



HAL
open science

Development of a candidate reference measurement procedure by ID-LC-MS/MS for total tau protein measurement in cerebrospinal fluid (CSF)

Chiara Giangrande, Hélène Vaneeckhoutte, Amandine Boeuf, Béatrice Lalere, Christophe Hirtz, Sylvain Lehmann, Milena Quaglia, Vincent Delatour

► To cite this version:

Chiara Giangrande, Hélène Vaneeckhoutte, Amandine Boeuf, Béatrice Lalere, Christophe Hirtz, et al.. Development of a candidate reference measurement procedure by ID-LC-MS/MS for total tau protein measurement in cerebrospinal fluid (CSF). *Clinical Chemistry and Laboratory Medicine*, 2023, 61 (7), pp.1235-1244. 10.1515/cclm-2022-1250 . hal-04598085

HAL Id: hal-04598085

<https://hal.science/hal-04598085>

Submitted on 3 Jun 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

Chiara Giangrande*, H el ene Vaneekhoutte, Amandine Boeuf, B eatrice Lalere, Christophe Hirtz, Sylvain Lehmann, Milena Quaglia and Vincent Delatour

Development of a candidate reference measurement procedure by ID-LC-MS/MS for total tau protein measurement in cerebrospinal fluid (CSF)

<https://doi.org/10.1515/cclm-2022-1250>

Received December 8, 2022; accepted February 2, 2023;

published online February 24, 2023

Abstract

Objectives: In clinical practice, tau protein measurement generally relies on immunoassays (IAs), whose major drawback is the lack of results comparability due to differences in selectivity and/or calibration. This underlines the importance of establishing a traceability chain for total tau (t-tau) measurements. The objective of this work is to develop a higher order candidate reference measurement procedure (RMP) for the absolute quantification of t-tau in cerebrospinal fluid (CSF).

Methods: To calibrate the candidate RMP and establish metrological traceability to the SI units, a primary calibrator consisting in a highly purified recombinant protein was sourced. Its purity was evaluated by liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS) and the protein mass fraction in solution was certified by amino acid analysis (AAA). An isotopically-labelled homologue was obtained to develop a candidate RMP by isotope dilution mass spectrometry (IDMS) for t-tau absolute quantification in CSF. Calibration blends and quality control (QC) materials were gravimetrically prepared and subjected to the same preparation workflow as CSF samples, followed by

LC-HRMS analysis in Parallel Reaction Monitoring (PRM) mode.

Results: A primary calibrator has been developed and an IDMS candidate RMP has been validated for CSF t-tau. The candidate RMP was used to certify t-tau concentration in three pools of CSF (low, medium, high).

Conclusions: The candidate RMP will pave the road towards global standardization of CSF t-tau measurements. Together with commutable Certified Reference Materials (CRMs), it will allow evaluating and improving the accuracy and comparability of results provided by IAs.

Keywords: CSF; ID LC-MS/MS; neurodegenerative diseases; reference method; SI-traceability; t-tau.

Introduction

Early and accurate diagnosis of neurodegeneration relies on different techniques, including cognitive tests, imaging and biomarkers measurement. However, the coexistence of coincidental symptoms for different neurodegenerative diseases makes the differential diagnosis challenging.

Tau is a soluble protein involved in the stabilization of microtubules and identified as the major constituent of neurofibrillary tangles, thus representing the main hallmark of a collection of neurodegenerative disorders also known as “tauopathies” [1], the most common of that being Alzheimer’s disease (AD).

t-Tau and phosphorylated-tau (p-tau) concentrations in CSF are usually employed for patients’ diagnosis and followup because they are correlated with disease progression. However, these two biomarkers can diverge in other pathological states, making t-tau a less specific biomarker for neuronal damage, whereas an increased level of p-tau can be specifically associated to the formation of paired helical filaments and thus to AD. Nevertheless, t-tau remains a key biomarker to define neurodegenerative processes, providing important information on the extent of the cognitive decline and closely correlating to the symptoms.

Chiara Giangrande and Helene Vaneekhoutte contributed equally to this work.

***Corresponding author: Chiara Giangrande**, Department of Biomedical and Organic Chemistry, Laboratoire National de M etrologie et d’Essais (LNE), Paris, France, Phone: +33 (0) 140433971, E-mail: chiara.giangrande@lne.fr

H el ene Vaneekhoutte, Amandine Boeuf, B eatrice Lalere and Vincent Delatour, Department of Biomedical and Organic Chemistry, Laboratoire National de M etrologie et d’Essais (LNE), Paris, France

Christophe Hirtz and Sylvain Lehmann, LBPC-PPC, Univ Montpellier, IRMB CHU Montpellier, INM INSERM, Montpellier, France. <https://orcid.org/0000-0001-6117-562X> (S. Lehmann)

Milena Quaglia, LGC, Teddington, UK

This is the reason why it has been included as a marker of neurodegeneration/neuronal injury (N) in the AT(N) biomarker system (A=amyloid deposition, T=pathologic tau, N=neurodegeneration), an unbiased framework established by the National Institute on Aging-Alzheimer's Association (NIA-AA), to serve as recommendations for grouping biomarkers and stratifying patients on the basis of biomarker profiles [2]. In this context, Delmotte et al. pointed out the importance of establishing cut-off value for t-tau since the AT(N) diagnostic criteria are dependent on the assay used for CSF fluid biomarker quantification and on the exact thresholds previously established [3]. The need for harmonization of CSF biomarkers in AD diagnosis was underlined by several authors in a review of the pre-analytical protocols, where an important heterogeneity in the definition of threshold values was reported for most of the AD markers including t-tau [4]. Although p-tau is a more specific biomarker in AD, t-tau remains an important biomarker of neuronal injury that is used to establish correlations with other neurodegenerative processes associated with dementia. This includes prion diseases [5, 6], where affected patients display a significant increase in CSF t-tau level that makes it suitable as diagnostic and prognostic marker of the Creutzfeldt-Jakob disease (CJD), often in combination with other clinically relevant biomarkers, e.g. neurofilament light chain [7].

In clinical practice, the measurement of t-tau and of the other neurodegenerative diseases biomarkers relies on IAs based on different antibodies, pre-analytical and analytical workflows, often leading to large inter-laboratory differences [8]. Several authors underlined the need for CRMs to reduce variability among different assays [9–11]. A source of measurement variability lies in the high structural heterogeneity of tau protein, thereby leading to differences in the selectivity of IAs [12].

