determined by the spacer, can differ by up to 10 % from the effective thickness, due to compressibility of the membrane. Also, the same non-ideal effects mentioned previously, can yield significant variations in the apparent effective thickness for nano-objects of the same or nearly the same size. For this reason, $w_{\text{eff}}$ should be established, if possible, using a calibrant that is closely matched with the target analyte. If this is not possible, then account should be taken of the potential bias in using an unmatched calibrant. Additionally, if an elution programme is utilized, yielding a variable cross flow rate over time, Formula (6) cannot be directly applied. In this case a discretization algorithm is required to relate $t_R$ to hydrodynamic size or diffusion coefficient as stated in 6.4.4.

For measurement of size using DLS, the hydrodynamic diameter should be measured across the full width at half maximum for each resolvable eluting peak. The quality of online DLS data declines significantly when insufficient particles are in the beam (as on the wings of an eluting peak) or when the particle size increases to the point that the residence time in the beam is insufficient to accurately capture the correlation decay function of the particles due to low diffusivity. The mean hydrodynamic diameter for a peak shall be calculated as the arithmetic mean of the data points obtained as described earlier. However, if the data indicates a clear upward trend, the arithmetic mean may not represent the



2) The cumulants method of DLS analysis yields a scattered light intensity-weighted harmonic mean particle diameter, which is commonly referred to as the "z-average". Size determined by methods based on different measurement principles produce different size parameters, which might differ significantly, even after reduction of the size distribution by fractionation. For example, the size obtained by DLS reflects the hydrodynamic properties of the constituent, whereas $r_g$ obtained by MALS is the mass weighted average distance of material from the centre of mass.

3) Since matched calibrants might not always be available, and because samples might contain multiple dissimilar constituents, the resulting retention-based particle size might, at best, be a rough estimate of the true value (i.e., a significant bias could exist).

true mean for the peak. Such a trend indicates that the population of nano-objects within the peak is polydisperse with larger particles exiting the channel after smaller ones, and their relative fractions might not be properly weighted.

The application of MALS for size analysis of nano-objects is generally limited on the lower size end where an angular dependence of scattered light is no longer evident (roughly greater than about 10 nm to 15 nm for detectors that use red light). The upper size limit exceeds the nano regime, and depends on the lowest angle of detection. The radius of gyration can be obtained from the slope $\left(r_g^2/3\right)$ of a log-log plot of scattered intensity versus $q^2$, where $q = (4\pi\eta/\lambda_0)\ sin\ (\theta/2)$ is the scattering vector magnitude, $n$ is the refractive index of the medium, $\lambda_0$ is the incident wavelength *in vacuo* and $\theta$ is the scattering angle. This is commonly referred to as a Guinier plot. Other data fitting models exist, including the widely-used Debye-Zimm formalism; full or partial Zimm plots are commonly used for polymeric materials to extract both the weight average molecular mass and $r_g$, but only when the mass concentration of the polymer is known and the MALS data can be extrapolated to zero scattering angle[39]. As with the Guinier plot, it is necessary for the polymer (particle) size to be sufficiently large to produce angular dependent scattering. A spherical-equivalent size can be obtained by simple transformation, $r = r_g/\sqrt{3/5}$ where $r$ is the geometric radius of a hard sphere. Relationships exist for other geometries, including hollow spheres, cylinders, tubes, ellipsoids and disks, which are readily available in published literature[40]. The analyst should be aware that optically active nano-objects, like gold, can yield an angular dependence that is not compatible with Guinier or Zimm analysis.

For optical-based measurements, the signal intensity depends on the amount of sample injected and on the optical properties of the analyte. The detector sensitivity should be set to obtain a strong peak signal for the analyte, in order to ensure accurate data processing.

NOTE 1 Size determined by methods based on different measurement principles might differ significantly, even after reduction of the size distribution by separation/pre-fractionation. For example, the size obtained by DLS reflects the hydrodynamic properties of a nano-object, whereas $r_g$ obtained by MALS is the root mean square radius reflecting the physical distribution of material around the centre of mass.

