A versatile, selective and “green” LC-DAD multi-active method with an extra: Method development, validation and routine application

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Overview

What to expect

- Traditional analysis of active substances
- Goals in the development of a LC multi-active method
- Choices made during method development
- Easy inclusion of new compounds
- Method validation and proficiency test results
- Application examples
- Use of the same chromatographic setup for the analysis of relevant impurities
- Conclusions and outlook

Traditional analysis of active substances I

Often similar but still individual methods for one compound

- Active substances are generally analysed by either HPLC or GC
- The traditional approach is to have a “dedicated” method for one active substance
- It is mostly unknown whether choices made during method development (selection of column type, mobile phase, etc.) were deliberate or more by chance/coincidence
- This results in (almost) all methods being different with a huge number of different columns, mobile phases, etc.
- Despite variations between methods many of them are quite similar
  (e.g. HPLC methods mainly rely on reversed phase columns, mostly C18)
- Generally no information how these methods perform for formulations with second/ third active substance

Traditional analysis of active substances II

An obstacle to efficient and cost-effective laboratory work

- Many laboratories need to analyse several or even a large number of different active substances in a variety of plant protection products
- (Strictly) following the prescriptions of the dedicated methods from CIPAC or manufacturers results in the need to have a large array of columns and mobile phases at hand in the laboratory
- The need for changing columns / mobile phases prohibits analysing different active substances in one sequence ⇨ highly inefficient
- Most traditional LC methods employ 4.6 mm ID columns with corresponding high flow rates (~ 1 ml/min) ⇨ consumption of large amounts of solvent and generation of corresponding amounts of waste

An alternative approach

Multi-active methods

- In other analytical areas the use of single-analyte or even group methods has long been abandoned, enabled by the use of highly selective detection systems (MS)
- Due to various factors (cost, availability, *precision*) the use of "true" (MS-based) multi-methods for the determination of active substances is not feasible
- Instead "multi-active methods" are an alternative to the traditional compound-dedicated methods
- Parallel establishment and implementation by several laboratories
- ESPAC method with LC and GC parts published as "free method" on CIPAC homepage

Goals in the development of a LC multi-active method

Our Wishlist I

- Focus on LC as no requirement for sufficient volatility and most active substances are (sufficiently) UV-active
- Highly versatile to cover a large number of different active substances from different chemical classes
- Cover a wide concentration range (sub g/kg to high g/kg)
- High selectivity to allow the analysis of plant protection products with more than one active substance and separate target compound from impurities and co-formulants
- Chromatographic setup should allow easy transfer between instruments (and laboratories) and pose no special requirements

Goals in the development of a LC multi-active method

Our Wishlist II

- "Green" method with reduced solvent consumption
- Good performance in terms of linearity, recovery and precision (comply with requirements of SANCO 3030/99 rev. 5)
- Fit for accreditation under ISO 17025

Choices made during method development

Instrumentation

- Standard HPLC with upper pressure limit of 400 bar
- Allows use of column with 3.5 µm particles to gain better efficiency than with 5 µm particles
- Column size of 150 x 2.1 mm selected:
  - no issues with extra-column volumes
  - lower flow-rate saves approx. 75% solvent compared to 4.6 mm ID column
- Single C18-column from big manufacturer to minimise potential supply problems
- Method established on both Agilent 1100 series and Agilent 1260 Infinity II instruments using a Waters XTerra™ column

Choices made during method development

Chromatography

- Isocratic elution for maximum resolution between closely related compounds, no need to worry about gradient delay time when transferring to different instrument
- Ratio of aqueous / organic mobile phase selected so that active substance elutes within 6 minutes
- Wash step to remove late-eluting constituents followed by re-equilibration
- Aqueous mobile phase: Water + 0.1% H_3PO_4 (alternative: water)
- Organic mobile phase: acetonitrile (alternative: methanol)
- Possible on both binary pump with solvent selection valve and quaternary pump

Choices made during method development

Detection

- Optimised wavelength for each active substance:
  - as high as possible for best selectivity
  - (local) maximum in spectrum
  - sufficient but not too high sensitivity
- Use of diode array detector to allow acquisition of spectra for enhanced identification confidence and peak purity assessment against library

Choices made during method development

Sample preparation / concentration ranges

- One-step sample preparation:
  - Weigh sample amount corresponding to 25 mg active substance in 50 ml flask
  - Add extraction solvent (mostly: acetonitrile) and extract in ultrasonic bath for 15 min
  - Fill up and filter turbid solutions through 0.2 µm filter
- This approach covers active substance contents of 20 – 1.000 g/l (g/kg), calibration range 0.375 – 0.625 mg/ml (75-125% of nominal amount); *"standard concentration"*
- For active substance contents of 0.5 – 20 g/l (g/kg) sample amount corresponding to 5 mg active substance, calibration range 0.05 – 0.15 mg/ml; *"low concentration"*
- Active substance contents < 0.5 g/l: analyse directly, adjust calibration range to cover 50-150% of nominal amount; *"very low concentration"*