Mass spectrometry plays an important role in the development of reference measurement procedures for value assignment of secondary CRMs that can be used to harmonize measurement results through common calibration to internationally agreed standards. Several LC-MS methods have been developed for the quantitation of CSF t-tau, relying either on single reaction monitoring (SRM) using triple quadrupole (TQ) equipments or PRM on HRMS systems, and different sample preparation procedures [13–18]. However, there is to date no protein-based primary calibrator available nor any higher order RMP for t-tau absolute quantification in CSF meeting the requirements of the ISO 15193 standard [19]. Here, we developed a candidate RMP by isotope dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS), which is not intended to be used as a clinical mass spectrometry proteomics method,

but as a RMP to standardize *in vitro* diagnostics (IVD) devices via an unbroken traceability chain. The method exploits the potential of PRM to detect a particular peptide to be used for the quantification of t-tau in CSF by using a recombinant protein as primary calibrator and its isotopically-labelled homologue as internal standard. The uncertainty of the method was evaluated on three CSF pools at different t-tau concentrations that might be used as secondary CRMs to harmonise results obtained with different analytical platforms with the final aim of standardizing diagnostic cut-offs for the different neurodegenerative diseases.

Materials and methods

Chemical and reagents

Ultra Performance liquid chromatography/mass spectrometry (UPLC/MS) grade acetonitrile (AcN), methanol (MeOH), formic acid 99% (FA) and trifluoroacetic acid (TFA) were from Biosolve (Dieuze, France). Perchloric acid (PCA) was from Fluka (Buchs, Switzerland).

A batch of recombinant tau-441 (hereafter referred to as r-tau) and a batch of its ¹³C and ¹⁵N labelled counterpart (r-tau*) were produced by Promise Advanced Proteomics (Grenoble, France). Three pools of frozen CSF were provided by the university hospital of Montpellier (LBPC-PPC, France). Artificial CSF (aCSF) consisted in a human serum (ref. H4522) from Sigma-Aldrich (Saint Quentin Fallavier, France) diluted 200 times in deionized water obtained from a Milli-Q purification system (Millipore, Guyancourt, France). During the sample preparation, Oasis® HLB μ Elution Plates 30 μ m (Waters, Saint-Quentin-en-Yvelines, France) and trypsin gold (mass spectrometry grade) from Promega (Charbonnières-Bains, France) were used.

For the amino acid quantification, CRMs from the National Metrology Institute of Japan (NMIJ, Tsukuba, Japan) were used for alanine (Ala), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), valine (Val) and proline (Pro) (CRM 6011-a, 6012-a, 6013-a, 6014-a, 6015-a, 6016-a). Their isotopically labelled counterparts were purchased from Cambridge Isotope Laboratories (Mass., USA). An amino acid solution (NIST SRM 2389a) from the National Institute of Standards and Technology (NIST, Md., USA) was used as a QC. Hydrolysis was performed by using 6N hydrochloric acid (HCl) from Sigma-Aldrich (Saint Quentin Fallavier, France).

Characterisation of the primary calibrator

The r-tau primary calibrator was purified by preparative high performance liquid chromatography (HPLC) using three orthogonal techniques: ion exchange, reverse-phase, and size-exclusion chromatography. Purity of the primary calibrator was then characterized by LC-HRMS on an Orbitrap QExactive Focus coupled to a Dionex UHPLC UltiMate® 3000 system (Thermo Scientific, Courtaboeuf, France). LC buffers consisted of 0.1% FA in water (A) and 0.1% FA in AcN (B). The LC-MS profile of 5 pmol of the intact r-tau was obtained by using a BioBasic® 4 C-4 column (300 Å, 5 μ m, 1 mm \times 150 mm, Thermo Scientific) at 45 C with a LC gradient from 20 to 80% of B in 80 min at 50 μ L/min. MS data were acquired at a resolution of 35,000. The LC-MS system was controlled by the XCalibur software (Thermo

Scientific) and data deconvolution was performed by using the Biopharma Finder software (Thermo Scientific).

The protein mass fraction was determined by AAA as reported in [20]. The mass fraction of the r-tau primary calibrator was certified by computing the average results from all six samples from all six amino acid results (Ala, Leu, Ile, Phe, Pro, Val). The uncertainty associated to the amino acid mass fraction was calculated according to the guides to the expression of uncertainty in measurement (GUM) approach [21] by combining uncertainties associated with i) weighings performed on gravimetrically spiked labelled amino acids in the sample solution, ii) weighings performed on gravimetrically prepared calibrators, iii) purity of the amino acid calibrators, iv) mass ratio derived from the calibration regression model, and v) precision of measurement results obtained from different hydrolysis replicates.

t-Tau Quantification in CSF by IA

The Lumipulse® G1200 instrument from Fujirebio (Tokyo, Japan) was used to estimate t-tau concentration in pools of CSF. The Innost hTAU-Ag uses established monoclonal antibodies (AT120, HT7 and BT2) targeting the mid domain of the protein thus allowing t-tau quantification. Total tau cartridges, along with their calibrators and QC samples, were used to quantify the three pools used in the present study.

t-Tau Quantification in CSF by LC-MS/MS

Preparation of the calibration blends: All the diluted solutions of r-tau and r-tau* used to prepare the calibration blends were prepared freshly in LoBind tubes (Eppendorf, Montesson, France) on the same day as the samples. Five calibration mixtures were prepared by gravimetrically spiking different amounts of r-tau ranging from ~1.4 to ~7.5 ng/g and a constant amount of ~3.2 ng/g of r-tau* in aCSF. The mass ratio between r-tau and r-tau* ranged from ~0.5 to ~2.0. Blank samples spiked with the internal standard (r-tau*) have been analysed. Seven supplementary solutions were prepared for the lower limit of quantification (LLOQ) determination, all having a ratio r-tau/r-tau* ratio close to one but decreasing concentrations of r-tau starting from the lowest calibration point, as follows: 1.45, 1.0, 0.75, 0.5, 0.3, 0.1 and 0.05 ng/g.

