Validation of analytical methods for contaminants in foods according to EU. Examples of screening and confirmatory methods for antibiotics and pesticides

Dario Compagnone

FoodQA Teramo 29 Jan 2018

Food Chemical Hazards

Dario Compagnone,
dcompagnone@unite.it

FoodQA Teramo 29 Jan 2018

Validation

Establishing documented evidence that provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes

"Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use."

There are many reasons for the need to validate analytical procedures. Among them are **regulatory requirements, good science, and quality control requirements.**

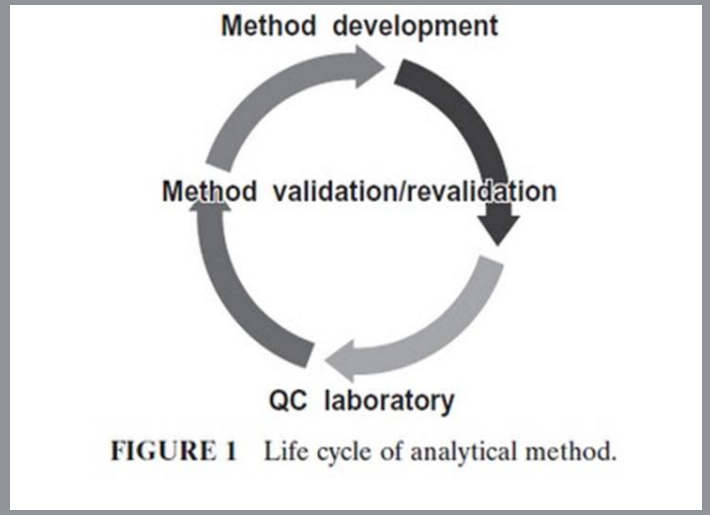


FIGURE 1 Life cycle of analytical method.

Typical validation characteristics which should be considered are:.

1) Accuracy
2) Precision
3) Specificity
4) Linearity
5) Range
6) Detection Limit
7) Quantitation Limit
8) Robustness/Ruggedness
9) Noise
10) Trueness
11) Sensitivity

Classifications of residues (contaminants)

Directive 96/23/CE

GROUP A — Substances having anabolic effect and unauthorized substances

(1) Stilbenes, stilbene derivatives, and their salts and esters
(2) Antithyroid agents
(3) Steroids
(4) Resorcylic acid lactones including zeranol
(5) Beta-agonists
(6) Compounds included in Annex IV to Council Regulation (EEC) No 2377/90 of 26 June 199)

Classifications of residues (contaminants)

Dir. 96/23/CE

GROUP B — Veterinary drugs and contaminants

(1)**Antibacterial substances, including sulphonomides, quinolones**
(2) Other veterinary drugs
(a) Anthelmintics
(**b) Anticoccidials, including nitroimidazoles**
(c) Carbamates and pyrethroids
(d) Sedatives
(e)Non-steroidal anti-inflammatory drugs (NSAIDs)
(f) Other pharmacologically active substances

(3) Other substances and environmental contaminants
(a) Organochlorine compounds including PcBs
(b) Organophosphorus compounds
(d) Chemical elements
(d) Mycotoxins
(e) Dyes
(f) Others

DECISION 2002/657/CE

Art. 1

The Decision states the rules for the analytical methods for the official methods of analysis

Art. 3

EU member states guarantee that the official samples will be assayed with analytical methods

- with documented instructions;
- following this the rules of this Decision;
- **validated according to the Decision.**

DECISION 2002/657/CE

Art. 6

The output of an analysis will be considered non-compliant if the decision limit (CCa) is exceeded with a confirmatory method

1. If a permitted limit has been established for a substance, the decision limit is the concentration above which it can be decided with a statistical certainty of 1 – a that the permitted limit has been truly exceeded.

2. If no permitted limit has been established for a substance, the decision limit is the lowest concentration level at which a method can discriminate with a statistical certainty of 1 – a that the particular analyte is present.

DECISION 2002/657/CE

Classification of analytical methods

Screening methods

Only those analytical techniques, for which it can be demonstrated in a documented traceable manner that they are validated and have a false compliant rate of < 5 % (β-error) at the level of interest shall be used for screening purposes in conformity with Directive 96/23/EC. In the case of a suspected non-compliant result, this result shall be confirmed by a confirmatory method.

Confirmatory methods

Confirmatory methods for organic residues or contaminants shall provide information on the chemical structure of the analyte. Consequently methods based only on chromatographic analysis without the use of spectrometric detection are not suitable on their own for use as confirmatory methods. However, if a single technique lacks sufficient specificity, the desired specificity shall be achieved by analytical procedures consisting of suitable combinations of clean-up, chromatographic separation(s) and spectrometric detection

Table 1

Suitable confirmatory methods for organic residues or contaminants

Measuring technique	Substances Annex 1 96/23/EC	Limitations
LC or GC with mass-spectrometric detection	Groups A and B	Only if following either an on-line or an off-line chromatographic separation Only if full scan techniques are used or using at least 3 (group B) or 4 (group A) identification points for techniques that do not record the full mass spectra
LC or GC with IR spectrometric detection	Groups A and B	Specific requirements for absorption in IR spectrometry have to be met
LC-full-scan DAD	Group B	Specific requirements for absorption in UV spectrometry have to be met
LC -fluorescence	Group B	Only for molecules that exhibit native fluorescence and to molecules that exhibit fluorescence after either transformation or derivatisation
2-D TLC - full-scan UV/VIS	Group B	Two-dimensional HPTLC and co-chromatography are mandatory
GC-Elektron capture detection	Group B	Only if two columns of different polarity are used
LC-immunogram	Group B	Only if at least two different chromatographic systems or a second, independent detection method are used
LC-UV/VIS (single wavelength)	Group B	Only if at least two different chromatographic systems or second, independent detection method are used.

DECISION 2002/657/CE

Table 9

Classification of analytical methods by the performance characteristics that have to be determined

		Detection limit CCß	Decision limit CCα	Trueness/recovery	Precision	Selectivity/ specificity	Applicability/ ruggedness/ stability
Qualitative methods	S	+	–	–	–	+	+
	C	+	+	–	–	+	+
Quantitative methods	S	+	–	–	+	+	+
	C	+	+	+	+	+	+

S = screening methods; C = confirmatory methods; + = determination is mandatory.

DECISION 2002/657/CE

Common criteria for analytical methods

Specificity/selectivity

Ability of a method to selectively detect the analyte. Interferences from the matrix must be studied with similar compounds and metabolites.

Recovery

Amount (%) of the analyte that is recovered during the analytical procedure, a recovery factor for each sample lot must be applied

DECISION 2002/657/CE

Performance criteria

Trueness

- Trueness means the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. Trueness is usually expressed as bias. Calculated using certified reference material or fortifiying samples

mass fraction	Tolerated range
≤ 1 µg/kg	da –50% a +20 %
> 1 µg/kg ÷ 10 µg/kg	da –30% a +10%
≥ 10 µg/kg	da -20% a +10%

DECISIONE 2002/657/CE

Performance criteria for analytical methods

Precision

Relative standard deviation (CV%), includes repeatability and reproducibility intra-lab

Concentration (µg/kg)	CV%
1	(*)
10	(*)
100	23
200	21
500	18
1000	16

DECISION 2002/657/CE

Ruggedness/robustnesss

Ruggedness means the susceptibility of an analytical method to changes in experimental conditions which can beexpressed as a list of the sample materials, analytes, storage conditions, environmental and/or sample preparation conditions under which the method can be applied as presented or with specified minor modifications. For all experimental conditions which could in practice be subject to fluctuation (e.g. stability of reagents, composition ofthe sample, pH, temperature) any variations which could affect the analytical result should be indicated.

Stability of the analyte in solution and in the sample

DECISION 2002/657/CE

Calibration curve

5 levels (including zero).

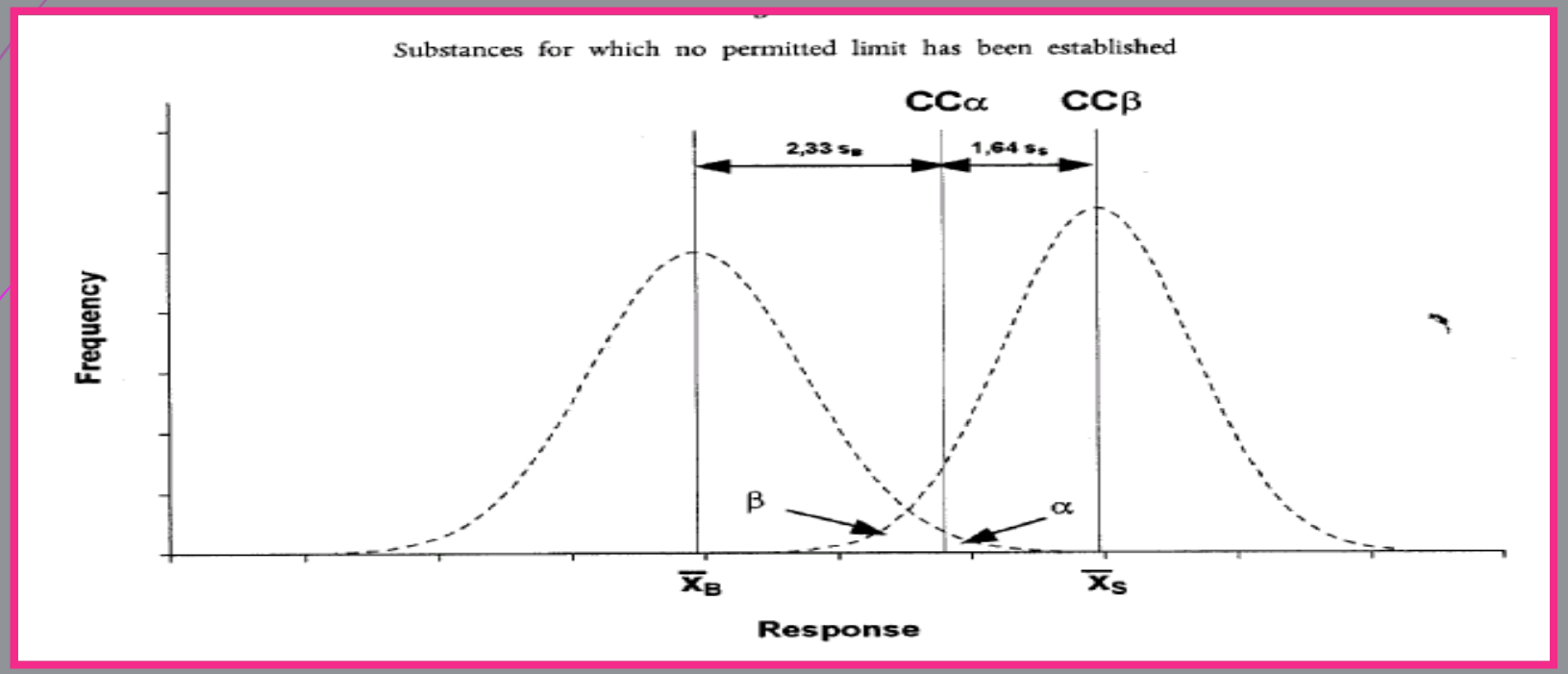
Establish acceptabilty criteria i.e

- ✓ determination coefficient $r^2 \geq 0.990$;
- ✓ ratio y/x; for each point the y/x ratio should be in the average (y/x) $\pm$ 10%

DECISION 2002/657/CE

CCα: compounds with no MRL simplified approach

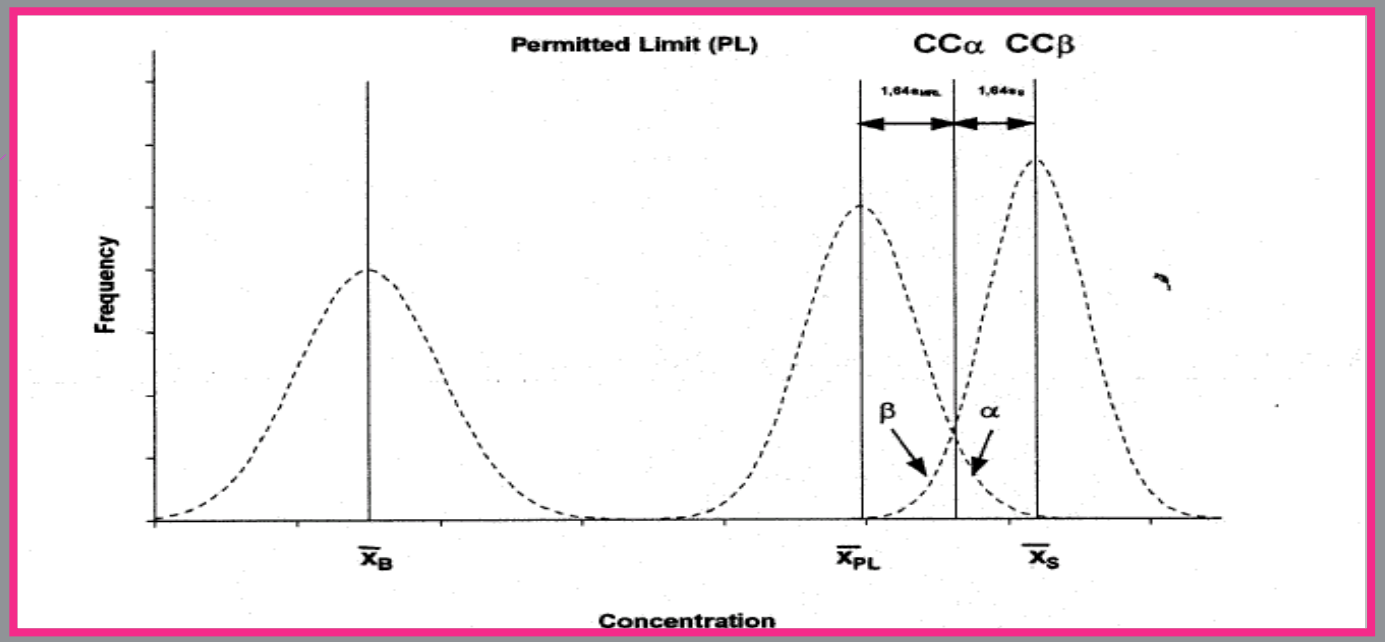
20 blank samples . Calculate the signal to noise ratio at the retention time of the analyte CCα = 3 S/N.



