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Analysis of tryptophan and tyrosine in cerebrospinal fluid by capillary electrophoresis and ''ball lens'' UV-pulsed laser-induced fluorescence detection

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Abstract

For the purpose of this study, we used a ''ball lens'' UV laser-induced fluorescence (LIF) detector comprising a pulsed laser and a collinear optical arrangement. The fluorescence signal is induced by a pulsed laser and detected by a photomultiplier tube. When coupling the high-frequency pulsed laser to the LIF detector we used, the electronics which is designed for continuous wavelength (CW) lasers, ''viewed'' the laser as a continuous source. Despite this mismatch between the laser and the ''ball lens'' UV LIF detector, the sensitivity we obtained with tryptophan is comparable to the one obtained with the best ''laboratory-made'' detector described in the literature which used a CW UV laser. Limits of detection of 0.15 n*M* for tryptophan and 50 n*M* for tyrosine were estimated. As an application of this technology, we studied tryptophan and tyrosine in cerebrospinal fluids (CSFs). The analysis is very simple and works on very small samples (5 μl). It consists of using a 10 m*M* 3-cyclohexylamino-1-propanesulfonic acid, 15 m*M* sodium tetraborate, pH 9.2 buffer and injecting CSF diluted 20 times in water prior to injection. 5-Hydroxyindoleacetic acid was used as an internal standard. The separation is completed in less than 12 min. The capillary electrophoresis method which we chose is rapid, resolutive and allows accurate measurements. Recovery experiments in CSFs show recoveries between 97 and 102%. We investigated 14 different CSFs from patients who suffered from neurological disorders. Most of the concentrations vary in a range of 1.7 to 3.7 μ*M* for Trp and 6.6 to 13.7 μ*M* for Tyr, which is in the range observed in the literature. One patient who suffers from Huntington disease had a higher concentration of Tyr at 17.3 μ*M*.

Keywords: Laser-induced fluorescence detection; Detection, electrophoresis; Tryptophan; Tyrosine; Amino acids

1. Introduction

Laser-induced fluorescence (LIF) coupled to capillary electrophoresis (CE) is one of the most powerful detection techniques to achieve low detection limits for biological compounds. Up to now, most of the studies have been done with continuous wavelength (CW) lasers in the visible spectrum to excite the molecules of interest. The analytes are excited in their native form or after derivatization by a fluorophore absorbing at the laser wavelength (for an

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exhaustive review, see Ref. [1]). Native fluorescence has been used for anthracyclines [2] or porphyrins [3]. LIF detection after derivatization has been used to analyze a broad range of molecules such as amino acids [4], acids, amines, and thiols [5,6].

For the past 10 years, CW UV lasers have also been used to study proteins [7], indole-containing molecules [8] and drugs [9]. Thanks to the recent commercialization of UV pulsed lasers, 266 nm lasers pulsing at 10 kHz have been used to detect polycyclic aromatic hydrocarbon metabolites in water [10], peptides and amino acids [11]. KrF pulsed excimer lasers (248 nm with variable frequency ranging from 550 to 2000 Hz) [12] and metal vapor NeCu (248.6 nm, 30 μs pulse, 122 Hz) [13] have also also been used. Paquette et al. have demonstrated that the sensitivity is directly proportional to the frequency of the laser [12]. All the different CW or pulsed lasers lead to similar results in the range of 0.1–1 n*M* for molecules containing Trp or indole functions. Most of the studies presented in the scientific literature were done with more or less sophisticated ''laboratory-made'' instruments. In all cases, these instruments were far from being user-friendly.

More and more studies are realized around the monitoring of variations of tyrosine (Tyr) and tryptophan (Trp) and their metabolites as a function of time [14]. It has been shown that Trp and Tyr and their metabolites are involved in different diseases such as Parkinson's disease [15], obsessive compulsive disorders [16] or schizophrenia [17]. The goal of the analytical biochemist is to propose simple methods to quantitate these analytes. In this study, our objective was to use the ''ball lens'' detector that we described some years ago [18] coupled to these low-cost high-frequency UV pulsed laser. A simple and rapid analytical method for clinical biochemistry laboratories is proposed to quantify Trp and Tyr.

