Fully automated dried spot analysis for the rapid quantitation of tramadol and its metabolites in various matrices

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Application benefits
- Fully automated dried spot analysis workflow with integrated software for seamless instrument control

- Flow-through desorption (FTD™) technology without manual punch-out and extraction of dried spot discs

- Intelligent Vision Camera (IVC™) for accurate spot recognition, positioning, barcode ID, sample traceability, and chain-of-custody

Goal
- Demonstrate the integrated workflow and benefits of the Thermo Scientific™ Transcend™ DSX-1 system, which combines a dried spot autosampler and UHPLC system to fully automate dried spot sampling and analysis.

- Highlight how innovative flow-through desorption (FTD™) technology from the dried spot autosampler allows direct desorption of the analytes from the dried spot cards to the UHPLC system that incorporates advanced Thermo Scientific™ TurboFlow™ technology to carry out online sample cleanup and liquid chromatography separation, which when combined with mass spectrometry (MS), allows for rapid quantitation of tramadol and its metabolites from various dried spot matrices.

Introduction
Tramadol is a synthetic opioid that is a centrally acting analgesic and widely prescribed to relieve acute or chronic pain. Pain management is achieved by a dual mechanism. Tramadol inhibits neurotransmitter reuptake, while its M1 metabolite, *O*-desmethyltramadol, activates the μ-opioid

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receptor.[1] The bioavailability of tramadol is around 70%, and approximately 10–30% is excreted unchanged in the urine. The distinct mechanism and lower risk of addiction compared to other opioids make it a popular choice for prescribers. However, misuse and emergency room visits are increasing. Many bioanalytical methods have reported the quantitation of tramadol or its metabolites in various biological matrices (blood, serum, urine, hair) using gas or liquid chromatography (GC or LC)-mass spectrometry.[2–4]

In a sport setting, tramadol has been on the World Anti-Doping Agency (WADA) monitoring program since 2012 and raised significant attention for its prevalence in cyclist communities in recent years. In a published 2017 sports monitoring report, 68% of urine samples containing tramadol were from cyclists.[5] While it is still in debate if tramadol use leads to performance improvements, an athlete may potentially experience fatigue and confusion from tramadol use, which can create dangerous situations. In 2019, Union Cycliste Internationale (UCI) banned the use of tramadol in professional cycling with penalties ranging from disqualification to a nine-month ban. UCI also provided suggested guidance to collect blood droplets of in-competition athletes randomly throughout an entire competition and submit them for laboratory analysis.[6] Therefore, implementation of dried blood spot (DBS) sample collection coupled with high throughput analysis to determine the presence of tramadol and metabolites (Figure 1) that are used as indicators for the presence or absence of drug intake is needed.[7–8]

Dried spot analysis, particularly DBS, has become an emerging technology in clinical research and toxicology applications such as inborn errors of metabolism, therapeutic drug monitoring research, forensic toxicology, and anti-doping.[9] It requires only a small volume of samples to be collected with the benefits of a less invasive sampling procedure, lower risk of infections, and ease of transportation and logistics. While dried spot analysis
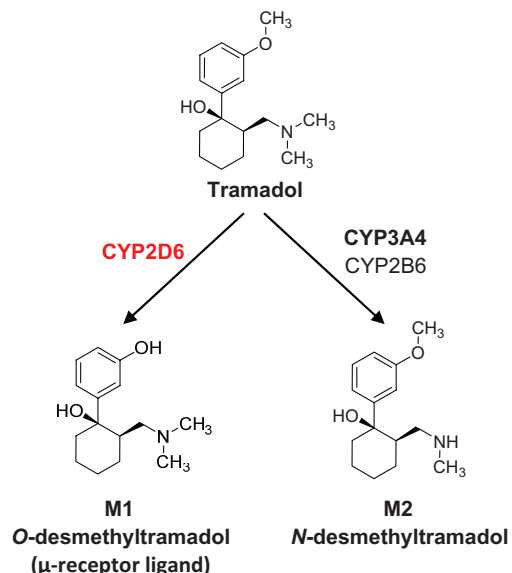

Figure 1. Metabolic pathway of tramadol and its major metabolites

has gained popularity over the years, the traditional workflow still utilizes manual disc-punching followed by a combination of sample preparation procedures, including various extractions, evaporation, and reconstitution for downstream analysis.[10] The whole process can take up to several hours.

