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Implementation of DRS in doping control

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Introduction

Several screening methods currently used in doping control rely on GC-MS. Using this technique stimulants, anabolic androgenic steroids, aromatase inhibitors, narcotics and other substances can be detected in the urine of athletes [1, 2]. Except for anabolic steroids, which have to be detected at an MRPL of 10 ng/ml according to the WADA guidelines, these substances can be detected using the GC-MS instrument in the full scan mode.

In our lab, at present the GC-MS data generated is interpreted by two independent reviewers by looking at two ion traces of diagnostic ions in a retention time window typically for the component. In case of a suspicious sample, the full scan spectrum aids in the decision to start a confirmation procedure. This process is time consuming and requires trained and concentrated staff to avoid reporting a false negative result.

The deconvolution reporting software (DRS), introduced in 2004, is a tool to produce an easy to read report based upon four different aspects. These are retention time, MSD chemstation software, AMDIS and the NIST library.

The goal of this study was to evaluate the possibilities of DRS in routine screening methods in doping control by implementing it in the GC-MS screening method for narcotics and stimulants.

Materials and Methods

All urine samples were extracted, derivatised and measured according to the procedure previously described by Van Thuyne et.al. [2]. Analysis was carried out on an Agilent 5973 mass selective detector directly coupled to a 6870 gas chromatograph. Interpretation of the results was performed by two scientists after which each sample was processed using the DRS present in the MSD Chemstation software (Revision D.03.00).

The sensitivity of the DRS software was examined by spiking 9 different components to negative urine in the range 10 – 500 ng/ml. these substances were: mephentermine,

amphetamine, methoxyphenamine, crotethamide, benzylpiperazine, MDEA, methylphenidate, pipradrol, morphine and fenethylline.
Afterwards 1366 routine samples were interpreted by both operators followed by the DRS software.

Results and Discussion

The goal of deconvolution is to extract a signal from a single compound out of a complex mixture of signals of (partially) coeluting substances. In this way, the mass spectrum of coeluting peaks can be purified allowing a better identification and confirmation of its structure. The tool to perform this process in the DRS software is AMDIS (automated mass spectral deconvolution and identification software). AMDIS works by grouping all extracted ions having the same peak apex and have a similar rise and fall of the ion trace. AMDIS has no correlation with peak integration.
In order to use the DRS software several steps have to be taken. The first one is to develop or reform an existing GC-method to a retention time locked method. As the identification is based upon both retention time and mass spectral criteria it is of utmost importance to keep the retention times constant. After locking the GC method a library has to be created and calibrated (i.e adding retention time data). The development of the library is done by injecting one or more reference mixtures and can be performed in two different ways. The first one relies on the MSD Chemstation software. Using this procedure all information present in the spectrum of the component is stored in the library. The second way is using the deconvoluted spectra obtained by AMDIS to create a library. Both methods have their advantages. Using the Chemstation software is much faster than using AMDIS. However using this approach all monitored ions, also those which are part of the background, are present in the reference spectrum. Using the deconvoluted spectrum results only in a limited number of ions, which can be attributed with a certainty of 100% to the reference component, which are being transferred in the library. Using amphetamine as an example, over 150 different m/z values were entered in the library using the Chemstation software while this number was restricted to 12 ions using the AMDIS approach. As a result, analysis of a spiked sample with MDA (500 ng/ml) results in a match factor of only 36% using the Chemstation software while using the AMDIS database a match factor of 98% is obtained. Every small ion present in the spectrum in the Chemstation library is taken into account to determine the match factor. The absence of these ions in the deconvoluted spectra because they are not present or belong to the

background (and therefore are filtered out) results in the higher match factor using AMDIS.

A

R.T.	Cas #	Compound Name	Agilent ChemStation Amount (ng)	AMDIS Match	R.T. Diff sec.	NIST Reverse Match	Hit Num.
8.9707	4764174	MDA-TMS		**36**	-0.252		

B

R.T.	Cas #	Compound Name	Agilent ChemStation Amount (ng)	AMDIS Match	R.T. Diff sec.	NIST Reverse Match	Hit Num.
8.9749	4764174	MDA-TMS		**98**	0.0		

Figure 1: DRS output of MDA using the Chemstation library (A) and the AMDIS library (B).