Sample preparation: 500 µL of CSF were gravimetrically spiked with the appropriate volume of r-tau* so as to reach a t-tau/r-tau* ratio close to one. The amount of r-tau* added to perform exact matching isotope dilution was based on an IA quantification performed beforehand on the CSF pools. The sample preparation was performed according to Bros et al. [14], except there was no protein oxidation step. The digestion was performed for 18 h at 37 °C and 500 rpm on a Thermomixer C from Eppendorf (Montesson, France). A time-course experiment was carried out to determine the optimal digestion time. The digestion was stopped with 5 µL of 10% FA solution before centrifugation and transfer to micro vials with inserts in order to be analysed by LC-MS/MS.

LC-MS analysis: The analyses were performed on the same system used for the characterization of the primary calibrator. LC separation was carried out on an Acclaim® PepMap®100 C18 column (250 × 1 mm, 1.7 µm, Thermo Scientific) heated at 60 °C. Mobile phases consisted in (A) 0.1% FA in water and (B) 0.1% FA in MeOH. Elution was performed with a

linear gradient from 2 to 50% of solvent B in 40 min at 40 µL/min. The column was then washed with 90% B for 4 min at 100 µL/min before being re-equilibrated during 14 min with 2% B. MS detection was performed with an electrospray (ESI) source in positive mode with a 4.20 kV spray voltage and a capillary temperature of 250 °C. The analyses were run in PRM mode, with a resolution of 35,000 and an isolation window of 1.5 *m/z*. Eleven peptides were monitored (see the list of peptides and transitions in the Table S1 of the Supplementary Material), and the collision energy was optimized for each peptide. Only the peptide 156–163 (GAAPPGQK) was used as a quantifier, with three transitions summed: 526.2984+ (y5); 299.1714++ (y6) and 263.6528++ (y5).

Data treatment was performed by Skyline (version 20.1.0.31).

Method validation: The analytical performance for t-tau quantification in CSF using a protein-based calibration approach was validated on matrix-matched materials according to the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) guidelines [22, 23]. The criteria for method validation include linearity, selectivity, trueness, precision, autosampler stability, LLOQ, and carryover.

Measurement equation and uncertainty estimation: A 5-point linear calibration model was constructed by plotting the ratio between the amounts of substance of r-tau and r-tau* of the calibration blends ($Q_{\text{calibrator}}$, obtained from weighing) to the ratio between the peak areas of the peptide 156–163 (GAAPPGQK) and its labelled counterpart (GAAPPGQK*) ($R_{\text{calibrator}}$). The measurement equation and uncertainty estimation are developed in the Document S1 of the Supplementary Material.

Results

The development of the candidate RMP for t-tau quantification in CSF was designed to ensure results traceability to the SI units through the use of a high quality protein primary calibrator and IDMS. The method was validated by using fit for purpose QC samples, consisting in the primary calibrator spiked in aCSF, as well as three CSF pool samples to be used as secondary CRMs.

Characterization and quantification of tau primary calibrator

An SI-traceable primary calibrator, consisting in a protein-based reference material corresponding to the recombinant form of tau-441 (the longest sequence for tau protein), was developed. An aliquot of the stock solution was analysed by LC-MS to assess the purity of the candidate primary calibrator and to evaluate the necessity for correcting the mass fraction obtained by AAA [24]. The total ion chromatogram (TIC) is presented in Figure 1A with the MS spectrum corresponding to the main chromatographic peak at 23.07 min

