Published in final edited form as:

J Chromatogr A. 2012 August 17; 1251: 54–62. doi:10.1016/j.chroma.2012.06.002.

Optimization for speed and sensitivity in capillary high performance liquid chromatography. The importance of column diameter in online monitoring of serotonin by microdialysis

Jing Zhang, Yansheng Liu, Andrea Jaquins-Gerstl, Zhan Shu, Adrian C. Michael, and Stephen G. Weber*

Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260, United States

Abstract

The speed of a separation defines the best time resolution possible in online measurements using chromatography. The desired time resolution multiplied by the flow rate of the stream of analyte being sampled defines the maximum volume of sample per injection. The best concentration sensitivity in chromatography is obtained by injecting the largest volume of sample that is consistent with achieving a satisfactory separation, and thus measurement accuracy. Taking these facts together, it is easy to understand that separation speed and concentration sensitivity are linked in this type of measurement. To address the problem of how to achieve the best sensitivity and shortest measurement time simultaneously, we have combined recent approaches to the optimization of the separation itself with an analysis of method sensitivity. This analysis leads to the column diameter becoming an important parameter in the optimization process. We use these ideas in one particular problem presented by online microdialysis sampling/liquid chromatography/electrochemical detection for measuring concentrations of serotonin in the dialysate. In this case the problem becomes the optimization of conditions to yield maximum signal for a given sample volume under the highest speed conditions with a certain required number of theoretical plates. It turns out that the observed concentration sensitivity at an electrochemical detector can be regulated by temperature, particle size, injection volume/column diameter and void time. The theory was successfully used for optimization of neurotransmitter serotonin measurement by capillary HPLC when sampling from a microdialysis flow stream. The final conditions are: 150 μm i.d., 3.1 cm long columns with 1.7 μm particle diameter working at a flow rate of 12 $\mu\text{L}/\text{min}$, an injection volume of 500 nL, and a temperature of 343 K. The retention time for serotonin is 22.7 s, the analysis time is about 36 s (which allows for determination of 3-methoxytyramine), and the sampling time is about 0.8 minutes with a perfusion flow rate of 0.6 $\mu\text{L}/\text{min}$.

Keywords

HPLC system optimization; high speed analysis; column diameter; detection sensitivity; microdialysis; serotonin

© 2012 Elsevier B.V. All rights reserved.

*412.624.8520, sweber@pitt.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

There are many important examples of flowing streams that are sampled periodically for analysis, for example microdialysis[1–3], microreactors[4–9], and even a chromatographic effluent as a first stage in a two- or three-dimensional separation[10–12]. When analysis of a flowing stream requires taking samples from the flowing stream, the sample volume is a key parameter in defining the attributes of the system. The time resolution of the measurement is related to the stream's flow rate and the sample volume. A larger sample volume dictates poorer time resolution. An exemplary demonstration of this is the early work from the Justice group[13] showing 1-minute time resolution in microdialysis at a time when the typical time resolution was 20 minutes[14–16]. They achieved this by taking 1 μL samples and using a 1 mm column ID for the separation in contrast to the typical 20 μL sample used with a then normal 4.6 mm ID column. The smaller column volume permitted the use of a smaller sample without decreasing the concentration of the analyte, dopamine, in the detector. The Justice group used off-line HPLC for analysis of the captured microdialysate. The problem becomes considerably more interesting if *online*, near-real time measurements are required.

When online analysis is required, the speed of the separation must equal or exceed the rate of sample acquisition. In this case, the time resolution of the measurement dictates the separation speed, or vice versa. Fortunately, there are developments in liquid chromatographic optimization that focus on achieving minimum time-per-plate that we can use to guide us [17–19]. These approaches allow workers to discover the combination of chromatographic parameters such as particle diameter d_p , column length L , and mobile phase linear velocity u for the smallest t_0 with a certain N (high speed separation). In addition, Carr *et al.*[20] demonstrated that an algebraic approach can be used to carry out the optimization, simplifying the process. But these strategies focus on the separation itself. They do not consider other figures of merit that may be important in the application of the separation to an analytical problem.

There is a need for an optimization approach that includes both separation and detection figures of merit. We face this problem when we consider the monitoring of serotonin (5-hydroxytryptamine, 5-HT) by microdialysis coupled to online HPLC. Our motivation is to improve significantly the speed of the chromatographic determination of 5-HT to improve the temporal resolution of the measurement. This is a challenge, because the concentrations expected for 5-HT in dialysate under resting conditions are less than 1 nM. The separation must be good enough to avoid interferences from solutes with much higher concentrations (by factors of $10^3 - 10^4$). Our group has successfully determined 5-HT with a retention time less than one minute in mouse microdialysate samples using capillary columns under high pressure, high temperature conditions[21]. In that work, 500 nL were injected onto a column with approximately 133 nL of fluid volume. This provided adequate concentration sensitivity. However, the large volume injection, while improving the analytical signal, decreased the number of theoretical plates. We therefore asked the question, "Knowing that a certain number of theoretical plates are sufficient for the separation, can we gain speed without compromising the detection limit?" We have a budget of excess plates (difference between plate number generated by the column with a small injection volume, N and number of theoretical plates achieved with the larger injection volume, N_a). What is the best way to spend the budget?

We will approach this problem conceptually in two steps. First, we recapitulate Carr's algebraic approach that determines N as a function of the void time, t_0 and the particle diameter, d_p , for fixed pump pressure and column temperature[20]. In this portion of the analysis we do not include the injection volume and the band spreading it creates, called

volume overload in the preparative chromatography literature[22, 23], nor do we include the column diameter. Based on work of Guiochon and Colon[24] and Mills et al.[25], we then incorporate the sacrifice in N caused by volume overload. Based on well-known detector properties[26–28], we incorporate how the concentration sensitivity of the method is altered by changes in the void time, particle diameter, and column diameter at fixed injection volume, pump pressure, and column temperature. We note that both concentration sensitivity, signal per unit concentration of analyte injected, and mass sensitivity, signal per unit mass injected, could be used as the “detection sensitivity”. In the application we are pursuing, the concentration sensitivity is more relevant.

