

PhD Dissertation



Élelmiszertudományi Kar

ANALYSIS OF PESTICIDES AND THEIR TRANSFORMATION
PRODUCTS IN FOOD WITH LC-(ESI+)MS TECHNIQUES

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PhD Dissertation

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The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.

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INTRODUCTION

Defending the plants and crops against pests is inevitable in recent agriculture production. An important part of this defense is the chemical plant protection, born around the 2nd world war. Paul Hermann Müller discovered the insecticidal effect of DDT that time (1939), followed by the extremely extensive use of this chemical. Owing to its effectiveness – that was used against malaria with success – DDT was very popular in that time, Paul Hermann Müller even received Nobel prize for his discovery in 1948.

In that time the only purpose of these chemicals was to eliminate the pests, regardless of their persistency, degradation time, capability of bioaccumulation *etc.* Writer Rachel Carson was one of the first oppositioners, who drew the attention to the enormous dangers meant by these chemicals. Among others, her bestseller book, *Silent Spring* led to the foundation of numerous environmental organizations, and to the regulation of pesticides that led to the ban of DDT.

DDT was followed by other chemicals, nowadays there are more than 1000 authorized pesticides on the market, the distribution and application of which fall under strict regulation. An important part of this regulation is the analysis of pesticide residues and their metabolites.

In order to determine the concentration of these residues analysts have to develop selective and multi-component methods. For such purposes gas or liquid chromatography (GC, LC) coupled to mass spectrometric (MS) detection are far by the most used instruments.

During my Phd work I tried to develop and validate LC-MS methods that can be used in ‘real-life’ practice, and to prove their applicability, I used them on real, commercial samples.

AIMS

The experiments presented in the dissertation can be divided into three main topics. My aims in these topics were as follows:

1. Development of a multi-component method for the determination pesticides residues in vegetable oils by LC-MS/MS technique. In this topic my aims were:
 - selection of components to analyze and optimizing their MS/MS detection
 - optimizing the chromatographic separation
 - testing the applicability of the 'modified-QuEChERS' sample preparation on vegetable oils
 - evaluating the effect of different d-SPE sorbent combinations on the method performance parameters
 - validation of the developed method
2. Development of an automatic, database-based screening method for the non-target analyzes of pesticide residues in food samples by LC-TOFMS technique. My aims in this topic were:
 - building an accurate-mass database containing the theoretical mass of numerous components and (if possible) their fragments'
 - setting the search parameters based on literature search
 - applying the screening method on real food samples
 - evaluation of the efficacy of the automated screening process by comparing the results from analyzes of the same samples with a dedicated LC-MS/MS and with an LC-TOFMS
3. Development of a systematic, database-based method for the screening of pesticides metabolites with LC-TOFMS technique. My aims in this topic were:
 - development of a quick, systematic metabolite-searching protocol by monitoring fragment ions using the database created in the prior topic
 - applying the developed method on real food samples
 - searching for pesticide metabolites in a non-targeted way
 - identification of the tentative pesticide metabolites found with the method

MATERIALS AND METHODS

During the tandem-MS measurement a hybrid linear ion trap triple quadrupole MS/MS instrument was used (Applied Biosystems 3200 QTRAP), containing a TurboV IonSpray ESI ion source operating in positive ion mode. The MS instrument was coupled to an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, USA), consisting of a vacuum degasser, a binary pump, an automatic sampler and a column thermostat. Nitrogen was used in the ion source and in the collision cell. Analyst 1.4.2. software was used to record and to process the data.

The LC-TOFMS measurements were carried out in the University of Jaén, (Jaén, Spain). For chromatographic separation an Agilent 1200 HLC system was used consisting of a vacuum degasser, an automatic sampler, a binary pump and a column thermostat. Mass spectrometric detection was carried out on an Agilent 6220 TOFMS instrument, equipped with an ESI ion source operated in positive ion mode. Mass correction was performed with the continuously introduced calibration solution, introduced with the dual-spray ESI source on low flow ($\sim 10 \mu\text{l min}^{-1}$). Agilent MassHunter B.04.00 Build 4.0.497.0 software was used to record and to process the mass spectrometric data.

For the preparation of the vegetable oil samples three modified versions of the QuEChERS method were used. The three methods differed in the d-SPE sorbents used in the clean-up step. Three different d-SPE sorbents were used: PSA, GCB and C_{18} sorbents in different combinations (PSA+ C_{18} +GCB, PSA+ C_{18} , and PSA). The LC-MS/MS measurement were carried out in MRM mode, recording two transitions for each component. For the optimization of MS/MS parameters individual standard stock solutions were used.

