Talanta 254 (2023) 124186

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Direct coupling of *fiber-in-tube* solid-phase microextraction with *tandem* mass spectrometry to determine amyloid beta peptides as biomarkers for Alzheimer's disease in cerebrospinal fluid samples

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ARTICLE INFO

Keywords:
Amyloid beta peptide
Alzheimer's disease
Biomarkers
Zwitterionic polymeric ionic liquids
Fiber-in-tube SPME
MS/MS

ABSTRACT

Current research efforts at neurological diseases have focused on identifying novel biomarkers to aid in diagnosis, to provide accurate prognostic information, and to monitor disease progression. This study presents the direct coupling of *fiber-in-tube* solid-phase microextraction to *tandem* mass spectrometry as a reliable method to determine amyloid beta peptides (Aβ38, Aβ40, and Aβ42) as biomarkers for Alzheimer's disease in cerebrospinal fluid (CSF) samples. To obtain the biocompatible *fiber-in-tube* SPME capillary, a PEEK tube segment was longitudinally packed with fine fibers [nitinol wires coated with a zwitterionic polymeric ionic liquid], to act as selective extraction medium. The *fiber-in-tube* SPME–MS/MS method integrated analyte extraction/enrichment and sample cleanup (exclusion of interferents) into one step. The method provided lower limits of quantification (LLOQ: 0.2 ng mL^{-1} for Aβ38 and 0.1 ng mL^{-1} for Aβ40 and Aβ42), high precision (CV lower than 11.6%), and high accuracy (relative standard deviation lower than 15.1%). This method was successfully applied to determine Aβ peptides in CSF samples obtained from AD patients (n = 8) and controls (healthy volunteers, n = 10). Results showed that Aβ42 levels in the CSF samples obtained from AD patients were significantly lower compared to healthy controls ($p < 0.05$). On the basis of the ROC analysis results, the Aβ42/Aβ40 ratio (AUC = 0.950, $p < 0.01$; 95%) performed significantly better than Aβ42 alone (AUC = 0.913, $p < 0.01$; 95%) in discriminating between AD patients and healthy controls and presented better diagnostic ability for AD. The novelties of this study are not only related to evaluating Aβ peptides as AD biomarkers, but also to demonstrating direct *online* coupling of *fiber-in-tube* SPME with MS/MS as a quantitative high-throughput method for bioanalysis.

1. Introduction

Diagnosing Alzheimer's disease (AD) generally requires extensive clinical evaluation, which is based on the main symptoms of the disease such as progressive memory loss and other cognitive deficits followed by behavioral abnormalities, inability to maintain daily activities, and increasing reliance on caregiver support. Although these symptoms often appear in the late stage of this neurological disease, the onset of AD pathology can occur decades earlier [1,2]. In this context, studies focusing on the detection of biomarkers that can identify individuals at risk of developing AD are important for prognostic purposes and for initiating preventive treatment to slow down the degenerative process. The primary neuropathological features of AD include β-amyloid (Aβ) plaque deposition in the cerebral cortex and hippocampus, neurofibrillary tangles formed by tau protein hyperphosphorylation, and neuroinflammation. In AD, amyloid plaques are predominantly composed of Aβ peptides (especially the Aβ42 isoform), derived from amyloid precursor protein processing by the concerted actions of β-secretase and the γ-secretase protease complexes [3]. During this catabolism, two main shorter Aβ isoforms, Aβ40 and Aβ38, can also be produced [4]. Determination of Aβ peptides in peripheral fluids (e.g., blood) is challenging because human serum albumin binds 95% of Aβ in blood plasma, thus their concentration (approximately 30 pg mL^{-1}) are 100-fold lower than in CSF [5]. The CSF has been the main biological fluid used for quantifying Aβ peptides. This matrix has more physical contact with the brain than any other biological fluid and can therefore reflect biochemical

Abbreviations: AD, Alzheimer's disease; Aβ, β-amyloid; CSF, cerebrospinal fluid; HSA, human albumin; VTMS, vinyltrimethoxysilane.

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https://doi.org/10.1016/j.talanta.2022.124186
Received 10 September 2022; Received in revised form 29 November 2022; Accepted 7 December 2022
Available online 9 December 2022

changes that occur inside the brain [6–9]. Because these amyloidogenic peptide levels can correlate with disease progression, Aβ42 concentrations and comparisons of the Aβ42/Aβ40 ratios have been accepted as AD biomarkers in the 2018 revision of the National Institute on Aging and Alzheimer's Association (NIA-AA) diagnostic guideline [10,11].

Aβ levels in the CSF have been measured by using several types of immunoaffinity methods including enzyme-linked immunosorbent assay (ELISA) and newer multiplexed techniques [12,13]. Immunoassays are sensitive, but they are time-consuming and expensive, require highly specific antibodies and reagents, present cross-reactivity and poor dynamic range, and are sensitive to matrix interferences. In this context, quantification methods based on *tandem* mass spectrometry (MS/MS) can overcome many of the problems associated with immunoassay methods. Ninety-six-well plate solid-phase microextraction with mixed-mode polymeric sorbent has been used to preconcentrate Aβ peptides in CSF samples followed by quantification by HPLC-MS/MS [14–18]. Despite the advances in MS/MS-based methods, Aβ peptide quantitation remains challenging because they are present at extremely low endogenous concentrations, tend to aggregate, and undergo nonspecific binding to surfaces, not to mention that matrix effects are introduced during electrospray ionization (ESI). Thus, developing new sample preparation strategies that can effectively clean up the extract from endogenous interferences and preconcentrate the analytes before MS/MS-based methods are performed is vital.