Easy inclusion of new compounds

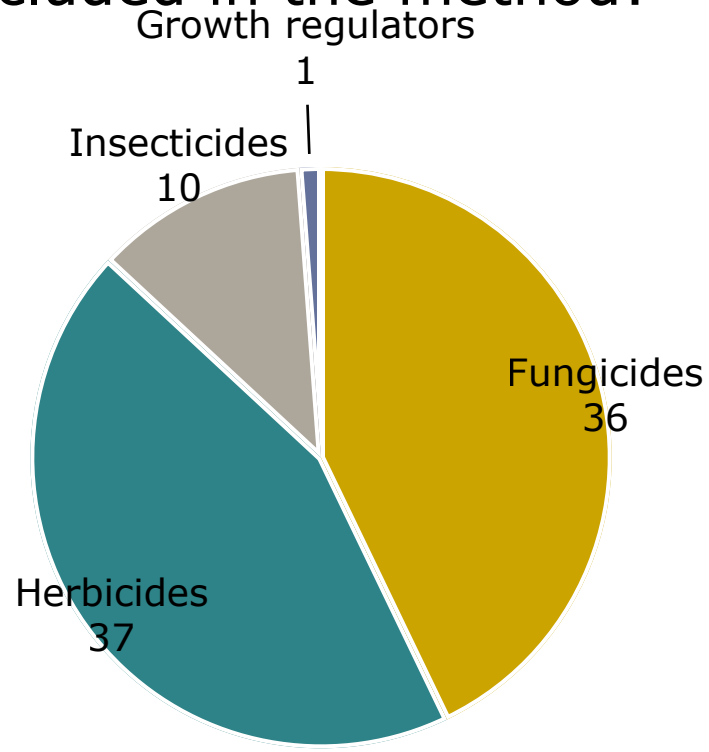
Scouting run and optimisation runs

- For the inclusion of a new active substance into the method the optimum percentage of organic mobile phase and detection wavelength need to be determined
- Scouting run: analyse 0.5 mg/ml standard with 20 min gradient from 5% to 100% organic mobile phase, acquire UV spectra from 210 to 400 nm
- Calculate initial isocratic conditions and determine initial wavelength
- Check initial settings:
  - Sufficient retention (k > 2) and retention time < 6 min
  - Peak height min. 200 mAU, max. 1000 mAU
- Adjust isocratic conditions and/or wavelength if required and re-check

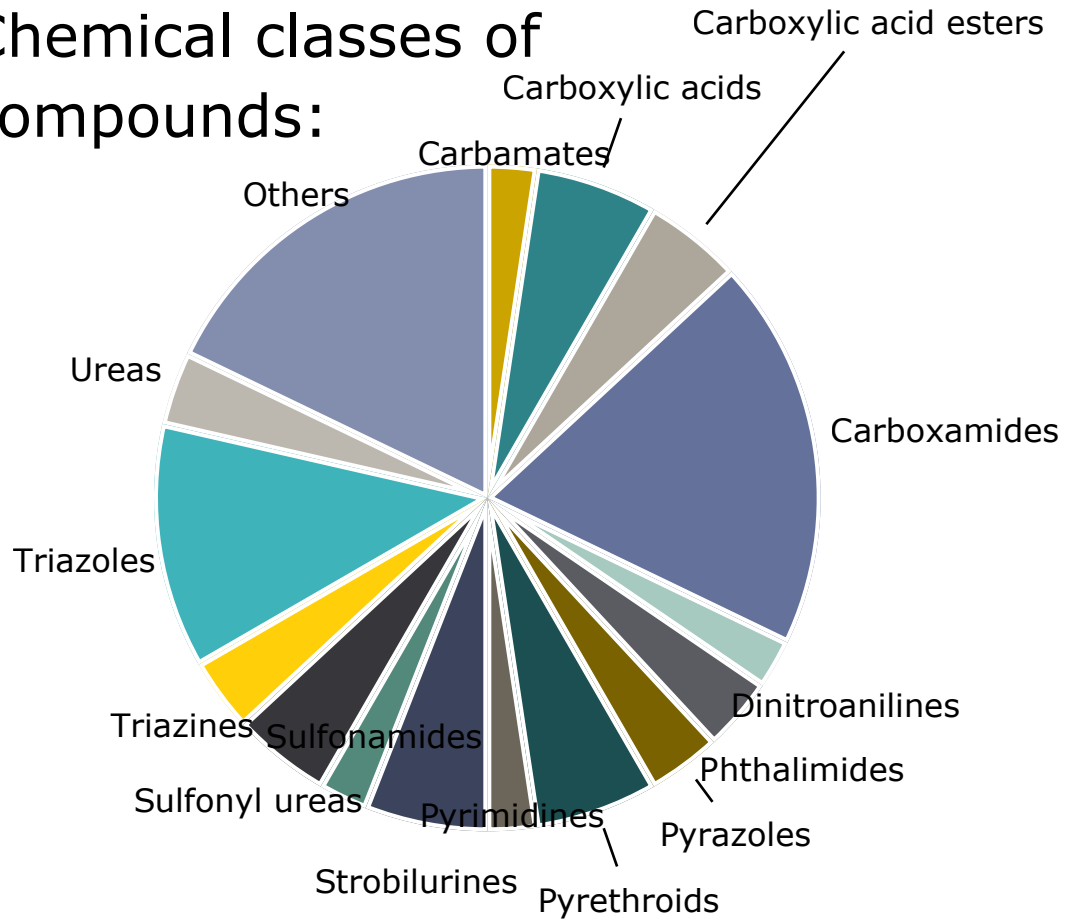
Method scope

Covered active substances

- To date our multi-active method covers more than 80 compounds
- Activities of compounds included in the method:



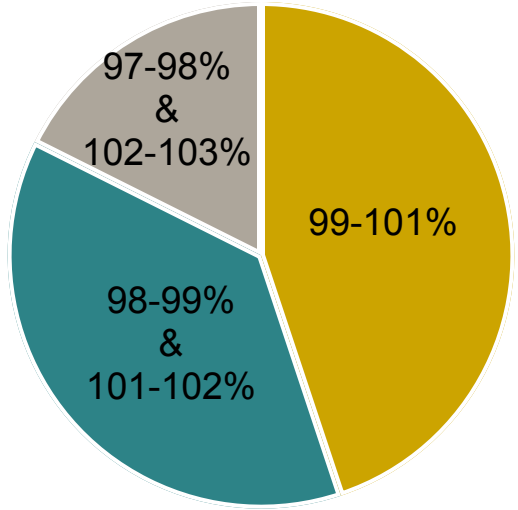
- Chemical classes of compounds:



Method validation

Linearity, recovery

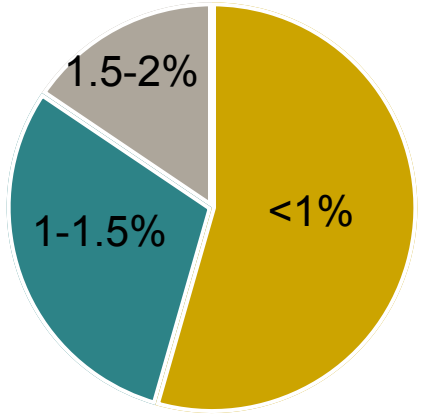
- Fully validated for 81 active substances @ standard concentration, 7 active substances @ low concentration and 2 active substances @ very low concentration
- Linearity: r ≥ 0,999 for all analytes
- Recovery:
  - within 97-103% in all cases for active substance contents @ standard concentration and low concentration
  - Between 98.7 % and 111.4% for two active substances @ 0.05 g/l (70-130%)