Fruit and vegetable samples analyzed during the LC-TOFMS measurements were prepared with the acetate-buffered QuEChERS sample preparation. In order to compare the results samples were analyzed both on the tandem-MS and on the LC-TOFMS instrument as well. During the MS/MS measurements a two-step analyzes was carried out. The first was the qualitative analyzes of the components. For this purpose a method combining the EPI and MRM mode of the instrument was used. The second step was the quantitative analyzes of the components in MRM mode. During the LC-TOFMS measurements mass spectra was recorded in full-scan mode using a 190V fragmentor voltage. Searching and identifying compound in the mass spectra was carried out in an automated, database-based fashion.

During the re-analyzes of positive samples the so-called 'fragmentation-degradation' technique was used in order to find tentative pesticide metabolites. The method is based on the monitoring of fragment ions: if on the chromatogram of a fragment ion (or a base molecule) more than one peak

can be observed that suggests the presence of a molecule with a similar structure (a possible metabolite). Searching of metabolites is carried out by checking the mass spectra in these retention times. This search is fostered by characteristic isotopic pattern (e.g. chlorine or sulphur atoms in the molecule).

RESULTS

During the analyzes of vegetable oils first choosing of components to analyze were chosen. When the list was complete (consisting of 50 compounds) optimization of their MS/MS detection was performed. Optimal parameters were successfully chosen for 44 out of the 50 selected compounds, six compounds were deleted from the method because of sensitivity issues.

During the optimization of the chromatographic separation the initial, linear gradient program with a run time of 45 minutes was successfully reduced to 35 minutes by inserting steps in the gradient program. With use of the optimized method selectivity increased, compound were better resolved from each other.

In the matrix effect study – as expected – the most moderate effects were observed when the most sophisticated clean-up procedure (PSA+C₁₈+GCB) was used. With this method moderate matrix effects were observed regardless of the oil sample. By reducing the efficiency of the clean-up more remarkable matrix effects were observed, especially in the case of sunflower and olive oils, while in rapeseed and palm oil no such difference could be observed between the methods.

Regarding the limit of detections (LODs) also the PSA+C₁₈+GCB sorbent combination proved to be the most suitable method. With this method in the 95% of the cases LOD values were less than 5 ppb. By omitting GCB and C₁₈ sorbents LODs worsened somewhat, though in the 77% of the cases they were still below 10 ppb. The observed LOD values were compared to the MRL values set by the EU in oil plants. In the case of six compound it was found that LOD values were greater than the MRL values showing that the methods were not sensitive enough for these compounds. It worth mentioning though that LOD values were compared to MRL values set in oil plant, not in vegetable oils (as no MRL values are set for oil commodities).

Results of the recovery study were in contradiction with that of former studies published earlier, as when the PSA+GCB+C₁₈ sorbent combination was used no acceptable recoveries could be obtained. The reason behind was the GCB sorbent as it adsorbed not only the matrix components but the analytes of interest too, resulting in very poor recovery values. By omitting this sorbent significantly higher recoveries could be achieved, while when C₁₈ was also omitted somewhat worse results were observed, especially in the case of palm oil. Based on these results it can be

concluded that in some cases sacrificing the efficiency of the clean-up step might be necessary in order to avoid the loss of the analytes of interest during sample preparation.

As a conclusion, with the use of PSA+C₁₈ sorbent combination I managed to develop a multi-component method that is suitable for the analyzes of 44 pesticides in vegetable oil matrices. The excessive adsorption ability of GCB sorbent can influence adversely the method performance, hence its use and the used quantity should be justified and to be optimized carefully.

The first step in the development of the database-based screening method was to create the list of analytes. To exploit the advantages of the TOFMS instrument (*i.e.* the theoretically unlimited number of components that can be analyzed simultaneously) wide scale of pesticides were covered by the method. For this purpose databases already published were used, supplemented with the results of an internet survey of pesticides. The database consists of approx. 1400 compound in its present state. The list then was filled up with chemical structure and theoretical mass-to-charge ratio data, and the database was created in .csv format. 850 out of the 1400 compounds are base pesticide molecules, 447 are fragment molecules and 99 are pesticide transformation products.

Parameters used in the automated search process were set up based on literature data: for minimal peak area 300 cps was set and for relative mass error ± 5 ppm was chosen.

