EURL-SRM - Analytical Observations Report

Concerning the following…

- **Compound(s)**: Meptyldinocap
- **Commodities**: Plant origin, animal origin
- **Extraction Method(s)**: CEN-QuEChERS, QuOil
- **Instrumental analysis**: LC-MS/MS

Analysis of Meptyldinocap by QuEChERS

followed by alkaline hydrolysis and LC-MS/MS measurement

Version 1.2 (August 2022)

Background information

Meptyldinocap is a contact fungicide with protective and curative activity. It is primarily used against powdery mildews in a variety of crops including cucurbits (e.g. melons, watermelons, pumpkins and zucchini), other fruiting vegetables (e.g. sweet peppers, chili peppers), various tree fruits (e.g. pome fruits, stone fruits, citrus and mango) as well as for the treatment of berries (e.g. grapes, and strawberries). Its fungicidal activity is based on the inhibition of spore germination by upsetting the electrochemical balance within the fungi cell.

Meptyldinocap (2,4-DNOPC), is a racemic mixture of two enantiomers of 2,4-dinitro-6-(methyl-heptyl)-phenyl crotonate. According to FAO, technical meptyldinocap typically also contains a small amount (~1.5%) of 2,4-dinitro-6-(1-ethylhexyl)phenyl crotonate as impurity. It has been introduced in 2007 after recognizing that it is the most active component of dinocap. Dinocap is a much more complex mixture and composed of six enantiomeric pairs of isomeric dinitrophenyl crotonates, with meptyldinocap constituting the most abundant component. The share of meptyldinocap in a typical dinocap mixture is ~22% (see Figure 1).

Isomers	Meptyldinocap	Dinocap
Meptyldinocap, 2,4-dinitro-6-(1-methylheptyl)phenyl crotonate	98.5%	22%
2,6-dinitro-4-(1-methylheptyl)phenyl crotonate	0%	11%
2,4-dinitro-6-(1-ethylhexyl)phenyl crotonate a	1.5%	22%
2,6-dinitro-4-(1-ethylhexyl)phenyl crotonate	0%	11%
2,4-dinitro-6-(1-propylpentyl)phenyl crotonate	0%	22%
2,6-dinitro-4-(1-propylpentyl)phenyl crotonate	0%	11%

Figure 1: Typical isomer composition of technical meptyldinocap and dinocap[1]

[1] FAO/JMPR: https://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation10/Meptyldinocap.pdf

Figure 2 shows an overview of the structural formulas and the nomenclature of the various components of dinocap and the corresponding phenols.

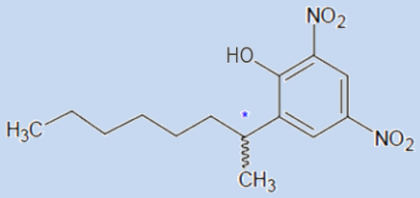
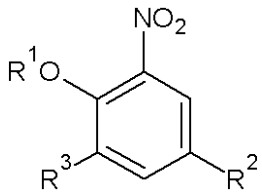
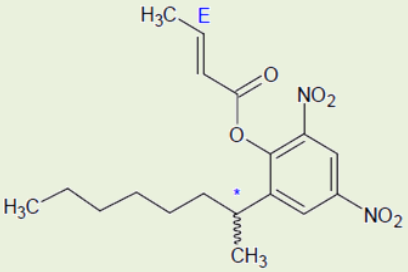
 **Meptyldinocap phenol**	 **General Formula crotonates / phenols**	 **Meptyldinocap**		
R1	**R2**	**R3**	**Name**	**Acronyms**
ESTERS ("Crotonates" = Crotonic acid esters = (E)-2-butenoic acid esters)				
C_4H_5O	**NO_2**	**C_5H_{18}**	**2,4-dinitro-6-(1-methylheptyl)-phenyl)-crotonate = Meptyldinocap;**	**2,4-DN-MH, DNOPC, 2,4-DNOPC**
C_4H_5O	NO_2	C_5H_{18}	2,4-dinitro-6-(1-ethylhexyl)-phenyl)-crotonate	2,4-DN-EH
C_4H_5O	NO_2	C_5H_{18}	2,4-dinitro-6-(1-propylpentyl)-phenyl)-crotonate	2,4-DN-PP
C_4H_5O	C_5H_{18}	NO_2	2,6-dinitro-4-(1-methylheptyl)-phenyl)-crotonate	2,6-DN-MH
C_4H_5O	C_5H_{18}	NO_2	2,6-dinitro-4-(1-ethylhexyl)-phenyl)-crotonate	2,6-DN-EH
C_4H_5O	C_5H_{18}	NO_2	2,6-dinitro-4-(1-propylpentyl)-phenyl)-crotonate	2,6-DN-PP
PHENOLS				
H	**NO_2**	**C_5H_{18}**	**2,4-dinitro-6-(1-methylheptyl)-phenol = meptyldinocap phenol**	**2,4-DN-MH-Ph, DNOP, 2,4-DNOP**
H	NO_2	C_5H_{18}	2,4-dinitro-6-(1-ethylhexyl)-phenol	2,4-DN-EH-Ph
H	NO_2	C_5H_{18}	2,4-dinitro-6-(1-propylpentyl)-phenol	2,4-DN-PP-Ph
H	C_5H_{18}	NO_2	2,6-dinitro-4-(1-methylheptyl)-phenol	2,6-DN-MH-Ph
H	C_5H_{18}	NO_2	2,6-dinitro-4-(1-ethylhexyl)-phenol	2,6-DN-EH-Ph
H	C_5H_{18}	NO_2	2,6-dinitro-4-(1-propylpentyl)-phenol	2,6-DN-PP-Ph

Figure 2: Overview of dinocap isomers and corresponding phenols.

Meptyldinocap is approved under Reg. 1107/2009/EC and currently authorized at national level in 14 EU Member States[2]. The current approval period of meptyldinocap expires in March 2025. In contrast, dinocap is no longer approved within the EU. It is however still in use elsewhere in the world.

Residues of dinocap and meptyldinocap in food are regulated separately. The wording of the residue definitions is as follows:

I. **D*inocap (sum of dinocap isomers and their corresponding phenols expressed as dinocap) (F)*[3]**
II. ***Meptyldinocap (sum of 2,4 DNOPC and 2,4 DNOP expressed as meptyldinocap)*[4]**

Both residue definitions include the corresponding phenol metabolites of the parent components.
The MRLs of dinocap and meptyldinocap differ and conflicts arising if the MRLs of meptyldinocap are applied on samples containing dinocap, or vice versa, need to be avoided.
To improve legal certainty, the residue definition for dinocap is accompanied by the note below:

"*Where only meptyldinocap or its corresponding phenol are detected but none of the other components constituting dinocap (including their corresponding phenols), the MRLs and residue definition of meptyldinocap are to be applied.*"

This means that the MRLs and the residue definition of dinocap are not applicable if only meptyldinocap and/or it corresponding phenol are detected or that the residue definition of meptyldinocap cannot be used if other isomers of dinocap or their corresponding phenols are detected.

For meptyldinocap, the MRLs are set at 0.1 mg/kg in cucumber, courgettes and watermelons, at 0.5 mg/kg in melons, at 1 mg/kg in grapes and at 3 mg/kg for strawberries. For commodities with no applications the MRLs are set at 0.05* mg/kg in most commodities and at 0.1* mg/kg in extract-rich commodities such as teas, herbs and spices.
For dinocap, despite the 12 components included in the residue definition, the MRLs of most commodities are set at the very low level of 0.02* mg/kg. For cereals, oily seeds, pulses and fresh herbs the MRLs are set at 0.05* mg/kg, and in extract-rich commodities at 0.1* mg/kg.

In the initial stages of our work with dinocap and meptyldinocap, we have observed a quick degradation of stock and working solutions when dissolved in pure acetonitrile[5]. Analysis of meptyldinocap (and dinocap) requires taking measures to ensure stability of meptyldinocap in standard solutions. This includes acidification (when acetonitrile is used) and keeping the standard solutions in a cool and dark place to minimize hydrolysis and photolysis. Still, small amounts of the free phenol are typically observed in standard solutions.

