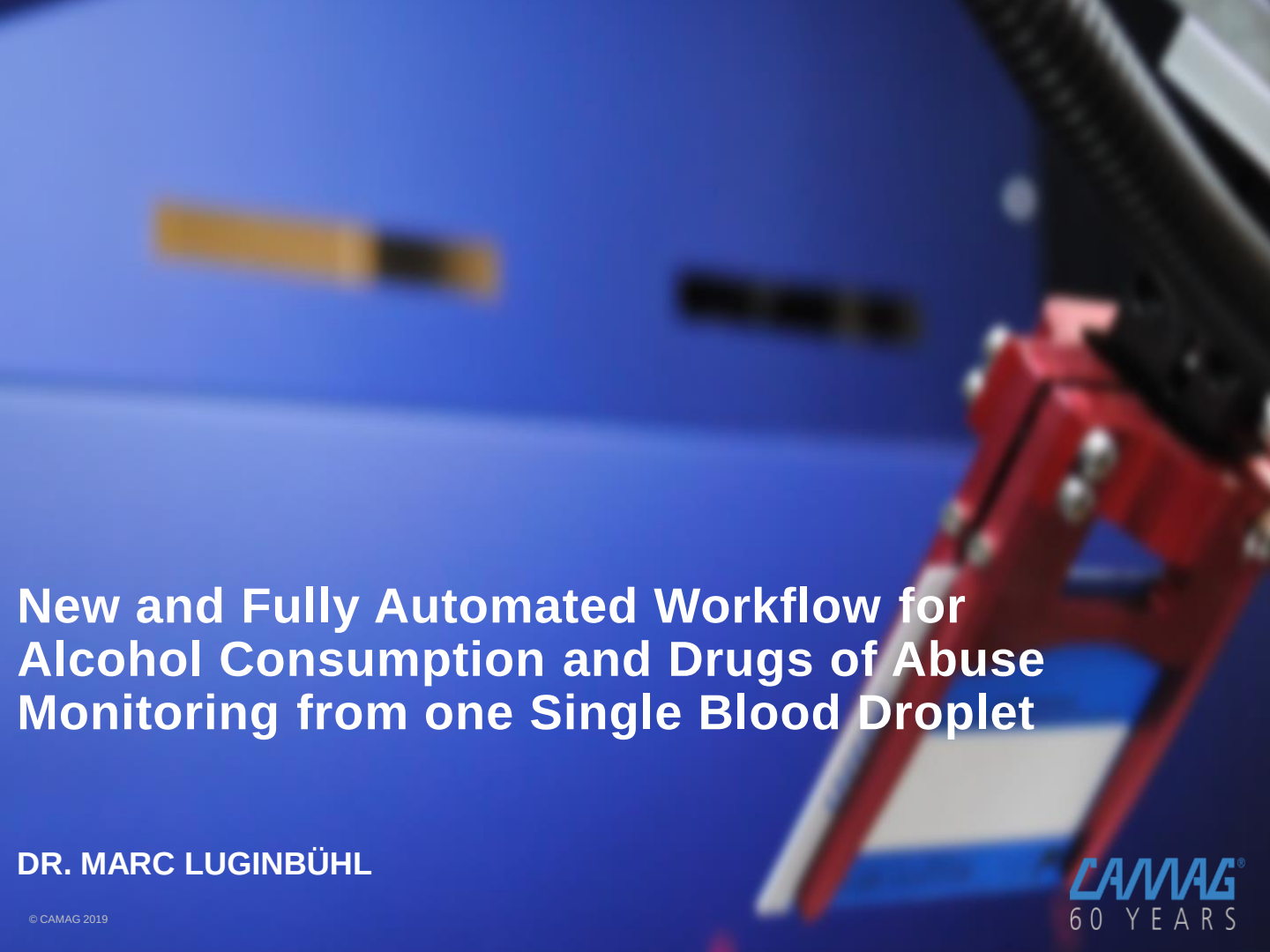
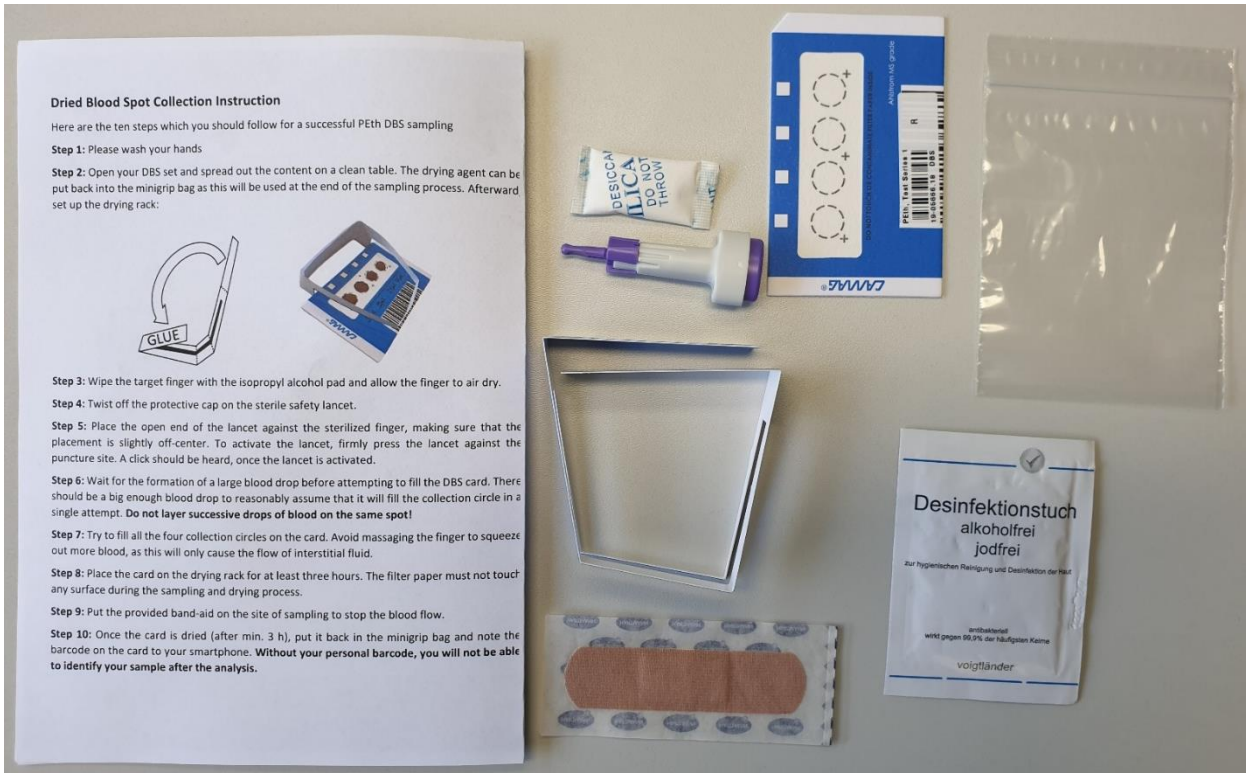


