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Freeze-Drying as Sample Preparation for Micellar Electrokinetic Capillary Chromatography – Electrochemical Separations of Neurochemicals in *Drosophila* Brains

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Abstract

Micellar electrokinetic capillary chromatography with electrochemical detection has been used to quantify biogenic amines in freeze-dried *Drosophila melanogaster* brains. Freeze drying samples offers a way to preserve the biological sample while making dissection of these tiny samples easier and faster. Fly samples were extracted in cold acetone and dried in a rotary evaporator. Extraction and drying times were optimized in order to avoid contamination by red-pigment from the fly eyes and still have intact brain structures. Single freeze-dried fly-brain samples were found to produce representative electropherograms as a single hand-dissected brain sample. Utilizing the faster dissection time that freeze drying affords, the number of brains in a fixed homogenate volume can be increased to concentrate the sample. Thus, concentrated brain samples containing five or fifteen preserved brains were analyzed for their neurotransmitter content, and five analytes; dopamine *N*-acetyltyramine, *N*-acetylserotonin, *N*-acetyltyramine, *N*-acetyldopamine were found to correspond well with previously reported values.

Keywords

Capillary electrophoresis; MEKC; drosophila brain; electrochemical detection; freeze drying; sample preparation

INTRODUCTION

Drosophila melanogaster, the fruit fly, develops from larva to sexually-mature adult relatively quickly (approximately 12–14 days), is easily maintained due to its small size, and contains functional genes orthogonal to 75% of all human genes and about 60% of genes responsible for diseases in humans.¹ Although the fly has a simpler nervous system than those of vertebrates, it is still capable of higher-order brain functions, such as learning and memory,^{2–4} and utilizes many of the same molecular components that mammals use within their nervous systems. Biogenic amines, including dopamine, serotonin, and tyramine, have been shown to have roles in physiological processes found in both mammalian and

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Drosophila systems.^{5–9} Octopamine is thought to control many of the same behaviors in the fly as norepinephrine regulates in mammals¹⁰ and is thus considered homologous to norepinephrine in mammals.⁵ Its simplicity combined with similarity to human systems has made flies one of the best models for the study of biological systems.¹¹ A plethora of genetic mutants of *Drosophila* have been developed to study neurodegenerative diseases, such as Alzheimer's disease,^{12–15} Huntington's disease,^{16–17} and Parkinson's disease.^{18–20} Yet, many of the neurochemicals associated with these diseases occur in minute amounts that can be difficult to detect. As such, it is essential to develop highly sensitive and accurate methods for this unique biological system.

Several methods have been developed and used to measure neurochemicals within *Drosophila*, including *in vivo* electrochemistry,^{21–25} high-performance liquid chromatography (HPLC),^{8, 26–28} and capillary electrophoresis (CE).^{29–34} Previous separation methods to study biogenic amines in *Drosophila melanogaster* have utilized a wide variety of sample homogenization techniques. Initial capillary electrophoresis procedures utilized large numbers of whole fly heads, homogenized with glass tissue homogenizers, and any high mass species were filtered with molecular filters.^{27–28, 30, 35} Although the increased sample size helps to reduce individual fly-to-fly variability, a large amount of red pigment from the eyes is left within the sample, which can overwhelm the analysis.³³ Mutants, such as the *white* mutant¹¹ which has a nullexpressed ABC transporter (ATP-binding cassette transporter), have been analyzed in an attempt to genetically remove the pigment signal;²⁶ however, care must be taken to assure that the mutant has similar activity as its wild-type counterpart. Dissection of individual brain regions is another approach that can be used to effectively and completely remove the eye-pigment,^{8, 26, 33} but dissections of fly heads can be slow and challenging.

Freeze-drying is a process where a sample is frozen and dehydrated under reduced pressure, forcing the water within the sample to sublime directly into the gas phase. This procedure has long been used as a method in biological research to preserve both the structure and molecular content of samples.³⁶ By quickly freezing the sample, the formation of large ice crystals, which can puncture cells, is prevented. When the water is removed by sublimation, the sample is left behind relatively undisrupted and preserved. Samples can then be collected and stored for later analysis. The small size of the fly and short life-cycle permit a large number of samples to be acquired, and preserved brains can be easily and quickly dissected after freeze-drying. As the process also helps to minimize enzyme activity,³⁷ other molecules found within the brain, such as proteins,³⁷ phospholipids,³⁷ and catecholamines,³⁸ have been quantitatively recovered and analyzed. However, the nature of the procedure makes it likely that it will be dependent on several variables related to sample drying and storage time.

