Screening, quantitation, and confirmation of drugs of abuse in oral fluid for drug-driving testing by liquid chromatography-high resolution quadrupole time-of-flight (LC-HR-QTOF) mass spectrometry

This work summarizes results obtained from the quantitation of 19 compounds classified as drugs of abuse or recreational drugs, along with their most relevant metabolites, from oral fluid (saliva) using liquid chromatography-high resolution quadrupole time-of-flight mass spectrometry (LC-HR-QTOF).

The proposed method is fast, sensitive, and extremely selective for detection and confirmation with samples of only a few ng/mL (ppb). Calibration equations were developed for all compounds under study, with a coefficient of determination (R^2) exceeding 0.996 and standard deviation for response factors (SDRF) below 15% within a calibration range between 5 and 400 ng/mL. A Method Detection Limit (MDL) of < 0.5 ng/mL was set for all drugs under study.

Keywords: Drugs of abuse, Elute UHPLC and impact II QTOF, Screening, Hystar 2.0, TargetScreener HR, Quantitation, TASQ 1.4, LC-QTOF, Saliva

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Introduction

Many studies [1] have linked alcohol or drug consumption to road traffic accidents. Chemically impaired operation of motor vehicles is a serious public health and safety issue around the world, as consumption of any drugs, both legal and illegal, that affect the central nervous system carries a high risk of causing fatal transportation accidents.

Many countries have introduced random roadside drug testing for operators of all types of vehicles (automobiles, motorcycles, transport trucks, etc.) with a view to addressing this problem. There is, however, no international consensus on drug consumption limits for drivers, as the impact of a given drug on an individual depends to a large extent on a number of factors, including drug interactions, consumption habits, metabolism, etc. Furthermore, the number of potential drugs of abuse is very large. While much of the attention is focused on road safety, the problem extends to operators of trains, aircraft, buses, and heavy motor equipment, among other "vehicles".

Consequently, legislation on drug consumption limits varies significantly from country to country. Some countries have a zero-tolerance approach, whereby the driver is penalized if the presence of any prohibited drug can be detected. The limits for certain drugs of abuse in certain countries are shown in Table 1.

Due to the significant increase in the need for roadside drug tests, a rapid, simple, and miniaturized (portable) method is required. The biological fluids that are conventionally used for drug testing in clinical and toxicology laboratories (blood, urine) are not suitable for this purpose. However, oral fluid (saliva) is a non-invasive alternative to blood and to urine, which

Table 1: Drugs limits for drivers in select countries

Mass Spectrometry			
Drug of abuse	**Europe [2]**	**UK [3,4]**	**United States [5]**
Amphetamines	25	250	Limits vary from state to state but some states have adopted zero tolerance limits
Methamphetamines	25	10	
Cocaine	20	10	
Cannabis (THC)	1	2	
Opiates	20	5	
Benzodiazepines	10	***	

may be substituted or adulterated. Oral fluid is also very easy to collect. Specialized medical personnel are not required for sample collection, offering a major advantage for on-the-spot roadside testing.

In 1984, Peel et al. [6] conducted the first investigations into the viability of using drivers' oral fluid for drug testing. Immunoassay kits which can detect up to 10 active compounds (opiates, amphetamines, barbiturates, benzodiazepines, cocaine, and tetrahydrocannabinol, amongst others) within a few minutes are now available for on-the-spot drugs of abuse testing. These kits include collection systems containing compressed absorbent material that expands when permeated with oral fluid. Once the adequate sample volume has been collected, the device provides a qualitative result by means of a colorimetric strip.

However, any tests returning a positive result must be verified in a laboratory using a reference technique for precise quantitation and confirmation of the detected drugs in order to be accepted as evidence in legal proceedings relating to traffic accidents, to validate road safety offenses, or to trigger criminal proceedings. Thus, a second oral fluid sample must be collected (typically 1 mL) with provisions to maintain sample integrity, including the addition of a buffer designed to prevent microbial growth, tamper-evident sealing, and transport under refrigerated conditions, with an unbroken chain of custody to the laboratory to be securely stored under appropriate refrigerated conditions until they are analyzed.