Under the optimum conditions determined by the analysis, the separation time for 5-HT was reduced to about 22.7 s while maintaining the column performance and concentration sensitivity. Further, we simulated online analysis by making repeated injections to demonstrate the feasibility of online analysis with a time resolution of 36 s.

2. Experimental

2.1 Chemicals

Disodium EDTA was purchased from Fisher Scientific (Fair Lawn, NJ). Sodium acetate and glacial acetic acid were purchased from J. T. Baker (Phillipsburg, NJ). Acetonitrile, 2-propanol, L-ascorbic acid (AA), serotonin hydrochloride, 3-methoxytyramine hydrochloride (3-MT), and sodium 1-octanesulfonate (SOS) were purchased from Sigma (St. Louis, MO). All the chemicals were used as received without any further purification. Ultra-pure water was obtained from a Millipore Milli-Q Synthesis A10 system (Bedford, MA).

2.2 Chromatographic system

Homemade capillary columns were slurry packed with 1.7 μm BEH C 18 reversed-phase particles (Waters, Milford, MA) using 100 or 150 μm i.d., 360 μm o.d. fused-silica capillaries as the column blank (Polymicro Technologies, Phoenix, AZ). Column frits were made by sintering 2 μm borosilicate particles (Thermo Scientific, Fremont, CA) which were pushed into the ends of the columns by pressing the capillary into the particle powder. A Model DSF-150-C1 air driven fluid pump (Haskel, Burbank, CA) was used for packing columns with a packing pressure of 12 000 psi. The packed capillary columns were connected directly to the injector and flushed overnight with mobile phase before use.

A UHPLC pump (Model nanoLC-Ultra 1D, Eksigent, Dublin, CA), with a maximum pump pressure of 10 000 psi, was used to deliver mobile phase in the flow rate range of 0.1–12 $\mu\text{L}/\text{min}$. A 10 port nanobore injector was used for consecutive injections (Valco Instruments, Houston, TX). Injection loops were fused silica capillaries. The typical sample volume was 500 nL. Column frits were made on the inlet side of the injection capillary to remove any particulates from dialysate samples and protect the column from being blocked.

Two homemade heating assemblies controlled the temperature of the injector and the capillary column. Two proportional-integral-derivative (PID) controllers (Model CT15122, Minco Products, inc., Minneapolis, MN) drove Kapton polyimide heat film (Minco Products, inc., Minneapolis, MN) to heat the column and injector separately. For the capillary column, the heat film covered the surface of an aluminum cylinder with insulation which transferred the heat to the capillary column. The temperature sensor was placed inside the aluminum block, and the signal was fed back to the controller. For the injector, a standoff assembly was used to insulate the actuator from the valve which was covered by the heat film with insulation so the electronic part of the injector was not heated. The temperature sensor was placed in contact with the surface of the valve. The precision of the temperature control is about 0.1 $^{\circ}\text{C}$ in the range from room temperature to 100 $^{\circ}\text{C}$.

Chromatographic condition: separations of serotonin were achieved using ion-pair reverse phase liquid chromatography. The mobile phase was prepared by mixing an aqueous buffer with acetonitrile with a 96:4 volume ratio. The aqueous buffer contains 100 mM sodium acetate, 0.15 mM disodium EDTA and 10.0 mM SOS. The pH of the buffers was adjusted to 4.0 with glacial acetic acid. The mobile phase was passed through a 0.22 μm Nylon filter (Osmonics, Minnetonka, MN).

Sample preparation: stock solutions of 1.0 mM analytes were prepared in 0.1 M acetic acid and stored frozen at -20°C . Analyte solutions with a desired concentration were prepared by successive dilution in 0.1 M acetic acid except for the final dilution in artificial cerebrospinal fluid (aCSF) with 7 μM AA which mimicked the sample matrix of the microdialysis sample. The aCSF solution contained NaCl 144 mM, KCl 2.7 mM, CaCl_2 1.2 mM, MgCl_2 1 mM, NaH_2PO_4 2 mM at pH 7.4.

2.3 Electrochemical detection

A homemade working electrode block was used with a BASi radial-style thin-layer auxiliary electrode. The 1 mm diameter glassy carbon working electrode (HTW Hochttemperatur-Werkstoffe GmbH, Thierhaupten, Germany) was sealed in a Kel-F block with Spurr low viscosity epoxy resin (Polyscience, Inc., Warrington, PA). Electrical contact was made by connecting the working electrode to a metal pin with silver epoxy H20E (Epoxy Technology Inc., Billerica, MA). The flow channel was defined by a 13 μm thick Teflon spacer. Before each day's experiments, the working electrode was wet polished with 0.05 μm γ -alumina slurry (Buehler Ltd., Lake Bluff, IL) and sonicated in Milli-Q water. The working electrode at 700 mV vs Ag/AgCl reference electrode was located directly opposite to the flow inlet. The detector was connected to the capillary column through a piece of 10-cm-long 25- μm -i.d. capillary tubing. Potential control and data collection were done by a BASi Epsilon potentiostat (West Lafayette, IN).