Method validation

Precision

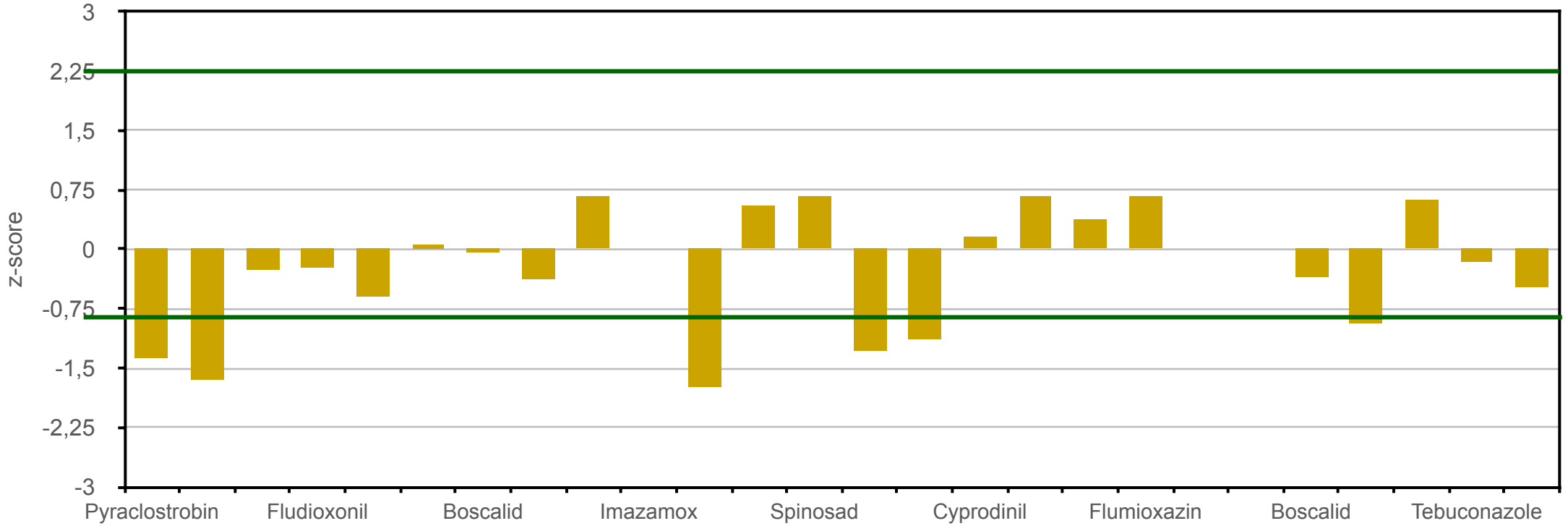
- The intra-laboratory precision was determined as measure for precision
- It was calculated from 2x3 replicates with two analysts performing the analysis three times each on two different days ⇨ unmodified Horwitz equation applies
- For a TC the Horwitz RSD = 2%, for a 0.5 g/kg active substance it is 6.3%
- In all cases, including very low concentrations, the precision was ≤2% !



Proficiency test results

Successful participations over many years

- In the last 5 years (2020-2024) we participated in 12 proficiency tests with 26 active substances in total (23 different ones)
- The obtained z-scores were all within ±2