Fiber-in-tube solid-phase microextraction (SPME) is an innovative sample preparation technique that combines the features of fiber SPME and *in-tube* SPME. In *fiber-in-tube* SPME, the analytes are extracted in a segment capillary that is longitudinally packed with fine fibers as the extraction medium. As a result, *fiber-in-tube* SPME exhibits higher extraction efficiency, longer lifetime (reusability), and reduced pressure drop compared to conventional *in-tube* SPME [19]. Our group has recently described a new crosslinked zwitterionic polymeric ionic liquid (zwitterionic PIL) coating for *fiber-in-tube* SPME [20], which exploited the zwitterionic PIL coating ability to establish ion-exchange and dispersive interactions that helped to preconcentrate (in the *offline* mode) the Aβ peptides selectively, which was followed by UHPLC–MS/MS quantification for protein binding studies. This *fiber-in-tube* SPME/UHPLC–MS/MS approach was applied to evaluate the human albumin (HSA) ability to bind to Aβ peptides in biological fluids.

Microextraction devices directly interfaced with MS/MS have redefined the analytical workflow by providing faster screening and quantitative methods for complex matrixes [21–23]. Moving forward, this study reports the direct coupling of *fiber-in-tube* SPME with MS/MS. The innovative *fiber-in-tube* SPME–MS/MS method is shown to combine the inherent specificity and biocompatibility of the zwitterionic PIL coating to obtain efficient sample cleanup and enrichment of Aβ peptides in the CSF samples. This study also reports the enhanced sensitivity achieved by using a *low-flow* ESI source compared to a conventional ESI source. Overall, this study shows that the *fiber-in-tube* SPME–MS/MS method provides sensitive Aβ peptide detection at trace levels without the need for chromatographic separation. Hence, the total analysis time is significantly shorter because the sample processing time is reduced, allowing high throughput analysis. Under optimized conditions, the *fiber-in-tube* SPME–MS/MS method can be successfully applied to evaluate Aβ peptides as AD biomarkers.

2. Experimental

2.1. Reagents and analytical standards

Human amyloid beta peptides (Aβ38, Aβ40, and Aβ42) and nitrogen-15 stable-isotope labeled amyloid beta peptides (internal standards (IS): $^{15}N_{51}$-Aβ38, $^{15}N_{53}$-Aβ40, and $^{15}N_{55}$-Aβ42) were purchased from rPeptide (Athens, USA). Polypropylene Protein LoBind tubes and LoRetention pipette tips were acquired from Eppendorf (Hamburg, Germany). Guanidine hydrochloride (≥99%), formic acid (≥98%), ammonium hydroxide (ACS reagent 28–30% NH_3 basis), 1,4-butane sultone (≥99%), vinyltrimethoxysilane (VTMS, 98%), 1,12-dibromododecane (98%), Dimethyl sulfoxide (DMSO), and HSA were purchased from Sigma-Aldrich (São Paulo, SP, Brazil). 2,2-azo-bis(isobutyronitrile) (AIBN; 98%) was acquired from Merck (São Paulo, SP, Brazil). Acetonitrile (HPLC grade) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Water purified in a Milli-Q system (Millipore, São Paulo, Brazil) was used to prepare the aqueous solutions.

2.2. Preparation of Aβ peptide stock solutions

One percent aqueous NH_4OH solution was added to the standards (lyophilized white powder) and sonicated for 30 s, to obtain the stock solutions (1 mg mL^{-1}). The diluted stock solutions were prepared by diluting the 1 mg mL^{-1} stock solutions with acetonitrile/0.1% aqueous NH_4OH solution (20:80, *v/v*) to 50 μg mL^{-1} (Aβ peptides) or 10 μg mL^{-1} (IS). These solutions were aliquoted in polypropylene tubes (LoBind, Eppendorf) and stored at −80 °C until they were analyzed.

2.3. Biological and artificial samples

Eighteen CSF samples (8 from AD patients and 10 from healthy controls), were obtained from patients assisted at the Ribeirao Preto Medical School Hospital, University of Sao Paulo, Brazil. All the participants provided a written informed consent. All the CSF samples were collected in agreement with the criteria established by the Ethics Committee of the Ribeirao Preto Medical School, which abides by the ethical principles of the Declaration of Helsinki. The CSF samples were collected by non-traumatic lumbar puncture procedure and immediately stored at −80 °C. The Aβ peptide concentrations in the CSF were determined by the *fiber-in-tube* SPME–MS/MS method. The Aβ42 concentration in the CSF was also determined by ELISA immunoassay (EUROIMMUN) according to the protocol recommended by the manufacturer.

Artificial CSF was prepared on the basis of a published study [24].