The laboratory testing process is quantitative in nature and includes a solid phase extraction (SPE) with a suitable solvent and subsequent concentration of the extract through evaporation in a nitrogen stream. The resulting extract is reconstituted in the mobile phase for subsequent LC-MS/MS analysis. The introduction of LCMS as a routine technique in toxicology laboratories has enabled significant progress to be made in drug screening in oral fluid. The high sensitivity and specificity of LCMS makes it possible to meet the low detection limits required under legislation using a minimum sample volume, as is the case for oral fluid, where it is usually possible to collect only a limited sample amount.

In addition to the analysis of the target parent drugs and pharmaceuticals included under legislation, a number of pharmacokinetic studies have recently been undertaken with a view to understanding the drug metabolites

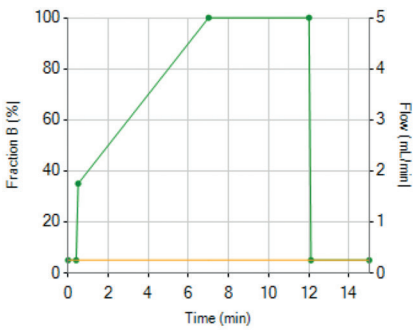
Table 2: Mass Spectrometry Method Conditions

Mass Spectrometer	Bruker impact II HR-QTOF
Source	ESI, Bruker Apollo II
Capillary	4500 V
End Plate Offset	500 V
Dry Gas	4 L/min at 220°C
Nebulizer	1 bar
Mass Range	30-1000 Da
MS Mode	bbCID (broad band Collision Induced Dissociation)
Acquisition Rate	1 Hz
Transmission Parameters	Optimized in stepping mode
Collision Energy	Variable ramp between 12.5 and 37.5 eV

Liquid Chromatograph	Bruker Elute™ UHPLC system
LC Column	Bruker Intensity Solo C18 100 x 2.1 mm (P/N:BRKHSC18022100)
Mobile Phase A	Water + 0.1% Formic Acid
Mobile Phase B	Methanol + 0.1% Formic Acid
Flow Rate	250 µL/min
Injection Volume	10 µL
Column Oven Temperature	35°C
Total Run Time	15 min
Software	Hystar 4.1/TASQ 1.4 processing software

LC gradient

Line:	Time (min):	Flow (mL/min):	%A:	%B:
01	0.00	0.25	95.0	5.0
02	0.4	0.250	95.0	5.0
03	0.5	0.250	65.0	35
04	7	0.250	0.0	100
05	12	0.250	0.0	100.0
06	12.1	0.250	95.0	5
07	15	0.250	95.0	5



generated by the human body for which testing would also be advisable. Furthermore, a large number of unmonitored new psychoactive substances (NPS) have recently entered the illegal drug market. These include potent designer hallucinogens, for which scientific data regarding their metabolism in humans is limited.

In this scenario, LC-HR-QTOF MS technology is an invaluable testing tool for both target drugs and for new synthetic designer drugs and their metabolites. In addition to the need to easily expand a laboratory's database of target compounds, high resolution and high mass accuracy are necessary for confident analyses, as provided by the Bruker impact II system used in this study. Figure 1 shows a high-resolution extracted ion chromatogram (hrEIC) of lorazepam and the overlay of the theoretical and experimental high-resolution spectra, showing perfect convergence.

Experimental

Oral fluids were provided by a local law enforcement agency following roadside testing. Samples were suitably processed prior to analysis. The system configuration used for this work and the established instrumental parameters are shown in Table 2 and 3. The analyzed drugs of abuse and the corresponding internal standards are shown in Table 6.