[2] **EU Pesticides Database (v.2.2) Active substance (europa.eu)** (accessed 20 January 2022)
[3] Commission Regulation (EU) No 1127/2014 of 20 October 2014 amending Annexes II and III to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for amitrole, dinocap, fipronil, flufenacet, pendimethalin, propyzamide, and pyridate in or on certain products
[4] Commission Regulation (EU) 2021/1864 of 22 October 2021 amending Annexes II, III and V to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for amisulbrom, flubendiamide, meptyldinocap, metaflumizone and propineb in or on certain products
[5] Back then, the quality of acetonitrile on the market was rather poor and acetonitrile obtained a certain basicity that was due to the production process. Many base-labile compounds such as captan, folpet, chlorothalonil and dicofol showed dramatic losses in pure acetonitrile.

The analysis of dinocap is additionally hampered by the non-availability of the analytical standards of the individual isomers of the parent compounds and the phenols and limitation in the chromatographic separation of the isomers.

GC-analysis:

GC analysis of meptyldinocap (and dinocap) is associated with a partial thermal degradation of the parent compounds to the respective phenols (see Figure 3). The degree of GC-degradation was found to be very dependent on matrix as well as on the condition of the GC liner and to affect the analytical robustness and accuracy of quantification of both parents and phenols. GC analysis offers a better chromatographic separation between the dinocap isomers compared to what is typically seen in LC-MS/MS and is useful for screening, confirmation of identity and as an additional supporting evidence in the distinction between dinocap and meptyldinocap. Unfortunately, meptyldinocap (2,4-DN-MH) is typically not separated from the 2,6-DN-MH isomer.

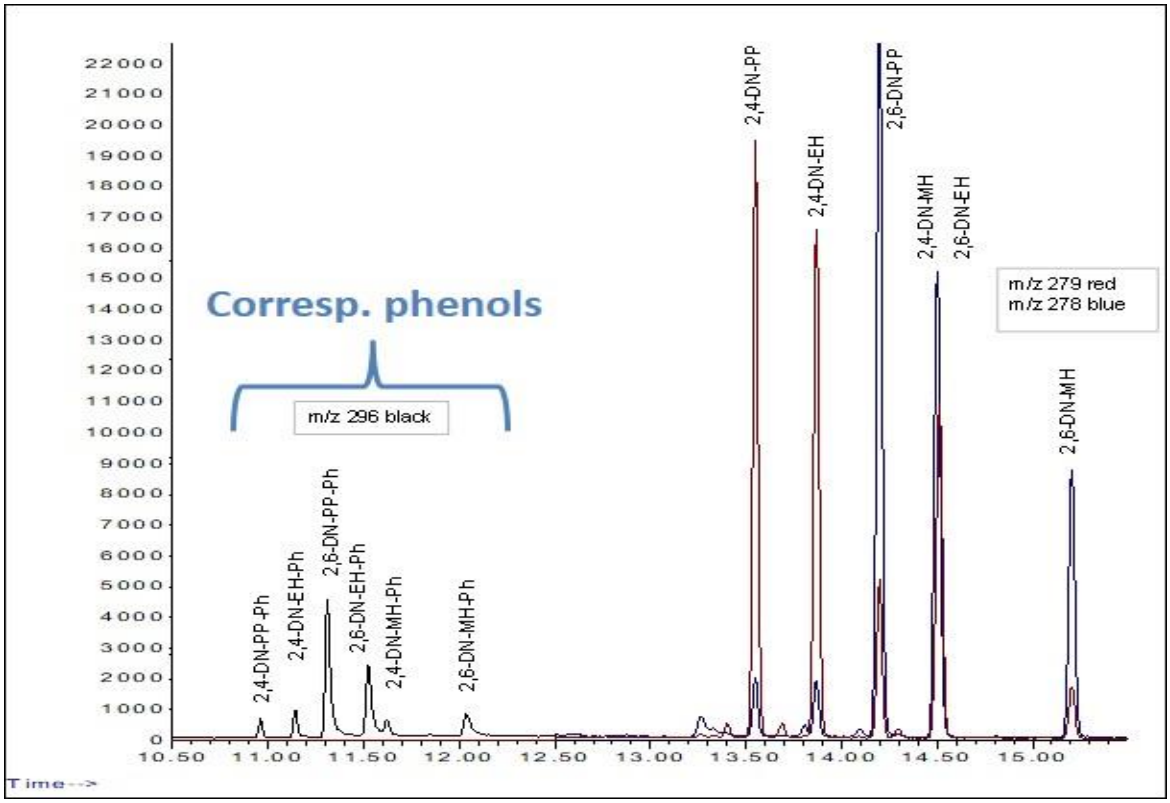


Figure 3: GC-MSD chromatogram of dinocap (CI-negative) mode.

In theory, GC-fluctuations could be corrected by using isotope labelled meptyldinocap as internal standard. Unfortunately, labelled meptyldinocap is currently not available but we have observed, that other dinocap isomers behave similarly during GC and therefore may be used to correct for fluctuations in GC analysis. In an experiment, an extract spiked with meptyldinocap and its propyl-pentyl-analogon (PP-dinocap)[6] was repeatedly injected in a GC-MSD (CI neg. mode). As can be seen in Figure 4 the signals fluctuated considerably (RSDs >20%), but the signal ratio against the PP-analogon was fluctuating comparably little (RSD 4.3%). The signals of the corresponding phenols (that

[6] Standards of (2,4-dinitro-6-(1-propylpentyl)-phenyl)-crotonate and 2,4-dinitro-6-(1-propylpentyl)-phenol were used. These were kindly donated by a former applicant of dinocap.

are present as impurities in standards of dinocap isomers and are also partly formed during injection) also fluctuate strongly, but also here the signal ratio is more stable (see Figure 5). Figure 6 shows the signals of meptyldinocap-phenol and PP-dinocap-phenol as well as their ratio when injected as such.

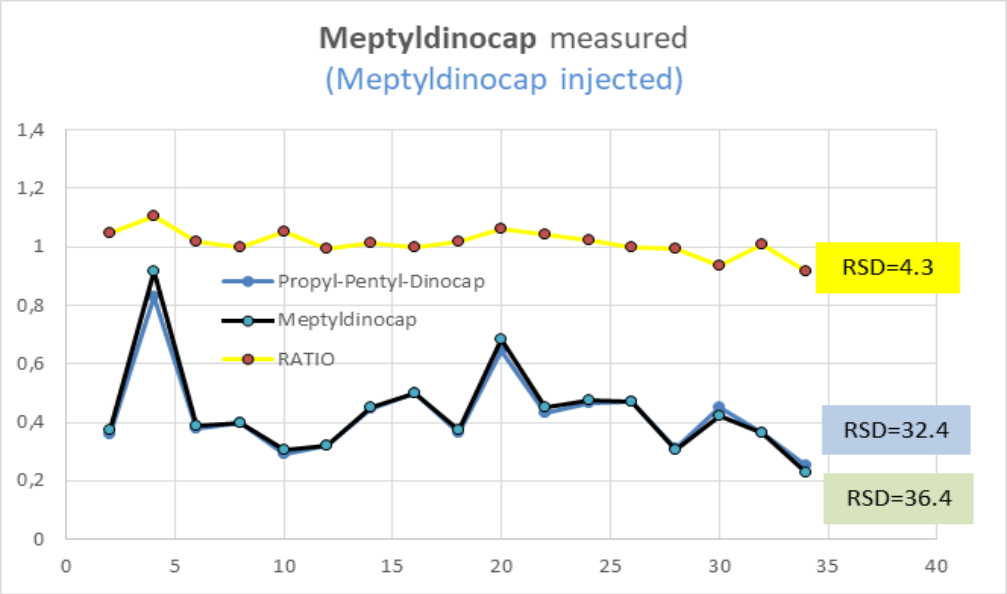


Figure 4: Detected fluctuations of GC-MSD (CI-neg.) signals of meptyldinocap and propyl-pentyl-dinocap (PP-dinocap) during a sequence of injections of the same extract spiked with meptyldinocap and PP-dinocap at 0.1 mg/kg each. The signal ratio between meptyldinocap and PP-dinocap is also shown. A factor was applied to the signals so that they can be plotted together with the ratio.