2. Materials and methods

The modular LIF detector used in this study is a ZetaLIF 266 (Picometrics, Ramonville, France). It is made of a collinear optical arrangement as described in Ref. [18]. Thanks to the ''ball lens'' used in this design, it is possible to illuminate all the inner diameter of the capillary. Fig. 1A is a drawing of the laser beam illuminating the capillary. Fig. 1B is the fluorescence emitted from the capillary and collected through the ''ball lens'' and the different optical filters. Thanks to its very high numerical aperture, the sapphire ''ball lens'' collects a much more fluorescence than a conventional lens. A pinhole allows obtaining a ''confocal effect'' which cancels the noise (reflections on the different areas of the ball lens and the capillary).

The CE system is a HP ^{3D}CE system (Agilent Technologies, Waldbron, Germany) with a capillary of 60 cm (53 cm effective length)×75 μm I.D. Because significant variations in the fluorescence intensity occurred when the temperature varied, the carrousel was thermostated at 17 °C and the capillary at 20 °C for all experiments. We used 10 s injections at 50 mB resulting in 62 nl injected.

All the chemicals were from Sigma (Saint Quentin, Fallavier, France).

For experiments on relative fluorescence in function of the pH and for the lowest detected concentration experiments, the electrolytes were 15 m*M* trisodium phosphate adjusted to pH 11.9, 11.0, 10.1, 9.1 using 5 *M* NaOH or phosphoric acid (17 *M*).

For the linearity experiments, and the determination of Trp and Tyr in cerebrospinal fluids (CSFs), we used 10 m*M* 3-cyclohexylamino-1-propanesulfonic acid (CAPS), 15 m*M* sodium tetraborate, pH 9.2 buffer. The voltage was set up at 15 kV resulting in a current of 27 μA. 5-Hydroxyindoleacetic acid (HIAA) was used as internal standard.

The 14 patients we tested suffered from various neurological pathologies. For diagnostic purposes, samples were collected. Then quantitative studies were carried out to measure Trp, Tyr and other aromatic amines on unused samples. Lumbar punctures were performed in the L3–4 or L4–5 interspace in the morning with the patient in a recumbent position. A 3-ml volume of CSF was collected and gently mixed to avoid a gradient effect.

3. Results

3.1. Optimization of the pH of the electrolyte

Tyrosine and tryptophan are pH-sensitive fluores-

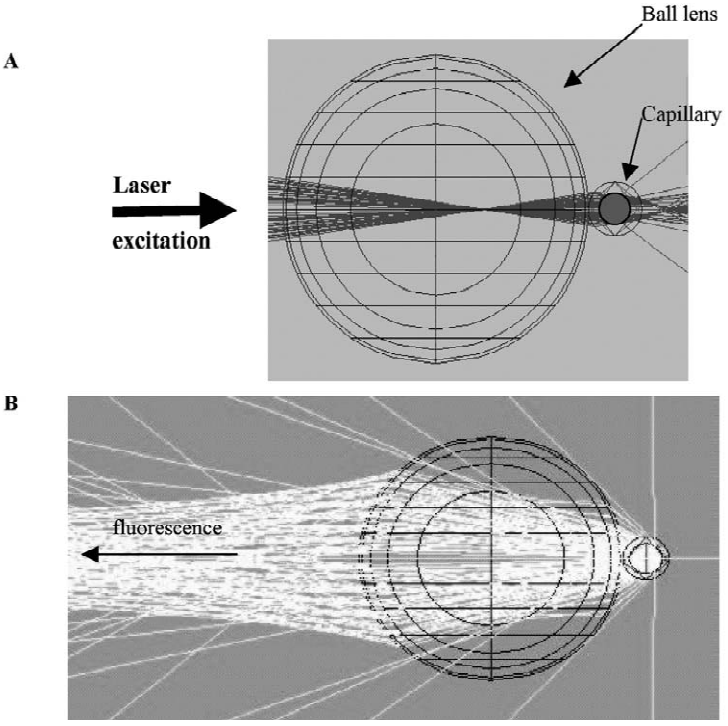

Fig. 1. Schematic of laser excitation (A) and fluorescence emission (B) light beams through the ball lens and the capillary for a ''ball lens'' LIF detector. Only the fluorescence emission going through the ball lens is shown.

cence molecules; consequently we decided to test the best buffer to optimize the pH at which we run the biological samples. Fig. 2 shows the signal-to-noise ratio of tryptophan at a concentration of 1 μ*M* and at different pH values. One can see that at pH 11, fluorescence intensity is optimum. However, to optimize the separation it is necessary to operate between pH 9 and 10.

