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Application Note

On-line SPE and determination of zearalenone from edible oil

Category	Food
Matrix	Edible oil
Method	HPLC
Keywords	Mycotoxins, on-line SPE, dynamic covalent hydrazine chemistry, DCHC, ZON, ZEA, zearalenone
Analytes	Mycotoxins, zearalenone
ID	VFD0132N_A_E

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Summary

This application note presents the development, optimization and validation of a new on-line HPLC method used for the separation and determination of zearalenone in edible oil samples. Zearalenone (ZON) is a non-polar mycotoxin and a common contaminant in cereal grain used for animal and human food. It exerts an estrogenic activity that modulates/disrupts endocrine function in animals and possibly humans. High concentrations of ZON are found in edible oils, especially in corn oil. The presented method combines two on-line SPE procedures: a chemical reaction on an SPE Hydrazine cartridge and a classic on-line SPE trapping process in reversed-phase mode. For the whole sample preparation and analysis process we obtain a recovery rate of 95 % ZON with a model system and methanol as matrix substitute. Adapting edible oil as a matrix we reach about 80 % recovery for ZON and the the hydrazine cartridges can be used up to 12 times. The adjusted on-line HPLC system consists of two KNAUER AZURA Assistants equipped with a high pressure switching valve set up of three valves and one SPE pump, plus a KNAUER AZURA 6.1L high pressure gradient pump and a fluorescence detector. The sample introduction is realized with an autosampler unit. The analysis of ZON is carried out on a Eurospher II C18P column.

Introduction

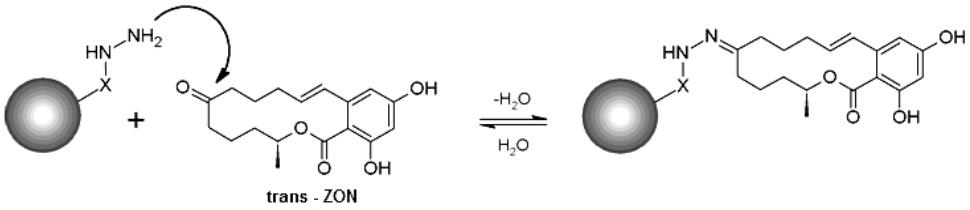
As estimated by the Food and Agricultural Organization of the United Nations 25 % of the global food are contaminated with mycotoxins.[1] Mycotoxins are secondary metabolites produced by mould fungus. The mycotoxin ZON, which is an intermediate catabolic product of filamentous fungi of the genus *Fusarium*, can be detected on almost all type of cereal. Although the overall toxicity is low, animal testings unraveled teratogenic, hepatotoxic, immonotoxic, genotoxic and cancerogenic effects.[2,3] ZON furthermore influences the tumor progression of hormonal sensitive tissues as it shows estrogen-like characteristics. Whereas in the past the crop has been assumed to be the main source of contamination with ZON, more recent studies identified especially maize, barley and soya germ oil as risky.[4] As a consequence the European Union introduced the critical value of 400 µg ZON/kg for refined maize oil.[5] The lipophilic character of ZON leads to much higher concentrations in liquid plant oils compared to solid cereal products. Corn oil shows peak values of up to 4600 µg/kg. Additionally there is evidence that about 60 % of all other tested edible oils also contain ZON.[6] Currently there is no standardized procedure to measure ZON in oil samples, so the EU is working

on a proposal (CEN-Mandat 520/2013).

As a consequence, a method to accurately determine the content of ZON in refined maize, that is salutary and can be performed with standard HPLC equipment is required. ZON with his apolar character is a component of edible oils, where fatty acid triglycerides dominate the matrix with 95–98 %. For a quantitative analysis of ZON the removal of the oil matrix is essential, as otherwise reliable measurements are impossible. The high amount of triglycerides make a separation of the non-polar ZON challenging. Due to their similar polarity values the classic solid phase extraction procedures for sample preparation are insufficient.