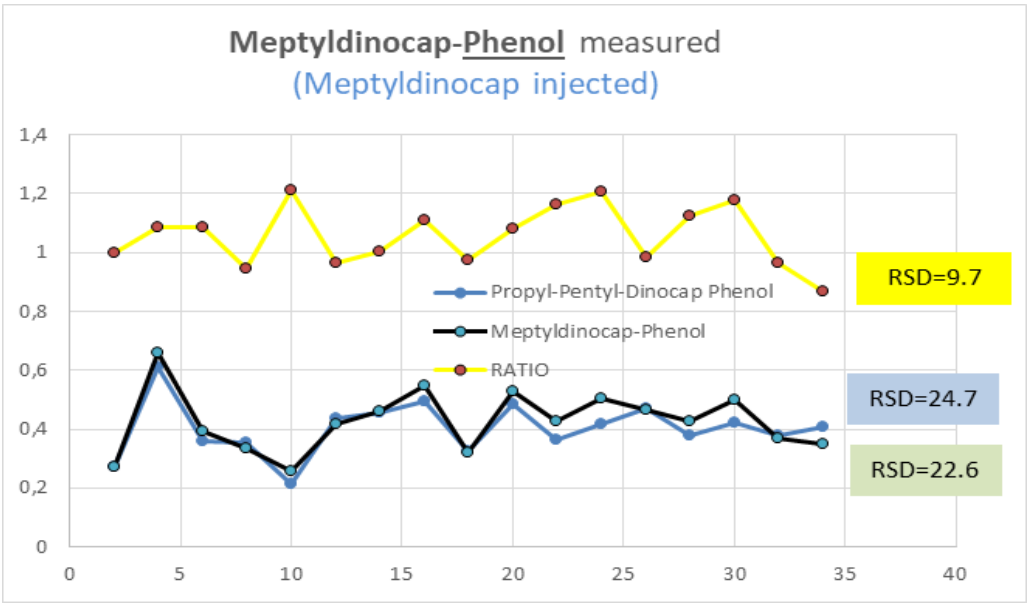


Figure 5: Detected fluctuations of GC-MSD (CI-neg.) signals of meptyldinocap phenol and PP-dinocap phenol during a sequence of injections of the same extract spiked with meptyldinocap and PP-dinocap at 0.1 mg/kg each. The signal ratio between the two phenols is also shown. A factor was applied to the signals, so that they can be plotted together with the ratio.

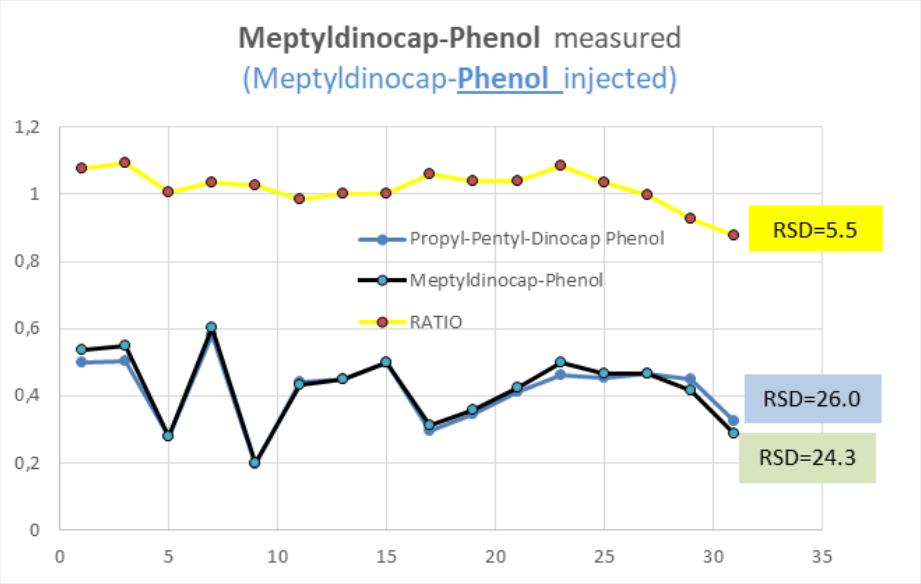


Figure 6: Detected fluctuations of GC-MSD (CI-neg.) signals of meptyldinocap phenol and PP-dinocap phenol during a sequence of injections of the same extract spiked with meptyldinocap phenol and PP-dinocap phenol at 0.1 mg/kg each. The signal ratio between the two phenols is also shown. A factor was applied to the signals so that they can be plotted together with the ratio.

LC-analysis:

Using typical reversed phase LC-columns, meptyldinocap is not well separated from 2,4-DN-PP, see Figure 7. All dinocap isomers experience an (in-source) fragmentation of the parents to the corresponding phenols so that the most intensive signal in the ESI (neg.) mode typically correspond to the phenols.

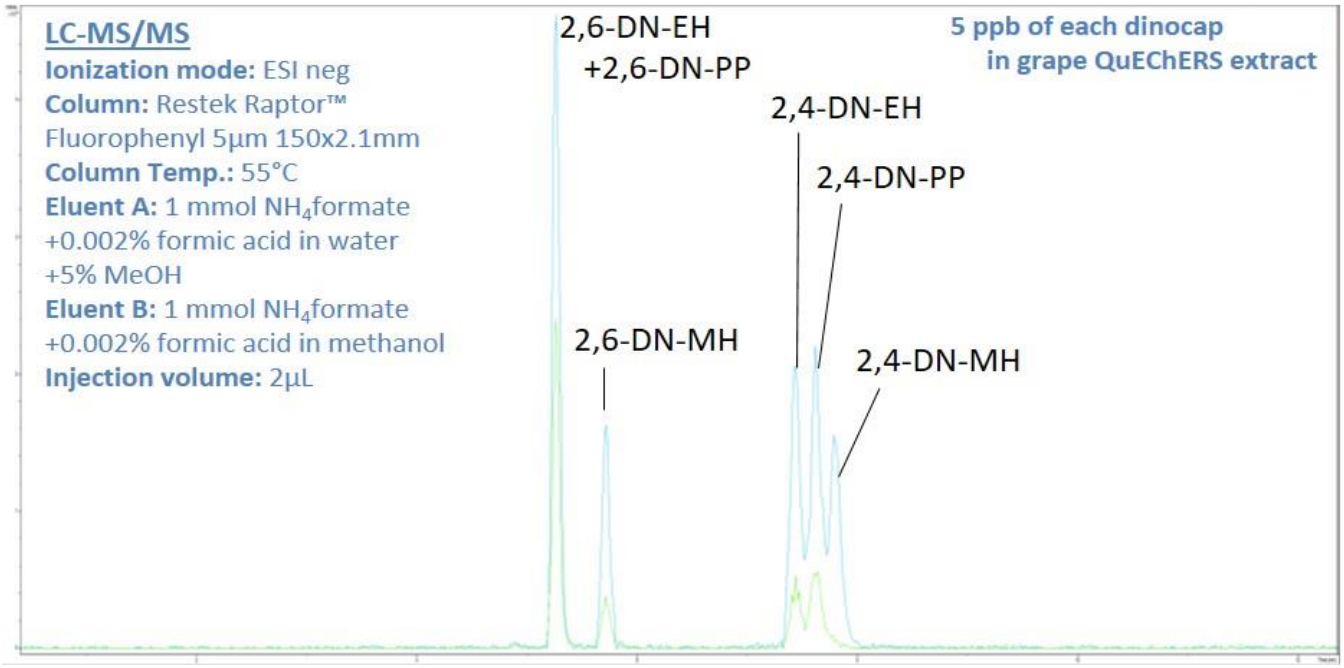


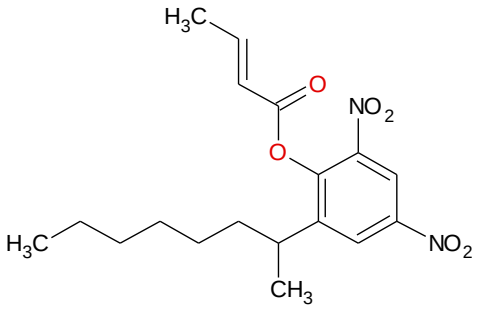
Figure 7: LC-MS/MS chromatogram of dinocap; nomenclature of the components see Figure 2.

The work presented here focuses on the analysis of meptyldinocap and meptyldinocap phenol using QuEChERS and LC-MS/MS on a standard C18 column both as such as well as following transformation of meptyldinocap to the corresponding phenol in order to enable the full residue definition based on one compound.
For the analysis of meptyldinocap (sum) a hydrolysis to the corresponding phenol (2,4-DNOP) is conducted on an aliquot of the QuEChERS extract followed by the analysis of the phenol by LC-MS/MS.