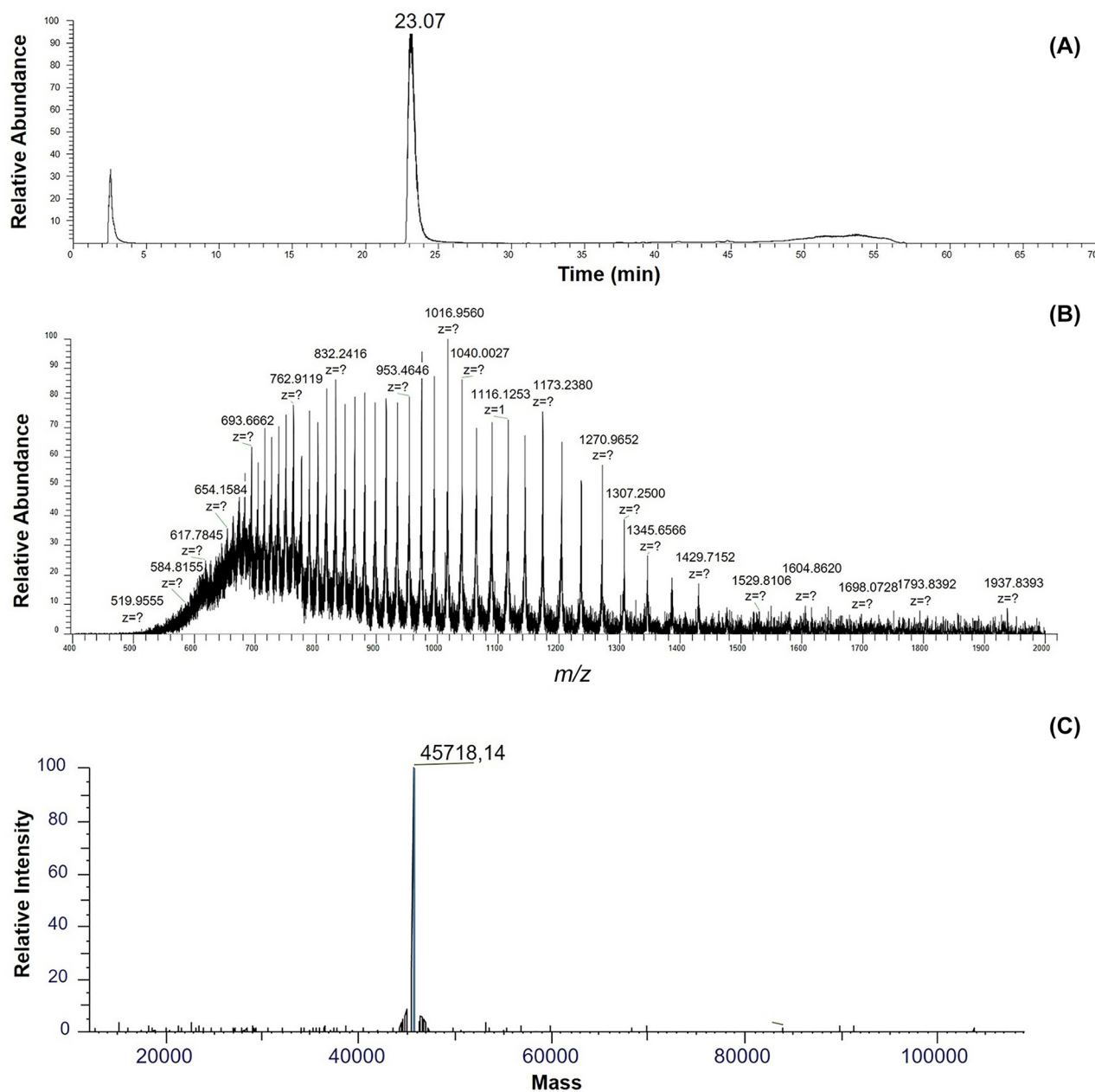


Figure 1: Characterisation of the r-tau primary calibrator. (A) Total ion chromatogram (TIC) obtained by injecting 5 pmol of r-tau primary calibrator. (B) Multi-charged mass spectrum corresponding to the peak at RT 23.3 min. (C) Deconvolution of the MS signal.

in Figure 1B. The deconvoluted MS signal fits to an average mass of 45,718.14 Da, that can be attributed to the sequence of tau-441 lacking the n-terminal methionine (theoretical mass: 45,718.39) with a final Δ_{mass} of -5.5 ppm (Figure 1C). No major impurity could be detected.

The protein content of the stock solution was SI-traceable quantified by gas-phase AAA. The results for each of the six stable amino acids are shown in Figure 2. A mass fraction of 232.1 ± 9.9 $\mu\text{g/g}$ ($k=2$) was certified for the stock solution of r-tau.

Quantification of t-tau in CSF

r-Tau was used as primary calibrator to quantify t-tau in CSF by using a bottom-up approach (Figure 3). A digestion time of 18 h was selected after a time-course analysis (see Figure S1, Supplementary material). The use of r-tau* as an internal standard allowed the accurate determination of t-tau concentration in CSF by determining the ratio between the GAAPPGQK peptide and its labelled counterpart. The spiking of the internal standard at the very beginning of the sample

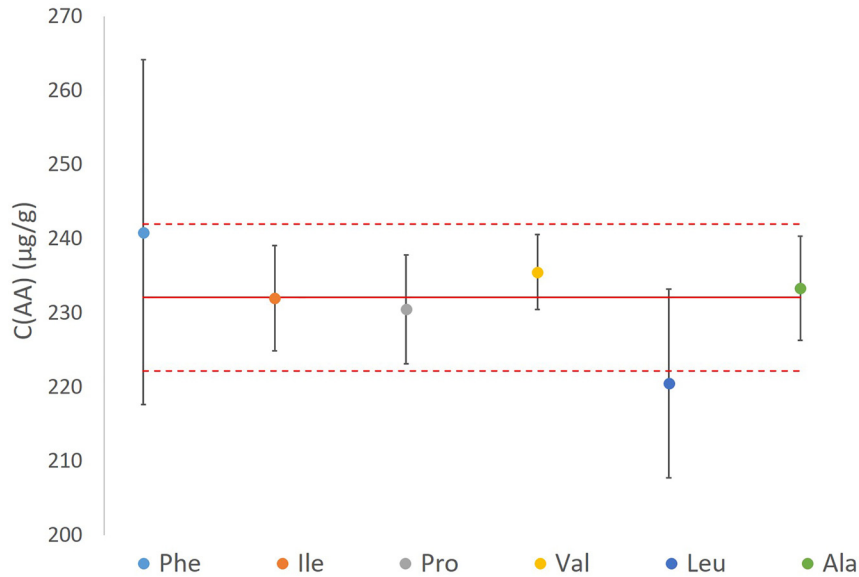


Figure 2: Results from the quantification of the r-tau stock solution by amino acids analysis. Each point represents the concentration of r-tau in $\mu\text{g/g}$ calculated for each amino acid (F, I, P, V, L, A), from six independent experiments. Error bars correspond to expanded uncertainties ($k=2$).

preparation workflow allowed to minimise the variations associated to the different steps of sample preparation, thus compensating for incomplete digestion and material loss [20]. Also, calibration blends and CSF samples were subjected to the same preparation.

t-tau Quantification was based on the peptide 156–163, whose identification was assured by PRM LC-MS/MS. Figure 4A depicts the extracted ion chromatograms of the unlabelled peptide 156–163 and its labelled form, showing the coelution of the two peptides at 5.6 min. The figure also shows the PRM MS/MS signal of the peptide (transition y_5^+ , y_6^{++} and y_5^{++}) obtained by the digestion of the recombinant protein spiked in aCSF (Figure 4B).

Method validation

Linearity

The regression model was linear over the range 1.4–7.5 ng/g with a Pearson regression coefficient >0.9998 obtained on six

different calibration curves gravimetrically prepared on six different weeks during the validation process. Figure 5 represents the mean calibration curve with error bars on the Q value (corresponding to the ratio among the r-tau and r-tau* obtained by gravimetry) and on the R value (area ratios of the unlabelled and labelled peptide 156–163).

Selectivity and specificity

Selectivity and specificity were evaluated by the analysis of blank samples (aCSF) spiked with the internal standard (r-tau*): no signal for 156–163 peptide was detected at the expected retention time by using the PRM-approach, excluding the presence of potential interfering substances in aCSF.

Carryover

No carryover was observed for 156–163 peptide in the blank runs after calibrators, QCs and CSF samples.

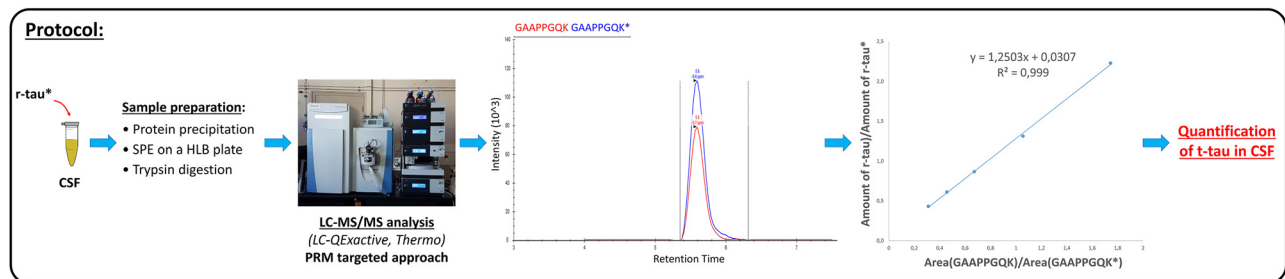


Figure 3: Sample preparation workflow for SI-traceable quantification of t-tau in CSF by an ID-LC-MS/MS method.