Method validation and results

For validation of the method (linearity, precision, and accuracy), blank oral fluid samples, previously analyzed and spiked with the compounds under study over a wide range of concentrations, were used. In addition, the samples were analyzed by LC-triple quadrupole (LC-TQ MS/MS) [1],

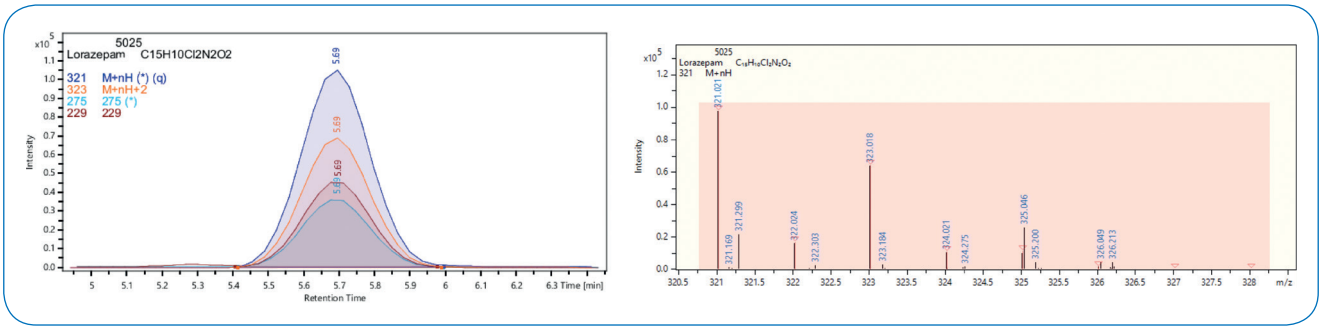


Figure 1: Left: hrEIC of lorazepam showing the precursor ion (blue) and several product ions. Right: Overlay of high resolution mass spectra for lorazepam: theoretical (red) and experimental (black)