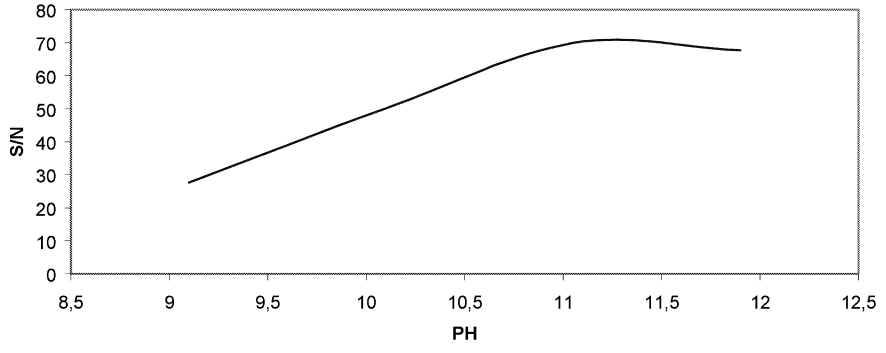

Fig. 2. Signal-to-noise ratio of a 1 μ*M* Trp solution analyzed at different pH with 15 m*M* trisodium phosphate adjusted to pH 11.9, 11.0, 10.1, 9.1 using 5 *M* NaOH or 17 *M* phosphoric acid. Voltage: 15 kV, 10 s hydrodynamic injection (50 mB). Capillary: 60 cm (53 cm effective length)×75 μm I.D. The detection was realized using the UV LIF ball lens detector (266 nm pulsed laser).

3.2. Comparison between the ''ball lens'' LIF detector, UV detector and other UV LIF detectors

In this part of the study, we wanted to compare the sensitivity we reached with our LIF detector to the sensitivity obtained by other scientists who used ''laboratory made'' UV LIF detectors. The result described in the literature was obtained with a detector which analyzed the fluorescence on a continuous basis [19] whereas we use a pulsed laser. The signal-to-noise ratios (S/N) are very close (data not shown) for 1 nM solution injected. S/N values are, respectively, 14 and 24. It indicates good performance for the ''ball lens'' detector. The LIF detector was also compared to UV absorption. Trp is detected at $5{\cdot}10^{-10}$ M and 10 μM, respectively, resulting in the same S/N (results not shown). A 20 000-fold increase in sensitivity was recorded. It can be noticed that the lowest quantitated concentration is 0.5 nM which at a $S/N=3$ gives a limit of detection (LOD) of 0.15 nM.

The ''ball lens'' LIF detector being a recent user-friendly system on the market, we decided to compare its sensitivity to other instruments described in the literature. Table 1 presents a comparison between the lowest detected concentration (LOD) of our system and the other optical arrangements described in different articles. We can easily notice that the sensitivity of the ''ball lens'' LIF detector is in the range of what was obtained by other authors, even though they used CW lasers.

3.3. Separation of standards

Standards of tyrosine, tryptophan, homovanilic acid (HVA), 4-hydroxy-3-methoxymandelic acid (HMMA) and HIAA were solubilized in water, and separated in CAPS, sodium tetraborate buffer. The electropherogram that was obtained is presented in Fig. 3. The calculated numbers of theoretical plates obtained on these standards are presented in Table 2. High values indicate a stacking effect. We checked the linearity of the response of the detector for Trp and Tyr using HIAA as internal standard. HVA and HMMA were also separated.

Calibration curves are linear in a range of 1 nM to 1 μM for tryptophan and 75 nM to 5 μM for tyrosine. As mentioned above, the LOD of Trp is 0.15 nM and 50 nM for Tyr.

To avoid the matrix effect, we realized a calibration curve using two different CSFs.

3.4. Analysis of tryptophan and tyrosine in cerebrospinal fluids

The calibration curves for tryptophan and tyrosine were realized by diluting the CSFs in standard solution. These two amino acids are in the range of 1–20 μM in CSFs. To get a good separation and a high number of theoretical plates, we decided to dilute the CSF by a factor of 20 in water, keeping this media in lower concentration of salts compare to the buffer and allowing stacking which results in

Table 1
Comparison of LODs of Trp in the literature and this study

Wavelength (nm)	Laser	LOD of Trp ($S/N=3$) (nM)	Optical arrangement and detection mean	Ref.
266	Pulsed frequency quadruple Nd:YAG	0.15	Collinear PMT	This work
248	Pulsed KrF 1000 Hz	13	Orthogonal PMT	[12]
266	Pulsed frequency quadruple Nd:YAG	2	Orthogonal PMT	[11]
275.4	Argon ion laser	3	Orthogonal PMT	[7]
284	Continuous Kr frequency doubled	0.2	Orthogonal CCD camera	[19]
248.6	NeCu pulsed laser	63	Sheath flow CCD	[13]

CCD=Charge-coupled device; PMT=photomultipler tube.

