Theses of the Ph.D. dissertation

Coupled mass spectrometric procedures for the determination of organic micropollutants from matrices of plant origin

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1 Introduction

In these days the importance of the determination of pesticide transformation products (TPs) or metabolites is continuously increasing due to the fact that some of these compounds are more persistent or possess higher toxicity than their corresponding parent compounds. The situation is similar for propylenethiourea (PTU, 4-methylimidazolidine-2-thione), which is the plant and animal metabolite and degradation product of propineb, the only *N,N'*-propylenebisdithiocarbamate type non-systemic fungicide. Since PTU has a higher polarity, it is the terminal residue to which consumers of produce treated with propineb are exposed. This concern associated with propineb and PTU is reflected by the commission directive 2006/125/EC and the 35/2004. (IV. 26.) EszCsM regulation.

Crnogorac et al. published an LC-MS[1] and an LCMS/MS[2] approach for the class based determination of "intact" dithiocarbamate fungicides (dimethyl-dithiocarbamates, ethylene-bis-dithiocarbamates and propylene-bis-dithiocarbamates), as the most frequent methods for residue analysis, which measure the released carbon disulfide after hot acid hydrolysis, do not distinguish between dithiocarbamate subclasses. To decrease the decay of the dithiocarbamates during extraction they used a special extraction medium (sodium hydrogen carbonate buffer and *DL*-penicillamine, 10 mM each at pH 12), which was also necessary to sufficiently solubilize the target compounds, because without sodium salts, dithiocarbamates forming polymeric chelates are almost insoluble in both water and organic solvents. Thus, it is reasonable to conclude that propineb and propylenethiourea, because of their different physicochemical properties, have to be analysed by single residue methods.

Due to the global widespread of fumonisin mycotoxin-producing fungi and the increasing popularity of modern corn based nutrition, the exposure of the population is continuously increasing. Particularly concerned are the vegetarians and those diagnosed with coeliac disease, as their nutrition is based solely on corn. Besides this, it is also important to note that organically grown products have to be considered carefully in the light of the mycotoxin question.

Depending on their chemical structures, the fumonisin type mycotoxins can be classified into four main groups (A, B, C and P), but toxicologically the most important ones

[1] Crnogorac, G. és W. Schwack, *Determination of dithiocarbamate fungicide residues by liquid chromatography/mass spectrometry and stable isotope dilution assay.* Rapid Communications in Mass Spectrometry, 2007. **21**(24): p. 4009-4016.

[2] Crnogorac, G., S. Schmauder, és W. Schwack, *Trace analysis of dithiocarbamate fungicide residues on fruits and vegetables by hydrophilic interaction liquid chromatography/tandem mass spectrometry*. Rapid Communications in Mass Spectrometry, 2008. **22**(16): p. 2539-2546.

are the FB analogues (FB_1, FB_2, FB_3 és FB_4). Based on the available reports, we can conclude that the incidence rate of B_1 type fumonisin mycotoxins in food samples is significant and growing, but nevertheless the number of research studies targeting FB_x analogues and isomers is rather low, although the required analytical instrumentations are readily available (LC-TOF/QTOF, LC-Trap). We can assume that by using the appropriate analytical instrumentation with suitable HPLC columns, additional isomers can be efficiently separated, detected and characterized. From the results obtained, the FB_1 isomer-producing ability of the *Fusarium* species could be further characterized.

2 Aims

The goals of the PhD work were the following.

1. The determination and characterization of previously not reported B_1 type fumonisin mycotoxin isomers in a solid rice culture inoculated with *F. verticillioides* by LC-TOF-MS.
    - Evaluation of a special HPLC column, manufactured for the separation of structural isomers;
    - Assignation of the FB_1 isomer peaks using accurate mass data;
    - Detection and characterization of previously reported higher molecular weight fumonisins.

2. The determination of propylenethiourea (PTU) in tomato and processed tomato based food commodities using LC-MS/MS.
    - Evaluation of the QuEChERS methodology for the analysis of PTU in tomato and processed tomato based food commodities (tomato puree and baby food with low fat content);
    - Optimization of the extraction and sample clean-up conditions;
    - Development of a novel chromatographic method for the effective separation of the matrix constituents from PTU.
    - Evaluation and characterization of the matrix effect in processed tomato based food commodities.
    - Validation of the method(s) according to the SANCO/10684/2009 guideline.