Analyte properties and analytical strategies

The physicochemical properties and additional information on meptyldinocap are shown in Table 1. and of its corresponding phenol (2,4-DNOP) in Table 2.

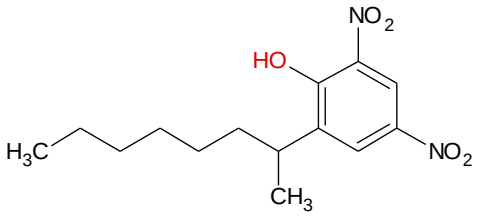
Table 1: Meptyldinocap at a glance

Meptyldinocap (CAS: 131-72-6, 1:1 mixture of RS isomers) Synonyms: 2,4-dinitro-6-(octan-2-yl)phenyl (2E)-but-2-enoate; 2,4-DNOPC Specification: mixture of (RS)-2-(1-methylheptyl)-4,6-dinitrophenyl crotonate (75-100 %) and (RS)-2-(1-methylheptyl)-4,6-dinitrophenyl isocrotonate (0-25 %); Note crotonic acid = (E)-2-butenoic acid, isocrotonic acid = (Z)-2-butenoic acid			
Parameter	Value	Notes	
Molecular Mass	364.398 g/mol		
Formula	$C_{18}H_{24}N_2O_6$		
Boiling point	Degrades at 200 °C		
Melting point	-22°C (thick liquid at room temperature)		
pKa	No ionizable atoms available		
LogP	6.3 6.55[7]	computed by chemicalize.com at 20.5 °C (pH 7); pH independent	
Water solubility	2.48 × 10^{-4} g/L[7] [8] = 0.25 mg/L	at 20°C (pH7) (virtually insoluble in water)	
Hydrolytic Stability	DT50 at 20 °C in the dark: 447 d @ pH4, 229 d @ pH5, 56 / 30 d @ pH7, and 0.7 / 9.3 @ pH 9. (Streelman,1981 , Winwick, T, 1998), **the compound is base-labile**		
Note of formulation	According to FAO[9] in a typical composition technical meptyldinocap contains ~1.5% of 2,4-dinitro-6-(1-ethylhexyl)phenyl crotonate as an impurity		
Residue definition EU	**Meptyldinocap** (sum of 2,4 DNOPC and 2,4 DNOP expressed as meptyldinocap), according to Reg.(EU) 2021/1864 **Dinocap** (sum of dinocap isomers and their corresponding phenols expressed as dinocap); *where only meptyldinocap or its corresponding phenol are detected but none of the other components constituting dinocap (including their corresponding phenols), the MRLs and residue definition of meptyldinocap are to be applied,* according to Reg.(EU) 1127/2014.		
Approved in …	AT, CY, CZ, EL, ES, FR, HR, HU, IT, MT, PT, RO, SI, SK		

7 https://www3.epa.gov/pesticides/chem_search/reg_actions/registration/fs_PC-036000_01-Sep-09.pdf
8 Conclusion on the peer review of the pesticide risk assessment of the active substance DE-126, referred to as meptyldinocap in Commission Decision 2006/589/EC - 2014 - EFSA Journal - Wiley Online Library
9 https://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation10/Meptyldinocap.pdf

Toxicity	ARfD: 0.12 mg/kg bw ADI: 0.016 mg/kg bw/day No classification according to Reg. (EC) 1272/2008, but dinocap is classified in cat. 4 as regards its acute toxicity, in cat. 1B as regards its preproduction toxicity, in cat. 2 as regards its specific toxicity to target organs at repeated exposure, in cat. 1 as regards its skin sensitizing properties, in cat. 1 as regards its acute hazard to the aquatic environment and in cat. 1 as regards its chronic hazard to the aquatic environment[10].
Other sources	Component of dinocap

Table 2: Meptyldinocap-phenol at a glance

2,4-Dinitrooctanylphenol (CAS: 3687-22-7) Other names: 2,4-dinitro-6-(octan-2-yl)phenol; 2,4-DNOP; Meptyldinocap-phenol		
Parameter	Value	Notes
Molecular Mass	296.323 g/mol	
Formula	$C_{14}H_{20}N_2O_5$	
Boiling point	-	
pKa	5.08 (acidic), computed by chemicalize.org	
LogP	pH dependent (5.0 at pH <4; 3,0 at pH >8) computed by chemicalize.org (► lipophilic enough at any pH for QuEChERS)	
Water solubility	pH dependent; very low solubility up to pH 9	
Stability	-	
Residue definition EU	See above	
Toxicity	-	
Other sources	Small amounts of the phenols are found even in fresh solutions of meptyldinocap	
Remark	Not to be confused with Di-N-octyl phthalate (DNOP) **https://echa.europa.eu/documents/10162/17233/dnop_echa_review_report_2010_6_en.pdf/c3aeee95-2a29-40e1-88a5-c79a79cd2835?t=1322595177704**	

Apparatus, Chemicals and Consumables

Chemicals and Materials

The used materials and apparatuses are listed in the QuEChERS (EN-15662) and QuOil (CEN/TS 17062:2019) standard procedures. Additional chemicals and materials used are listed in Table 3.

Table 3: Additional used chemicals for the alkaline hydrolysis following QuEChERS extraction

Chemical	Purity, Assay	Brand/Source	Article No.
Ammonium hydroxide solution	25 %, EMSURE®, for analysis	Merck Chemicals	105432
Acetic acid	96 %, EMSURE®, for analysis	Merck Chemicals	100062

Disclaimer: Names of companies are given for the convenience of the reader and do not indicate any preference by the EURL-SRM towards these companies and their products

[10] Reg. (EC) No 1272/2008 of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Reg. (EC) 1907/2006, amended by Commission Delegated Regulation (EU) 2021/1962 of 12 August 2021

Analytical standards

Exemplary suppliers of the used analytical standards are shown in Table 4.

Table 4: Sources of analytical standards

Compounds	Details on standards used		Provider
Meptyldinocap	Purity: Supplier Code:	96.2 % C14895000	Dr. Ehrenstorfer GmbH
Meptyldinocap-phenol (2,4-DNOP)	Purity: Supplier Code:	98.5 % C14895050	Dr. Ehrenstorfer GmbH

Disclaimer: Names of companies are given for the convenience of the reader and do not indicate any preference by the EURL-SRM towards these companies and their products

Stock and working solutions:

Taking the purity of the standard substances into account, stock solutions (at e.g. 1 mg/mL) are prepared in acetonitrile in the case of meptyldinocap phenol and in acetonitrile containing 0.4% acetic acid in the case of meptyldinocap. The solutions are sufficiently stable in the refrigerator for up to 36 months. Working solutions of meptyldinocap phenol and meptyldinocap at the required concentrations are prepared in acetonitrile and acetonitrile containing 0.4% acetic acid respectively and may be stored in the refrigerator until use. In case of a mixed standard, use acidified acetonitrile as a solvent.

Sample Preparation

Homogenization: The samples are homogenized by cryogenic milling using dry ice according to Document Nº SANTE/12682/2019.

Sample preparation: The samples are extracted according to QuEChERS (citrate-buffered; EN-15662) method without applying dSPE-cleanup. High oil content commodities are extracted according to the QuOil method (CEN/TS 17062:2019).

Chlorpyrifos-D_{10} or propyzamide-D_3 (100 µL of a 10 µg/mL solution in ACN, each substance) may be used as internal standards.

For the derivatization step, transfer an aliquot of 1000 µL into a vial, add 25 µL of 25% ammonia solution (75 µL in case of dry commodities or commodities of animal origin) and let the vial standing for at least 12 h at room temperature (e.g. overnight) or let it react for 2 h at 60° C. The hydrolysate is "neutralized"[11] with 25 µL of concentrated acetic acid (75 µL in the case of dry commodities and commodities of animal origin).

NOTE: In case a precipitate is formed after "neutralization", but the hydrolysate is clear, you can proceed directly with LC-MS/MS. In case of turbidity, centrifuge and decant if possible or pass the extract through a syringe filter (e.g. 0.45 µm pore size).

[11] The 25% ammonia is 14.5N and conc. acetic acid 17.5 N. For the neutralization of 25 µL 25% ammonia ~ 21 µL of acetic acid are needed. By adding 25 µL of acetic acid the final solution is slightly acidic. For the neutralization of 75 µL 25% ammonia ~ 66 µL of acetic acid are needed.

