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Automated method for analysis of tryptophan and tyrosine metabolites using capillary electrophoresis with native fluorescence detection

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Abstract

Capillary electrophoresis (CE) with laser-induced native fluorescence (LINF) detection offers the ability to characterize low levels of selected analyte classes, depending on the excitation and emission wavelengths used. Here a new automated CE-LINF system that provides deep ultraviolet (DUV) excitation (224 nm) and variable emission wavelength detection was evaluated for the analysis of small molecule tryptophan- and tyrosine-related metabolites. The optimized instrument design includes several features that increase throughput, lower instrument cost and maintenance, and decrease complexity when compared with earlier systems using DUV excitation. Sensitivity is enhanced by using an ellipsoid detection cell to increase the fluorescence collection efficiency. The limits of detection ranged from 4 to 30 nmol/L for serotonin and tyrosine, respectively. The system demonstrated excellent linearity over several orders of magnitude of concentration, and intraday precision from 1–11% relative standard deviation (RSD). The instrument's performance was validated via tryptophan and serotonin characterization using tissue extracts from the mammalian brain stem, with RSDs of less than 10% for both metabolites. The flexibility and sensitivity offered by DUV laser excitation and tunable emission enables a broad range of small-volume measurements.

Introduction

The latest human metabolome database [1] reports more the 6,800 identified metabolites, demonstrating the demand for selective and information-rich analytical techniques capable of examining chemically complex environments that consist of lipids, proteins, peptides, salts and, the focus of this work, small molecule metabolites. Techniques for measuring the abundance and distribution of these metabolites within the brain are made challenging by its complex morphology and the often vanishingly small quantities of analytes and volumes available for analysis. While universal characterization approaches such mass spectrometry (MS) [2] and nuclear magnetic resonance (NMR) [3] offer great flexibility, the selectivity provided by separations hyphenated to electrochemical (EC) and laser-induced fluorescence (LIF) detection offers advantages for investigating defined analyte classes, in applications ranging from clinical analysis to basic research.

Capillary electrophoresis (CE) is a separation method well-suited for the analysis of trace-level signaling molecules from neural systems due to its small sample-volume requirements

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(nanoliter to femtoliter), high separation efficiencies (more than 10^6 theoretical plates), and online sample concentration techniques, as reviewed by Lapainis et al. [4]. A variety of detection modalities are available for use with CE (e.g., EC [5], ultraviolet (UV) absorbance [6], LIF [7,8], NMR [9], and MS [10]). Oftentimes, the universal nature of these approaches and the high chemical information content they yield complement many studies. However, the selective detection provided by EC and LIF allows trace level characterization, even from incompletely separated complex samples, as only a subset of the molecules within the sample are detected. Both labeled and label-free methods can be used for LIF detection schemes. Labeling target molecules by derivatization with highly fluorescent dyes can achieve high sensitivities, but it is difficult to achieve efficient labeling of small-volume samples without causing analyte dilution. In addition, non-specific and incomplete reactions can make analysis challenging, particularly within chemically complex sample environments and with limited sample volumes, such as those examined in the present work.

Selective detection can target a defined subset of molecules with a specific molecular property such as native fluorescence or electrochemical activity. The compounds targeted here are metabolites of tryptophan (Trp) and tyrosine (Try), two important analyte classes that include a variety of signaling molecules, including neurotransmitters, neuromodulators and trophic factors. The distribution and tissue-specific levels of these metabolites are correlated with functional, behavioral, and pathologic states. For example, serotonin, a neurotransmitter derived from Trp, is a known modulator of mood. It is also involved in physiological functions such as circadian rhythm, neurogenesis, memory, learning, and body temperature regulation, and has been implicated in pathologies including depression, schizophrenia, and bipolar disorder [11]. One quantitative method for the analysis of these metabolites, along with Trp- and Tyr-containing peptides and proteins, is native fluorescence detection. These species contain aromatic rings that undergo $S_0 \rightarrow S_1$ or $S_0 \rightarrow S_2$ transitions upon deep ultraviolet (DUV) excitation (200–300 nm) and have quantum yields ranging from 0.13–0.28, which are sufficient to take advantage of their native fluorescence as a means of detection at trace levels [12–14]. Thus, laser induced native fluorescence (LINF) detection is an attractive choice for both traditional and microchip CE systems [15].

