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ORIGINAL ARTICLE

MALDI TOF mass spectrometry of selected mycotoxins in barley

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Summary
A novel method for the simultaneous determination of the trichothecene mycotoxins deoxynivalenol (DON), nivalenol (NIV), 3-acetyl-deoxynivalenol (3-ADON), and 15-acetyl-deoxynivalenol (15-ADON) in barley and malt extracts has been developed using Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI TOF MS). This technique enables highly sensitive and fast analysis and/or detection using very small samples. In this work several matrices were examined and the most suitable ones were identified. Statistical analysis was carried out to verify the ability of the system to determine the mycotoxins in a real sample. Test for the accuracy of the method, repeatability, limit of detection (LOD) and limit of quantification (LOQ) were studied. Moreover, the accuracy of the method was confirmed by comparing analytical data to certified values from reference materials for those mycotoxins. In addition, a comparison of the analytical parameters for the determination of DON, 3-ADON, 15-ADON, and Nivalenol was carried out. This work opens up the possibility of very sensitive determination of the selected mycotoxins in barley, malt, cereals and food.

Keywords: malt – deoxynivalenol – nivalenol – 3-acetyl-deoxynivalenol – 15-acetyl-deoxynivalenol – MALDI TOF MS

INTRODUCTION

Trichothecene mycotoxins, such as deoxynivalenol (DON), nivalenol (NIV), 15-acetyldeoxynivalenol (15-ADON) and 3-acetyldeoxynivalenol (3-ADON) are produced by a fungus, e.g. by *Fusarium culmorum* and *Fusarium graminearum* (Sweeney and Dobson 1998, Olson et al. 2002).

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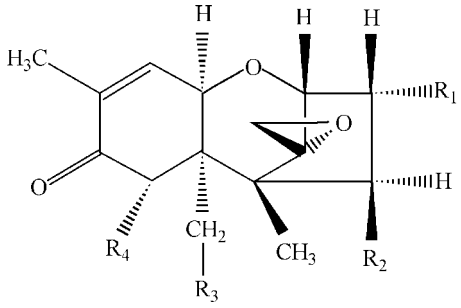
Dedicated to memory of Dr. Pavla Havlová († 13/6/2006).

Contamination of cereals and feeds with these mycotoxins are known to be associated with several diseases in humans and animals (Ueno 1983), such as vomiting, haemorrhage, anaemia and diarrhoea (Trucksess et al. 1996, Berger et al. 1999). They are potent inhibitors of protein synthesis and this that can predispose animals to other diseases and mask the underlying toxicoses (Prelusky et al. 1994). DON is the most frequently detected trichothecene in grain samples such as wheat, corn and barley but NIV, 3- and 15-ADON can also be found there (Trucksess et al. 1995, Razzazi-Fazeli et al. 1999). Chemical structures of the mycotoxins investigated in the present paper are shown in Fig. 1. Maximum tolerated levels for DON, the most commonly detected type B trichothecene, in food commodities are usually in the range from 500 to 1,000 μg/kg (Van Egmond and Jonker 2004), the maximum level of DON in

raw cereals allowed by the European Union is 1,250 µg/kg (http://www.fsai.ie).

The trichothecene mycotoxins are nonvolatile, low-molecular-weight (MW 250–550) compounds (Cole and Cox 1981). This group of mycotoxins is relatively insoluble in water but highly soluble in acetone, ethyl acetate, chloroform, dimethyl sulfoxide (DMSO), ethanol, methanol, and propylene glycol (Cole and Cox 1981). Purified trichothecenes generally have a low vapour pressure, but they do vaporize when heated in organic solvents. Extraction of trichothecene mycotoxins from fungal cultures with organic solvents yields a yellow brown liquid, which, if allowed to evaporate, forms a greasy yellow crystalline product.



Trichothecene	R1	R2	R3	R4
Deoxynivalenol (DON)	OH	H	OH	OH
Nivalenol (NIV)	OH	OH	OH	OH
15-O-Acetyl-4-Deoxynivalenol (15-ADON)	OH	H	OAc	OH
3-Acetyldeoxynivalenol (3-ADON)	OAc	H	OH	OH

Fig. 1. **Basic structure of some selected trichothecenes.**

Rapid, simple and efficient assays are necessary to determine the contamination of raw materials of barley, corn and wheat. Several different methods for the determination of trichothecene mycotoxins have been published based on GC with electron capture detection (ECD), GC-MS and LC-MS (Razzazi-Fazeli et al. 2002, Fuchs et al. 2003) The use of HPLC with UV detection for the determination of NIV and DON has some sensitivity limitations due to the lack of UV absorption by these molecules.