After the optimisation of the AMDIS library several options can be selected to customise the layout of the output including a quantification using the Chemstation software and a reconfirmation of the detected substances using a comparison with the NIST library. However, not all substances are present as their TMS derivatives in the NIST library or not all substances are added in the NIST with their trivial names. Therefore the reconfirmation using NIST was skipped from the processing method. However, for unknown samples this can be a helpful tool.

In order to optimize the cut-off match value used by the DRS negative urine samples were spiked with 9 different components in a concentration range between 10 and 500 ng/ml. The selection of the components was made based upon their full scan spectrum. TMS derivatised morphine shows a good spectrum for deconvolution as it possesses numerous ions over its complete mass spectrum with medium to high relative intensities. The other selected components however only have a poor mass spectrum. These substances are stimulants showing only one abundant fragment ion with a low specificity (common to numerous amines) and a small molecular ion. The goal of selecting these substances was to investigate what the influence of a (small) matrix interference could be on the DRS match value obtained. This value should be optimised in such a way that all substances can be detected at a concentration as low as possible without the risk of a false positive result. This was the case when applying a cut-off value of 45% at a concentration of 50 ng/ml where a false positive result for methoxyphenamine was obtained (Figure 2).

Setting the cut-off value to 65% would result in false negative results for MDEA and morphine. At a concentration of 100 ng/ml, which is below the MRPL of all substances in this

screening method, all nine components are detected using a cut-off of 65%. Therefore this value of 65% was used. In addition, a maximal deviation in retention time of 6 seconds compared to the reference library was applied. This makes retention time locking of utmost importance.

		Agilent	AMDIS		NIST	
R.T.	Compound Name	ChemStation Amount (ng)	Match	R.T. Diff sec.	Reverse Match	Hit Num.
3.6341	Mephentermine		91	-4.1		
4.7247	Amphetamine-TMS		88	-2.3		
8.4520	**Methoxypenamine-TMS**		**45**	**2.9**		
9.2010	Crotethamide		79	-1.7		
9.2239	Benzylpiperazine-TMS		91	-1.5		
10.0936	MDEA-TMS		49	-1.3		
10.5285	Methylphenidate-TMS		73	-1.0		
11.8508	Pipradrol-TMS		79	2.0		
13.4639	Morphine-bis-TMS		56	-1.2		
14.4412	Fenethylline		86	-0.6		

Figure 2: DRS output of negative urines spiked with 9 components at 50 ng/ml using a cut-off of 45%.

In order to test the capabilities of the DRS a library containing more than 100 components was created to implement in the screening method for stimulants and narcotic agents. The DRS interpretation of the data was performed after both operators had reviewed the chromatograms of the samples in order to avoid influence of the DRS output on the operators conclusion. The DRS did not produce any false negative results. All positive results observed by the operators were also picked out by the DRS. In total 105 samples contained one or more substances identified in both ways. However, the DRS picked out three additional positive samples which remained unnoticed by the analysts.

The first case was a sample positive for amphetamine. Normally amphetamine elutes at a retention time of 4.78 minutes and the ions m/z 116 and m/z 192 are monitored. Figure 3.A shows the ion traces in a QC sample. As can be seen a systematic interference elutes at the end of this retention time window which can also be observed in a negative urine (Figure 3.B).

With this knowledge, both operators interpreted Figure 3.C as negative. However, due to a large interference at the beginning of the chromatogram retention times were shifted with approximately 0.1 minutes. Because the peak apex was found within an interval of 0.1 minutes from the expected retention time the DRS recognised this sample as an amphetamine positive sample which was confirmed during a consecutive B-analysis.