In this paper, we present methodology to couple the freeze-dry procedure to micellar electrokinetic chromatography (MEKC) with amperometric electrochemical detection (EC) for the analysis of biogenic amines in *Drosophila*. The effects of drying and storage time, and water extraction time have been examined. The data presented show that the freeze-dry procedure is reliable under controlled conditions for fly brain samples leading to improvement in detection signal in populations of dissected fly brains.

EXPERIMENTAL SECTION

Reagents

Sodium hydroxide (NaOH) was obtained from Fluka (Buchs, Switzerland) and perchloric acid (HClO₄) from Riedel-de Haën (Morristown, NJ, USA). *N*-acetylaminedopamine, *N*-acetylamineoctopamine, and *N*-acetylaminetyramine were obtained from the National

Institutes of Mental Health (NIMH) chemical synthesis and drug supply program (Research Triangle Park, NC, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). All standards were prepared weekly as 10 mM stock solutions in 0.1 M perchloric acid, stored at 4 °C, and diluted to the desired concentration with additional 0.1 M perchloric acid.

***Drosophila* Maintenance and Preparation**

Canton-S wild-type *Drosophila melanogaster* strains were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN, USA). Male flies were cultured on standard potato meal/agar medium and collected for homogenization 4 to 5 days after emerging from pupal cages.

Flies were prepared for CE experiments using methods adapted from protocols previously described.^{29, 37–38} Freeze-dried fly brain samples were prepared by collecting flies in 15-mL centrifuge tubes, plunging them into liquid nitrogen, and vortexing them for 60 s to separate the heads from the body. Heads were counted, collected, and placed in a 10 mL round bottom flask filled with 10 mL acetone. The flasks were then frozen in liquid nitrogen and thawed in a water bath three times before being stored in the freezer (–80 °C) for 3 to 7 days. Upon removal from the freezer, the acetone was evaporated with a rotary evaporator at the lowest speed setting and a cold-water bath. The fly heads were then placed on the stage of an Olympus SZ40 stereoscope (Melville, NY, USA). Using small dissecting forceps (Fine Science Tools, Heidelberg, Germany), each cuticle was cracked and the freeze-dried brain extracted.

For fresh-tissue dissections, fly brains were removed using methods described previously.^{33, 39} Briefly, flies were immobilized on ice for 10 s, dipped in a bath of cold 95% ethanol for 30 s to remove their waxy coating, and transferred to a petri dish containing 0.1 M perchloric acid on ice. Fly brains were then dissected using small, dissecting forceps making sure to eliminate any remnants of the air sacs, eyes, or cuticle debris. All fresh brain dissections were performed in less than 5 min to minimize enzymatic degradation.

Dissected brains were collected in 200-μL PCR tubes (VWR International, West Chester, PA, USA) with 1.0 μL of 0.1 M perchloric acid/25–100 μM catechol (internal standard) and were centrifuged (Galaxy Mini, VWR International) at 10,000 g for 30 s. The heads were then ultrasonicated (Sonics & Materials, Inc, Satigny, Switzerland) with a 2-mm microtip at 100% amplitude for 120 s in a cold-water bath. The sample was centrifuged for 30 s and promptly injected onto the capillary for separation and analysis.

Instrumentation and Procedures

The CE system with end-column amperometric EC detection utilized in this study was built in-house and has been described previously.⁴⁰ Briefly, 45 to 50 cm of fused-silica capillary with an outer diameter of 151 μm and an inner diameter of 14 μm (Polymicro Technologies, Phoenix, AZ, USA) was used. To enhance microelectrode placement, the capillary inner diameter at the detection end was enlarged via HF etching as previously described.⁴⁰ Briefly, approximately 2 mm of polyimide coating was burned off from the capillary to expose the fused silica. The exposed portion of the capillary was placed in HF for 15 min with a pressure of 3.5 MPa (N₂) through the capillary. Subsequently, the same segment of capillary was placed in a sodium bicarbonate solution to neutralize the acid and then in water to remove excess salt. Capillaries were filled with separation buffer, using a stainless steel reservoir with applied N₂ pressure (3.5 MPa).³² Injections were performed electrokinetically at 5 kV for 5 s to inject ~1 nL of sample from the homogenate.

