**SSPT 39 - Otte - Analysis of pesticides in tobacco by LC-MS/MS: specific matrix effects on quantification for different tobacco grades**

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## Summary

Agrochemicals are being used worldwide to protect agriculture products including tobacco against a wide range of pest and plant diseases. During the last years methodologies for the analysis of agrochemical residues have been established at different industry and contract laboratories and a large variety of procedures for extraction, clean-up, separation and detection were set up.

The common analytical method using GC and different detectors, e.g. NPD, ECD and MSD allows the determination of agrochemical residues in tobacco at very low concentration levels.

However, in the last years a tendency to use more polar agrochemicals can be observed. The well-established analysis by GC is less suitable for polar compounds; thus there is a demand for an alternative technique. As a consequence, the LC-MS/MS technique has been implemented by more and more laboratories. The extracts for LC-MS/MS analysis are obtained by extraction, gel permeation chromatography (GPC) and dissolving in LC eluent. In this work the determination of selected agrochemical classes in tobacco will be demonstrated.

Different tobacco grades (air cured, flue cured and oriental) were analysed and it was demonstrated that individual tobacco grades differ significantly in their matrix effects. The sample preparation including a dilution step (1:50) reduces matrix effects significantly with minor effects on sensitivity. The LOQs determined for the different sample preparations are comparable.

## 1. Introduction

Due to the complexity of the tobacco matrix and the different physicochemical properties of agrochemicals, the analysis of residues in tobacco is a challenging task. A combination of multi-residue methodologies (GC and LC) must be applied to analyse more than 800 agrochemical residues.

Commonly, gas chromatography (GC) has been used as the main analytical technique. The coupling of the GC with a mass spectrometer (ion trap) provided the opportunity to screen up to 200 agrochemicals and their metabolites in one analytical run. For those agrochemicals

that are not amenable to the GC due to their thermal instability or insufficient volatility, LC-MS/MS is the analytical method of choice. In general, liquid chromatography (LC) is an effective technique for separating thermally labile polar agrochemicals while MS/MS allows their identification and quantification. This technique enables analysis of agrochemicals at very low concentration levels in the presence of interfering compounds.

However, this highly sensitive and selective technique is not applicable for the direct determination of agrochemicals in complex matrices without sample preparation. Therefore, clean-up steps are essential for tobacco samples. Matrix interferences in the sample extracts may result in the occurrence of false positive results and incorrect quantitation.

The sample preparation applied in this study is based on the DFG S 19 method which includes a very effective extraction and clean up step. As described in the S 19 method, the analytes are obtained in an organic solution after clean up by gel permeation chromatography (GPC). Since this solvent is not applicable to LC, a solvent exchange is necessary for LC-MS/MS detection. This last step of sample preparation might be useful to reduce interfering matrix effects in the sample extract.

In this study, the effect of different tobacco grades on matrix interferences was examined. The analytical method was optimised in order to reduce the matrix effects observed.

## 2. Experimental

### Studied material

Three different tobacco grades (air cured, flue cured, oriental) and a tobacco blend low in agrochemical residues have been selected for this study.

### Sample preparation (extraction and clean up based on DFG S19 methodology)

The tobacco samples were ground and homogenised. A representative portion of 15 g was weighed and mixed with 100 mL water. To this solution, 200 mL of a *cyclo*-hexane / ethyl acetate (1:1) mixture and 20 g of sodium chloride were added. After filtration, an aliquot of 200 mL was extracted with 100 mL *cyclo*-hexane / ethyl acetate (1:1) mixture and 20 g sodium chloride. The aqueous phase was removed and discarded. Sodium sulphate (25 g) was added to the organic layer and mixed well. Subsequently, the organic phase was filtrated over sodium sulphate and the eluate was evaporated to 2 mL. This residue was solved in 15 mL of a *cyclo*-hexane / ethyl acetate (1:1) mixture and 2 g of sodium sulphate were added. An aliquot of 10 mL was subject to the clean up step by GPC.