New and Fully Automated Workflow for Alcohol Consumption and Drugs of Abuse Monitoring from one Single Blood Droplet

DR. MARC LUGINBÜHL

CAMAG® 60 YEARS

Minimally invasive sampling



Dried Blood Spot Collection Instruction

Here are the ten steps which you should follow for a successful PEth DBS sampling

Step 1: Please wash your hands

Step 2: Open your DBS set and spread out the content on a clean table. The drying agent can be put back into the minigrip bag as this will be used at the end of the sampling process. Afterward set up the drying rack:

Step 3: Wipe the target finger with the isopropyl alcohol pad and allow the finger to air dry.

Step 4: Twist off the protective cap on the sterile safety lancet.

Step 5: Place the open end of the lancet against the sterilized finger, making sure that the placement is slightly off-center. To activate the lancet, firmly press the lancet against the puncture site. A click should be heard, once the lancet is activated.

Step 6: Wait for the formation of a large blood drop before attempting to fill the DBS card. There should be a big enough blood drop to reasonably assume that it will fill the collection circle in a single attempt. **Do not layer successive drops of blood on the same spot!**

Step 7: Try to fill all the four collection circles on the card. Avoid massaging the finger to squeeze out more blood, as this will only cause the flow of interstitial fluid.

Step 8: Place the card on the drying rack for at least three hours. The filter paper must not touch any surface during the sampling and drying process.

Step 9: Put the provided band-aid on the site of sampling to stop the blood flow.

Step 10: Once the card is dried (after min. 3 h), put it back in the minigrip bag and note the barcode on the card to your smartphone. **Without your personal barcode, you will not be able to identify your sample after the analysis.**

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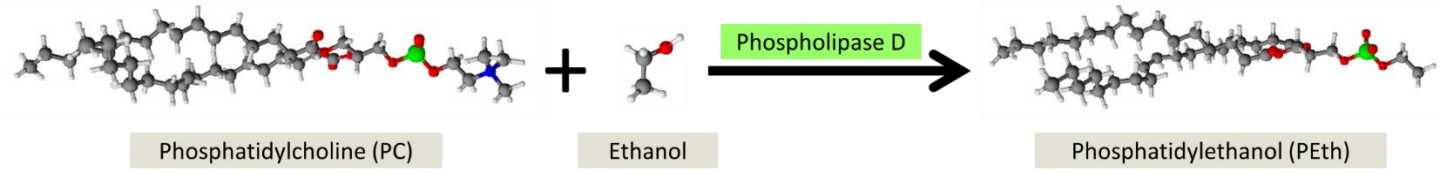
Fully Automated Determination of Phosphatidylethanol