NOTE 2 The ratio of the radius of gyration (from MALS) to the hydrodynamic diameter (from DLS), $2r_g/d_H$, is a shape-sensitive characteristic parameter, referred to commonly as the shape factor[41]. The magnitude of this parameter depends on the architecture of the analyte. This ratio decreases with increasing 'compactness' of the analyte. For example, the ratios obtained for a hard sphere and a long thin rod are 0,778 and > 2,0, respectively, with prolate and oblate ellipsoids falling between these values.[42][43] Shape factors for particles (excluding macromolecules) greater than about 1,5 suggest higher aspect ratio morphologies.

## 8.3 Online concentration analysis

### 8.3.1 General

An appropriate online evaluation method should be selected to measure the concentration of fractionated nano-objects during elution, if concentration measurements are required for an application. This analysis may be based on mass or number concentration, but is more commonly mass-based. Because intensity of scattered light is dependent on both concentration and particle size/shape, it is not considered an appropriate evaluation method. However, under conditions where size/shape is constant (uniform), scattering intensity can be directly related to the mass or number concentration of scatterers.

### 8.3.2 Mass-based methods

Appropriate and commonly used mass-based methods include inductively coupled plasma-optical emission spectrometry (ICP-OES), inductively coupled plasma-mass spectrometry (ICP-MS) and UV-Vis optical absorption (See References [44] to[51]).

ICP-OES, ICP-MS and UV-Vis are generally sensitive to the mass of analyte present. ICP-OES and ICP-MS have very low detection limits (down to ng/l regime depending on the instrument and element) and are element specific. UV-Vis detection limits depend on the absorptivity and scattering properties of

the analyte, and are not element specific; UV-Vis works best for optically active materials that exhibit a characteristic absorption band (e.g. SPR) or where only a single absorbing species is present. Linearity of absorption (extinction) should be confirmed for the analyte over the relevant concentration range. Available UV-Vis detectors include devices that use a single selectable wavelength, multiple selectable wavelengths and full spectrum analysis [diode array detector – (DAD)].

For element-specific concentration, ICP-MS and ICP-OES offer the highest specificity and lowest limits of detection. ICP-OES provides simultaneous multi-element (up to 60) detection at trace levels; metallic and some non-metallic elements can be detected by ICP-OES, limited by emission interferences. ICP-MS can detect 83 elements at trace and ultratrace levels, and although analysis time can be very fast, the number of different elements analysed simultaneously (via peak hopping) is generally limited to a few. ICP-MS offers the additional capacity to detect specific isotopes of the same element, allowing isotope ratio analysis. Important light elements, specifically carbon, nitrogen and oxygen, are not traditionally detected by MS or OES, and some elements (e.g. sulfur, calcium, selenium) can have relatively high limits of detection or require ancillary use of a collision or reaction cell. Additionally, there are polyatomic mass interferences in ICP-MS that limit the detection of certain elements and require additional steps to mitigate (e.g. use of a reaction or collision cell). In general, standard liquid chromatographic introduction systems used for ICP-OES and ICP-MS can be adopted for coupling to AF4 or CF3; the liquid flow rate should be adjusted for compatibility with the detector in accordance with the manufacturer's recommendations.

Online AF4-coupled quantification of elemental carbon using ICP-MS with a helium collision cell and or an organic carbon analyser designed for use with liquid chromatographic systems, have been demonstrated at limits of detection in the single mg/l range[53], raising the possibility of detecting and quantifying carbon based nano-objects and other carbon-containing species using an AF4 based hyphenated method.

For quantitative results, each of these methods requires calibration with traceable analytical standards or certified reference materials. For ICP-OES and ICP-MS, calibration is achieved by injection of standard solutions containing elements (or mass isotopes) of interest using an injection valve placed between the last online flow-through detector, or the exit port of the fractionation channel if no other detectors are used, and the sample nebulizer interface just in front of the plasma. For UV-Vis calibration, suspensions containing known mass concentrations of the analyte are injected into the fractionation channel. For AF4, elution without cross flow should be used for calibration purposes.

The signal intensity depends on the amount of sample injected. The detector sensitivity should be set to obtain a strong peak signal for the analyte, to ensure accurate data processing. The linear relationship between solute concentration and peak height (or area) should be maintained by keeping the sensitivity at the same setting.