### Sample preparation for the Matrix-matched calibration

For this study the GPC eluate of each tobacco grade sample was separated into three portions. The first portion (1 mL) was evaporated to dryness and re-solved in 3 mL of ammonium formiate solution 10 mM in methanol/water (20:80). The extract was filtrated and transferred into a LC vial. The second portion (300 µL) was transferred into a tube and 2700 µL ammonium formiate solution 10 mM in methanol/water (20:80) was added. After filtration the sample was transferred into a LC vial. The third portion (60 µL) was transferred into another tube and 2940 µL ammonium formiate solution 10 mM in methanol/water (20:80)

was added, filtrated and the transferred into a LC vial. The experimental set-up appears from **Fig. 1.**

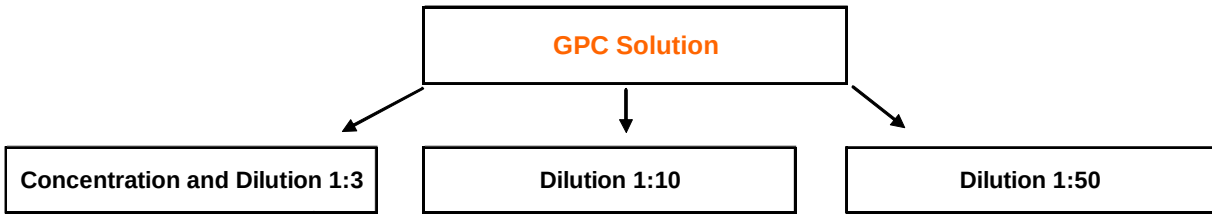


**Fig. 1:** Experimental set-up: Dilution steps following sample preparation according to S19.

Each of the solutions prepared this way were spiked with standard solution at four concentration levels (15, 30, 150 and 600 µg/L).

**Instrumentation (Liquid chromatography-mass spectrometry)**

The LC system consisted of an Agilent 1100 liquid chromatograph equipped with a vaccum degasser, a solvent delivery compartment with high pressure mixing camber, an autosampler and a column oven. The injection volume was 20 µL. The separation of the agrochemicals was performed using a C 18 Synergi Fusion-RP analytical column, 50 × 2 mm (i.d.), 4 µm (phenomenex). The separation column was protected by a guard column, C 18 Synergi Fusion-RP, 4 × 2 mm (i.d.) (phenomenex). The total flow rate adjusted to 0.2 mL/min. The mobile phase consisted of a 10 mM ammonium formiate solution in methanol/water (20:80; pH: 4.1) (Eluent A) and methanol (Eluent B). Initial gradient was 100 % Eluent A, decreasing to 10% A over 11 min. This proportion was held until 23 min and return to the initial condition in 2 min. The column was then re-equilibrated for 10 min. The total run time was 35 min. The retention times appear from **Table 1**. An integral switching valve on the mass spectrometer was used to divert the LC- Flow to waste for the first 2.0 min.

**Table 1:** LC-MS/MS conditions: molar weight, precursor ion, primary and secondary traces, DP, CE for each agrochemical and retention time.

