Edit Trace of Manuscript 10.1021/acs.analchem.8b01962

Dried Blood Spheroids for Dry-~~state~~State Room Temperature Stabilization of Microliter Blood Samples

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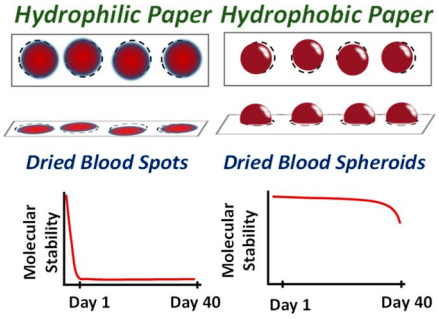
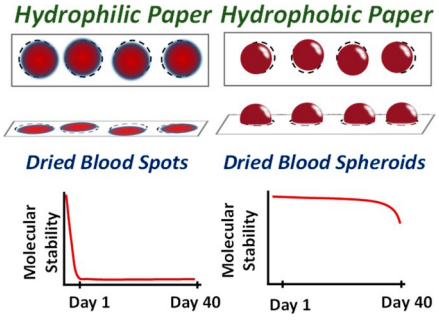
It is well -known that 2D dried blood spots on paper ~~offers~~offer a facile sample collection, storage, and transportation of blood. However, large volume requirements, possible analyte instability, and difficult sample recovery plague this method, lowering confidence in analyte quantification. For the first time, we demonstrate a new approach using 3D dried blood spheroids for stabilization of small volume blood samples, mitigating these effects without cold storage. Blood spheroids form on hydrophobic paper, preventing interaction between the sample and paper substrate, eliminating all chromatographic effects. Stability of the enzyme alanine transaminase and labile organic compounds such as cocaine and diazepam were also shown to increase in the spheroid by providing a critical radius of insulation. On-surface analysis of the dried blood spheroids using paper spray mass spectrometry resulted in sub-ng/mL limits of detection for all illicit drugs tested, representing ~~an~~1 order of magnitude improvement compared with analysis from 2D dried blood spots.



Abstract Graphic



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F ~~For~~or more than a century, blood samples have been collected onto paper substrates as dried blood spots (DBS),[1] and yet all noted advantages are still valid today—, including the simplification of sample collection, transportation, storage, and processing. Interests in DBS have been rekindled in recent years due to the emergence of personalized healthcare. This includes the recent introduction of an on-demand diagnostic strategy,[2,3] which is expected to enable timely initiation of treatment and long-term disease monitoring within the context of a medical home and subspecialty center. These efforts are significant for newborn screening programs,[4−7] low-cost analytical diagnostic method development for use in resource-limited settings,[8−13] environmental research,[14,15] and drug analysis.[16−18] Consequently, aspects of DBS need improvement, including the preservation of labile compounds during storage and reduction of sample volume requirements, while also maintaining the simplicity of the approach.

As a facile method, the basic precautions for DBS collection include limiting sample exposure to moisture, sunlight, and heat. As ~~will be~~ shown in this study, however, exposure of DBS to ambient air can also substantially affect analyte integrity.[19] ~~Since~~Because the current major focus of DBS collection is ~~re-testing~~retesting at a reference laboratory (which may be a part of external quality assessment plan), it has become critical to know the exact volume of blood in the sample to enable effective comparison to results recorded at the testing site. This seemingly simple task is complicated by the following: (i) volcanic effects—~~that~~, which cause a concentration gradient in DBS with higher analyte concentrations detected toward the edge; (ii) chromatographic effects—, the choice of paper substrate impacts DBS sampling by altering blood diffusion and adsorption; ~~and~~ (iii) hematocrit effects—, varied red blood cells in patients' blood (e.g., anemic sample) cause variable blood diffusion on paper, altering the volume sampled in a punch. Standard protocol to mitigate unknown sample volumes is to collect unnecessarily large volumes (>70 μL) and punch a standard area from the sample. Other specific solutions include mathematical calculations ~~20−23~~, [20−23] the use of radioactive chemical tracers,[23,24] or ~~via~~ prediction of hematocrit level in blood in separate/independent experiments.[25]

Herein~~,~~ —we report a new paper-based blood (<30 μL) collection platform that is based on three-dimensional (3D) dried blood spheroids as opposed to the traditional two-dimensional (2D) DBS sample collection procedure. This new dried blood sample collection procedure uses functionalized hydrophobic paper substrates (prepared in-house) to overcome major challenges associated with the traditional DBS procedure. The advantages and attributes of this approach are ~~fourfold~~4-fold: (i) blood sample applied on the hydrophobic paper forms a spherical drop due to a mismatch in surface energies, which dries to yield a dried blood spheroid. Experiments have shown that hydrolytically labile chemicals such as cocaine and diazepam trapped in the 3D dried blood spheroid are stabilized, compared with storage ~~done~~performed under the porous DBS conditions where a major portion of the sample becomes susceptible to oxidative stress from ambient air; (ii) decreased blood~~-~~—paper interaction enables easy analyte extraction, a reduction in the required sample volume, and improvements in analyte quantification; (iii) ~~because~~ the origin of volcanic, chromatographic, and hematocrit effects can all be traced to a common source—, uneven biofluid/analyte adsorption —; controlling wetting on hydrophobic paper enables simple validation of results; ~~and~~ (iv) the hydrophobic paper also ~~afford a~~affords direct mass spectrometry (MS) detection via paper spray (PS) ionization[2,26−32] for sensitive analyte quantification. I~~n~~—situ extraction of illicit drugs (methamphetamine, benzoylecgonine, amphetamine, and cocaine) from the dried blood spheroids resulted in a sub-ng/mL limit of ~~detections~~detection using ethyl acetate spray solvent.