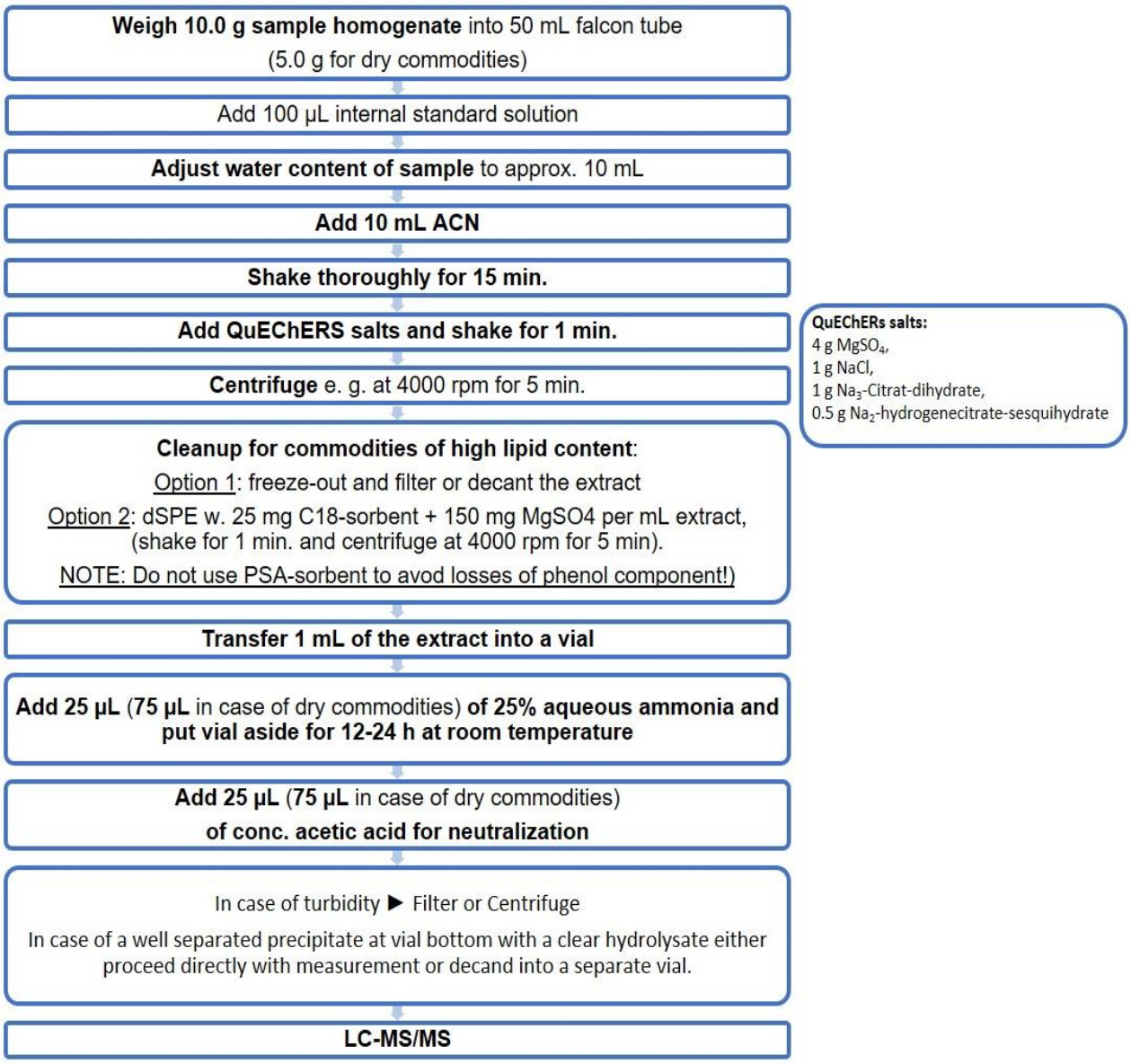


Figure 8: Method at a glance of the simple alkaline hydrolysis step after QuEChERS extraction for the determination of 2,4-DNOP

Measurement:

The extract is directly subjected to LC-MS/MS separation and measurement of 2,4-DNOP. Exemplary LC-MS/MS conditions are given in Table 5.

Table 5: LC-MS/MS details of a fast and a slow method for the analysis of 2,4-DNOP

Instrument parameters	Conditions					
LC-MS/MS system used	Waters Acquity UPLC®-system; MS: Sciex QTrap 5500+					
Column/temperature	Waters Acquity BEH C_{18}, 2.1x100 mm, 1.7 µm					
Pre-column	Van Guard BEH C_{18}, 1.7 µm					
Column temperature	40 °C					
Eluent A	0.01% acetic acid in Water + 5 % ACN					
Eluent B	0.01% acetic acid in ACN					
Injection volume	2 µL					
Gradient FAST NOTE: With this fast gradient, meptyldinocap elutes close to the earlier eluting 2,4-DNOP. As both compounds share the same MRMs **the peak of meptyldinocap gets interfered by the tailing of 2,4-DNOP**. If the signals of the peaks are comparable, separation is sufficient, but if the 2,4-DNOP signal is much higher than that of meptyldinocap (typically the case if both components are present in similar concentrations) interference becomes inacceptable.	**%A**	**Flow [mL/min]**	**Time [min]**			
	95	0.5	0.0			
	60	0.5	0.5			
	10	0.5	3.0			
	10	0.5	7.0			
	95	0.5	7.1			
	95	0.5	11.0			
Gradient SLOW	**%A**	**Flow [mL/min]**	**Time [min]**			
	95	0.5	0.0			
	10	0.5	15			
	95	0.5	15.1			
	95	0.5	19.0			
Acquired mass transitions (m/z)	**Compound**	**Mass transitions and their MS-parameters**				
		Q 1 (m/z)	**Q 3 (m/z)**	**DP[1] (V)**	**CE[2] (V)**	**CXP[3] (V)**
	2,4-DNOP	295	194	-70	-38	-9
		295	193	-70	-40	-9
		295	134	-70	-72	-5
	BNPH (IS)	301	137	-45	-16	-7
	Propyzamid-D_3 (IS)	257	231	-70	-20	-1
Ionisation mode	ESI negative					
Ion Source Parameters	**Curtain Gas Flow**	35 psi				
	Ion Spray Voltage	-4500 V				
	Temperature	470 °C				
	Nebulizer Gas Flow	60 psi				
	Heater Gas Flow	70 psi				

1) DP: Declustering Potential; 2) CE: Collision Energy; 3) CXP: Cell Exit Potential

Validation:

Using QuEChERS extraction and measurement via **LC-MS/MS ESI (neg)**, **Meptyldinocap** as such (without any transformation) and **Meptyldinocap phenol** were validated in tomatoes at 0.02 mg/kg and at 0.005 mg/kg respectively. The recovery rates obtained are shown in Table 6.

Table 6: Recoveries and relative standard variations (RSDs) obtained for meptyldinocap and meptyldinocap phenol (2,4-DNOP) in tomatoes using QuEChERS and LC-MS/MS in ESI (neg) mode, n = 5

Matrix	Spiking level (mg/kg)	Mass trace	Calculation using matrix-matched calibration					
			w/ ISTD Propyzamide-D_3		w/ ISTD BNPH		w/o ISTD	
			Mean Rec.	RSD	Mean Rec.	RSD	Mean Rec.	RSD
Meptyldinocap	0.02	295/194	97 %	7 %	99 %	6 %	106 %	7 %
		295/193	95 %	10 %	96 %	9 %	102 %	10 %
		295/134	97 %	5 %	98 %	6 %	105 %	5 %
Meptyldinocap phenol	0.005	295/194	95 %	2 %	103 %	1 %	103 %	1 %
		295/193	95 %	1 %	104 %	2 %	104 %	2 %
		295/134	94 %	1 %	102 %	1 %	102 %	1 %

Using QuEChERS extraction and measurement via LC-ToF ESI (neg), **Meptyldinocap** was validated in grapes at 0.02 and 0.1 mg/kg both as such (direct analysis) as well as following transformation **to Meptyldinocap phenol** (2,4-DNOP). The recovery rates obtained are shown in Table 7.

