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Reliable identification and quantification of trichothecenes and other mycotoxins by electron impact and chemical ionization-gas chromatography–mass spectrometry, using an ion-trap system in the multiple mass spectrometry mode Candidate reference method for complex matrices

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Abstract

Highly toxic mycotoxins like the trichothecenes can be found as contaminants from the metabolism of fungi in food and food preparations. They can be identified and quantified with great accuracy by GC/MS-measurements. Reliable analytical methods are urgently needed because such mycotoxins are not only toxic substances occurring in nature but also are in the list of biological weapons (e.g. T2-toxin, HT-2-toxin) and have some potential for terroristic attacks. By using GC/MS in the EI- and NCI- or PCI-mode and MS^n-measurements with a 30 m Rtx 5MS fused-silica capillary column it is possible to identify and quantify all relevant mycotoxins either as underivatized substances or as their TMS-derivatives in extracts from food, food preparations or beverages with very complex matrix-derived background. This method can also be used to determine free ricinine as a biological marker for ricine in terroristic attacks. So laborious and time-consuming steps of sample-preparation can often be diminished. The LOD is in the range of 10–50 pg and the LOQ with linear calibration curves is in the range of 50–5000 pg. The high specificity of these methods helps not only to detect the existence of intentional terroristic or natural food contamination but also to avoid faulty alarm with unnecessary panic in the public. Furthermore, these methods have a high potential in ameliorating the safety of basic food and food products.

Keywords: Mycotoxins; T2-toxin; HT-2-toxin; Nivalenol; Patulin; Ricinine; Bioterrorism; Food safety

1. Introduction

Some mycotoxins like the trichothecenes belong to those substances which are suspected to have a strong potential for terroristic attacks or biological warfare. Therefore, they are posted on many lists world-wide and also on the lists of the German law to control war-weapons [1] in the group of controlled biological warfare substances. Because the analysis of suspected terroristic material often is confronted with complex matrices [2] reliable methods of identification and quantification of mycotoxins like the trichothecenes are essential to react properly and in short time to identify the substances with readily available analytical equipment [3]. The meanwhile wide-spread availability of ion-trap MS-instruments did encourage us to develop a method using the MS^n-technique for selected trichothecenes and other mycotoxins or the biological marker ricinine found in crude preparations of the extremely hazardous proteotoxin ricin which due to its thermolability is not amenable to GC/MS-analysis.

2. Experimental

2.1. Chemicals and devices

Unless otherwise specified, all reagents and solvents were of analytical grade from Merck, Darmstadt, Germany.

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N-Methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) and *N*-trimethylsilyl-imidazole (TSIM) were purchased from Macherey und Nagel, Düren, Germany. All trichothecene toxins and patulin were from Sigma, Taufkirchen, Germany. Due to the high toxicity of the trichothecenes and other mycotoxins their handling in the laboratory has to be done under special care to avoid every contact with the skin or inhalation of substance dusts during handling of the neat substances and/or their derivatives. Ricinine was purchased from Latoxan, Valence, France. All stock solutions of the highly toxic mycotoxins were stored in the safe and light protected amber glass Certan® ampoule-vials from Promochem, Wesel, Germany. Extrelut® columns were purchased from Merck, Darmstadt, Germany.

2.2. GC/MS-conditions

EI- and CI-GC/MS-measurements have been done with the GCQ-system (ThermoFinnigan, Dreieich, Germany) working with the GCQ-Xcalibur-software version 2.2. The working conditions for the GCQ-system were as follows. GC-conditions: fused-silica column Restek Rtx 5MS with 5 m Integra-Guard, 30 m × 0.25 mm, d.f. = 0.25 μm; injection port: 285 °C, splitless injection, oven: 120 °C for 1 min, 40 °C/min to 295 °C, final temperature for 15 min; He-flow 30 cm/s, constant velocity; transfer-line: 298 °C; MS-conditions: source temperature 170 °C, 0.58 s/scan; multiplier 1275 V; acquisition start time 2.5 min. Reactant gas for the CI-measurements was CH_4. The trap working conditions for the MS^n-mode were: excitation voltage: 1.00–10.00 V, q: 0.45; depending on the selected ions. Table 2 shows selected parent and product-ions which have been used for confirmation purposes.

2.3. Derivatization procedures

The reference substances were derivatized by reaction with TSIM at room temperature for 1 h. In most cases all OH-groups of the trichothecenes are reacting completely to form the TMS-derivatives. For deoxynivalenol minor amounts of the partially substituted compound like the di-TMS-derivative could be observed when the derivatization was done with MSTFA (60 °C for 20 min) instead of TSIM. Patulin is completely derivatized by MSTFA (60 °C for 20 min).