For UV-Vis detection, the measured absorbance can be affected by particle scattering, which increases as the 6th power of the radius (at a given wavelength). Generally, the UV-Vis signal is not strictly proportional to mass concentration, though the scattering effect can be sufficiently insignificant to ignore this nonlinearity (e.g. metal particles exhibiting strong surface plasmon resonance). A scattering correction should be applied or it should be demonstrated that the scattering contribution is negligible. The effect of scattering is more prominent following fractionation relative to batch measurements due to the population averaging in the latter.

### 8.3.3 Number-based methods

Particle number-based online methods for concentration measurement coupled to FFF have been rarely reported in the literature, but are likely to increase in the future. For that reason, they are briefly mentioned here with the acknowledgement that these are emerging methods based on relatively new measurement technologies. Two techniques have been reported as online particle counting detectors with FFF, namely single particle ICP-MS (spICP-MS)[54] and particle tracking analysis (PTA)[55]. Each has certain limitations and advantages.

Generally, spICP-MS (ICP-MS operated in time-resolved mode with dwell times of 10 ms or less) is applicable to particle mass concentrations in the ng/l regime (optimally in the range from about $10^6$ to

$10^8$ particles/l) and to particle diameters above roughly 20 nm (dependent on the detected element). The coupling for spICP-MS to AF4 or CF3 is essentially the same as that used for ICP-MS. However, the calibration process is more complicated, as the particle transport efficiency into the plasma has to be accurately determined, and an event-mass-to-size conversion performed. This requires a two-step calibration typically involving standard ionic solutions of the element of interest and a reference nanoparticle suspension of known particle size and mass concentration. Since the analysis involves transport of particles through the FFF channel, it is also necessary to determine the number-based recovery (with and without the applied field) for the analyte particles.

PTA is a particle counting technique that uses optical-based tracking and detection. Although PTA can be coupled online to the output from AF4 or CF3, it is operated in a stop-flow mode or using extremely low flow rates compatible with the particle tracking technology. The results of each analysis interval (yielding number concentration and number-based hydrodynamic size) can then be plotted versus retention time and integrated to obtain the total particle concentration for the fractogram. Theoretically, calibration is not necessary to obtain particle concentration or size, but there are other measurement and material factors that can affect the results of the analysis. Additionally, the need for stop-flow or low-flow operation requires a more complicated coupling and control system relative to spICP-MS, but PTA can operate at relatively higher particle concentrations (mg/l range, roughly $10^9$ to $10^{12}$ particles/l) where spICP-MS would not be appropriate. PTA is limited on the lower particle size range by the optical scattering properties of the material; the particles need to scatter sufficient light for the system to detect and track individual particles (typically > 10 nm). At the upper size limit, particles need to generate sufficiently long tracks to derive a meaningful analysis; as the rate of diffusion slows with increasing size, the range of motion detected during analysis is reduced.

## 8.4 Online material identification or composition

In addition to the measurement of nano-object size and concentration, measurement of sample composition (or material identification) constitutes the third principal type of online analysis for application to FFF. Relative to batch composition measurements, analysis of fractionated samples yields size (AF4 and CF3) or density (CF3) differentiated compositional information.

The selection of an appropriate online detector and method depends on the nature of the sample, the type of information required and detector availability. UV-Vis absorbance (using specific wavelengths or a DAD) is an economical approach that works well if material identification is the principal objective. In this case, optically active nano-objects can be identified based on their characteristic absorbance peaks. For example, nanorods and spherical nanoparticles of similar diameter can be fractionated and identified by monitoring the known longitudinal and transverse surface plasmon resonance bands. UV-Vis absorbance can also be used online to differentiate between the target analytes (nano-objects) and other species (e.g. micelles, organic colloids). In order to use UV-Vis as a detector for material identification, some prior knowledge about the sample is generally required, since absorbance is not element specific. UV-Vis is frequently utilized in a qualitative manner to confirm the presence or absence of a species of interest.