Until recently, several technical challenges have limited the use of CE separations coupled to LINF detection. Many instruments have been large, costly, and/or high maintenance, thereby limiting the wide-spread application of CE systems using native fluorescence [16–23]. DUV excitation sources were typically full-frame ion lasers such as argon ion (257, 305, and 275 nm) [24–26] and frequency quadrupled Nd:YAG (266 nm) [27] lasers. In an effort to produce more accessible instrumentation, stable DUV sources such as light emitting diodes [28,29] and hollow-cathode metal vapor lasers [19], which are smaller, lower cost, and require reduced maintenance, have replaced older excitation sources in CE-LINF applications. CE-LINF using DUV excitation is complicated by the high amount of scatter and background fluorescence with on-column excitation. Sheath-flow cells achieve off-column excitation and detection with impressive performance [8,30], but they require more complex liquid handling compared to more traditional CE-LIF systems. As has been the case with most analytical techniques, ongoing improvements can contribute to making a system more robust, versatile, and higher throughput, which should expand its use.

Here, we describe a prototype of an automated CE-LINF instrument used for the analysis of mammalian central nervous system (CNS) tissues, and evaluate the analytical figures of merit for several neuroactive metabolites of tryptophan and tyrosine. When designing the new system, the criteria included automated sampling, DUV excitation, and flexible emission wavelength detection, packaged into a format compatible with existing CE systems. While having features in common with our previous systems [19,24,31], this

instrument offers several new features: a small DUV excitation source, on-column excitation, an elliptical fluorescence collection cell to increase the collection efficiency, and automated sample and injection systems for higher throughput analyses. The system uses a pulsed He-Ag metal vapor laser with a 224 nm output for excitation, and tunable emission wavelength selection via a spectrometer, followed by detection with a photomultiplier tube. The components of the instrument are relatively low-cost and can be installed on an existing automated CE instrument, allowing increased reliability and reproducibility. This new instrument demonstrates competitive analytical figures of merit compared with significantly larger, more complex native fluorescence-based systems.

In order to demonstrate the applicability and performance of the automated CE-LINF system compared to our earlier CE-wavelength-resolved (WR)-LINF system, we examined rat brain stem tissue using both instruments. In addition to its ability to target selected metabolites, the prototype instrument, can be used to investigate a wide range of other analyte classes via native fluorescence detection, including proteins and peptides containing Tyr and Trp residues.

Experimental

Chemicals, Solutions, and Materials

The following standards were used: epinephrine, norepinephrine, dopamine, tyramine, tryptamine, 5-hydroxy indole acetic acid, melatonin, tryptophan, tyrosine, N-acetyl serotonin (Sigma Aldrich, St. Louis, MO, USA) and serotonin (Alfa Aesar, Ward Hill, MA, USA). Glacial acetic acid, citric acid monohydrate, and methanol were purchased from Sigma Aldrich. Chemicals for standards were purchased at the highest purity possible. Ultrapure water for stock solutions and standards was obtained from an Elga PureLab Prima water filtration system (Elga LLC, Woodridge, IL, USA) with a purity of 18.2 MΩ. Fused silica capillaries were obtained from Polymicro (Phoenix, AZ, USA).

The extraction media consisted of 49.5/49.5/1, methanol (LC-MS grade)/water/glacial acetic acid (99%) by volume. The background electrolyte (BGE) for separations was made by dissolving 5.25 g of citric acid monohydrate (25 mM, pH 2.75) in 1.0 L of ultrapure water and sonicated to dissolve and degas. Sodium hydroxide (0.1 N), used for capillary conditioning, was obtained from Beckman Coulter Inc. (Brea, CA, USA).

Preparation of Standards

Individual, high concentration stocks for standards were prepared by weighing 1–2 mg on a microbalance (Mettler Toledo; Columbus, OH, USA) and dissolving the analyte into the extraction media. Stocks were stored at –80°C until dilution to the working concentrations as indicated herein.