The development of multimycotoxin methods, allowing the detection and determination of several groups of co-occurring mycotoxins with signal chromatographic runs is highly desirable. The complex composition of food and feed matrices as well as the wide range of the physical and chemical properties of mycotoxins requires selective and sensitive detection techniques, such as mass spectrometry.

In the field of *Fusarium* mycotoxins there are some GC-MS methods for the simultaneous determination of trichothecene (Onji et al. 1998, Tanaka et al. 2000). However, the need to derivatise the samples prior to GC analysis and the huge technical improvements in the field of LC-MS has increased the number of methods using LC-MS. Still, the number of LC-MS or LC-MS/MS methods for the simultaneous determination of more than one family of mycotoxins is very limited.

The Matrix-assisted laser/desorption ionization time of flight mass spectrometry (MALDI-TOF MS) instrumentation is a rather new technique developed two decades ago (Tanaka et al. 1988). It enables highly sensitive and rapid analysis at femtomole or even attomole levels of organic and/or high molecular biomolecules without their destruction. Another advantage of MALDI-TOF MS over conventional techniques is its ability to analyze samples within a few minutes and using only small sample. Thus, the MALDI-TOF MS method could offer the possibility for fast and sensitive detection of mycotoxins in barley, barley malt and effluent, etc.

The aim of this paper is to study the ionization processes of mycotoxins with the goal of developing a fast, simple and accurate MALDI-TOF MS for the simultaneous detection and semi quantitative determination and identification of the trichothecene mycotoxins in barley and malt extracts.

MATERIALS AND METHODS

Chemicals

Deoxynivalenol (DON), 15-O-acetyl-4-deoxynivalenol from *Fusarium* cultures (15-ADON), 3-acetyldeoxynivalenol from *Fusarium* (3-ADON) and nivalenol (NIV) standards (99% purity) were obtained from Sigma–Aldrich (Steinheim, Germany). 2, 5-dihydroxybenzoic acid (DHB), hydrazine hydrate, and sodium azide were purchased from Sigma–Aldrich (Steinheim, Germany). Diamond powder of 0–1 μm grain size was obtained from Saint-Gobain Advanced Ceramics, s. r. o. (Turnov, Czech Republic). HPLC-grade acetonitrile were purchased from Merck (Darmstadt, Germany). Distilled water used to prepare all solutions was double-distilled in a quartz apparatus supplied by Heraeus Quartz-schmelze (Hanau, Germany).

Reference Material

Standard non gushing barley malt (Carlsberg Research Laboratory, Denmark) was used as a blank reference sample.

Real samples of barley and malt

Barley of species Kompact no. 156 and 161, malt of species Kompact no. 673 and 678, (VÚPS, a.s., Brno, Czech Republic)

Mycotoxin Sample Preparation

Stock solutions of DON, NIV, 3-ADON and 15-ADON (concentrations 1 mg/ml) were made up in acetonitrile from commercial standards.

A sample of Carlsberg malt was spiked with DON in concentrations 500, 600, 1500 and 5000 μg/kg of malt. After drying, the sample was extracted with Extraction and Clean-up Procedures.

Malt sample preparation

Procedure of malting

Malting of barley consists of 3 parts: maceration, germination and kilning. Total time of malting was 144 hours.

Conditions: content of water before germination was 45 %, temperature of water during maceration and germination was 14.5 °C, temperature of water during kilning was from 30 °C at the start to 80 °C at the end.

Extraction and clean-up procedures

The extraction and clean-up procedure for barley and/or malt are important, because barley and/or malt represent complex matrix. Therefore, an adapted extraction procedure was used from the literature (Blechová et al. 2006).