~~EXPERIMENTAL SECTION~~Experimental Section

Standards and Solutions

Standard solutions (1.0 mg/mL) of benzoylecgonine, cocaine, amphetamine, and (±)-methamphetamine were obtained from Cerilliant (Round Rock, TX). All solvents, α-ketoglutaric acid disodium salt hydrate, and ~~L~~l ~~-Alanine~~alanine, were purchased from Sigma-Aldrich (St. Louis, MO). Human blood was purchased from Innovative Research (Novi, MI). Whatman filter paper (24 cm, grade 1) and Guthrie card (grade 903) was purchased from Whatman (Little Chalfont, England). For detection of illicit drugs from human blood, standards were directly spiked into whole blood and analyzed without further treatment. Phosphate-buffered saline (PBS) tablets were purchased from AMRESCO (Solon, OH). Lyophilized alanine aminotransferase from human liver was purchased from Lee Biosolutions (Maryland Heights, MO).

Hydrophobic Paper Preparation

Using a digital template, paper triangles were cut from filter paper with an Epilog Legend 36EXT laser with 15% power at 1000 Hz. ~~Pre-cut~~Precut paper was treated in a vacuum desiccator with 0.5 mL of trichloro(3,3,3-trifluoropropyl) silane. Untreated paper was not subject to this reaction. Paper triangles were 9.5 mm base × 16.6 mm height, and paper/polymer rectangles were 4 mm base ~~x~~× 20 mm height to maintain similar surface area. Whole blood samples were pipetted onto the paper surface and allowed to dry overnight unless otherwise stated.

Mass Spectrometry

Mass spectra were recorded using Thermo Scientific Velos Pro LTQ linear ion trap mass spectrometer (San Jose, CA). MS parameters used were as follows: 200 °C capillary temperature, ~~3~~three microscans, and 60% Slens voltage. Spray voltage was 5 kV unless otherwise specified. Thermo Fisher Scientific Xcalibur 2.2 SP1 software was applied for MS data collecting and processing. Tandem MS with collision-induced dissociation (CID) was utilized for analyte identification. For the CID tests, 1.5 Th (mass/charge units) for the isolation window and 35 (manufacturer's unit) of normalized collision energy ~~was~~were chosen ~~for the CID tests.~~

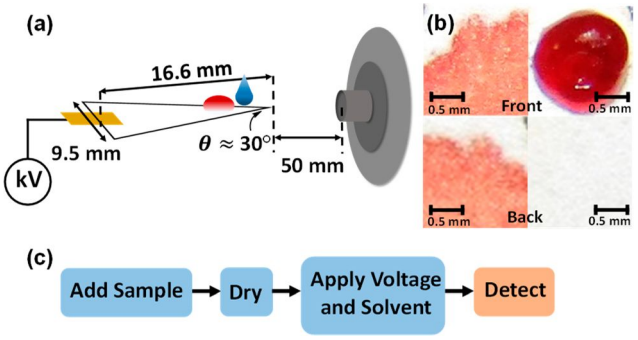
Thermal Analysis Simulation Model

Transient natural thermal transfer analysis (natural convection and conduction) was carried out using SolidWorks Simulation 2014 (SolidWorks Corp., ~~Massachusetts~~Waltham, ~~USA~~MA). A sphere (radius 5 mm) and circular disc (radius 5 mm, depth 1 mm) were drawn in CAD to approximate the geometry of the blood spheroid and DBS, respectively. A finite element analysis was applied to the CAD model using the thermal analysis FFEPlus solver with the following parameters: triangular mesh with 10234 nodes and 6929 elements each of size 0.378 mm, 30 °C object initialization temperature, 40 °C bulk ambient temperature, with air as the natural convection medium with a heat transfer coefficient of 25 W/(m2·°C). Transient analysis was conducted over 90 ~~seconds~~s with 3 ~~seconds~~ measurement intervals. Thermal properties were chosen so as to approximate whole human blood: 3617 J/kg/°C specific heat capacity, 0.492 W/m-°C thermal conductivity, and 1060 kg/m^3 density.

Alanine Transaminase (ALT) Stability

For ALT stability test, 30 µL of human whole blood containing 500 U/L ALT was dried on 10 mm ~~x~~× 10 mm grade 1 30 ~~minutes~~min treated paper and untreated paper and stored for a given number of days. After storage, blood was extracted in 100 µL of 1XPBS, grinded with ~~pipette~~pipet tip and allowed to soak 5 ~~minutes~~min in ice and then centrifuged 1500 rcf for 2 ~~minutes~~min. ~~2 µL~~Two microliters of 1 M ~~L~~L -alanine and 2 µL of 1 M α-ketoglutarate were added to the blood sample and allowed to react for 20 ~~minutes~~min at 37 °C. A 10 ~~µL~~microliter aliquot ~~of~~ was pipetted onto untreated grade 1 paper triangle and allowed to dry 5 ~~minutes~~min. Analysis of pyruvate products in the blood sample present on the paper triangle was achieved using 20 µL of ethyl acetate and 5 kV spray voltage. Pyruvate signal at *m*/*z* 87 was monitored in negative mode and compared with a solvent related ion at *m*/*z* 59.