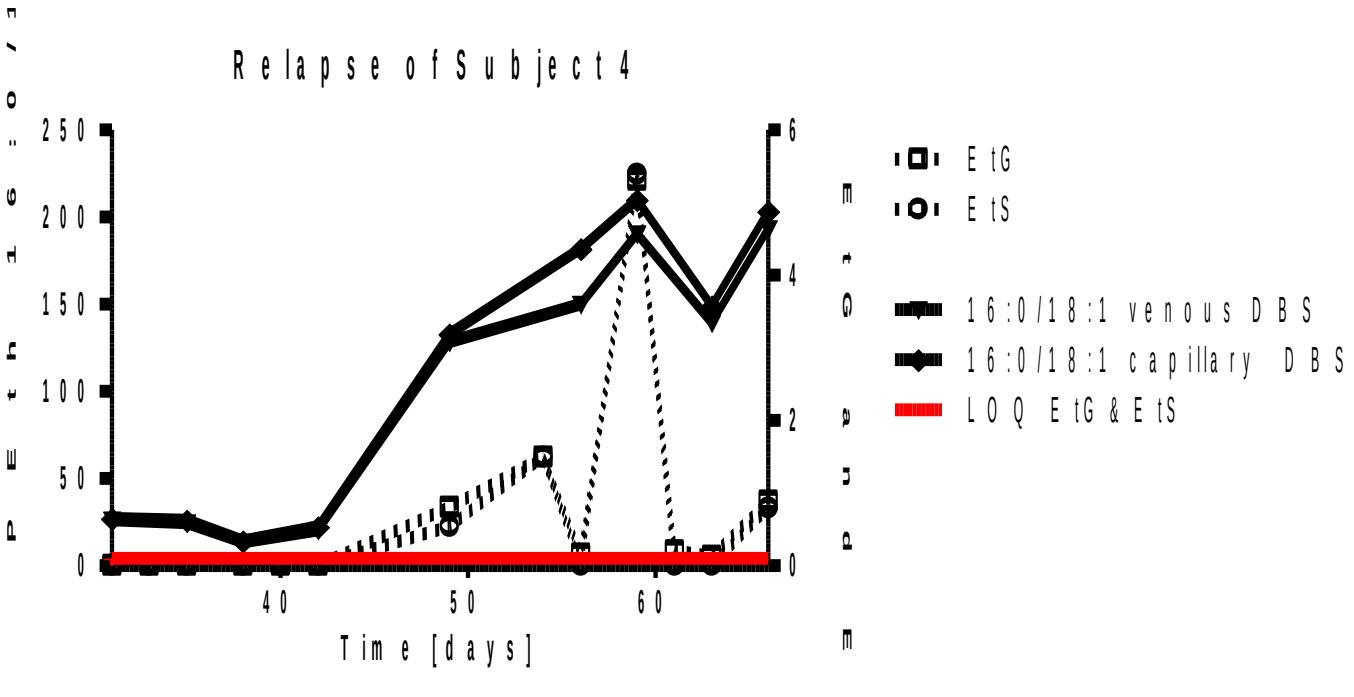
Formation of Phosphatidylethanol



- PEth is a group of phospholipids that serve as direct alcohol marker
- PEth 16:0/18:1 and PEth 16:0/18:2 are most abundant in human blood
- PEth is used for the assessment of alcohol consumption behaviour
- PEth has a half-life of 3-12 days
- PEth is not stable in liquid blood, unless stored at -80ºC
- Dried Blood Spots are the matrix of choice for PEth

Gnann et al. - Formation of phosphatidylethanol and its subsequent elimination during an extensive drinking experiment over 5 days. Alcohol Clin Exp Res. 2012
Schröck et al. - Phosphatidylethanol (PEth) detected in blood for 3 to 12 days after single consumption of alcohol—a drinking study with 16 volunteers. Int J Legal Med. 2017
Gnann et al. - Identification of 48 homologues of phosphatidylethanol in blood by LC-ESI-MS/MS. Anal Bioanal Chem. 2010

Applications for Phosphatidylethanol
Alcohol Withdrawal and Dishabituation Therapy



 Luginbühl et al. - Monitoring of direct alcohol markers in AUD patients during withdrawal treatment and successive rehabilitation, Drug Test Anal. 2019

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General Experimental Setup
Fully Automated PEth Determination

Required Setup

- CAMAG DBS-MS 500 autosampler
- 2× binary LC-MS pump
- Mass spectrometer (e.g. Shimadzu 8050)
- Switching valve

Luginbühl et al. - Fully Automated Determination of Phosphatidylethanol 16:0/18:1 and 16:0/18:2 in Dried Blood Spots. J Anal Toxicol. 2019.