Recent ideas for sample preparation aim on the reversible formation of a covalent bond, which is formed between the target metabolite ZON and a reactive chemical group on a solid phase. Once the metabolite is fixed, the non-reactive impurities can be removed. The whole procedure is named dynamic covalent hydrazine chemistry (DCHC), see Figure 1.[7] The reversible hydrazine-hydrazone reaction makes recycling and reuse of the solid phase cartridge possible.

Figure 1:
Reaction of zearalenone with a sulfonyl-hydrazine functionalized carrier material



Experimental:

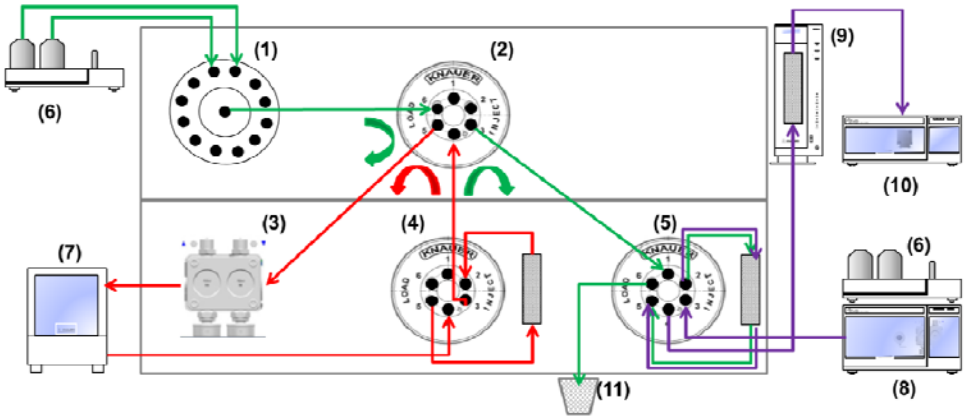
Sample preparation

The edible oil has to be diluted in heptane. There to 0.70 to 0.74 g oil were diluted up to 1 ml with heptane.

Method

Most challenging is the chemical equilibrium of this hydrazine-hydrazone reaction, as it takes about 60 min. Usually on-line SPE methods use physisorption and this process is done within seconds. For the DCHC of ZON a novel system for the on-line SPE has been developed (see Figure 2). This system enables a sample flush cycle process several times through the hydrazine-cartridge (see Figure 2, red arrows as cycle). Once ZON is linked to the hydrazine solid phase, the circulation valve opens and different eluents are pumped through the system to flush any remaining oil matrix out of the system and to convert the system to aquatic conditions (see Figure 2, green and red arrows without grey C18-trap cartridge). During ZON coupling and washings steps the HPLC is constantly running on a low flow rate to keep the C18-trap cartridge and the analytical column in “ready” modus (see Figure 2, purple arrows with grey C18-trap-cartridge). Before ZON cleavage using a diluted acetone solution, the C18-trap cartridge is connected to the sample circuit (green and red arrows with grey C18-trap cartridge, Figure 2). Acetone substitutes ZON from the hydrazine-cartridge and binds with its own carbonyl group to this phase. The released ZON is collected and concentrated on the C18-trap cartridge (green and red arrows with grey C18-trap cartridge, Figure 2). This second step is a classical on-line SPE step based on physisorption. Before the analytical measurement can start, the acetone should be removed from the C18-trap cartridge to avoid interferences in the baseline. After cleaning the C18-trap cartridge is incorporated into the HPLC cycle of the system, which correlates with an injection (see Figure 2, purple arrows with grey C18-trap cartridge). With an ACN-Water gradient the target compound ZON, now concentrated on the C18-trap cartridge, starts to elute on the Eurospher II C18P column. The target compound is detected by fluorescence. The hydrazine cartridge is regenerated during the analytical measurement of ZON

and thus prepared for the next sample preparation including measurement. With the described on-line procedure 10-15 runs are possible on one hydrazine cartridge.