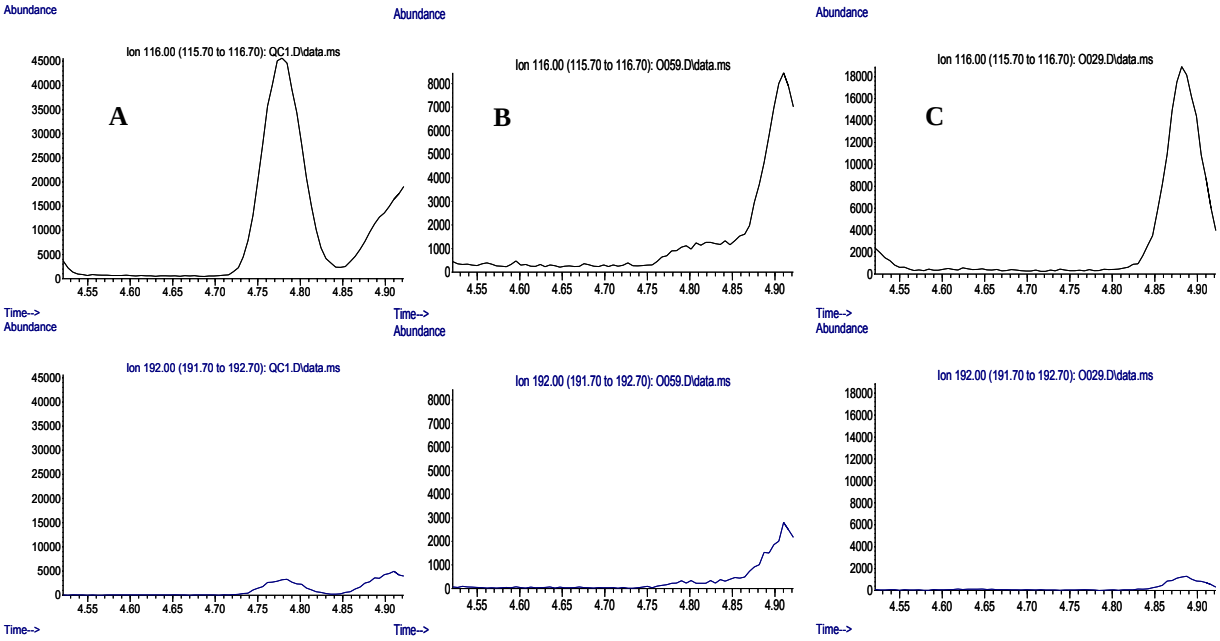

Figure 3: Ion traces for amphetamine in a QC-sample (A), negative sample (B), DRS positive sample (C).

The second case was a sample positive for methylphenidate. Figure 4 shows the ion traces as printed in the chromatogram and the corresponding mass spectrum. The lack of the detection of m/z 91, diagnostic for methylphenidate, and difference between the obtained spectrum and a reference spectrum lead to the conclusion that this sample was negative. However, DRS identified methylphenidate which was later confirmed by the detection of ritalinic acid in the diuretic screening method.