Ten grams of blended homogenous representative malt or barley sample were extracted with acetonitrile-water solution (84:16, v/v) for 1 h. For the purification of crude extract a special column-the Mycosep®225 Trich (supplied by Romer Labs®) was used. This column offers the best results for analysis of type trichothecenes and provides the best recoveries for DON (Radová et al. 1998, Francisco et al. 2005). The crude grain extract (5 ml) was transferred into this column. A 2 ml volume of the purified extract was then dried in a rotary vacuum evaporator and the residue was dissolved in 400 μl acetonitrile.

Instrumentation

Mass spectrometer, Axima-CFR instrument (Kratos Analytical, Manchester, UK) with software launchpad for proteomics V2.0.3 (Kratos Analytical Shimadzu Corporation, England) was used for the analysis. Nitrogen laser (337 nm) from Laser Science Inc. was used. Reflectron positive mode was used. The samples were analysed after the vacuum in the ion source fell below 10^{-4} Pa. Mass spectra of MALDI were accumulated from 300, occasionally 600, laser shots. Laser power was calculated from the arbitrary units given by the software (0-180 range) to the real laser power values (0-6 mW) using a linear relationship provided by the manufacture.

Sample preparation

The matrix (0.5, 1 μl) was prepared as a usual solution (or suspension), deposited onto the sample spot on the MALDI target, then (3 × 1 μl) of sample were added, mixed, and allowed to dry in an air stream.

Statistical analysis

The following steps were tested, which should document the ability of the system to determine the mycotoxins in the real sample: instrumental precision (repeatability), method accuracy, resolution, limit of detection, limit of quantification. The average, standard deviation, relative standard deviation, and confidence interval were determined and Grubbs test (Eckschlager et al. 1980) was done. For confidence interval the significance level $\alpha = 0.05$ was used.

RESULTS AND DISCUSSION

First, using mycotoxin standards, the challenge was to find an appropriate matrix for ionization of trichothecene mycotoxins (DON, NIV, 15-ADON and 3-ADON). The first tests were carried out with 3-ADON.

Influence of different matrices on ionization of mycotoxins

The ionization of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON) and nivalenol (NIV) using different matrices was studied by

MALDI-TOF mass spectrometry and the results were statistically evaluated. Saturated solutions from 2, 5-dihydroxybenzoic acid (DHB) in acetonitrile and 0.2% hydrazine hydrate were prepared as a matrix; also fine powdered synthetic diamond and sodium azide (NaN_3) were prepared as a suspension in acetonitrile. The standards were dissolved in acetonitrile (concentration 1 mg/ml).