| Agrochemical | Molar weight | Precursor ion | Primary trace | | | Secondary trace | | |
|---|---|---|---|---|---|---|---|---|
| | | | Mass (m/z) | DP (V) | CE (V) | Mass (m/z) | DP (V) | |
| Azoxystrobin | 403 | [M+H]+ | 404 372 | 64 | 18 | 404 344 | 60 | |
| Oxamyl | 219 | $[M+NH_4]+$ | 237 72 | 38 | 20 | 237 90 | 41 | |
| Methomyl | 162 | [M+H]+ | 163 88 | 63 | 13 | 163 106 | 59 | |
| Carbaryl | 201 | [M+H]+ | 202 145 | 34 | 14 | 202 127 | 37 | |
| Oxadixyl | 278 | [M+H]+ | 279 219 | 44 | 17 | 279 132 | 44 | |
| Metalaxyl | 279 | [M+H]+ | 280 192 | 41 | 25 | 280 220 | 43 | |
| Linuron | 249 | [M+H]+ | 249 160 | 46 | 23 | 249 133 | 45 | |
| Propoxur | 209 | [M+H]+ | 210 111 | 36 | 19 | 210 168 | 49 | |
| Carbendazim | 191 | [M+H]+ | 192 160 | 50 | 24 | 192 132 | 46 | |
| Thiophanate-methyl | 342 | [M+H]+ | 343 151 | 64 | 28 | 343 160 | 65 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Thiodicarb | 355 | [M+H]+ | 355 88 | 42 | 24 | 355 108 | 45 | |
| Imidacloprid | 256 | [M+H]+ | 256 175 | 58 | 25 | 256 209 | 51 | |
| Aldicarb | 190 | $[M+NH_4]+$ | 208 116 | 28 | 9 | 208 89 | 28 | |
| Aldicarb-sulfoxid | 206 | [M+H]+ | 207 89 | 40 | 16 | 207 132 | 40 | |
| Aldicarb-sulfon | 222 | $[M+NH_4]+$ | 240 76 | 38 | 18 | 240 86 | 30 | |
| Ethiofencarb | 225 | [M+H]+ | 226 107 | 40 | 24 | 226 164 | 40 | |
| Ethiofencarb-sulfoxid | 241 | [M+H]+ | 242 107 | 57 | 26 | 242 185 | 48 | |
| Ethiofencarb-sulfon | 257 | $[M+NH_4]+$ | 275 107 | 33 | 27 | 275 201 | 31 | |

The MS/MS detection was performed on a Q Trap 2000 instrument (Applied Biosystems). The mass spectrometer was operated with TurboIonspray® source in the positive mode (ESI +). The specific parameters for ionisation were as follows: curtain gas (CUR) 30 a.u., ionspray voltage, 4500 V; temperature of the turbo heater gas, 400 °C; nebuliser gas (GS1) 60 a.u., turbo gas (GS2) 60 a.u. Nitrogen was used as the curtain gas, nebuliser gas and turbo gas. The exhaust gas and curtain gas regulators were set at 3.5 bar each. The GS1/GS2 regulator was set at 6.5 bar. Unit mass resolution settings were used for Q1 and Q3. The analytical dependent parameters, declustering potential (DP) and collision energy (CE) were optimised for each compound. The data were acquired using Analyst software, version 1.4 and appear from **Table 1**.

## 3. Results and Discussion

### LC-MS/MS analysis

The LC-MS/MS performance gave sharp peaks for all analytes **(Fig. 2).** A fronting was observed by early eluted compounds, when organic solutions were used as a solvent. The retention time ranged from 2.2 to 14.1 min. Some compounds were detected in the protonated form of the molecule and other as adduct ions. For each compound, two transitions were detected. The transition with the highest MRM response was detected as a quantifier and the second transition was used as a qualifier to confirm the identity of the compound.