Separations were performed in 25 mM borate buffer containing 50 mM SDS and 2% 1-propanol and adjusted to a pH of 9.52 with 0.1 M NaOH.

Amperometric EC was carried out with a two-electrode format in which a 5- μ m diameter, 500 μ m long, cylindrical, carbon-fiber microelectrode was held +0.75 V versus a Ag/AgCl reference electrode (Harvard Apparatus, Holliston, MA, USA). Detection current was measured using a Keithley model 427 current amplifier (Cleveland, OH, USA) and recorded at 10 Hz using LabView 8.0 (National Instruments, Austin, TX, USA) software, written in-house. The electropherograms were exported to ascii format and loaded into the Origin software (v8.5, Originlab, New Brunswick, MA, USA) for baseline correction, peak picking and peak integration using the implemented peak analyzer function. The peak integration data were exported to MS Excel (v.2010) for statistical analysis by means of student's t-test (unpaired, two-tailed). Significance testing was set for a value of $p < 0.05$.

Safety Considerations

A safety interlock box was built in house and utilized to protect the user from high voltage. Since HF can cause severe burns, it was used with extreme care in a fume hood with proper eye, skin and ventilation precautions.

RESULTS AND DISCUSSION

Freeze Drying *Drosophila* Heads

Morphologically examined freeze-dried fly heads are well preserved with no apparent fractures within the cuticle or pieces missing from the head (Figure 1a). When microforceps are used to disrupt and to remove the cuticle (Figure 1c), it is easy to crack and separate, having become significantly more brittle than a non-freeze-dried specimen, making dissections faster and simpler to perform. Once the cuticle is opened, the central brain and optic lobes (Figure 1d) are easily lifted out and collected for analysis by MEKC-EC.

In non-freeze-dried samples, the cuticle is quite springy and resistant to puncture. Therefore, significant time is spent positioning the forceps to grab and hold the head properly before being able to open the cuticle. The two halves of the cuticle must be pulled apart from one another with the force applied being enough to completely expose the brain but gentle enough to ensure that the brain regions are left intact. In fresh specimens, care must also be taken to not disrupt the compound eyes whose pigment upon agitation can dissolve into the dissecting solution and cover the brain. Even after a full dissection, many air sacs from these regions can still surround the brain, which can impede visual confirmation that the brain is indeed whole. Dissection in a liquid may also lead to variability in brain samples, regardless of care taken, as the volume of dissecting solution associated with the freshly dissected brain can be highly variable at such low volumes.

In contrast to fresh dissections, freeze-dried heads can be easily used with a pair of forceps to remove enough of the cuticle and to reach the brain without the need for a dissecting liquid. The surrounding air sacs attached to the brain tissue and inner wall of the cuticle are disconnected after freeze drying allowing the intact brain to be easily lifted from the shell of the cuticle. If pieces of the compound eye are attached to the optic lobes (Figure 1c), these pieces are generally large flakes that can easily be lifted off the brain sample with forceps. Brains can then be transferred from the air to the sample buffer without possible further dilution.

Following dissection and extraction, samples are separated and analyzed by MEKC and amperometric electrochemical detection (Figure 2). A standard sample containing 13 biogenic amine neurotransmitters was first separated (Figure 2a–I). Subsequent analysis of

the fly samples (5 freeze-dried brains in 5 μL of 25 μM catechol/0.1 M HClO_4) allows identification of different neurotransmitters based on their migration time (Figure 2b). Moreover the experimental system has proven to be very robust and reproducible in terms of peak area and peak migration time (Figure 2a-II–IV).

Effect of freeze drying on signal intensity

Separations of a single hand-dissected brain (Figure 3a) have been compared to a single freeze-dried brain (an example is shown in Figure 3b). As with the hand-dissected brain sample, the large, overloading peaks from the eyes are eliminated in the freeze-dried brain sample. Despite a slightly noisier electrode, peaks associated with neurochemicals in the freeze-dried sample are clearly visible and appeared to be of similar intensity to those identified in the hand-dissected sample (*vide infra*). Some immediate differences are found with the unidentified peaks in the separation profiles between the two sample preparation methods. Some peaks, such as that at 525 s, are found to have a decreased intensity in the freeze-dried brain sample.

