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DEVELOPMENT AND VALIDATION OF A GC-MS METHOD FOR THE SIMULTANEOUS QUANTITATION OF ZEARALENONE AND ITS METABOLITES IN WATER

OPRACOWANIE I WALIDACJA METODY GC-MS DO RÓWNOCZESNEGO OZNACZANIA ZEARALENONU I JEGO METABOLITÓW W WODZIE

Abstract: The method of simultaneous determination of zearalenone and its metabolites in water with use of GC-MS chromatography was evaluated. The solid phase extraction SPE was used for analytes separation from water and as an enrichment method. The influence of compounds concentration and water sample volume on analytes recovery was investigated. The chromatographic analysis was preceded by conversion of compounds to trimethylsilyl ethers derivatives. The developed SPE-derivatization-GC/MS method allow to separate quaternary mixture of mycoestrogens with the quantitative analysis in water, when their concentration is greater than 0.2–0.5 ng/dm^3. The repeatability of the results was from 1 to 8 %. The recovery of compounds exceeded 60 % for samples contains from 50 to 200 ng/dm^3 of mycotoxins. The concentration of analytes and volume of the sample (100–500 cm^3) did not have an influence on the extraction output. The developed method can be applied to analyze water samples containing mycoestrogens at the level of ng/dm^3.

Keywords: zearalenone, metabolites, mycoestrogens, SPE, GC-MS, determination, water analysis

The group of biologically active organic micropollutants effected aqueous environment has recently been widened with mycotoxins, which, despite of toxic properties, demonstrate also an estrogenic activity [1]. This is the reason they are also named mycoestrogens. In literature, the most commonly described toxin is zearalenone (ZON) (toxin F-2), which is produced by fungi of kind *Fusarium* living on grains, especially on corn and products, which are produced from it [2]. The increase of feminizing effect of ZON is observed among animals, when the mycotoxin concentration in fodder is greater than 0.06 mg/kg of animal body mass/day [3]. Analogically, the consumption of the toxin with food and water may also have an influence on humans.

The modified form of zearalenone produced via fermentation is zearalanone (ZAN). The compound is used in USA as an anabolic, which stimulates the mass growth of

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cattle [1]. Zearalanone can also be applied as a regulator for plant growth [4]. However, in Europe, including Poland, ZAN is totally forbidden to be used. However, *in vivo* studies on animals and humans have revealed the possibility of direct transformation of natural zearalenone into zearalanone [5–6]. In analyzed urine samples metabolites of ZON were identified, including α-zearalenol (α-Zol) and β-zearalenol (β-Zol) (primary metabolites of ZON) and α-zearalanol (α-Zal) (zeranol) and β-zearalanol (α-Zal) (taleranol) (secondary metabolites of ZON) [6]. Compounds from the group of secondary ZON metabolites can be transformed into ZAN. The transformation pathways of zearalenone are presented in Fig. 1.

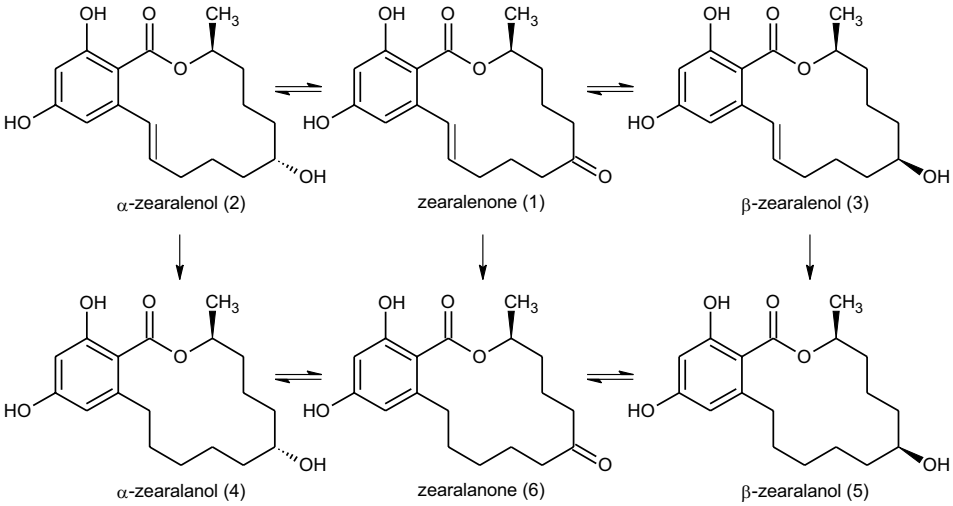

Fig. 1. Structures of zearalenone and its metabolites [1]

The presence of zearalenone, zearalanone and their metabolites is restrictively checked in food and fodder samples [3]. However, the data about the appearance of those compounds in aqueous environment is very limited. There are several papers which discuss the presence of ZON and ZAN in surface water [1–2, 7–8], as well as in influent and effluent streams of municipal wastewater treatment plants [1–2, 8–9]. The concentration of those compounds in aqueous environment was estimated at the level from 0 to 60 ng/dm^3 [1–2, 7–9]. In Poland, according to the study of Gromadzka et al [2] the concentration of zearalenon in selected water samples did not exceed 43.7 ng/dm^3.