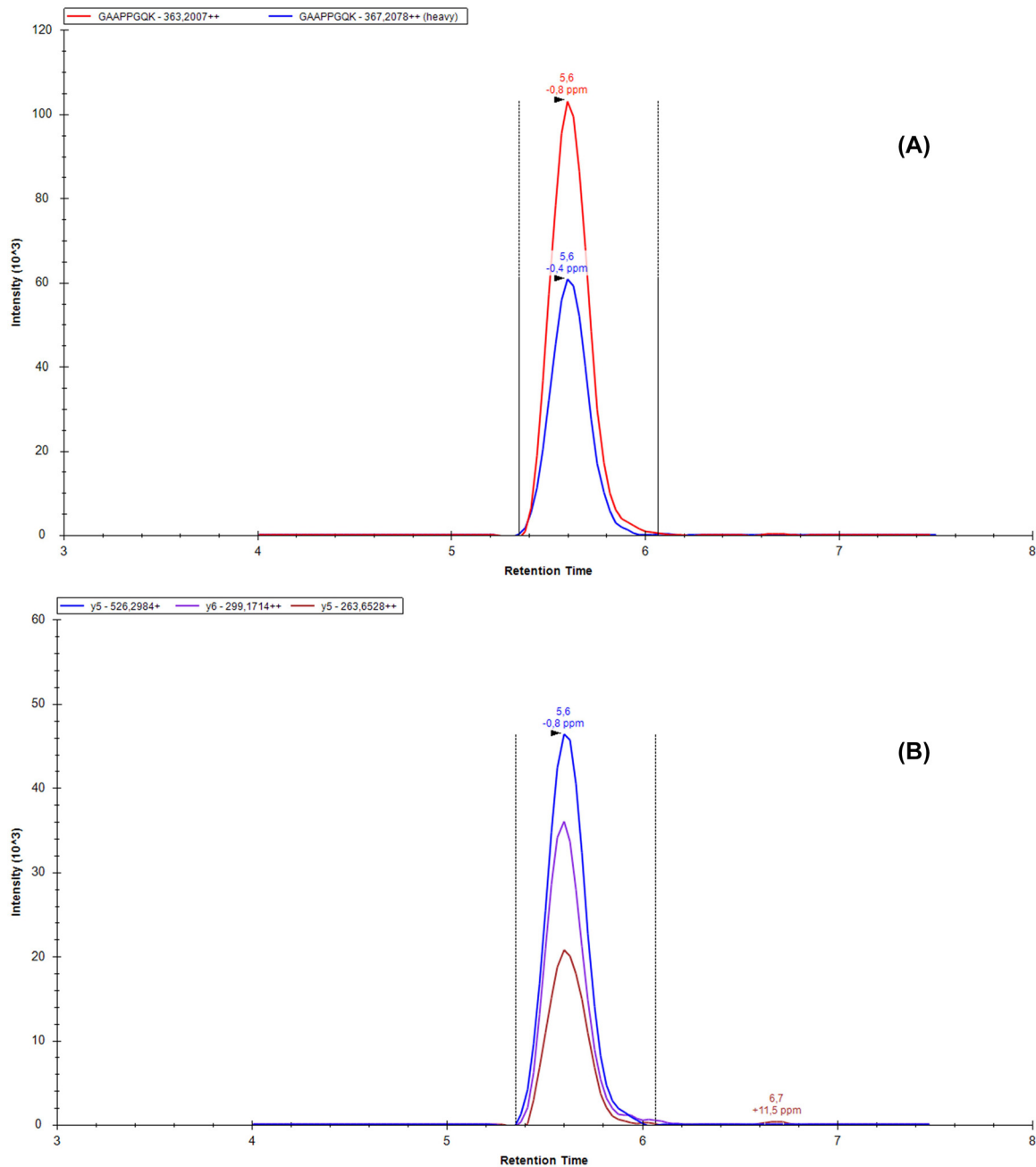


Figure 4: PRM signal of the 156–163 peptide for t-tau quantification in CSF. (A) Extracted ion chromatogram obtained by measuring aCSF gravimetrically spiked with r-tau and r-tau* and showing the co-elution of the two peptides. (B) Extracted ion chromatogram of the PRM signal of the peptide 156–163 showing the transitions used for t-tau quantification.

Autosampler stability

One of the QC samples was injected twice 14 days apart to evaluate the autosampler stability at +7 °C. The bias from the initial concentration was <20%.

LLOQ

To determine the LLOQ of the method, seven solutions having a ratio r-tau/r-tau* around one were prepared in aCSF starting from the r-tau concentration of the lowest

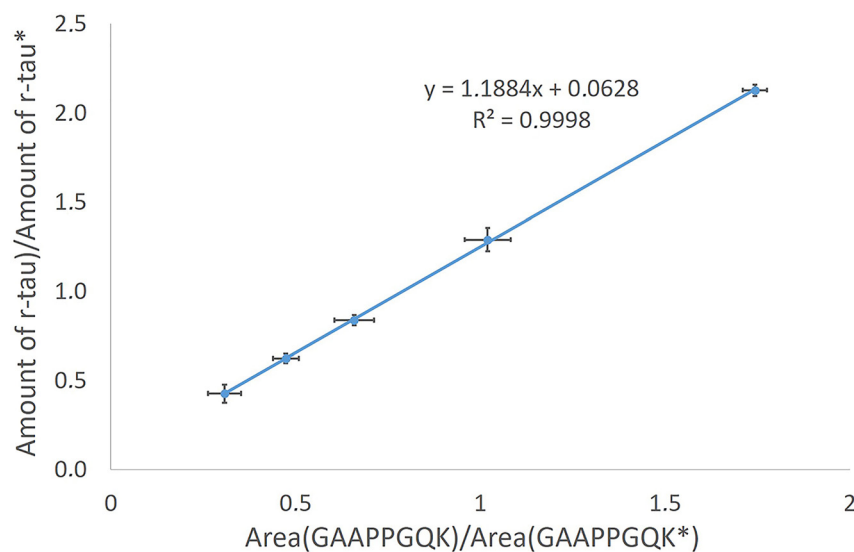


Figure 5: Mean calibration curve obtained from six independent sets of calibration blends. On each point, the error bars show the variability observed on Q and the area ratios during the experiments.

calibration point (1.45 ng/g) down to 50 pg/g. The limit of quantification was fixed at 0.5 ng/g in order to have the peptide detected and quantified with a coefficient of variation among three individual injections <10%.