Figure 1. (a) Experimental setup using paper triangles. (b) Image showing a 4 µL of dried blood spot/spheroid on an untreated (left) and treated (right) paper substrates, including the front (top) and back (bottom). Scale bars show 0.5 mm. (c) Workflow of direct on-surface dried blood analysis.



~~RESULTS AND DISCUSSION~~Results and Discussion

Sample Collection on Treated Paper

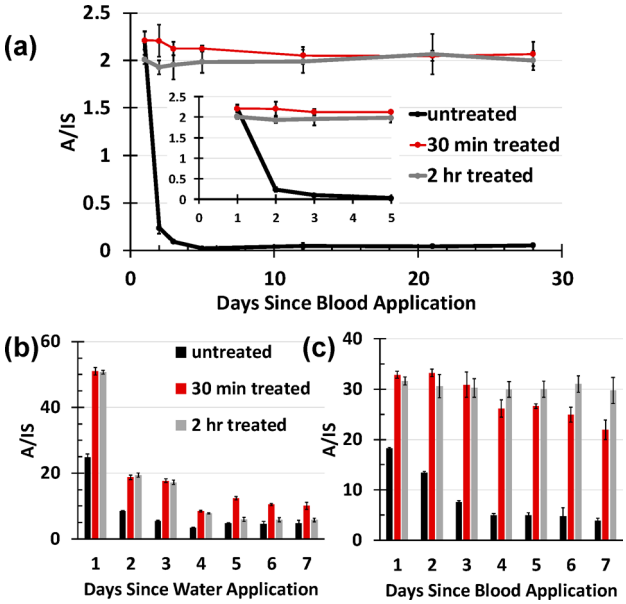
To enable dried blood spheroid collection, we converted ~~pre-cut~~precut hydrophilic filter papers into hydrophobic paper substrates through a gas-phase silanization procedure.[26] Because this approach utilizes a gas-phase preparation procedure to impact changes in surface properties, many of the physicochemical characteristics (e.g., color, weight, porosity, tensile strength, malleability, flammability) of the filter paper remain unchanged. However, wettability of the paper is altered controllably by varying silane vapor exposure time. As a result of the lowered surface energy, aqueous-based samples such as blood and serum bead when applied onto the hydrophobic paper~~,~~ and, as a consequence, form 3D spheroids (Figure 1 b) upon drying due to concentration-driven self-assembly of ~~bio-macromolecules~~biomacromolecules in the biofluid. Only the outermost layer of the dried blood spheroid is exposed to air

during storage, preserving the integrity of majority of analytes inside the dried blood. Hydrophobic polymers may be used for dried blood spheroid preparation, but direct analysis of the stored blood samples may be limited by the instability of the polymer substrate in the presence of the organic solvents. The porous cellulose core in the hydrophobic paper is friendly to organic solvents, enabling direct extraction and ionization of organic compounds present in the dried blood samples for subsequent online detection by MS.

The overall workflow for blood collection and analysis from hydrophobic paper is ~~as~~ illustrated in Figure 1 c, where 4 µL of blood was typically deposited onto the hydrophobic paper~~r~~, and dried for a specified time, and analytes were detected using hydrophobic PS MS. Using ethyl acetate as spray solvent, small organic compounds (e.g., amphetamine and methamphetamine) were selectively extracted and detected from blood samples dried on hydrophobic paper (Figure 1 a).

As observed from Figure 1 b, the fresh blood penetrated to the back of the untreated paper forming a blood spot of area 0.13 ± 0.05 cm^2, with ~~>2X~~2× relative standard deviations between samples. In contrast, no traces of blood were observed on the back of the hydrophobic paper. Instead, the entire 4 µL of blood volume bead, which rested on top of the hydrophobic paper, confined to a reproducible area of 0.013 ± 0.002 cm^2 (Figure S1). Although not the focus of the current work, the whole dried blood spheroid could be punched for subsequent extraction without regard to volcanic, chromatographic or hematocrit effects.

Figure 2. Stability of (a) cocaine in dried blood, (b) neat dried diazepam prepared in water, and (c) diazepam in dried blood. Both dried blood spots (untreated) and spheroids (treated) samples were stored under ambient conditions at 25 °C. Internal standard was spiked into the spray solvent to normalize between samples/days. Error bars show one standard deviation.



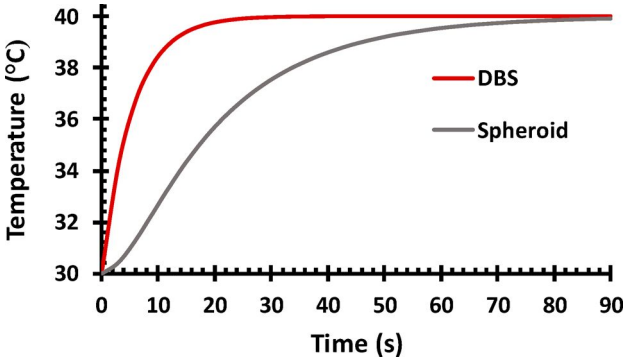
Stability of Organic Compounds in Ambient Air