This technical note demonstrates a fully automated workflow for dried spot analysis using the Transcend DSX-1 system. The DSX-1 system eliminates the need for manual disc-punching and extraction and reduces human error. The innovative integration of sample preparation into online analysis utilizing a revolutionary dried spot autosampler capable of flow-through desorption, followed by a two-dimensional TurboFlow UHPLC-MS/MS analysis is suited to handle sample cleanup from various biomatrices, separation, and identification (Figure 2). Rapid quantitation of tramadol and metabolites in dried urine, blood, serum, and saliva samples was achieved with linearity from 5 to 400 ng/mL that meets different cut-off needs depending upon the regulations.
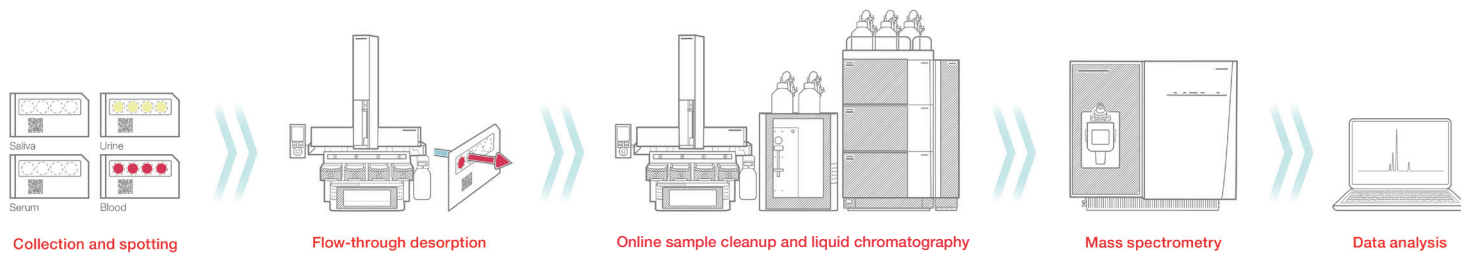

Figure 2. Automated dried spot analysis: Transcend DSX-1 system combined with the TSQ Altis MD series mass spectrometer

Experimental

Sample preparation

Tramadol and metabolites (O-desmethyltramadol, N-desmethyltramadol) were spiked into blood, serum, urine, and saliva samples in a concentration range from 5 to 400 ng/mL. A 6 µL aliquot of sample was spotted on three of the four spots on each DBS card (Qiagen FTA™ DMPK-Type C, Ahlstrom AutoCollect™, or Perkin Elmer 226 Bioanalysis RUO). The fourth spot was reserved for the autosampler cleaning step. The sample cards were dried at room temperature and placed directly onto the autosampler cardholder.

Fully automated sample extraction

The dried spot module (DSM) was attached to the front end of the LC-MS system and configured with a standard size clamp head to desorb a 6 mm spot from the card. Internal standards (IS, tramadol-^{13}C-d$_3$, O-desmethyltramadol-d$_6$, N-desmethyltramadol-d$_3$) were delivered via the built-in IS loading pump in the DSM to apply a precise amount onto each spot. Elution was performed using water containing 0.1% formic acid in water (v/v) at a flow rate of 1 mL/min for 30 s with HotCap™ enabled at 100 °C, followed by 20 s drying using an internal air compressor to flush the residual mobile phase. Individual spots were photographed with the IVC™ before and after each analysis to check the presence of a spot, adjust the position for extraction, and verify the occurrence of extraction. After the desorption process, the clamp head was rinsed with the 0.1% formic acid in water (v/v) (wash 1) and acetonitrile/isopropanol/acetone (30/30/40) (v/v/v) (wash 2).