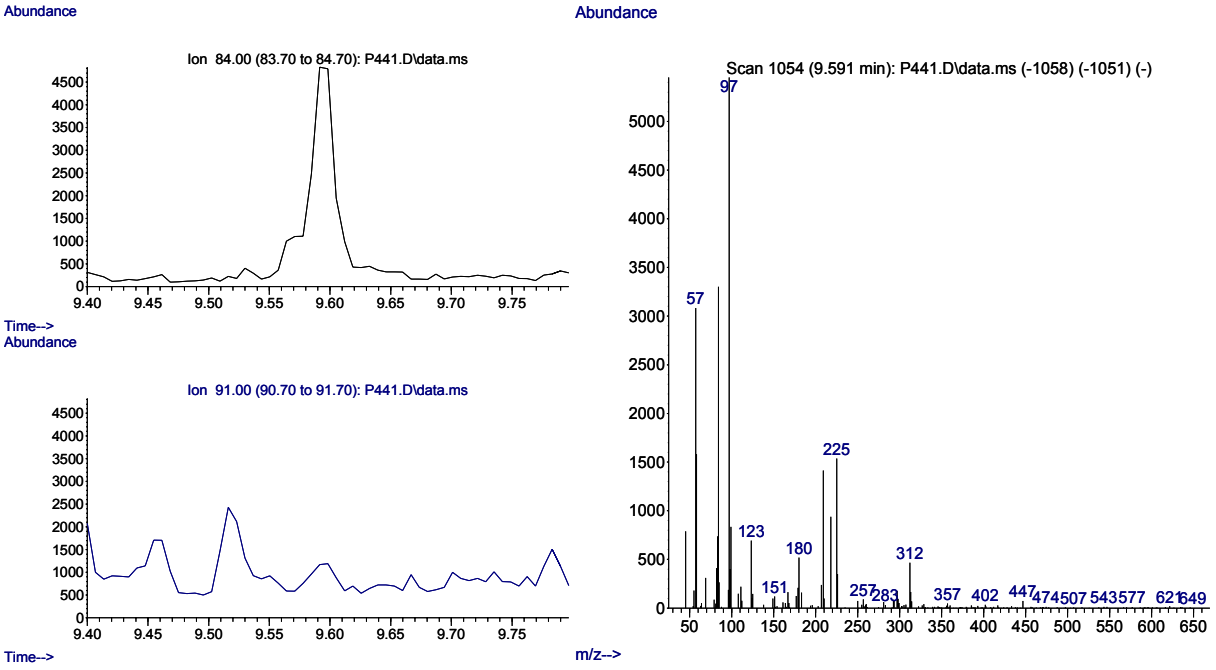

Figure 4: Ion traces for methylphenidate and corresponding spectrum for the peak at RT 9.59 min.

The third sample was positive for morphine. Because of the structural resemblance between morphine and hydromorphone both substances elute within a small time interval and have the same diagnostic ions monitored in the screening method. At MRPL level both substances are separated by 0.06 minutes as can be seen in Figure 5.B.

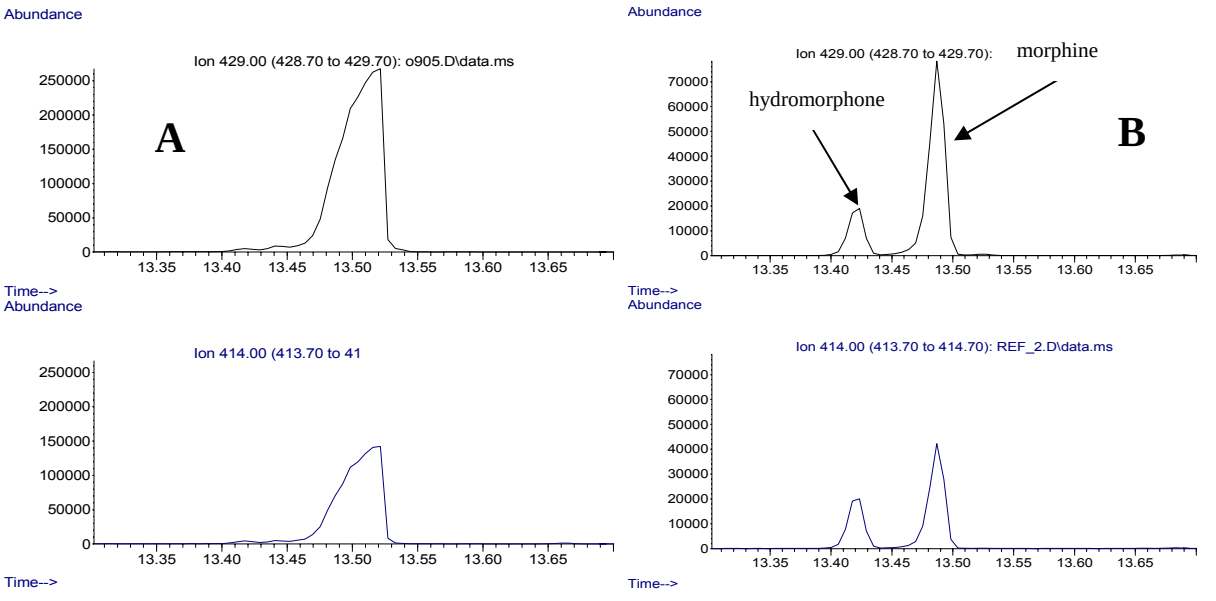

Figure 5: Positive screening result for morphine (A) and QC sample for morphine/hydromorphone (B).

