Instrumental approach to eliminate the hematocrit issue using online DBS analysis

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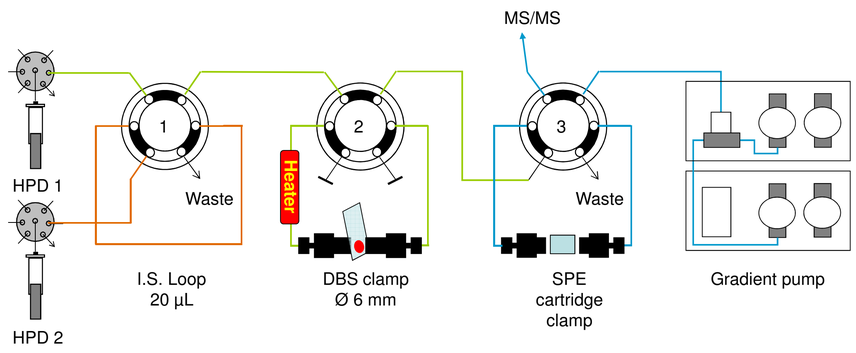
Introduction

Blood hematocrit (HCT) variation has emerged as one of the most important issues in Dried Blood Spot (DBS) Analysis. HCT variations influence blood spot size, and consequently cause variations in assay precision when only a fraction of the blood spot is used for analysis ("partial spot analysis"). The most obvious solution to this problem is using the entire spot for analysis ("full-spot analysis") This would completely eliminate the issue of varying spot size. However, not only spot size but also *recovery* of the analyte is influenced by HCT. A strategy toward a solution here would be to apply more rigorous desorption conditions, aiming at re-dissolution of the entire blood sample. The downside of this approach is that more unwanted matrix components are re-dissolved as well, requiring extra sample clean-up. Flow-through desorption of DBS coupled online to SPE and (LC)MS/MS combines total workflow automation with efficient front-end sample clean-up and seems perfectly positioned to follow the above strategy successfully. We have explored this approach for full-spot analysis; desorption conditions, including pH, organic content, temperature and pre-wetting were optimized to achieve maximum dissolution of dried blood.

Online DBS-SPE-MS/MS



The experimental Flow-Through Desorption (FTD) device for DBS cards comprised a card clamp, a sliding holder for positioning the card in the clamp and two switching valves. Valve 2 is used for switching the clamp online or offline with the solvent flow to the SPE cartridge and valve 1 for loop injection of internal standard. High Pressure Dispenser 1 (HPD, Spark Holland) delivers activation and conditioning solvent to the SPE cartridge (DBS clamp offline) and desorption solvent via the DBS card (DBS clamp online). The same HPD delivers wash solvent to the cartridge to remove matrix components from the SPE cartridge after trapping (DBS clamp offline). HPD 2 is used to deliver the internal standard solution to the 20 µL injection loop on valve 1. The loop is switched into the desorption flow stream simultaneously with the start of the desorption. A standard SPE cartridge exchange system (ACE, Spark Holland) is used to provide a new SPE cartridge for every analysis and to switch the SPE cartridge between trapping and elution position. A heater placed upstream of the clamp allows heating of the desorption solvent up to 80 °C. A gradient pump system (Symbiosis LC pump, Spark Holland) elutes the trapped components from the SPE cartridge to the mass spectrometer. The entire system is controlled via SparkLink version 3.10 SP3 instrument control software (Spark Holland).

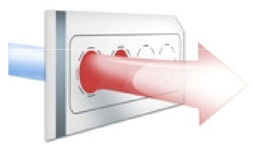
Experimental conditions

Preparation of blood samples

Human blood (K_3-EDTA) was spiked with haloperidol, amitriptyline and verapamil at 50 ng/mL (50 µL 1000 ng/mL standard solution in 40% ACN is added to 950 µL blood). For the (approximate) determination of the HCT value, blood was centrifuged for 10 minutes at 2500 rpm. Subsequently, the HCT value was calculated by dividing the volume of blood cells by the total blood volume. Blood with different hematocrit values (0.3, 0.4, 0.5, 0.6 and 0.7) was prepared by the addition of plasma or red blood cells to blood.