DECISION 2002/657/CE

CCα: compounds with MRL simplified approach

Use 20 blank samples fortified at MRL, the concentration at MRL + 1.64 the standard deviation is the CCα.



DECISION 2002/657/CE

Detection capability (CCβ)

Detection capability (CCβ) means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1 – β.

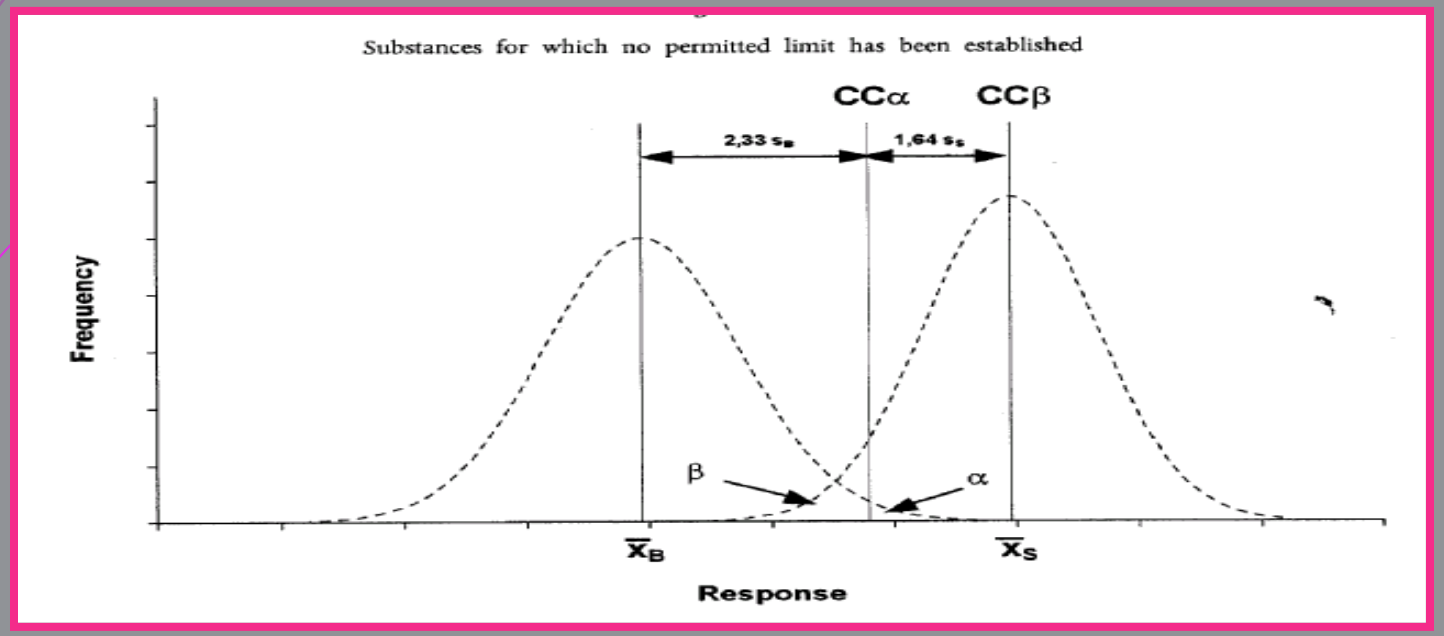
In the case of substances with an established permitted
limit, this means that the detection capability is the concentration at which the method is able to detect permitted
limit concentrations with a statistical certainty of 1 – β.

Beta (β) error means the probability that the tested sample is truly non-compliant, even though a compliant measurementhas been obtained (false compliantdecision).

DECISION 2002/657/CE

CCβ: compounds with no MRL simplified approach

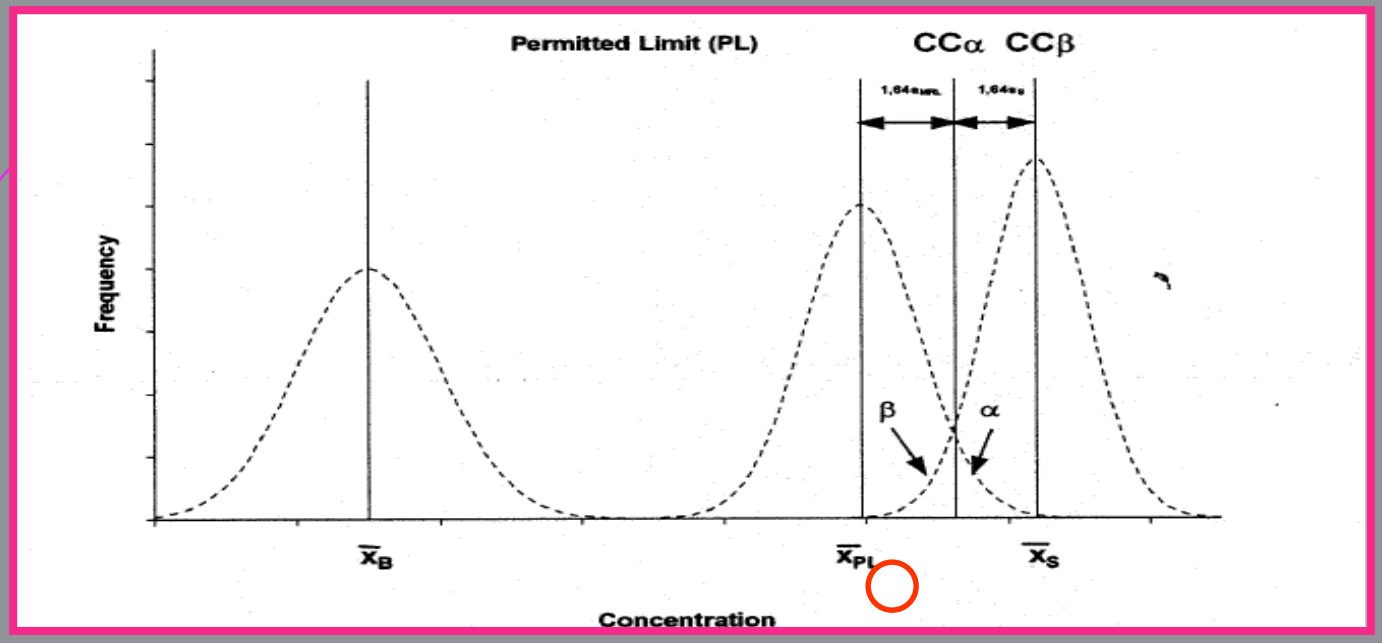
20 blank samples fortified at the decision limit. CCβ is the concentration of CCα + 1.64 the standard deviation of the intra-laboratory reproducibility (β = 5%).



DECISION 2002/657/CE

CCβ: compounds with MRL simplified approach

20 blank samples fortified at the decision limit. CCβ is the concentration of CCα + 1.64 the standard deviation of the intra-laboratory reproducibility (β = 5%).

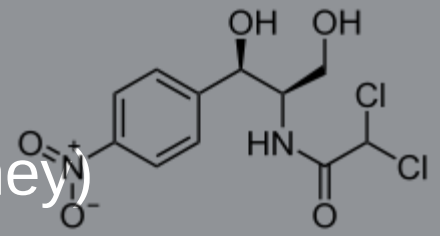


Validation of a screening ELISA method for bacteriostatic antibiotic chloramphenicol CAP

- cut,
- Precision;
- Specificity/Selectivity;
-Robustness/Stability

minimum required performance limit (MRPL),

CAP (0,3 µg/kg) (meat, acquacolture, eggs, milk, honey)



Validation of elisa for cap

Sample preparation

- CAP extraction from meat (muscle), eggs and honey has been achieved with acetone/dichloromethano (1:1, v/v), followed by a purification on alumina SPE (muscle and egg) or C_{18} (honey).
- milk sample were treated in 2 different ways as suggested by the producer of the CAP ELISA kit (Euro-Diagnostica B.V).
- 50 μl of the final solution have been used in the ELISA .

Validation plan

CCβ: 20 blanks for each type of sample (muscle, egg, honey, milk) added at the MRPL (0,3 µg/kg)*.

Specificity/Selectivity: 20 representative blank samples for each type (bovine, ovine, swine, poultry species included in the National Residues Plan) + egg milk honey from different sources and production process. Samples of bovine muscle fortifiedhave been fortified with 0,3 µg/kg di CAP and with tiamphenicol (TIF) e Florfenicol (FF), a concentrazions corrispondent at their (MLR) per il muscolo and 5 x LMR (50-250 µg/kg for TIF and 200-1000 µg/kg for FF), *

*S. Hooijerink et al. Analytica Chimica Acta 483 (2003) 51

*S. Impens et al. Analytica Chimica Acta 483 (2003) 153.

Validation

- **Precision/Recovery**: for each type of matrix, fortified 18 blanks at 0,30-0,45-0,60 µg/kg (6 replicates each level).
- **LOD/LOQ**: 3 x SD of the blank (LOD) 10 x SD of the blanks (LOQ) *.
- **Robustness**: Youden* approach introducing "minor changes" in some parameters of the estraction procedureto 7 variables shown in **Tabella 1**.

Variable selezionata	Unità	Abbrev.[a]	Livello "alto"	Livello "basso"
% Diclorometano miscela estrazione	%	A,a	55	45
% Metanolo miscela eluente SPE	%	B,b	85	75
Età cartuccia SPE	-	C,c	Vecchia	Nuova
Modalità eluizione SPE	-	D,d	Sempre bagnata [b]	Lasciata asciugare [c]
Volume eluizione SPE	ml	E,e	6.5	5.5
Temperatura evaporazione estratto finale	°C	F,f	55	45
Modalità evaporazione estratto finale	-	G,g	A secco, subito ripreso	A secco + 5 min

*EURACHEM Guide, The Fitness for Purpose of Analytical Methods, 1998.
*W.J. Youden, E.H. Steiner. Statistical Manual of AOAC (Association of Official Analytical Chemists), (1975) 33.

Robustness

Selected variables for the test

Variable selezionata	Unità	Abbrev.[a]	Livello “alto”	Livello “basso”
% Diclorometano miscela estrazione	%	A,a	55	45
% Metanolo miscela eluente SPE	%	B,b	85	75
Età cartuccia SPE	-	C,c	Vecchia	Nuova
Modalità eluizione SPE	-	D,d	Sempre bagnata [b]	Lasciata asciugare [c]
Volume eluizione SPE	ml	E,e	6.5	5.5
Temperatura evaporazione estratto finale	°C	F,f	55	45
Modalità evaporazione estratto finale	-	G,g	A secco, subito ripreso	A secco + 5 min

Robustness

8 samples added with CAP at 0,3 µg/kg. Recovery was evaluated according to the following experimental design .

Variabile	**Esperimento ≠**							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
% Diclorometano miscela estrazione	A	A	A	A	a	a	a	a
% Metanolo miscela eluente SPE	B	B	b	b	B	B	b	b
Età cartuccia SPE	C	c	C	c	C	c	C	c
Modalità eluizione SPE	D	D	d	d	d	d	D	D
Volume eluizione SPE	E	e	E	e	e	E	e	E
Temperatura evaporazione estratto finale	F	f	f	F	F	f	f	F
Modalità evaporazione estratto finale	G	g	g	G	g	G	G	g
Risultato osservato	**s**	**t**	**u**	**v**	**w**	**x**	**y**	**z**

CCβ - LOD - LOQ

CAP was detected in all samples. CCβ was then < 0,3 µg/kg, the method is then able to detect CAP aat the MRPL with an error β < 0,05 (**Tabella 3**).