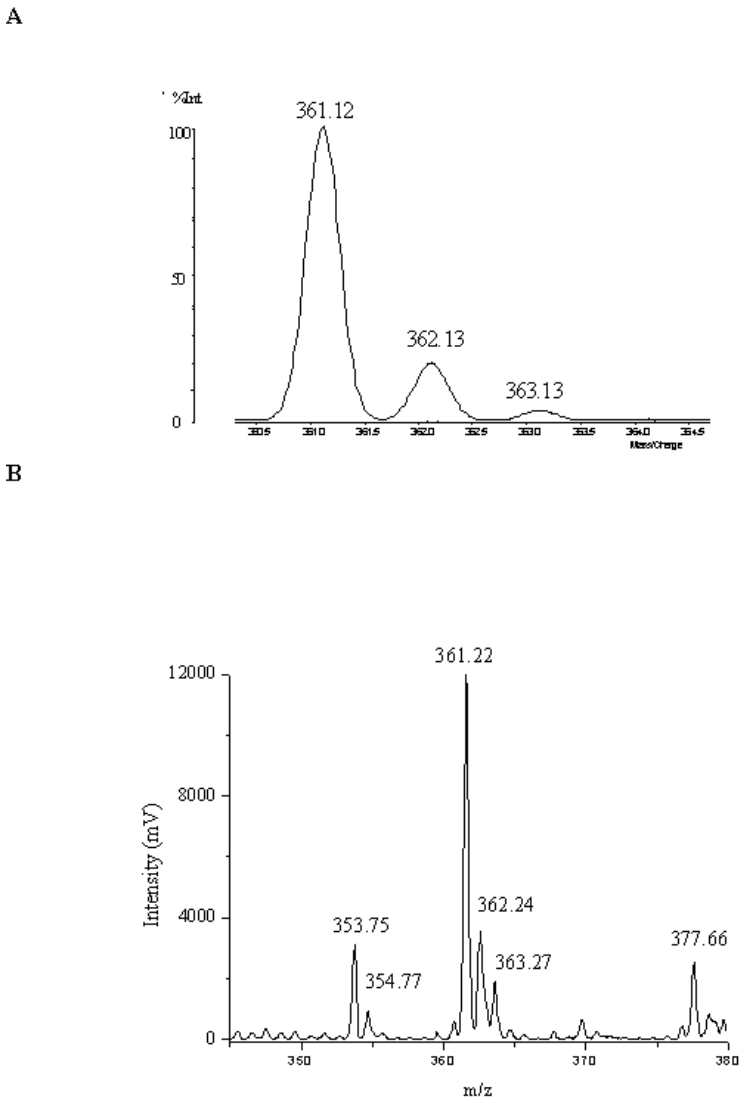


Fig. 2. **Comparison of isotopic patterns concerning theoretical (A) and experimental (B) spectra of 15-ADON.**

The 1 µl of matrix was deposited on the target and allowed to dry. Then the sample (3 µl) was added onto the matrix. After deposition of the sample over the dried matrix it was left in an air stream to dry and then introduced to the mass spectrometer (Blechová et al. 2006). It was found that for all matrices studied the ionization of DON, 3-ADON and NIV) leads to the formation of $DON.Na^+$ cation. The correctness of this conclusion was proved by a computer modelling of the mass spectra. Fig. 2 shows a comparison of theoretical and experimental spectra concerning $3\text{-}ADON.Na^+$ ion.

The matrices were examined and compared to determine the optimum ionization for the sample because individual matrices have different ionization at different energies of the laser. Fig. 3 demonstrates the relationship of different laser energy levels and their influence on the ionization (peak intensity) of 3-ADON.

The highest intensity was observed using fine powdered synthetic diamond at laser energy 140-

150 (relative units), which is also the case for other matrices. Example of Mass spectra of pure malt standard Carlsberg (A) and spiked standard with DON (B) to final concentration 500 µg/kg (0.6 µg/ml) is shown in Fig. 4.

Statistical analysis

For the analysis of mycotoxins (DON, NIV, 15-ADON and 3-ADON) 4 matrices were used. (Diamond, sodium azide, DHB and hydrazine). For statistical evaluation the standard solutions in concentration 1 ug.ml^{-1} and 100 µg.ml^{-1} were used. The statistical analysis was done for all the matrices. With respect to deficient statistic parameters (resolution, detection limit, etc.) detailed statistic evaluation was done only for the diamond and sodium azide matrices.

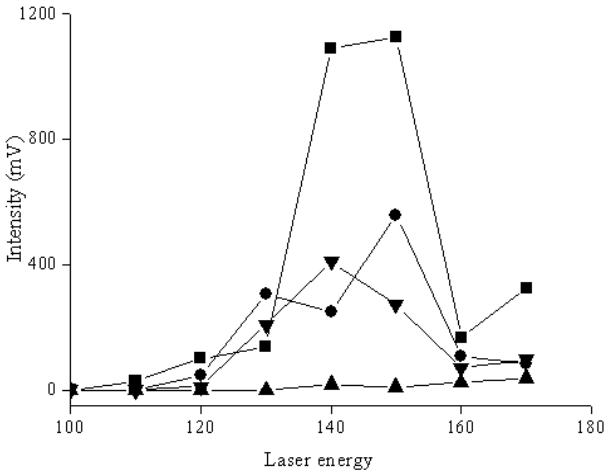


Fig. 3. **The relation of peak intensities for ionization on laser energy of 3-acetyldeoxynivalenol (at m/z =338.4 Da).**
Matrices: ■ Fine powdered synthetic diamond (suspension)
● DHB – saturated solution in ACN
▼Hydrazine hydrate (0.2%)
▲ Sodium azide (suspension)