Online sample cleanup-liquid chromatography

Automated online cleanup and chromatographic separation were performed on a UHPLC platform enabled with TurboFlow™ technology, consisting of a Thermo Scientific™ Cyclone™-P column (0.5 × 50 mm) followed by a biphenyl analytical column (3 × 50 mm, 2.6 µm). The TurboFlow method was configured in *Focus* mode where an extra loop was filled with specific solvent composition optimized for efficient elution of analytes off the TurboFlow column prior to entering the analytical column. The analysis process and flow path are mainly divided into four steps (Figure 3):

1. Sample loading onto the TurboFlow column

2. Eluting of analytes from the TurboFlow column and transferring to the analytical column

3. Separating of analytes on the analytical column and washing of the TurboFlow column

4. Equilibration of the the analytical column, refilling of the transfer loop, and equilibration of the TurboFlow column

The mobile phases and detailed gradients used in this technical note are described in Table 1.
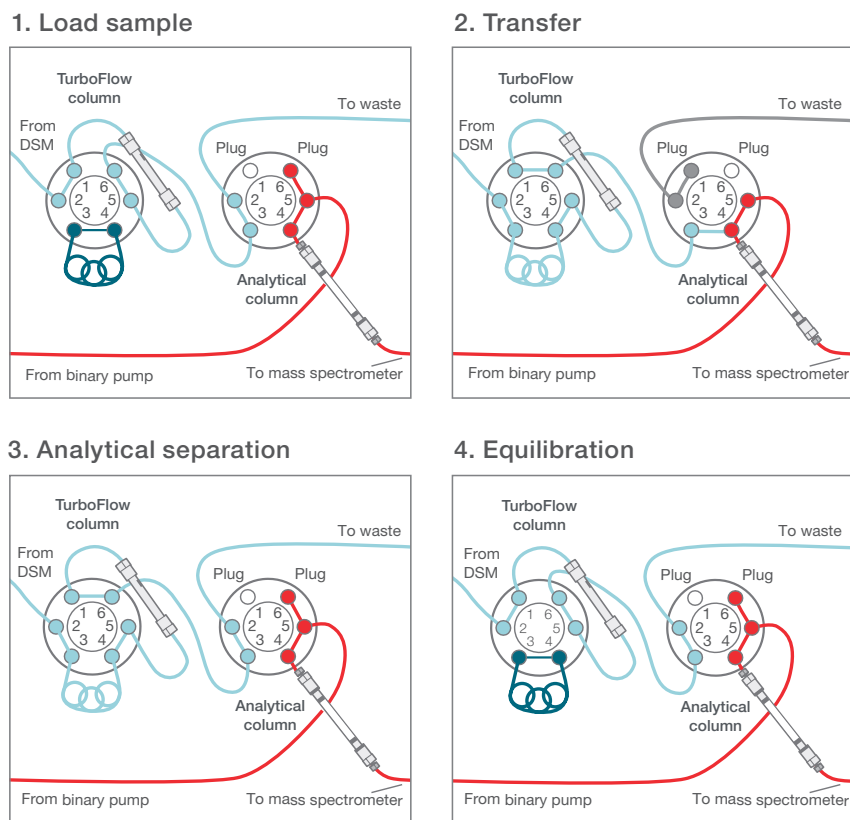

Figure 3. TurboFlow technology for sample cleanup (Focus mode)

Table 1. Liquid chromatography conditions

Time (min)	TurboFlow column						Analytical column		
	Flow rate*	% A	% B	%C	Tee	Loop	Flow rate*	%A	%B
0	1.0	100	—	—	====	out	0.5	100	—
0.5	0.1	100	—	—	T	in	0.4	100	—
1.5	2	—	—	100	====	in	0.5	85	15
2.5	2	100	—	—	====	in	0.5	25	75
4.5	2	—	100	—	====	in	0.5	—	100
5.5	2	—	100	—	====	in	0.5	—	100
6.5	2	100	—	—	====	out	0.5	95	5
7	2	100	—	—	====	out	0.5	100	—
8	2	100	—	—	====	out	0.5	100	—
Mobile phase	A: 0.1% formic acid in water B: 10 mM ammonium formate in methanol C: 40% acetonitrile: 40% isopropanol: 20% acetone (v/v/v)						A: 10 mM ammonium formate in water B: 10 mM ammonium formate in methanol		

* Flow rate (mL/min)

Mass spectrometry

Analysis was performed using a Thermo Scientific™ TSQ Altis™ MD Series mass spectrometer equipped with a Thermo Scientific™ OptaMax™ NG ion source with a heated electrospray ionization (HESI) probe, operating in positive ion mode (3.5 kV). Other source conditions are listed in Table 2. Selected reaction monitoring (SRM) transitions of compounds, optimized collision energy, and RF lens are shown in Table 3.