DBS

Filter card:	Whatman FTA™ DMPK-C Card
Sample:	5 µL blood was spotted on the card using a volumetric micropipette
Desorption:	Standard: 1 mL water 0.2% FA at 2 mL/min Harsh: see text
Clamp diameter:	6 mm
Clamp flush:	1 mL 80/20 acetonitrile/water 0.2% FA at 5 mL/min 1 mL water 0.2% FA at 5 mL/min

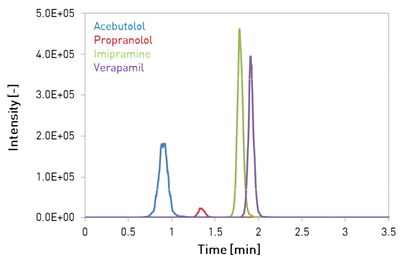


Combined SPE/LC

Cartridge:	HySphere C18HD 10x2 mm, 7 µm
Conditioning:	1 mL acetonitrile at 5 mL/min
Equilibration:	1 mL water 0.2% FA at 5 mL/min
Sample transfer:	1 mL water 0.2% FA at 2 mL/min
Cartridge wash:	1 mL 5/95 acetonitrile/water 0.2% FA at 2 mL/min
Clamp flush:	1 mL 80/20 acetonitrile/water 0.2% FA at 5 mL/min 1 mL water 0.2% FA at 5 mL/min
Elution:	Solvent: A) water 0.2% FA; B) acetonitrile 0.2% FA Post-column flow-split to 200 µL/min

Elution gradient:

time (m:s)	flow (mL/min)	A %	B %
00:01	1.0	95	5
00:05	1.0	95	5
01:45	1.0	75	25
02:50	1.0	75	25
02:51	1.0	95	5
03:10	1.0	95	5



Example of an online DBS-SPE-MS/MS separation for blood spiked at 250 ng/mL

ESI-MS/MS with Acquity TQD (Waters) in positive mode

MS/MS settings						
Compound	**Parent (m/z)**	**Daughter (m/z)**	**General settings**			
Verapamil	455.3	165.2	Capillary voltage	2.00 kV	Source temp	150 °C
Haloperidol	376.1	165.1	Cone voltage	40 V	Desolvation temp	350 °C
Amitriptyline	278.1	90.8	Cone gas	50 L/hr	Desolvation gas	500 L/hr
Acebutolol	337.2	116.1	Collision gas	0.20 mL/min	Dwell time	0.05 s
Imipramine	281.3	85.9				
Propranolol	260.3	116.1				

Measuring recovery from a blood spot

For reasons of consistency we used the same method for recovery measurement as developed for partial spot analysis. Recovery of analyte from the blood spot was measured by performing a number of consecutive analyses of the same blood spot. It is assumed that the sum of the analyte signals of all analyses from one blood spot represent (near) 100% recovery if the signals of the last analyses are a negligible fraction of the first. The recovery of the method (the first analysis) is then calculated as percentage of the accumulated recovery from all consecutive runs. See example below.

Desorption	Recovery (%)		
Consecutive runs	**Haloperidol**	**Amitriptyline**	**Verapamil**
1	**75.7**	**77.9**	**77.3**
2	21.9	20.4	20.7
3	1.9	1.7	1.6
4	0.3	0.2	0.3
5	0.2	0.0	0.1
sum	100	100	100

Recovery of the method

Results and Discussion

Precision of full-spot analysis

Before measuring recoveries under varying conditions, the precision of the whole spot analysis method was checked. No internal standard was used here.

Precision as %RSD (n = 6)		
Haloperidol	**Amitriptyline**	**Verapamil**
3.2	1.9	3.4

Effect of hematocrit on recovery

Recovery is significantly lower for blood spots with high HCT values as is evident from the table below.