Accreditation & routine application

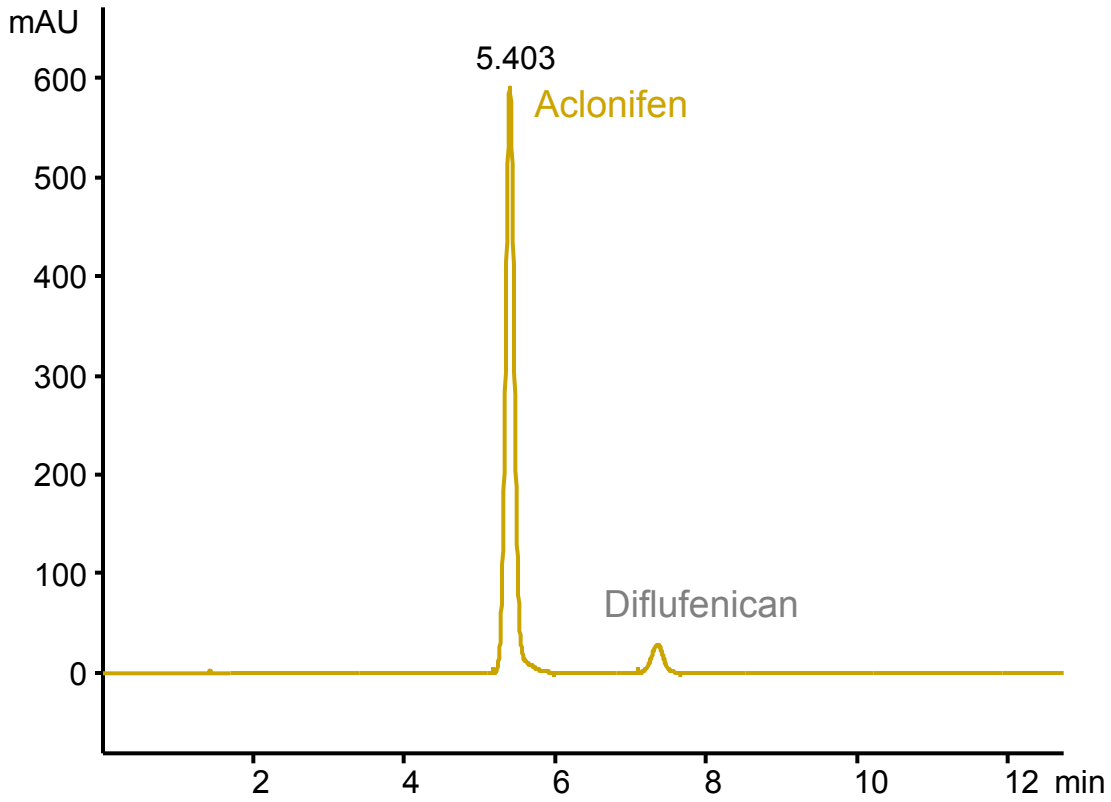
A method fit for official controls

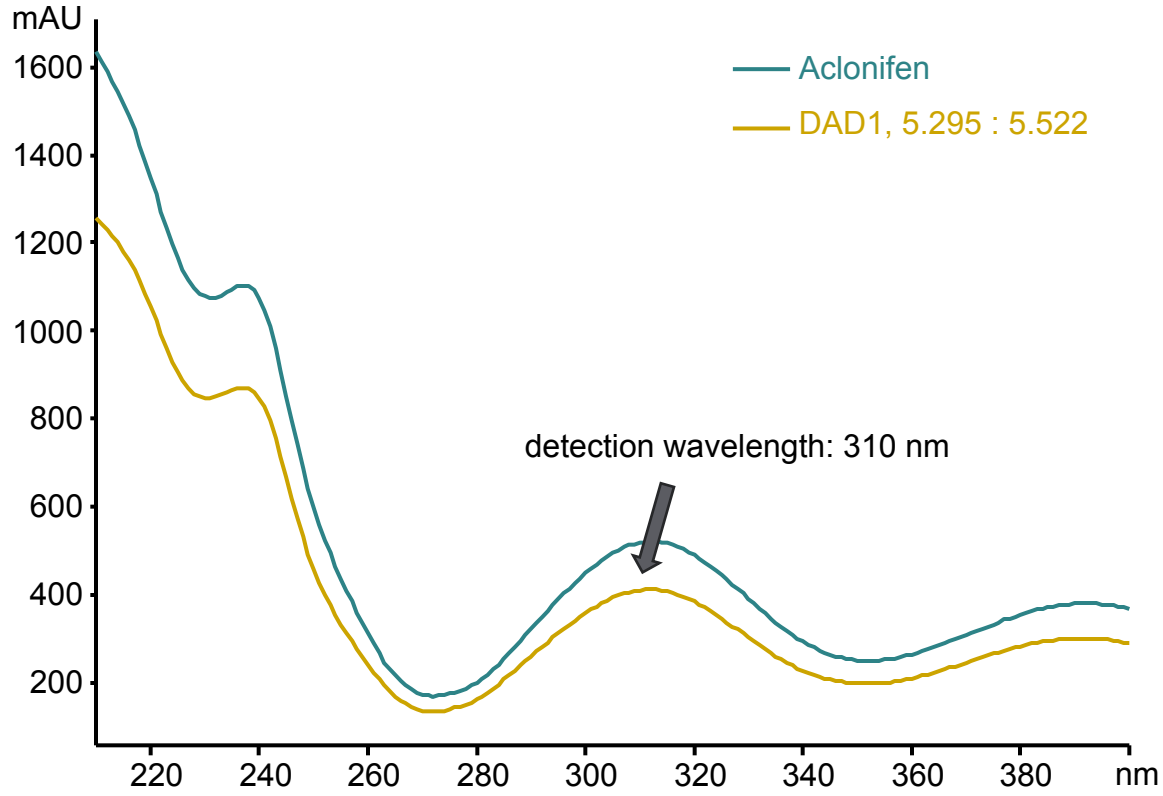
- Our multi-active method has been accredited for more than 5 years under the scope of our laboratory's ISO 17025 accreditation
- The method has been used routinely in the course of official market control for many hundreds of samples covering a huge variation of formulation types
- Very few difficulties were encountered, e.g. co-elution of two active substances in a plant protection product containing four active substances could only be solved by changing to methanol and substantially extending the isocratic run-time (but still without any need to change the chromatographic setup!)
- If challenges occur they are in almost all cases related to extraction problems