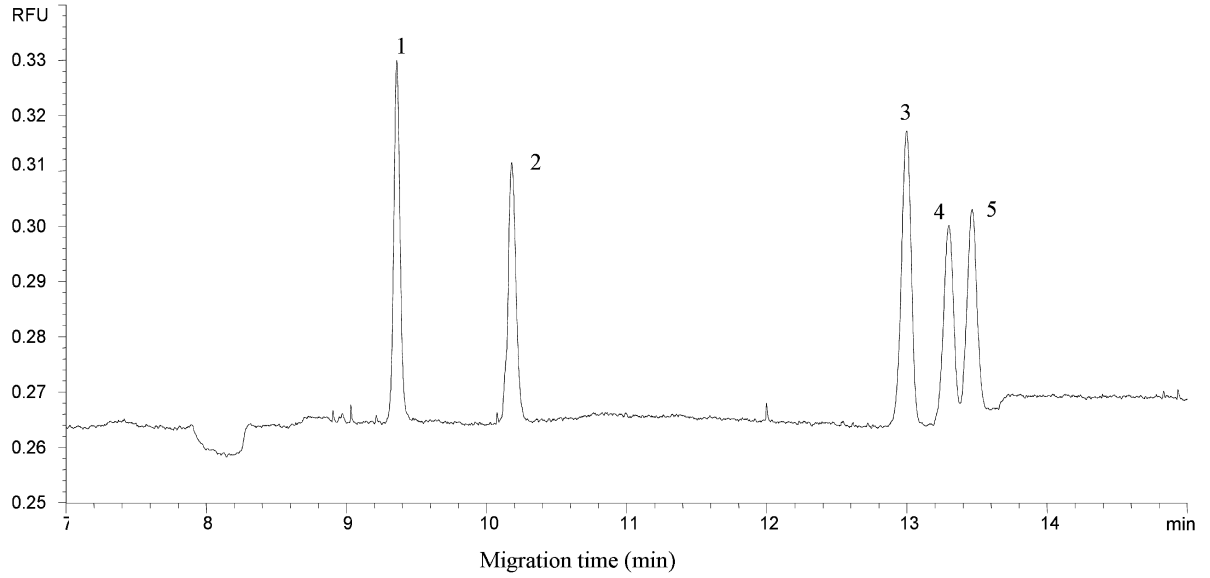

Fig. 3. Electropherogram of Trp (1), Tyr (2), hydroxyindole acetic acid (3), homovanillic acid (4), 4-hydroxy-3-methoxymandelic acid (5) separated with 10 m*M* CAPS, 15 m*M* sodium tetraborate, pH 9.2 buffer. Voltage: 15 kV, current: 27 μA, 10 s hydrodynamic injection (50 mB). Capillary: 60 cm (53 cm effective length)×75 μm I.D. The detection was realized using the UV LIF ball lens detector (266 nm pulsed laser).

very thin peaks. Very small volumes of CSF were analyzed. We worked on 5-μl samples which were diluted in 95 μl of Tyr and Trp solutions at known concentrations (with HIAA, 0.1 μ*M*). The data presented in Table 3 shows two calibration curves called ''added quantities'' which are the added quantities according to the ratio of area of Trp or Tyr to area of HIAA. Very nice calibration curves were obtained for both with r^2 above 0.996. Moreover, Table 3 reports the concentration of the initial CSF solutions estimated from the calibration curves, assuming that they were linear as we have demonstrated above. To simplify the calculation of the concentration for all the CSF samples we analyzed, two calibration curves called ''concentrations in the CSF sample'' showing the concentration of the CSF sample in function of the ratio of Trp or Tyr area to HIAA area are presented. As we can notice, the lowest injected quantities are 7.7 and 29.1 fmol, respectively, for Trp and Tyr in the diluted CSF.