Table 7: Recoveries and relative standard variations (RSDs) obtained for meptyldinocap in grapes with measurement using Bruker compact QTOF in ESI (neg) mode, n = 5

Spiking level (mg/kg)	m/z (monoisotopic)	Sum formula [M-H]-	Calc. via matrix-matched calibration	
			Meptyldinocap (Meptyldinocap following hydrolysis to 2,4-DNOP)	
			Mean Rec.	RSD
0.02	295.1299 Q 209.0204 193.0255	$C_{14}H_{19}N_2O_5$ $C_8H_5N_2O_5$ $C_8H_5N_2O_4$	93 % (93 %)	8 % (4 %)
0.1			105 % (100 %)	5 % (9 %)

Validation experiments for meptyldinocap following transformation to its corresponding phenol 2,4-DNOP were conducted for all four main matrix groups. Meptyldinocap was spiked in quintuplicate to 10 g portions (high water content commodities), 5 g (dry commodities) and 2 g (high oil content commodities) of sample homogenate.
All samples were extracted using the citrate buffered QuEChERS approach with the exception of the peanuts that were extracted by the QuOil method.
Matrix-matched calibration solutions as well as internal standards (BNPH and propyzamide-D3) were used. The obtained recovery rates are shown in Table 8. Exemplary chromatograms are shown in Figure 9 and Figure 10.

Table 8: Recoveries and relative standard variations (RSD) of the validation of meptyldinocap, measured as 2,4-DNOP after alkaline hydrolysis, in various matrices at 0.005 mg/kg, n = 5

Matrix	Spiking level (mg/kg)	Mass trace	Calculation using matrix-matched calibration						Amount of aqueous ammonia solution (25%) added
			w/ ISTD Propyzamide-D_3		w/ ISTD BNPU[12]		w/o ISTD		
			Mean Rec.	RSD	Mean Rec.	RSD	Mean Rec.	RSD	
Cucumber	0.005	295/194	83 %	10 %	80 %	17 %	82 %	8 %	**+ 25 µL** (per mL extract)
		295/193	87 %	9 %	85 %	15 %	85 %	6 %	
		295/134	95 %	15 %	95 %	25 %	94 %	14 %	
Grapes	0.005	295/194	83 %	10 %	77 %	8 %	80 %	7 %	
		295/193	89 %	11 %	83 %	9 %	86 %	8 %	
		295/134	75 %	8 %	70 %	7 %	72 %	4 %	
Whole wheat flour	*0.005*	*295/194*	*77 %*	*15 %*	*98 %*	*8 %*	*85 %*	*11 %*	
		295/193	*90 %*	*11 %*	*110 %*	*5 %*	*96 %*	*6 %*	
		295/134	*83 %*	*14 %*	*101 %*	*8 %*	*88 %*	*9 %*	
Peanut butter	*0.005*	*295/194*	*73 %*	*9 %*	*72 %*	*13 %*	*70 %*	*9 %*	
		295/193	*79 %*	*16 %*	*79 %*	*15 %*	*77 %*	*16 %*	
		295/134	*81 %*	*17 %*	*83 %*	*23 %*	*80 %*	*17 %*	
Bovine liver	0.005	295/194	83 %	2 %	91 %	10 %	87 %	12 %	
		295/193	83 %	3 %	91 %	10 %	86 %	12 %	
		295/134	83 %	2 %	91 %	10 %	86 %	13 %	
Whole wheat flour	0.005	295/194	85 %	2 %	89 %	4 %	89 %	3 %	**+ 75 µL** (per mL extract)
		295/193	89 %	2 %	92 %	3 %	92 %	2 %	
		295/134	87 %	6 %	89 %	4 %	89 %	6 %	
Peanut butter	0.005	295/194	83 %	6 %	86 %	6 %	85 %	7 %	
		295/193	80 %	4 %	82 %	3 %	81 %	4 %	
		295/134	86 %	4 %	87 %	3 %	87 %	2 %	

[12] BNPU= 1,3-bis(4-nitrophenyl)urea (a component of nicarbazine).

Exemplary chromatograms of the validation experiments:

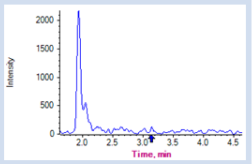
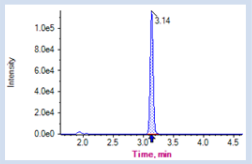
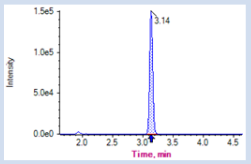
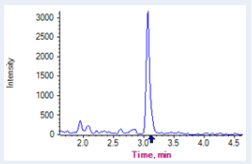
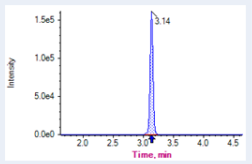
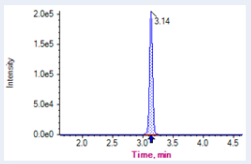
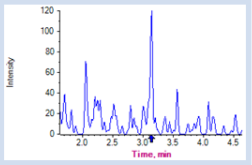
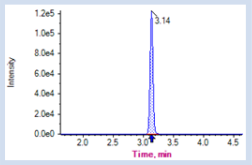
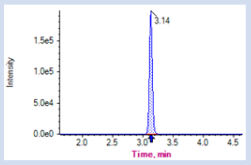
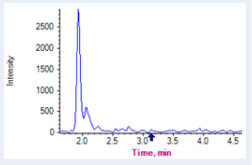
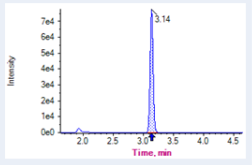
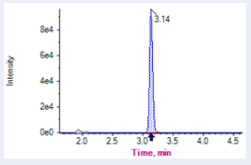
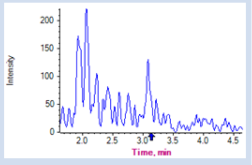
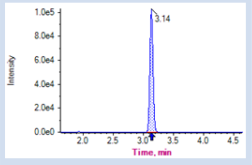
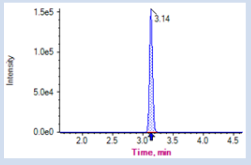
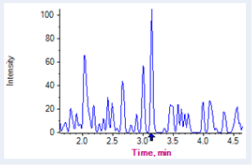
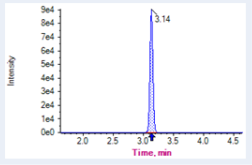
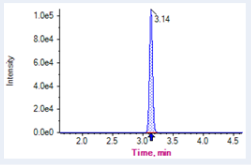
Substance spiked	Matrix	Level (mg/kg)	Mass transition	Matrix blank	Matrix calibration (60 % level)	Recovery
Meptyldinocap	Cucumber	0.005	295/194			
			295/193			
			295/134			
Meptyldinocap	Grapes	0.005	295/194			
			295/193			
			295/134			

Figure 9: Selected chromatograms of the conducted validation of meptyldinocap following hydrolysis to 2,4-DNOP, in cucumber and grapes at 0.005 ppm (the fast LC-method was employed)

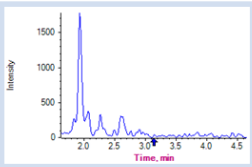
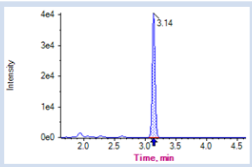
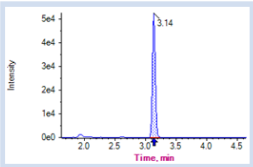
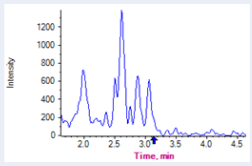
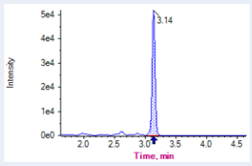
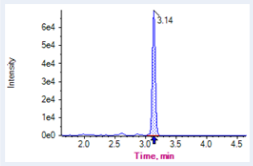
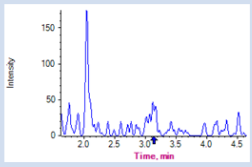
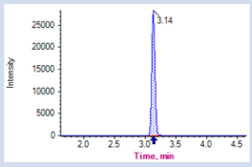
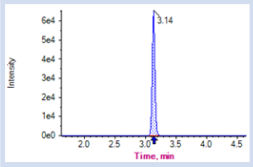
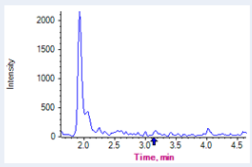
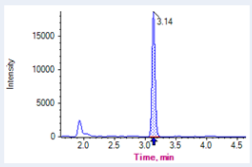
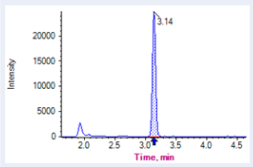
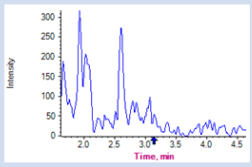
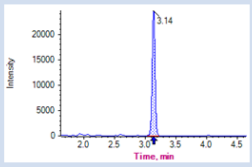
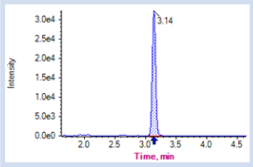
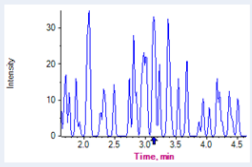
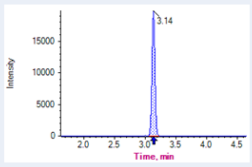
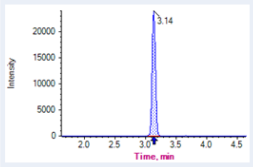
Substance spiked	Matrix	Level (mg/kg)	Mass transition	Matrix blank	Matrix calibration (60 % level)	Recovery
Meptyldinocap	Wheat flour	0.005	295/194			
			295/193			
			295/134			
Meptyldinocap	Peanut butter	0.005	295/194			
			295/193			
			295/134			