To investigate the possibility of reducing oxidative stress during dried blood spheroid storage, we selected cocaine and diazepam as models of hydrolytically labile organic compounds. Cocaine and diazepam (2 µg/mL each ~~of cocaine and diazepam~~) were spiked separately into whole human blood, and 4 µL aliquots were spotted onto the as-prepared hydrophobic paper and stored in ambient air for a maximum of 28 days. Similar blood samples were stored using the conventional DBS method on untreated, hydrophilic paper. Results from these experiments are summarized in Figure 2 , which indicate that cocaine (Figure 2 a) and diazepam (Figure 2 c) trapped inside the 3D dried blood spheroid are stabilized compared with storage done under the porous DBS conditions. About 90% of cocaine is hydrolyzed within a day of storage on untreated hydrophilic paper (~~insert~~inset, Figure 2 a). Separate offline extraction experiments showed that cocaine was hydrolyzed into benzoylecgonine metabolite (Figure S2). Therefore, we investigated the

stability of benzoylecgonine under DBS versus dried blood spheroid conditions (Figure S3). Here, greater than 95% of benzoylecgonine was oxidized (via the direct addition of oxygen) after the 1212th day of storage under the typical DBS condition. In contrast, a stable ion signal was detected for benzoylecgonine stored in the spheroid even after the 46th day. Unlike cocaine, the signal loss for diazepam on DBS was gradual, and 21% of the analyte remained in the DBS after one1 week (untreated, Figure 2 c). As expected, the signal was relatively stable when stored using the dried blood spheroid methodology.

To ensure that the decomposition processes observed here are due to oxidative stress from atmospheric air, we performed a control experiment in which both DBS and dried blood spheroids were stored in a vacuum desiccator for 15 days. Under this airtight condition, both cocaine and benzoylecgonine were found to be stable in DBS and spheroids (Figure S4). This confirms our hypothesis that analyte degradation is due to ambient air oxidation. The involvement of oxygen is also confirmed by the direct detection of O_2 adducts (+32 Da increase) in MS analysis (Figure S5). To investigate a possible "wall effect" (i.e., critical radius of insulation) in the stabilization of analytes in dried blood spheroids, the stability of neat, dry diazepam (prepared in water, as opposed to blood) on both treated (no spheroid was formed) and untreated paper substrates were compared and found to be similar (Figure 2 b). That is, neat diazepam analytes gradually degraded at comparable rates on both hydrophobic and hydrophilic paper. The stability of diazepam was restored when prepared in blood and dried as a spheroid (Figure 2 c). Ion intensities recorded from treated paper were relatively higher than those from untreated paper because of higher ionization efficiency of hydrophobic paper substrates.[26] Collectively, these results suggest that the creation of 3D spheroid from a viscous sample like blood is essential in preventing oxidation in air, and that the interior of the spheroid was protected by providing a possible critical radius of insulation [33,34] that increases the spheroid's resistance to thermal conduction and oxidative degradation. Finite element analysis of thermal energy flux from surrounding ambient air for spheroid and DBS approximated geometry confirms the spheroid's enhanced thermal protection over a given time period (Figure S6). The reduced surface area-to-volume ratio of the spheroid limits bulk exposure to the ambient environment (Figure 3), which also improves resistance to oxidative degradation over time.

Figure 3. Heat transfer transient simulation analysis. Both blood storage geometries (DBS versus spheroid) had an initial temperature of 30 °C and were subjected to a constant ambient air temperature of 40 °C. Temperature is measured at the geometric center for each case.

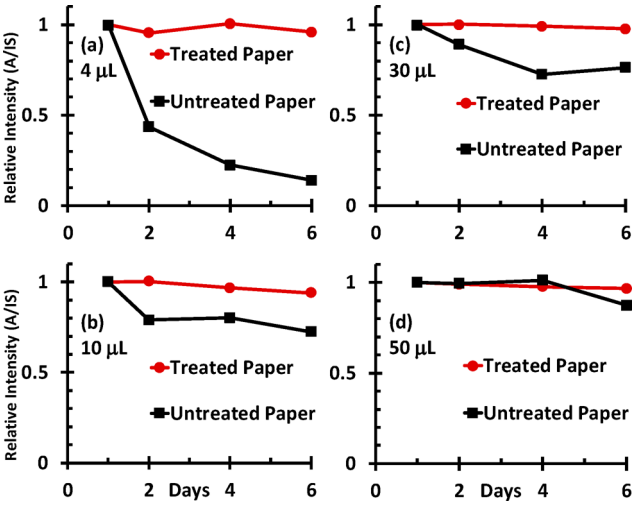


To investigate the ability of the spray solvent to extract the target analyte from the interior of the dried blood spheroid despite this protective layer, we prepared a two-layered dried blood spheroid consisting of an inner blood layer spiked with 1 ppm of cocaine and an outer blood layer containing no the cocaine analyte. The two blood layers were physically separated by a thin, porous hydrophobic barrier, which served to prevent mixing and the physical diffusion of cocaine from inner layer into the outer blood layer of the spheroid (see Figure S7 and other details in SupplementalSupporting Information). During online MS analysis of this multi-layeredmultilayered blood sample using ethyl acetate spray solvent, we observed a signal due to cocaine at *m/z* 182 in an MS/MS experiment. This result suggests that the spray solvent does not only sample from the surface of the dried blood spheroid but is also able to penetrate the outer blood layer during which the stable analyte is extracted and ionize. In this case, the depth of spray solvent penetration was estimated to be at least 0.59 mm, which represents 34% of the total spheroid radius.