Obviously the routine sample shown in Figure 5.A contains morphine. A confirmation procedure pointed out that the concentration was higher than 50 µg/ml. According to the DRS

software this sample contained also hydromorphone. Because the ion traces in the macro are scaled to the highest peak detected in a retention time window, small amounts of a substance closely eluting to a highly concentrated peak remain unnoticed. The presence of hydromorphone in this sample could be confirmed. The presence of hydromorphone in the urine of people taking high amounts of morphine has been described in the past [3].
Besides the presence of other doping substances matrix interferences can also hamper the detection of substances of interest. As described previously the use of ethyl acetate as extraction solvent for a comprehensive method for the detection of anabolic steroids, narcotic agents and stimulants was hampered because of a large interference at the retention time of several stimulants including amphetamine [4] (Figure 6). This interference has been identified as glycerol and can also be detected in smaller amounts using diethyl ether (cfr Figure 3.A). Applying the DRS on a sample spiked at 500 ng/ml and using ethyl acetate as extraction solvent results in a match factor of 89% proving the applicability of the DRS to identify target substances in complex matrices.

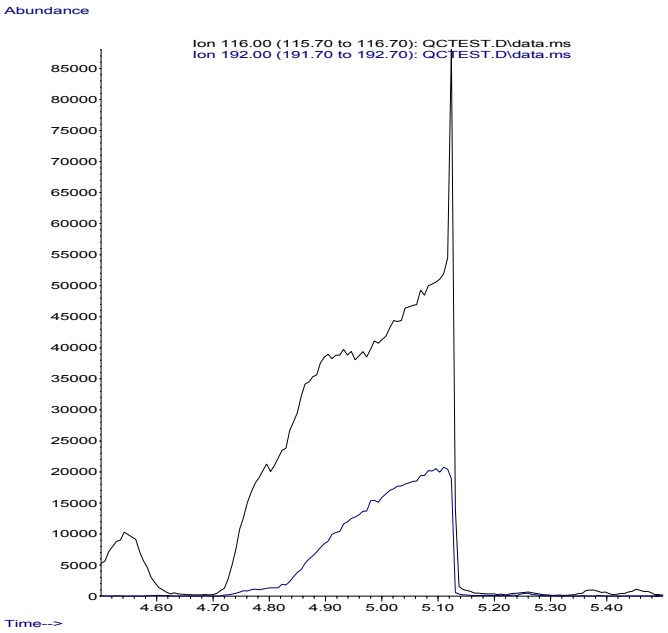

Figure 6: Extracted ion chromatogram for amphetamine using ethyl acetate as extraction solvent.

The deconvolution software is developed to operate in the full scan mode. However, in doping control anabolic androgenic steroids have to be detected using selected ion monitoring because of the low level excreted in urine. At present analytical equipment offer the possibility of combining SIM and scan runs in one analytical run due to improved electronics allowing a faster data transfer. However, the DRS is designed to operate using only one dataset making the use in SIM/scan impossible. Its use in a method using only SIM however

is possible. For this purpose at least three diagnostic ions have to be selected for each component to avoid numerous false positive results. Because only a selected number of ions are used, the resulting match factors are also higher. This requires different settings compared to a processing method for scan data. This optimisation has been performed for approximately 20 different anabolic steroids using a match factor cut-off value of 85%. Using this processing method identification of dehydrochloromethyltestosterone in a WADA PT-sample was possible.

Conclusion

The DRS software is a tool which combines quantitation and identification based upon self created libraries and the NIST library. During this study it was proven that it can be implemented in routine doping control and is very reliable. Therefore, the current system of reviewing obtained data, i.e. 2 analysts looking at chromatograms, can be reduced to one person and the DRS software. However, in order to use the DRS software in SIM/scan methodology several improvements have to be made including the incorporation of more diagnostic ions for anabolic androgenic steroids monitored in the SIM mode.

Acknowledgements

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