2.4. Preparation of fiber-in-tube SPME capillary

The synthesis of the monomers ([$VIm^+C_4SO_3^-$] and [$(VIm)_2C_{12}$]2[Br]) (Fig. S1) and their *in-situ* polymerization onto nitinol wires were carried out according to the procedures described in our previous work [20]. Briefly, nitinol wires were pretreated with hydrogen peroxide (72 °C for 3 h), which was followed by functionalization with VTMS (85 °C for 5 h). For the polymerization process, [$VIm^+C_4SO_3^-$] and [$(VIm)_2C_{12}$]2 [Br] (2:1, w/w) were dissolved in DMSO/methanol (60:40, *v/v*) solution (monomer + crosslinker corresponded to 2% (w/v) of solvent solution). This mixture was maintained in ultrasonic bath and subsequently degassed under nitrogen stream. Then, AIBN (2.4 mg) was added to the reaction mixture. The functionalized wires were packed into the fused-silica capillary, which was filled with the reaction mixture. These capillaries were sealed and placed in an oven under inert atmosphere at 60 °C for 36 h. Finally, twenty-two nitinol fibers coated with the zwitterionic PIL stationary phase were packed longitudinally into the same length of the PEEK tube segment (0.762-mm i. d., 150-mm length), to obtain the *fiber-in-tube* SPME capillary.

2.5. MS/MS conditions

The analyses were carried out on a Waters ACQUITY UPLC H-Class system coupled to the Xevo® TQ-D *tandem* quadrupole (Waters Corporation, Milford, MA, USA) mass spectrometer equipped with a Z-spray and *low-flow* electrospray ionization (*low-flow* ESI) source operating in the positive mode with Selected Reaction Monitoring (SRM). The source and the operating parameters were optimized as follows: capillary voltage, 3.50 kV; source temperature, 150 °C; desolvation temperature, 500 °C; and desolvation gas flow, 1000 L h^{-1} (N_2, 99.9% purity). Argon (99.9999% purity) was used as collision gas. The fragments, the cone

energy, and the collision energy were optimized for each analyte, as shown in Table 1. Instrument control, peak detection, and integration were carried out with a MassLynx 4.1 Data System.

2.6. Optimization of fiber-in-tube SPME–MS conditions

Online extractions were carried out by direct coupling of the *fiber-in-tube* SPME capillary to the MS/MS system. In this method, one end of the *fiber-in-tube* SPME–MS/MS capillary was connected to the LC system six-port valve, whereas the other end was connected to the MS/MS system selector valve. The mobile phase (quaternary HPLC pump, QSM) was percolated through the capillary, while the selector valve was responsible for directing the effluent from the capillary to the waste (preconcentration step) or to the *low-flow* ESI (elution/desorption step) (Fig. S2).

The main *fiber-in-tube* SPME–MS extraction conditions including the mobile phase composition and the time for the different steps (preconcentration, extractive phase cleanup, and desorption) were evaluated (factor-by-factor approach). The best conditions were chosen on the basis of the absolute peaks areas of the chromatograms obtained during these experiments.

In the optimized condition, the pretreated sample solution (400 μL) was injected via LC (autosampler, QSM) and percolated (at 80 μL min^{-1}) through the capillary by using a mobile phase consisting of an aqueous formic acid solution (0.4%, *v/v*) (Channel A). After the sample was percolated, the extractive phase cleanup step was carried out (for 1 min) by using a mobile phase consisting of aqueous formic acid solution (0.4%, *v/v*) (Channel A)/acetonitrile (Channel B) (95:5, *v/v*). In the preconcentration and cleanup stages, the selector valve was in the waste position.

A mobile phase composed of aqueous NH_4OH solution (0.3%, *v/v*) (Channel C)/acetonitrile (Channel D) 20:80 (*v/v*) was used to desorb the analytes. During the desorption step, the selector valve was switched to the *low-flow* ESI source.

Finally, the mobile phase was returned to 100% aqueous formic acid solution and left in that condition for 2 min to recondition the extractive capillary.

2.7. Analytical validation of the fiber-in-tube SPME–MS/MS method

The *fiber-in-tube* SPME–MS/MS method was validated on the basis of the international guidelines issued by the European Medicine Agency (EMA) and the Food and Drug Administration (FDA) [25,26]. The proposed method was analytically validated with artificial CSF containing 5% rat plasma as a surrogate matrix. The calibration curves were plotted by linear regression of the ratio between the Aβ peptides and the internal standard peak areas (Y) *vs* the Aβ peptide concentrations (X, ng mL^{-1}). To establish linearity, five concentrations of diluted stock solutions (prepared in triplicate) were used. The lower limit of quantitation (LLOQ) corresponded to the lowest concentration of the calibration curve that could be quantitatively measured with acceptable precision and accuracy (within 20%).

Table 1
Ion transitions, instrument settings, and retention times for each studied Aβ peptide.

Analytes	Precursor ion (*m/z*)[a]	Product ion (*m/z*)	Product ion i.d.	DP (V)	CE (eV)	t_R (min)
Aβ38	1033.4	1000.2	b36	45	20	6.6
$^{15}N_{51}$-Aβ38[b]	1046.3	1012.1		45	22	6.6
Aβ40	1082.7	1053.3	b39	55	20	6.6
$^{15}N_{53}$-Aβ40[b]	1096.3	1066.6		45	23	6.6
Aβ42	1128.7	1078.1	b40	55	25	6.6
$^{15}N_{55}$-Aβ42[b]	1142.9	1090.7		50	24	6.6

[a] Precursor $[M+H]^{4+}$.
[b] Internal standard; DP: declustering potential; CE: collision energy; t_R: retention time.