The qualitative and quantitative analysis of mycoestrogens in water environment is based on chromatographic techniques, among which liquid chromatography tandem mass spectrometry (LC-MS-MS) is the most popular [1, 7–9]. The aim of the study was the development of a procedure of parallel indication of zearalenone and its metabolites (α-Zol, β-Zol and ZAN) in water by means of solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS). The chromatographic analysis was preceded by the conversion of compounds into trimethylsilyl ethers derivatives.

Materials and methods

Apparatus, materials, reagents

Zearalenone (ZON), zearalanone (ZAN), α-zearalenol (α-Zol), β-zearalenol (β-Zol), mirex (IS), *N*,*O*-bis(trimethylsilyl)trifloroacetamide (BSTFA), trimethylchlorosilane (TMCS) and dithioerythritol (DTE) were purchased from Sigma-Aldrich (Poland). Methanol and acetonitrile, both of reagent grade, were supplied by POCH S.A. (Poland). SPE Supelclean^{TM} ENVI-18 Supelco tubes (6 cm^3, 1 g of the phase) and a Varian SPE chamber were used during the solid phase extraction.

Their base and working solutions of mycoestrogens had concentrations of 1 mg/cm^3 and 10 ng/mm^3 (μL), respectively, and they were added to deionized water and tap water in selected amounts.

Solid Phase Extraction SPE

The separation of mycoestrogens from a water sample (of volume 100–500 cm^3) was carried out in SPE C_{18} extraction tubes (Supelclean^{TM} ENVI-18), which were firstly washed with (5 cm^3) of acetonitrile (ACN) and conditioned with water (5 cm^3). The extract was eluted with 4 cm^3 of ACN. The removal of solvent from the eluate was made by nitrogen stripping and the conversion of analytes was performed.

Silylation reaction

The silylation reaction of mycoestrogens was made with the use of ternary mixture of BSTFA/TMCS/DTE composed in ratio of 1000:10:2 (v/v/w). The reaction took 5 min under the temperature of 90 ^oC. Conditions of the reaction were selected according to Kinani et al studies [10].

GC-MS analysis

The analysis was made with the use of gas chromatograph with mass detector GC-MS of ion trap type (model Saturn 2100 T by Varian). The device was equipped with VF-5ms column of dimensions 30 m × 0.25 mm × 0.25 μm (the film thickness). The detailed parameters of the chromatographic analysis are represented in Table 1.

Table 1

GC-MS conditions for mycoestrogens analysis

Instrument	Varian Saturn 2100T GC-MS
Parameters for GC	
Carrier gas	Helium (purity > 99.999 %)
Carrier gas flow rate	1.4 $cm^3\ min^{-1}$
Injector temperature	300 ^oC

Table 1 contd.

Instrument	Varian Saturn 2100T GC-MS
Injected volume	3 mm^3
Injection mode	splitless
Oven program	Initial temperature of 140 ^oC, hold 0.5 min, 20 $^oC\ min^{-1}$ to 280 ^oC, hold 5.5 min
Parameters for MS	
Ionization	Electron Ionization EI, 70 eV
Ion trap temperature Ion source temperature	200 ^OC

Results and discussion

The analytical method was based on the selected ions monitoring (SIM) presented in Table 2. More than two ions were selected for registration of particular compounds in order to increase the sensitivity of identification. The chromatogram of the mixture of standards of mycoestrogenes derivatives is displayed in Fig. 2.

Table 2

Selected ions for derivatives [m/z] in SIM and retention time

Compound	Ions for derivatives [m/z]	Retention time [min] ($t_R \pm SD$*, n = 12)	*C.V.*** (%, n = 12)
Mirex (IS)	272, 235, 187, 119	9.2506 ± 0.023	0.246
ZAN	449, 432, 406, 308	10.462 ± 0.026	0.245
ZON	444, 430, 306, 150	10.903 ± 0.027	0.249
α-Zol	446, 432, 414, 306	10.964 ± 0.178	0.620
β-Zol	446, 432, 414, 306	11.173 ± 0.030	0.265

* Standard deviation; ** Coefficient of variation.