Automated DBS Processes

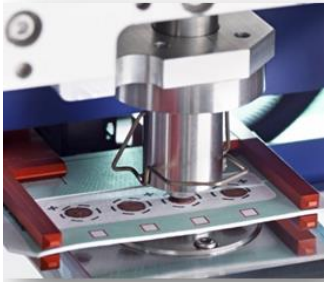
Optical card recognition

- Photo documentation
- Spot recognition
- Barcode reader
- Recognition of spot size, roundness, and center.

Internal standard application

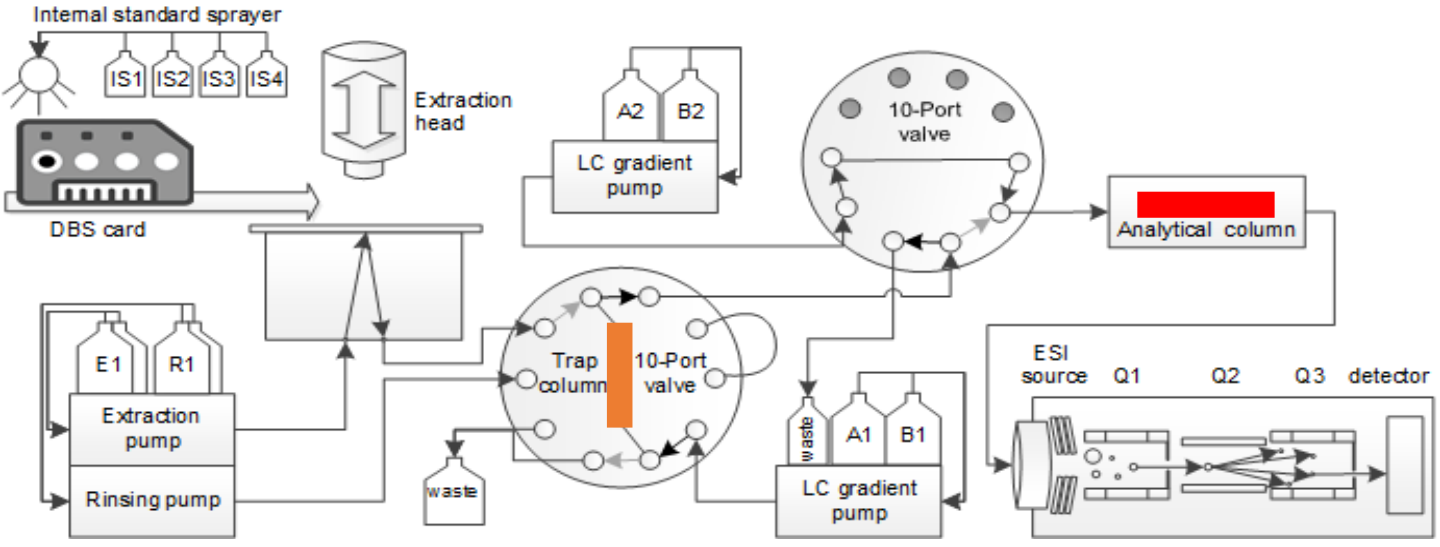
- Spray application of internal standard compensates for extraction bias
- 5-40 µL

Extraction



- Extraction under pressure
- 4 mm Ø subpunch
- Variable volume
- Wash station

Detailed Instrumental Setup



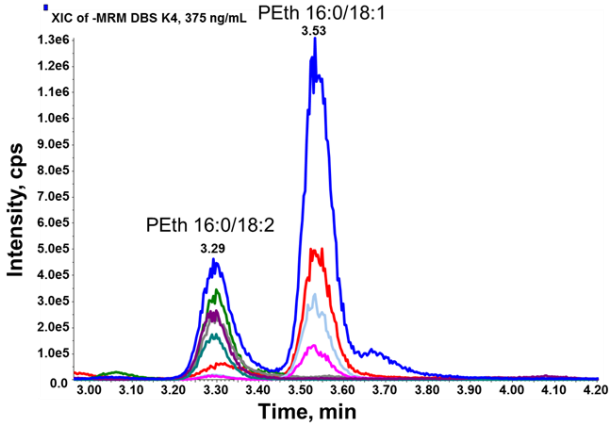
Trapping column: Synergy Polar RP, 20 mm
Analytical column: Luna RP-C5, 50 mm

Sample preparation:	2 min
LC-MS/MS run :	5 min
Total runtime, overlapped:	**5 min**

Luginbühl et al. - Fully Automated Determination of Phosphatidylethanol 16:0/18:1 and 16:0/18:2 in Dried Blood Spots. J Anal Toxicol. 2019.