An online fluorescence detector can be used to identify fractionated analyte species if the species of interest are native fluorophores or can be tagged with fluorescent markers. Fluorescence offers higher specificity and lower limits of detection (comparable to mass spectrometry) relative to UV-Vis absorbance, but is more limited in its application for nano-object analysis. Fluorescence sensitivity is limited by the intensity of the excitation light, quantum efficiency of the fluorophore, saturation (out of the linear range) and quenching. The excitation and emission wavelengths need to be determined or known a priori in order to apply this method; the method should also account for potential interferences from other fluorophores present.

For elemental composition and material identification, ICP-MS and ICP-OES offer the highest specificity and lowest limits of detection. ICP-OES, with simultaneous multi-element trace analysis, is broadly applicable to the analysis of complex or naturally derived nano-objects. ICP-MS provides ultratrace analysis of specific element isotopes. Historically, neither method has been used to quantify the important light elements carbon, nitrogen and oxygen, and both methods have spectral or isotopic interferences that limit the detection of specific elements. Detection of carbon isotopes using ICP-MS with a helium collision cell or total carbon using a total organic carbon analyser (requires online

acidification and oxidation to $CO_2$), have recently proved successful in combination with AF4 as previously mentioned. The capacity to detect carbon at single mg/l levels has important and growing application for FFF-based methods, though a number of technical issues (e.g. high carbon backgrounds, inorganic carbon, and matrix effects) can limit or complicate its use.

### 8.5 Off-line analysis (Fraction collection)

Fractions can be collected following separation by AF4 or CF3 and used for off-line analysis. The advantage of fraction collection is a wider range of available detection modalities compared with online detection. Fractions can be collected using an automated fraction collector or by manual collection based on specified elution time intervals. Off-line results can then be matched with the retention time as traced by an appropriate online detector (typically UV-Vis or MALS); in this case, a single responsive wavelength or scattering angle is sufficient to monitor elution.

Off-line analysis includes a wide range of potential methods, and is limited only by the volume and concentration of analyte present after fractionation. The quantity (volume of fraction collected) can be increased, as necessary, by combining fractions from multiple experiments performed under identical conditions. The concentration of analyte can be increased if necessary by centrifugation or filtration of combined fractions.

The most common and useful off-line analysis methods are as follows:

a) Electron microscopy (transmission or scanning) to image fractionated nano-objects and to measure or confirm physical dimensions; requires deposition of material from the fraction onto an appropriate substrate followed by removal of any residual mobile phase. Environmental electron microscopes with specialized cell designs can permit analysis of samples under a gas-controlled environment and in a liquid phase; the magnification or resolution is generally reduced from instruments operated *in vacuo*. A microscope equipped with an energy dispersive spectrometer can also provide semiquantitative or qualitative information on elemental composition of samples.

b) Atomic force microscopy to image or measure height displacement of fractionated nano-objects after deposition onto an appropriate substrate; measurements can be detected after removal of mobile phase (dry) or in presence of mobile phase using a liquid cell.

c) Single particle ICP-MS to measure the mass (or mass based size) of individual nano-objects and their number concentration within each fraction.

d) ICP-MS and ICP-OES to measure the concentration and elemental composition of fractionated nano-objects; this typically does not require pre-digestion, but results can be improved by dissolution in a stable acid medium.

e) UV-Vis-NIR to characterize the optical absorbance properties of fractionated nano-objects; off-line measurement generally provides a broader wavelength range compared with online detectors.

## 9 Qualification, performance criteria and measurement uncertainty

### 9.1 System qualification and quality control

#### 9.1.1 Basic system qualification

Basic system qualification (instrument performance verification) should be conducted using a monodisperse spheroidal test material (preferably a certified reference material or test materials with established traceability to reference materials or the SI) of known composition, uniformity and size, under conditions (including mobile phase composition) appropriate for the stability and testing of said material. The availability of certified reference materials with assigned values based on the same measurement principle and methodology obtained via FFF might be limited. In this case, monodisperse PSL spheres with a stated size coefficient of variation no greater than 5 %, with mean particle size



determined preferably by DLS or electron microscopy, if necessary, can be used for light scattering based detection. The nominal mean diameter should be not smaller than 20 nm and not larger than 200 nm.