Volume Effects

The effect of blood volume (4–50 µL) on analyte stability was also investigated using filter paper (grade 1) and Guthrie card (grade 903). In general, the thicker Guthrie card offered better protection for cocaine trapped within the thick fiber core compared with the thin filter paper. When stored on untreated Guthrie card, the stability of cocaine in DBS increased with increasing blood volume, consistent with the formation of a protecting thick blood film.[35] Significant degradation of cocaine was observed below 30 µL of blood (Figure 4). This volume-dependent data provides a new critical insight regarding how labile analytes may be stabilized in a thick blood film formed by large volume of blood in conventional dried blood sampling. Interestingly, the new dried blood spheroid method presents an alternative using only microliter blood samples. For example, creation of spheroids on treated Guthrie cards restored the stability of cocaine, even in 4 µL blood volume in accordance with our findings using filter paper (Figure 2 a). Formation of spheroid from larger (>50 µL) blood volumes becomes increasingly difficult.

Figure 4. (a) 4 µL, (b) 10 µL, (c) 30 µL, and (d) 50 µL of 5 ppm cocaine in human whole blood ~~was~~were stored on a grade 903 card, and a 6.5 mm punch was placed on top of a grade 1 triangle. ~~20 µL~~Twenty microliters of 5 ppm cocaine d3 internal standard in ethyl acetate was used a spray solvent with 5 kV spray voltage during analysis.



Quantification in Dried Blood Spheroids

Using hydrophobic filter paper, we quantified the presence of cocaine, benzoylecgonine, amphetamine, and methamphetamine stored in the dried blood spheroids. The initial investigations involved the use of untreated paper and hydrophobic paper triangles treated with silane vapor at 5, 30, 120, 240, 720, and 1440 min exposure times. These samples were analyzed with ethyl acetate as the spray solvent, and the absolute intensities of the fragment ions derived from collision-induced dissociation were quantified. Overall, the paper treated for 30 and 120 min produced the highest intensity responses and were selected for further testing (Figures S8–S10).

These responses were found to be influenced by (i) drug binding affinity to the paper surface versus its solubility in the spray solvent (partitioning)~~;~~, (ii) ionization efficiency—the impact of treatment time on PS performance~~;~~, and (iii) extraction efficiency of the analyte from the dried blood. Treatment time was not observed to affect analyte ionization due to comparable wetting of ethyl acetate on all paper triangles, which produced protonated ions via electrospray-based mechanism (as opposed to electrostatic ionization). Properties of the blood spheroids appeared identical (e.g., size and interaction with paper surface) on all paper treated for >30 min. Therefore, partitioning of the analyte between the paper and the solvent (~~post-extraction~~postextraction) is expected to be the major contributing factor affecting ion yields from treated paper substrates. This partitioning factor is in turn controlled by the log *P* of drug and paper treatment time (Table 1). For example, cocaine is the most hydrophobic drug tested (log*P* 2.28), hence, gave a higher ion intensity on paper with a shorter treatment time (i.e., less hydrophobic paper substrate: Figure S9). Similarly, benzoylecgonine, the most hydrophilic drug tested (Log*P*– 0.59), showed a higher ion signal on paper with a longer treatment time (i.e., more hydrophobic). These results may be explained by that fact that molecules with

high log *P* values prefer hydrophobic medium (and vice versa), and thus binding capacity is low on paper substrates prepared over shorter treatment times, enabling enhanced ion yield from such surfaces.

In this case, not only did the ion signal last approximately twice as long when hydrophobic paper was used, but limits of detection (LODs) as low as 0.12 ng/mL (corresponding to ~~10X~~10× reduction in LOD and LOQ for amphetamine on 30 min treated paper) were observed, with enhanced linearity ($R^2 > 0.999$; Table 1 , and Figures S11~~–~~–S14).

Compared with untreated paper substrates, the lower LODs calculated for hydrophobic paper are mainly attributed to the following: (i) ~~the~~The inability of the blood sample to wet through the paper~~—~~; the fact that the aqueous-based blood samples are unable to wet through the fiber core of the porous hydrophobic paper and spread suggests interactions between drug and the paper surface prior to extraction is decreased. This results in a greater number of free drug analytes available in the dried spheroid, increasing analyte signal~~;~~. (ii) ~~the~~The more uniform spot size for the dried spheroids ~~–~~; this contributes to the observed quantitative abilities (i.e., lower LODs and improved linearity) by creating a more reproducible extraction area and decreasing variations in analyte signal~~; and~~ . (iii) ~~the~~The decreased analyte binding capacity to the paper ~~post extraction~~postextraction. That is, redistribution of extracted analyte back into the hydrophobic paper is reduced compared with hydrophilic paper substrates.