3 Experimental

3.1 Separation and identification of B_1 type fumonisin mycotoxin isomers

3.1.1 Production and extraction of fumonisins

Long-grain rice (50 g, Uncle Ben's) and HPLC grade water (50 mL) were added to Erlenmeyer flasks (500 mL) and kept at room temperature overnight, and the excess water was then decanted off. The flasks were autoclaved at 121°C for 15 min on each of 2 consecutive days and then inoculated with 5 mL of the conidial suspensions of *F. verticillioides* Fv16 and thermostated at 28°C in the dark. The cultures were shaken once daily for the first 3 days in order to distribute the inoculum and to prevent the grains from adhering. After 4weeks, the cultures were frozen and freeze-dried, grounded to a fine meal and stored in a deep freezer (-80°C) until analysis. Freeze-dried rice culture material (1 g) was homogenized and extracted in a polypropylene centrifuge tube (30 mL) with a mixture (8 mL) of MeOH/H_2O (75/25, v/v), using an UltraTurrax T25 high-speed homogenizer at 9500 rpm for 4 min. After extraction, the sample was centrifuged at 10000*g* for 10 min and membrane-filtered through a 0.2 mm PTFE membrane into a HPLC autosampler vial.

3.1.2 LC-MS-TOF conditions

Separations were performed with a YMC-Pack J'sphere ODS H80 (250mm x 2.1mm, 4 mm) HPLC column at a mobile phase flow rate of 0.2 mL/min using an Agilent 1200 Series LC system. Solvent A was water containing 0.1% (v/v) formic acid and solvent B was MeCN containing 0.1% (v/v) formic acid. The gradient elution was started with 24% B, which was increased linearly to 40% B at 79 min, and then to 100% B at 94 min, the latter being maintained for 10 min. The column temperature was maintained at 40°C and the injection volume was 1µL.

The exact masses of the FB_1 isomers were determined with an Agilent 6210 time-of-flight mass spectrometer. The instrument was operated with a dual-nebulizer ESI source in positive ion mode. ESI parameters were the following: drying gas (N_2) flow and temperature: 10 L/min and 350°C, respectively; nebulizer gas (N_2) pressure: 20 psig; capillary voltage: 3500 V. TOFMS parameters: fragmentor voltage: 170 V; skimmer potential: 70 V; OCT 1 RF Vpp: 250 V. Two reference masses (*m/z* 121.050873 and 922.009798) were used to recalibrate the mass axis during the chromatographic analysis. Full-scan mass spectra were acquired over the m/z range 100–1700 at an acquisition rate of 250 ms/spectrum. Agilent

MassHunter Data Acquisition TOF/Q-TOF B.02.00. and Qualitative Analysis B.03.01. softwares were used for data analysis.

3.2 Synthesis and impurity profiling of the internal standard

3.2.1 Synthetic procedures

4,5-Dimethylimidazolidine-2-thione was prepared (Fig. 1.) using a modified version of the literature procedure. Commercially available diacetyl was reacted with benzylamine to give the appropriate dibenzyl-butanediimine. This product was then subjected to Pd-catalyzed hydrogen-reduction to furnish the final precursor diamine. Subsequent reaction with carbon-disulfide resulted in the desired thione. Due to the diastereomeric mixture of the diamine, formed in the reduction step the product was also composed of a mixture of diastereomers, including the meso and the two enantiomeric forms. The diastereomers appeared as two separate bands in the NMR spectra as well as under certain conditions in the HPLC chromatogram.

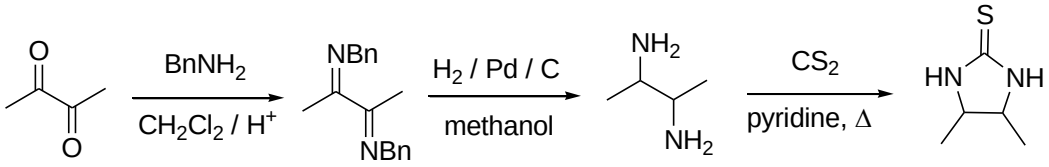

Fig. 1. Synthetic protocol of 4,5-dimethylimidazolidine-2-thione

3.2.2 Assay and impurity profiling of the internal standard

Separations were performed using an Agilent Technologies 1200 series LC with an Agilent ZORBAX Eclipse Plus Phenyl-Hexyl RRHT column (50 x 4.6 mm i.d., 1.8 µm) at a flow rate of 0.8 mL min^{-1}. Column temperature was 25 °C and injection volume was 3 µL. Gradient elution was applied using aqueous 5 mM ammonium formate as mobile phase A and methanol:acetonitrile (1:1 v/v) binary mixture as mobile phase B with the following method: 0 min 0% B; 6.5 min 65% B; 12 min 65% B; 17 min 0% B. UV signal was recorded at 235 nm.