The purpose of this qualification is to confirm the proper function of the FFF system under optimal conditions. Measured particle size (DLS, hydrodynamic diameter, $d_H$) should be compared with the known or stated particle size of the test material. For online DLS measurements, the mean $d_H$ obtained by averaging across the eluting peak at FWHM should be used for comparison; the relative standard deviation of the mean should be lower than 5 %.

The response of all detectors should be recorded where appropriate (e.g. UV-Vis absorbance, MALS intensity). Once conditions have been optimized for the test material (i.e. a specified method established), the test material and method can be used in the future to monitor system stability or to identify significant deviations from the initial state that would recommend further investigation. This qualification should be performed after the initial instrument installation and at regular intervals thereafter for quality control purposes. Failure of the qualification indicates a problem with the test material suspension, the preparation of the test sample or mobile phase, or the instrument.

To ensure SI-traceable results, relevant instrument, detector or test parameters should be measured with traceably calibrated tools (e.g. cell temperature, laser wavelength and scattering angle). This is typically performed by the manufacturer during production, prior to delivery or periodically as part of a maintenance programme.

For ICP-MS or ICP-OES detection, a suitable metal containing particle should be used, again preferably a reference material. In this case, the purpose of qualification is to demonstrate that all components of the integrated FFF system are performing as per manufacturer's specifications. Data obtained from an optimized method using a suitable reference material can be used in the future to determine if significant changes in the system have occurred. Any significant changes should be investigated or required maintenance performed to regain the previous performance level.

For more routine quality checks performed under conditions relevant to a specific experiment or method, any test material that is sufficiently homogeneous and stable can be used (preferably one similar in nature to the experimental materials under evaluation or the actual experimental material itself) in order to identify significant changes in the system (e.g. contamination or fouling of the membrane in AF4, subtle changes in pressure, loss of detector sensitivity) during the period of the experiments. For this purpose, $R_s$ and $t_R$ should be determined under these conditions, as should any relevant detector response. By maintaining a record of these runs (e.g. in a control chart), any significant change in the system that might influence measurement/method performance can be detected, tracked, and addressed as necessary.

Pressure within the FFF system, in particular the channel pressure, should be monitored on a regular basis, e.g. by noting its value in an instrument logbook at the start of each measurement day. These values should be within the stated operational range of the instrument. Sudden or chronic increases in pressure might indicate a malfunction, a clogged membrane or that something is blocking flow inside the fluid handling components of the system (tubing, valves, flow controllers, etc.). Instruments are typically equipped with high pressure alarms, but a gradual increase in pressure within the operational range may indicate a problem that should be addressed.

### 9.1.2 Focusing performance

For AF4, the proper location of the focusing point during band focusing is critical, which means that the split of the flow rates entering the channel from opposite ends are balanced so that the focusing zone is positioned near the widest point of the trapezoidal channel geometry, typically at 10 % to 15 % of the channel length downstream of the inlet port. The position can be established by measuring at least one of the flow rates (e.g. inlet flow) or by injection of a visible (coloured) analyte under optimal conditions. Visible inspection of the focus band (i.e. uniformity and narrowness) can be used to confirm proper performance with respect to quality of focusing and integrity of the channel. A poorly formed or diffuse band indicates the presence of one or more performance issues, such as the focusing flows not operating properly, a leak along the channel edge, or the focusing point located too far from the point of injection. Lack of acceptable focus performance should be addressed by adjustment of the focus flows,

replacement of the membrane, or reseating of the channel. Failure to obtain a stable uniform focused band after applying these corrective actions might require valve or flow control maintenance or lastly vendor intervention. For the purpose of visual inspection of the focus band, blue dextran, a high molar mass glucose polymer containing a covalently bound blue dye, in a mobile phase of deionized water, has frequently been used, but other colour-producing molecular species or nano-objects can also serve this purpose so long as they do not interact with the membrane and other surfaces. It is also possible to use a low molar mass species such as bromophenol blue, which is small enough to penetrate the membrane and underlying frit and thus easily flushed out of the channel via the membrane after focusing[56].