2.4. Extraction procedure for ricinine or patulin from cola-beverages, soft drinks, water or clear juices

The extraction of all these matrices can easily be achieved by using Extrelut®-3 glass columns. One milliliter of the liquid suspected for contamination is made alkaline by adding some drops of a 2 m KOH to reach a pH of 14. Patulin can be extracted completely without modifying the pH due to its neutral character. This solution is poured onto an Extrelut® column and extracted with 3 × 5 ml of $CHCl_3$. In all cases, an additional step of drying the clear extracts with Na_2SO_4 was not necessary. When the extract was evaporated to dryness with a rotary evaporator or a Vortex-Evaporator the remainder was carefully reconstituted in 100 μl of acetonitrile (or derivatized with 100 μl MSTFA and addition of 100 μl acetonitrile for patulin determination) and used for the GC/MS measurements. Recovery of ricinine and patulin from spiked samples was 95 ± 5% and 85 ± 10% for spiked amounts of up to 200 ng/ml. Extraction efficiency for ricinine was the same for water, tonic water and Coca Cola®. Due to the good solubility of caffeine and quinine in $CHCl_3$ it is possible to use this extraction procedure additionally for the quantitative determination of these substances in soft drinks. For the ex-

Table 1
Mass spectrometric key-fragments for different ionization modes

Substance	Key-fragments (m/e) and ionization mode		
	EI-mode	NCI-mode	PCI-mode
Patulin	53, 55, 110, 136	136, 154, 108, 137	155, 81, 99, 71
Patulin-TMS	73, 170, 183, 75, 226	136, 108, 226, 123	227, 73, 255, 137
Ricinine	164, 121, 149, 82	149	ND[a]
15-Acetoxyscirpenol-di-TMS	159, 73, 141, 91, 285, 131	121, 195, 306, 245, 246	319, 159, 73, 379
3-Acetyldeoxynivalenol-di-TMS	73, 287, 75, 117, 289, 105	230, 290, 482, 362, 229	289, 467, 377, 287
15-Acetyldeoxynivalenol-di-TMS	73, 75, 193, 197, 117, 77	135, 267, 215, 268, 216	407, 193, 393, 73
Deoxynivalenol-tri-TMS	73, 393, 333, 392, 259, 512	297, 298, 305, 215, 299	407, 497, 514, 269
Deoxynivalenol-di-TMS	73, 187, 321, 215, 261, 440	ND[a]	ND[a]
Diacetoxyscirpenol-TMS	91, 124, 73, 244, 105, 75	165, 59, 123, 245, 305	379, 183, 229, 319
Fusarenon X-tri-TMS	73, 141, 75, 185, 273, 275	297, 298, 321, 207, 135	555, 465, 273, 405
HT-2-toxin-di-TMS	73, 185, 157, 466, 143, 175	197, 89, 101, 212, 567	317, 185, 467, 303
Neosolaniol-di-TMS	73, 193, 75, 245, 244, 185	213, 179, 231, 226, 197	197, 317, 377, 467
Nivalenol-tetra-TMS	73, 261, 289, 245, 259, 75	297, 298, 303, 207, 299	585, 495, 289, 273
T2-toxin-TMS	244, 73, 290, 245, 185, 75	101, 213, 197, 244, 89	197, 317, 377, 287
T-2-triol-tri-TMS	73, 185, 157, 275, 143, 292	ND[a]	ND[a]
Verrucarol-di-TMS	73, 159, 91, 105, 143, 187	107, 122, 398, 168, 89	185, 231, 195, 169

[a] ND: not determined.

traction of patulin from beverages like apple juice ethylacetat has already been described by others [4].

2.5. Extraction of trichothecenes from different matrices

For the extraction of the trichothecenes many procedures have been developed in the past [5–8]. Isotopomeres are recommendend as internal standards and have been already used successfully [9]. It is highly recommended to extract clean and unadulterated material additionally to the contaminated samples to gain blank GC/MS-runs for comparison purposes.