Tissue Extraction and Quantitation

Brain stem tissue was dissected from a Sprague Dawley rat (Harlan, Inc., Indianapolis, IN, USA). Euthanasia was performed in accordance with animal use protocols approved by the University of Illinois Institutional Animal Care and Use Committee, and local and federal regulations. The brainstem was surgically removed immediately after animal decapitation by sharp guillotine. The excised brain structure was flash frozen in dry-ice cooled isopentane, transferred into individual capped plastic tubes, and stored at –80°C until extraction. Extraction media was added to the weighed tissue at a concentration of 5 µL/mg of wet weight. The tissue was manually homogenized with the extraction media and allowed to extract for 90 min at 4°C [32], centrifuged at 16,000 g for 15 min, and the supernatant

filtered using an Amicon 10 KDa molecular weight cut-off filter (Millipore; Billerica, MA, USA) at 4°C. Samples were stored –80°C until analysis.

Working curves were established using dilutions of high stock standards at concentrations of 1.00 μ M, 500 nM, 250 nM, 125 nM, and 62.5 nM. Each concentration was analyzed in duplicate on the same day as the tissue analysis; linear regression analysis was used to determine the working equation.

CE Separation Conditions

The CE separations for both the automated and laboratory-built systems were performed using a BGE of 25 mM citric acid (pH 2.5) and an applied voltage of +30 kV (18–20 μ Amp). The BGE was filtered using a 0.2 μ m surfactant-free, cellulose acetate syringe filter (Nalgene; Rochester, NY, USA) immediately prior to use. Between each separation, the capillary was conditioned for 1 min with 0.1 N of NaOH at 15 psi and then rinsed with BGE for 2 min at 30 psi. Total analysis time, including capillary conditioning, was approximately 10 min for each analysis.

Automated CE-LINF System

A prototype CE-LINF detection system was provided by Beckman Coulter, Inc.; the fundamental design of the LINF detector has been previously described in application with an high performance liquid chromatography (HPLC) system and was installed on a modified automated CE system (Beckman Coulter, Brea, CA, USA) [33]. Briefly, a hollow-cathode, He-Ag laser operating at 224 nm is used as the excitation source. The incident angle for excitation is set at 30° to reduce scatter and power loss. Fluorescence is collected using a patented elliptical cell with the capillary and the spectrometer entrance slit positioned at the two foci [34] (Fig. 1).

We used a polyimide-coated, fused silica capillary, 50 μ m inner diameter (ID) $\times$ 365 μ m outer diameter (OD) and 48 cm in length, with a detection window created at 37 cm. The coating at the detection window was removed using a capillary window maker (MicroSolv; Eatontown, NJ, USA) in lieu of the more traditional open flame method to prevent excessive damage to the capillary surface. The bare fused silica of the detection window was cleaned by sonication for 10 min in ethanol and any remaining debris removed using optical lens paper (Thor Labs; Newton, NJ, USA). Care was taken to clean and protect the surface of the detection window as contaminants and/or defects both on and in the surface of the capillary can increase background and scattering significantly, particularly when using DUV radiation.

CE-WR-LINF System

The limits of detection (LODs) and analyte identification results from the automated CE-LINF instrument were compared with those from a laboratory-built CE-WR-LINF system [23,35]. Briefly, this instrument uses 264 nm excitation (frequency double of the 528 nm fundamental) from an Innova FreD 300C argon ion laser (Coherent, Inc; Santa Clara, CA, USA). We used a separation capillary with a 50 μ m ID $\times$ 150 μ m OD and a length of 50 cm, and post capillary excitation within a sheath-flow cell containing 25 mM citric acid. The resulting fluorescence spectra were imaged onto a liquid nitrogen-cooled charge-coupled device by a flat-field corrected spectrograph.

Automated System Optimization

In order to determine the optimal detection wavelength for the analytes of interest, the fluorescence emission was scanned using the automated CE system. Each analyte was dissolved at a concentration of 10 μ M in ultrapure water and flowed through the capillary

using a positive pressure of 15 psi. Fluorescence spectra were scanned from approximately 200–600 nm and the resulting spectra were normalized to the peak intensity after background subtraction (Fig. 2). Subsets of these analytes that had peak intensities at similar wavelengths were separated into two sets of standards for evaluation—Set A: serotonin, tryptophan, and tryptamine, and Set B: tyrosine, dopamine, epinephrine, norepinephrine, and tyramine. Both sets were evaluated using a grating position of optimal fluorescence for the individual set of analytes, as indicated by the vertical lines in Fig. 2.