The accuracy (relative standard error, RSE) and the precision (coefficient of variation, CV) of the *fiber-in-tube* SPME–MS/MS method were evaluated with artificial CSF samples (blank samples) spiked with the Aβ peptides at different concentrations (LLOQ, low, medium, and high) of the quality control (QC) solutions, with three replicates at each level.

The selectivity of the developed method was determined by comparing the SRM chromatograms of the blank samples spiked with QC solutions at the concentration corresponding to the LLOQ with the non-spiked blank sample chromatogram. The response of co-eluting interferences should be less than 20% of the response of the analytes and less than 5% of the peak area of the IS.

Carryover was evaluated by the peak area of the analyte in a blank sample that followed an injection of a QC solution at the highest concentration, corresponding to the upper limit of quantitation (ULOQ), which should be less than 20% and 5% of the analyte signals in the LLOQ and IS chromatogram, respectively.

The capillary reusability and long-term stability were evaluated on the basis of the extraction efficiency over multiple extractions.

2.8. Sample pretreatment

An aliquot of the CSF sample (200 μL) was added to a protein LoBind tube containing 100 μL of guanidine hydrochloride (5 mol L^{-1}). This tube was vortexed and agitated at room temperature for 45 min. Then, 100 μL of 1.2% formic acid aqueous solution was added, and the tube was vortexed. Next, 400 μL of the diluted sample was injected in the *fiber-in-tube* SPME–MS/MS system.

2.9. Statistical analysis

The data were analyzed by using the software SPSS version 20.0 for Windows and Minitab® Statistical. Pearson correlation was used to analyze the relationship between the Aβ42 concentration in the CSF determined by *fiber-in-tube* SPME–MS/MS and ELISA. Pairs of AD patient groups *vs* control were compared by using the Mann-Whitney *U* test. Receiver Operating Characteristic (ROC) curves analyses were performed to compare the diagnostic value of the Aβ42 concentration and the Aβ42/Aβ40 and Aβ42/Aβ38 ratios. The ROC curves were drawn by plotting the true-positive fraction (sensitivity) against the false-positive fraction (1 – specificity).

3. Results and discussion

3.1. Optimization of fiber-in-tube SPME-MS/MS conditions

To obtain maximum extraction efficiency within short analysis time, we evaluated the *fiber-in-tube* SPME parameters including the mobile phase composition, the time for the different steps (preconcentration, extractive phase cleanup, and desorption), and the mobile phase flow rate. The mobile phase used for the preconcentration and desorption steps flowed in the same direction through the capillary (*flow-through mode*).

In acidic media, Aβ peptides (see chemical structures on Fig. S3) can undergo cation-exchange and dispersive-type interactions with the zwitterionic PIL coating [20]. Therefore, we evaluated formic acid solution concentrations ranging from 0.1 to 0.8% (*v/v*) during the preconcentration step. The extraction efficiency increased with increasing formic acid solution concentration until 0.4% (Fig. 1a). This concentration was sufficient to impart the analytes with positive charge, thereby strengthening the interactions with the zwitterionic PIL coating. The next step was to optimize the desorption/elution step. Considering the extraction mechanism, the mobile phase pH and strength influenced

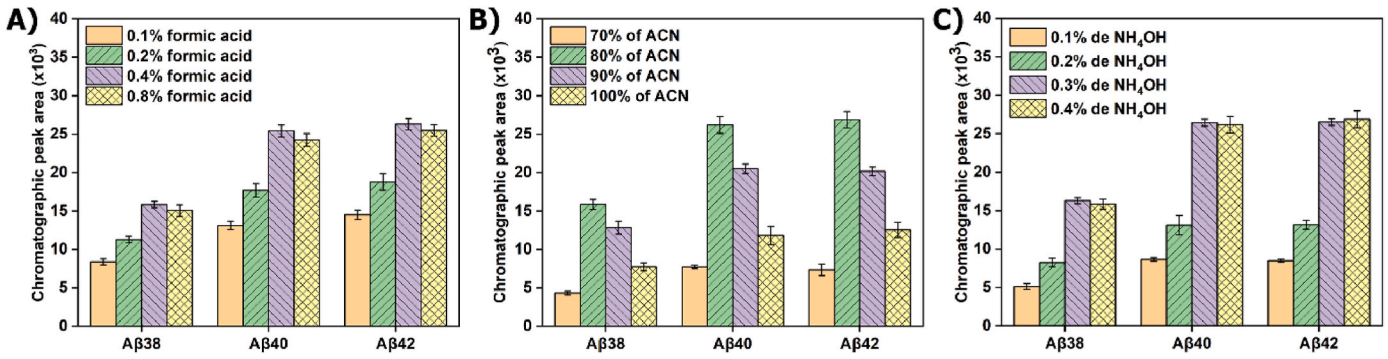

Fig. 1. Optimization of the parameters of the *fiber-in-tube* SPME–MS/MS method: (a) concentration of formic acid in the extraction solution, (b) acetonitrile (ACN) content in the desorption solution, and (c) concentration of ammonium hydroxide in the desorption solution.

analyte desorption. We kept the NH_4OH solution concentration constant and examined acetonitrile ratios ranging from 70 to 100% (*v/v*) in the desorption solution. We found that 80% acetonitrile provided the highest extraction efficiency for all the analytes (Fig. 1b). The organic-rich mobile phase increased analyte ionization due to enhanced desolvation (ESI source), so the NH_4OH solution ratio influenced this ionization. Next, we kept the acetonitrile ratio at 80% and evaluated the NH_4OH concentration from 0.1 to 0.5%. An increase in the NH_4OH concentration to 0.3% raised the analyte desorption efficiency (Fig. 1c). Thus, we used acetonitrile/0.3% NH_4OH solution (80:20, *v/v*) in the subsequent experiments.