2.4 Animals and surgical procedures

All procedures involving animals were carried out with the approval of the Institutional Animal Care and Use Committee of the University of Pittsburgh. Male Sprague-Dawley rats (Hilltop; Scottsdale, PA) (250–375 g) were anesthetized with isoflurane (2% by volume) (Halocarbon Products Corporation; North Augusta, SC) and wrapped in a homoeothermic blanket (EKEG Electronics; Vancouver, BC, Canada) to maintain a body temperature of 37°C . The rats were placed in a stereotaxic frame (David Kopf Instruments; Tujunga, CA). Holes were drilled through the skull in the appropriate positions to expose the underlying dura and brain tissue. The dura was removed with a scalpel to allow for placement of the microdialysis probe into the brain tissue with minimal disruption to the surrounding blood vessels.

2.5 Guide cannula implantation

Rats underwent surgical preparation three days prior to microdialysis probe placement. Surgery was performed under aseptic conditions. Animals were positioned in a stereotaxic frame according to the atlas of Paxinos and Watson[29]. In addition to the holes in the skull for passage of a guide cannula (BASI, West Lafayette, IN) into the brain, four additional holes were drilled to accept four jeweler's screws. The cannula was anchored in place with acrylic dental cement. Animals were housed individually until they recovered from the surgery.

2.6 Microdialysis

Four millimeter membrane microdialysis probes were purchased (30 KDa, MD-2204, BASi, West Lafayette, IN) and conditioned prior to insertion by flushing with aCSF for 1 hour. FEP tubing 0.65 mm OD $\times$ 0.12 mm ID was used to connect the inlet and outlet ends of the microdialysis probe to a 1.0 mL gas-tight syringe. Microdialysis probes were lowered into the striatum using flat-skull coordinates (0.75 mm anterior to bregma, 2.5 mm lateral from midline and 7.0 mm below dura) over a 30 minute period. All probes were perfused with aCSF with 7 μ M of AA, pH 7.4, at a rate of 0.586 μ L/min. After a 2-h equilibration period, two baseline samples were collected at 1-h each and analyzed by HPLC. All baseline samples contained a final concentration of 0.05 M acetic acid. To elicit spreading depression (high K^+ concentrations), the microdialysis probe was perfused with 120 mM K^+ aCSF (NaCl 26.7 mM, KCl 120.0 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 1 mM, NaH_2PO_4 2 mM) in 7 μ M of AA for 20 minutes at rate of 0.586 μ L/min. During this time, 5 samples were collected at 20 minutes each in tubes containing 0.05 M acetic acid. After 20 minutes the perfusate was switched back to aCSF in 7 μ M of AA till the end of the experiment. Two additional samples were collected at 1 hour post high K^+ perfusion. All samples were placed on dry ice until analyzed by HPLC later on the same day, usually within an hour.

3. Results and discussion

3.1 Theory for optimization of a chromatographic system operating at maximum pressure and with a choice of particle diameter

This section is a brief recapitulation of Carr et al. [20] to establish certain relationships among variables. In that work, and Poppe's work, one can find the optimum values of column length L and interstitial linear velocity u_e to give the maximum N once d_p , t_0 , and P are specified. Alternatively, one could find the optimum values of L and u_e to give the minimum t_0 once d_p , N , and P are specified.

Equation 1 gives interstitial linear velocity u_e as a function of void time t_0 and column length L :

$$u_e = \frac{u}{\lambda} = \frac{L}{\lambda t_0} \quad (1)$$

where λ is the ratio of interstitial and total porosity. Interstitial linear velocity u_e is used here in accordance with Carr's work [20] and for its relevance to mass transport in relationships between plate height and mobile phase velocity. Equation 2 gives length, L , as a function of pressure drop over the whole column, P , particle diameter, d_p , and u_e .

$$L = \frac{d_p^2 P}{\phi \eta(T) u_e} \quad (2)$$

Here, ϕ is the flow resistance factor (relating specifically to u_e), and $\eta(T)$ is the mobile phase viscosity at temperature T . Then u_e and L can be expressed as functions of t_0 and d_p (and other parameters) below derived from eqs. 1 and 2.

$$u_e(t_0, d_p, T) = \sqrt{\frac{P}{\phi \eta(T) \lambda t_0}} d_p \quad (3)$$

$$L(t_0, d_p, T) = \sqrt{\frac{P\lambda t_0}{\varphi\eta(T)}} d_p \quad (4)$$

For the best chromatographic performance, we will operate at a certain maximum pressure,

$$P = P_{\max} \quad (5)$$

The plate number generated by a column with length L and velocity u_e is shown in eq. 6,

$$N = \frac{L}{H} = \frac{L}{hd_p} = \frac{L}{d_p(A + \frac{B}{v} + Cv)} = \frac{L}{d_p(A + \frac{BD_m(T)}{u_e d_p} + \frac{Cu_e d_p}{D_m(T)})} \quad (6)$$

Where H is the plate height, h is the reduced plate height, v is the reduced linear velocity, A , B , C are dimensionless terms in the van Deemter equation expressed in terms of reduced velocity, and $D_m(T)$ is the solute diffusion coefficient in the mobile phase at temperature T . Using eqs. 3–5 in 6 leads to eq. 7.

$$N(t_0, d_p, T) = \frac{\sqrt{\frac{P_{\max}\lambda t_0}{\varphi\eta(T)}}}{A + \frac{BD_m(T)}{d_p^2} \sqrt{\frac{\varphi\eta(T)\lambda t_0}{P_{\max}}} + \frac{Cd_p^2}{D_m(T)} \sqrt{\frac{P_{\max}}{\varphi\eta(T)\lambda t_0}}} \quad (7)$$

Note that eq. 7, which is equivalent to eq. 13 of [20], is now a function of t_0 , d_p and $P_{\max}$, as well as other parameters. As in Carr et al. [20], we use the van Deemter equation instead of Knox equation for algebraic simplicity. Using reduced A , B , and C is convenient because it allows equation (7) explicitly to show variables (e.g., d_p) that are important in optimization, and simplifies the application of a final result to a column with known parameters. Below we show that the fits of the two equations to the data are statistically the same. This equation could be used to determine the number of theoretical plates achievable with a particular d_p and t_0 (with the remaining parameters: $D_m(T)$, $\eta(T)$, λ , A , B , C , φ , and $P_{\max}$ known). Column length and mobile phase velocity are calculated using eqs. 3 and 4. In the current context, we do not expect to realize N theoretical plates; rather, we expect some increased band spreading from volume overload which we accept for achieving a certain sensitivity of the analysis. In this case, we will realize an apparent theoretical plate number, N_a , that will be sufficient for the separation but lower than the intrinsic plate number of the column, N , because of the volume overload required to maximize detection sensitivity.