3.5. Method validation

The concentrations of Trp and Tyr in the literature

Table 2
Number of theoretical plates, migration times (min) slopes, intercepts and correlation coefficients (r^2) of calibration curves (concentrations expressed in n*M*) and range of tyrosine, tryptophan, homovanilic acid (HVA), 4-hydroxy-3-methoxymandelic acid (HMMA) and 5-hydroxyindoleacetic acid (HIAA) diluted in water

	Number of theoretical plates	Migration time (min)	Slope of calibration curve ($\cdot 10^{-4}$)	Intercept of calibration curve	r^2	Range (n*M*)
Trp	608 400	9.4	5.9	0.043	0.9913	1–100
Tyr	460 600	10.2	6.7	0.089	0.9916	10–1000
HIAA	452 400	13.0	–	–	–	–
HVA	473 500	13.3	–	–	–	–
HMMA	486 800	13.5	–	–	–	–

Table 3
Calibration curves of ''added quantities'' (in mol added to the sample of 5 μl and diluted to 100 μl) of Trp and Tyr in a CSF sample, basal concentrations of Trp, Tyr (RSD, %, $n=5$) and calibration curve of ''concentration in the CSF sample'' of Trp and Tyr in 5 μl

	''Added quantities''			''Concentration in the CSF sample''				
	Slope	Intercept	Basal amount (pmol)	Slope	Intercept	r^2	Range (μM)	Basal concentration (μM)
Trp	$6.0 \cdot 10^{10}$	0.78	13 (3.2%)	0.3	0.02	0.9946	0.02–23	2.5 (3.2%)
Tyr	$7.1 \cdot 10^{9}$	0.33	47 (5.9%)	3.6	0.00	0.9923	0.2–30	9.4 (5.9%)

Calibration curves present the variation of Trp area on HIAA area following the added concentrations or the real concentration in the 5 μl sample.

are quite variable [14,15,20]. They are estimated to be in the ranges 1–2 and 5–9 μM, respectively. To validate the quantification that we obtained with our method, we realized recovery experiments on two different CSF samples. Table 4 presents the recoveries obtained by adding 95 μl of Trp and Tyr solutions at different concentration to 5 μl of CSF (resulting in 20× diluted CSF). The recoveries are between 97 and 102%, which validates the recommended quantitative method. The RSDs are below 5.9% ($n=3$), which indicates that the results obtained with this simple method are good.

3.6. Study of cerebrospinal fluids

Fourteen CSFs from different patients were studied. Fig. 4 presents an electropherogram of a cerebrospinal fluid diluted 20 times in water (5 μl CSF sample in 95 μl water). The observed concentrations are summarized in Table 5. The results are comparable to the concentrations of Trp and Tyr presented elsewhere [15]. The RSDs are in a range of 2.0 to 9.6%. ($n=5$). In these patients who suffered from neurological disorders, we noticed that most of the time the concentrations of Trp and Tyr are above 1.4 and 8.5 μM [15], respectively. The increase of these two amino acids corresponds to neurological pathologies. For example, patient H who suffers from Huntington's disease has a very high level of tyrosine at 17.3 μM.

4. Conclusion

In this study, we developed a UV LIF detector working with a pulsed laser. The electrically converted fluorescence signal is long and the frequency of the laser is high (10 kHz). In consequence, we used the detector as we usually use it with CW lasers. The sensitivity of the UV LIF detector is comparable to the best ''laboratory-made'' detector we found in the literature. The use of a ball lens makes the detector very easy to use. There is no need for sophisticated adjustments of the laser beam in front of the capillary. The results show that pulsed laser associated to the ball lens detector give the same sensitivity than other detector and continuous UV laser. Moreover, this detector offers a linear

Table 4
Recovery experiments on two different CSF samples

CSF	Experimental concentration of Trp (μM)	Theoretical concentration of Trp (μM)	Recovery	Experimental concentration of Tyr (μM)	Theoretical concentration of Tyr (μM)	Recovery
Sample 1	2.5 (3.2%)	–	–	9.4 (5.9%)	–	–
Sample 2	2.2 (3.0%)	–	–	9.3 (0.4%)	–	
Sample 1	11.7 (2.8%)	12	0.98	18.4 (3.0%)	18.9	0.97
Sample 2	11.5 (2.1%)	11.7	0.98	19.0 (5.8%)	18.8	1.01
Sample 1	21.9 (3.7%)	21.5	1.02	27.6 (3.2%)	28.2	0.98
Sample 2	21.2 (1.2%)	21.2	1.00	28.0 (1.6%)	28.3	0.99

Concentrations are in μM. RSDs are in parentheses ($n=3$).