Figure 10: Selected chromatograms of the conducted validation of meptyldinocap following hydrolysis to 2,4-DNOP, in wheat flour and peanut butter at 0.005 ppm (the fast LC-method was employed)

Miscellaneous Observations:

Stability of meptyldinocap during sample extraction and in the final extract:

The stability of meptyldinocap during sample preparation was briefly studied in the case of tomato (slightly acidic commodity). Tomato homogenates were spiked with meptyldinocap in different ways:
a) in frozen condition with immediate extraction; or
b) in thawed condition at RT, followed by a standing time of 2h at RT before extraction

Additionally, a blank extract was spiked and left standing for 24 h at room temperature before measurement. Degradation to the phenol was negligible in all cases. This correlates with information regarding the hydrolytic degradation of meptyldinocap, which is reportedly slow at low pH[13]. Further experiments regarding the stability of meptyldinocap using high pH commodities, will follow.

Measurement of parent meptyldinocap and the composition of its analytical standard:

Meptyldinocap parent shows very poor signals in the LC-MS/MS ESI (pos) mode[14] (data not shown) and moderately sensitive signals in the ESI (neg) mode with the most intensive signals deriving from its ion-source fragment the meptyldinocap phenolate that serves as parent ion. Meptyldinocap phenol as such can be detected with excellent sensitivity under the same conditions. Meptyldinocap and meptyldinocap phenol can thus be analyzed in the same mass trace, but care is needed to ensure that the two peaks separate chromatographically (see Figure 11).

Typically, the injection of meptyldinocap standards results in two LC peaks within the same mass trace: one deriving from the in-source fragmentation of meptyldinocap and the other one deriving from the phenol, which is contained as an impurity (typically 1-2%) within meptyldinocap stock and working solutions. Using a C_{18} column the phenol elutes earlier, see Figure 12.

Despite being by far underrepresented in the meptyldinocap mixture (meptyldinocap:2,4-DNOP ratio ~ 80:1 in this case), **the phenol impurity shows a more sensitive signal than meptyldinocap**. This suggests a much better ionization rate of the phenol as it is already present in the parent ion form and does not need to be formed through in-source fragmentation as in the case of meptyldinocap.
Instrument tuning is conducted using a standard containing the phenol as impurity with the phenol producing more ions than the parent thus influencing the autotune much more than the parent. To gain better sensitivity for meptyldinocap parent, special tuning of MS parameters involving **chromatographic separation between meptyldinocap and its phenol would be needed**. The declustering potential is expected to play some role but also the ion-source temperature, with higher temperatures possibly promoting the ion-source fragmentation.

[13]Streelman, D.R., 1981. Hydrolysis Study of Karathane (dinocap). Rohm and Haas Technical Report Number 36F-81-14. (ER 13.5). Unpublished; https://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation98/dinocap.pdf

[14] Meptyldinocap parent was measured as ammonium adduct. No peaks could be detected even at concentrations of 0.1 µg/mL in ACN.

IMPORTANT ADVICE: Where meptyldinocap standards are injected, that contain relatively high amounts of 2,4-DNOP (as an original impurity or formed through degradation in solution) this may lead to misinterpretations and erroneous peak allocation.

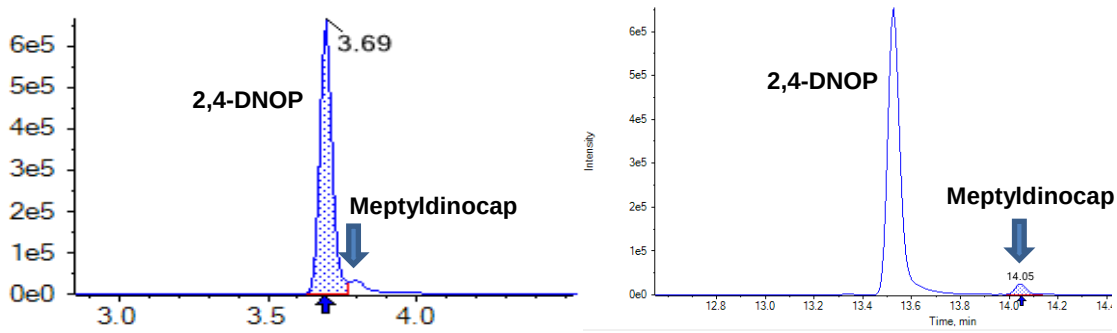


Figure 11: Chromatograms showing the separation between meptyldinocap and 2,4-DNOP using the fast and the slow LC-MS/MS gradient (see Table 5). 2,4-DNOP at 0.005 and meptyldinocap at 0.02 µg/mL were injected.

All in all, the procedure involving chemical transformation of meptyldinocap to its phenol (2,4-DNOP) in the sample extracts via alkaline hydrolysis, enables a much more sensitive analysis, compared to the analysis of the two components separately, with meptyldinocap (parent) being the limiting component in terms of overall sensitivity.