By using an artificial CSF sample spiked with the analytes and acetonitrile/0.4% aqueous formic acid solution (5:95, *v/v*), we evaluated the capillary cleanup time from 0.5 to 2.0 min (Fig. S4a). A cleanup time of 1.0 min gave the highest extraction efficiency. The cleanup time of 0.5 min resulted in the lowest extraction efficiency and the largest variation in the results probably because incomplete cleanup of the matrix interferents suppressed analyte ionization.

We maintained the optimized parameter values constant and evaluated the mobile flow rates of 50 and 80 μL min^{-1} (Fig. S4b). A flow rate of 50 μL min^{-1} afforded higher chromatographic peak areas. Nevertheless, this flow rate led to higher chromatographic peak dispersion, which resulted in chromatograms with lower signal-to-noise ratio and sensitivity. Thus, we adopted the mobile phase flow rate of 80 μL min^{-1} in subsequent assays.

3.3. Analytical validation

Aβ peptides are endogenous substances. Therefore, we used artificial CSF+5% mouse plasma as a surrogate matrix while developing and validating the analytical method. Addition of 5% mouse plasma to artificial CSF decreased Aβ peptide (human) binding to the CSF albumins, to increase the sensitivity of the method [14]. The Aβ peptides found in mice and humans are different due to three amino acid mutations (R5G, Y10F, H13R) [27]. Thus, the mouse Aβ peptides do not interfere in the MS/MS transitions of the human Aβ peptides.

In the pretreatment procedure, we treated the samples with guanidine hydrochloride (a denaturant) followed by dilution with formic acid solution. The high guanidine hydrochloride concentration caused Aβ peptide denaturation from various aggregates of oligomeric and polymeric forms to soluble monomeric peptide [17]. We used the formic acid dilution to minimize enzymatic activities and non-specific interactions of the Aβ peptides with the surface of the tube and the CSF proteins and to induce charge before *fiber-in-tube* SPME [14,15].

Regarding analytical validation (Table 2), the *fiber-in-tube* SPME-MS/MS method was linear from 0.20 to 10.00 ng mL^{-1} for Aβ38 and from 0.10 to 10.00 ng mL^{-1} for Aβ40 and Aβ42. These linear intervals were based on the endogenous Aβ peptide concentrations in the CSF samples. The coefficients of determination were higher than 0.99, and the calculated *p*-values (*Lack-of-fit* Test) were higher than 0.05.

Table 2
Analytical validation parameters.

Analyte	Analytical curve (ng mL^{-1})	LLOQ (ng mL^{-1})	QC[a] (ng mL^{-1})	Precision CV (%)		Accuracy RSD (%)	
				Intra-assay	Inter-assay	Intra-assay	Inter-assay
Aβ38	0.2–10	0.2	0.2	6.1	7.6	1.2	−7.0
			0.3	9.2	9.1	0.7	−2.2
			5.0	11.4	11.4	4.8	3.1
			8.0	9.6	9.2	−6.4	5.4
Aβ40	0.1–10	0.1	0.1	5.4	7.6	15.1	−5.9
			0.3	1.0	8.6	1.5	6.0
			5.0	6.8	8.4	3.5	1.3
			8.0	8.6	3.1	−1.4	−9.7
Aβ42	0.1–10	0.1	0.1	6.8	11.6	6.2	−7.2
			0.3	7.3	8.4	4.1	12.2
			5.0	8.1	9.1	12.9	5.3
			8.0	3.3	6.8	7.4	−8.9

[a] QC: quality control.

The intra- and inter-assay precision yielded CV values ranging from 1.0 to 11.6%. The intra- and inter-assay accuracy presented RSE values ranging from −9.7 to 15.1%.

We evaluated the matrix effect by comparing the slopes of the calibration curves obtained with human CSF and artificial CSF. According to *Student's t-test*, the slopes were not statistically different (at significance level of 0.05%). These results not only proved that artificial CSF was a suitable surrogate matrix, but also indicated that human CSF had no significant matrix effects.

A comparison between the chromatogram of the blank sample spiked at the LLOQ and the blank sample chromatogram certified that the method was selective: endogenous compounds did not coelute with any of the analytes (Fig. 2). In addition, there was no significant carryover in the blank chromatograms.

We also evaluated the *fiber-in-tube* SPME device reusability. The reduced system back-pressure and the high chemical and physical stability of the zwitterionic PIL coating allowed the capillary to be reused over 90 times (extractions) without significant extraction efficiency loss (CV less than 15%).

Different studies have reported that Aβ concentrations in fresh CSF are stable at room temperature for up to 2 days, at 4 °C for up to 14 days, and at −80 °C for up to 1 month [28,29]. Samples can go through up to 4–5 freeze/thaw cycles without the measured concentration being significantly affected [28,30].