Parameter	Eggs	Muscle	Honey	Milk[c]	Milk[d]
Media bianchi (µg/Kg)[a]	0,0047	0,0074	0,025	0,082	0,041
LOD (µg/Kg)	0,0076	0,018	0,063	0,22	0,11
LOQ (µg/Kg)	0,014	0,044	0,151	0,54	0,28
Recupero ± SD (%)[b]	70,9±8,2	78,3±13,7	98,6±16,3	83,1±19,5	106,4 ± 9,8
CCβ (µg/Kg)	< 0,3	< 0,3	< 0,3	< 0,3	< 0,3

[a] 20 representative blanks

[b] 20 blanks fortified at RMPL (0,3 µg/kg)

[c] Procedure a)

[d] Procedure b)

Precision/Recovery

CV (5,5-17,3%) and mean recovery (78,2-107,5%) were satisfactory for all the matrices/concentrations

Cochran test and ANOVA dimostrated that precision and recovery did not vary in the0,3-0,6 μg/kg range (p=0.05).

Matrice	Livello aggiunta[a] (μg/Kg)	Ripetibilità (CV%)	Recupero (%)
Eggs	0,30	5,5	80,6
	0,45	11,8	78,2
	0,60	15,9	89,7
	Globale[b]	**13,0**	**82,8**
Muscle	0,30	6,7	78,2
	0,45	6,5	91,7
	0,60	12,8	88,7
	Globale[b]	**9,7**	**88,0**
Honey	0,30	16,9	96,2
	0,45	10,3	103,0
	0,60	17,3	98,9
	Globale[b]	**14,7**	**99,4**
Milk	0,30	10,7	96,2
	0,45	7,4	103,0
	0,60	13,5	107,5
	Globale[b]	**10,6**	**105,0**

[a] 6 replicates for each level

[b] Total dta 18 replicates at 3 levels

Robustness

Data obtained from high level and low levels were subtracted to the mean value obtained, the difference was expressed as % recovery . The critical t value (2-sided) was acceptable in all cases

Variabile	Differenza (D) % Recupero (valore assoluto)	Valore di *t*
% Diclorometano miscela estrazione	5,3	0,55
% Metanolo miscela eluente SPE	7,1	0,73
Età cartuccia SPE	5,3	0,55
Modalità eluizione SPE	5,0	0,52
Volume eluizione SPE	3,6	0,37
Temperatura evaporazione estratto finale	14,5	1,50
Modalità evaporazione estratto finale	0,8	0,08

$$t = \frac{\sqrt{n} \cdot |D|}{\sqrt{2} \cdot SD}$$

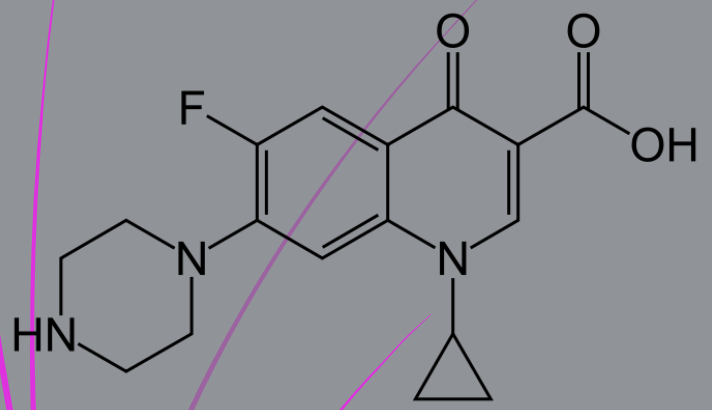
n = 4 (numero di replicates per level/parameter) e and CV = 13,7% (**t critical value (2-sided) = 2,09**, ν = 20-1, 95% probability

Specificity/Selectivity

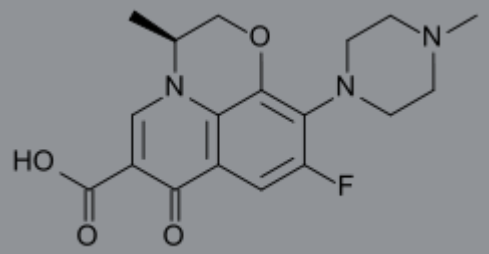
No relevant effect for the samples added with TIF and FF on the CAP data. For milk liquido/liquid extraction with etil-acetat was selected because more reproducible.

The method is validated !

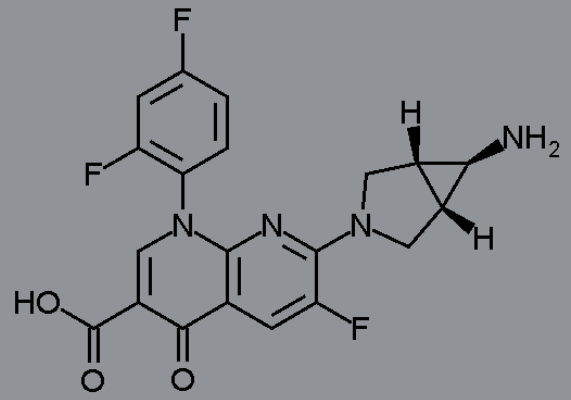
Quinolones in animal feed category B1



ciprofloxacin



levofloxacin



trovafloxacin

Quinolones: Reg. (UE) n.37/2010

Analite	Specie [a]	MLR (µg/kg)
Danofloxacin	bovine, ovine, poultry other species	200 100
Difloxacin	bovine, ovine, poultry , swine other species	400 300
Enrofloxacin	All the species	100
Flumequin	bovine, ovine, poultry, swine, fish	200 400 600
Marbofloxacin	bovine, swine	150
Oxolinic Acid	All the species	100
Sarafloxacinn	*Salmonidae*	30
[a] not for species producing eggs for human consumption		

analytical procedure

ESTRACTION[a]: 5 g of sample + 20 ml (+20 ml) di methanol/phosphoric acid 1% (40:60, v/v);
20 ml dried under at 50°C to evaporate methanol.

PURIFICATION: OASIS HLB (500mg/3ml) conditioned with 2 ml methanol and 2 ml water;
wash with 5 mlmetafphosphoric acid 1% and 5 ml water ;
eluition with 5 ml di 30% ammonia /methanol (5:95, v/v).

ENRICHMENT: solvent evapration and dilution in 0.1%.formic acid

INSTRUMENTAL Analysis: HPLC-MS/MS.

[a] on muscle samples 100 µg/kg norfloxacin-d5 (SI) are added;
on eggs 10 µg/kg norfloxacin-d5

HPLC-MS/MS

- HPLC Column: X-TERRA C18 100 x 2,1 mm, 3,5 µm, Waters
- Flow rate 0.2 ml/min, injection volme 10 µl
- Source API – ESI +
- Analyser Quadrupole
- MRM (Multi Reaction Monitoring) modality
- Two fragmented ion for each analyte
- Quantitative analysis on higher intensity ion
- Gradient

time (min)	acetonitrile	formic acid 0.1%
0	2	98
5	70	30
9	70	30
10	2	98
25	2	98

Instrumental Linearity

- 5 concentration levels x 3
- Levels selected according to the validation levels establisehed for each type of sample
- Calibration curves built using analyte area/IS area *vs* concentration

fortification levels muscle

Analyte	0.5 LMR (µg/kg)	1 LMR (µg/kg)	1.5 LMR (µg/kg)
Marbofloxacin	75	150	225
Ciprofloxacin	50	100	150
danofloxacin [a]	50	100	200
Enrofloxacin	50	100	150
difloxacin [b]	150	300	400
oxolinic acid	50	100	150
flumequin [c]	200	400	600

[a] MLR danofloxacin 100-200 µg/kg

[b] MLR difloxacin 300-400 µg/kg

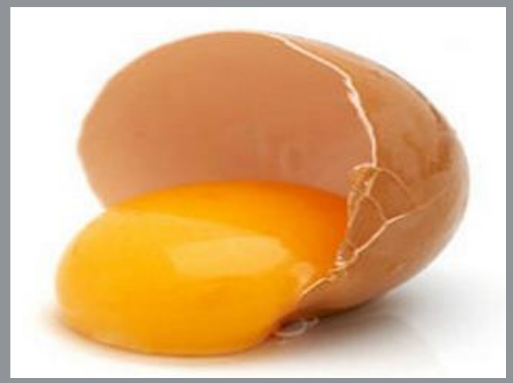
[c] MLR flumequin 200-400-600 µg/kg

fortification levels muscle unauthorised compounds

Analyte	C_0 (µg/kg)	2 C_0 (µg/kg)	3 C_0 (µg/kg)
norfloxacin	10	20	30
lomefloxacin	10	20	30
sarafloxacin	10	20	30
Nalidixic acid	10	20	30

Fortified levels eggs

- Quinolones are not allowed even in traces in eggs
- fortified levels 5-10-20 µg/kg for all the analytes



Validation Plan

Validation plan	
Procedure	n.repetitions/ levels
I	6
II	6
III	6

- Verification of the normality of the data - test Shapiro Wilk test
- Verification of outliers - Grubbs test
- Variance analysis (ANOVA)
- Recoveries calculated by calibration curve in solvent
- CV%
- Calibration curves in matrices

Validation data muscle

Analyte	fortified level (µg/kg)	Recovery% (n=18)	CV (%RSD) n=18
marbofloxacin	75-150-225	97-103-99	11-9-4
norfloxacin	10-20-30	97-102-99	16-14-8
Ciprofloxacin	50-100-150	98-102-99	12-10-5
Danofloxacin	50-10-200	91-107-99	23-16-7
Lomefloxacin	10-20-30	95-105-98	13-12-7
Enrofloxacin	50-100-150	100-100-100	9-8-5
Sarafloxacin	10-20-30	98-101-99	7-8-5
Difloxacin	150-300-400	98-102-99	8-8-10
Oxolinic acid	50-100-150	99-101-96	7-9-11
nalidixic acid	10-20-30	99-101-100	11-10-7
flumequin	200-400-600	97-103-99	13-12-7

Validation data eggs

Analyte	fortified level (µg/kg)	Recovery% (n=18)	CV (%RSD) n=18
marbofloxacin	5-10-15	100-99-100	8-9-7
norfloxacin	5-10-15	101-99-100	5-4-3
ciprofloacin	5-10-15	98-102-99	9-9-5
danofloxacin	5-10-15	100-100-100	17-17-14
lomefloxacin	5-10-15	96-104-99	11-9-11
enrofloxacin	5-10-15	99-101-100	12-15-11
sarafloxacin	5-10-15	95-105-98	16-13-10
difloxacin	5-10-15	96-104-98	18-15-12
oxolinic acid	5-10-15	100-100-100	20-21-13
Nalidixic acid	5-10-15	102-98-101	17-16-16
flumequin	5-10-15	100-100-100	13-12-17

Calculation of CCa and CCβ for compounds with MLR

$CCa = MLR + 1.64\ SD_{r,MLR}$

d where $SD_{r,\ MLR}$ is the intra-laboratory standad deviation at MRL

$CC\beta = CCa + 1.64\ SD_{r,CCa}$

where $SD_{r,CCa}$ is the inta-laboratory standard deviation at CCa. We are assuming that DS between MLR e CCa increases linearly with concentration, (CV% is constant). Thus:

$CC\beta = CCa + 1.64\ (CV\%_{pooled} \times CCa/100)$

where $CV\%_{pooled}$ is the combination of CV% observed at MRL and CV% at 1.5 LMR

Calculation of CCa and CCβ for unauthorised compounds

$CCa = C_0 + 2.33\ DS_{r,C0}$

where $DS_{r,C0}$ is the intra-lab standard deviation at the C_0 level

$CC\beta = CCa + 1.64\ DS_{r,CCa}$

where $DS_{r,CCa}$ intra-lab standard deviation at CCa. intra-lab standard deviation We are assuming that DS between C_0 and CCa increases linearly with concentration, thus:

$CC\beta = CCa + 1.64\ (CV\%_{pooled} \times CCa/100)$

where $CV\%_{pooled}$ is the combination of CV% at C_0 and CV% at $2C_0$

CCα and CCβ

Analyte	Muscle		eggs	
	CCα	CCβ	CCα	CCβ
marbofloxacin	173	194	6.0	6.8
norfloxacinn	14	17	5.6	6.1
ciprofloxacin	116	132	6.0	6.9
danofloxacin	126	151	6.9	8.8
lomefloxacin	13	16	6.3	7.5
enrofloxacin	113	126	6.4	7.7
sarafloxacin	32	35	6.9	8.7
difloxacin	339	390	7.1	9.2
oxolinic acid	115	135	7.4	9.8
nalidixic acid	13	15	7.0	9.0
flumequin	234	282	6.6	8.0

Robustness

Minor changes :

- 7 potential critical factors ;
- Tests were run on 8 negative fortified samples, using Youden approach, each parameter was varied within 10%;
- Compounds were fortified at MRL or C_0 ;