The decrease in some peak intensities in the freeze-dried samples might originate from the freeze-drying process leading to removal of these analytes as the brain is drying in the rotary evaporator. An experiment (not shown) with standards showed that 10–12 % of dopamine of the concentration of 10 mM and 25 μM was lost in the rotary evaporator step. Also, three peaks from 600 to 650 s have been found in the freeze-dried sample but are not present in the hand-dissected sample. These new peaks might be attributed to less degradation of the sample with shorter dissection time. Importantly at this stage, these peak variations are found for only unidentified electroactive analytes.

Effect of time for drying and storing on freeze-dried brain samples

Previous work carried out using freeze-drying methodologies for *Drosophila* have involved storing the frozen (in acetone) fly heads for 7–10 days before removing them from the freezer to evaporate the acetone.^{37–38} Heads were then placed on filter paper to let the acetone evaporate,³⁷ or dried under a vacuum (0.1 Torr) for 8 to 72 h.³⁸ As some enzymatic activity might still take place after the freeze-drying process³⁷ or oxidation of analytes could occur from exposure to air, a faster drying approach has been investigated here. Samples of five frozen *Drosophila* heads have been stored for 3 days at -80°C and dried for 120, 60, 30, 20, 10, and 5 min. The acetone is completely evaporated after approximately 10 min. Samples dried for 5 min are found to be too wet with acetone, and as such the cuticle is too pliable to see any improvement in dissection time. Conversely, samples dried for 120, 60, and 30 min are found to have cuticles easy to open, but only a small amount of “white powder” is found on the inside of the cuticle. These brains have completely lost their shape and cannot be collected for analysis. It is possible that the extended application of low pressure begins to sublime structural brain chemicals causing the brain to crumble and lose its structure. Brain samples dried for 10 and 20 min drying periods have intact structures, are dry, and can be used for further investigation. To insure intact brain structures the shorter 10-min time was chosen.

Previous freeze-drying approaches for fly brains^{37–38} also involved longer storage times (with freezer times of 7–10 days before drying). This long storage time was used to allow the acetone to slowly extract water and other hydrophilic molecules from the cells. Here, when five-brain samples were stored for 3 or 7 days using the method presented and then dissected, the optic lobes of extracted brains from the 7 days of storage were orange in appearance (see Figure 1b) suggesting that the pigment was extracted from the eyes into the brain area even though frozen. When these samples have been analyzed using MEKC-EC, some peaks apparent in the 3-day samples were diminished in the 7-day samples, whereas

others increased from barely detectable in the 3-day samples to prominent in the 7-day samples. One example where this effect is clearly apparent is the peak corresponding to tyrosine and the doublet that occurs after the internal standard, catechol (~900 s) (Figure 4a–b). For tyrosine a decrease in peak area by 91.6% was observed in between samples stored for 3 days and 7 days ($p < 0.05$, $n = 3$, student's t-test). At the same time, the peaks in the unknown doublet increased by factor of 3.7 (Peak 1) and a factor of 22.3 (Peak 2) ($p < 0.001$, $n = 3$, student's t-test, Figure 4 c–e) when sample storage time is increased from 3 to 7 days. These observations suggest that the tyrosine is degraded during prolonged storage for more than 3 days, whereas the increase in the unknown peaks might be the result of degradation product formation. Samples stored for 3 days or less has been found to have no discoloration and have been used for the remainder of the study.

Measurement of Neurochemicals in Freeze-Dried Wild-type *Drosophila* Brain Homogenates

The small size (~5 mm) and short total life cycle (~30 days) of *Drosophila* allows multiple flies to be homogenized into larger sample sizes, which not only helps to balance the variability between individual flies but helps to concentrate the samples with more heads per unit volume. However, dissection of brains from many non-freeze-dried flies is problematic in time. As discussed above, it is difficult to break apart the cuticle, and exposure to the dissecting solution for periods greater than 15 min has been found to lead to brain swelling and increased tissue tearing from osmotic pressure of the buffer into the cells. Moreover, hand-dissected brains are not easily preserved and can have intact enzymatic activities that could degrade analytes of interest. As such, a separation of samples containing more than 7 dissected brains has been too difficult to carry out, even by those highly skilled in dissections.