Blood Chemistry and Stability of Alanine Transaminase

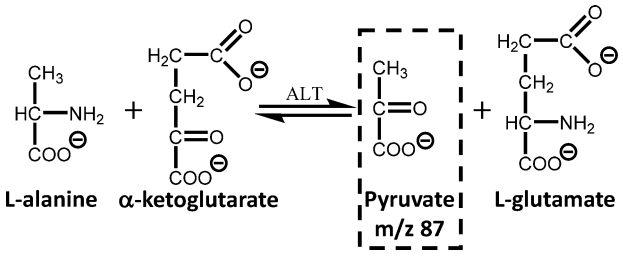
With a drying time of 30 min for the spheroids compared with 5 min for DBS, it was necessary to investigate possible changes in blood chemistry, especially pertaining to enzymatic activity (note: all samples were dried overnight before analysis as is typically done). [36,37] Alanine transaminase (ALT) was used for this study, where the enzyme was spiked into whole blood, and an offline extraction was performed from the dried samples. ~~L~~L ~~alanine~~Alanine and α-ketoglutarate were added to the enzyme extract, and the production of pyruvate (*m*/*z* ~~–~~87) was monitored (Scheme 1). A 30% reduction in ALT activity was observed after the first day of dry storage in the spheroid (Figure 5), which then remained stable over the course of 10 storage days. When stored in the DBS condition, while stability appeared identical to spheroids in days ~~1~~ ~~–~~4, day 7 showed a drop-off of pyruvate production, where about 25% of ALT activity remained at the end of the 10 ~~days~~day storage period. These results are consistent with reported enzymatic decay in DBS, [37] ~~–~~ [39] where gradual loss of enzyme activity is observed in almost all cases upon blood drying. These results suggest the difference in initial blood drying time for spheroid versus DBS will not cause significant changes in blood chemistry during the drying step and the initial period of storage (up to 4 days with regards to ALT). On the contrary, prolonged and dry-state stabilization of ALT in blood could be useful in, for example, diagnosing liver injury from remotely collected blood samples. This condition often occurs in patients that take several medications at once,[40] especially in people with HIV and tuberculosis, which are most prevalent in the developing world.

Table 1. Limits of Detection (LOD) and Quantification (LOQ) of ~~drugs~~Drugs in ~~dried blood~~Dried Blood on ~~triangles.~~Triangles

~~Analyte~~	~~Log~~*P*	LODs (LOQs) in dried blood ~~(~~[K](ng/mL)[T] ~~ng/mL)~~[a]		
~~Untreated Paper Triangle~~analyte	[K]log *P* [T]	untreated paper triangle	~~30~~[K]30 min[T] ~~Minute Treated Paper Triangle~~treated paper triangle	~~2~~[K]2 h[T] ~~Hour Treated Paper Triangle~~treated paper triangle
~~Amphetamine~~amphetamine	1.80	~~4.4~~[K]4.4 (9.7)[T] ~~(9.7)~~	~~0.12~~[K]0.12 (1.3)[T] ~~(1.3)~~	~~0.11~~[K]0.11 (0.69)[T] ~~(0.69)~~
~~Methamphetamine~~methamphetamine	2.24	~~7.9~~[K]7.9 (9.1)[T] ~~(9.1)~~	~~0.34~~[K]0.34 (1.8)[T] ~~(1.8)~~	~~1.7~~[K]1.7 (4.2)[T] ~~(4.2)~~
~~Cocaine~~cocaine	2.28	~~3.5~~[K]3.5 (19)[T] ~~(19)~~	~~0.37~~[K]0.37 (0.57)[T] ~~(0.57)~~	~~1.0~~[K]1.0 (1.7)[T] ~~(1.7)~~
~~Benzoylecgonine~~benzoylecgonine	~~–~~0.59	~~3.7~~[K]3.7 (7.9)[T] ~~(7.9)~~	~~0.48~~[K]0.48 (0.79)[T] ~~(0.79)~~	~~0.49~~[K]0.49 (1.6)[T] ~~(1.6)~~

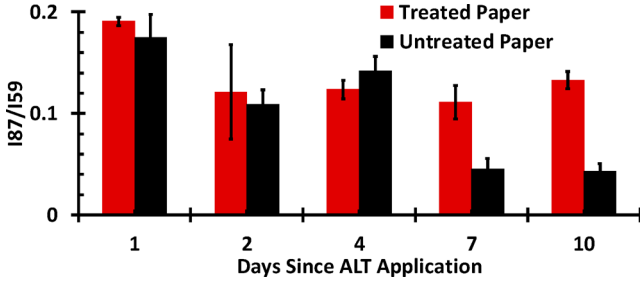
[a]LODs were calculated from respective calibration curves using signal corresponding to (S_{blank}) + 3 × σ_{blank}; LOQs were calculated from respective calibration curves using signal corresponding to (S_{blank}) + 10 × σ_{blank} ~~;~~where (S_{blank}) is the average blank signal and σ_{blank} is the standard deviation of the signal from ~~3~~three replicates.

Scheme 1. Catalytic ~~action~~Action of ~~alanine aminotransferase~~Alanine Aminotransferase[a] ~~Reactants L-alanine and α-ketoglutarate were added to blood samples containing ALT, and pyruvate was monitored at *m/z* 87 in the negative ion mode.~~



[a]Reactants L-alanine and α-ketoglutarate were added to blood samples containing ALT, and pyruvate was monitored at *m/z* 87 in the negative ion mode.

Figure 5. Alanine transaminase (ALT) stability in dry-state ambient conditions stored using 10 µL dried blood spheroids (treated) and dried blood spots (untreated). ALT activity was monitored by the production of pyruvate. Both dried blood spots (untreated) and ~~spheroids~~spheroid (treated) samples were stored under ambient conditions at 25 °C. Error bars show one standard deviation.