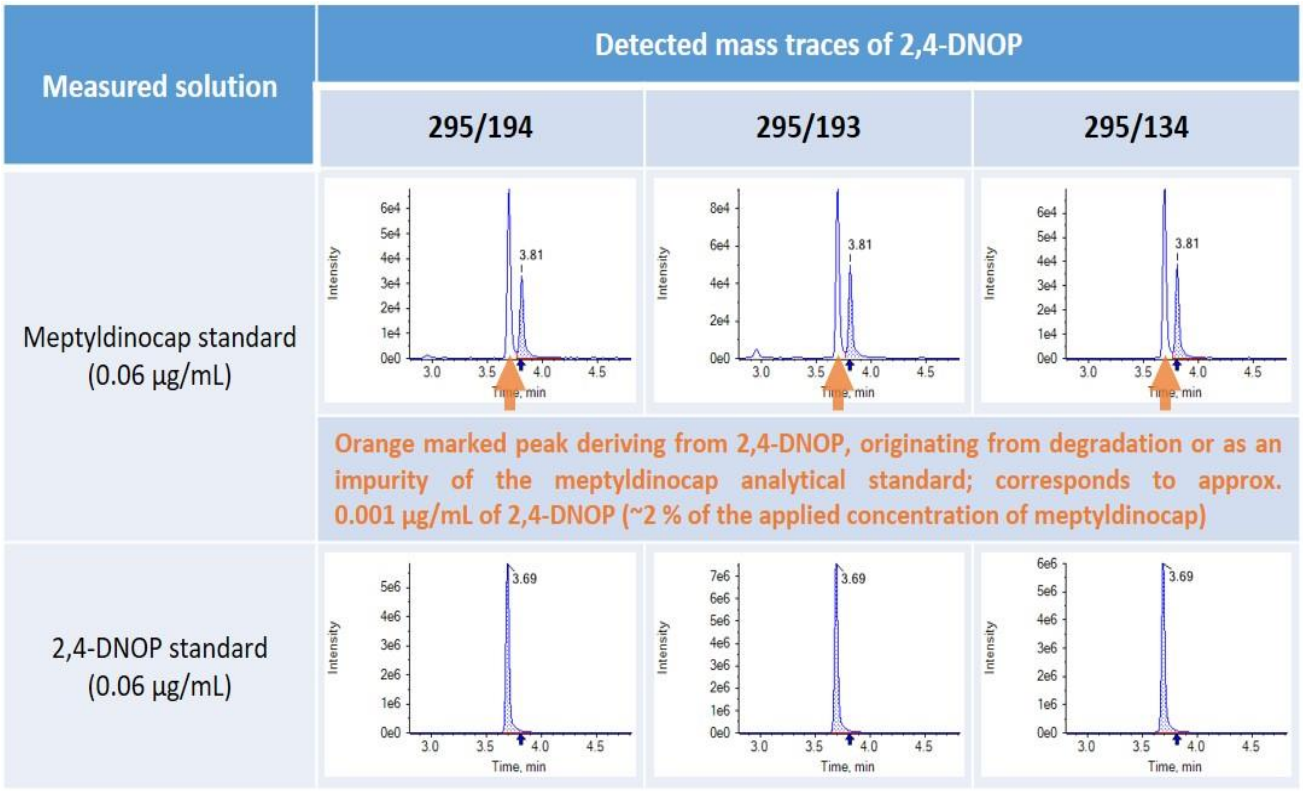


Figure 12 Chromatograms of meptyldinocap, measured as its in-source fragment 2,4-DNOP. These chromatograms were generated by the fast method.

Optimization of the hydrolysis conditions:

To optimize the hydrolysis conditions at room temperature, 10 µL of 25% aqueous ammonia solution was added to 500 µL QuEChERS extract spiked with meptyldinocap as well 2,6-DN-MH[15]. The vials were put into the autosampler at room temperature, and analyzed repeatedly via LC-MS/MS at regular intervals (30 min), to record progression of hydrolysis.
As shown in Figure 13, quantitative hydrolysis of dinocap esters in QuEChERS grape extract was achieved at room temperature within 3 hours in the case of 2,6-DN-MH-PC and within 12 hours in the case of 2,4-DN-MH-PC (meptyldinocap). The faster hydrolyzability of the 2,4 congeners compared to that of the 2,6 congeners was also observed for other dinocap isomers. This can be explained by steric reasons, as in the case of 2,4-congeners the large octyl chain is in ortho position to the phenolic moiety whereas in the case of the 2,6-congeners it is in para position.
Overall, meptyldinocap was found to be a suitable compound for optimizing the hydrolysis conditions.

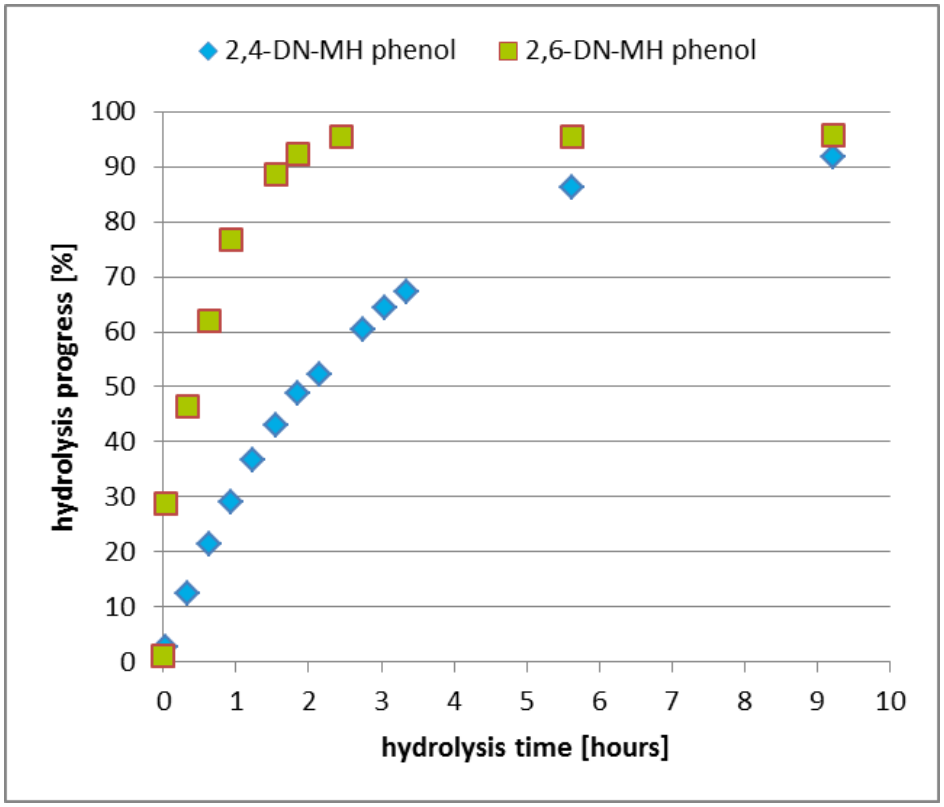


Figure 13: Progression of hydrolysis in grape extract at room temperature, shown exemplary using 2,4-DN-MH-PC (blue)and 2,6-DN-MH-PC (green). Quantifier m/z 295/134 for 2,4-DN-MH and m/z 295/209 for 2,6-DN-MH

The other 2,6-DNOPCs' and 2,4-DNOPCs' behavior was identical to that of their corresponding 2,6-DN-MH- and 2,4-DN-MH-PCs, respectively. 2,6-DNOPCs thus hydrolyzed significantly faster than 2,4-DNOPCs; this is likely the result of the greater distance of the bulky alkyl sidechain from the ester bond in 2,6-DNOPCs, as compared to 2,4-DNOPCs.

[15] 2,6-dinitro-4-(1-methylheptyl)-phenyl)-crotonate (was donated by a former applicant of dinocap)

Intermediate Conclusions and Outlook:

A simple and sensitive method for the analysis of meptyldinocap (sum), involving transformation of meptyldinocap to the corresponding phenol (2,4-DNOP) in sample extracts was developed.
Following extraction via QuEChERS or QuOil the extracts are subjected to a simple alkaline hydrolysis overnight. Following neutralization, measurement is conducted via LC-MS/MS in the ESI(neg) mode on a C_{18} column.
A direct measurement of meptyldinocap and 2,4-DNOP individually via LC-(ESI-neg)-MS/MS is also possible. Meptyldinocap undergoes fragmentation to the phenol within the ion source. Parent and phenol can thus be analyzed in one chromatographic run and are even detected within the same MRM-traces. Unfortunately, the detection sensitivity of meptyldinocap (via its in-source fragment) is rather poor, which compromises overall sensitivity of the method. The approach involving alkaline hydrolysis to 2,4-DNOP is much more sensitive overall.
Validation of meptyldinocap, following its conversion to the corresponding phenol (2,4-DNOP), was successful in cucumber, grapes, wheat flour, peanut butter and bovine liver at 0.005 mg/kg. Based on preliminary experiments, parent meptyldinocap remains stable during QuEChERS extraction.
Meptyldinocap residues in samples may derive either from the use of meptyldinocap or from dinocap (contains meptyldinocap as a component) and, less likely, from the use of both. Typically, dinocap is well distinguishable from meptyldinocap as it shows a more complex LC-MS/MS peak pattern. Formally, the residue definition of meptyldinocap will apply if no other components of dinocap or their corresponding phenols are detected. A chromatographic separation of all components of dinocap (parents and phenols) is thus important but unfortunately, this is mostly not the case with standard. LC- and GC- separation methods.
Further work will focus on improving the chromatographic separation of all dinocap components, to enable proper quantitative analysis of dinocap (sum). The availability of analytical standards for all components, the six parents and the six phenols, is however a prerequisite for this.

History

Action	When	Document Version
Initial LC-MS/MS Experiments	Nov – Dec 2014	
Initial Hydrolysis Experiments	Jan – August 2016	
Preparation of poster	May 2016	Procedure involving hydrolysis in vial: https://www.eurl-pesticides.eu/userfiles/file/EurlSRM/EPRW2016_Lemke_PD_060_Dinocap.pdf
Further Experiments	Jan – Feb 2017	
Hydrolysis Validation Experiments	March 2018	
Observation document placed on-line	Jan 2022	V1
Observation document updated: Wrong slow gradient was proposed in table 5	March 2022	V1.1
Correction of some typos	August 2022	V1.2