Robustness – experimental design on muscle

selected parameter	Unit	High/low	Centered value	High	low
%MeOH in the etraction mixture	%	A,a	40	44	36
T of enrichment	°C	B,b	50	55	45
SPE OASIS lot	-	C,c	-	080A38157A	084038263A
pH washing SPE	pH	D,d	3.0	3.1	2.9
% ammonia in elution mixture	%	E,e	5.0	5.5	4.5
Volume of the elution mixture	ml	F,f	5.0	5.5	4.5
% of formic acid in mobile phase	%	G,g	0.10	0.11	0.09

The method was robust CV was similar in all cases to intra-lab CV

Dioxins, dioxin-like PCBs

6.4.2017 EN Official Journal of the European Union L 92/9

COMMISSION REGULATION (EU) 2017/644

of 5 April 2017

laying down methods of sampling and analysis for the control of levels of dioxins, dioxin-like PCBs and non-dioxin-like PCBs in certain foodstuffs and repealing Regulation (EU) No 589/2014

Chemical Elements (metals)

29.3.2007 EN Official Journal of the European Union L 88/29

COMMISSION REGULATION (EC) No 333/2007

of 28 March 2007

laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs

Micotoxins

L 70/12 EN Official Journal of the European Union 9.3.2006

COMMISSION REGULATION (EC) No 401/2006

of 23 February 2006

laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs

(Text with EEA relevance)

Pesticides

ANALYTICAL QUALITY CONTROL AN METHOD VALIDATION PROCEDURES FOR PESTICIDE RESIDUES ANALYSIS IN FOOD AND FEED

Supersedes Document No. **SANTE/11945/2015. Implemented by 01/01/2018**

Pesticides

The key objectives are:

- to provide a harmonized, cost-effective quality assurance and quality control system across the EU
- to ensure the quality and comparability of analytical results
- to ensure that acceptable accuracy is achieved
- to ensure that false positives or false negatives are avoided
- to support compliance with, and specific implementation of ISO/IEC 17025 (accreditation standard)

C4 Sample comminution should ensure that the sample is homogeneous enough to ensure that sub-sampling variability is acceptable. If this is not achievable, the use of larger test portions or replicate portions should be considered in order to be able to obtain a better estimate of the true value. Upon homogenization or milling, samples may separate into different fractions, e.g. pulp and peel in the case of fruits, and husks and endosperm in the case of cereals. This fractionation can occur because of differences in size, shape and density. Because pesticides can be heterogeneously distributed between the different fractions, it is important to ensure that the fractions in the analytical test portion are in the same ratio as in the original laboratory sample. It is advisable to store in a freezer a sufficient number of sub-samples or analytical test portions for the number of analyses/repeated analyses that are likely to be required.

Pooling of samples

C5 Pooling of individual samples or sample extracts may be considered as an option for the analyses of commodities with a low frequency of pesticide residues (e.g. organic or animal products), provided that the detection system is sensitive enough. For example, when pooling 5 samples, the limit of quantification (LOQ) or screening detection limit (SDL) must be at least 5 times lower than the reporting limit (RL).

Pesticides

Clean-up, concentration/reconstitution and storage of extracts

C8 A clean-up, or dilution step may be necessary to reduce matrix interferences and reduce contamination of the instrument system leading to an improved selectivity and robustness. Clean-up techniques take advantage of the difference in physicochemical properties (e.g. polarity, solubility, molecular size) between the pesticides and the matrix components. However, the use of a clean-up step in a multi-residue method can cause losses of some pesticides.

C9 Concentration of sample extracts can cause precipitation of matrix-components and in some cases losses of pesticides. Similarly, dilution of the extract with a solvent of a different polarity can also result in pesticide losses because of decreased solubility (e.g. dilution of methanol or acetonitrile extracts with water).

C10 To avoid losses during evaporation steps the temperature should be kept as low as is practicable. A small volume of a high boiling point solvent may be used as a "keeper". Foaming and vigorous boiling of extracts, or dispersion of droplets, must be avoided. A stream of dry nitrogen or vacuum centrifugal evaporation is generally preferable to the use of an air stream for small-scale evaporation, as air is more likely to lead to oxidation or the introduction of water and other possible contaminants.

PESTICIDES

C13 Nowadays, selective detectors for GC (ECD, FPD, PFPD, NPD) and LC (DAD, fluorescence) are less widely used as they offer only limited specificity. Their use, even in combination with different polarity columns, does not provide unambiguous identification. These limitations may be acceptable for frequently found pesticides, especially if some results are also confirmed using a more specific detection technique. In any case, such limitations in the degree of identification should be acknowledged when reporting the results.

PESTICIDES

C17 Multi-level calibration (three or more concentrations) is preferred. An appropriate calibration function must be used (e.g. linear, quadratic, with or without weighing). The **deviation of the back-calculated concentrations of the calibration standards** from the true concentrations, **using the calibration curve** in the relevant region should not be more than ±20%.

C18 Calibration by interpolation between two levels is acceptable providing the difference between the 2 levels is not greater than a factor of 10 and providing the response factors of the bracketing calibration standards are within acceptable limits. The response factor of bracketing calibration standards at each level should not differ by more than 20% (taking the higher response as 100%).

C19 Single-level calibration may also provide accurate results if the detector response of the analyte in the sample extract is close to the response of the single-level calibration standard (within ±30%). Where an analyte is spiked to a sample for recovery determination purposes at a level corresponding to the LCL, recovery values <100% may be calculated using a single point calibration at the LCL. This particular calculation is intended only to indicate analytical performance achieved at the LCL and does not imply that residues <LCL may be determined in this way.

PESTICIDES

Calibration using derivative standards or degradation products

C31 Where the pesticide is determined as a derivative or a degradation product, the calibration standard solutions should be prepared from a "pure" reference standard of the derivative or degradation product, if available. Procedural standards should only be used if they are the only practical option.

Use of various internal standards

C32 An internal standard (IS) is a chemical compound added to the sample test portion or sample extract in a known quantity at a specified stage of the analysis, in order to check the correct execution of (part of) the analytical method. The IS should be chemically stable and/or typically show the same behaviour as of the target analyte.

PESTICIDES

Routine recovery check

C40 Where practicable, recoveries of all target analytes should be measured within each batch of analyses. If this requires a disproportionately large number of recovery determinations, the number of analytes may be reduced. However, it should be in compliance with the minimum number specified in Table 2. This means, that at least 10% of the representative analytes (with a minimum of 5) should be included per detection system.

Table 2. Minimum frequency of recovery checks (quantitative method performance verification)

	Representative analytes	**All other analytes**
Minimum frequency of recovery checks	10% of representative analytes (at least 5) per detection system, in each batch of analyses	Within a rolling programme to include all other analytes at least every 12 months, but preferably every 6 months
	Within a rolling programme covering all representative analytes as well as representative commodities from different commodity groups, at least at the level corresponding to the reporting Limit	At least at the level corresponding to the reporting limit

Table 4. Identification requirements for different MS techniques[2]

MS detector/Characteristics		Acquisition	Requirements for identification	
Resolution	**Typical systems (examples)**		**minimum number of ions**	**other**
Unit mass resolution	Single MS quadrupole, ion trap, TOF	full scan, limited m/z range, SIM	3 ions	S/N ≥ 3[d] Analyte peaks from both product ions in the extracted ion chromatograms must fully overlap. Ion ratio from sample extracts should be within **±30% (relative)** of average of calibration standards from same sequence
	MS/MS triple quadrupole, ion trap, Q-trap, Q-TOF, Q-Orbitrap	selected or multiple reaction monitoring (SRM, MRM), mass resolution for precursor-ion isolation equal to or better than unit mass resolution	2 product ions	
Accurate mass measurement	High resolution MS: (Q-)TOF (Q-)Orbitrap FT-ICR-MS sector MS	full scan, limited m/z range, SIM, fragmentation with or without precursor-ion selection, or combinations thereof	2 ions with mass accuracy ≤ 5 ppm[a, b, c]	S/N ≥ 3[d] Analyte peaks from precursor and/or product ion(s) in the extracted ion chromatograms must fully overlap. Ion ratio: see D12

[a] preferably including the molecular ion, (de)protonated molecule or adduct ion
[b] including at least one fragment ion
[c] < 1 mDa for m/z < 200
[d] in case noise is absent, a signal should be present in at least 5 subsequent scans

PESTICIDES

G. Analytical method validation and performance criteria

Quantitative methods

G1 Within-laboratory method validation should be performed to provide evidence that a method is fit for the intended purpose. Method validation is a requirement of accreditation bodies, and must be supported and extended by method performance verification during routine analysis (analytical quality control and on-going method validation). Where practicable, all procedures (steps) that are undertaken in a method should be validated.

G2 Representative matrices may be used to validate multi-residue and single-residue methods. As a minimum, one representative commodity from each commodity group as described in Annex A must be validated, depending on the intended scope of the method. When the method is applied to a wider variety of matrices, complementary validation data should be acquired, e.g. from on-going QC during routine analyses. An example of a practical approach to the validation procedure is presented in Appendix A.

Table 5. Validation parameters and criteria

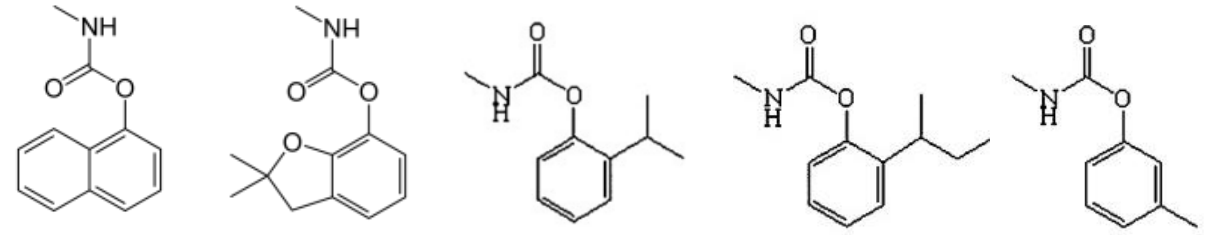
Parameter	What/how	Criterion	Cross reference to AQC document
Sensitivity/linearity	Linearity check from five levels	Deviation of back-calculated concentration from true concentration ≤±20%	C14-C19
Matrix effect	Comparison of response from solvent standards and matrix-matched standards	*	C22-C24
LOQ	Lowest spike level meeting the method performance criteria for trueness and precision	≤ MRL	G6
Specificity	Response in reagent blank and blank control samples	≤30% of RL	C42
Trueness (bias)	Average recovery for each spike level tested	70-120%	G3,G6
Precision (RSD_r)	Repeatability RSD_r for each spike leveltested	≤ 20%	G3, G6
Precision (RSD_{wR})	Within-laboratory reproducibility, derived from on-going method validation / verification	≤ 20%	G3, G6
Robustness	Average recovery and RSD_{wR}, derived from on-going method validation / verification	See above	G6, C40-C44
Ion ratio	Check compliance with identification requirements for MS techniques	Table 4	Section D
Retention time		±0.1 min.	D2

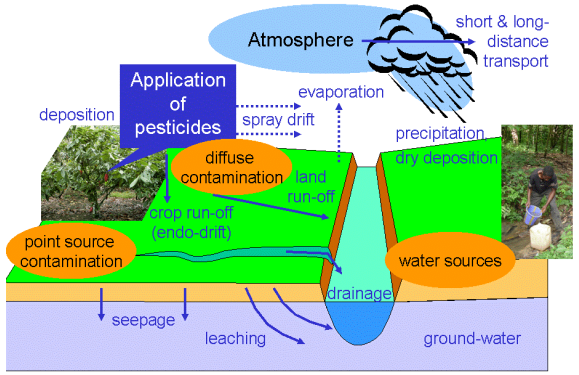
* in case of more than 20% signal suppression or enhancement, matrix-effects need to be addressed in calibration (C22-C30)

Target analytes: Screening of pesticides in food

Phenoxyl-Type N-Methylcarbamates

Carbaryl **Carbofuran** **Isoprocarb** **Fenobucarb** **Metolcarb**





European Food Safety Authority, EFSA Journal (Annual Report) 11 (3) (2013) 3130 (*EFSA Journal 2013;11(3):3130)*

Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA). (2016). Rapporto nazionale pesticidi nelle acque. Dati 2013-2014. Rapporti, 244/2016. ISBN: 978-88-448-0770-2.

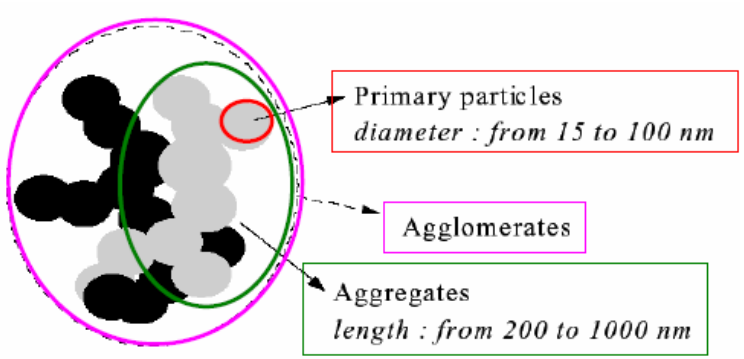
Carbon Black

- Produced by the incomplete combustion **of heavy petroleum products**
- Reinforcing filler used in **rubber** compounds (also used as black pigment)
- Bulk CB is used in various applications for **electronics**

.