Trueness and precision

Trueness was estimated using two different approaches. The first approach consisted in treating each calibration blend as an unknown sample in order to determine the trueness of its back-calculated concentration. The relative deviation between the value obtained from the gravimetric preparation (theoretical value based on the AAA on the recombinant protein) and the value obtained from the calibration curve (experimental value) was determined for each calibration blend. The mean bias for all five calibration blends was under 4% (the acceptance criteria from the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines was fixed at 20% at the LLOQ and 15% for the other levels). The results are shown in Table S2 of the Supplementary material. The second approach consisted in evaluating recovery using six independent QC samples prepared by spiking r-tau and r-tau* in aCSF with a ratio r-tau/r-tau* close to 1. On the six samples, the recovery, calculated as the concentration obtained with the calibration curve on the concentration obtained by gravimetric dilution $\times 100$, was of 98.7% in average (SD=3.3, n=6) with the inter-day bias ranging between -6% and +2% of the experimentally determined mean concentration.

The value of inter-day precision calculated as %CV of the results obtained using the five calibration blends was dependent on the concentration level and ranged from

1.1% for the first calibration point (7.4 ng/g) to 4.4% for the last one (1.4 ng/g) (see Table S3, Supplementary Material for details). For the six QC samples, it was 3.3%. Precision was also evaluated on the three CSF pools intended to be used as CRMs. The intra-day precision was 7.6% for the low pool, 3.7% for the medium pool and 8.8% for the high pool. The inter-day precision was estimated at 6.4% for the low pool, 3.3% for the medium pool and 5.1% for the high pool.

Application to the measurement of CSF samples

In order to assess the applicability of the RMP to CSF samples, the developed method was applied to three pools of frozen CSF corresponding to three different concentration ranges (low, medium, high). The analysis were carried out in triplicates on three different weeks. Individual results are illustrated in Supplementary Material (Table S3) while Figure 6 compares the concentrations obtained by ID LC-MS/MS and the concentrations measured by IA, pointing out considerable differences among the two methods. The ID-LC-MS/MS method measures a concentration 9.3-fold higher for the pool low, 6.7-fold higher for the pool medium and 5.7-fold higher for the pool high. An investigation was conducted to identify the main sources of uncertainty of the RMP. As illustrated in Figure 7A, precision represents an important contribution in the uncertainty budget (~20–50%). The most important source of uncertainty was the uncertainty associated with calibration blends preparation, which is in turn due for around 70% to the determination of the concentration of the primary calibrator (Figure 7B).

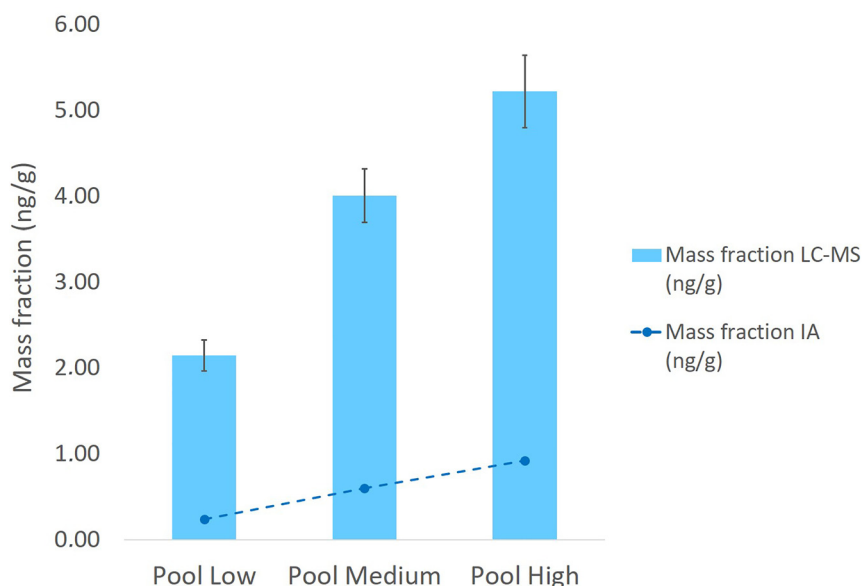


Figure 6: t-Tau concentration of the three CSF pools determined by using the ID LC-MS/MS (column chart) compared to the concentrations determined by immunoassay (dots). Expanded uncertainty is represented by error bars.