(1) assistant-I left: solvent selection valvel
(2) assistant-I center circulation valve
(3) assistant-II left : pump for ZON-SPE-system
(4) assistant-II center : valve with hydrazine-cartridge
(5) assistant-II right : valve with C18 trap-cartridge
(6) solvents
(7) autosampler
(8) HPLC-pump (high-pressure gradient)
(9) column thermostat with analytical C18P-column
(10) fluorescence detector
(11) waste

Figure 2: Composition and connection scheme of system

Method for the analytical step

Column	Eurospher II C18P 100-3, 150 x 3mm
Eluent A	Water
Eluent B	Acetonitrile
Flow rate	0.7 ml/min
Column temperature	40 °C
Gradient	from 30 % B to 60 % B in 20 min
Detection	Fluorescence (excitation 276 nm, emission 456 nm)

Method for on-line SPE

1. SPE cartridge	Sulfonyl hydrazine functionalized carrier material
2. Trap cartridge	Eurospher II 100-3 C18P, 30 x 3 mm
Column temperature	40 °C
Injection	100 µL
SPE solvents and flows	see table 1

Table 1
Individual steps of on-line SPE run

Step	Description	Time [min]	Flow [ml/min]
1	Cartridge conditioning with methanol, injection of sample and coupling of ZON by circulatory pumping	70	0.75
2	Wash SPE-cartridge with 2-propanol and flush system down to 15 % acetonitrile (not shown in Fig. 2)	20	0.75
3	Release of ZON by switching to 20% acetone	30	0.075
4	Elute ZON from SPE-cartridge onto trapping column and wash out acetone with 15% acetonitrile*	10	0.75
5	Focused elution of ZON onto analytical column (gradient from 30 % acetonitrile to 60 % acetonitrile in 26 min, 7 min re-equilibration)/parallel regeneration of SPE-cartridge using methanol:acetic acid 95:5 (v/v)	34/130	0.75

*After the sample is eluted onto the analytical column the SPE cartridge can be recycled in parallel.

Results

The high selectivity and enrichment of ZON from edible oils, compared with other sample preparation procedures leads to a quite low detection limit without coelution. The functionality of this on-line method was validated with a ZON standard in methanol. The SPE step comprises hydrazine-functionalized particles to which ZON is coupled and cleaned from oil matrix via different washing steps and solvents. The decoupling of the covalent bonded ZON directly into the HPLC-FLD system is accomplished using a diluted acetone solution.

The recovery rate of the methanol-based sample is 95%. The recovery rate for corn oil is about 80%, which is consistent with the demands of the European Union (70–120% recovery rate).[8] Controls, using ZON-free oils as a blank did not show any unspecific peaks in the fluorescence chromatogram within the retention time window of the ZON compound (Figure 3). The determined calibration values show excellent linearity. The calibration range was calculated and measured in the range fromf 5 ng up to 100 ng (Figure 4). As the hydrazine-hydrazone reaction is reversible the SPE cartridge can be reused at least 12 times (Figure 5).