Fully Automated Determination of Phosphatidylethanol

- Analytes
  - PEth 16:0/18:1
  - PEth 16:0/18:2
  - PEth 16:0/18:1-D_5
  - PEth 16:0/18:2-D_5
- Range: 20-1500 ng/mL (six-point calibration)
- QC: 20, 45, 1180 ng/mL
- Extraction: 40 µL (40 µL/min)
  - (34.5/15/50/0.05, H2O/MeCN/2-Propanol/FA)

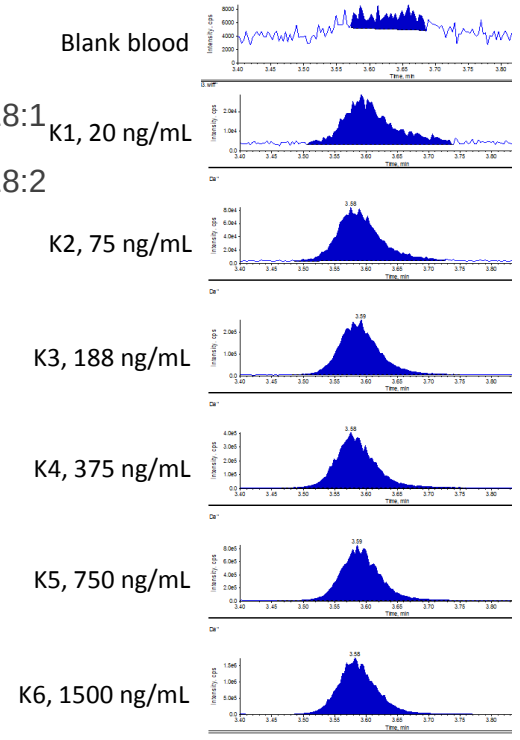


Compound	Q1 [m/z]	Q3 [m/z]	Dwell time [msec]	DP [volts]	EP [volts]	CE [volts]	CXP [volts]	RT [min]
PEth 16:0/18:1 SRM 1	701.3	255.2	20	-32	-10	-40	-14	3.53
PEth 16:0/18:1 SRM 2	701.3	281.3	20	-20	-10	-40	-14	
PEth 16:0/18:1–D_5 SRM 1	706.3	255.3	20	-20	-10	-40	-14	
PEth 16:0/18:1–D_5 SRM 2	706.3	281.1	20	-32	-10	-40	-14	
PEth 16:0/18:2 SRM 1	699.5	279.4	20	-5	-10	-40	-14	3.29
PEth 16:0/18:2 SRM 2	699.5	255.3	20	-5	-10	-40	-14	
PEth 16:0/18:2–D_5 SRM 1	704.5	279.5	20	-5	-10	-40	-14	
PEth 16:0/18:2–D_5 SRM 2	704.5	255.3	20	-5	-10	-40	-14	

Luginbühl et al. - Fully Automated Determination of Phosphatidylethanol 16:0/18:1 and 16:0/18:2 in Dried Blood Spots. J Anal Toxicol. 2019.

Validation Parameter

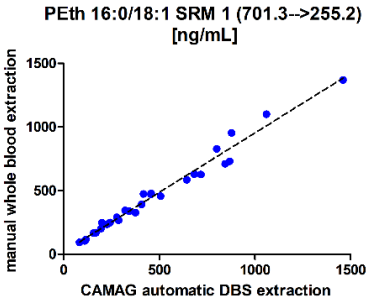
- Linearity:
  - 0.9980±0.0016 (range: 0.9954-0.9996) for PEth 16:0/18:1
  - 0.9974±0.0005 (range: 0.9953-0.9982) for PEth 16:0/18:2
  - extended linear range up to 2500 ng/mL with a correlation coefficient of at least 0.9975
- **LOQ at 20 ng/mL**, LOD at 10 ng/mL
- Accuracy and precision within ±15%
- Mean extraction efficiency: 88%

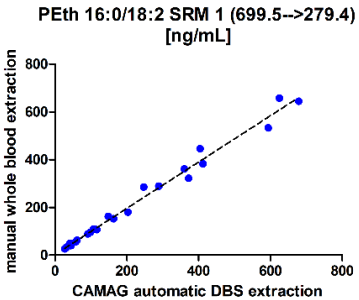


 Luginbühl et al. - Fully Automated Determination of Phosphatidylethanol 16:0/18:1 and 16:0/18:2 in Dried Blood Spots. J Anal Toxicol. 2019.

Whole Blood Extraction vs Fully Automated DBS Analysis

- **Manual whole blood analysis**
  - Prepare sample labels for extraction containers
  - Label all containers
  - Pipette sample into Eppendorf tube
  - Add extraction solvent
  - Add internal standard and close tubes
  - Shake
  - Centrifuge
  - Transfer into glass vial
  - Evaporate solvent
  - Reconstitute
  - Cap samples
  - Build batch
  - Inject





- **Fully automated DBS analysis**
  - Add card into autosampler
  - Build batch
  - Hit play

Figure 1 comparison of the automated CAMAG DBS extraction to manual whole blood extraction for 28 samples from AUD patients. (data points above LOQ)

- **Whole blood, PEth 16:0/18:1, Slope: 0.9349±0.02824 (R=0.9768)**
- **Whole blood, PEth 16:0/18:2, Slope: 0.9706±0.02339 (R=0.9863)**

Luginbühl et al. - Fully Automated Determination of Phosphatidylethanol 16:0/18:1 and 16:0/18:2 in Dried Blood Spots. J Anal Toxicol. 2019.