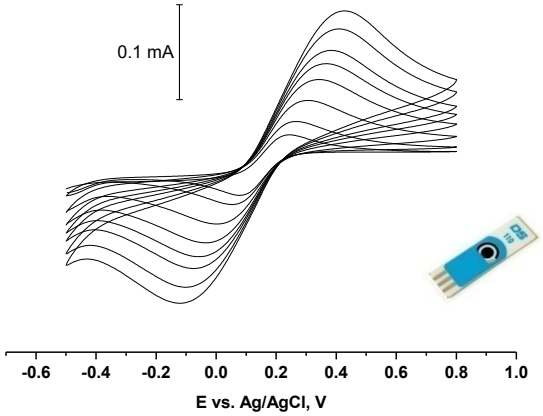
An industrially manufactured
colloidal carbon material
in the form of spheres and of their fused aggregates
with sizes below 1000 nm.

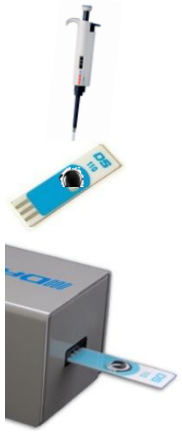
IUPAC Compendium of Chemical Terminology 2nd Edition (1997)



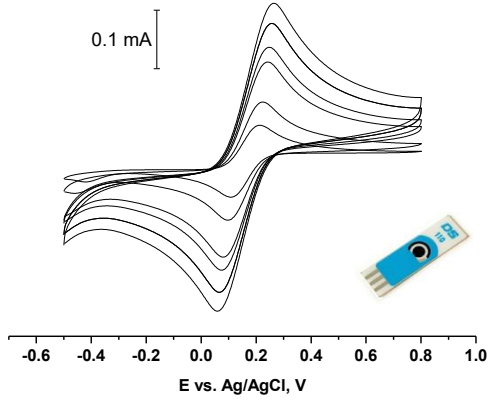
(B) SPE-CBNPs electrochemical (vs. DRP 110 GPH)

Carbon SPE

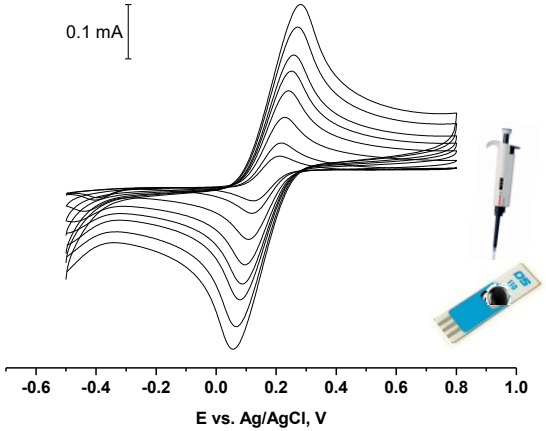


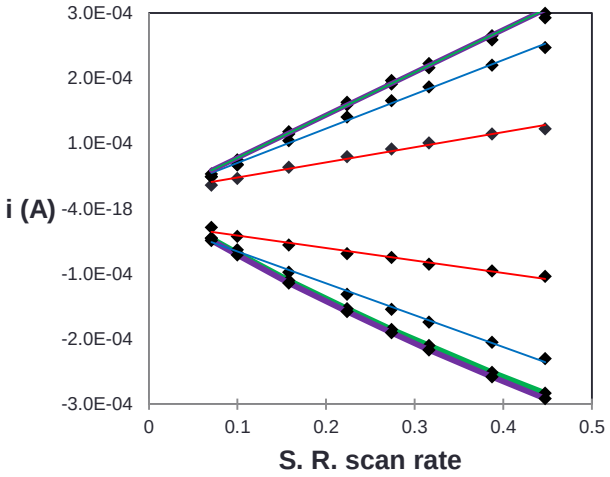


Graphene/Carbon SPE (DS110-GPH)

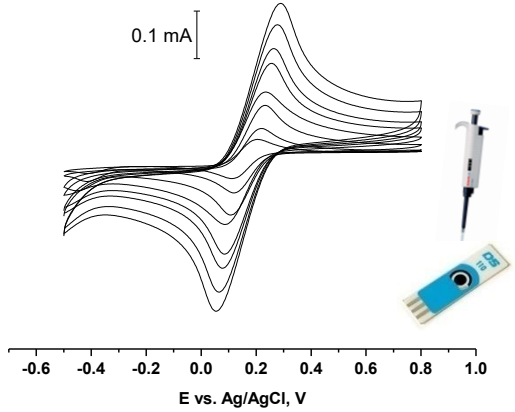


Carbon-CB SPE



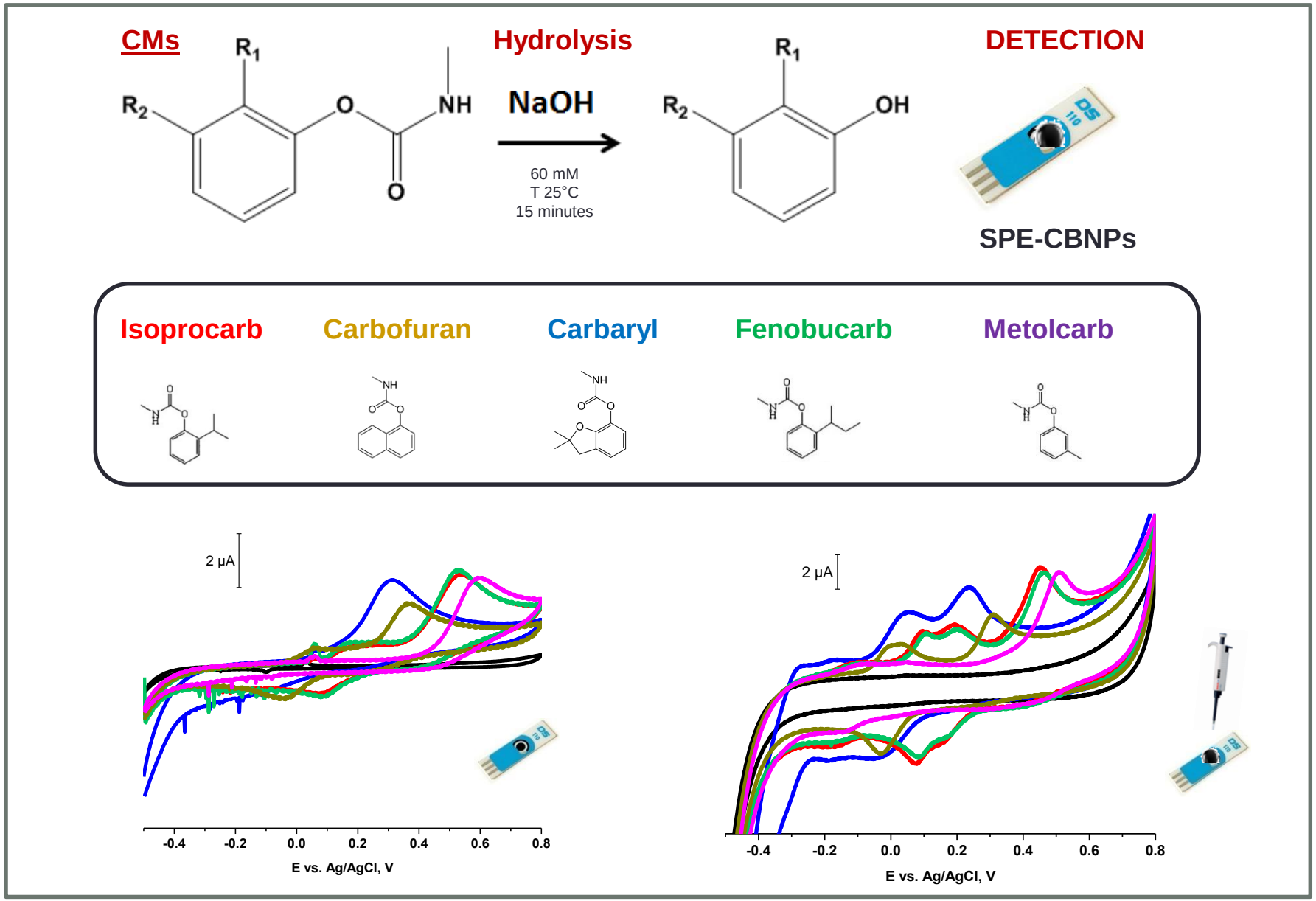


Graphene/Carbon-CB SPE (DS110-GPH)

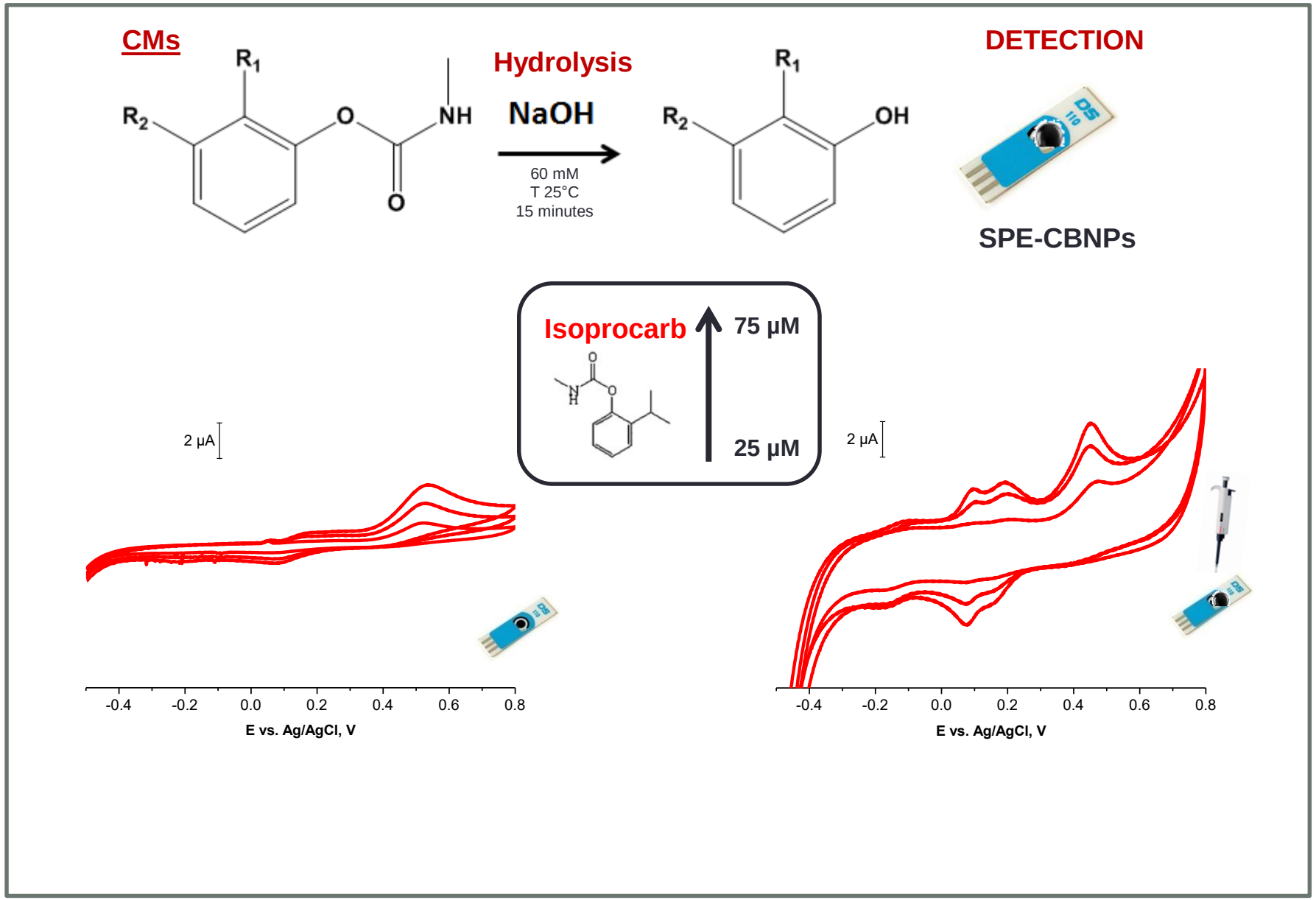


CB modified SPE demonstrates a better electron transfer.