3.2 Column diameter: an important variable

The following discussion is based on work by Mills et al.[25] and Colin and Guiochon[24]. We turn our attention to the degradation of column performance by volume overload in an isocratic chromatography system. Based on the column's intrinsic properties, given by variance σ_{col}^2 and additional variance from overload, σ_{ol}^2 , we define the apparent number of theoretical plates, N_a . Equation 8, describes the relationship between N_a and the intrinsic plate number generated by the column, N :

$$N_a = N(1 - \frac{\sigma_{ol}^2}{\sigma_{ol}^2 + \sigma_{col}^2}) = N(1 - \theta^2) \quad (8)$$

where θ^2 is the fractional contribution to the observed band variance from the extra-column effect. Other contributions to extra-column peak variance such as postcolumn reactor volume[30] are not included here. We need the relationship for the volume-based variance of the peak, σ_v^2 , based on the number of theoretical plates in terms of retention volume, V_r , (eq. 9)

$$\sigma_v^2 = \frac{V_r^2}{N} \quad (9)$$

Equation 10 relates injection volume to the sacrifice in column efficiency represented by θ and other column parameters [24]. It arises from using eq. 10 in the definition of θ with the assumption that σ_{ol}^2 is small compared to σ_{col}^2

$$V_{inj} = \frac{K\theta\pi\epsilon d_c^2 L(k'+1)}{4\sqrt{N}} \quad (10)$$

V_{inj} is the injection volume which gives a particular contribution (θ) to the band dispersion. K is a constant depending on the injection technique, ϵ is column porosity, L is the column length, and k' is the retention factor of the solute. Eq. 10 is valid when the sample solvent and the injection solvent are the same. Mills *et al.* [25] analyzed the situation in which the sample solvent is weaker than from mobile phase. Mills *et al.* showed that there are two effects to consider: one is the fact that the elution strength of the sample solvent is low resulting in a low velocity of the injected front. This effect requires that the term $(k'+1)$ in eq. 11 be replaced by $(k_0'+1)$ where k_0' is the value of the retention factor in the sample solvent. The other effect is that of the step gradient that occurs when the mobile phase reaches the column, compressing the injected solute zone. This effect requires multiplication

of eq. 10 by the ratio $\frac{(k_0'+1)}{(k'+1)}$. Thus, we find that the injection volume is

$$V_{inj} = \frac{K\theta\pi\epsilon d_c^2 L(k_0'+1)^2}{4\sqrt{N}(k'+1)} \quad (11)$$

From eq. 11, we can find a relationship for θ which when used with eq. 8 gives rise to the relationship between achieved plate number and injection volume

$$N_a = N - \frac{16(k'+1)^2 N^2 V_{inj}^2}{K^2 \pi^2 \epsilon^2 (k_0'+1)^4 L^2 d_c^4} = N - \frac{N^2 V_{inj}^2}{X^2 L^2 d_c^4} = N - \frac{N^2 V_{inj}^2}{X^2 \frac{t_0}{\tau(T)} d_p^2 d_c^4} \quad (12)$$

Where

$$X = \frac{K\pi\epsilon (k_0'+1)^2}{4(k'+1)}$$

and

$$\frac{\varphi\eta(T)}{P_{\max}\lambda} = \tau(T)$$

Eq. 12 shows that the column diameter and volume injected as well as t_0 and d_p are used to define N_a , whereas the intrinsic power of the column, expressed as N , only depends on t_0 and d_p (and other parameters that for a particular solute, mobile phase, and temperature are constants: X , $P_{\max}$, $\tau(T)$, ϕ , $D_m(T)$, and the terms in the van Deemter equation).

The foregoing is completely general. In the context of the problem we posed in the introduction, N_a is a known quantity – the number of theoretical plates required for quantitative determination of the analyte. Thus, we derive eq. 13 from eqs. 4, 8, 11 and 12. This expression for d_c depends on t_0 , d_p , T , and V_{inj} (and other parameters that for a particular solute, mobile phase, and temperature are constants: X , $P_{\max}$, $\tau(T)$, ϕ , $D_m(T)$, and the terms in the van Deemter equation).

$$d_c(t_0, d_p, V_{inj}, T) = \sqrt{\frac{4\sqrt{N}(k'+1)V_{inj}}{K\theta\pi\epsilon L(k_0'+1)^2}} = \sqrt{\frac{V_{inj}}{X\sqrt{\frac{t_0}{\tau(T)}d_p}} \frac{N(t_0, d_p, T)}{\sqrt{N(t_0, d_p, T)} - N_a}} \quad (13)$$

3.3 Influence of chromatographic optimization on sensitivity

We now consider the sensitivity of the detector and its relationship to the chromatography. We use an electrochemical detector, but we will derive the necessary relationships in general terms. We use the symbol i generically for “signal”. The instantaneous signal depends on the mobile phase flow rate F_m to some power, α .