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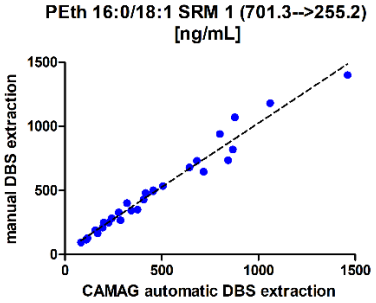
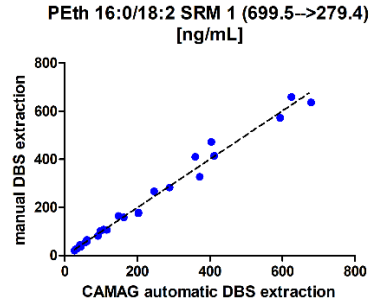
Manual Whole Spot DBS vs Fully Automated DBS Analysis

- **Manual DBS analysis**
  - Prepare sample labels for extraction containers
  - Label all containers
  - Cut DBS, fold, and place into Eppendorf tube
  - Add extraction solvent
  - Add internal standard and close tubes
  - Shake
  - Centrifuge
  - Transfer into glass vial
  - Evaporate solvent
  - Reconstitute
  - Cap samples
  - Build batch
  - Inject





- **Fully automated DBS analysis**
  - Add card into autosampler
  - Build batch
  - Hit play

Figure 1 comparison of the automated CAMAG DBS extraction to manual DBS extraction for 28 samples from AUD patients.(data points above LOQ)

- **Manual DBS, PEth 16:0/18:1, Slope: 0.9995±0.03644 (R=0.9666)**
- **Manual DBS, PEth 16:0/18:2, Slope: 1.006±0.02542 (R=0.9849)**

Luginbühl et al. - Fully Automated Determination of Phosphatidylethanol 16:0/18:1 and 16:0/18:2 in Dried Blood Spots. J Anal Toxicol. 2019.

Fully Automated, Library Based Screening

The Advantages of Screening in DBS

- Minimally invasive sampling process
- Sampling is possible on site, without healthcare professionals
- Blood is the matrix of choice to tell if somebody is under the influence
- DBS are stable at room temperature
- No biohazard label
- Reduced storage and shipping costs
- Fully automated processes are available

Stock photos from pexels.com

Forensic Toxicology Database for LC-MS/MS By Shimadzu

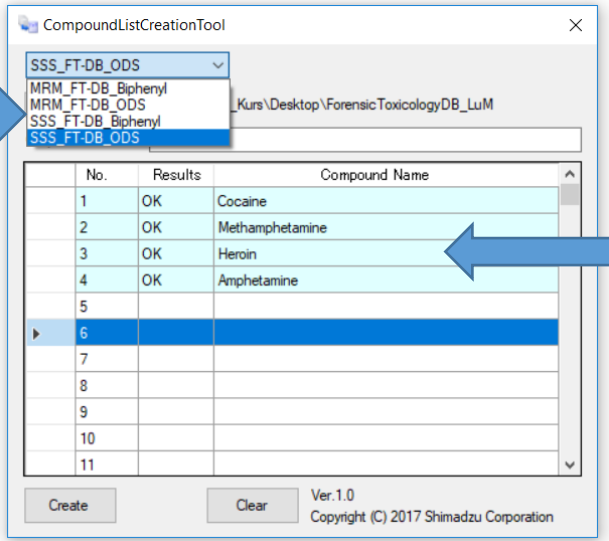
- **MRM & Spectral Library Database**

- **Two separation conditions available**
  - **ODS** (Phenomenex Kinetex XB-C18 (2.1 mmI.D. x 100 mmL., 2.6um))
  - **Biphenyl** (Restek Raptor Biphenyl (2.1 mmI.D. x 100 mmL., 2.7um)

- The ODS method contains information on 1,250 compounds and the Biphenyl method contains 1,281 compounds.

Compound List Creation Tool

MRM or Spectral Library Screening?