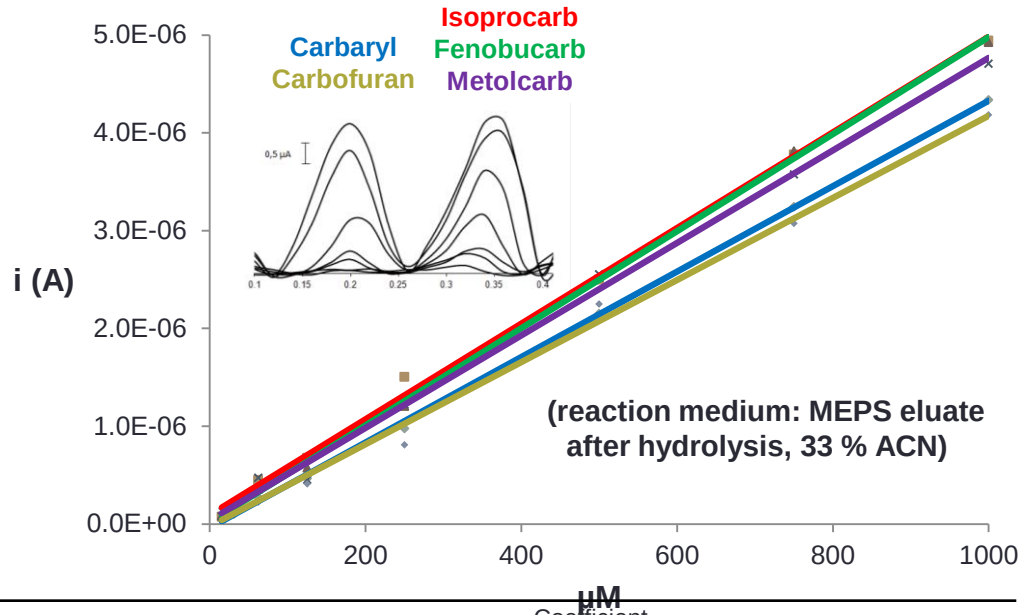
(B) SPE-CBNPs vs. CMs



(B) SPE-CBNPs vs. CMs



(B)SPE-CBNPs CMs Calibration, Reproducibility and Fouling resistance



Analyte	Linear range (µmol L^{-1})	Regression equation (Y=am + b)	Coefficient of determination (r^2)	Detection limit (µmol L^{-1})	Quantification limit (µmol L^{-1})
Isoprocarb	0.1-100	y = 3E-08x + 5E-09	**0.9971**	0.6	**0.7**
Carbofuran	0.1-100	y = 6E-08x - 1E-08	**0.9999**	0.4	**0.5**
Carbaryl	0.1-100	y = 6E-08x + 2E-08	**0.9983**	0.4	**0.5**
Fenobucarb	0.1-100	y = 3E-08x - 8E-09	**0.9996**	0.6	**0.7**
Metolcarb	0.1-100	y = 6E-08x + 4E-08	**0.9980**	0.3	**0.4**

Peak intensity (RSD, n=7): < 0.9 %

Peak potential (RSD, n =7): < 4,8 %

Inter electrode reproducibility (RSD, n=10): < 6.6 % p.i and < 3,4 % p.E.

Fouling (peaks RSD):
DPV (n = 30, 250 µM) 96 % v.s.32 %
CV (n = 20, 500 µM) 94 % v.s 15 %

food matrix analysis
(wheat, wheat flour)

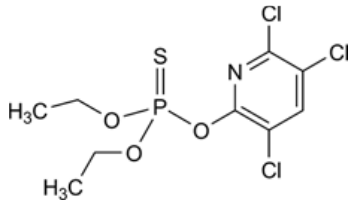
SPE-CBNPs

ANALYTICAL TARGETS FOR LC-MS-MS DETERMINATION OF PESTICIDES

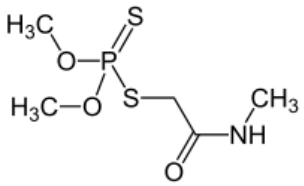
MULTIPLE CONTAMINATION

MULTICLASS ANALYSIS

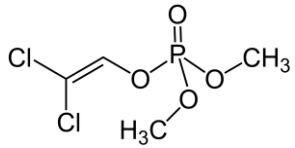
ORGANOPHOSPHATES



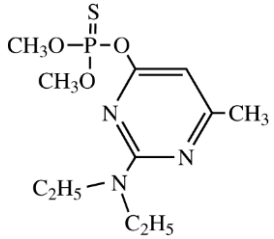
Chorpiriphos meth



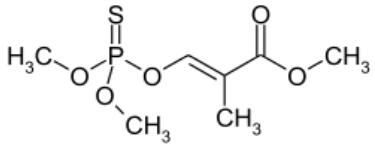
Dimethoate



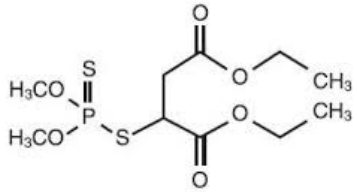
Dichlorvos



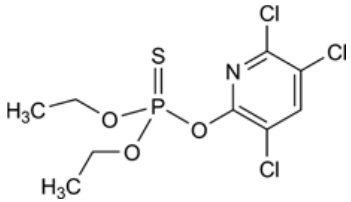
Pirimiphos Meth



Methacriphos

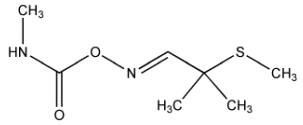


Malathion

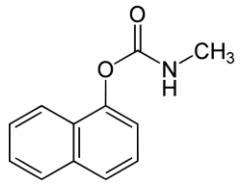


Chlorpiriphos ethyl

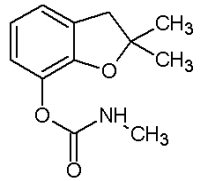
CARBAMATES



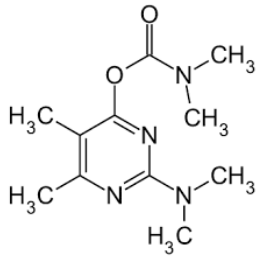
Aldicarb



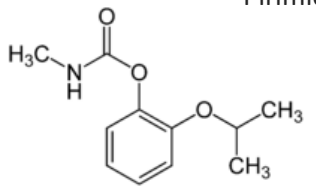
Carbaryl



Carbofuran

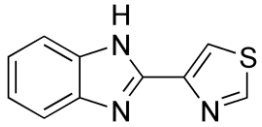


Pirimicarb

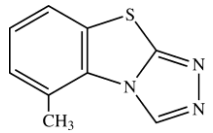


Propoxur

AZOLINE DERIV

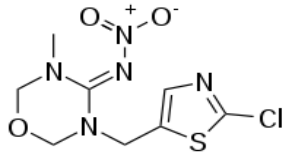


Thiabendazole

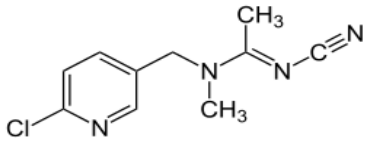


Tricyclazole

NEONICOTINOIDS

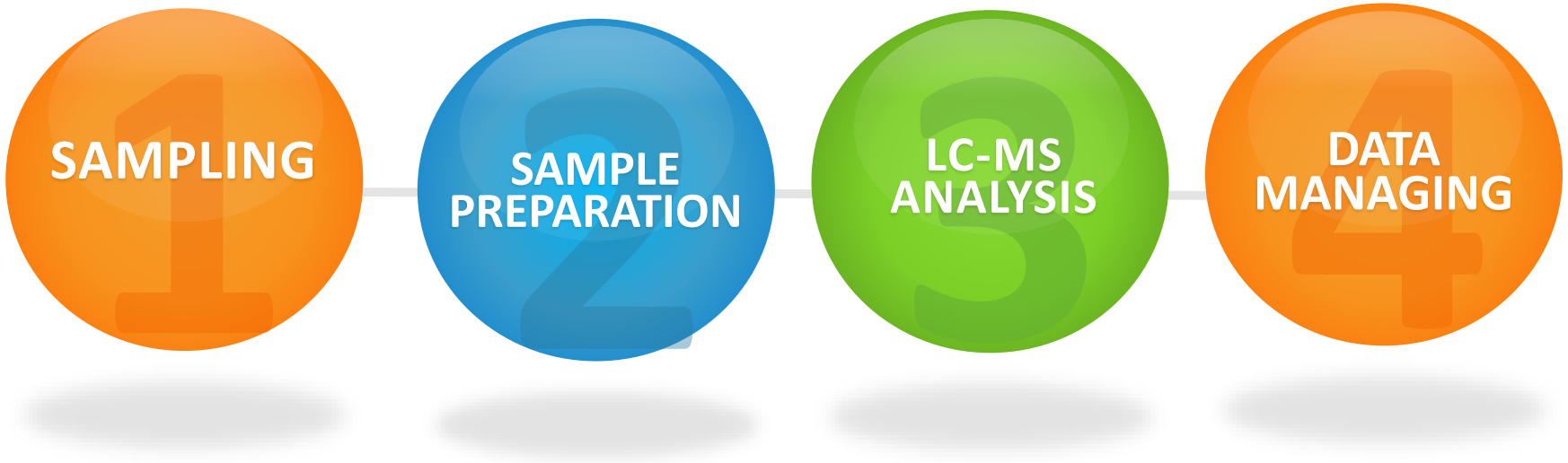


Thiametoxam



Acetamiprid

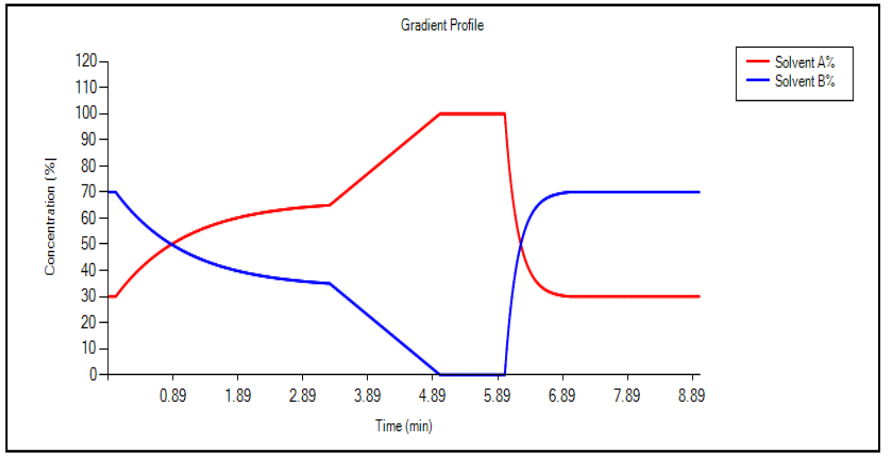
BIOANALYTICAL PROCEDURE



SAMPLE PREPARATION IS A CRITICAL AND TIME-CONSUMING STEP

LIQUID CHROMATOGRAPHY

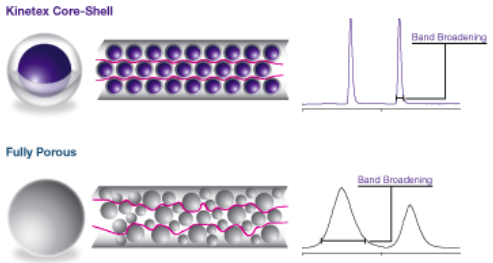
GRADIENT SCHEME



Fase A: MeOH 5mM HCOOH

Fase B: H_2O MilliQ 5mM HCOOH

- **Curved Increase of Phase A from 43% to 65% in 3,3 min**
- **Lineare increase of Phase A from 65% to 100% in 1,7 min**
- **Reconditioning for 2 min**