Application examples

Aclonifen-containing product

- Chromatogram and UV spectrum match with library

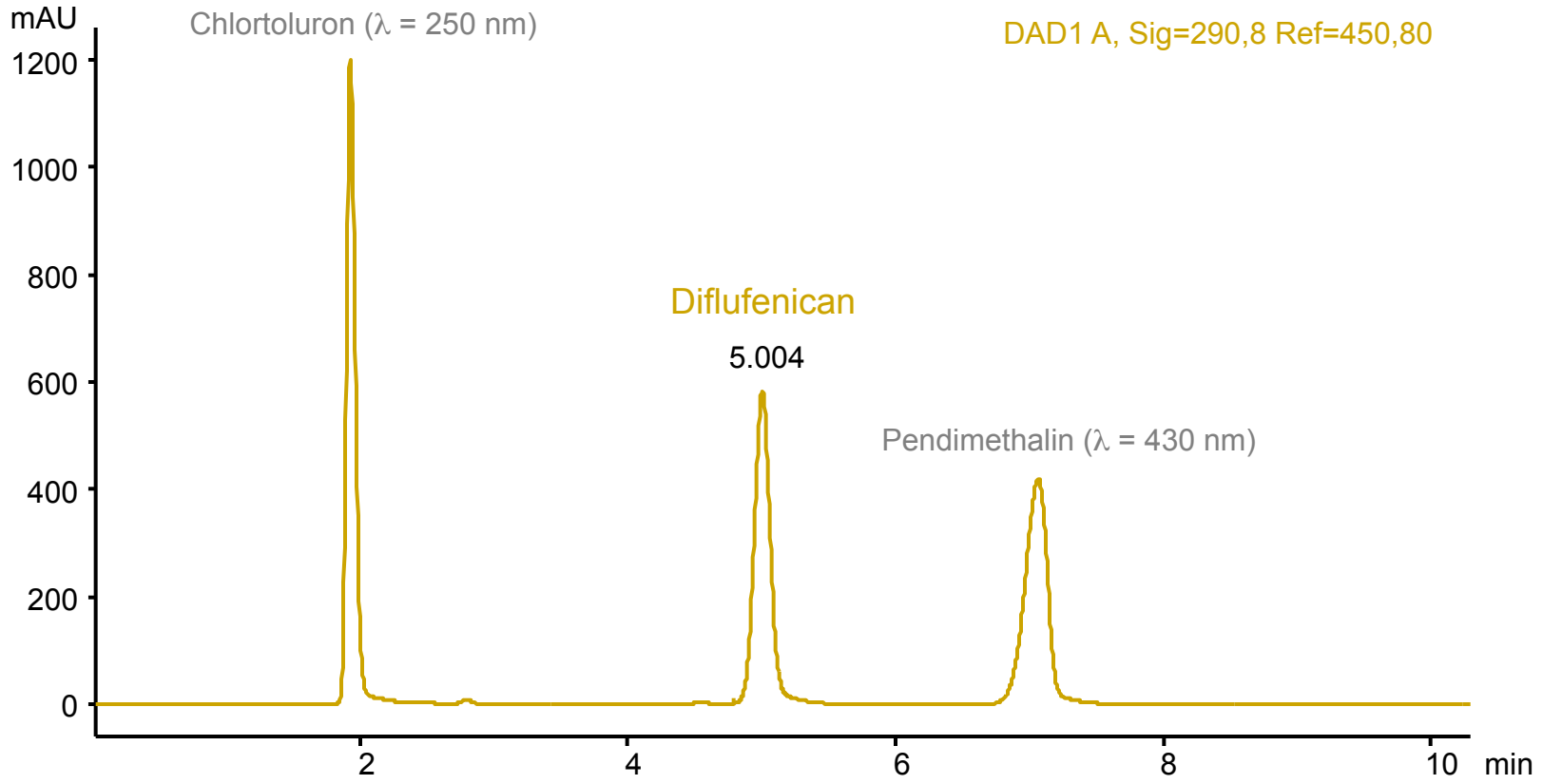




Application examples

Plant protection product with three active substances I

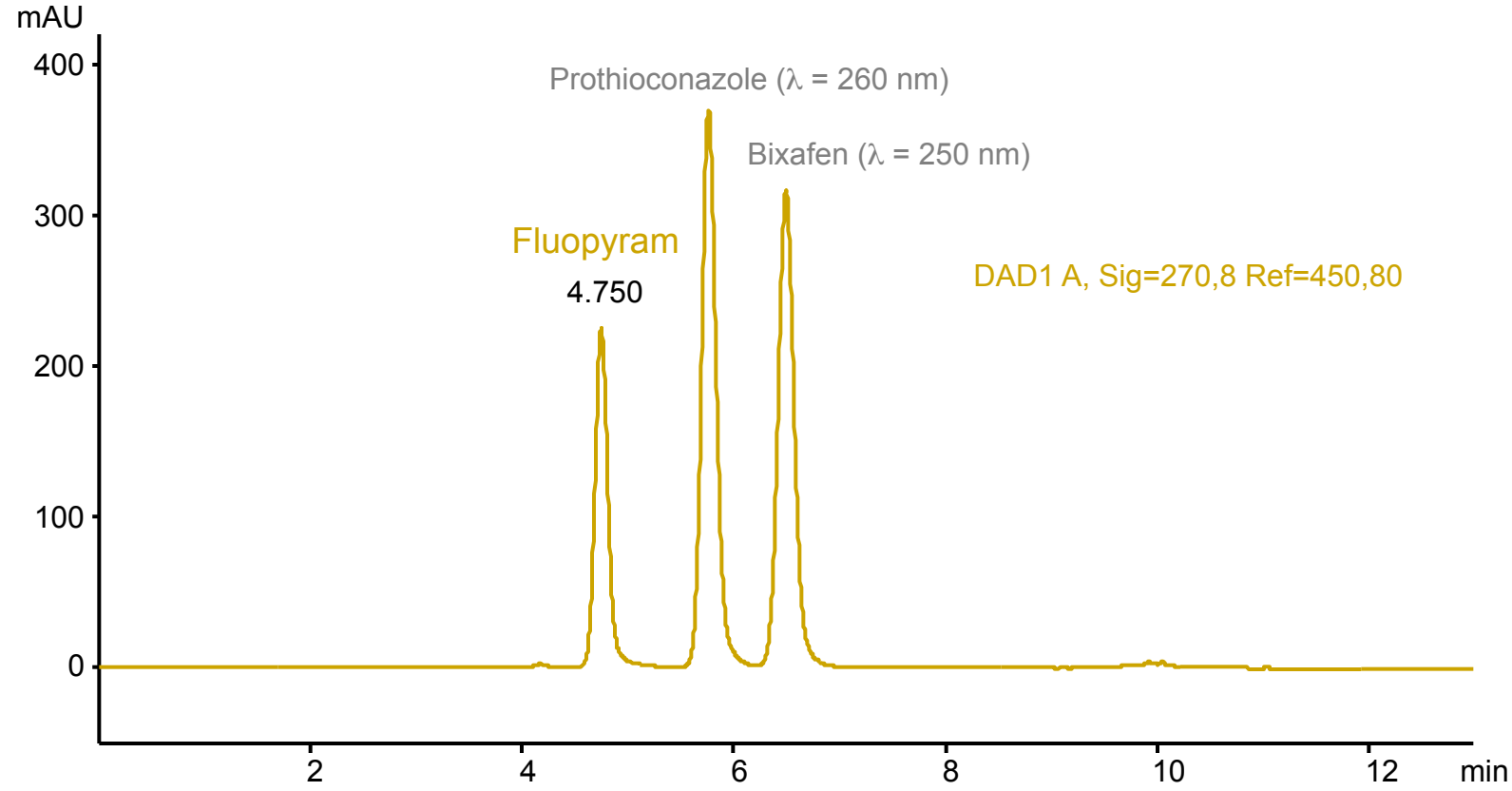
- Chromatogram of suspension concentrate containing chlorotoluron (250 g/l), diflufenican (40 g/l) and pendimethalin (300 g/l)