$$i = kC_{in}F_m^\alpha \quad (14)$$

where k is a sensitivity factor that need not concern us and C_{in} is the analyte concentration in the mobile phase entering the detector. The observed concentration sensitivity is the ratio of the signal to the concentration in the injected sample

$$S = \frac{i}{C_s} = \frac{kC_{in}F_m^\alpha}{C_s} \quad (15)$$

where C_s is the analyte concentration. Eq. 15 expresses the well-known fact that the observed concentration sensitivity is influenced by chromatographic band spreading and may be dependent on the flow rate of the chromatographic mobile phase. We can relate the concentration in the detector, conceptually, to the number of moles of analyte injected into the column and the peak volume. The peak volume is proportional to the volume standard deviation, σ_v , regardless of the details of the peak shape. We do not lose insight from calculating proportionalities rather than equalities, so we can drop the unknown detector sensitivity factor and the proportionality between peak standard deviation and peak volume that depends on peak shape.

$$S \propto \frac{C_{in}F_m^\alpha}{C_s} \quad (16)$$

We can express C_{in} as being proportional to the moles injected divided by the peak volume,

$$\frac{C_s V_{inj}}{\sigma_v} \quad S \propto \frac{C_s V_{inj} F_m^\alpha}{C_s \sigma_v} = \frac{V_{inj} F_m^\alpha}{\sigma_v} \quad (17)$$

The volume standard deviation can be expressed in terms of theoretical plate count and retention volume as in eq. 9, except that here we are considering the peak width in an actual analysis, so N_a replaces N . In addition, we write the retention volume as $t_0(k' + 1)F_m$.

$$S \propto \frac{\sqrt{N_a} V_{inj} F_m^\alpha}{t_0(k' + 1)F_m} \quad (18)$$

And finally, combining the flow rate terms, we have eq. 19.

$$S \propto \frac{\sqrt{N_a} V_{inj} F_m^{\alpha-1}}{t_0(k' + 1)} \quad (19)$$

What remains is to replace flow rate with its equivalent in terms of other variables (here ϵ is the total column porosity):

$$F_m = \frac{u_e \lambda \pi \epsilon d_c^2}{4} \propto u_e \lambda \epsilon d_c^2 \propto \sqrt{\frac{P \lambda}{\varphi \eta(T) t_0}} d_p \epsilon d_c^2 \propto \sqrt{\frac{1}{\tau(T) t_0}} d_p \epsilon d_c^2 \quad (20)$$

$$S \propto \frac{\sqrt{N_a} V_{inj} \left(\sqrt{\frac{1}{\tau(T) t_0}} d_p \epsilon d_c^2 \right)^{\alpha-1}}{t_0(k' + 1)} \quad (21)$$

For the electrochemical detector under many conditions the value of α is 1/3 [26–28]. Thus, for the electrochemical detector, the sensitivity is

$$S_{el}(t_0, d_p, V_{inj}, T) \propto \frac{\sqrt{N_a} V_{inj} \tau(T)^{1/3}}{t_0^{2/3} (k' + 1) (d_p \epsilon d_c(t_0, d_p, V_{inj}, T))^2} \quad (22)$$

In eqs. 22, sensitivity is a function of the independent variables t_0 , d_p , V_{inj} and T . To achieve any combination of t_0 , d_p and T , we will work at the column length and velocity determined by eqs. 3 and 4. This length and velocity will generate N theoretical plates (eq 7) in the limit of small injection volume. For an injected sample volume V_{inj} and required apparent plate number N_a , we will work at the column diameter determined by equation 13. The incorporation of column diameter d_c adds another degree of freedom for the optimization.

3.4 Experimental determination of fixed parameter values

Table 1 shows the fixed parameters, how they were determined, and the parameter values. Eq. 2 shows that the slope of a P vs u_e plot will yield the column flow resistance factor for a column at 70 °C (constant d_p , $\eta(T)$ and L). This value, relating to mobile phase linear

velocity, u , is 462. Thus, the flow resistance factor, ϕ , relating to u_c is 289. The A , B , and C terms were experimentally determined by building up a van Deemter plot (incorporating reduced variables) followed by regression (Figure 1, Table 2). The van Deemter and Knox fits are both good and have essentially identical values of R^2 . From our previous experience, N_a is 2200 for sufficient separation. Thus, A , B , C , ϕ and N_a are determined by experience or experiment. P_{max} , 41.37 MPa (6000 psi), is governed by our equipment. Although the maximum pressure capability of the pump is 10,000 psi, the pressure generated from the pump's internal flow path is as high as 2100 psi at a flow rate of 12 $\mu\text{L}/\text{min}$. In consideration of this and that we want to work at a pressure a little lower than the pressure limit, P was chosen as 6000 psi in the calculation. Typical values of λ are in the range of 0.6–0.7 so here we use 0.625. For viscosity, we used the form of an empirical relationship for viscosity as a function of composition and temperature [31]. A nonlinear regression of that empirical function with the apparent integral viscosities at 400 bar for water-acetonitrile between 20 and 100 °C from Billen *et al.* [32] resulted in eq. 23

$$\eta = 10^{(-2.533 + (742/T - 0.452X_{acn} + (235/T)X_{acn} + 1.573X_{acn}^2 - (691/T)X_{acn}^2))} \quad (23)$$

where X_{acn} is the volume fraction of acetonitrile in the mobile phase. The diffusion coefficient D_m of 5-HT is $5.4 \times 10^{-6} \text{ cm}^2/\text{s}$ at 298 K [33]. D_m at other temperatures is calculated based on empirical equation 24.

$$D_m(T) = 5.4 \times 10^{-6} \times \frac{T \times \eta(298)}{298 \times \eta(T)} \text{ cm}^2/\text{s} \quad (24)$$

Finally, we have experimentally determined the effect of injection volume on efficiency based on equation 12. A regression of N_a on V_{inj}^2 will reveal the lumped parameter X which can then be used in optimization (Figure 2). The regression yields the relationship (V_{inj} is in nanoliters)

$$N_a = 6005 - 0.0187V_{inj}^2 \quad (R^2 = 0.928) \quad (25)$$

and

$$X = \frac{K\pi\epsilon(k'_0 + 1)^2}{4(k' + 1)} = 87.94$$

at 343 K. Our primary conclusions are for $T = 343 \text{ K}$, so X can be considered as a constant. In practice, changes in temperature are often accompanied by changes in mobile phase composition, so that k' may be more or less constant. However, k'_0 will be temperature dependent. Thus, X is temperature dependent in general.