~~CONCLUSION~~Conclusion

In summary, hydrophobic paper substrate enables a 3D dried blood spheroid collection platform, eliminating chromatographic effects associated with 2D dried blood spot samples ~~whilst~~while decreasing the sample volume required. Dried blood spheroids increase stability for labile compounds against oxidative stress, extending the lifetime of alanine transaminase, diazepam, cocaine, and benzoylecgonine from days to several weeks under ambient conditions without cold storage. Manipulation of surface energy of the paper and organic spray solvent allows selective extraction of target analytes, which increases extraction efficiency of cocaine, benzoylecgonine, amphetamine, and methamphetamine from the dried blood spheroids, resulting in sub-ng/mL limits of detection. Because of its close resemblance to DBS, the implementation of dried blood spheroid sample collection in clinical settings can be accomplished with no changes in blood collection procedures.

Supporting Information

~~Supporting Information~~

~~The Supporting Information is available free of charge on the ACS Publications website.Analyte degradation and stability tests, heat conduction, and calibrations/quantification.~~The Supporting Information is available free of charge on the ACS Publications website ~~(PDF~~ at DOI: 10.1021/acs.analchem.8b01962~~)~~.

Analyte degradation and stability tests, heat conduction, and calibrations/quantification (PDF)

Acknowledgments. This research was supported by the Ohio State University start-up funds to A.B-T., Eli Lilly Young Investigator Award to A.B-T. by Eli Lily and Co., and by Iowa State University through startup funds and

Black and Veatch faculty fellowship to M.T. S.M. acknowledges support from EPSRC (EP/R007500/1) and via the EPSRC-ESRC Centre for Doctoral Training on Quantification and Management of Risk & Uncertainty in Complex Systems & Environments.
The authors declare no competing financial interest.

References

(1) Schmidt, V. *Clin. Chem.* **1986**, *32*, 213–21510.1016/B978-0-08-034186-6.50041-4.
(2) Chen, S.; Wan, Q.; Badu-Tawiah, A. K. *J. Am. Chem. Soc.* **2016**, *138*, 6356–635910.1021/jacs.6b02232.
(3) Seidenberg, P.; Nicholson, S.; Schaefer, M.; Semrau, K.; Bweupe, M.; Masese, N.; Bonawitz, R.; Chitembo, L.; Goggin, C.; Thea, D. M. *Bull. World Health Organ.* **2012**, *90*, 348–35610.2471/BLT.11.100032.
(4) Crossle, J. R.; Elliot, R. B.; Smith, P. A. ~~*The*~~ *Lancet* **1979**, *313*, 472–47410.1016/S0140-6736(79)90825-0.
(5) Zytkovicz, T. H.; Fitzgerald, E. F.; Marsden, D.; Larson, C. A.; Shih, V. E.; Johnson, D. M.; Strauss, A. W.; Comeau, A. M.; Eaton, R. B.; Grady, G. F. *Clin. Chem.* **2001**, *47*, 1945–1955.
(6) Li, Y.; Scott, C. R.; Chamoles, N. A.; Ghavami, A.; Pinto, B. M.; Turecek, F.; Gelb, M. H. *Clin. Chem.* **2004**, *50*, 1785–179610.1373/clinchem.2004.035907.
(7) Gelb, M. H.; Turecek, F.; Scott, C. R.; Chamoles, N. A. *J. ~~Inherit.~~ Inherited Metab. Dis.* **2006**, *29*, 397–40410.1007/s10545-006-0265-4.
(8) Sherman, G. G.; Stevens, G.; Jones, S. A.; Horsfield, P.; Stevens, W. S. *JAIDS, J. ~~Acquir.~~ Acquired Immune Defic. Syndr.* **2005**, *38*, 615–61710.1097/01.qai.0000143604.71857.5d.
(9) Sia, S. K.; Linder, V.; Parviz, B. A.; Siegel, A.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **2004**, *43*, 498–50210.1002/anie.200353016.
(10) Nemiroski, A.; Christodouleas, D. C.; Hennek, J. W.; Kumar, A. A.; Maxwell, E. J.; Fernandez-Abedul, M. T.; Whitesides, G. M. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 11984–11989 10.1073/pnas.1405679111.
(11) Fu, E.; Liang, T.; Spicar-Mihalic, P.; Houghtaling, J.; Ramachandran, S.; Yager, P. *Anal. Chem.* **2012**, *84*, 4574–457910.1021/ac300689s.
(12) Yager, P.; Edwards, T.; Fu, E.; Helton, K.; Nelson, K.; Tam, M. R.; Weigl, B. H. *Nature* **2006**, *442*, 412–41810.1038/nature05064.
(13) Li, M.; Tian, J.; Al-Tamimi, M.; Shen, W. *Angew. Chem., Int. Ed.* **2012**, *51*, 5497–550110.1002/anie.201201822.
(14) McDade, T. W.; Williams, S.; Snodgrass, J. J. *Demography* **2007**, *44*, 899–92510.1353/dem.2007.0038.
(15) Burse, V. W.; Deguzman, M. R.; Korver, M. P.; Najam, A. R.; Williams, C. C.; Hannon, W. H.; Therrell, B. L. *Biochem. Mol. Med.* **1997**, *61*, 236–23910.1006/bmme.1997.2603.
(16) Manicke, N. E.; Abu-Rabie, P.; Spooner, N.; Ouyang, Z.; Cooks, R. G. *J. Am. Soc. Mass Spectrom.* **2011**, *22*, 1501–150710.1007/s13361-011-0177-x.
(17) Edelbroek, P. M.; van der Heijden, J.; Stolk, L. M. L. *Ther. Drug Monit.* **2009**, *31*, 327–33610.1097/FTD.0b013e31819e91ce.
(18) Hoogtanders, K.; van der Heijden, J.; Christiaans, M.; Edelbroek, P.; van Hooff, J. P.; Stolk, L. M. L. *J. Pharm. Biomed. Anal.* **2007**, *44*, 658–66410.1016/j.jpba.2006.11.023.
(19) Golbahar, J.; Altayab, ~~D.D~~D. D.; Carreon, E. *J. Med. Screen.* **2014**, *21*, 5–910.1177/0969141314525367.
(20) Denniff, P.; Spooner, N. *Bioanalysis* **2010**, *2*, 1385–139510.4155/bio.10.103.
(21) Vu, ~~D.H~~D. H.; Koster, R. A.; Alffenaar, ~~J.W.C~~J. W. C.; Brouwers, J. R. B. J.; Uges, D. R. A. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2011**, *879*, 1063–107010.1016/j.jchromb.2011.03.017.
(22) Youhnovski, N.; Bergeron, A.; Furtado, M.; Garofolo, F. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 2951–295810.1002/rcm.5182.
(23) Wagner, M.; Tonoli, D.; Varesio, E.; Hopfgartner, G. *Mass Spectrom. Rev.* **2016**, *35*, 361–43810.1002/mas.21441.
(24) Mei, J. V.; Zobel, S. D.; Hall, E. M.; De Jesús, V. R.; Adam, B. W.; Hannon, W. H. *Bioanalysis* **2010**, *2*, 1397–140310.4155/bio.10.73.
(25) Capiau, S.; Wilk, L. S.; Aalders, M. C. G.; Stove, C. P. *Anal. Chem.* **2016**, *88*, 6538–654610.1021/acs.analchem.6b01321.
(26) Damon, D. E.; Davis, K. M.; Moreira, C. R.; Capone, P.; Cruttenden, R.; Badu-Tawiah, A. K. *Anal. Chem.* **2016**, *88*, 1878–188410.1021/acs.analchem.5b04278.
(27) Wang, H.; Liu, J.; Cooks, R. G.; Ouyang, Z. *Angew. Chem., Int. Ed.* **2010**, *49*, 877–88010.1002/anie.200906314.
(28) Damon, D. E.; Maher, Y. S.; Yin, M.; Jjunju, F. P. M.; Young, I. S.; Taylor, S.; Maher, S.; Badu-Tawiah, A. K. *Analyst* **2016**, *141*, 3866–387310.1039/C6AN00168H.