Application examples

Plant protection product with three active substances II

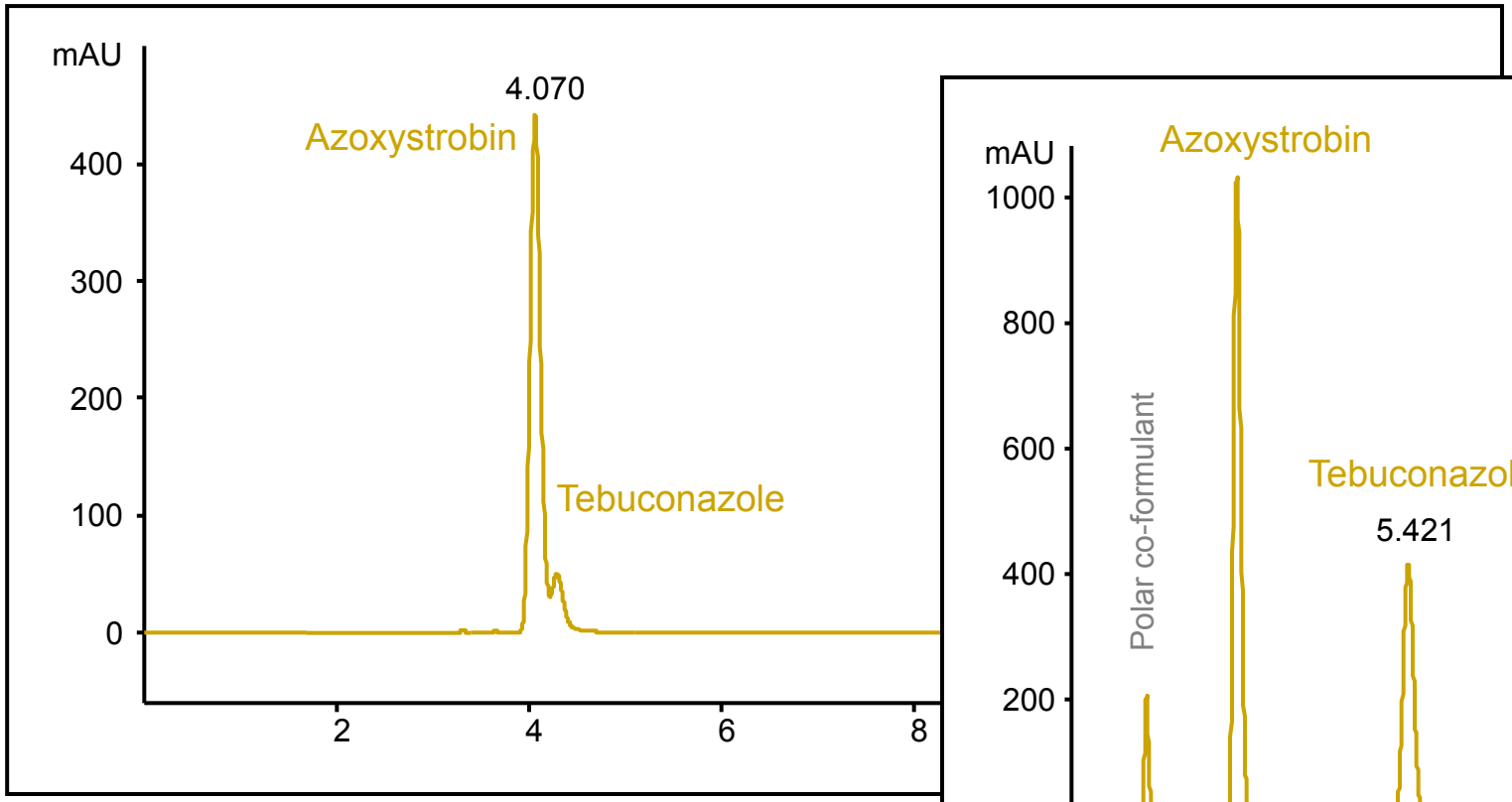
- Chromatogram of emulsion concentrate containing bixafen (65 g/l), fluopyram (65 g/l) and prothioconazole (130 g/l)