3.5 Finding the optimum conditions

Now that we have all the parameters, we will first explore briefly the variables T , V_{inj} and d_p . Fig. 3 encapsulates, with just a few curves, the effect of these variables on sensitivity and speed according to equation 22. Note that each curve has a clear maximum, dropping off steeply to the short time side. We find the maximum sensitivity at a t_0 that is near the minimum accessible value. Thus, striving for maximum sensitivity does not require a

significant sacrifice in speed. Comparison of the green and red curves shows that the higher temperature (383 K) permits a higher sensitivity and a potentially faster separation in comparison to the lower temperature (343 K) all else being equal. Comparison of the red and black curves shows that a modest change (red: 500 nL; black: 250 nL) in injected volume decreases the sensitivity, but has little effect on the speed. Comparison of the red (1.7 μm d_p) and blue (2.6 μm d_p) curves show that the smaller particle diameter improves speed and sensitivity. Therefore, we can conclude that with the goal of higher sensitivity and speed, we should work at conditions with the highest practical T , largest V_{inj} and smallest d_p . Prior work guided us to choose 70 °C (343 K) as the column temperature which is the highest working temperature with good column selectivity and stability[21]. Although larger sample volume benefits the detection sensitivity, it also relates to the time resolution of the analysis. Here, a sample volume of 500 nL was used based on experience which provides a sampling time resolution of less than 1 minute for typical microdialysis flow rates. Also, the sub-2 μm porous particles are currently the smallest commercially available porous particles which is our best choice. Given the pragmatic constraints mentioned above, we will choose to operate at $T = 343$ K, use 1.7 μm particles, and inject 500 nL.

The column diameter is a key parameter in this optimization. In the current context where we seek optimum conditions, it is not independently variable. It depends, like sensitivity, on t_0 , d_p , V_{inj} , and T (as well as the parameters in Table 1). For a given temperature and injection volume, there is a single value of S_{el} corresponding to a pair of values (t_0 , d_p). The same is true of column diameter. Thus, it is instructive to consider S_{el} and d_c as a function of t_0 given d_p , V_{inj} , and T . Figure 4a shows how sensitivity (with V_{inj} and T fixed at the values stated above) depends on column diameter and void time. Each curve corresponds to a different particle diameter. It is hard to visualize the curve in three dimensions, so we have plotted in Fig. 4b the projections of each curve on the three two-dimensional planes. Table 3 shows the range of laboratory parameters that will provide a sensitivity within 3% of the maximum.

Some general observations may be made. Smaller particle diameter results in higher sensitivity at optimum conditions. The column diameter is an important variable. Note that in Figure 4 significant sensitivity is lost by using columns with a diameter of 500 μm . On the other hand, when the column diameter is too small the column length must increase to avoid volume overload and maintain N_a . This is reflected in the simultaneous decrease in sensitivity and increase in void time when column diameter becomes small. Note that the void time is completely independent of the column diameter for column diameters larger than some value that depends on the particle diameter. This reflects the point where volume overload is negligible, *i.e.*, $N = N_a$.

3.6 Application of the optimized system to microdialysis

We use 1.7 μm BEH C 18 particles. Considering both sensitivity and speed from Fig. 4 and Table 3, the column diameter is 150 μm with a length of 3.1 cm. Figure 5 demonstrates results from this column. The total analysis time for 5-HT and 3-MT was reduced to about 0.6 minutes with t_0 of 1.5 s and a plate number of 2450. The flow rate is 12 $\mu\text{L}/\text{min}$ and the reduced linear velocity v is 32. The total pressure is 8354 psi while the back pressure generated by the column is 6204 psi. We note that the plate count and column pressure are close to the values specified in the optimization (2200 and 6000 psi, resp.). This supports the reproducibility of our column packing, as Figs. 1 and 2 are from different columns created before packing the optimally sized columns.

We first applied the system to offline measurements. The system proved excellent within-day retention and sensitivity reproducibility. The average retention time of 5-HT peaks in standards and dialysate samples from one typical day-long experiment is 22.5 ± 0.1 s based

on 47 injections. One concern often expressed about electrochemical detection is a slow decrease in sensitivity commonly attributed to electrode fouling. The change in the active electrode surface area and surface properties may cause this sensitivity drift. Therefore, the detection sensitivity at the beginning and end of a whole day's experimentation (typically 7–9 hours, 50–80 injections) was investigated. The sensitivity loss for 5-HT is in the range of 2–5%. This relatively small sensitivity change of about 0.5% per hour can be ignored for experiments requiring an hour or less. For longer experiments, pre- and postcalibration should be used.

The electrochemical detector responds linearly in the concentration range from 0.5 nM to 100 nM (5-HT: $R^2=0.9992$; 3-MT: $R^2=0.9994$). Therefore, one point at the very high concentration (100 nM) was used for 5-HT and 3-MT concentration calibration. Limits of detection for 5-HT and 3-MT are 0.3 nM and 1.0 nM respectively (signal three times greater than rms noise).