The outlet of the UV detector was directly connected to the ESI source of a TOF MS (Agilent Technologies 6210A Time-of-Flight) equipped with a dual-nebulizer ESI source operated in positive ion mode. Two reference masses (*m/z* 121.050873 and 922.009798) were used. The following operation conditions were used: gas temp: 350 °C; drying gas flow (N_2):

13 L/min; nebulizer (N_2): 60 psig; VCap: 3500 V; acquisition rate: 250 ms/spectrum; fragmentor: 175 V; skimmer: 65 V; OCT 1 RF Vpp: 250 V. Agilent MassHunter Data Acquisition TOF/Q-TOF B.02.00. and Qualitative Analysis B.03.01. softwares were used for data analysis.

3.3 Determination of PTU in tomato samples by LC-MS/MS

3.3.1 Sample preparation protocol and HILIC-ESI-MS/MS conditions

Tomatoes, obtained from a local market, were chopped and homogenized in a commercial blender. Prepared samples were stored at -18 °C, until analysis. Blended tomato samples (10 ± 0.1 g) were weighed into 50 mL centrifuge tubes. The extraction involved the addition of 10 mL acetonitrile followed by 50 µL of the IS solution containing 10 µg/mL 4,5-dimethylimidazolidine-2-thione. The tube was sealed and shaken by hand for 1 min. After shaking a mixture of 4 g magnesium sulfate, 1 g sodium chloride, 1 g trisodium citrate dihydrate and 0.5 g disodium hydrogencitrate sesquihydrate was added to induce phase separation and partitioning. The tube was sealed and immediately shaken vigorously by hand for 1 min and centrifuged for 5 min at 5000 rpm.

For the clean-up of the extracts dispersive SPE was employed. The acetonitrile extract (6 mL) was transferred into a 10 mL polypropylene centrifugation tube containing 150 mg PSA and 900 mg $MgSO_4$. The tube was sealed and immediately shaken vigorously by hand for 1 min and centrifuged for 5 min at 5000 rpm. 1 mL of the supernatant was filtered through a 0.45 µm regenerated cellulose membrane filter and transferred into an autosampler-vial to be used for LC-MS/MS analysis. Agilent MassHunter Data Acquisition QQQ B.02.01., Qualitative Analysis B.03.01. and Quantitative Analysis B.03.02. softwares were used for data acquisition and analysis.

Column:	SeQuant ZIC-pHILIC column (150 mm × 2.1 mm i.d., 5 µm) including a ZIC-pHILIC pre-column (20 mm × 2.1 mm i.d., 5 µm)
Eluent A:	5 mM ammonium acetate
Eluent B:	Acetonitrile
Gradient profile:	0 min 90% B; 1 min 90% B; 11 min 50% B; 16 min 50% B; 21 min 90% B
Flow rate:	0.4 mL/min
Column temperature:	30 °C
Injection volume:	3 µL
Analysis time:	21 min
Drying gas temperature:	325 °C
Drying gas flow (N_2):	5 L/min
Nebulizer (N_2):	40 psig
Sheath gas temperature:	400 °C
Sheath gas flow (N_2):	12 L/min
Nozzle voltage:	300 V
Capillary voltage:	3500 V
ΔEMV:	500 V

Table 1. HILIC-ESI-MS/MS conditions

Compound	Precursor ion [m/z]	Fragment ion [m/z]	Fragmentor voltage [V]	Collision energy [V]	Dwell time[ms]
PTU	117.1	41.1	90	20	60
		58.2	90	11	60
IS	131.1	72.0	100	13	60
		55.1	100	20	60

Table 2. The acquired MRM transitions

3.4 Analysis of PTU in tomato based baby food and tomato puree by LC-MS/MS

3.4.1 Sample preparation protocol and RP-ESI-MS/MS conditions

Representative baby food and tomato puree samples purchased were "ready to eat" and analysed as such. A sub-sample of 10.0 g (tomato puree or baby food) was weighed into a polypropylene centrifuge tube. In the case of tomato puree, as the moisture content was below 80 m/m%, 3 mL water was added, leading to a total water content in the tube of approximately 10 g. The already detailed sample preparation protocol was used (section 3.3.1.) with the following deviations: 80 µL instead of 50 µL of the internal standard solution (10 µg/mL, 4,5-dimethylimidazolidine-2-thione) was added only to the tube containing baby

food and the clean-up procedure was carried out with the optimized amount of 150 mg PSA, 150 mg C_{18} and 900 mg $MgSO_4$. The acquired MS/MS transitions and the collision energy and fragmentor voltage values correspond to those detailed in section 3.3.1.