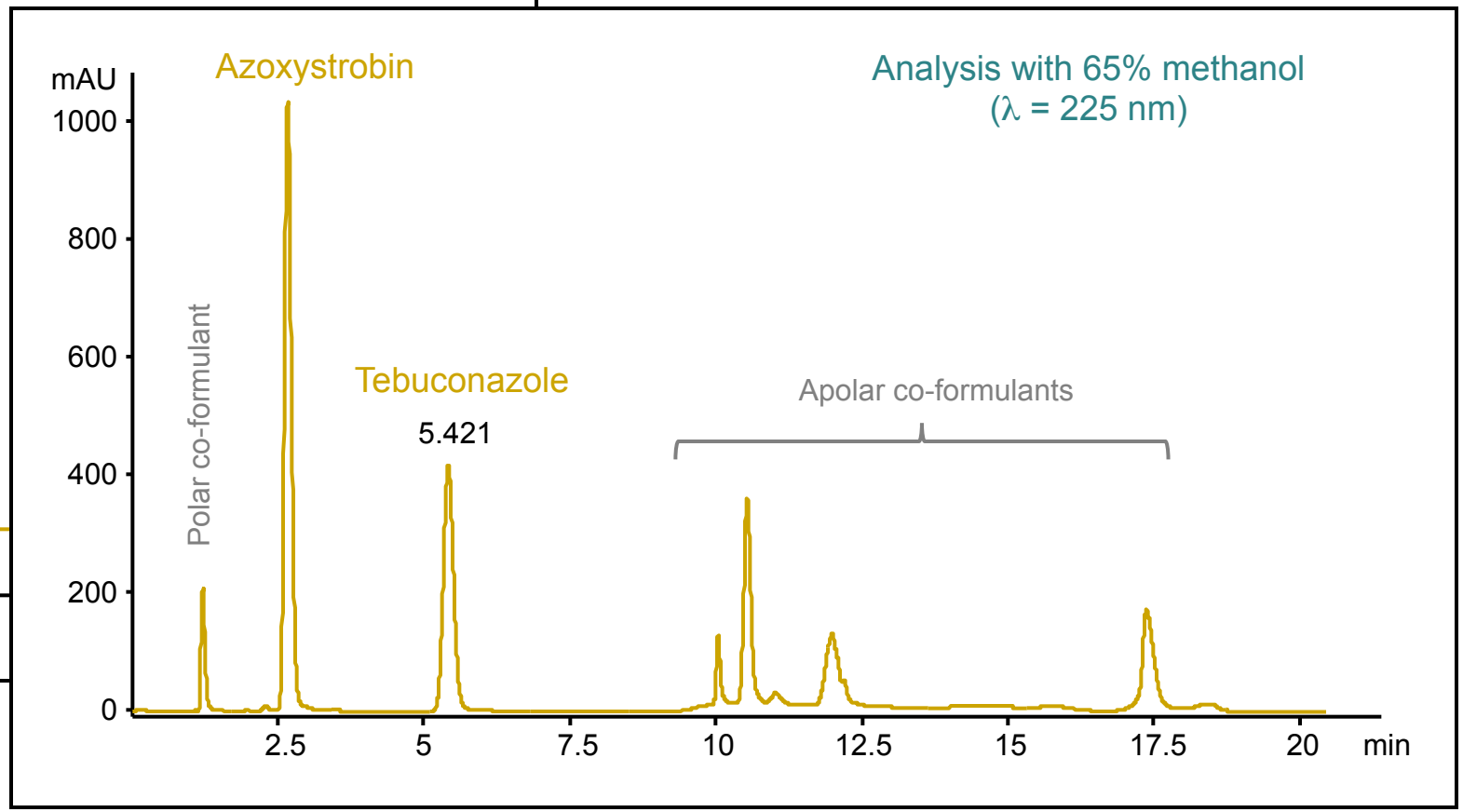
Application examples

Solving co-elution problems by switching organic mobile phase

- Emulsion concentrate containing azoxystrobin and tebuconazole



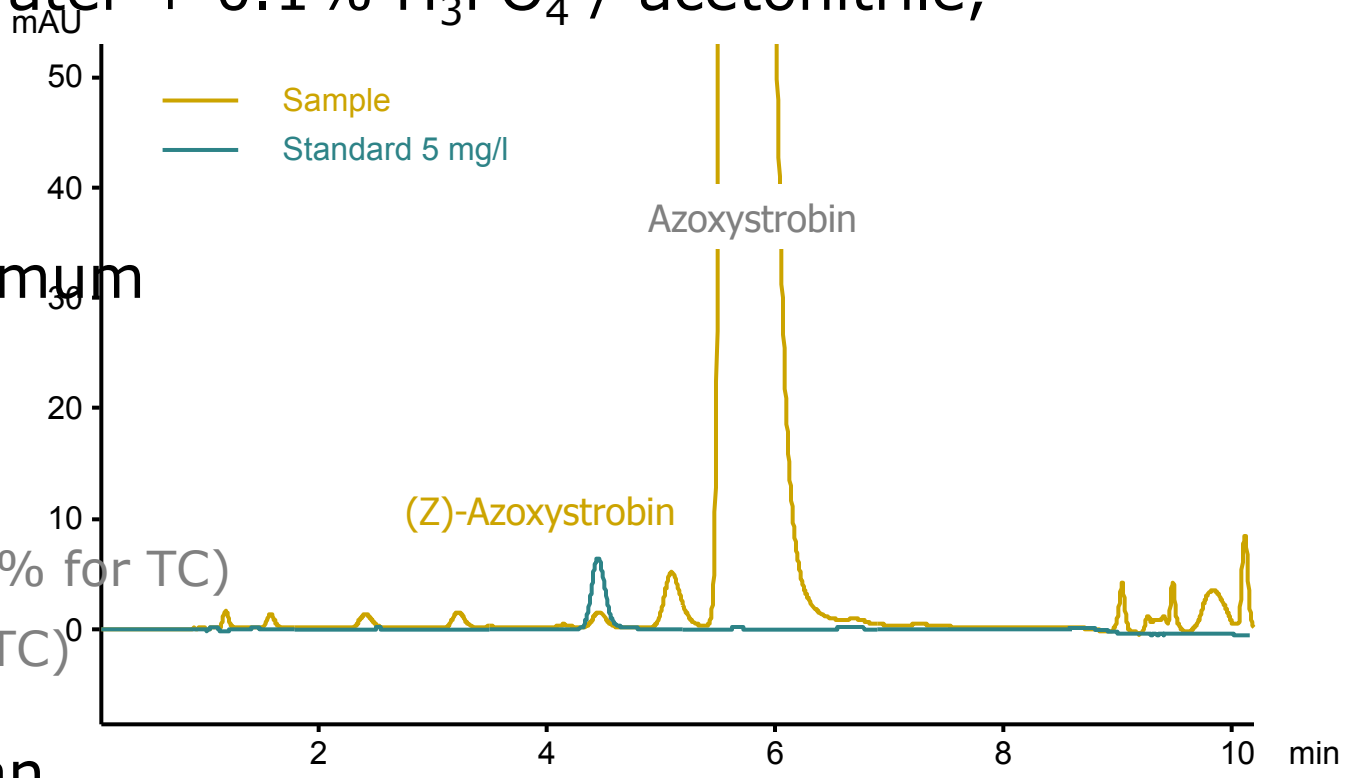
Analysis with 50% acetonitrile
(λ = 270 nm)



Analysis of relevant impurities

Use of same chromatographic setup

- The relevant impurity (Z)-Azoxystrobin can be analysed with the same setup
  (same column, mobile phases: water + 0.1% H_3PO_4 / acetonitrile, isocratic elution)
- Calibration range: 5 – 200 mg/l
  (corresponds to 5-200% of maximum level (25 g/kg azoxystrobin))
- Excellent performance:
  recovery: 94.7 – 96.2 % (90-110 % for TC)
  precision: RSD ≤ 1.6% (3.5% for TC)
- ➢ Active substance and impurity can be analysed in same sequence