3.4. Comparison between the fiber-in-tube SPME–MS/MS method with other literature methods

We compared the proposed method with other literature methods for

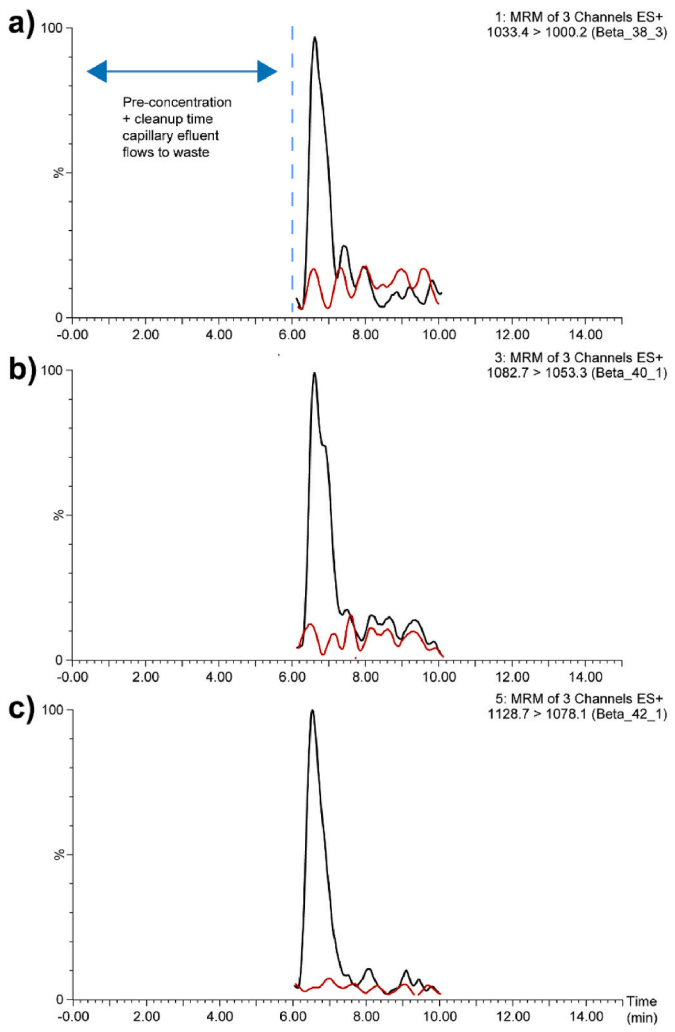

Fig. 2. Chromatograms obtained by using *fiber-in-tube* SPME–MS/MS for an artificial CSF sample spiked with Aβ38 (a), Aβ40 (b), and Aβ42 (c) peptides at the LLOQ (black line) and blank artificial CSF (red line) obtained after analysis at the ULOQ. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Aβ peptide determination in CSF samples. Table S1 highlights the main parameters of the methods including the sample preparation technique, sample volume, linear range, analytical system, and number of analyzed samples.

Compared with other methods that used μ-elution SPE plate as sample preparation [14–18] the *fiber-in-tube* SPME–MS/MS method offered advantages including *online* sample procedure and minimal number of steps. These advantages contribute to high throughput analysis and reduced organic solvent consumption. Moreover, the *fiber-in-tube* SPME reusability an advantage over the μ-elution SPE plate.

Compared with *offline fiber-in-tube* SPME/UHPLC–MS/MS [20], *on-line fiber-in-tube* SPME-MS/MS not only required smaller CSF sample volume, but also reduced the total analysis time significantly.

Despite the superior benefits and the novelties of the proposed method, direct coupling of *fiber-in tube* SPME to MS/MS is challenging, particularly when it comes to determining large molecules such as Aβ peptides at trace levels in biological fluids (complex samples). However, the characteristics of the zwitterionic PIL coating and the analytical system helped us to circumvent these difficulties. The zwitterionic PIL coating provided adequate selectivity and extraction capacity and efficient cleanup performance for excluding interferents from the biological matrix and/or minimizing ion suppression. In addition, we used the *low-flow* ESI probe. Fig. 3 shows the Aβ peptide chromatographic peak areas for the *fiber-in-tube* SPME–MS/MS method carried out by using conventional ESI (ESI source needle i. d. = 120 μm) compared to the *low-flow* ESI probe (i.d. = 60 μm). The *low-flow* ESI probe increased sensitivity by twofold. The ESI source needle i. d. directly influences the droplet size in the spray. Larger diameters produce larger droplets, whilst smaller diameters produce finer droplets [31]. Finer droplets increase the analyte ionization efficiency. The *low-flow* ESI source also showed higher stability for the $[M+4H]^{+4}$ ion signal during infusion of the individual analyte solutions. Therefore, the *low-flow* ESI probe ensured more sensitive and reproducible analyses.

3.4. Determination of Aβ38, Aβ40, and Aβ42 in CSF from Alzheimer's patients

We successfully applied the *fiber-in-tube* SPME–MS/MS approach to determine the Aβ38, Aβ40, and Aβ42 concentrations in the CSF samples (eight samples from AD patients and 10 samples from healthy controls) for clinical studies. To compare the performance of the *fiber-in-tube* SPME–MS/MS and immunoassay (reference technique) methods, we also analyzed all the samples by Aβ42 ELISA (Fig. S5a). Although the Aβ42 concentrations in the CSF samples determined by *fiber-in-tube* SPME –MS/MS were higher than the values obtained by ELISA, there was a strong positive linear correlation (0.95, Pearson's r) between both methods [32]. These results were consistent with findings from previous studies that compared LC–MS/MS with immunoassay methods [33,34].