In the experiments presented here, freeze-drying is used to alleviate these obstacles by preserving and making the dissections faster on average. To demonstrate the applicability of using this method in bioanalysis, biogenic amines and metabolites were separated and quantified in homogenates containing 15 freeze-dried brains of Canton-S *Drosophila*. Analyte peak identification has been performed using spiked samples and comparisons made to separations of 50 μM standards run before and after the homogenate separation. Figure 5 shows electropherograms for a 50 μM standard and the corresponding separation of a homogenate of 15 *Drosophila* brains extracted in 1 μL (50 μM catechol/0.1 M HClO_4) that has been run directly after the standard sample. Peaks corresponding to *N*-acetylserotonin (peak 5), *N*-acetyltyramine (6), *N*-acetyldopamine (7), L-DOPA (8), and tyramine (10) are easily identifiable and have intensities far above baseline (50–200 pA). Octopamine is sometimes found to co-elute with another, unidentified peak that occurs just before 500 s, which prevents it from being repeatedly quantified. These separations demonstrate that MEKC-EC can be used to analyze biogenic amines and metabolites in freeze-dried multi-brain samples.

Dopamine (1), salsolinol (2), *N*-acetyloctopamine (3), and octopamine (4) have also been analyzed in the freeze-dried multi-brain samples. These peaks are difficult to identify, as they appear not to concentrate as much as the other analytes. Peaks corresponding to dopamine and *N*-acetyl octopamine are identifiable but had intensities close to baseline (<10 pA), while salsolinol was not clearly discernable from the baseline. It is not entirely clear why dopamine, salsolinol, and *N*-acetyl octopamine are less concentrated under these conditions. One possibility is that the acetone, which appears to help in extraction of several analytes, might lead to loss of the more volatile analytes as they evaporate with the acetone. This is further supported by the separation times as all three of these species elute within 50 s of one another and are the first species eluted. Hence, these analytes interact with the SDS micelle pseudo-stationary phase similarly and appear to have similar physical properties. Thus, they might be expected to have similar losses. Quantitative information has been

acquired from four independent preparations of five homogenized freeze-dried Canton-S brains. Dopamine, *N*-acetyloctopamine, *N*-acetylserotonin, *N*-acetyltyramine, *N*-acetyldopamine, have been quantified. The average values and SEMs for each of these analytes per brain are reported in Table 1. Values for each of these neurochemicals are very consistent across four different sets of samples demonstrating the reproducibility of the freeze-dried procedure for these separations. Values for L-DOPA and tyramine were also quantified, but the levels were considerably higher than found in the literature leading to the possibility that these peaks are co-eluting with other substances and are not included here at this time as we are examining freeze-dried sample preparation.

CONCLUSIONS

The use of controlled freeze-drying has been shown as an improved sample preparation method for the analysis of fly brain homogenates. This procedure is significantly faster compared to whole brain dissection. Utilizing the faster dissection time with freeze-drying, the number of brains in a fixed homogenate volume can be increased to concentrate the sample. Experiments were carried out with one to fifteen freeze-dried brains. When five brains were used, five analytes, dopamine, *N*-acetyloctopamine, *N*-acetylserotonin, *N*-acetyltyramine, *N*-acetyldopamine were found to all correspond well with previously reported values from dissected brains that had not been freeze dried.

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REFERENCES

1. Rubin GM, Yandell MD, Wortman JR, Gabor GL, Miklos, Nelson CR, Hariharan IK, Fortini ME, Li PW, Apweiler R, Fleischmann W, Cherry JM, Henikoff S, Skupski MP, Misra S, Ashburner M, Birney E, Boguski MS, Brody T, Brokstein P, Celniker SE, Chervitz SA, Coates D, Cravchik A, Gabrielian A, Galle RF, Gelbart WM, George RA, Goldstein LSB, Gong F, Guan P, Harris NL, Hay BA, Hoskins RA, Li J, Li Z, Hynes RO, Jones SJM, Kuehl PM, Lemaitre B, Littleton JT, Morrison DK, Mungall C, O'Farrell PH, Pickeral OK, Shue C, Vosshall LB, Zhang J, Zhao Q, Zheng XH, Zhong F, Zhong W, Gibbs R, Venter JC, Adams MD, Lewis S. *Science*. 2000; 287:2204–2215. [PubMed: 10731134]
2. Wustmann G, Rein K, Wolf R, Heisenberg M. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*. 1996; 179:429–436.
3. Spatz HC, Emanns A, Reichert H. *Nature*. 1974; 248:359–361. [PubMed: 4206649]
4. Quinn WG, Harris WA, Benzer S. *Proceedings of the National Academy of Sciences of the United States of America*. 1974; 71:708–712. [PubMed: 4207071]
5. Schwaerzel M, Monastirioti M, Scholz H, Friggi-Grelín F, Birman S, Heisenberg M. *J. Neurosci*. 2003; 23:10495–10502. [PubMed: 14627633]
6. Li H, Chaney S, Roberts IJ, Forte M, Hirsh J. *Curr. Biol*. 2000; 10:211–214. [PubMed: 10704417]
7. Kume K, Kume S, Park SK, Hirsh J, Jackson FR. *J. Neurosci*. 2005; 25:7377–7384. [PubMed: 16093388]
8. McClung C, Hirsh J. *Curr. Biol*. 1999; 9:853. [PubMed: 10469593]
9. Bergquist J, Sciubisz A, Kaczor A, Silberring J. *J Neurosci Methods*. 2002; 113:1–13. [PubMed: 11741716]
10. Hoyer SC, Eckart A, Herrel A, Zars T, Fischer SA, Hardie SL, Heisenberg M. *Current Biology*. 2008; 18:159–167. [PubMed: 18249112]
11. Morgan TH. *Science*. 1910; 32:120–122. [PubMed: 17759620]