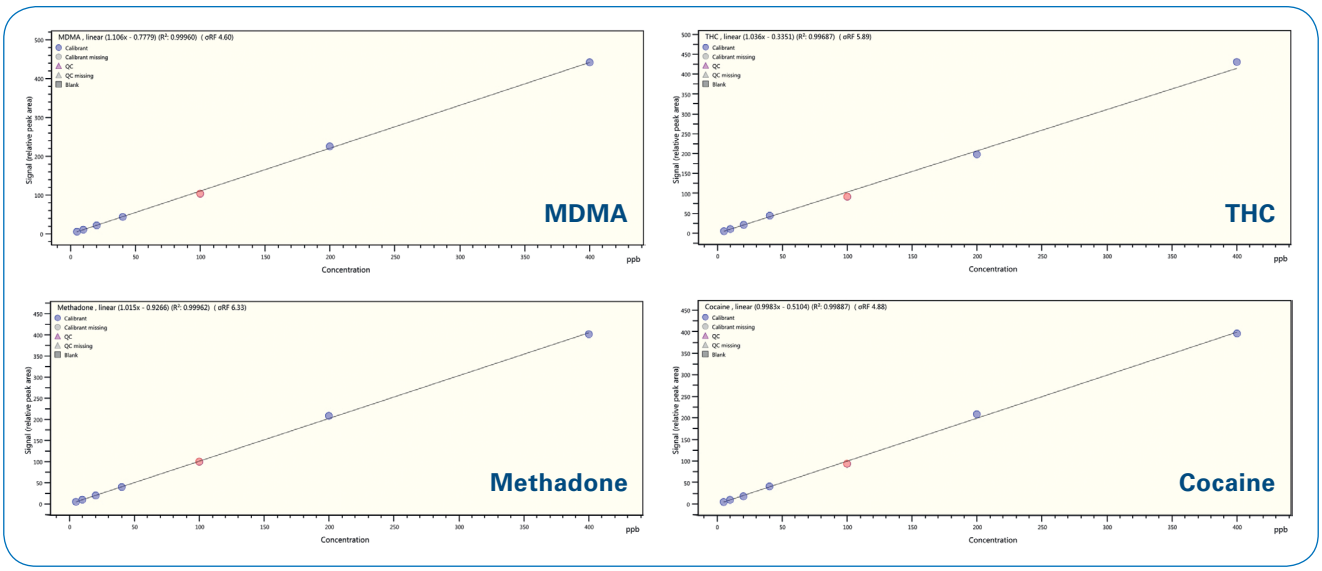


Figure 2: Calibration curves for representative drugs of abuse under study

the standard reference technique, and a comparison of the results obtained using both techniques (LC-TQ MS/MS and LC-HR-QTOF MS/MS) was performed. Both systems use MS/MS for analysis; however, the impact II HR-QTOF system uses FullScan and MS/MS modes simultaneously, using FullScan mode for rapid identification and quantitation and MS/MS for compound identity confirmation.

[1] An EVOQ™ LC-TQ MS/MS system (Bruker Daltonics) was used

Linearity

For the linearity study, different oral fluid samples containing various concentrations (between 5-400 ng/mL) of the drugs and metabolites under study were prepared for analysis. Internal standards were added to all samples uniformly at 100 ng/mL prior to extraction.

Example calibration curves for representative compounds under study are shown in Figure 2.

Table 4 summarizes the results of the linearity study. For all analyzed compounds, the coefficient of determination (R^2) is ≥ 0.995 and the standard deviation for response factors (SDRF) is ≤ 15%.

Sensitivity: Method Reporting Limits (MRL) and Method Detection Limits (MDL)

Conventionally, limits of detection have been estimated using the mean of the signal-to-noise ratio for a chromatographic peak with regard to the background of a blank sample and then applying different statistical calculations.

However, modern LC-HR-QTOF mass spectrometry systems frequently generate very low background, with near-zero values for many compounds. Any statistical calculation with near-zero values would clearly generate inaccurate estimates of limits of detection that are far from the analytical reality.

In this work, the established MRLs correspond to the low calibration limits shown in Table 4. An MRL = 5 ng/mL was established for all analyzed compounds. These MRLs are the lowest concentrations that

can be reliably quantitated meeting the linearity, precision, and accuracy criteria established in the method and following the entire sample preparation process. Thus, these are robust limits for routine use which could be defended to any judicial authority.

MDL values were experimentally determined. For this evaluation, oral fluid samples which had been previously analyzed to confirm the absence of any interference were prepared with 0.5 ng/mL of the analyzed compounds: This concentration is ten times lower than the set MRL for all compounds. Table 5 summarizes the area values measured for each compound at 0.5 ng/mL.

It should be noted that each of the following criteria must be met for the identification of a compound by HR-QTOF mass spectrometry and to establish its MDL: 1) retention time convergence, 2) presence of the quantitation ion and all defined product/fragment ions, 3) mass accuracy and 4) isotopic fidelity (convergence between the experimental and theoretical profiles).

These strict criteria allow for a high degree of confidence in the establishment of MDLs and the avoidance of false positives.

The area values for the 0.5 ng/mL concentration (Table 5) indicate that the majority of the analyzed compounds can be readily detected even below this level.

Figure 3 shows the hrEIC chromatograms for example compounds at the set MRL = 5 ng/mL.

Precision and accuracy: comparison between LC-HR-QTOF MS/MS and LC-TQ MS/MS

The oral fluid samples were also analyzed to verify the precision of the measurement of typical drugs of abuse in relation to the reference method using liquid chromatography triple quadrupole mass spectrometry (LC-TQ MS/MS). The samples were extracted, analyzed using LC-TQ MS/MS, and refrigerated for approximately three weeks prior to analysis by LC-HR-QTOF MS/MS. The differences between the concentrations detected in each case are shown in Table 7.