Insert target compounds to build compound list

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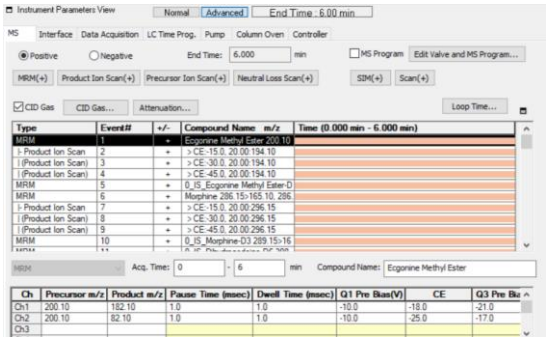
Compound List Creation Tool

Test - Notepad

File Edit Format View Help

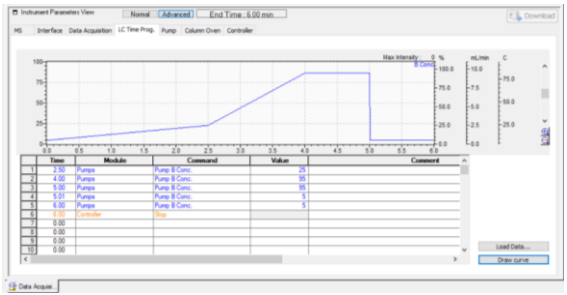
Name	Type	m/z(1)	m/z(2)	Ret. Time	Unit	Ref.(1) m/z(1)	Ref.(1) m/z(2)	Ref.(1) Ratio	Ref.(1) Allowance	Ref.(1) Rel Allowance	Conc.(1)	Band	ProcessStartTime	ProcessEndTime	Ref. Mode	Pk. Select	Calc.
ange(2) Minimum Repeat Time		Exec Condition	Order	Intensity	Dependent Scan Start m/z(1)	Dependent Scan End m/z(1)	Dependent Precursor Ion m/z(1)	Dependent Start Time(1)	Dependent End Time(1)	Dependent Acquisition Mode(1)	Depen						
t Intensity(2)	Dependent Scan	Start m/z(3)	Dependent Scan End m/z(3)	Dependent Precursor Ion m/z(3)	Dependent Start Time(3)	Dependent End Time(3)	Dependent Acquisition Mode(3)	Dependent Polarity(3)	Dependent Event Time(3)	Dependent Com							
ependent Scan End m/z(5)		Dependent Precursor Ion m/z(5)	Dependent Start Time(5)	Dependent End Time(5)	Dependent Acquisition Mode(5)	Dependent Polarity(5)	Dependent Event Time(5)	Dependent Comment(5)	Dependent Interface Voltage(5)	Depen							
Cocaine	3	304.15	182.25	4.409		304.15	82.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1913	0.00E+00	0.00E+00	0.00E+00	0.00E+00	
Methamphetamine	3		150.12	91.05	3.466		150.12	119.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	3481	0.00E+00	0.00E+00	0.00E+00	0.00E	
Heroin	3	370.16	58.15	4.31		370.16	44.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2729	0.00E+00	0.00E+00	0.00E+00	0.00E+00	
Amphetamine	3		136.1	91.05	3.36		136.1	119.05	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1309	0.00E+00	0.00E+00	0.00E+00	0.00E	

Import custom target list into the default screening LC-method



Start optimization:

- LC parameters
- MS compound window



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Chromatogram

MS/MS Spectrum with three different collision energies (CE)

MRM Transitions for the quantification

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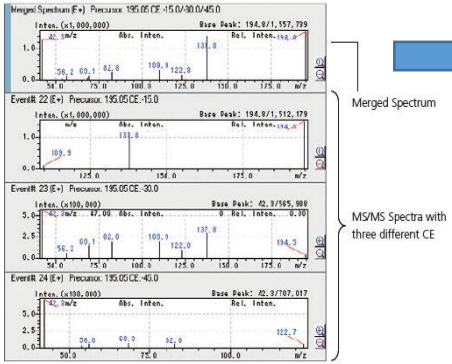
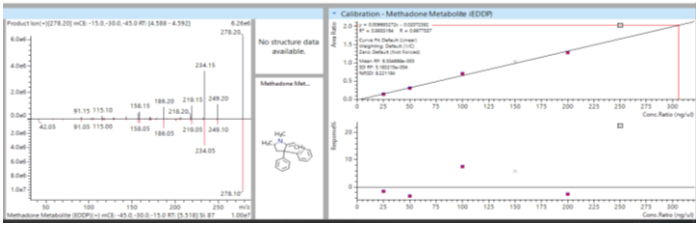
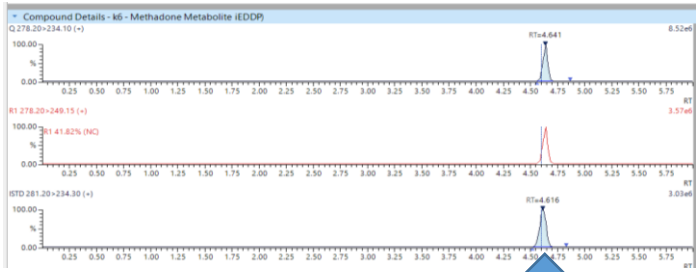
Compound Identification by Merged Spectra







Merged spectra using three MS/MS spectra with three different CE helps to increase the confidence in reporting

Qualifier, Quantifier, and IS

https://www.shimadzu.com/an/lcms/forensic_toxicology-database/index.html

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Library Search

Library Hits - k6 - Methadone Metabolite iEDDP)