In order to determine the sample volume for optimal reproducibility, varying volumes of standard Set A were pressure-injected and the RSD calculated. Solutions were injected using a pressure of 0.5 psi and the duration was varied from 3–25 s, with total injected sample volumes ranging from 4 to 33 nL; each injection volume was repeated five times. The relative standard deviation (RSD) for both peak height and area were calculated for tryptamine, serotonin and tryptophan. The injected volume with the lowest RSD for the analytes was 20 nL, and this volume was used for subsequent quantitative studies for both performance evaluation and tissue analysis.

Data Processing and Analysis

Data was processed using Origin 8.0 (OriginLab Corporation; Northhampton, MA) for smoothing, background subtraction, and peak quantitation. The Savitzky-Golay smoothing method was used to reduce the high-frequency noise from the electropherograms using a window of five data points and a 2nd order polynomial fitting. The peak integration analysis function of the Origin software was used to subtract the background and quantify several peak parameters: migration time, peak height, full width at half maximum, and peak area.

Evaluation of Analytical Performance

The LODs were defined as the concentration of analyte with a signal-to-noise ratio of 3, with the signal as the peak fluorescence intensity of the particular analyte peak, and the noise as the standard deviation of the background immediately preceding the peak. Each analyte was analyzed individually using a standard at a concentration of 500 nM and the data processed as described in the Data Processing and Analysis section above. Concentrations of 30, 20, 15, 10, and 5 nM for both standard sets were prepared from high-concentration stocks for the determination of the limits of quantitation (LOQs). Each concentration was analyzed at $n = 5$. Measurements were considered quantitative at a particular concentration of analytes if the RSD was less than 20%. Within-day, day-to-day, and total precision were evaluated over a period of five consecutive days using three concentrations of both standard sets: 1.25 μM , 312 nM, and 78 nM. The same standards at each concentration were analyzed in triplicate each day. Linearity was evaluated from 29 nM to 20 μM using mixed standards. Both 1st and 2nd order regression was used to determine the linearity of the peak area.

Results and Discussion

Design and Application

CE-LINF as a detection method has proven to be useful approach that is applicable to a variety of fields of research. It has been used for discovering new serotonin metabolites [36], characterizing single cells [17,23], elucidating pharmaceutical metabolism [37], enabling environmental monitoring [38], and allowing multicolor sequencing [39–41]. The success of these previous systems and methods provided the motivation to adapt the key concepts into the design of a less expensive and automated instrument, thereby enabling routine and economical applications. Obstacles to the general application of CE-LINF have been the cost and complexity of DUV excitation sources, which have been primarily limited to large-

frame ion and quadrupled Nd:YAG lasers. The hollow-cathode HeAg laser (emitting at 224 nm) used here costs five to ten times less than more traditional full-frame ion laser DUV sources, and is economically competitive with other fluorescence-based excitation sources used in commercial CE detection systems using longer wavelengths. Excitation at 224 nm increases the sensitivity of catecholamines by accessing the $S_0 \rightarrow S_2$ transition, which has a higher absorbance cross section than more traditional DUV excitation wavelengths (e.g., 257, 264, 266, 287 nm) that use the $S_0 \rightarrow S_1$ transition [12].

Sheath-flow detection cells are typically used for off-column excitation due to the high level of background and power losses associated with Rayleigh scattering in DUV [30,42]. The system described here is enhanced by using on-column excitation at an incident angle of 30° to reduce the scatter, and a patented ellipsoid excitation cell (Fig. 1) to collect a larger fraction of emitted photons compared with traditional collection optics, making on-column excitation competitive with the more complicated, off-column sheath-flow setups. An added benefit of this excitation and detection setup is greater ease in optical alignment. While the ellipsoidal cell has been used previously with HPLC [33], we expand its use here by integrating the flow cell with CE instrumentation. This new system for CE handles a 1000-fold smaller sample volume than used in LC, and so offers improved compatibility with the volume-limited sample characteristic of most neuronal analyses. This configuration allows for the application of LINF detection on existing automated CE instrumentation, poising the technique for ready implementation by laboratories currently using automated CE systems.