Column:	Agilent ZORBAX Eclipse Plus Phenyl-Hexyl RRHT column (50 x 4.6 mm i.d., 1.8 µm)
Eluent A:	0.1% acetic acid
Eluent B:	acetonitrile:methanol 1:1 (V/V)
Gradient profile:	0 min 5% B; 3.0 min 20% B; 5.0 min 80% B; 6.0 min 80% B; 5 min post time
Flow rate:	0.5 mL/min
Column temperature:	30 °C
Injection volume:	1 µL
Analysis time:	11 min
Drying gas temperature:	350 °C
Drying gas flow (N_2):	5 L/min
Nebulizer (N_2):	40 psig
Sheath gas temperature:	400 °C
Sheath gas flow (N_2):	12 L/min
Nozzle voltage:	300 V
Capillary voltage:	3500 V
ΔEMV:	500 V

Table 3. RP-ESI-MS/MS conditions

4 Summary of the new scientific results and achievements included in the thesis

1. 28, previously not reported, isomers of fumonisin B_1 mycotoxin in a solid rice culture inoculated with *F. verticillioides* were separated and detected by LC-TOF-MS.

2. The presence of previously reported, but not characterized, higher molecular weight fumonisin B_1 isomers (2-2-2 isomer) with a third acyl group (palmitoyl, linoleoyl and oleoyl) was successfully confirmed using LC-TOF-MS.

3. The QuEChERS methodology was adapted for the determination of propylenethiourea (PTU) in tomato and processed tomato based food commodities (tomato puree and baby food with low fat content). Additionally two new chromatographic methods were developed for the determination of PTU using triple quadruple detection: HILIC-ESI-MS/MS and RP-ESI-MS/MS.

4. The impurity profiling (using LC-UV-TOF-MS technique) of a new internal standard was reported, which is structurally similar to PTU. Furthermore a widely used approach in bioanalytical[3] studies was also adapted to assess the matrix effect in more detail and to evaluate the applicability of the internal standard.

[3] Matuszewski, B.K., M.L. Constanzer, és C.M. Chavez-Eng, *Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC-MS/MS*. Analytical Chemistry, 2003. **75**(13): p. 3019-3030.

5 Publications

Papers:

- *Identification of the first fumonisin mycotoxins with three acyl groups by ESI-ITMS and ESI-TOFMS following RP-HPLC separation: palmitoyl, linoleoyl and oleoyl EFB1 fumonisin isomers from a solid culture of Fusarium verticillioides* – Bartók, T., L. Tölgyesi, Á. Mesterházy, M. Bartók, és Á. Szécsi, – Food Additives & Contaminants: Part A, **27**(12), 1714-1723. *doi:10.1080/19440049.2010.521958*

- *Detection and characterization of twenty eight isomers of fumonisin B1 (FB1) mycotoxin in solid rice culture infected with Fusarium verticillioides by reversed-phase high-performance liquid chromatography/electrospray ionization time-of-flight and ion trap mass spectrometry* – Bartók, T., L. Tölgyesi, A. Szekeres, M. Varga, R. Barta, Á. Szécsi, M. Bartók és Á. Mesterházy – Rapid Communication in Mass Spectrometry, **24**(1), 35-41. *doi:10.1002/rcm.4353*

- *Determination of Propylenethiourea, the Main Metabolite of Propineb, in Tomato by HILIC-MS* – Tölgyesi, L,. P. Kele, K. Torkos – Chromatographia, 2010. **71**(S1): 75-80. *doi:10.1365/s10337-010-1617-7*

- *High-Performance Liquid Chromatography - Tandem Mass Spectrometry Analysis of Propylenethiourea in Tomato Based Baby Food and Tomato Puree* – Tölgyesi, L. és K. Torkos. – *Submitted*

Conference posters:

- *Determination of the Main Metabolite of Propineb, Propylenethiourea, in Foods of Plant Origin by High Performance Hydrophilic Interaction Liquid Chromatography Tandem Mass Spectrometry* (L. Tölgyesi; 8*th* Balaton Symposium, 2009, Siófok)

- *High Performance Liquid Chromatography Tandem Mass Spectrometry analysis of Propylenethiourea in Tomato Based Baby Foods and Tomato Puree* (Elválasztástudományi Vándorgyűlés, 2010, Tapolca – Tölgyesi, L. és M. Ripszám)