#	Compound ...	Lib. SI	Theory MW	Formula	Class	Lib. Filename	RT	Adduct Ion	Precursor Ion	MS Stage	Ionization	Collision En...	Instrument	Data Filena...	Acquired By	Acquired
1	Methadone ...	87	277.1830	C20H23N	----	Library_FT-...	5.518	----	278.2000	2	ESI	----	LCMS-TQ	----	----	1/1/1601 1:...
2	4-F-alpha-P...	66	277.1842	C17H24FNO	----	Library_FT-...	5.443	----	278.2000	2	ESI	----	LCMS-TQ	----	----	1/1/1601 1:...
3	Venlafaxine	66	277.2042	C17H27NO2	----	Library_FT-...	5.299	----	278.2000	2	ESI	----	LCMS-TQ	----	----	1/1/1601 1:...
4	Maprotiline	61	277.1830	C20H23N	----	Library_FT-...	6.284	----	278.2000	2	ESI	----	LCMS-TQ	----	----	1/1/1601 1:...
5	Amitriptyline	58	277.1830	C20H23N	----	Library_FT-...	6.368	----	278.2000	2	ESI	----	LCMS-TQ	----	----	1/1/1601 1:...

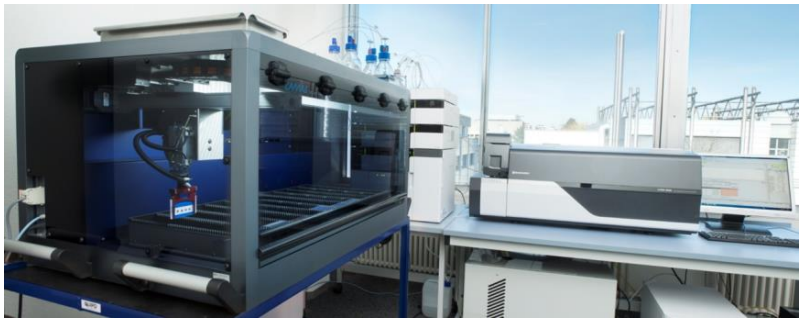
Library search based on similarity index increases confidence

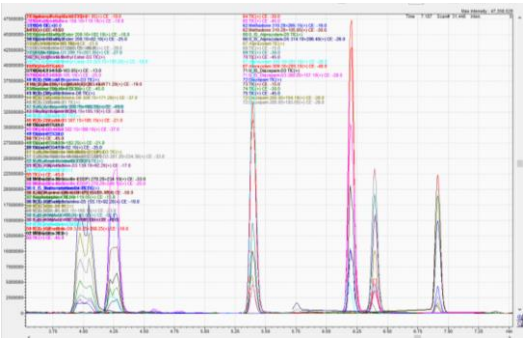
Compound identification by

- Retention time
- Multiple MRM transitions (Qualifier/Quantifier)
- Merged Spectra (three different CE)
- Library Search/Similarity Index
- Internal Standard

Method Example

- Shimadzu 8050 coupled with a DBS-MS 500 autosampler
- ODS method, total runtime of 6 min
- Ex sol (20 µl): 50-50 MeOH-H_2O, 0.1%FA, 10 mM Ammonium Formate





Quantification of Target Compounds

Six-point calibration (25, 50, 100, 150, 200, 250 ng/mL) in blank blood, one QC each

Target	Internal Standard	Linearity	QC 25 ng/mL	QC 125 ng/mL
▪ Codeine	IS Codeine-D3	r=0.994	25.7	113
▪ Dihydrocodeine	IS Dihydrocodeine-D6	r=0.976	25.7	110
▪ Amphetamine	IS Amphetamine-D3	r=0.997	26.9	125
▪ Methamphetamine	IS Methamphetamine-D5	r=0.996	27.9	123
▪ MDMA	IS MDMA-D5	r=0.993	25.3	135
▪ Cocaine	IS Cocaine-D3	r=0.991	27.1	143
▪ Benzoyl Ecgonine	IS Benzoyl Ecgonine-D3	r=0.995	29.4	131
▪ Ecgonine Methylester	IS Ecgonine Methylester-D3	r= 0.995	27.2	137
▪ Methadone	IS Methadone-D9	r=0.991	25.3	148
▪ Methadone Metabolite (iEDDP)	IS Methadone Metabolite (IS iEDDP)-D3	r=0.991	31.6	143
▪ Alprazolam	IS Alprazolam-D5	r=0.975	24.8	126
▪ Diazepam	IS Diazepam-D3	r=0.981	18.0	120

Monitoring of two MRM transitions + Spectra for Library identification

And there is much more you can do!

- Vitamin D
- Nicotine and Cotinine
- Ivermectin
- Antiepileptic Drugs
- Antiretroviral Drugs
- New Born Screening
- Testosterone Panels

Thank you for your Attention!

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