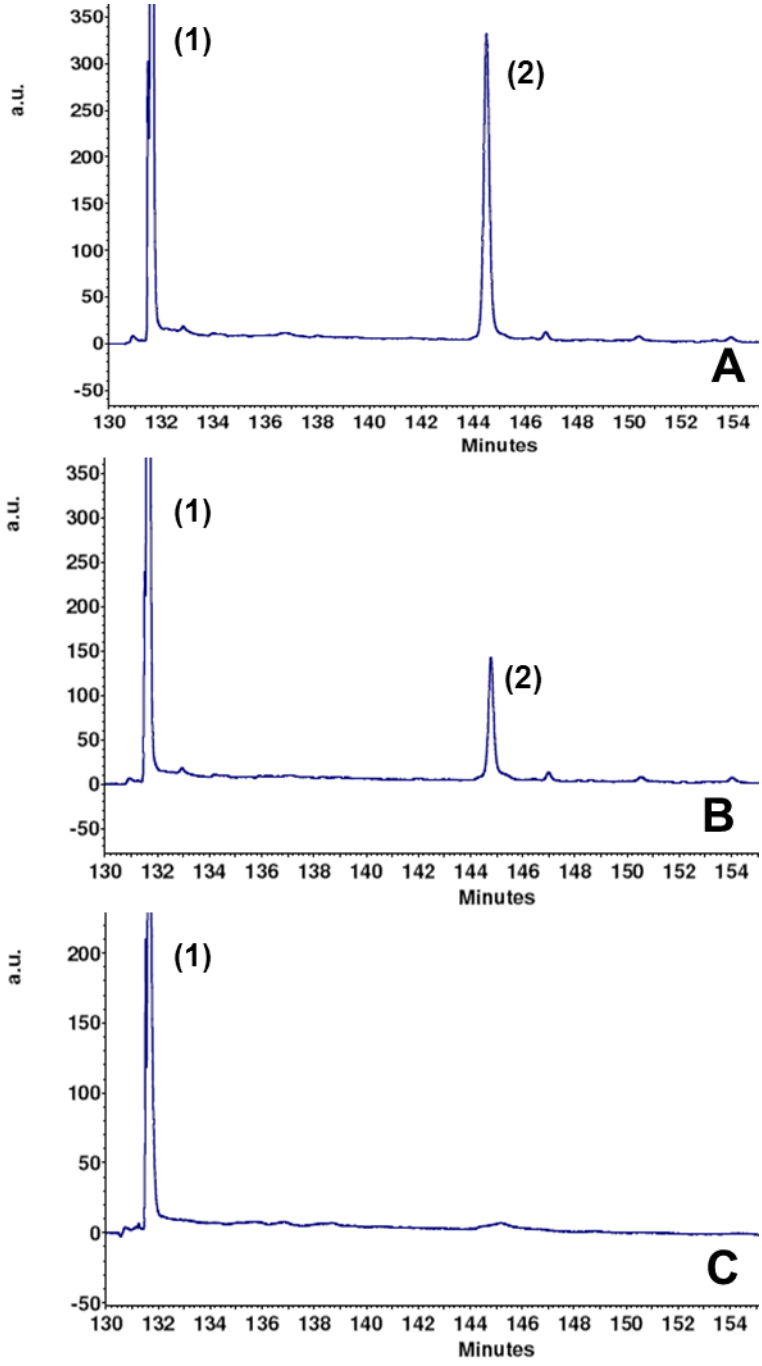


Figure 3:
Chromatogram of ZON (2) and injection peak (1)

A: 470 ng/ml ZON in methanol with a recovery rate of 95 %.

B: 250 µg/kg ZON in corn oil a recovery rate of 80 %.

C: Canola oil without ZON (blank control).