(29) Maher, S.; Jjunju, F. P. M.; Damon, D. E.; Gorton, H.; Maher, Y. S.; Syed, S. U.; Heeren, R. M. A.; Young, I. S.; Taylor, S.; Badu-Tawiah, A. K. *Sci. Rep.* **2016**, *6*, 35643 10.1038/srep35643.
(30) Yang, Q.; Wang, H.; Maas, J. D.; Chappell, W. J.; Manicke, N. E.; Cooks, R. G.; Ouyang, Z. *Int. J. Mass Spectrom.* **2012**, *312*, 201–207 10.1016/j.ijms.2011.05.013.
(31) Ren, H.; Wang, J.; Liu, Z.; Zhang, M. N.; McLuckey, Z.; Ouyang. *Chromatographia* **2013**, *76*, 1339–1346 10.1007/s10337-013-2458-y.
(32) Wang, H.; Ren, Y.; McLuckey, M. N.; Manicke, N. E.; Park, J.; Zheng, L.; Shi, R.; Cooks, R. G.; Ouyang, Z. *Anal. Chem.* **2013**, *85* (23), 11540–11544 10.1021/ac402798m.
(33) Sahin, A. Z.; Kalyon, M. *Heat Mass ~~Transf.~~ Transfer* **2004**, *40*, 377–382 10.1007/s00231-003-0471-7.
(34) Aziz, A. *Heat ~~Transf.~~ Transfer Eng.* **1997**, *18*, 61–91 10.1080/01457639708939897.
(35) Alfazil, A. A.; Anderson, R. A. *J. Anal. Toxicol.* **2008**, *32*, 511–515 10.1093/jat/32.7.511.
(36) Hamilton, J.; Jones, I.; Srivastava, R.; Galloway, P. *Clin. Chim. Acta* **2012**, *413*, 1207–1210 10.1016/j.cca.2012.03.019.
(37) de Castilhos, C. D.; Mezzalira, J.; Goldim, M. P. S.; Coelho, J. C. *Clin. Biochem.* **2011**, *44*, 922–926 10.1016/j.clinbiochem.2011.03.138.
(38) Fujita, M.; Brindle, E.; Shofer, J.; Ndemwa, P.; Kombe, Y.; Shell-Duncan, B.; O'Connor, K. A. *Clin. Chem.* **2007**, *53*, 1972–1975 10.1373/clinchem.2007.093104.
(39) Hamilton, J.; Jones, I.; Srivastava, R.; Galloway, P. *Clin. Chim. Acta* **2012**, *413*, 1207–1210 10.1016/j.cca.2012.03.019.
(40) Wang, K.; Zhang, S.; Marzolf, B.; Troisch, P.; Brightman, A.; Hu, Z.; Hood, L. E.; Galas, D. J. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 4402–4407 10.1073/pnas.0813371106.