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ software.

Results and discussion

Samples spotted on the dried spot cards were submitted for direct analysis without additional treatments. Total run-time is approximately 8 minutes including extraction and detection. Thermo Scientific™ Aria™ MX, an integrated software, is used to control all instrument parameters for sample desorption and LC separation to facilitate the ease of use and parallelism among instruments for maximum productivity.

Table 2. Mass spectrometer source settings

Parameter	Setting	Parameter	Setting
Polarity	Positive	Cycle time (seconds)	0.4
Sheath gas (Arb)	50	Q1 resolution (FWHM)	0.7
Aux gas (Arb)	10	Q3 resolution (FWHM)	1.2
Sweep gas (Arb)	0	Source fragmentation	0
Ion transfer tube temperature (°C)	325	Chromatographic peak width (seconds)	6
Vaporizer temperature (°C)	350	CID gas (mTorr)	1.5

Table 3. Optimized SRM transitions, collision energies (CEs), and RF lens for tramadol and metabolites

Analyte	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)	RF lens (V)
Tramadol[1]	264.2	58.2	54	37
Tramadol[2]	264.2	42.3	17	37
Tramadol-^{13}C-d$_3$	268.2	58.2	17	37
O-desmethyltramadol[1]	250.2	58.1	18	35
O-desmethyltramadol[2]	250.2	42.1	55	35
O-desmethyltramadol-d$_6$	256.2	64.1	18	35
N-desmethyltramadol[1]	250.2	44.0	14	35
N-desmethyltramadol[2]	250.2	232.0	8	35
N-desmethyltramadol-d$_3$	253.3	47.0	14	35

1: quantifier; 2: qualifier

Table 4. Precision and accuracy data in different dried spot matrices

Concentration (ng/mL)	Whole blood			Saliva			Serum			Urine			Standard		
	% Diff	% RSD	% CV	% Diff	% RSD	% CV	% Diff	% RSD	% CV	% Diff	% RSD	% CV	% Diff	% RSD	% CV
Tramadol															
5	18.5	5.6	7.8	3.1	8.8	6.9	4.3	5.0	5.4	-9.1	4.7	3.5	11.2	6.3	8.0
10	-3.1	15.4	18.6	3.0	10.3	9.1	0.9	5.4	5.6	9.6	13.8	12.1	0.5	4.4	4.9
25	-3.0	5.2	5.6	-1.6	6.8	6.5	0.1	5.8	5.9	8.9	12.3	11.7	-7.5	5.4	5.7
50	-6.2	8.5	8.8	1.3	8.3	8.1	-3.2	7.7	7.7	8.7	9.1	8.8	-4.4	7.0	7.2
100	-4.2	6.5	6.6	-0.3	7.4	7.3	0.2	9.6	9.7	10.9	4.8	4.7	-1.4	8.5	8.6
200	-3.5	7.9	7.9	-1.4	8.1	8.0	-1.3	5.5	5.5	0.9	8.0	7.9	0.9	8.0	8.0
400	3.6	7.9	7.9	2.3	7.6	7.6	1.0	8.7	8.7	-4.8	5.8	5.8	0.8	7.1	7.1
O-Desmethyltramadol															
5	16.1	6.1	9.2	7.8	6.9	6.4	2.8	6.3	7.2	-5.0	4.3	3.6	6.6	6.1	7.3
10	-3.3	14.9	18.7	4.2	9.1	8.7	-0.5	4.9	5.2	3.8	13.6	12.5	-0.6	4.2	4.6
25	-1.6	4.9	5.3	-4.7	4.6	4.5	-0.1	4.7	4.8	4.6	11.1	10.7	-4.9	4.7	4.9
50	-5.5	8.1	8.5	-3.9	6.1	6.0	-1.5	7.6	7.7	5.5	5.4	5.3	-2.1	6.4	6.6
100	-4.0	6.0	6.1	-1.9	7.5	7.4	1.1	9.4	9.5	8.7	4.4	4.3	-0.4	5.9	6.0
200	-3.1	7.9	7.9	-0.9	9.0	9.0	-1.0	5.7	5.7	-0.9	7.9	7.8	1.6	5.3	5.3
400	3.3	7.4	7.5	3.1	7.9	7.9	0.4	8.0	8.0	-2.6	5.5	5.5	-0.2	6.0	6.0
N-Desmethyltramadol															
5	18.4	5.8	9.1	8.6	7.4	7.9	4.1	5.8	6.8	-4.5	6.0	5.4	12.6	5.9	9.0
10	-2.3	15.1	19.3	2.4	7.3	7.6	0.7	5.7	6.2	1.2	2.9	2.7	0.5	5.0	6.1
25	-3.3	6.0	6.6	-4.1	4.1	4.2	-0.3	5.4	5.6	-2.0	1.1	1.1	-8.3	6.9	7.5
50	-6.4	8.6	9.0	-1.1	7.0	7.0	-2.7	8.0	8.1	3.7	2.2	2.2	-5.5	8.8	9.2
100	-4.5	6.0	6.1	-2.4	7.2	7.2	0.7	9.9	10.0	8.9	0.5	0.5	-1.3	8.6	8.8
200	-3.5	8.5	8.6	-1.6	7.8	7.8	-1.7	5.9	5.9	1.5	5.1	5.0	1.2	7.6	7.7
400	3.7	8.0	8.0	3.9	8.2	8.2	1.0	9.2	9.2	-3.2	3.5	3.5	0.8	8.5	8.6