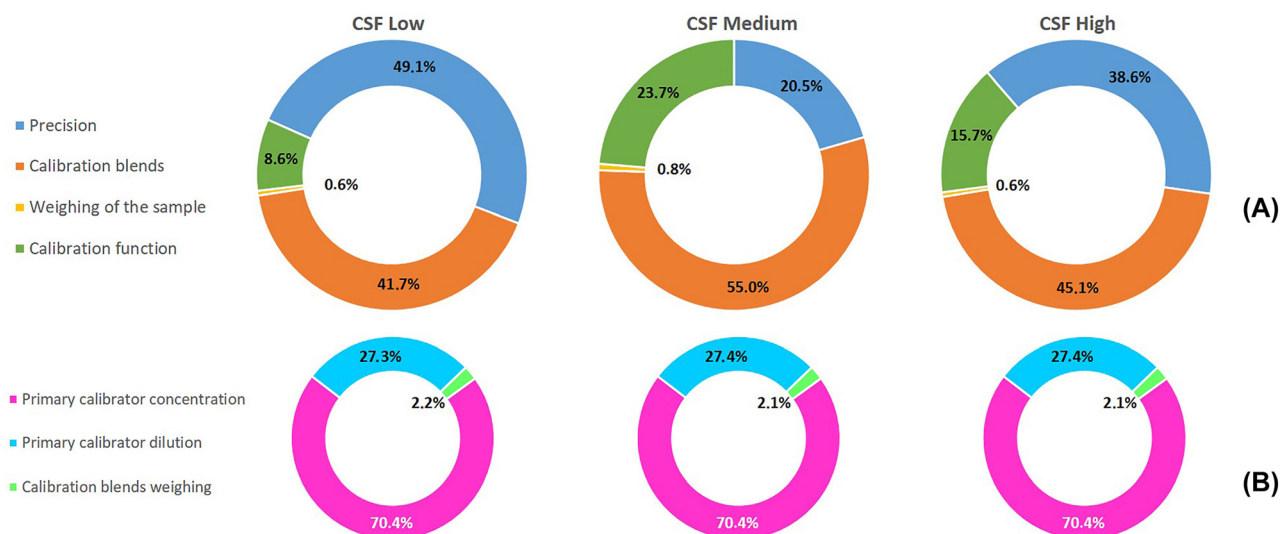


Figure 7: (A) Uncertainty breakdown on the three CSF pools. (B) Breakdown of the standard preparation component.

Discussion

For the first time, a higher order candidate RMP for the SI-traceable quantification of t-tau in CSF was developed and validated through an ID LC-MS/MS approach and the use of a SI-traceable primary calibrator fully characterized by AAA and impurity profile. No major impurities justifying to correct the amino acids mass fraction were detected, and this was confirmed by the agreement among the mass fraction values obtained from different amino acids. However, the presence of minor impurities difficult to detect due to the complexity of intact protein MS spectra cannot be excluded, but this was already taken into account in the final

uncertainty budget with the uncertainty between the results from the different amino acids.

Figure S2 shows the full chromatogram of the 11 peptides. Although all these peptides were monitored by the LC-MS method, only the 156–163 peptide (GAAPPGQK) was used for t-tau quantification. It is quite unusual for a large protein to be quantified by only one peptide but in this case, the choice was justified by the fact that this peptide is not subjected to post-translational modifications [14, 15] and gives a linear calibration curve (Figure S3, Supplementary Material). Moreover, this peptide is the only one exhibiting a comparable PRM signal for the endogenous peptide obtained from CSF digestion and the peptide obtained by r-tau

digestion (Figure S4, Supplementary Material), confirming the unsuitable behaviour of other peptides in quantifying CSF endogenous t-tau. The use of a PRM approach allowed to get rid of the presence of interfering compounds thanks to the specificity of the chosen transitions for the quantifier and its labelled form and to the coelution of the endogenous peptide and the internal standard.

The RMP was validated with a LLOQ of 0.5 ng/g, which is in agreement with [16] and covers t-tau concentration in the CSF of healthy donors (data not shown). Recovery studies carried out to evaluate the performance of the analytical method allowed the detection of occasional instability problems on the standard solutions. They were solved by modifying the sample preparation workflow by preparing all the calibration, QC and sample materials on the same day. The ID-LC-MS/MS method gave higher results than IA for all CSF samples. This result is in agreement with other studies [13, 14], showing a good correlation between IA and LC-MS methods, but higher concentrations for the latter. That can be explained by a calibration bias and/or poor antibody affinity in IA. These discrepancies could be due to the use of different materials in the calibration of IAs (not SI-traceable). In addition to this, very often, the calibration material for IAs is constituted by peptide epitopes, whereas here we have developed a method using protein material, which better mimics the behaviour of the endogenous protein. Another reason for the different affinity could be ascribed to the involvement of structural aspects in the binding in a way that only particular conformers could be able to bind to the antibodies. Our antibody-free method is not influenced by structural aspects and gives a more realistic value for t-tau content in CSF. The discrepancies between IAs and the RMP could be solved by performing a virtual recalibration of IVD kits with SI-traceable certified reference materials (CRMs). For all the samples, relative expanded uncertainty ($k=2$) was under 10% (Table S3, Supplementary Material). Given the size of tau protein and the low concentrations in CSF, this can be considered suitable for the value assignment of candidate CRMs to be used for the evaluation of the accuracy of commercial IA. As seen in the Results section, the uncertainty is mostly due to precision and to the determination of the concentration of the primary calibrator. Measurement uncertainties could thus be reduced by increasing the number of replicate analysis on the CSF and the number of hydrolysis replicates in amino acid analysis. Recently, alternative approaches have also shown great potential for the value assignment of primary calibrators for tau protein [25]. However, it is worth-noting that whatever approach is used, impurity identification and quantification remain the main challenge in the

characterisation of a primary calibrator for such a large protein. In this regard, the application of the top-down approaches to the analysis of the intact protein calibrator would be beneficial and could potentially result in the identification of impurities whose contribution should be corrected. This may improve the agreement between the different amino acids and reduce uncertainties associated with the concentration of the primary calibrator.

In conclusion, the establishment of a traceability chain for t-tau quantification will allow improving the lack of harmonization between different IA, through the development of CRMs and RMPs. This candidate RMP will support the activities of the International Federation of Clinical Chemistry and Laboratory Medicine working group on CSF proteins (IFCC WG-CSF) and especially evaluate the feasibility for standardizing t-tau IAs. In collaboration with reference laboratories taking part in IFCC WG-CSF, the candidate reference method will be used to certify t-tau concentration in matrix-based CRMs with which all t-tau IAs will be recalibrated. A prerequisite to ensure that these materials can be used as secondary calibrators will be to evaluate their commutability to ensure that they mimic real patients' samples.

Research funding: This work was supported by the European Metrology Programme for Innovation and Research (EMPIR) joint research projects [15HLT04] "Neuromet" and [18HLT09] "Neuromet2" which have received funding from the EMPIR program co-financed by the Participating States and the European Union's Horizon 2020 research and innovation program. The project was also supported by the Association "France Alzheimer et maladies apparentées".

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest.

Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: The local Institutional Review Board deemed the study exempt from review.