When one tags an analyte with a fluorophore, the fluorescence properties of the tagged analyte tend to be dictated by the characteristics of the particular fluorescence tag. Native fluorescence, with its inherent information-rich nature, allows individual species to be identified based on the differing maxima of their fluorescence spectra. Small changes in the functional groups near the aromatic rings result in subtle changes in the fluorescence spectra, creating clusters of fluorescence maxima, as shown in Fig. 2. Reconfiguration of the ring structure during metabolism can shift the fluorescence maxima substantially, as is the case for kynurenine and kynurenic acid. As Fig. 2 illustrates, even though the fluorescence spectra for the metabolites of interest are broad and have maxima centered around similar wavelengths, ~ 300 nm for tyrosine metabolites and ~ 350 nm for tryptophan metabolites, analytes can be differentiated based on their spectral properties. For optimal performance this requires that either the entire spectra be acquired, or a predefined, optimal wavelength be determined and monitored for the analytes of interest.

We divided the metabolites of interest into two sets according to their fluorescence maxima: Set A (tyrosine metabolites) and Set B (tryptophan metabolites). The two detection wavelengths for each set were chosen to correspond as close to the maximum fluorescence for as many of the analytes as possible from each cluster. The selected grating position for each is indicated by vertical lines in Fig. 2. Some of the analytes for which fluorescence spectra are included in Fig. 2 were insufficiently fluorescent at the chosen detection wavelengths and thus, were excluded from the performance studies, specifically, kynurenine, kynurenic acid, phenylalanine, and L-DOPA. The electropherograms for each set are shown in Fig. 3.

System Performance

The system was evaluated for several figures of merit for each mixed standard set: LOD, linearity, LOQ, and precision across concentration ranges relevant to levels that are typical for biological systems. LODs for the prototype system and our own laboratory-built, WR instrument [35] are given in Table 1 for comparison of their performance. The CE-WR-LINF system was somewhat more sensitive for indolamines (tryptophan, serotonin, tryptamine), likely due to a larger absorbance cross section for the $S_0 \rightarrow S_1$ at 264 nm for

indolamines than catecholamines. There was a substantial, more than 5-fold improvement in the detection limits for the catecholamines with the automated CE-LINF over the WR system, also likely due to an increased $S_0 \rightarrow S_2$ absorbance cross section, in this case at 224 nm.

Typically, fluorescence demonstrates linearity across many orders of magnitude, with the limiting factor being the method used for detection. Here, the gain settings were optimized for the detection of low concentration analytes, which limits the dynamic range, but is optimal for the analytes of interest in CNS tissues. Even so, as summarized in Table 2, a minimum of two orders of magnitude of linearity was observed for the analytes with higher fluorescent yields (serotonin, tryptamine, tyramine, tryptophan) and three orders of magnitude for analytes with lower fluorescent yields (dopamine, epinephrine, norepinephrine, tyrosine).

One of the many advantages of an automated CE system is the level of precision that can be achieved by limiting several of the more common sources of variation inherent to laboratory-built systems. The automated system we describe offers temperature-controlled sample and capillary environments and pressure-controlled sample injections, two variables that can substantially degrade reproducibility. Here, the variation associated with sample introduction was minimized by optimizing the pressure-injected volume. Fig. 4 clearly shows that the most reproducible injection volume is approximately 20 nL. Because LODs were not significantly dependent on injection volume, they were not taken into consideration when optimizing the injection volume. The intraday precision ranged from 1–11% for the lowest concentrations, and 1–2% percent for the highest concentrations (Table 3). As expected, the total precision over five days was higher than intraday, but the data trending (not shown) showed a slight drift toward lower total signal, indicating a degradation of the standards or minor changes in alignment. However, even with the minor change in signal, the precision was still excellent and comparable with many clinical assay performance characteristics.