2.6. Quality characteristics

2.6.1. Reproducibility of retention times

The GC/MS-conditions mentioned above result in high reproducibility of retention times for all measured substances. Even those substances with higher retention times are eluted with very narrow retention time (t_r) windows. The within-day measured values (n = 10) for patulin-TMS and for ricinine for example were x_{tr} = 5.42 ± 0.01 min and x_{tr} = 6.88 ± 0.01 min, respectively. So the daily t_r-variation always was below ±0.5% and the day-to-day retention time variation never exceeded ±2.0% during a working period of more than 6 months for all substances under study.

2.6.2. Limit of detection (LOD) and limit of quantification (LOQ) and calibration

The GC/MS limit of detection (S/N >3 for the base peak) for the TMS-derivatives of all mycotoxins was 50 pg and the limit of quantification (S/N >10 for the base peak) was >50 pg. These values were measured with the pure substances and with splitless injection of 1 μl-solutions in acetonitrile. The calibration-curves for the measurement of the TMS-derivatives of selected mycotoxins were linear up to an amount of 5000 pg. Underivatized ricinine also gave a linear calibration curve for the range of 50–2200 pg (R^2 = 0.9999). For the biological marker ricinine the recovery from beverages like Coca Cola®, tonic water or water have been determined to be >95% with an added amount of 100 ng/ml. The GC/MS limit of detection for ricinine isolated from water, Coca Cola® or soft drinks was determined with 50 pg and the