References

1. Arendt T, Stieler JT, Holzer M. Tau and tauopathies. *Brain Res Bull* 2016; 126:238–92.
2. Jack CR, Jr., Bennett DA, Blennow K, Carrillo MC, Dunn B, Haeberlein SB, et al. NIA-AA research framework: toward a biological definition of Alzheimer's disease. *Alzheimers Dement* 2018;14:535–62.
3. Delmotte K, Schaeferbeke J, Poesen K, Vandenberghe R. Prognostic value of amyloid/tau/neurodegeneration (ATN) classification based on diagnostic cerebrospinal fluid samples for Alzheimer's disease. *Alzheimer's Res Ther* 2021;13:84.

4. Delaby C, Teunissen CE, Blennow K, Alcolea D, Arisi I, Amar EB, et al. Clinical reporting following the quantification of cerebrospinal fluid biomarkers in Alzheimer's disease: an international overview. *Alzheimers Dement* 2022;18:1868–79.
5. Bruzova M, Rusina R, Stejskalova Z, Matej R. Autopsy-diagnosed neurodegenerative dementia cases support the use of cerebrospinal fluid protein biomarkers in the diagnostic work-up. *Sci Rep* 2021;11:10837.
6. Hermann P, Haller P, Goebel S, Bunck T, Schmidt C, Wiltfang J, et al. Total and phosphorylated cerebrospinal fluid tau in the differential diagnosis of sporadic Creutzfeldt-Jakob disease and rapidly progressive Alzheimer's Disease. *Viruses* 2022;14:276.
7. Zerr I, Villar-Piqué A, Hermann P, Schmitz M, Varges D, Ferrer I, et al. Diagnostic and prognostic value of plasma neurofilament light and total-tau in sporadic Creutzfeldt-Jakob disease. *Alzheimer's Res Ther* 2021;13:86.
8. Mattsson N, Andreasson U, Persson S, Arai H, Batish SD, Bernardini S, et al. The Alzheimer's Association external quality control program for cerebrospinal fluid biomarkers. *Alzheimers Dement* 2011;7:386–95.e6.
9. Kuhlmann J, Andreasson U, Pannee J, Bjerke M, Portelius E, Leinenbach A, et al. CSF A β 1-42 – an excellent but complicated Alzheimer's biomarker – a route to standardisation. *Clin Chim Acta* 2017;467:27–33.
10. Andreasson U, Kuhlmann J, Pannee J, Umek RM, Stoops E, Vanderstichele H, et al. Commutability of the certified reference materials for the standardization of β -amyloid 1-42 assay in human cerebrospinal fluid: lessons for tau and β -amyloid 1-40 measurements. *Clin Chem Lab Med* 2018;56:2058–66.
11. Korecka M, Shaw LM. Mass spectrometry-based methods for robust measurement of Alzheimer's disease biomarkers in biological fluids. *J Neurochem* 2021;159:211–33.
12. Kang JH, Korecka M, Toledo JB, Trojanowski JQ, Shaw LM. Clinical utility and analytical challenges in measurement of cerebrospinal fluid amyloid- β (1-42) and τ proteins as Alzheimer disease biomarkers. *Clin Chem* 2013;59:903–16.
13. McAvoy T, Lassman ME, Spellman DS, Ke Z, Howell BJ, Wong O, et al. Quantification of tau in cerebrospinal fluid by immunoaffinity enrichment and tandem mass spectrometry. *Clin Chem* 2014;60:683–9.
14. Bros P, Vialaret J, Barthelemy N, Delatour V, Gabelle A, Lehmann S, et al. Antibody-free quantification of seven tau peptides in human CSF using targeted mass spectrometry. *Front Neurosci* 2015;9:302.
15. Barthélemy NR, Gabelle A, Hirtz C, Fenaille F, Sergeant N, Schraen-Maschke S, et al. Differential mass spectrometry profiles of tau protein in the cerebrospinal fluid of patients with Alzheimer's disease, progressive supranuclear palsy, and dementia with Lewy bodies. *J Alzheimers Dis* 2016;51:1033–43.
16. Viodé A, Epelbaum S, Benyounes I, Verny M, Dubois B, Junot C, et al. Simultaneous quantification of tau and α -synuclein in cerebrospinal fluid by high-resolution mass spectrometry for differentiation of Lewy body dementia from Alzheimer's disease and controls. *Analyst* 2019;144:6342–51.
17. Pottiez G, Yang L, Stewart T, Song N, Aro P, Galasko DR, et al. Mass-spectrometry-based method to quantify in parallel tau and amyloid β 1-42 in CSF for the diagnosis of Alzheimer's disease. *J Proteome Res* 2017;16:1228–38.
18. Zhou M, Haque RU, Dammer EB, Duong DM, Ping L, Johnson ECB, et al. Targeted mass spectrometry to quantify brain-derived cerebrospinal fluid biomarkers in Alzheimer's disease. *Clin Proteomics* 2020;17:19.
19. ISO 15193:2009. In vitro diagnostic medical devices — measurement of quantities in samples of biological origin — requirements for content and presentation of reference measurement procedures; 2009. [Online]. Available from: <https://www.iso.org/standard/42021.html>.
20. Huynh HH, Delatour V, Derbez-Morin M, Liu Q, Boeuf A, Vinh J. Candidate high-resolution mass spectrometry-based reference method for the quantification of procalcitonin in human serum using a characterized recombinant protein as a primary calibrator. *Anal Chem* 2022;94:4146–54.
21. ISO/IEC Guide 98-3:2008. Uncertainty of measurement (Part 3. Guide to the Expression of Uncertainty in measurement GUM:1995); 2010. [Online]. Available from: <https://www.iso.org/standard/50461.html>.
22. FDA. Bioanalytical method validation guidance for industry; 2018. [Online]. Available from: <https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf>.
23. EMA. ICH guideline M10 on bioanalytical method validation; 2019. [Online]. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/draft-ich-guideline-m10-bioanalytical-method-validation-step-2b_en.pdf.
24. Josephs RD, Martos G, Li M, Wu L, Melanson JE, Quaglia M, et al. Establishment of measurement traceability for peptide and protein quantification through rigorous purity assessment—a review. *Metrologia* 2019;56:044006.
25. Lemke N, El-Khatib AH, Tchpilov T, Jakubowski N, Weller MG, Vogl J. Procedure providing SI-traceable results for the calibration of protein standards by sulfur determination and its application on tau. *Anal Bioanal Chem* 2022;414:4441–55.

Supplementary Material: This article contains supplementary material (<https://doi.org/10.1515/cclm-2022-1250>).