The applicability of the screening method was tested by analyzing 29 fruit and vegetable samples. The results were compared to those obtained with an MS/MS instrument. Using the tandem-MS instrument pesticide residues were found in 130 cases in the 29 samples. 81% of these compounds (105 of 130) were detected with the TOF instrument as well. In 25 cases compound were detected only with the tandem-MS instrument. The reason behind could be (i) the somewhat higher sensitivity of the MS/MS instrument; (ii) the time elapsed between the two measurements: first the tandem-MS, then few month later the TOFMS measurements were performed. During that time some analytes could degrade despite the low temperature conditions. (iii) The use of different membrane filters: for the tandem-MS measurements PTFE filters were used, while during the TOFMS measurements nylon filter was used. In lower concentration levels such difference might influence a binary yes/no decision. It worth mentioning though that 20 out of this 25 cases the concentration of analytes were lower than 0.02 mg kg⁻¹. It occurred only in five cases that compound presenting in higher concentration escaped the detection by the TOFMS instrument. Two of them was chlormequat chloride pesticide, that can be explained with the above mentioned reasons. In three cases compounds in high concentration (0.65, 1.18 and 3.52 ppm; cyprodinil, propamocarb and azoxystrobin, respectively) escaped the automatic detection. The reason behind is the limited dynamic range of the TOFMS instrument. Compounds in high concentration saturate the detector worsening the mass accuracy so compounds fall out of the ± 5 ppm tolerance level.

During the comparison study in 24 cases occurred that compounds were detected only with the automated screening method. Three of them were base pesticide molecules (cyproconazole, chlorantraniliprole and fluopicolide) not analyzed (implicitly not detected) in the MS/MS method. It worth mentioning that cyproconazole was found in a head lettuce sample, a commodity that should not contain any detectable residue of this compound. Four compounds were metabolites identified earlier, that were also omitted by the MRM method. Interestingly, eight compounds were detected only with TOFMS despite that they were included in the dedicated MRM method. The reason behind can be the high sensitivity of TOFMS instrument in full-scan mode, facing a somewhat decreased sensitivity on the other side in the MRM-EPI method (more than 300 compounds monitored). Furthermore, checking the MRM transitions in the MRM method revealed that in the case of two compounds (iprodione and penconazole; both containing two chlorine atoms) MS/MS optimization was carried out to the less intensive M+2 ion.

Based on the results of the comparison study it can be concluded that the automated, database-based screening process can be used successfully in wide-scale screening methods, showing good agreement with the results of the dedicated tandem-MS method. It can be seen also that the somewhat lower sensitivity of TOFMS is compensated by its capability for non-target analyses, successfully detecting not expected residues (*e.g.* the case of cyproconazole).

Search for metabolites was carried out by analyzing the previously mentioned 29 'positive' fruit and vegetable samples, seeking the metabolites of the pesticides found earlier. Understandably, because of its 'target analysis' nature, no metabolites were found with the MS/MS instrument. Reanalyzing them with TOFMS revealed the presence of such residues in 29 cases. This high number shows the applicability of the searching developed method; on the other hand it draws the attention to the frequency of metabolite residues found in food samples, justifying the necessity of the development of metabolite searching methods.

Most of the metabolites found by the developed method were compounds reported earlier, it worth mentioning though that, according to the searching protocol reported, in all cases relying on literature data was a later step after detection in order to identify the metabolite detected (not the other, also applicable though reverse way, *i.e.* searching metabolites found in literature in a targeted way).

A specific type of degradation products (glycoside derivatives) were found in a relatively high amount (in some cases in higher concentration than that of the parent compound). Generally glycoside transformation products are considered to be less toxic owing to their higher polarity. However, because of this feature their recovery from samples might be lower using conventional sample preparations, leading to the underestimation of their concentration and the threat meant by

them to consumers. This possibility along with the lack of toxicological surveys of glycoside metabolites draws the attention to the monitoring of these kinds of compounds.

One of the advantage of the searching method is that the presence of metabolites can be revealed quickly and easily only by monitoring fragment ions. However, this is also a drawback, since (apart from some special cases) it strongly relies on the fragment ions. Some of the drawbacks can be eliminated by including the most fragment ions in the accurate-mass database.

Some of my results to highlight: 2nd phase degradation product of iprodione was found, a metabolite not reported earlier; an in-house made metabolite standard (fenhexamid-glycoside) was used for qualitative and quantitative analyses.

The large number of metabolites found during the study showed that the limits of pesticide analyses should be expanded. Wide-scale screening methods are needed to complement the results of conventional 'target' methods in order to provide more realistic results. I think during my work I managed to develop a method that meets the above mentioned requirements, and stands it ground among the method reported earlier.