Analysis of Mammalian CNS Tissue Extracts

The quantitation of trace levels of analytes from tissue extracts presents some challenges, namely, successful extraction while minimizing effects from interfering proteins and peptides. By adding methanol to the extraction media, we not only preserved the analytes from enzymatic degradation by deactivation of the enzymes, but sufficiently lowered the conductivity to provide field-amplified stacking in order to improve LODs and increase separation efficiencies. While CE is tolerant of complex sample environments, excess amounts of proteins can coat the walls of the capillary, degrading performance. This was mitigated in three ways. First, the total amount of protein present in the sample was reduced by using a 10 kDa molecular weight cut-off filter. Second, a low pH separation buffer was chosen. At low pH, the silanol groups on the capillary surface are protonated, limiting the attraction between the peptides and the surface. Finally, conditioning of the capillary surface with 0.1 N of NaOH between each analysis removed any contaminants. The amount of sodium hydroxide used to condition the capillary was kept at a minimum so as to preserve the capillary and reduce the frequency of replacement. Flushing the capillary for 1 min at 15 psi with 0.1 N of NaOH yielded reproducible electropherograms for the tissue extract.

The analyses of rat brain stem tissue demonstrated the ability of the automated CE-LINF instrument and described method to achieve reproducible quantitation from CNS tissue extracts. Comparison electropherograms, extracted at the same wavelength for the tissue extracts, are shown for both the automated CE-LINF (Fig. 5a) and WR-CE-LINF (Fig. 5b) systems. A full wavelength-resolved electropherogram is shown in Fig. 6. The peaks quantified using the automated CE system are labeled in Fig. 5a: serotonin and tryptophan,

and one indolamine-like, unidentified peak. Results for the automated system include serotonin, 1.25 ($\pm$ 0.12) nmol/g; tryptophan, 10.6 ($\pm$ 0.37) nmol/g; and the signal for an unidentified peak, 3.64 ($\pm$ 0.42) A.U. While the two electropherograms are similar, the minor differences between them can be attributed to differences in the fluorescence responses between the two excitation wavelengths. The utility of tunable emission wavelength monitoring is further demonstrated by the wavelength-resolved electropherogram in Fig. 6. The varying analyte bands detected here have spectra ranging from 300–500 nm. Selecting optimal wavelengths for specific analytes of interest simplifies the electropherogram, reducing potentially interfering signals.

Conclusions

We introduce an automated, cost-effective, and sensitive CE-LINF platform for the routine analysis of tryptophan and tyrosine metabolites in complex sample matrixes. The ability to monitor a particular wavelength of fluorescence emission enables fast and efficient data processing compared with WR systems, which result in more complex data requiring correspondingly more complex data analysis methods. In contrast to charge-coupled devices or array detection platforms, using a single wavelength for detection lowers instrument cost and complexity. The system is made more versatile by a programmable spectrograph that allows the optimal detection wavelength to be selected and tailored to the analytes of interest. We look forward to the commercial availability of this instrument for both research and clinical applications. While metabolite detection has been emphasized here, CE-LINF with DUV is applicable to many areas, including general characterization of peptides and proteins [25,29,43,44]. The advantages of fluorescence include its fairly constant response for many Try and Trp-containing proteins and ease of quantitation. We expect the use of LINF to greatly expand with the availability of this detection modality for CE.

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Fig. 1. Schematic of the ellipsoidal collection cell. The excitation point and entrance to the monochromator are positioned at the two foci of the ellipsoid, allowing for collection of photons not emitted directly towards the monochromator and increasing the total signal collected compared with more traditional detector configurations. The relative sizes of the components are not represented for clarity

Fig. 2. Fluorescence profiles for tyrosine metabolites (**a**) and tryptophan metabolites (**b**) were obtained using the automated CE-LINF instrument by scanning the emission spectra of the analyte being flowed through the capillary. The differences in fluorescence maxima for the various metabolites require selection of a wavelength to monitor, corresponding as close as possible to the maxima of the analytes of interest. Vertical lines denote the grating position for detection for each set of metabolites

Fig. 3. Stacked electropherograms of standard set A (tryptamine, serotonin, and tryptophan) and B (tyramine, dopamine, epinephrine, norepinephrine, and tyrosine) from the automated CE-LINF system. Electropherograms were monitored at the optimal wavelengths determined for each set

Fig. 4.