By applying the *fiber-in-tube* SPME–MS/MS method, we determined mean Aβ38, Aβ40, and Aβ42 concentrations of 1693 ± 1006, 7449 ± 2685, and 558 ± 231 pg mL^{-1} in the AD patients and of 1674 ± 365, 7023 ± 1614, and 942 ± 167 pg mL^{-1} in the healthy controls, respectively. In this study, the Aβ38 and Aβ40 levels in the CSF obtained from AD patients and healthy controls did not differ significantly (at significance level of 0.05, Figs. S5b and S5c). However, the AD patients had lower Aβ42 concentration than the healthy controls (Fig. 4a), which could be attributed to the fact that a lower amount of the peptide was able to diffuse into the CSF due to Aβ42 aggregation into fibrils and plaques in the brain of AD patients. Various clinical studies have established that reduced CSF Aβ42 levels indicate the presence of this peptide in fibrils and plaques in the brain of AD patients. Strozyk et al. analyzed *postmortem* samples and suggested that lower Aβ42 levels reflected neuropathological processes implicated in amyloid-related pathologies [9]. Additionally, Tapiola et al. described that AD patients with a disease lasting two years or less at baseline had more pronounced

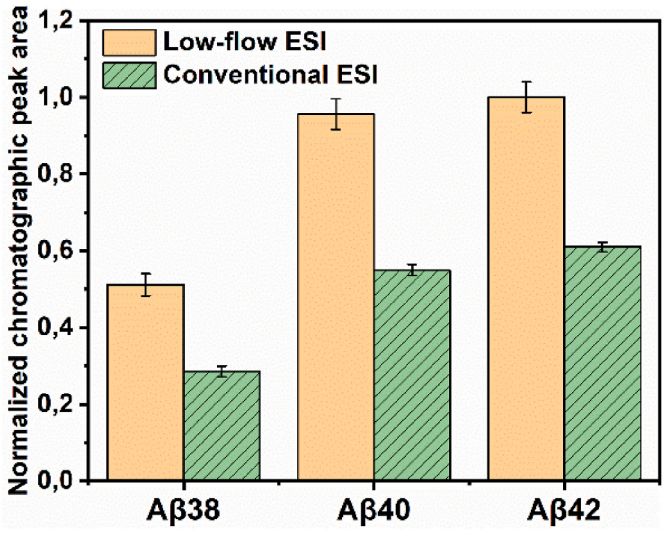

Fig. 3. Comparison of the results obtained by using conventional ESI *vs low-flow* ESI probe with the *fiber-in-tube* SPME–MS/MS method.

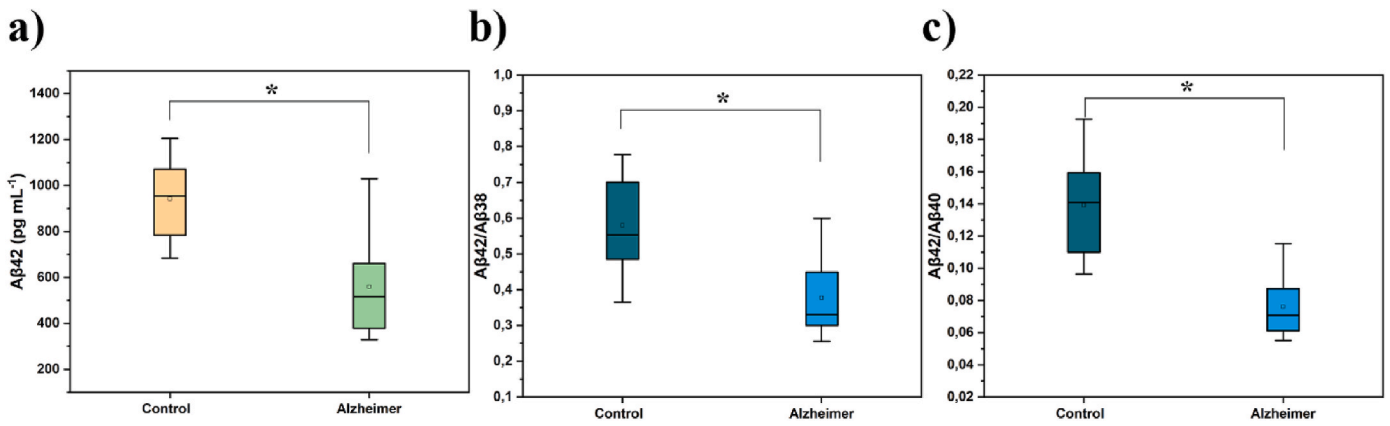

Fig. 4. Distribution of the (a) Aβ42 peptide concentration and the (b) Aβ42/Aβ38 and (c) Aβ42/Aβ40 ratios for the healthy controls and AD patients measured by the *fiber-in-tube* SPME–MS/MS method. Mann-Whitney Test at significance level of 0.05. *$p < 0.05$.

decrease in Aβ42 concentrations compared to patients with the disease for over two years at baseline ($P < 0.05$) [35].