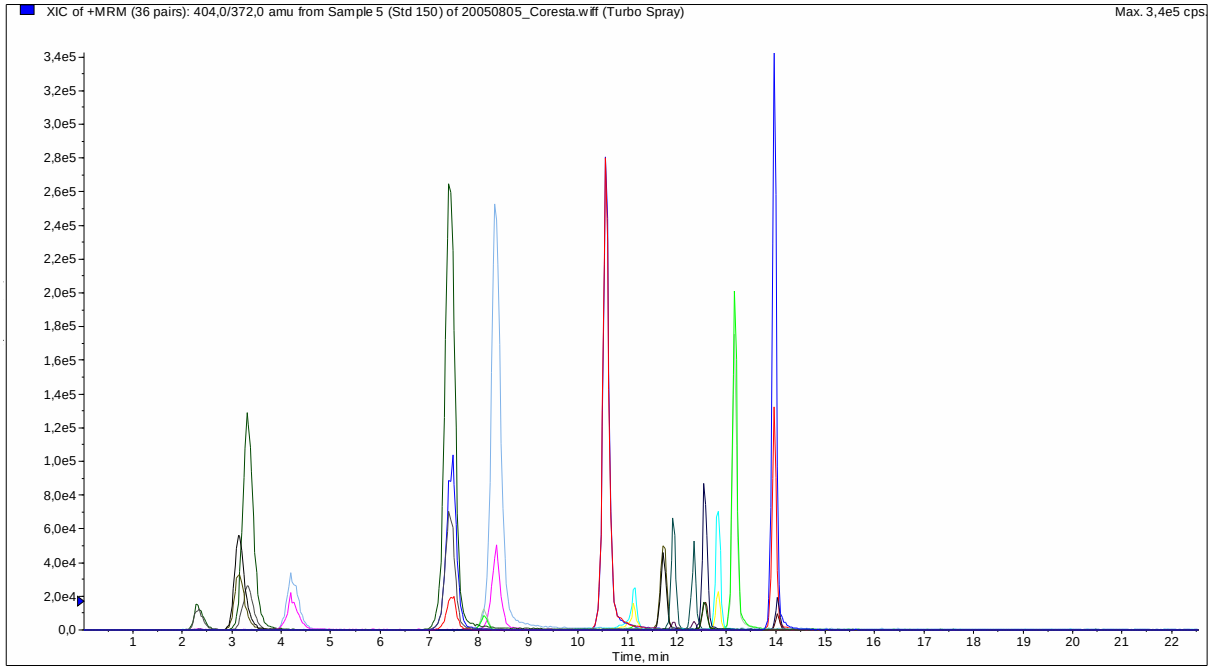


**Fig. 2:** Chromatogram of the standard solution (150 µg/L) includes 18 agrochemicals and their metabolites

**Matrix-matched calibration and matrix effect**

It is well known that the MS-MS response is dependent on the eluent and matrix interferences entering the interface and the detector. Co-eluting substances could be the reason for quantification problems caused by compound specific suppression or enhancement. The signal suppression is observed for most of the early eluted compounds. Matrix interferences could be eliminated by optimisation of sample preparation, dilution of sample extracts or variation of the chromatographic conditions. A relatively high buffer concentration in the LC-eluent could prevent signal suppression. Other methods can also be applied to compensate the signal suppression, e.g. standard addition, matrix-matched standards or internal standards.

Usually, a matrix-matched calibration is the method of choice for the quantification of agrochemicals. The disadvantage of the matrix-matched calibration is the requirement of using a matrix standard with the same matrix compounds as the analysed sample. Due to the complexity of the tobacco matrix, the selection of an appropriate matrix standard is very important since tobacco products consist of different tobacco types (orient, air cured, flue cured). In this study an effective sample preparation and a matrix-matched calibration was performed.

In the present study the matrix effect of different tobacco grades was investigated by comparing pure standard solutions (in Eluent A) with matrix-matched standards. The relative responses of selected agrochemicals are shown in **Table 2**. Significant differences were detected for methomyl, oxamyl and ethiofencarb (s. **Table 2**).

**Table 2:** Selected response ratios (response matrix-matched standard / response solvent standard) for the sample preparation characterised by concentration and 1:3 dilution. Tobacco matrices include oriental, air cured, flue cured and blended tobacco.

| Compound | Ratio (matrix-matched std./solvent std.) | | | |
|---|---|---|---|---|
| | oriental | air-cured | flue-cured | blend |
| Azoxystrobin | 0,2 | 0,3 | 0,2 | |
| Oxamyl | 0,5 | 0,4 | 1,0 | |
| Methomyl | 0,8 | 0,7 | 1,8 | |
| Carbaryl | 0,2 | 0,2 | 0,4 | |
| Oxadixyl | 0,3 | 0,2 | 0,4 | |
| Metalaxyl | 0,2 | 0,5 | 0,6 | |
| Linuron | 0,1 | 0,2 | 0,2 | |
| Propoxur | 0,2 | 0,2 | 0,4 | |
| Thiodicarb | 0,1 | 0,2 | 0,3 | |
| Imidacloprid | 0,7 | 0,6 | 0,9 | |
| Σ Aldicarb | 0,3 | 0,3 | 0,6 | |
| Σ Ethiofencarb | 0,8 | 0,8 | 1,8 | |

In this work it could be demonstrated, that different tobacco grades show different matrix effects. In routine analysis it is not practicable to carry out a separate matrix-matched calibration for each individual tobacco grade. Therefore, matrix effects should be minimised, so that only one matrix-matched calibration is required for all tobacco grades and products.