12. Wittmann CW, Wszolek MF, Shulman JM, Salvaterra PM, Lewis J, Hutton M, Feany MB. *Science*. 2001; 293:711–714. [PubMed: 11408621]
13. Iijima K, Liu H-P, Chiang A-S, Hearn SA, Konsolaki M, Zhong Y. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:6623–6628. [PubMed: 15069204]
14. Finelli A, Kelkar A, Song H-J, Yang H, Konsolaki M. *Molecular and Cellular Neuroscience*. 2004; 26:365–375. [PubMed: 15234342]
15. Iijima-Ando K, Iijima K. *Brain Structure and Function*. 2009; 214:245–262. [PubMed: 19967412]
16. Kazemi-Esfarjani P, Benzer S. *Science*. 2000; 287:1837–1840. [PubMed: 10710314]
17. Lee W-CM, Yoshihara M, Littleton JT. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:3224–3229. [PubMed: 14978262]
18. Auluck PK, Chan HYE, Trojanowski JQ, Lee VMY, Bonini NM. *Science*. 2002; 295:865–868. [PubMed: 11823645]
19. Feany MB, Bender WW. *Nature*. 2000; 404:394–398. [PubMed: 10746727]
20. Botella JA, Bayersdorfer F, Schneuwly S. *Neurobiology of Disease*. 2008; 30:65–73. [PubMed: 18243716]
21. Vickrey TL, Condron B, Venton BJ. *Anal. Chem*. 2009; 81:9306–9313. [PubMed: 19842636]
22. Borue X, Cooper S, Hirsh J, Condron B, Venton BJ. *J. Neurosci. Methods*. 2009; 179:300–308. [PubMed: 19428541]
23. Borue X, Condron B, Venton BJ. *Journal of Neurochemistry*. 2010; 113:188–199. [PubMed: 20070864]
24. Makos MA, Kim Y-C, Han K-A, Heien ML, Ewing AG. *Anal. Chem*. 2009; 81:1848–1854. DOI: [PubMed: 19192966]
25. Makos MA, Han K-A, Heien ML, Ewing AG. *ACS Chem. Neurosci*. 2009; 1:74–83. [PubMed: 20352129]
26. Hardie SL, Hirsh J. *J. Neurosci. Methods*. 2006; 153:243–249. [PubMed: 16337274]
27. Borycz J, Vohra M, Tokarczyk G, Meinertzhagen IA. *J. Neurosci. Methods*. 2000; 101:141–148. [PubMed: 10996374]
28. Borycz J, Borycz JA, Kubow A, Lloyd V, Meinertzhagen IA. *Journal of Experimental Biology*. 2008; 211:3454–3466. [PubMed: 18931318]
29. Ream PJ, Suljak SW, Ewing AG, Han K-A. *Anal. Chem*. 2003; 75:3972–3978. [PubMed: 14632107]
30. Paxon TL, Powell PR, Lee H-G, Han K-A, Ewing AG. *Anal. Chem*. 2005; 77:5349–5355. DOI: [PubMed: 16097779]
31. Powell PR, Paxon TL, Han K-A, Ewing AG. *Anal. Chem*. 2005; 77:6902–6908. DOI: [PubMed: 16255588]
32. Kuklinski NJ, Berglund EC, Engelbrektsson J, Ewing AG. *Electrophoresis*. 2010; 31:1886–1893. [PubMed: 20446293]
33. Kuklinski NJ, Berglund EC, Engelbrektsson J, Ewing AG. *Anal. Chem*. 2010; 82:7729–7735. [PubMed: 20738098]
34. Fang H, Vickrey TL, Venton BJ. *Anal. Chem*. 2011; 83:2258–2264. [PubMed: 21322586]
35. Powell PR, Paxon TL, Han K-A, Ewing AG. *Anal. Chem*. 2005; 77:6902–6908. DOI: [PubMed: 16255588]
36. Lowry OH. *J. Histochem. Cytochem*. 1953; 1:420–428. [PubMed: 13118123]
37. Fujita SC, Inoue H, Yoshioka T, Hotta Y. *Biochem. J*. 1987; 243:97–104. [PubMed: 3111462]
38. Ramadan H, Alawi AA, Alawi MA. *Cell Biol. Int*. 1993; 17:765–771. [PubMed: 8220304]
39. Wu JS, Luo L. *Nat. Protocols*. 2006; 1:2110–2115.
40. Sloss S, Ewing AG. *Anal. Chem*. 1993; 65:577–581.