The quantitation workflow is evaluated with the built-in IS delivery module. Therefore, extraction efficiency and analyte recovery can be compared among various matrices (data not shown). Spiked samples from five matrices (blood, saliva, serum, urine, water) were spotted in replicates onto various vendors' cards to evaluate the performance of the automated workflow and card compatibilities. Accuracy and precision data that combined different vendors' cards are listed in Table 4, with all analytes within acceptance limits (% RSD and % CV below 20%). To evaluate the reproducibility of the MS response, peak areas of the IS (tramadol-^{13}C-d$_3$) were analyzed from over 70 injections of various matrices, and the % RSD were observed to be below 15% (Figure 4).
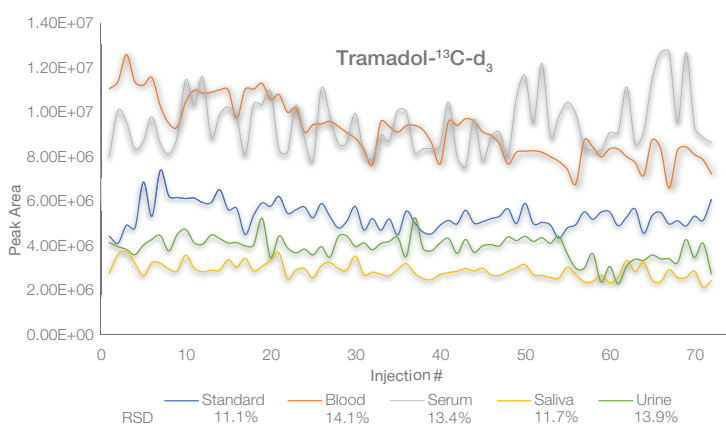