Conditions applied for the chromatographic analysis allowed to separate all silyl derivatives of mycoestrogens present in the mixture. Chromatographic peaks corresponding to particular components of the mixture had different times of retention (Table 2, Fig. 2).

The crucial parameter of the qualitative analysis is the retention of peak derived from the sample and from the standard, that is retention time. The very precise identification method is the comparison of retention times coming from sample analysis with those obtained for standards. The variation of retention times, which were obtained during many chromatograph analyses, was insignificant. That could be confirmed by low values of standard deviation of this parameter for all investigated compounds as well as by the high precision of measurements certificated by values of variability coefficient (*C.V.*), which varied from 0.245 to 0.620 % (Table 2).

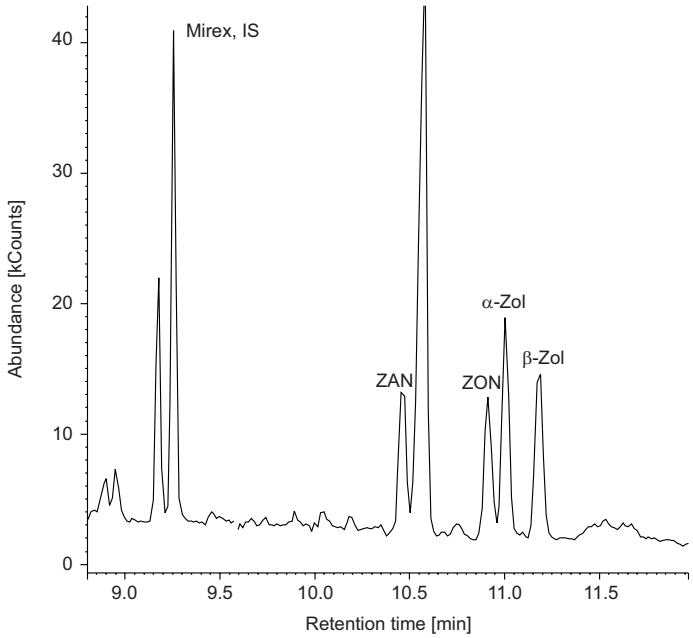

Fig. 2. Chromatogram GC-MS derivatives of mycoestrogens after SPE (concentration of the compounds 50 ng/dm^3, volume of the sample 500 cm^3)

In the quantitative analysis of mycoestrogens content in water the repeatability of results is very important. This analysis was made using peaks areas of mass ions (m/z) of mycoestrogens derivatives (Table 2), which were corrected with areas obtained for the constant content of the internal standard (IS-mirex). The precision of the response of the mass detector was calculated for two different concentrations of mycoestrogens derivatives introduced onto the chromatographic column (Table 3). The *limit of detection* (*LOD*) as well as linear response of the mass detector were also determined for mycoestrogens concentrations in the range from 1 ng/3 mm^3 to 15 ng/3 mm^3.

Table 3

The limit of detection, precizion and linearity of mass detector response

Compound	Limit of detection (*LOD*)* [ng/μg]	Concentration		Correlation coefficient (R^2)**
		3 ng/3 mm^3	12 ng/3 mm^3	
		C.V. [%]		
ZAN	0.3	10	2.0	0.980
ZON	0.1	10	0.3	0.982
α-Zol	0.1	5.5	1.9	0.997
β-Zol	0.1	0.5	0.1	0.998

* Defined as the mass of analyte injected producing a peak with a signal-to-noise ratio (*S*/*N*) of 3; ** Linear ranges of the calibrations curve: 1 ng/3 mm^3 to 15 ng/3 mm^3.

The repeatability (expressed as *C.V.*) of most of GC-MS analysis results varied from 0.1 to 5.5 %, what confirmed the good precision of the measurements. However, during the analysis of ZON and ZAN the obtained repeatability was equal to 10 % for the lower concentration of these compounds, what is shown in Table 3. Linear correlation coefficients (R^2) of calibration curves exceeded 98 % for all investigated compounds. The limit of detection of the method was estimated at the level of 0.1 ng/μg except from ZAN, for which it was equal to 0.3 ng/μg.

Values of parameters characterizing the quantitative analysis using the SPE--derivatization-GC/MS procedure are presented in Table 4.