Figure 1.

Image of (a) a freeze-dried fly head and (b) a dissected brain specimen containing both optic lobes and central brain that has been extracted for a time longer than optimum in acetone. The discoloration, found especially within the optic lobes, is thought to be from extracted eye pigment. (c) The cuticle after the dissected brain showing no brain parts, and (d) a dissected undamaged brain specimen containing both optic lobes and central brain. Visually the freeze-dried head does not appear to be different from a non-dried counterpart

Figure 2.

Separation and identification of neurotransmitters in fly brain. **(a)** Electropherogram of common biogenic amine standard (I) and replicate separations of neurotransmitters in fly head populations (5 brains/sample II–IV), demonstrating the reproducibility of MEKC based separation. **(b)** Migration time based identification according to the standard separation (top) in three technical replicates showing repeatable detection of multiple neurotransmitters including octopamine (1), N-acetyl octopamine (2) and tyramine (3).

Figure 3. Electropherograms of (a) a single hand-dissected *Drosophila* brain and (b) a single freeze-dried *Drosophila* brain dissolved in 25 μ M catechol/0.1M HClO₄. The internal standard peak, catechol, is identified with an arrow in each electropherogram. A picture of the sample is provided as an insert for each sample.

Figure 4.

Effect of sample storage on neurotransmitter stability. **(a–b)** Electropherograms of neurotransmitters in fly heads (5 brains/sample $n = 3$) after 3 **(a)** and 7 **(b)** days storage at -80°C . Change of neurotransmitter amount over time of storage days, showing degradation of tyrosine (Tyr, $*p < 0.05$) (shown in **c**) as well as increase of two unknown peaks (in **d**, **e**) as a result of sample degradation ($***p < 0.001$). Y-axis denotes relative peak area normalized to internal catechol standard.

Figure 5.

Electropherogram of (a) a freeze-dried dissected homogenate of 15 brains, and (b) the accompanying 50 μ M standard separation used to identify the following peak identities: (1) dopamine, (2) salsolinol, (3) *N*-acetyloctopamine, (4) octopamine, (5) *N*-acetylserotonin, (6) *N*-acetyltyramine, (7) *N*-acetyldopamine, (8) L-DOPA, (9) catechol, and (10) tyramine. The buffer used here is slightly different compared to the buffer used for Figure 2 and 4 thereby changing elution order of catechol and tyramine.

Table 1

Quantification of dopamine, *N*-acetyloctopamine, *N*-acetylserotonin, *N*-acetyltyramine, *N*-acetyldopamine in homogenates of five freeze-dried brains. Values reported for each analyte include the femtomole amount of the analyte (C) and mean $\pm$ the SEM for the four fly samples.

Fly culture	Dopamine (fmol)	<i>N</i> -acetyl octopamine (fmol)	<i>N</i> -acetyl serotonin (fmol)	<i>N</i> -acetyl tyramine (fmol)	<i>N</i> -acetyl dopamine (fmol)
1	22	10	554	630	1140
2	15	10	526	658	1020
3	29	7	562	687	1090
4	11	12	588	594	1190
Mean $\pm$ S.E.M.	19 $\pm$ 4	9 $\pm$ 1	560 $\pm$ 10	640 $\pm$ 20	1110 $\pm$ 40