With the conditions and method established, we then determined 5-HT in rat brain microdialysate (Fig. 6). The total analysis time is about 36 s with 5-HT eluting at 22.7 s and 3-MT eluting at 33.3 s. The basal 5-HT and 3-MT concentrations are 0.67 nM and 2.62 nM. Confirmation of the 5-HT peak was done by spiking samples with an equal volume of a 5 nM standard (Fig. 6). Confirmation of the peak identity can also be done using the physiological response to elevated extracellular K^+ concentration. Samples were collected before, during and after high K^+ stimulation at 20-min intervals (Figure 7). The increased extracellular K^+ concentration induced a significant increase of the 5-HT concentration in the dialysate[34].

The temporal resolution of the 5-HT separation is now 36 s with 500 nL dialysate samples. This makes it possible to imagine online measurements every 36 s with a perfusate flow rate of 0.83 μ L per minute (*i.e.*, 500 nL/0.6 minutes). Since our goal is online analysis, the feasibility of continuous analysis was done by making 5 consecutive injections of basal dialysate samples with 0.6-min time intervals (Figure 8). The average 5-HT concentration was 0.66 ± 0.03 nM and the average 5-HT peak retention time was 22.7 ± 0.1 s based on the five injections. The small standard error shows excellent reproducibility of sample loading, separation and detector response which are important parameters for online analysis. There are no late-eluting peaks from early injections that impact later injections.

4. Conclusions

The optimization of an HPLC system generally pursues the speed or efficiency of a separation. However, the theory regarding optimization, as valuable as it is, does not optimize conditions for an isocratic trace analytical method based on chromatography. Specifically, we take into account the ubiquitous procedure of on-column preconcentration as part of the overall optimization. A sample volume that is too large will sacrifice the separation speed, separation efficiency and analysis temporal resolution when sampling from flowing streams at a constant rate. A sample volume that is too small may result in undetected compounds. We demonstrate that the column diameter is an important parameter in the optimization of conditions to attain high speed and the best possible sensitivity for a given sample volume with a certain required number of theoretical plates. For an electrochemical detector, the maximum concentration sensitivity is achieved with a particular t_0 and corresponding d_c . Smaller particle size and higher temperature provides smaller optimum t_0 and greater sensitivity which means higher speed and better detection limit. The theory was successfully used for optimization of serotonin measurements using online microdialysis coupled with capillary HPLC-EC. Careful optimization is required because our goal is to improve temporal resolution, but we must not reduce the method's

concentration sensitivity since the sample size is limited and concentration detection limit is important. With the optimized system, we demonstrated basal 5-HT measurements in rat microdialysate samples offline in 36 s. The consecutive analysis provides strong support for successful online analysis.

Acknowledgments

We appreciate the financial support from the National Institute of Mental Health through grant MH083134 (SGW). We also thank Dr. Ed Bouvier of Waters Corp. for the gift of packing materials.

References

1. Schultz KN, Kennedy RT. *Annu Rev Anal Chem.* 2008; 1:627–661.
2. Perry M, Li Q, Kennedy RT. *Anal Chim Acta.* 2009; 653:1–22. [PubMed: 19800472]
3. Davies MI, Cooper JD, Desmond SS, Lunte CE, Lunte SM. *Adv Drug Delivery Rev.* 2000; 45:169–188.
4. Cukalovic A, Monbaliu JCMR, Stevens CV. *Top Heterocycl Chem.* 2010; 23:161–198.
5. Fang H, Xiao Q, Wu F, Floreancig PE, Weber SG. *J Org Chem.* 2010; 75:5619–5626. [PubMed: 20666502]
6. Frost CG, Mutton L. *Green Chem.* 2010; 12:1687–1703.
7. Weiler A, Junkers M. *Pharm Technol.* 2009; S6:S10–S11.
8. Wiles C, Watts P. *Spec Chem Mag.* 2009; 29:40–41.
9. Wiles C, Watts P. *Adv Chem Eng.* 2010; 38:103–194.
10. Davis JM, Stoll DR, Carr PW. *Anal Chem.* 2008; 80:461–473. [PubMed: 18076145]
11. Potts LW, Stoll DR, Li X, Carr PW. *J Chromatogr A.* 2010; 1217:5700–5709. [PubMed: 20673902]
12. Stoll DR. *Anal Bioanal Chem.* 2010; 397:979–986. [PubMed: 20369231]
13. Newton AP, Justice JB Jr. *Anal Chem.* 1994; 66:1468–1472. [PubMed: 8017633]
14. Nagata T, Nishiyama A, Yamato T, Obata T, Aomine M. *Nutr Neurosci.* 2011; 14:96–105. [PubMed: 21756530]
15. Morita M, Nakayama K. *Psychiatry Clin Neurosci.* 2011; 65:246–253. [PubMed: 21507131]
16. Kleijn J, Cremers TIFH, Hofland CM, Westerink BHC. *Neurosci Res.* 2011; 70:334–337. [PubMed: 21420449]
17. Knox JH, Saleem M. *J Chromatogr Sci.* 1969; 7:614–622.
18. Poppe H. *J Chromatogr A.* 1997; 778:3–21.
19. Desmet G, Clicq D, Gzil P. *Anal Chem.* 2005; 77:4058–4070. [PubMed: 15987111]
20. Carr PW, Wang X, Stoll DR. *Anal Chem.* 2009; 81:5342–5353. [PubMed: 19505090]
21. Liu Y, Zhang J, Xu X, Zhao MK, Andrews AM, Weber SG. *Anal Chem.* 2010; 82:9611–9616. [PubMed: 21062014]
22. Neue, UD. *HPLC Columns: Theory, Technology, and Practice.* Wiley-VCH; 1997. p. 51
23. Poole, CF.; Schuette, SA. *Contemporary Practice of Chromatography.* Elsevier; Amsterdam: 1984. p. 866
24. Guiochon, G.; Colin, H. *Microcolumn High-Performance Liquid Chromatography.* Elsevier; Amsterdam: 1984. p. 1
25. Mills MJ, Maltas J, Lough WJ. *J Chromatogr A.* 1997; 759:1–11.
26. Weber SG. *J Electroanal Chem Interfacial Electrochem.* 1983; 145:1–7.
27. Elbicki JM, Morgan DM, Weber SG. *Anal Chem.* 1984; 56:978–985. [PubMed: 6742436]
28. Weber SG, Long JT. *Anal Chem.* 1988; 60:903A–904A. 906A–913A.
29. Paxions, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates.* Academic Press; New York: 1998.
30. Xu H, Weber SG. *J Chromatogr A.* 2009; 1216:1346–1352. [PubMed: 19150071]