Table 4

Efficiency of mycoestrogens extraction and accuracy of the SPE-derivatization-GC/MS method

Compound	Sample	Concentrations [ng/dm^3]						Limit of quantification LOQ** [ng/dm^3]
		200		100		50		
		Recovery [%]*	*RSD** [%]*	Recovery [%]*	*RSD** [%]*	Recovery [%]*	*RSD** [%]*	
ZAN	Deionized water	61	8	63	4	62	1	0.5
	Tap water	69	1	68	1	75	3	0.5
ZON	Deionized water	69	4	71	4	77	4	0.5
	Tap water	62	7	76	8	80	8	0.3
α-Zol	Deionized water	80	3	60	2	83	5	0.3
	Tap water	60	3	63	1	72	1	0.2
β-Zol	Deionized water	92	1	64	1	68	2	0.3
	Tap water	62	1	65	1	64	1	0.3

* Relative standard deviation; ** $S/N > 10$, The recoveries and precision were examined by replicates analysis (n = 4, volume of the sample 500 cm^3).

The efficiency of extraction and measurements precision were determined by quadruple repetition of the whole procedure in which two types of water (deionized and tap water) were used. The concentration of mycoestrogens varied from 50 to 200 ng/dm^3. Obtained results allowed to determine the efficiency of the extraction of mycoestrogens. Values of this parameter were in the range from more than 60 % to more than 90 % depending on the compound extracted. The precision of the method can be noted as good. The influence of the impurities content in water on the extraction efficiency was negligible. Similarly, the volume of the water sample from which compounds were extracted did not effected the extraction process, what is presented in Table 5. The repeatability (expressed as *relative standard deviation*, *RSD*) of the results obtained during the developed method was satisfying. The calculated *RSD* values were in the range from 1 to 8 %. The lower limit of analysis of mycotoxins concentration in water varied from 0.2 to 0.5 ng/dm^3 depending on the investigated matrix.

Table 5

The effect of sample volume on the recovery of mycoestrogens

Compound	Concentration [ng/dm^3]	Sample volume [cm^3]		
		500	250	100
		Recovery (*RSD*) [%]*		
ZAN	200	61 (*8*)	63 (*4*)	62 (*1*)
ZON		69 (*4*)	68 (*3*)	64 (*8*)
α-Zol		80 (*3*)	80 (*2*)	93 (*5*)
β-Zol		92 (*1*)	94 (*1*)	98 (*2*)

* The recoveries and precision were examined by replicates analysis (n = 4).

Conclusions

1. The applied chromatographic conditions are suitable to separate compounds in quaternary mycoestrogenes mixture and to run the effective qualitative and quantitative analysis. The limit of detection of the GC-MS method was estimated at the level of 0.1 ng/mm^3 except from zearalenone for which it was equal to 0.3 ng/mm^3.

2. The developed SPE-derivatization-GC/MS method allow to determine the concentration of mycoestrogens in water at the level from 0.2 to 0.5 ng/dm^3, depending on the applied matrix (deionized and tap water). The carried out analysis of water samples containing from 50 to 200 ng/dm^3 of mycoestrogens characterized with satisfied value of results repeatability equal from 1 to 8 %. The recovery of mycoestrogens exceeded 60 % and the compounds concentration and volume of the sample did not have an influence on the value of investigated parameter.

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OPRACOWANIE I WALIDACJA METODY GC-MS DO RÓWNOCZESNEGO OZNACZANIA ZEARALENONU I JEGO METABOLITÓW W WODZIE

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Abstrakt: Ocenie poddano metodę równoczesnego oznaczania zearalenonu i jego metabolitów w wodzie z użyciem chromatografii GC-MS. Ekstrakcja do fazy stałej SPE wykorzystana została jako metoda wydzielania i wzbogacania analitów z wody. Badano wpływ stężenia i objętości próbki wody na odzysk związków. Z kolei przed chromatograficznym oznaczeniem mykoestrogeny przeprowadzono w etery trimetylosiliowe. Metoda SPE-*derywatyzacja*-GC/MS umożliwia rozdział czteroskładnikowej mieszaniny mykoestrogenów i ich ilościowe oznaczenie w wodzie na poziomie stężeń 0,2–0,5 ng/dm^3. Powtarzalność oznaczeń była w zakresie 1–8 %. Wyznaczony odzysk związków dla stężenia w zakresie 50–200 ng/dm^3 przekraczał 60 %. Nie obserwowano znacznego wpływu stężenia analitów jak i objętości próbki wody (100–500 cm^3) na wydajność ekstrakcji. Przedstawioną metodykę można więc zastosować do kontroli analitycznej obecności mykoestrogenów w środowisku wodnym na poziome stężeń ng/dm^3.

Słowa kluczowe: zearalenon, metabolity, mykoestrogeny, SPE, GC-MS, oznaczanie, analiza wody