In the majority of cases, no significant difference between the two methodologies was observed, with a coefficient of variation below 20%. However, LC-HR-QTOF MS/MS analysis detected traces of certain drugs that were not detected by LC-TQ MS/MS.

Lastly, extractions were performed for a reference material (oral fluid containing 25 ng/mL of methadone) to verify the precision and accuracy of the proposed methodology. This reference oral fluid was extracted in

Table 4: Results of the linearity study

Compounds	RT (min)	R^2	RSD RF (%)	Working calibration range (ng/mL)
6-O-Monoacetylmorphine (MAM)	2.70	0.996	9.74	5-200
Alprazolam	5.74	0.996	8.08	5-400
Amphetamine	3.09	0.999	5.10	5-400
Benzoylecgonine	3.34	0.995	8.03	5-400
Clonazepam	5.29	0.997	5.99	5-400
Cocaethylene	3.97	0.998	12.55	5-400
Cocaine	3.53	0.999	4.88	5-400
Codeine	2.60	0.999	8.10	5-400
Diazepam	6.38	0.999	5.74	5-400
Ecgonine methyl ester	1.64	0.999	11.26	5-400
Ketamine	3.33	0.999	5.43	5-400
Lorazepam	5.69	0.999	6.61	5-400
Lormetazepam	5.93	0.995	6.01	5-400
MDA	3.04	0.995	15.34	5-400
MDMA	3.02	0.999	4.60	5-400
Methadone	5.53	0.999	6.33	5-400
Methamphetamine	3.08	0.998	4.22	5-400
Morphine	2.40	0.998	7.51	5-400
THC	8.53	0.997	5.89	5-400

Table 5: Experimentally determined Method Detection Limits (MDLs)

Compounds	Area 0.5 ng/mL
6-O-Monoacetylmorphine (MAM)	2.179
Alprazolam	32.282
Amphetamine	87.731
Benzoylecgonine	9.148
Clonazepam	2.825
Cocaethylene	15.531
Cocaine	40.923
Codeine	7.271
Diazepam	31.266
Ecgonine methyl ester	8.117
Ketamine	25.280
Lorazepam	765
Lormetazepam	4.852
MDA	34.991
MDMA	26.061
Methadone	41.715
Methamphetamine	28.844
Morphine	5.802
THC	17.962