Current commercial systems, if properly maintained in accordance with the manufacturer's recommendations, do not require frequent experimental confirmation of the focusing band quality. Periodic quality checks of the focusing band quality are recommended (e.g. as part of the Operation Qualification during an annual preventive maintenance), and should also be conducted if performance issues are observed.

### 9.1.3 Flow rate of the carrier liquid

Flow rates introduced or exiting the fractionation channel should be known with a maximum relative standard uncertainty of 2 %. In case of AF4, the rates during inlet flow, focus flow and sample injection flow (if applied) should be qualified individually. If the carrier flow path is changed in each mode, the flow rates should be measured in each mode. In the case of CF3, potential carrier liquid leak in the rotation axis should be considered. In this case, the rate of carrier flow downstream of the rotation axis seal should be measured. Checking the pump flow rate and back pressure at the same position is also recommended. These qualifications should be performed by the manufacturer prior to or during installation. The manufacturer can provide periodic qualifications to ensure proper continuing performance of the system. Follow the manufacturer's recommendations for ensuring correct operational performance.

### 9.1.4 Separation field

To qualify the separation field, the characterizing parameter of the field should be qualified. For AF4, accuracy of the cross flow rate should be measured or calculated. In this case, the relative standard uncertainty of the flow rate should not exceed 1 %. For CF3, accuracy and repeatability of the rotation frequency of the channel should be measured. In this case, the relative standard deviation of the rotation frequency should be 2 % or lower.

## 9.2 Method performance criteria

### 9.2.1 Recovery

Analyte recovery should be evaluated for most applications, unless the experimental objective is qualitative in nature (e.g. how many components are present in the sample). Analyte recovery is calculated as shown below:

$$\text{Analyte recovery}, R(\%) = \frac{A_S}{A_D} \times 100 \tag{16}$$

where

$A_S$ is the peak area of the eluted sample; and

$A_D$ is the peak area of sample directly injected into the detctor

For the denominator in Formula (16), a more convenient alternative to direct injection is to repeat the elution programme used to obtain the numerator, but without the application of the fractionation (cross) field (as described in 6.2). It is also possible to inject the sample without both focusing and cross flow, which should yield a result that is similar to direct injection into the detector. As a general rule, $R$(%) should be ≥ 70. For high performance separations, $R$(%) ≥ 90 would be considered acceptable.

Recovery should be evaluated and reported, whenever it is relevant to the objective of the analysis or the development of a method. The method of recovery determination should be reported.

### 9.2.2 Selectivity

If test materials of similar composition to the target nano-object are available over a range of sizes, evaluate size selectivity of the method according to:

$$\text{Selectivity}, S_{\mathrm{d}} = \left|\frac{d\log(t_{\mathrm{R}})}{d\log(d)}\right| = \left|\frac{d\log(D)}{d\log(R)}\right| \tag{17}$$

where

$d$ is the known or independently measured particle diameter of each test material;

$t_{\mathrm{R}}$ is the retention time;

$D$ is the diffusion coefficient; and

$R$ is the retention ratio.

The equivalent diffusion-based selectivity is shown on the far right of Formula (17). For symmetrical flow FFF, the maximum value of $S_d$ is 1, while for AF4 $S_d$ can exceed unity. For CF3 the maximum value is 3; CF3 offers relatively high size selectivity (and therefore fractionation power) for materials that are appropriate for CF3 analysis[57].

### 9.2.3 Retention

The retention ratio, $R$, assesses the efficiency of the fractionation method, where efficiency is defined as the balance between speed of analysis and resolution. The magnitude of this ratio also reflects the strength of interaction between the applied field and the analyte. For AF4, the optimal range is approximately $0{,}03 \leq R \leq 0{,}2$ (valid when $t_R >> t_0$). The retention ratio should be evaluated and reported for all target analytes (see Formula (3)).