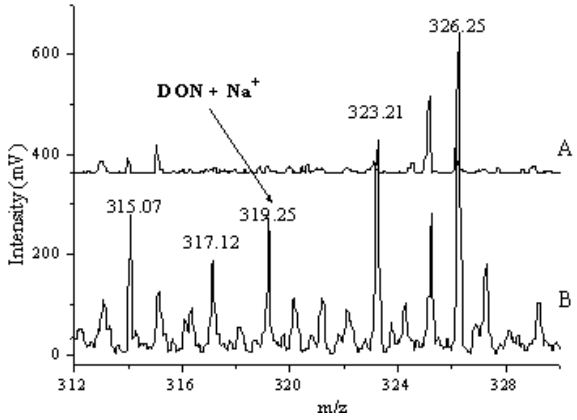


Fig. 4. **Mass spectra of pure malt standard Carlsberg (A) and spiked standard with DON (B) to final concentration 500 µg/kg** (0.6 µg/ml). Conditions: matrix sodium azide, laser energy 140 (arbitrary units).

Table 1. **Statistical evaluation of MALDI mass spectrometry method with standard mycotoxins**

Sample of mycotoxin (matrix)	Repeatability R_1 [%]		Repeatability R_2 [%]		Accuracy of Mr mass/charge [‰]		Resolution		LOD s/n = 3 µg.ml^{-1}	LOQ s/n = 10 µg.ml^{-1}
	1 spot [1]	1 spot [2]	10 spots [1]	10 spots [2]	10 spots [1]	10 spots [2]	10 spots [1]	10 spots [2]	1 spot [1]	1 spot [1]
DON (diamond)	19	9	23	10	0.18	0.23	3400 ± 500	3800 ± 200	1.2 ± 0.2	4.0 ± 0.7
DON (azide)	30	3	50	3	0.21	0.18	3800 ± 500	3700 ± 200	0.6 ± 0.4	2.2 ± 0.9
NIV (diamond)	41	8	68	20	0.05	0.16	1400 ± 200	1500 ± 400	0.8 ± 0.2	2.7 ± 0.7
NIV (azide)	16	30	45	30	1.08	1.03	2100 ± 200	2290 ± 280	0.9 ± 0.1	2.9 ± 0.4
15-ADON (diamond)	16	40	25	50	0.39	0.47	2300 ± 400	1900 ± 300	0.13 ± 0.05	0.4 ± 0.2
15-ADON (azide)	40	10	19	10	0.61	0.95	1200 ± 200	2100 ± 100	0.9 ± 0.2	2.9 ± 0.8

[1] Concentration of individual mycotoxins was 1 µg.ml^{-1}
[2] Concentration of individual mycotoxins was 100 µg.ml^{-1}
$R_{1,2}$ Relative standard deviation

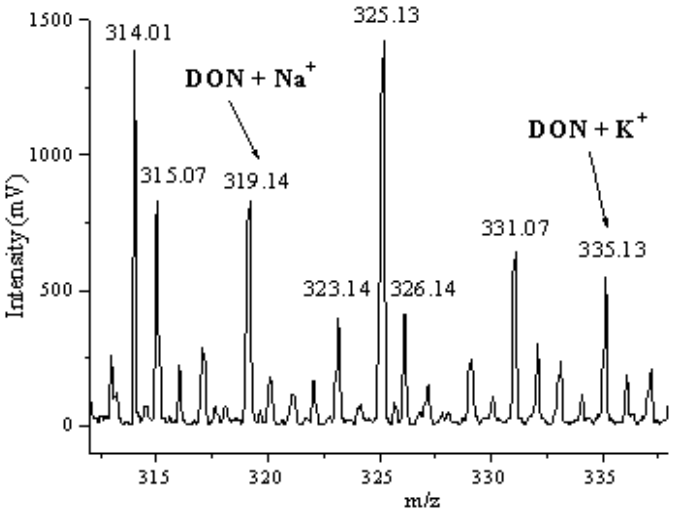


Fig. 5. **Mass spectrum of the extract from malt (Kompact 673) infected with *Fusarium*, none protected.** Conditions: matrix sodium azide, laser energy 140 (arbitrary units).