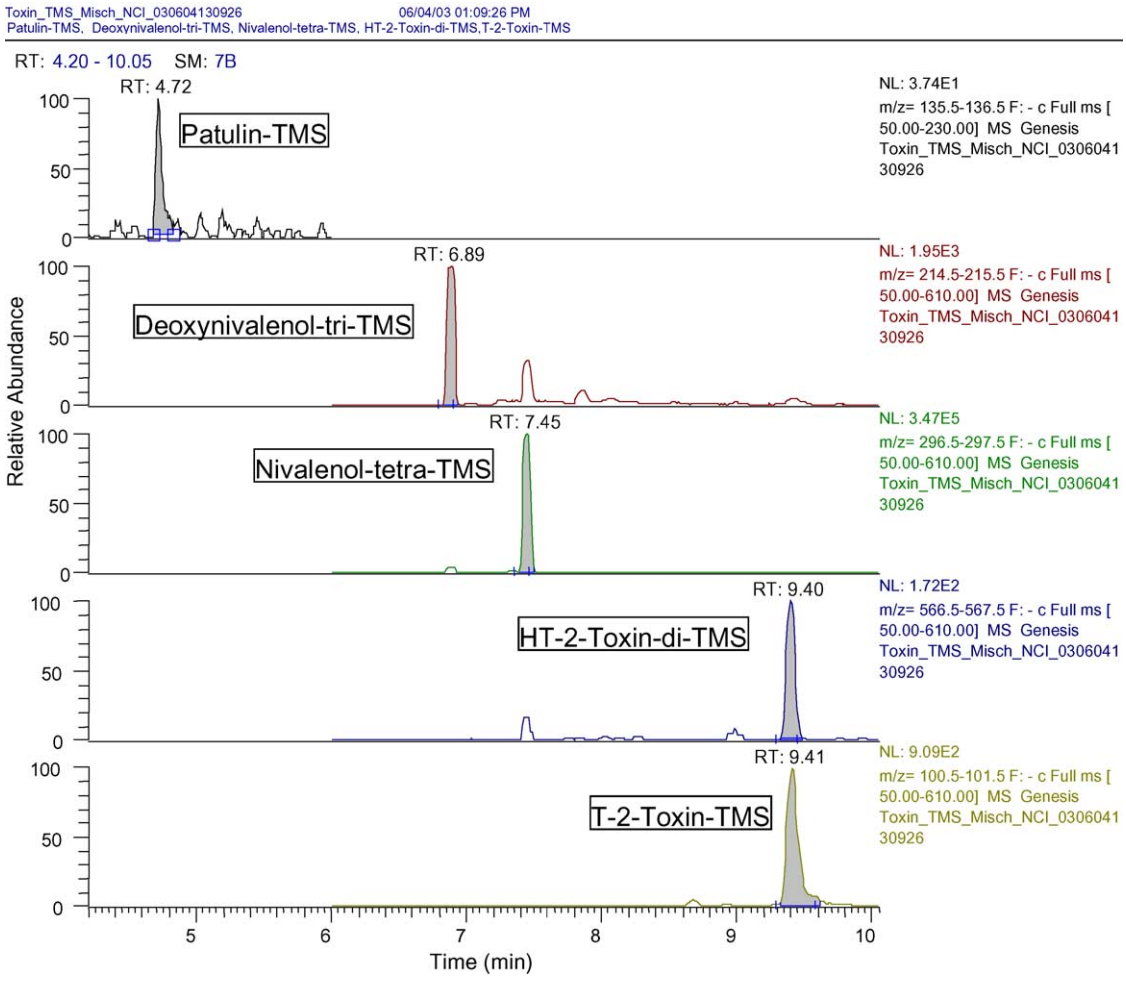

Fig. 1. Gaschromatographic separation of reference substances patulin-TMS (10 ng), deoxynivalenol-tri-TMS (2 ng), nivalenol-tetra-TMS (2 ng), HT-2-toxin-di-TMS (10 ng), and T2-toxin-TMS (5 ng), measured in the NCI-mode with extracted MS key-fragments; injected in splitless mode.

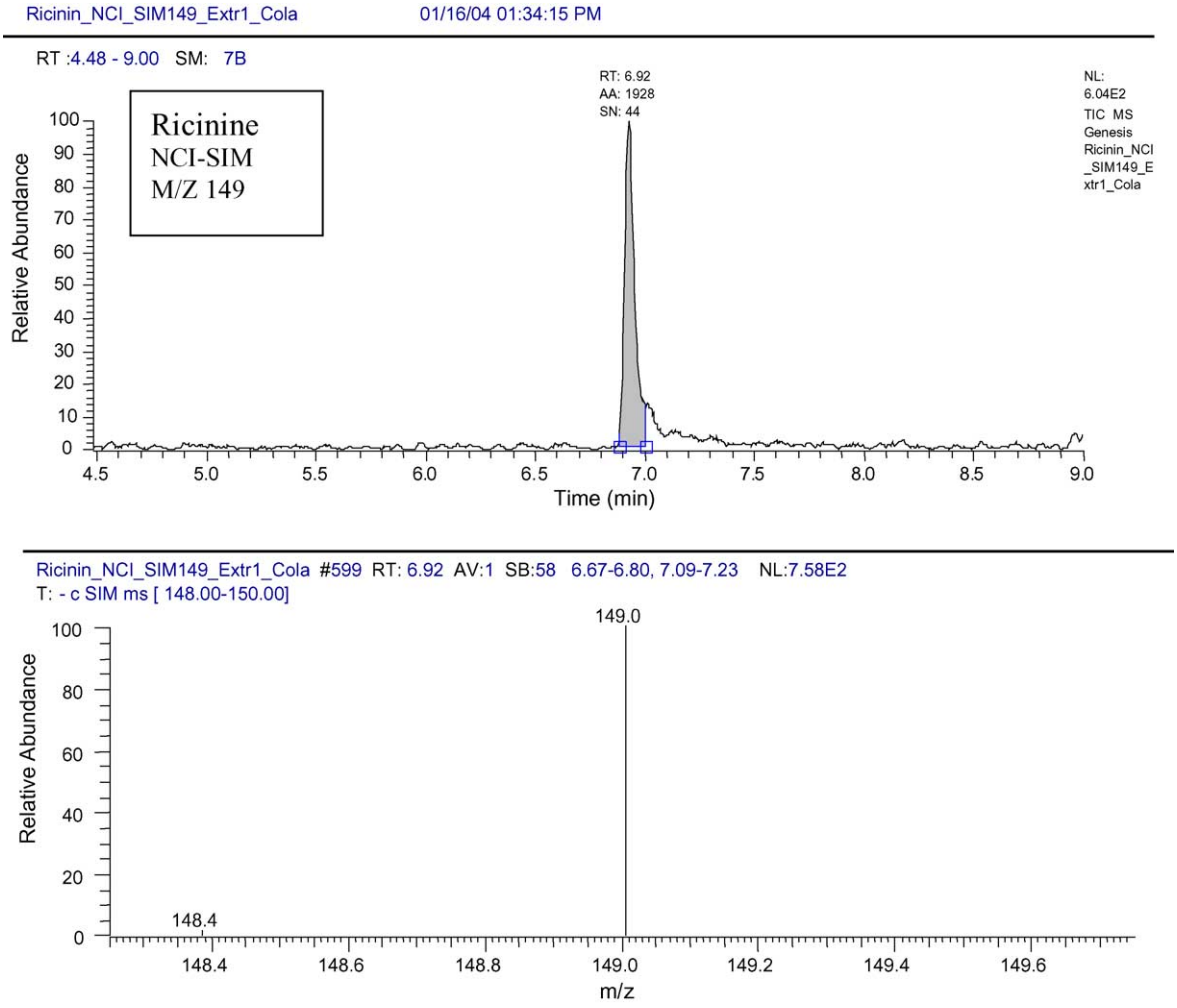

Fig. 2. Gaschromatographic separation of extracted ricinine from a Coca Cola® sample, ricinine amount 850 pg/1 μl acetonitrile.