Figure 4. Peak area response of tramadol internal standard over 70 runs

	Standard	Blood	Serum	Saliva	Urine
RSD	11.1%	14.1%	13.4%	11.7%	13.9%

Calibration curves for individual analytes were generated using a weighting factor of 1/x from a lower limit of quantitation (LLOQ) of 5 ng/mL to an upper limit of quantitation (ULOQ) of 400 ng/mL, with R^2 values >0.99 and % RSD of internal standards <20% in all matrices. Datasets from dried blood spots are demonstrated in Figure 5 as an example; representative chromatograms from the LLOQ and images for pre-post analysis are also shown in Figure 6. Sample capacity is expanded with a 96-card cassette allowing 288 samples to be analyzed in a high throughput manner.
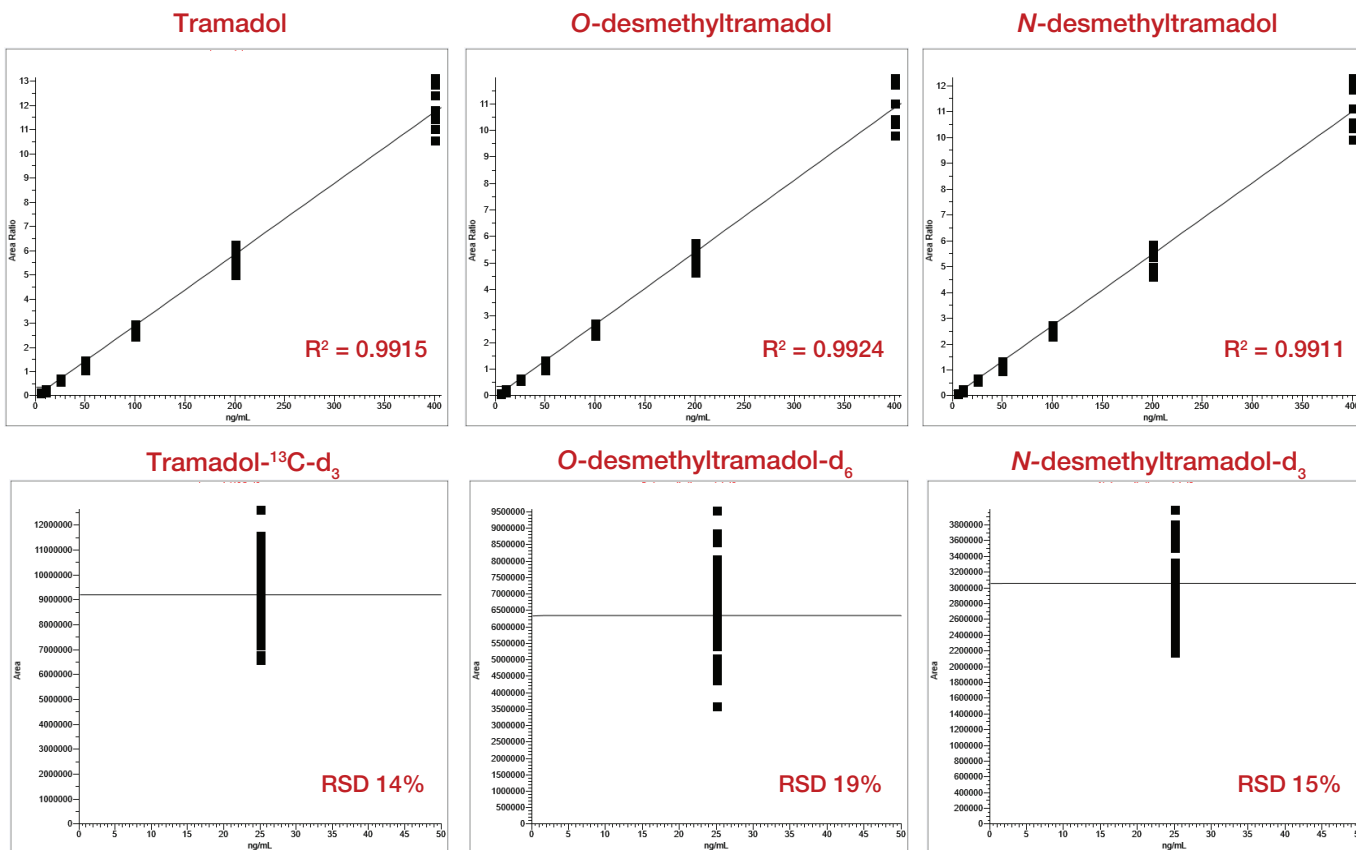
Figure 5. Calibration curves and internal standards in dried blood spots dataset (N = 9, combined vendor cards)