31. Guillarme D, Heinisch S, Rocca JL. *J Chromatogr A*. 2004; 1052:39–51. [PubMed: 15527119]
32. Billen J, Broeckhoven K, Liekens A, Choikhet K, Rozing G, Desmet G. *J Chromatogr A*. 2008; 1210:30–44. [PubMed: 18834987]
33. Gerhardt G, Adams RN. *Anal Chem*. 1982; 54:2618–2620.
34. Jackson D, Abercrombie ED. *J Neurochem*. 1992; 58:890–897. [PubMed: 1371152]

Highlights

Our goal is system optimization for speed and concentration sensitivity.

Concentration sensitivity depends on the injection volume.

There is an optimum column diameter for a given injection volume.

T , d_p , V_{inj}/d_c and t_0 regulate concentration sensitivity for a required N_a .

Serotonin (~ 0.5 nM) in microdialysate samples ($0.5 \mu\text{l}$) in less than 30 s.

Figure 1.
The reduced plate height vs. reduced velocity (interstitial) generated from a column.

Figure 2.

Apparent plate number measured at different injection volumes. Column: 100 μm inside diameter, 5.0 cm long capillary column packed with 1.7 μm BEH C 18 particles. Mobile phase: 100 mM sodium acetate, 0.15 mM disodium EDTA, 10.0 mM SOS, pH=4.0, mixed with 4% (v/v) acetonitrile. Flow rate: 4.0 $\mu\text{L}/\text{min}$. Column temperature $T = 343\text{ K}$.

Figure 3.
Effect of particle size, injection volume and column temperature on concentration sensitivity and separation speed (void time t_0).

Figure 4. Concentration sensitivity as a function of void time t_0 and column diameter d_c with different particle sizes. Left: a line for each particle diameter in three dimensions. Right: projections of each of the latter curves in each of the two-dimensional planes.

Figure 5.

Typical chromatogram of a 500 nL standard injection sample. Red: standard sample containing 2 nM 5-HT, 2 nM 3-MT and 7 μ M AA. Black: blank aCSF solution. Column: 150- μ -i.d., 3.1-cm-length capillary column packed with BEH C 18 particles. Mobile phase: 100 mM sodium acetate, 0.15 mM disodium EDTA, 10.0 mM SOS, pH=4.0, mixed with 4% (v/v) acetonitrile. Flow rate: 12.0 μ L/min. Column temperature: 70 $^{\circ}$ C. Electrochemical detection.

Figure 6.

Determination of basal 5-HT and 3-MT in rat brain microdialysate. Separation conditions were the same as in Figure 5. Black: blank aCSF solution. Red: basal microdialysate sample collected from striatum of rat brain with a perfusion flow rate of 0.6 μ L/min. Blue: 1:1 volume ratio mixture of basal microdialysate sample and 5 nM standard.

Figure 7. Monitoring of 5-HT concentration followed by high K^+ stimulation. Administration: 120 mM K^+ aCSF solution for 20 min. Samples were collected and analyzed offline. Separation and sampling conditions were the same as in Figure 6.

Figure 8.

Chromatograms of samples with consecutive injections. Injection intervals: 0.6 minutes. Separation and sampling conditions were the same as in Figure 6.

Table 1

Fixed parameters involved in the optimization.

Parameter	Determination	Value
ϕ	pressure vs u_e at T=343 K	289
A, B, C	h vs v at T=343 K	See Table 2
N_a	experience	2200
$P_{\max}$	instrument	41.4 MPa (6 kpsi)
λ	literature	0.625
D(298K)	literature	$5.4 \times 10^{-10} \text{ m}^2\text{s}^{-1}$ (T=298 K)
$\eta(T)$	literature	See eq. 24
X	N_a vs V_{inj} at T=343 K	87.94, See Fig. 2

Table 2

Nonlinear fit of the van Deemter plot using theoretical equations.

	A	B	C	R ²	equations
van Deemter	1.4 ± 0.2	9.4 ± 0.4	0.12 ± 0.01	0.968	$h = 1.4 + 9.4/v + 0.12v$
Knox	0.8 ± 0.1	10.3 ± 0.3	0.08 ± 0.01	0.960	$h = 0.8v^{1/3} + 10.3/v + 0.08v$

Table 3

Optimum chromatographic parameters' range for 97–100% of the maximum sensitivity with different particle size with 500 nL injection volume and working at 343 K.

$d_p/\mu\text{m}$	Sensitivity	$u_e/\text{cm/s}$	L/cm	t_0/s	$d_e/\mu\text{m}$
1.0	7.48–7.71	1.8–1.4	1.2–1.4	0.67–1.00	237.8–194.9
1.7	5.82–6.00	2.0–1.7	3.0–3.6	1.49–2.18	148.0–122.3
2.6	4.57–4.71	2.1–1.8	6.6–7.9	3.13–4.53	98.9–82.3
3.5	3.82–3.94	2.2–1.8	11.6–14.0	5.39–7.78	74.4–61.9
5.0	3.06–3.16	2.2–1.8	23.3–27.9	10.56–15.17	52.5–43.8