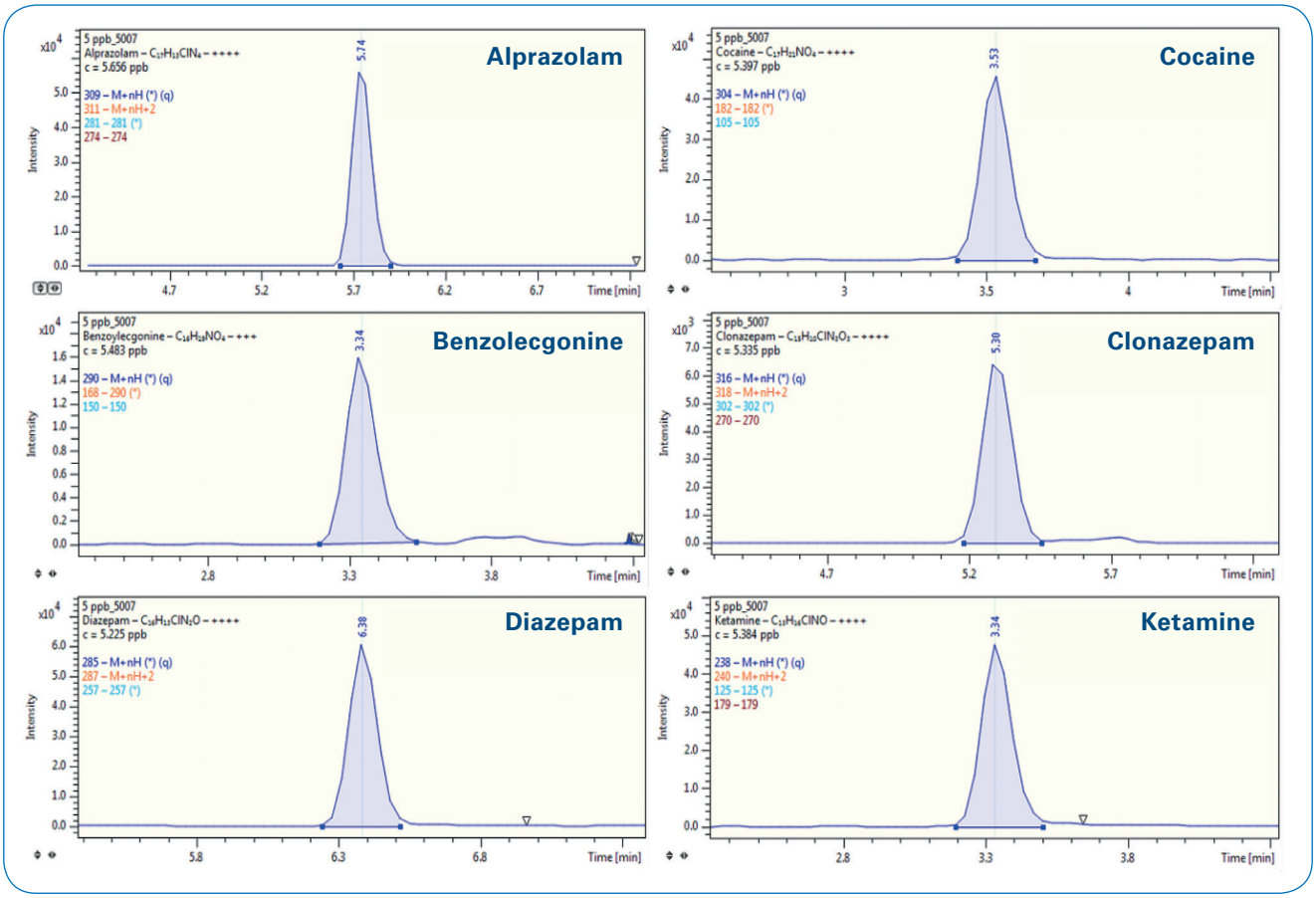


Figure 3: MRL - hrEIC chromatograms for example compounds at 5 ng/mL

Table 6. Drugs of abuse quantified in oral fluids and associated internal standards (indicated in italics)

6-O-Monoacetylmorphine (MAM)	Cocaethylene	Ketamine	MDMA
6-O-Monoacetylmorphine D3	*Cocaethylene D8*	*Ketamine D4*	*MDMA D5*
Alprazolam	Cocaine	Lorazepam	Methadone
Alprazolam D5	*Cocaine D3*	*Lorazepam D4*	*Methadone D3*
Amphetamine	Codeine	Lormetazepam	Methamphetamine
Amphetamine D6	*Codeine D3*	*Lormetazepam D5*	*Methamphetamine D11*
Benzoylecgonine	Diazepam	MDEA	Morphine
Benzoylecgonine D3	*Diazepam D5*	*MDEA D5*	*Morphine D3*
Clonazepam	Ecgonine methyl ester	MDA	THC
Clonazepam D4	*Ecgonine methyl ester D3*	*MDA D5*	*THC D3*