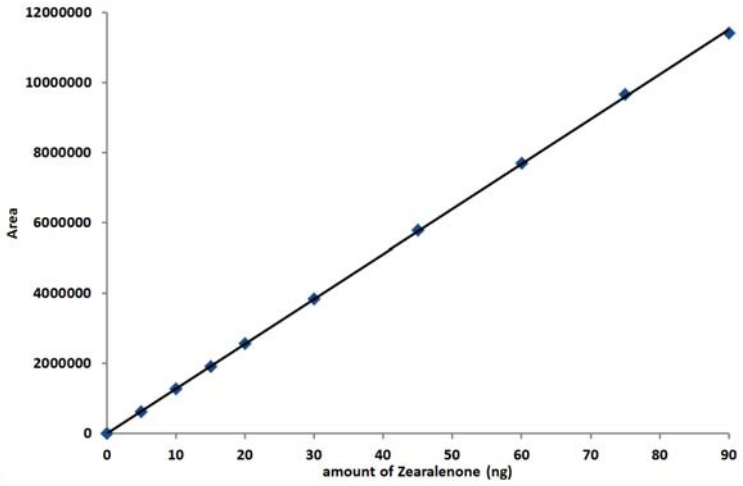


Figure 3:
Calibration of ZON

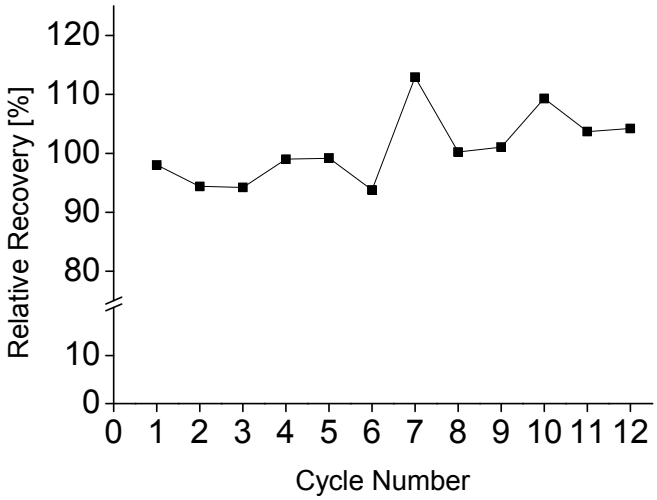


Figure 5:
Recoveries obtained from the analysis of spiked ZON samples depending on the number of presenting recycling passes

Method performance

Calibrated range:	0-90 ng zearalenone
Linearity (r^2)	0,99998
Limit of detection	1.5 ng
Peak area precision	< 1 %

Conclusion

The presented technique enables a highly selective and automatable measurement of ZON in edible oils in the range of 10-1000 µg (zearalenone)/kg (oil) for an injection volume of 100 µL. A higher range is possible by applying higher injection volumes. The used Hydrazine cartridge with a column bed length of 100 x3 mm ID can tolerate injection volumes of edible oil samples up to 500 µl. This new sample preparation procedure is superior to liquid extraction and GPC sample preparation steps for the determination of ZON in edible oil with a detection limit of 25 µg/kg. In contrast to the immunoaffinity columns, hydrazine-functionalized SPE columns can be reused more than 10 times.
The recovery of the on-line SPE method is in the range of 80% and higher for edible oil samples.
Investigation of spiked and original oil samples with ZON levels of around 80 µg/kg showed the advantage of this method. Thedemonstrated SPE-HPLC on-line coupling requires only the insertion of liquid oil samples to monitor the contamination level of zearalenone in these oil samples.

References

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Physical properties of recommended analytical column

Stationary phase	Eurospher II C18P 100-3
USP code	L1
Pore size	100 Å
Pore volume	0.8 ml/g
Specific surface area	320 m^2/g
Particle size	3 µm
Form	spherical
Surface area	320 m^2/g
% C	20
Endcapping	yes
Dimensions	150 x 3mm
Order number	15XE182E2G

Physical properties of recommended trap cartridge

Stationary phase	Eurospher II C18P 100-3
USP code	L1
Pore size	100 Å
Pore volume	0.8 ml/g
Specific surface area	320 m^2/g
Particle size	3 µm
Form	spherical
Surface area	320 m^2/g
% C	20
Endcapping	yes
Dimensions	30 x 3mm
Order number	03CE182E2G

Physical properties of recommended hydrazine cartridge

Stationary phase	Silia Bond® Tosyl Hydrazine
USP code	L1
Pore size	60 Å
Pore volume	0.7 ml/g
Specific surface area	480-550 m^2/g
Particle size	40-63 µm
Form	spherical
Dimensions	100 x 3mm
Order number	upon request

Recommended instrumentation

This application requires a high pressure gradient HPLC system with autosampler, degasser, column thermostat, fluorescence detector and two assistants equipped with valves and additional solvent pump for the on-line SPE. Other configurations are also available. Please contact KNAUER to configure a system that is perfect for your needs.

Description	Order No.
AZURA pump P6.1L HPG	DPH35EA
AZURA Assistant ASM 2.1L one 12 port multiposition valve, 1/8 one 6 port 2 position injection valve 1/16	DYFAHNLX
AZURA Assistant ASM 2.1L one pump P 4.1S two 6 port 2 position injection valves 1/16	DYBAHNHN
AZURA autosampler 3950	A50070
AZURA column thermostat CT 2.1	A05852
Fluorescence detector RF-20 Axs	A59201
AZURA Eluent tray E 2.1L	AZC00

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