Hence, the GPC solutions of the different tobacco grades were diluted 1:10 and 1:50. The identification of the matrix effects was also determined in the relative response. The results are shown in **Table 3** and **4**.

**Table 3** and **4:** Selected response ratios between matrix-matched standard and solvent standards. Different dilution steps were applied for tobacco matrices including oriental, air cured, flue cured and blended tobacco.

| Compound | Tobacco grade: oriental | | | Tobacco grade: a | |
|---|---|---|---|---|---|
| | Sample preparation | | | Sample prepa | |
| | concentration | dilution 1:10 | dilution 1:50 | concentration | dilution 1 |
| Azoxystrobin | 0,2 | 0,3 | 0,6 | 0,3 | 0,5 |
| Oxamyl | 0,5 | 0,3 | 1,2 | 0,4 | 0,3 |
| Methomyl | 0,8 | 0,0 | 0,4 | 0,7 | 0,1 |
| Carbaryl | 0,2 | 0,3 | 0,6 | 0,2 | 0,4 |
| Oxadixyl | 0,3 | 0,4 | 0,8 | 0,2 | 0,5 |
| Metalaxyl | 0,2 | 0,5 | 0,8 | 0,5 | 0,8 |
| Linuron | 0,1 | 0,1 | 0,6 | 0,2 | 0,4 |
| Propoxur | 0,2 | 0,4 | 0,7 | 0,2 | 0,5 |
| Thiodicarb | 0,1 | 0,3 | 0,7 | 0,2 | 0,4 |
| Imidacloprid | 0,7 | 0,7 | 1,4 | 0,6 | 1,0 |

| | | | | | |
|---|---|---|---|---|---|
| S Aldicarb | 0,3 | 0,4 | 1,0 | 0,3 | 0,5 |
| S Ethiofencarb | 0,8 | 0,9 | 1,2 | 0,8 | 1,0 |

| Compound | Tobacco grade: oriental | | | Tobacco grade: a | |
|---|---|---|---|---|---|
| | Sample preparation | | | Sample prepa | |
| | concentration | dilution 1:10 | dilution 1:50 | concentration | dilution 1 |
| Azoxystrobin | 0,2 | 0,4 | 0,8 | 0,2 | 0,5 |
| Oxamyl | 1,0 | 0,4 | 1,3 | 0,5 | 0,4 |
| Methomyl | 1,8 | 0,1 | 1,0 | 0,8 | 0,1 |
| Carbaryl | 0,4 | 0,5 | 0,8 | 0,2 | 0,5 |
| Oxadixyl | 0,4 | 0,6 | 0,8 | 0,3 | 0,5 |
| Metalaxyl | 0,6 | 0,8 | 1,0 | 0,3 | 0,7 |
| Linuron | 0,2 | 0,3 | 0,7 | 0,2 | 0,3 |
| Propoxur | 0,4 | 0,5 | 0,8 | 0,2 | 0,5 |
| Thiodicarb | 0,3 | 0,6 | 0,8 | 0,2 | 0,5 |
| Imidacloprid | 0,9 | 1,1 | 1,4 | 0,4 | 0,8 |
| S Aldicarb | 0,6 | 0,7 | 1,1 | 0,4 | 0,6 |
| S Ethiofencarb | 1,8 | 1,2 | 1,3 | 0,8 | 1,0 |

As the result of these investigations, the matrix effects were reduced significantly by performing a (1:50) dilution of the sample extract. Therefore, only one matrix-matched calibration is required.

For the selected agrochemicals mentioned in **Table 3** and **4**, limits of quantification (LOQs) have been determined for two different sample preparations (concentration /dilution 1:3 and dilution 1:50, respectively). For each procedure, an LOQ of 0.1 mg/kg has been calculated.

## 4. Conclusion

In this study, the effect of different tobacco grades on the matrix interference was examined. The analytical methodology was optimised in order to reduce the matrix effects observed.

It was demonstrated that individual tobacco grades differ significantly in their matrix effects. However, sample preparation including a dilution step (1:50) reduces matrix effects significantly with only minor effects on sensitivity. The LOQs for agrochemicals determined with the different method (dilution 1:50 and concentration-dilution 1:3) were comparable.

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