Analysis of formaldehyde

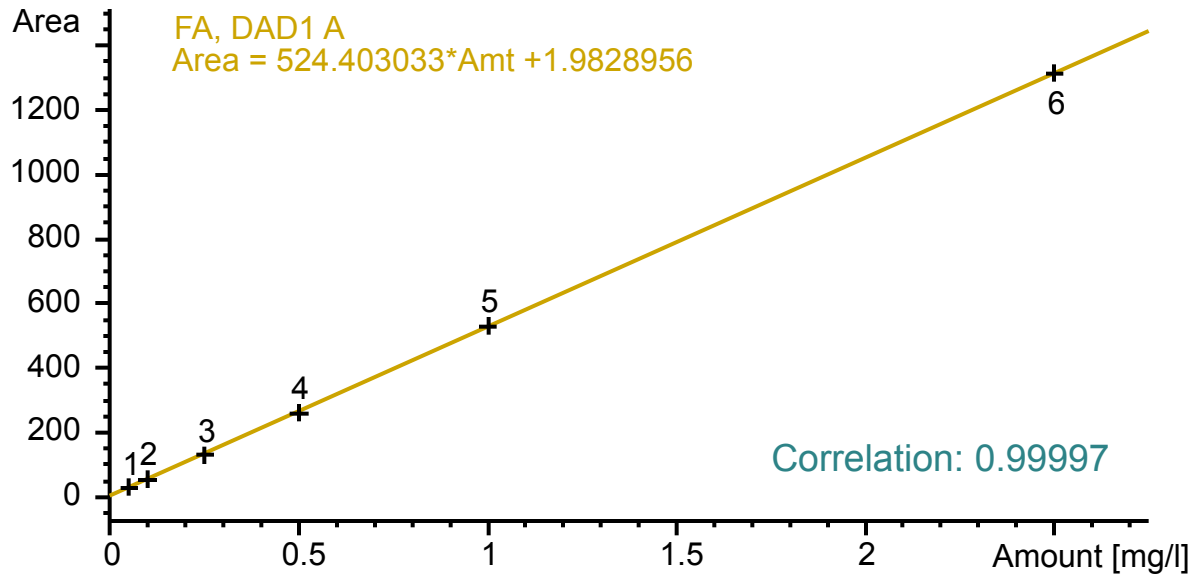
A challenging analyte of various potential origins

- Formaldehyde is a relevant impurity (of glyphosate) and at the same time a forbidden co-formulant according to EU Regulation 2021/383
- Formaldehyde may also be present as unreacted monomer of different polymers, e.g. urea-formaldehyde polymer (Pergopak™), as well as from formaldehyde-releasing preservatives, e.g. bronopol, benzyloxymethanol
- Various methods of analysis, but all require derivatisation
- A classical derivatisation reaction uses 2,4-dinitrophenylhydrazine
- When sample contains compounds that may release (additional) formaldehyde, longer and varying standing times after derivatisation can lead to increased (wrong) results for formaldehyde

Analysis of formaldehyde

Inline derivatisation & use of multi-active chromatographic setup

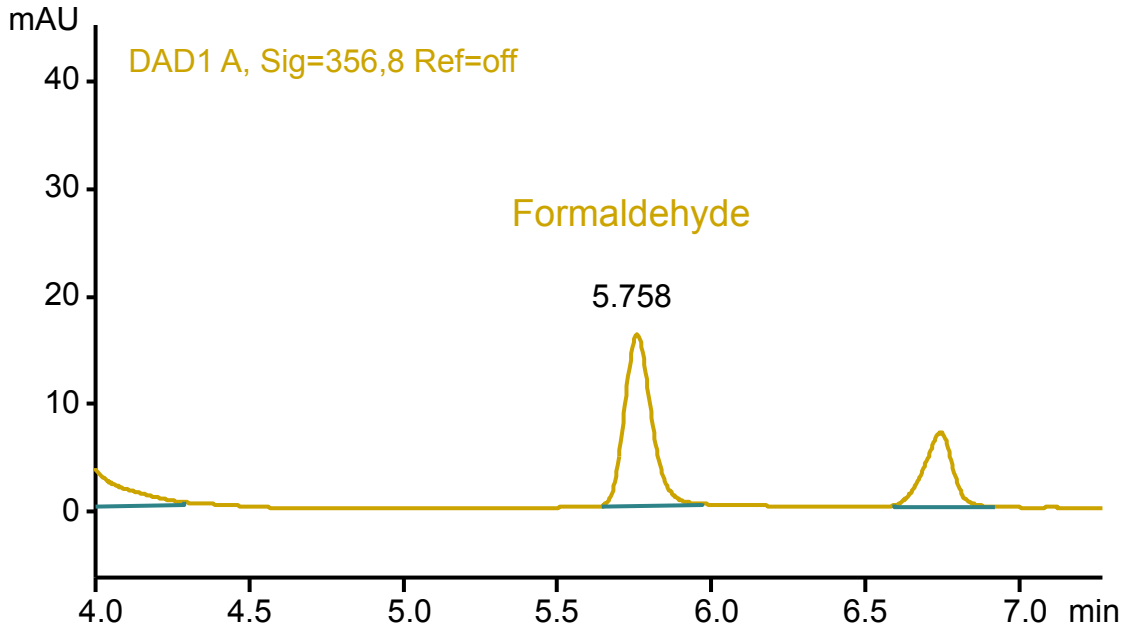
- An elegant solution is to perform the derivatisation in the HPLC injector (“inline”)
- Sample and derivatisation reagent are drawn into autosampler loop, mixed and left to react before the derivative is then injected ⇨ fully automated and economical
- HPLC-UV analysis using column and mobile phase components of multi-active method, employing a 16 min gradient
- Despite miniaturisation (2 µl derivatisation reagent) excellent linearity was obtained



Analysis of formaldehyde

Inline derivatisation & use of multi-active chromatographic setup

- The method was successfully validated:
  - LOQ = 0.05 g/kg (0.005% w/w)
  - Recovery: 99.5 – 102.5 %
  - Precision: ≤ 2.1%
- Five-fold analysis of a plant protection product containing the preservative benzyloxymethanol (Preventol D 2):
  formaldehyde: 0.029% (w/w)
  relative standard deviation: 1.16%
- ➢ See also poster by Christian Mink



Conclusions I

All goals achieved

- The developed multi-active method fulfils all requirements including:
  - Analytical performance (selectivity, versatility, concentration range, precision)
  - Ease of use (single well-defined setup, 24/7 runs possible, transferability)
  - Reduced environmental impact (lowered solvent consumption)
- Performance proven in routine application and proficiency tests over many years
- Two relevant impurities can also be analysed with the same instrumental setup, including a derivatisation method, further improving laboratory efficiency
- Limitations of approach only encountered rarely so far (e.g. separate method for glyphosate, active substances for which enantioselective analysis is required)

Conclusions II

The future of active substance analysis

- A multi-active method is far superior to individual compound-dedicated methods in terms of efficiency, while delivering the same analytical performance
- Further efficiency gains, e.g. by using UHPLC, would in principle be possible
- Laboratories not yet using a multi-active method should consider implementation
- The future of active substance analysis lies with one/two multi-active methods, with the necessity of dedicated single-compound methods limited to special cases

THANK YOU!

My colleagues involved in establishing and running this method

especially Barbara Steffl

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