Optimization of injection volumes. Varying volumes of standard Set A were injected by applying pressure at 0.5 psi for varying durations. While there was minimal variation in the LODs between the different injection volumes, the intra-day precision was substantially improved at a volume of ~20 nL.

Fig. 5. Typical electropherograms for the same brain stem tissue extract from the (a) automated prototype CE-LINF instrument provided by Beckman Coulter, Inc. and the (b) laboratory built, wavelength-resolved instrument. The differences in capillary lengths between the two instruments account for the differences in migration times. The identified peaks from serotonin and tryptophan were aligned for comparison. While the electropherograms are similar, the differences can be attributed to individual analyte responses to the differing excitation wavelengths. Time scales have been aligned using known peaks (numbered) Key: 1 – Unidentified, but quantified peak, 2 – serotonin, 3 – tryptophan, 4 – tyrosine

Fig. 6.

Analysis of the same tissue extract shown in Fig. 5 using wavelength-resolved native fluorescence detection. Several of the peaks can be identified by comparison of spectral and migration times to standards. The unidentified peaks are likely other tryptophan metabolites or tryptophan-containing peptides/proteins. Key: 1 – Unidentified, but quantified peak, 2 – serotonin, 3 – tryptophan, 4 – tyrosine, * – unidentified peak

Table 1

Comparison of the detection limits ($S/N = 3$) of the automated CE-LINF platform with the CE-WR-LINF system. The LODs for the tryptophan metabolites with the automated system are comparable to the wavelength-resolved system and show a 5-fold improvement for tyrosine metabolites

Analyte	Automated System (nmol/L)/(Attomoles) (224 nm)	Wavelength-Resolved System (nmol/L)/(Attomoles) (264 nm)
Tryptophan	18/342	5/50
Serotonin	4/76	1/10
5-HIAA	13/247	50/500
Melatonin	6/114	NA
NAS	9/171	NA
Tyrosine	15/285	88/880
Dopamine	16/304	36/360
Epinephrine	16/304	94/940
Norepinephrine	30/570	91/910

Table 2

Evaluation of linearity for selected standards. Typical linearity descriptors are included, along with the coefficient for the 2nd order polynomial, which indicates a negligible 2nd order component to the fit

Analyte	Linear Range (nM)	R-Squared (1 st order regression)	Intercept	Slope	2nd Order Coefficient
Serotonin	39–1,250	0.9997	-0.12	0.0083	1.7e-05
Tryptophan	39–2,500	0.9951	0.16	0.0045	-4.5e-07
Tryptamine	39–1,250	0.9998	-0.09	0.0104	3.7e-07
Dopamine	39–20,000	0.9993	-0.02	0.0006	2.6e-09
Epinephrine	39–20,000	0.9995	0.10	0.0008	-2.7e-09
Norepinephrine	39–10,000	0.9991	0.10	0.0014	-1.2e-08
Tyramine	39–5,000	0.9959	0.07	0.0016	-7.2e-08
Tyrosine	39–10,000	0.9979	0.16	0.0016	-2.3e-08

Summary of within-day, day-to-day, and total precision for the seven metabolite standards used to evaluate the analytical merits of the methods used in this study

Table 3

	Serotonin	Tryptamine	Tryptophan	Tyramine	Dopamine	Epinephrine	Norepinephrine
Level 1 (78 nM)	Within-Day	1%	2%	8%	6%	11%	11%
	Day-to-Day	14%	12%	13%	20%	36%	24%
	Total	14%	13%	15%	21%	38%	26%
Level 2 (312 nM)	Within-Day	2%	2%	4%	2%	6%	4%
	Day-to-Day	9%	9%	10%	9%	10%	9%
	Total	9%	9%	10%	9%	11%	10%
Level 3 (1.25 μM)	Within-Day	1%	1%	2%	2%	1%	1%
	Day-to-Day	4%	3%	3%	6%	8%	7%
	Total	4%	3%	3%	7%	8%	8%