Although the Aβ40 levels in the CSF obtained from AD patients did not change substantially, some studies have established the efficacy of comparing the CSF Aβ42/Aβ40 and Aβ42/Aβ38 ratios [36–38]. These ratiometric analyses are more reliable than solely observing Aβ42 concentrations because they compensate for intra-individual fluctuations within AD patients [11].

As expected, the AD patients had lower CSF Aβ42/Aβ40 and Aβ42/Aβ38 ratios than the healthy controls (Fig. 4b and 4c). These results confirmed the efficacy of these ratiometric analyses in accurately diagnosing and distinguishing AD patients from healthy patients.

In the total cohort (N = 18), the Aβ42/Aβ40 ratio (AUC 0.950) was superior to Aβ42 (AUC 0.913) and the Aβ42/Aβ38 ratio (AUC 0.869) in distinguishing between cases with or without AD pathology (Fig. 5). By using *cut-off* values of 723 pg mL^{-1} for Aβ42 and 0.10 for the Aβ42/Aβ40 ratio, the analyses yielded 87.5% sensitivity and 90.0% specificity in distinguishing patients with AD pathology from those without AD, as shown in Table S2.

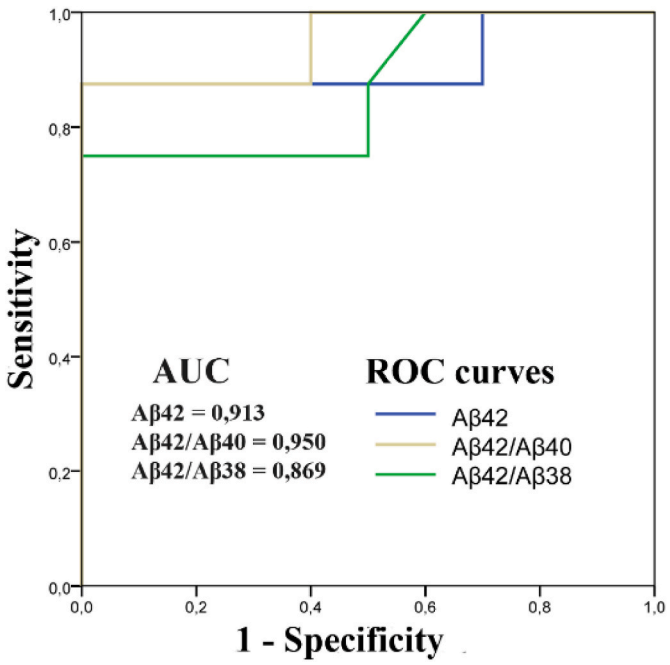

Fig. 5. Characteristic receiver-operating curves of Aβ42 and the Aβ peptide ratios (Aβ42/Aβ40 and Aβ42/Aβ38) for distinguishing AD patients from healthy patients (controls), $p < 0.05$.

4. Conclusion

This work described effective direct coupling of *fiber-in-tube* SPME to an MS/MS system equipped with a *low-flow* ESI probe. The selective capillary packed with nitinol wires coated with zwitterionic PIL in the *fiber-in-tube* SPME system provided adequate biocompatibility, allowing sample cleanup and enrichment of Aβ peptides from CSF samples.

The *fiber-in-tube* SPME–MS/MS system with microflow configuration showed higher ionization efficiency than the conventional ESI probe, increasing the sensitivity of the method.

On the basis of the analytical validation parameters evaluated here, mainly linearity, the proposed *fiber-in-tube* SPME–MS/MS method proved adequate for determining Aβ peptides in CSF samples. The total analytical process was completed in less than 10 min per sample, which represented a significant enhancement in the overall assay throughput.

The ratiometric analysis (CSF Aβ42/Aβ40) was more reliable than solely observing Aβ42 concentrations because it compensated for intraindividual fluctuations within the patients. The Aβ42 results obtained by using the *fiber-in-tube* SPME–MS/MS method exhibited a significant positive linear correlation with the results obtained by using the ELISA immunoassay.

Ongoing work is being devoted to evaluating direct coupling of the *fiber-in-tube* SPME capillary with CapLC and NanoESI-MS systems. We expect that these miniaturized systems will provide higher sensitivity and LLOQ values compatible with Aβ peptide levels in plasma samples. Advantages of blood over CSF include easier access, minimally invasive sampling, cost-effectiveness, and suitability of the procedure for repeated analysis in longitudinal studies.

Credit author statement

Israel D. Souza: Conceptualization, Methodology, Synthesis, Experimental analysis, Data analysis, Investigation, Writing – original draft. **Jared L. Anderson:** Conceptualization, Methodology, Writing – review & editing. **Vitor Tumas:** Conceptualization, Resources. **Maria Eugênia C. Queiroz:** Resources, Supervision, Conceptualization, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to acknowledge FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, processes 2020/00126–8, 2019/19485–0, and 2017/02147–0) and INCT-TM (465458/2014–9) (Instituto Nacional de Ciência e Tecnologia Translacional em Medicina) for financial support. J.L.A. acknowledges funding from the Chemical Measurement and Imaging Program at the National Science Foundation (Grant No. CHE-2203891).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2022.124186.

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