Recovery (%)			
HCT	**Haloperidol**	**Amitriptyline**	**Verapamil**
0.3	90.2	91.1	91.5
0.7	63.9	67.7	68.1

Effect of spot aging on recovery for high-hematocrit samples?

Recovery seems to decrease with spot aging for blood with high HCT values as is indicated by the recovery measurements below.

Recovery (%) after one day storage			
HCT	**Haloperidol**	**Amitriptyline**	**Verapamil**
0.3	90.8	92.0	91.7
0.7	64.7	68.6	68.5

Recovery of the test compounds from blood spots with HCT 0.3 and 0.7; after one day storage. Desorption at standard conditions.

Recovery (%) after 4 days storage			
HCT	**Haloperidol**	**Amitriptyline**	**Verapamil**
0.3	88.3	88.6	90.2
0.7	59.0	55.3	57.8

Recovery of the test compounds from blood spots with HCT 0.3 and 0.7 after four days storage. Desorption at standard conditions.

Rationale to reduce the hematocrit effect on recovery

If the amount of sample left behind on the card after desorption varies with HCT (or other variables, such as aging, paper type etc), then the best way to reduce the effect of such variations is to make that residue as small as possible. In other words: if more than 95% of the sample is desorbed, independent of HCT, aging, etc, then there is only 5% of the recovery that can vary with HCT, aging, etc.

Harsh desorption conditions

In order to achieve high recovery, independent of HCT, "harsh" desorption conditions must be applied. Apart from testing the influence of flow rate and solvent composition, we also added heat as a variable to enhance desorption, using a capillary heater upstream the DBS clamp. After a large number of experiments to optimize recovery, the following desorption conditions were determined as optimal:.
1 mL H2O 0.2% FA at 2 mL/min at 80°C (set point of heater) followed by 1 mL 5% ACN 0.2% FA at 2 mL/min at 80°C . Approximately 95% recovery was achieved for high HCT after 4 days of storage.

Recovery (%) after 4 days of storage (HCT 0.7)		
haloperidol	**amitriptyline**	**verapamil**
94	96	95

Recovery from blood spots with 0.7 HCT , after 4 days of storage using the final "harsh" desorption conditions.

Validation using harsh desorption conditions

Harsh desorption conditions result in more matrix to co-desorb with the analytes. Although the combined online SPE/LC will remove most matrix components, there is an increased risk of interference. In order to check for such interferences the DBS-SPE/LC-MS/MS method was briefly validated using the harsh desorption conditions. ("normal" (HCT not measured) human blood was spiked with acebutolol, imipramine and verapamil as test compounds at 0.1 – 1000 ng/mL (50 µL standard solution in 40%ACN is added to 950 µL blood). Other conditions as described under "Experimental conditions". No internal standard was used.

Validation check using harsh desorption conditions		Acebutolol	Imipramine	Verapamil
Recovery (%)	125 ng/mL	100.0	99.8	99.6
Linearity (R^2)	1-800 ng/mL	> 0.99	> 0.99	> 0.99
Precision (% RSD; n = 6)	1 ng/mL 25 ng/mL 500 ng/mL	9.9 4.3 3.3	7.9 2.2 1.0	17.6 3.9 2.1
Accuracy (%) 3 pts cal line 2, 50, 150 ng/mL	1 ng/mL 25 ng/mL 500 ng/mL	120 98 91	85 101 103	103 100 103
Carry-over	A blank blood spot was measured immediately after a 800 ng/mL blood spot sample	< LOD	< LOD	< LOD
Matrix effects (% signal loss)	A std mix of analytes was injected in eluate from a blank blood spot and compared with same std. mix injected with clamp in by-pass position	< 5	-16	< 5

Conclusion

- Recovery from blood spots is negatively affected by high hematocrit values.
- Aging of the blood spot seems to worsen this effect.
- Harsh desorption conditions, including heating, increase recovery to > 90% independent of HCT and aging.
- The added clean-up function of online DBS-SPE-MS/MS effectively removes the inevitable extra matrix.
- Full-spot DBS analysis with online desorption can be performed without influence from varying HCT on assay results.

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