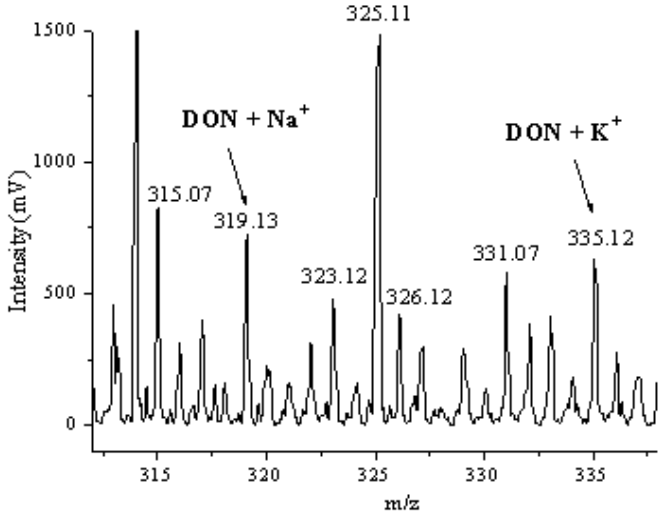


Fig. 6. **Mass spectrum of the extract from malt (Kompact 678) infected with *Fusarium*, protected by fungicides (Horizon 0,5 l/ha + Mirage 0.75 l/ha).** Conditions: matrix sodium azide.

Test of repeatability

Each standard of mycotoxin was repeatedly analyzed from one spot (R_1) and from 10 spots (R_2) and the intensity of the peak was statistically evaluated (Table 1). $R_1 < 10$ the sample on the Maldi target was homogenous. ($R_{1,\ 2}$ = relative standard deviation).

The homogeneity of a sample in the spot of a MALDI slide can only be observed in high concentration of standard (100 $\mu g.ml^{-1}$). The Matrix sodium azide gave a good repeatability for NIV and 15-ADON. Diamond Matrix is suitable for the mycotoxin DON (Table 1).

Test of method accuracy

Each standard of mycotoxin was repeatedly analyzed from 10 spots (Table 1). This parameter was determined as the rate between (M_r theoretical – Mr experimental) and M_r theoretical.

For the determination of DON both matrices have a high accuracy of molecular mass.

Resolution (Resolving power)

This parameter determines the quality of the spectra. The resolution (R) was determined as a

rate $R = M_r / \Delta l$, where M_r = molecular weight and Δl = half the width of the peak.

The diamond and azide matrices show good resolution for DON and NIV. In the case of 15-ADON, the resolution at low concentration (1 $\mu g.ml^{-1)}$ shows good results with the diamond.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD was determined as a rate s/n = 3 and LOQ as a rate s/n = 10, where s = signal (intensity of the peak) and n = signal noise.

The sodium azide matrix is recommended for the determination of DON and NIV. The diamond is recommended for the determination of 15-ADON and NIV.

Determination of DON in real sample of barley and malt

Four real samples of barley and malt were analysed and the quantity of DON was determined by the MALDI method. Samples of protected and non protected barley and malt infected by *Fusarium* were analysed. The calibration curve of the DON standard was made (R^2 = 0.932). Figure 5 shows the mass spectrum of malt (Kompact 673) infected with *Fussarium*, none protected. From the calibration curve a 507.1 ± 8.6 μg/kg (0.63 μg/ml) level of DON was found. This value is comparable with the level of DON determined by HPLC (Institute of Chemical Technology – ICT, Department of Food Chemistry and Analysis, Prague, Czech Republic) which was 779.5 ± 124.1 μg/kg (0.97 μg/ml). The content of DON in the original Kompact 678 barley was not detected by MALDI mass spectrometry and was below DL.

Fig. 6 shows the mass spectrum of malt (Kompact 678) infected with *Fusarium*, protected by fungicides. From the calibration curve a 393.5 ± 8.6 μg/kg (0.49 μg/ml) level of DON was found. The value is comparable with the level of DON determinated by HPLC (ICT, Department of Food Chemistry and Analysis, Prague, Czech Republic) which was 528.0 ± 84.0 μg/kg (0.66 μg/ml). The content of DON in the original Kompact 678 barley was not detected by MALDI mass spectrometry and was below DL.

CONCLUSIONS

In this work, the optimal conditions for the determination of mycotoxins by MALDI mass spectrometry were found. Both matrices (diamond and sodium azide) are suitable for the determination of mycotoxins.

The sodium azide matrix shows a low standard deviation and high resolution. For the determination of DON in the real sample, the matrix sodium azide was chosen as the best. The limit of detection for DON is 0.6 $\mu g.ml^{-1}$

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