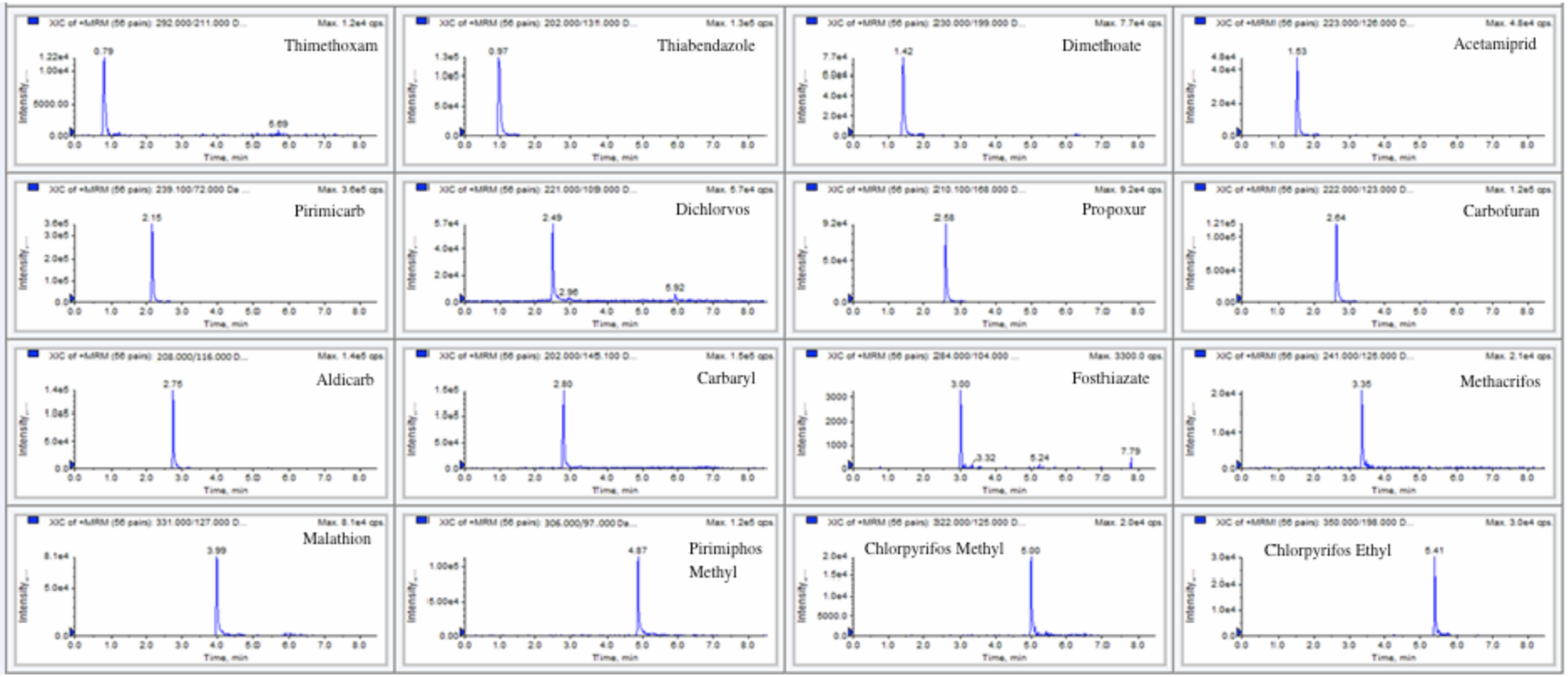
Column Phenomenex Kinetex XB-C18, 100x2,1 mm

Instrumental parameters for MS/MS detection

ANALYTE	Q1 (amu)	Q3 (amu)	DP (V)	EP (V)	CE (V)	CXP (V)	t_R (min)
Thiamethoxam	292,0	211,0	60	10	17	8	0.79
		181,0			30	6	
Thiabendazole	202,0	131,0	65	8	35	9	0,97
		175,0			34	9	
Dimethoate	230,0	125,0	53	5	28	12	1,42
		199,0			13	7	
Acetamiprid	223,0	126,0	80	5	27	6	1,53
		56,0			32	5	
Pirimicarb	239,1	72,0	88	8	37	7	2,15
		182,0			22	9	
Dichlorvos	221,0	109,0	74	5	22	5	2,49
		127,0			37	8	
Propoxur	210,1	111,0	53	7	11	16	2,58
		168,0			20	8	
Carbofuran	222,0	123,0	34	4	31	15	2,64
		165,0			16	7	
Aldicarb	208,0	116,0	12	3	12	5	2,75
		89,0			23	11	
Carbaryl	202,0	145,1	14	9	12	11	2,80
		117,1			36	9	
Fosthiazate	284,0	104,0	150	10	22	10	3,00
		228,0			14	12	
Methacrifos	241,0	209,0	70	11	14	13	3,35
		125,0			19	15	
Malathion	331,0	127,0	70	9	17	8	3,99
		99,0			33	10	
Pirimiphos methyl	306,0	108,0	26	9	20	14	4,87
		67,0			29	15	
Chlorpyrifos methyl	322,0	125,0	65	4	21	9	5,00
		290,0			23	10	
Chlorpyrifos ethyl	350,0	97,0	15	9	25	5	5,41
		198,0			18	4	