Row	Sample	Analyte	SampleType	MRSQ	RT Score	ΔRT [min]	m/z Score	m/z exp.	m/z meas.	Δm/z [mDa]	mSigma Score	mSigma	Ions Score	Area	Quantity
1	Material Referencia A	Methadone	SAMPLE		++	-0.01	++	310.2165	310.2156	-0.98	++	14.5	++	3303579	23.4 ppb
2	Material Referencia A	Methadone	SAMPLE		++	-0.00	++	310.2165	310.2162	-0.37	++	15.5	++	3361822	23.4 ppb
3	Material Referencia A	Methadone	SAMPLE		++	-0.01	++	310.2165	310.2155	-1.02	++	15.9	++	3451508	24.5 ppb
4	Material Referencia B	Methadone	SAMPLE		++	-0.00	++	310.2165	310.2155	-1.06	++	16.3	++	3423992	23.9 ppb
5	Material Referencia B	Methadone	SAMPLE		++	-0.00	++	310.2165	310.2153	-1.25	++	16.6	++	3446792	23.2 ppb
6	Material Referencia B	Methadone	SAMPLE		++	-0.01	++	310.2165	310.2172	0.65	++	17.9	++	3353802	23.3 ppb

Figure 4: Reproducibility of the measurement in a reference oral fluid taken in two separate extractions

Table 7: Comparison of results obtained by the accredited LC-TQ MS/MS reference method and by LC-HR-QTOF MS/MS

Sample	Compounds Detected	Results LC-TQ MS/MS (ng/mL)	Results LC-HR-QTOF MS/MS (ng/mL)	CV (%)
Sample #1	Cocaine	34	28	18
	Benzoylecgonine	18	17	6
	Ecgonine methyl ester	365	305	16
	THC	n.d	5.1	
Sample #2	Cocaine	515	513	<1
	Benzoylecgonine	892	802	10
	Ecgonine methyl ester	414	346	16
	Morphine	144	132	8
	Codeine	9	10	11
	MAM	29	22	24
	THC	n.d	3.1	
Sample #3	Cocaine	8	9	13
	Ecgonine methyl ester	n.d	2.9	
	Benzoylecgonine	n.d	2.1	
	THC	17	17	<1

duplicate and analyzed by both LC-TQ MS/MS and LC-HR-QTOF MS/MS to assess the precision of the concentration measurement. Further, both extractions were analyzed via triplicate injections into the LC-HR-QTOF MS/MS system in order to establish the accuracy of the measurement.

The LC-TQ MS/MS methodology resulted in an average value of 24.98 ng/mL and LC-HR-QTOF MS/MS analysis resulted in an average value of 23.6 ng/mL, with a standard deviation between the six injections of both extractions of 0.5%. This assumes a coefficient of variation of 5.53%.

Conclusion

The proposed methodology for analysis of drugs of abuse by LC-HR-QTOF MS/MS meets and even exceeds the detection limits achieved by LC-TQ MS/MS, the standard reference technique in process laboratories for drug testing. The results obtained using both techniques are comparable with a coefficient of variation < 20%. Using LC-HR-QTOF MS, compound screening and quantitation can be made within the same run, reducing analysis time.

High resolution mass spectrometry, such as that provided by the impact II HR-QTOF MS system, provides additional discriminatory criteria for compound identification, such as isotopic profile, mass accuracy, and multiple product and fragment ions (both in MS and MS/MS modes). These offer further security in preventing false positives.

While the detection and quantitation of compounds studied within this work include the most common drugs of abuse and their key metabolites, the power and scope of this analytical platform extends much further. The option to conduct subsequence (retrospective) analysis on previously analyzed samples offers additional versatility and flexibility for the defense of the results in a legal setting or for appropriate medical treatment. Further, this system enables the detection of other factors which may result in driver impairment, including over-the counter medications or workplace chemicals (e.g., solvents or pesticides). The integrated software of the impact II HR-QTOF MS system also enables users to add new compounds and metabolites as needed, and Bruker's available TargetScreener HR database [7] includes over 2000 compounds of toxicological[2], pharmaceutical, and forensic interest. With capabilities for both rapid targeting and extensive screening and quantitation from a variety of complex matrices including food, oral fluids, urine, serum or environmental samples, LC-HR-QTOF MS technology can provide reliable, comprehensive analyses for today's analytical laboratories.

[2] Includes toxins and metabolites previously included with Bruker's ToxScreener database

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