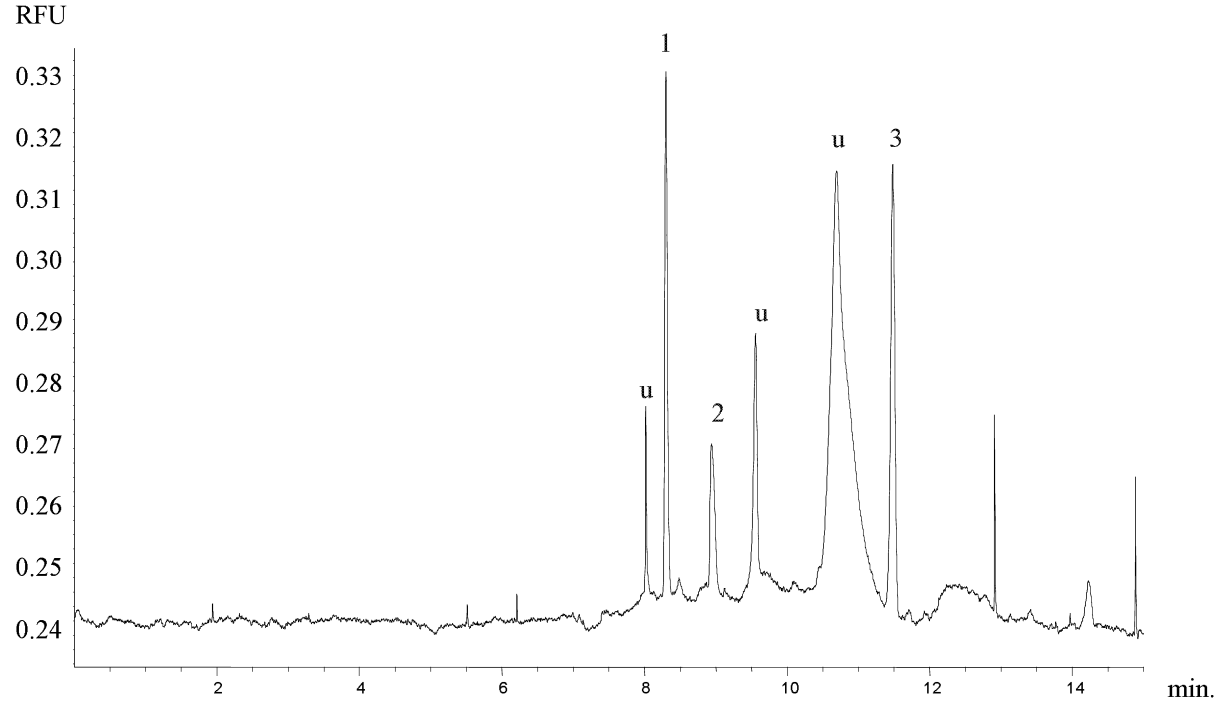

Fig. 4. Electropherogram of diluted CSF sample (×20 in water) Trp (1), Tyr (2), hydroxyindole acetic acid (3), (u) are unidentified compounds. The separation was realized using 10 m*M* CAPS, 15 m*M* sodium tetraborate, pH 9.2 buffer. Voltage: 15 kV, current: 27 μA, 10 s hydrodynamic injection (50 mB). Capillary: 60 cm (53 cm effective length)×75 μm I.D. The detection was realized using the UV LIF ball lens detector (266 nm pulsed laser).

Table 5
Quantitation of Trp and Tyr in 14 neurological pathological CSFs

Sample	Trp (μ*M*)	Tyr (μ*M*)	Sample	Trp (μ*M*)	Tyr (μ*M*)
A	1.9 (4.3)	10.1 (3.1)	H	3.2 (6.7)	17.3 (7.7)
B	1.7 (3.3)	6.7 (4.4)	I	3.2 (3.8)	13.4 (5.1)
C	2.6 (2.0)	6.6 (2.0)	L	3.1 (8.2)	13.4 (9.1)
D	2.1 (2.7)	7.3 (2.7)	N	2.7 (2.7)	11.0 (7.9)
E	2.5 (4.3)	11.7 (6.5)	O	2.5 (3.8)	8.8 (9.6)
F	2.5 (3.1)	12.1 (4.5)	P	3.7 (3.9)	13.7 (7.5)

RSDs are in parentheses (n=5).

response for tryptophan and tyrosine in the ranges of 1 n*M* to 1 μ*M* and 75 n*M* to 5 μ*M*, respectively. The same linearity was observed for these amino acids diluted in cerebrospinal fluid. The CE method which was chosen is rapid, results in a high number of theoretical plates and gives accurate measurements. The CSFs we studied, came from patients suffering from neurological disorders, which may explain the higher concentration levels of Trp and Tyr in most of the CSFs.

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