limit of quantification for the base peak (m/z = 149) was found to be 100 pg by using the NCI-SIM-mode and splitless injection of 1 μl-solutions in acetonitrile. MS^n-measurements using m/z = 164 as parent-ion and a specific product-ion (e.g. m/z = 135) clearly can enhance the specificity of the determination. Using GC/MS-suitable capillary columns with a low background-noise level and properly cleaned ion-volumes are essential to achieve low levels of detection and quantification. Contaminated ion-volumes always result in heavy reduction of sensitivity.

3. Results and discussion

Mycotoxins from the group of trichothecenes and patulin are readily derivatized to their TMS-derivatives and can be separated by 30 m fused-silica capillary columns like Restek Rtx 5MS. Fig. 1 shows the gas chromatographic separation of patulin-TMS, deoxynivalenol-tri-TMS, HT-2-toxin-di-TMS, nivalenol-tetra-TMS and T2-toxin-TMS in the NCI-mode. As can be seen from Fig. 1 HT-2-toxin-di-TMS and T2-toxin-TMS form a critical pair which are not sufficiently separated on the Rtx 5MS column. Their identification and quantification can be achieved using the MS/MS-mode. Table 1 shows the MS-key fragments which can be used for the detection and quantification of ricinine and the TMS-derivatives of pat-

Table 2
Parent- and product-ions for qualitative and quantitative MS/MS-determinations

Substance	MS/MS-mode under EI-condition	
	Parent-ion	Product-ions
Patulin-TMS	136	108, 98, 104, 85
Ricinine	164	135, 134, 121, 149
Deoxynivalenol-tri-TMS	512	393, 333, 392, 496
Deoxynivalenol-tri-TMS	393	333, 259, 260, 305
Nivalenol-tetra-TMS	510	407, 317, 361, 289
Nivalenol-tetra-TMS	482	392, 362, 379, 377
T2-toxin-TMS	290	259, 274, 275, 257
T2-toxin-TMS	244	229, 214, 173
T2-toxin-TMS	185	142, 141, 157, 170
HT-2 toxin-di-TMS	466	287, 303, 284, 288
HT-2 toxin-di-TMS	185	142, 157, 141, 129

ulin and the trichothecenes in the EI- and NCI- or PCI-mode. As can be seen from Fig. 2 ricinine is easily detected by the NCI-SIM-mode (m/z = 149) with an amount of 850 pg/μl acetonitrile after the extraction from a Coca Cola®-sample.

The specificity of all EI-determinations can be greatly enhanced by looking additionally on the mass spectra gained by using the NCI- or PCI-mode. When it is necessary to analyze extracts from very complex matrices it is highly recommended to select parent-ions from EI-, NCI- or PCI-derived mass spectra as candidates for MS^n-measurements. Table 2 shows selected mass spectrometric signals which are suitable for the MS/MS- or MS^n-mode to identify and quantify the derivatives of the trichothecenes definitely in complex matrices like foodstuff, beverages or soil samples. The high specificity of MS/MS or MS^n-measurement offers the opportunity to get clear mass spectra in the low picogramme-range which can help to overcome possible disturbances in the normally registered EI-mass spectra by background peaks from matrix derived substances. This is of great value in the analysis of complex matrices of daily life products which could be contaminated intentionally or with naturally occurring mycotoxins.

4. Conclusions

By using the combined information of retention times and MS-data in different ionization modes like EI, NCI, PCI and the MS/MS-mode with ion-trap instruments it is possible to detect and to quantify all above mentioned thermostable mycotoxins from the group of the trichothecenes as their TMS-derivatives. The reproducibility and specificity of the GC/MS-determinations with safe and reliable detection and quantification can avoid faulty alarm in the public with all their negative consequences not only in the context of 'homeland security'-analyses but also in conventional analytical work. The gained results with the above mentioned methods can also be used in judicial proceedings when questions of food safety are involved. Natural but potentially harmful contamination with toxins from Byssochlamys-, Fusarium-, Cephalosporium-, Stachybotrys, Penicillium- and Trichoderma-species [10,11] can easily occur and should be avoided by using appropriate technologies of food production.

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