#	RT	Analyte	Intensity (NL)
1	3.18	O-desmethyltramadol	5.83E5
		O-desmethyltramadol-d$_6$	1.97E6
2	3.33	Tramadol	1.87E6
		Tramadol-^{13}C-d$_3$	5.26E6
3	3.35	N-desmethyltramadol	4.85E5
		N-desmethyltramadol-d$_3$	1.67E6
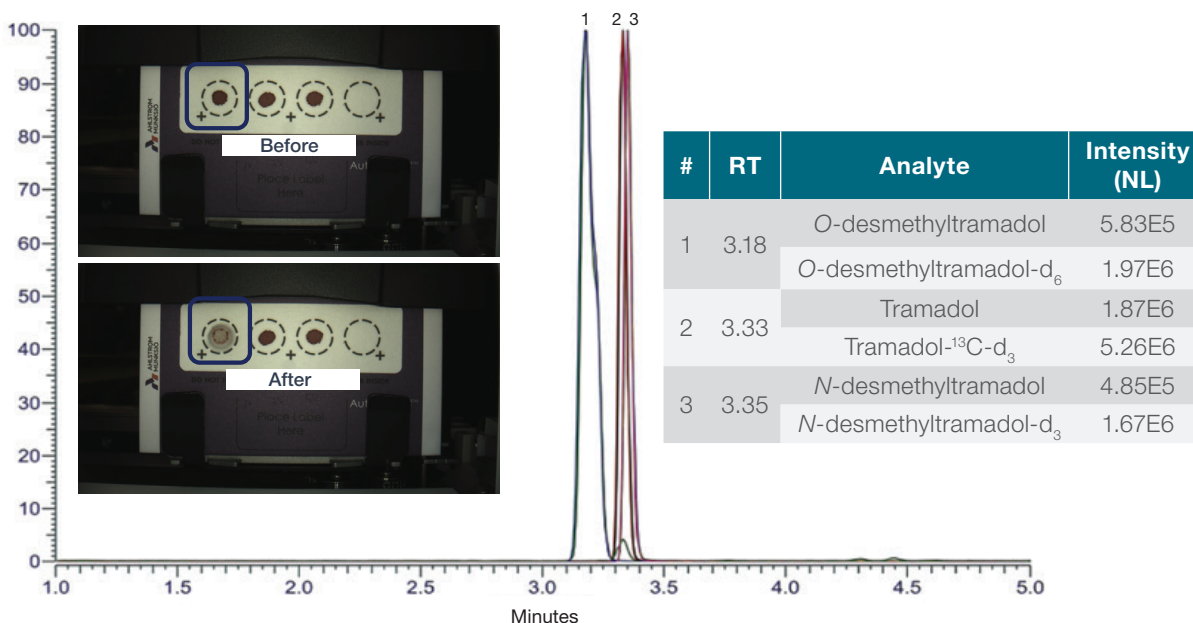
Figure 6. Chromatograms of LLOQ in dried blood spot and pre-post analysis images

Conclusion

Tramadol and its metabolites have been analyzed in various biological matrices, such as urine, saliva, blood, or serum, for purposes of therapeutic monitoring, drug abuse, and sports doping. Dried specimens offer several advantages including analyte stability, ease of sampling, and convenience of logistics when it comes to transfer and storage. Conventional dried spot analysis methods involve lengthy sample pre-treatments needing manual punchouts that require additional labor, time, and resources. The Transcend DSX-1 system combines intelligent dried spot direct sampling technology with TurboFlow 2D-LC-MS/MS to provide a fast, robust, and highly integrated platform that enables online sample extraction, separation, and data analysis while maximizing the sample throughput and cost-effectiveness and minimizing the complexity in tedious sample preparation procedures.

Quantitation of tramadol and its metabolites was evaluated in several dried matrices for reproducibility and robustness across large sample sets, as well as compatibility using several card suppliers. The analytical method described here offers a concerted and automated solution to achieve a fast analytical run for targeted quantitation of individual dried spot samples, sample authenticity, and security to maintain chain-of-custody.

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