### 9.2.4 Resolution

The resolution factor or fractionation power assesses the performance of a method with respect to its capacity to separate two adjacent eluting peaks representing two monodisperse populations. Where multiple eluting components are present, the ratio of the difference in retention time, $\Delta t_R$ (for each analyte pair), to the average peak width, $W_{\text{avg}} = \frac{w_1 + w_2}{2}$ should be evaluated and reported. For a Gaussian peak, $w = 4\sigma$, where $\sigma$ is the standard deviation and the full width at half maximum, FWHM = 2,354σ. Therefore:

$$\text{Resolution factor}, R_{\mathrm{s}} = 1{,}18\frac{\Delta t_{\mathrm{R}}}{\langle w_{\mathrm{FWHM}}\rangle} \tag{18}$$

where the angle brackets indicate an average.

A resolution factor, $R_S$, less than unity indicates substantial overlap, while a value of 1,5 indicates fully resolved peaks. If peaks are clearly separated in time, with no apparent overlap, it is not necessary to calculate $R_S$; simply reporting $R_S > 1{,}5$ is sufficient.

## 9.3 Method precision and measurement uncertainty

Method precision depends on the nature of the sample and the analytical methods utilized, and should be evaluated under repeatability conditions at a minimum (i.e. same analyst, same instrument, same location, same method, and same conditions, over short time intervals). Evaluation of intermediate

precision (i.e. same location, and replicate measurements on the same or similar analyte over an extended period of time and perhaps including other changes) is highly recommended to achieve a higher level of validation. Evaluation of reproducibility (i.e. independent test results obtained using the same method on the same or similar analyte in different test facilities with different analysts using different equipment) is necessary when the objective is to achieve the highest level of validation (e.g. for regulatory purposes).

Evaluate precision by repeating the fractionation experiment under repeatability conditions. Determine the mean and relative standard deviation for $t_R$, $R$ and all relevant measurands (e.g. hydrodynamic size, radius of gyration). The relative standard uncertainty should not exceed 5 % for $t_R$ or $R$. If conditioning of the channel is required to obtain consistent results, data acquired during conditioning should be excluded from the precision evaluation.

If possible, an uncertainty budget should be constructed[58], with the objective of evaluating the combined standard uncertainty for an FFF method as described in, for instance, ISO/IEC Guide 98-3 or ISO 21748.

The appropriate evaluation of uncertainties for routine online analytical measurements, including determination of $t_R$ or nano-object size based on $t_R$ calibration, should include, if possible, contributions from the following sources: repeatability (precision under repeatability conditions), intermediate precision, calibration and other detector-related factors (e.g. baseline stability, limit of detection), and band broadening effects. The latter may be quantified using highly uniform test materials under the same or similar measurement conditions. Refer to ISO/IEC Guide 98-3.

# 10 General procedures for measurement of samples

## 10.1 General

Since the principal objective of FFF is to separate constituents for online or off-line analysis, or for further application or processing, a suitable FFF method should be developed to separate the size-, density- or mass-distributed nano-object components in a sample. Although method development and validation are central to successful separation, general measurement procedures can be specified for both AF4 and CF3. 10.2 to 10.4 provide such general procedures applicable to any AF4 or CF3 method. To properly implement these general procedures, Clauses 6 and 7 provide guidance on key aspects of method development and validation for AF4 and CF3, respectively, while Clause 9 provides guidance and procedures for instrument qualification and determination of performance criteria such as recovery. Refer to these clauses for details regarding each step in the general procedures.

FFF can also be used as a preparative technique, for instance to obtain a narrow size band within a polydisperse material for other purposes. Preparative methods are not within the scope of this document, but generally follow the basic premises set out herein with respect to optimizing and defining the separation process.

## 10.2 Calibration of retention time for online size analysis

### 10.2.1 Calibration of the AF4 channel

If desired or required, effective thickness of the channel should be determined as follows, assuming that system qualification (see 9.1) has already been performed.

1. Choose a suitable sample containing a uniform size population that can be well separated in the channel to be used, e.g. polystyrene latex spheres in a size range of 20 nm to 200 nm diameter. Size or diffusion coefficient should either be known in advance or measured online using DLS. Use FFF theory to calculate flow conditions to elute the sample well separated from the void peak.

2. Inject sample of known volume into the channel containing mobile phase; mobile phase should be a simple formulation such as 1 mmol/l NaCl to 5 mmol/l NaCl or other appropriate simple monovalent salt.