NOVEL SCIENTIFIC RESULTS

1. A multi-component LC-MS/MS method was developed for the analyses of pesticides in vegetable oil samples. During method validation the following conclusions were drawn:

- using more clean-up sorbent decreased the effects caused by matrix components, and the cleaning capability of sorbents is commodity-dependent
- analysis of the 44 pesticides can only be achieved with the use of matrix-matched calibration
- LODs of the method depend on the clean-up procedure and in most cases meet the requirements set by the EU in oil plant commodities
- in contrary to the results found in studies reported earlier the PSA+C₁₈+GCB sorbent combination caused very low recoveries, caused by the excessive adsorption capability of GCB sorbent (especially in the case of pesticides with planar structure)
- the simplified sample preparation resulted in less clean extracts, on the other significantly better recovery values were obtained

2. A wide-scale, database-based LC-TOFMS screening method was developed.

- an accurate-mass database was built consisting of approx.. 1400 compound including the fragment ions of base pesticide molecules as well
- the applicability of the method was tested on 29 fruit and vegetable samples. Pesticide residues were found in 105 cases
- results of the automated search were compared to those obtained with an LC-MS/MS instrument. Result were in good agreement, furthermore as the benefit of the non-target analysis residues not expected or unknowns can be detected as well, justifying the applicability of LC-TOFMS techniques in wide-scale screening methods

3. A novel LC-TOFMS method was developed for the detection of pesticide metabolite residues in food samples by the automated monitoring of fragment ions.

- the applicability of the method was tested on 29 fruit and vegetable samples
- the method can be applied successfully in the analysis of unknown metabolites in non-targeted measurements
- 2nd phase degradation product of iprodione was detected, identified and reported for the first time
- an in-house made metabolite standard (fenhexamid-O-glycoside) was used for the first time in qualitative and quantitative analyses

LIST OF PUBLICATIONS

National and international conference abstracts:

Polgár László: Módszerfejlesztés növényvédő szerek meghatározására növényi olajokból HPLC-(ESI+)MS/MS technikával

(XXIX. OTDK Konferencia, Élelmiszertudomány szekció, 2009. április 6-8., Gödöllő)

Polgár László, Fodor Péter, Juan F. García-Reyes: Tömeg-spektrometriás keresőmódszer fejlesztése növényvédő szerek és bomlástermékeik kimutatására zöldség- és gyümölcsmintákból pontos-tömeg adatbázis felhasználásával

(TÁMOP-4.2.1/B-09/1/KMR-2010-0005 "Kihívások és megoldások a XXI. század élelmiszertudományában" alprojekt záró konferencia, 2012. január 18-19., Budapest)

Kmellár Béla, **Polgár László,** Abrankó László, Fodor Péter: Mátrixhatások zöldség-gyümölcs termékek növényvédőszer maradékainak HPLC-ESI-(QQQ)-MS/MS technikával történő meghatározása során

(Hungalimentaria konferencia, 2011. április 19-20., Budapest)

Kmellár Béla, **Polgár László,** Abrankó László, Fodor Péter: Combination of screening and quantitation method for the determination of 300 target pesticides by HPLC-MS/MS: achievements and pitfalls

(2nd Latin American Pesticide Residue Workshop konferencia, 2009. június 8-11., Santa Fé, Argentína)

Polgár László, Kmellár Béla, Fodor Péter: Comparison of three QuChERS-based methods for the determination of multiclass pesticides from vegetable oils by HPLC-(ESI+)MS/MS

(8th European Pesticide Residue Workshop konferencia, 2010. június 20-24., Strasbourg, Franciaország)

Polgár László, Kmellár Béla, Fodor Péter: Evaluation of the effect of pear stone cells on the recovery of multi-class pesticides

(7th Aegean Analytical Chemistry Days konferencia, 2010. szeptember 29. – október 3., Lesvos, Görögország)

Polgár László, Juan F. García-Reyes, Juan C. Dominguez-Romero, Abrankó László, Dernovics Mihály, Fodor Péter: Systematic screening of multiclass pesticides and their metabolites in food samples using accurate-mass database combined search of parent molecules and diagnostic fragment ions

(13^{as} Jornadas de Análisis Instrumental konferencia, 2011. november 14-16., Barcelona, Spanyolország)

Peer reviewed journal articles:

Polgár László, Kmellár Béla, Fodor Péter (2012): *Evaluation of the effect of pear stone cells on the recovery of multi-class pesticides.*
Analytical Letters, 45 (5-6) 563-569
(IF₂₀₁₂: 0,965)

Polgár László, Kmellár Béla, Juan F. García-Reyes, Fodor Péter (2012): *Comprehensive evaluation of the clean-up step in QuEChERS procedure for the multi-residue determination of pesticides in different vegetable oils using LC-MS/MS.*
Analytical Methods, 4 (4) 1142-1148
(IF₂₀₁₂: 1,547)

Bienvenida Gilbert-López, Lucía Jaén-Martos, Juan F. García-Reyes, Marina Villar-Pulido, **Polgár László**, Natividad Ramos-Martos, Antonio Molina-Díaz (2012): *Monitoring of pesticide residues in fruit-based soft drinks from the EU market using liquid chromatography-mass spectrometry*
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(IF₂₀₁₂: 4,531)