72

XIC (extracted-ion currents) of the selected analytes

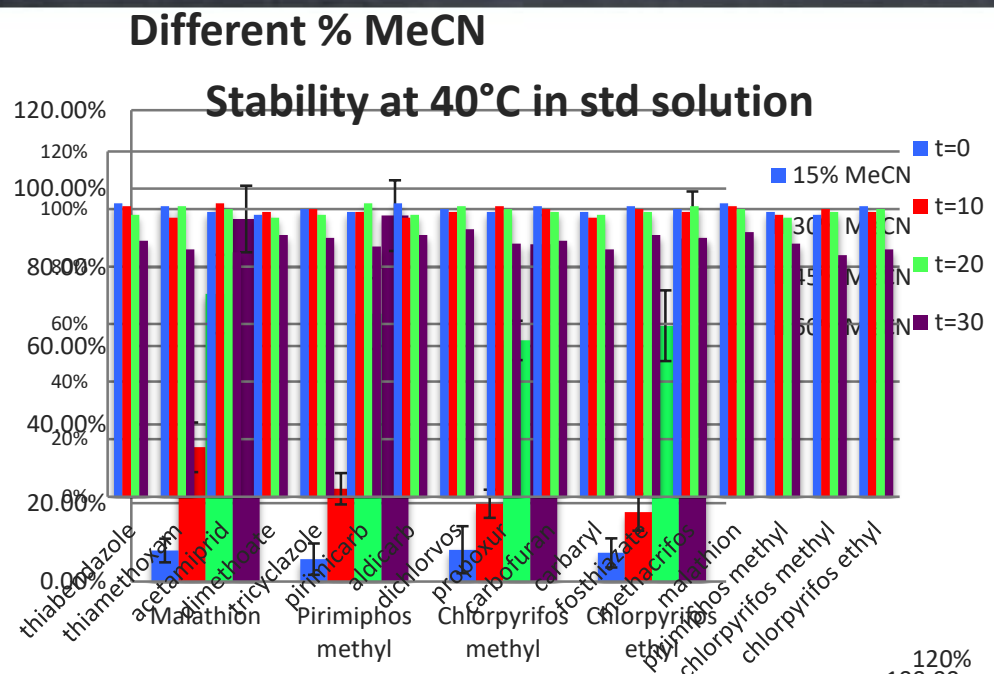


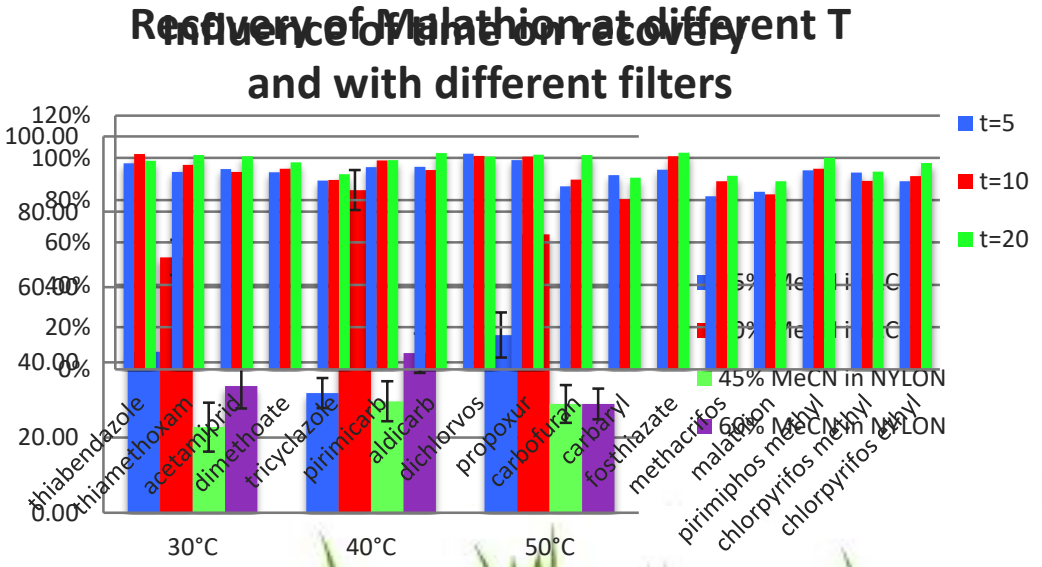
Extraction of pesticides from wheat

- Solvent
- Temperature
- Ultrasounds
- Centrifugation
- Filtration
- Stability

EXTRACTION OF PESTICIDES FROM WHEAT

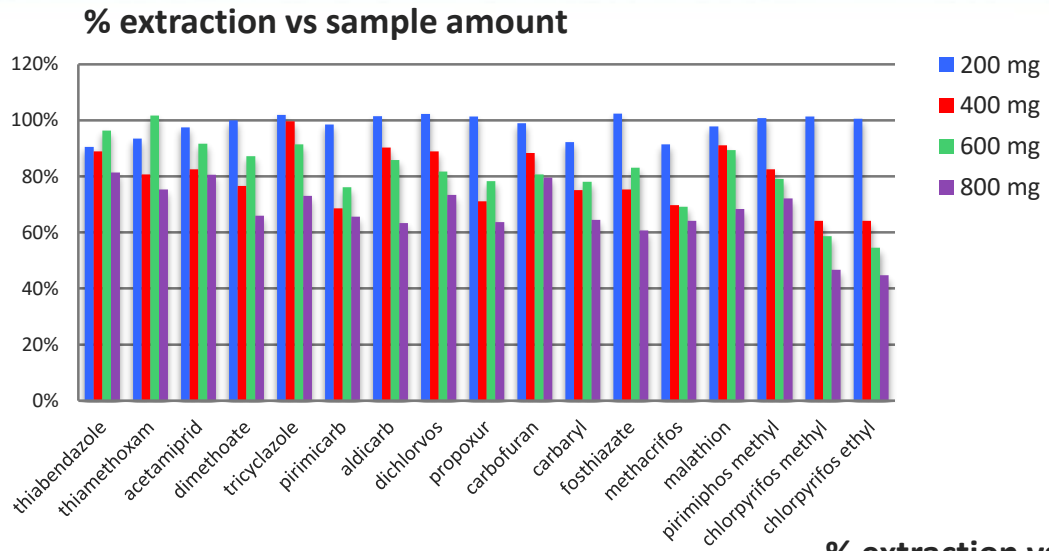
RECOVERY

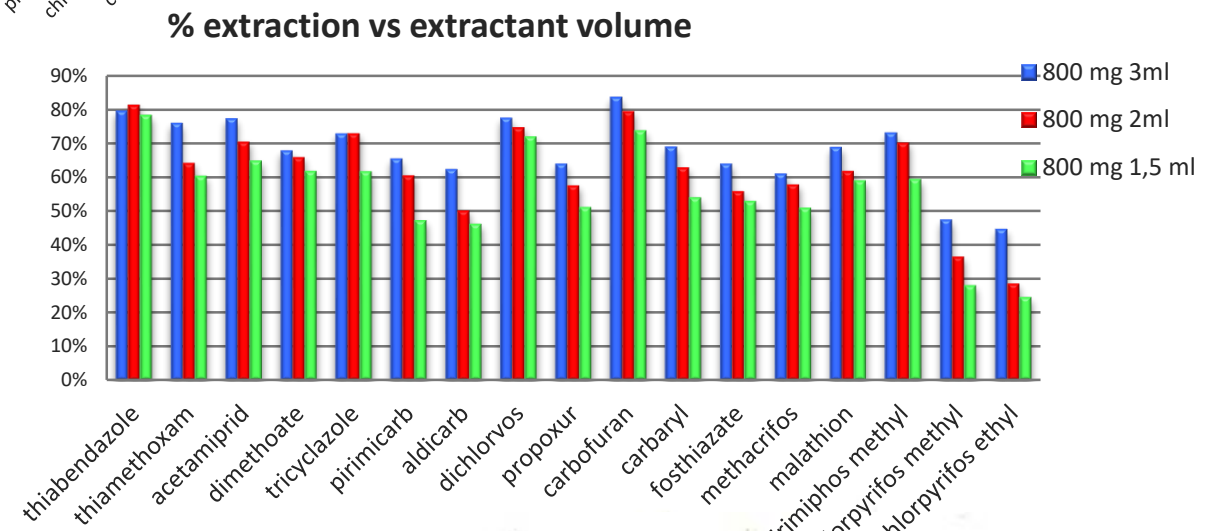




EXTRACTION OF PESTICIDES FROM WHEAT

ENRICHMENT

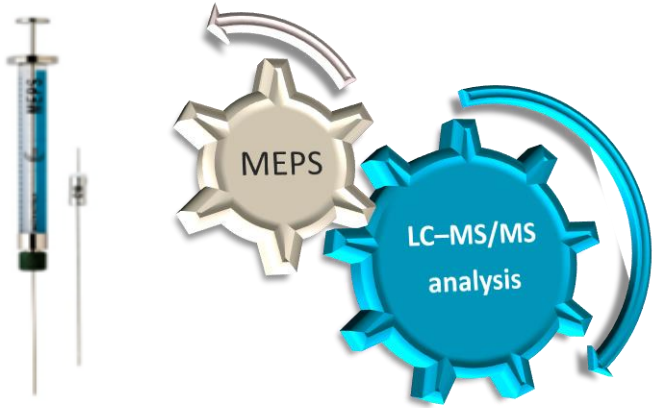




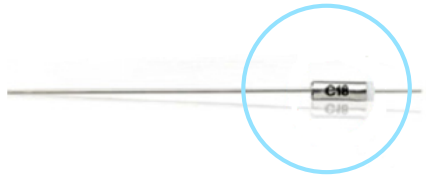
2

Clean-up of pesticides from wheat extract

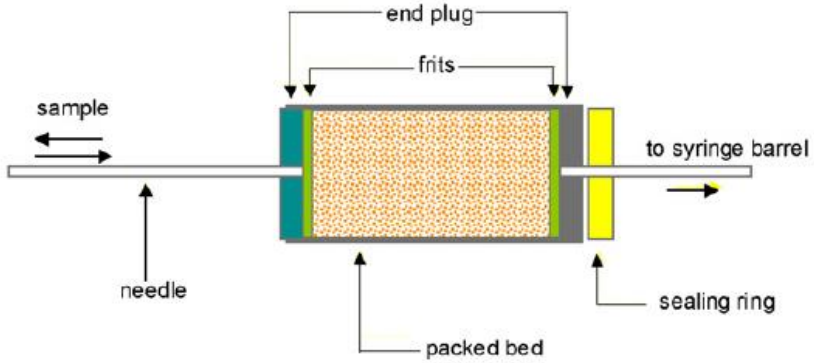
Micro Extraction on Packed Sorbent



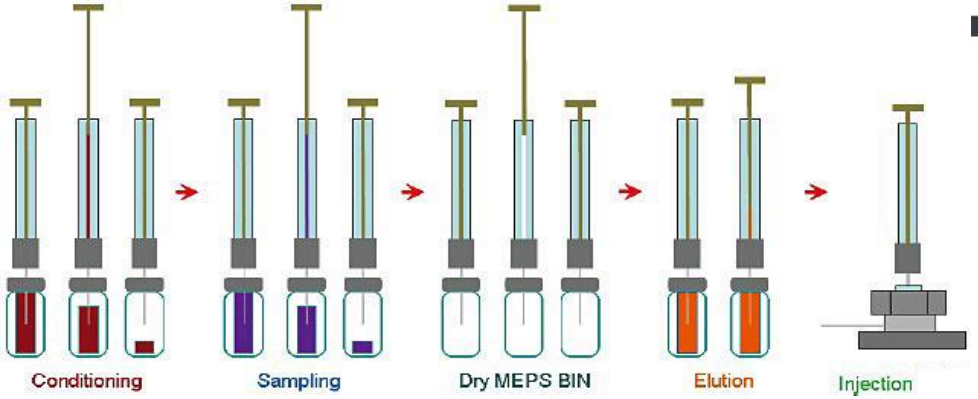
Micro Extraction on Packed Sorbent (MEPS)



- Few mg of stationary phase
- Reduction of sample volume to 10-100 µL
- Reduction of organic solvent needed

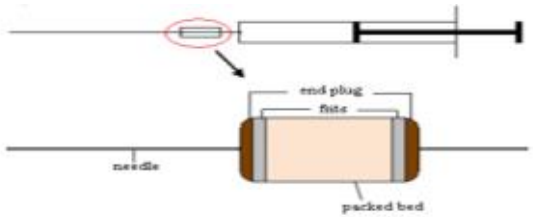


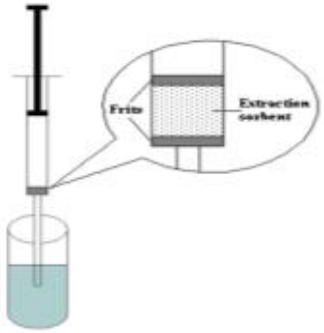
CLEAN-UP ➔ MEPS (Micro Extraction by Packed Sorbent)



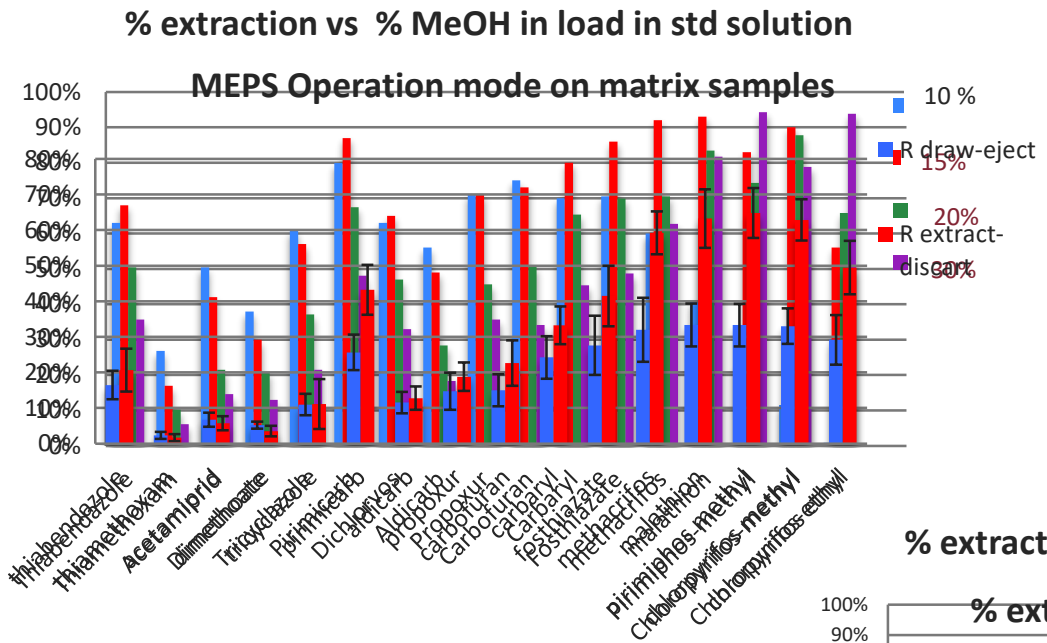
FEATURES

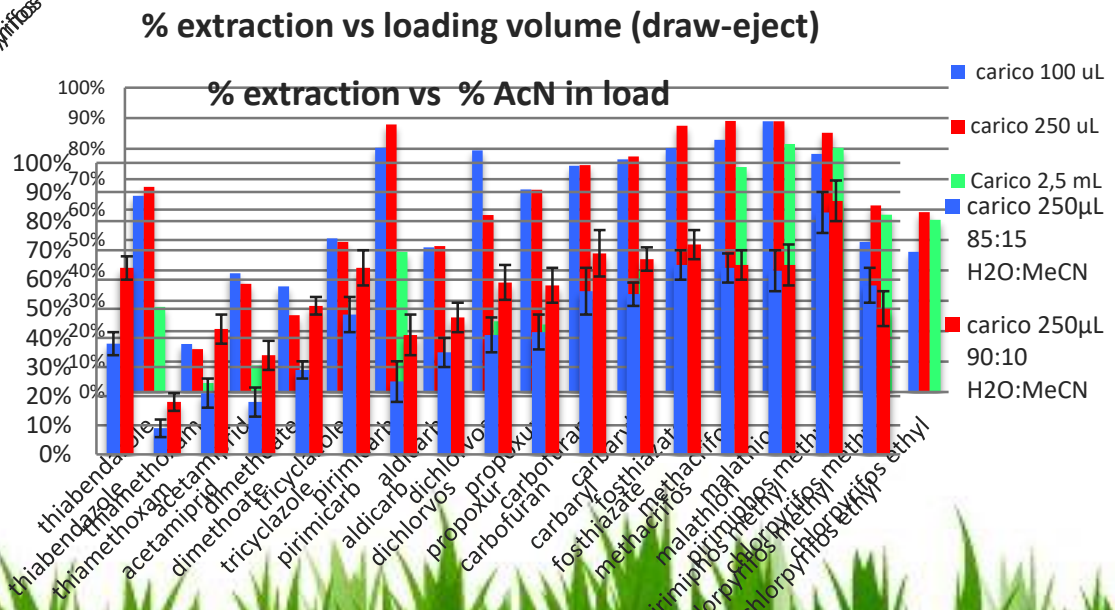
- Miniaturized SPE
- Multiple extractions :
  - ✧ Draw-eject
  - ✧ Extract-discart
- Can be used for 50 /100 samples





CLEAN-UP ➔ MEPS (Micro Extraction by Packed Sorbent)





SAMPLE PREPARATION PROCEDURE

1g wheat+1,5mL extraction mixture (60%MeCN+acetate buffer)

Sonication 5 min

Thermostatic bath (40°C 5 min)

Centrifugation
10000rpm
5 min 20°C

Filtration, 500µL of eluate+2,5mL acetate buffer

Centrifugation
10000rpm
5 min 4°C

MEPS C18

- Activatione:100µL MeOH
- Conditioning: H_2O/MeCN 90:10
- Load: 3 mL extract-discart
- Wash: 100 µL H_2O
- Elution: 100 µL MeOH

UHPLC-MS/MS

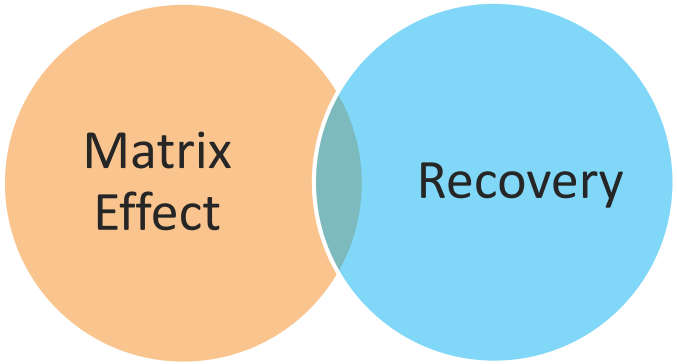
VALIDATION DATA

Analyte	Equation	r^2	Internal standard	LOD (mg/Kg)	LOQ (mg/Kg)	MLR (mg/Kg)
Thiabendazole	$y=361x+3,2x10^{-3}$	0,9992	Thiabendazole NH d_6	1.10^{-4}	3.10^{-4}	5.10^{-2}
Acetamiprid	$y=751x+3,14x10^{-3}$	0,9994	Thiabendazole NH d_6	2.10^{-4}	5.10^{-4}	5.10^{-1}
Dimethoate	$y=922x+4,46x10^{-3}$	0,9991	Thiabendazole NH d_6	1.10^{-2}	1.10^{-3}	3.10^{-2}
Tricyclazole	$y=534x+2,79x10^{-3}$	0,9996	Thiabendazole NH d_6	2.10^{-4}	5.10^{-4}	5.10^{-2}
Pirimicarb	$y=4x10^{-3}x+3,31x10^{-4}$	0,9987	Chlorpyrifos diethyl-d_{10}	3.10^{-5}	$1.\ 10^{-4}$	5.10^{-1}
Aldicarb	$y=743x+6,75x10^{-3}$	0,9983	Thiabendazole NH d_6	2.10^{-5}	5.10^{-5}	2.10^{-2}
Carbofuran	$y=907x+5,22x10^{-3}$	0,9994	Thiabendazole NH d_6	2.10^{-4}	5.10^{-4}	2.10^{-2}
Dichlorvos	$y=110x+1,16x10^{-3}$	0,9972	Thiabendazole NH d_6	7.10^{-5}	2.10^{-4}	1.10^{-2}
Propoxur	$y=912x+7,13x10^{-3}$	0,9984	Thiabendazole NH d_6	1.10^{-4}	3.10^{-4}	5.10^{-1}
Carbaryl	$y=837x+3,92x10^{-3}$	0,9992	Thiabendazole NH d_6	3.10^{-4}	1.10^{-3}	5.10^{-1}
Fosthiazate	$y=1,27x10^{-3}x+5,1x10^{-3}$	0,9997	Chlorpyrifos diethyl-d_{10}	3.10^{-4}	5.10^{-5}	2.10^{-2}
Methacrifos	$y=532x+2,29e003$	0,9998	Chlorpyrifos diethyl-d_{10}	1.10^{-5}	4.10^{-5}	5.10^{-2}
Malathion	$y=564x+5,55e003$	0,9995	Chlorpyrifos diethyl-d_{10}	2.10^{-5}	5.10^{-5}	8
Pirimiphos Methyl	$y=1,16x10^{-3}x+4,32x10^{-3}$	0,9992	Chlorpyrifos diethyl-d_{10}	2.10^{-5}	5.10^{-5}	5
Chlorpyrifos Methyl	$y=24,5x-47,2$	0,9961	Chlorpyrifos diethyl-d_{10}	7.10^{-4}	2.10^{-3}	3
Chlorpyrifos Ethyl	$y=139x-8,8$	0,9992	Chlorpyrifos diethyl-d_{10}	2.10^{-4}	5.10^{-4}	5.10^{-2}

VALIDATION DATA

Pesticide	RSD(%) intra-day			RSD(%) inter-day			Accuracy (%)		
Concentration level	½ MRL	MRL	1,5 MRL	½ MRL	MRL	1,5 MRL	½ MRL	MRL	1,5 MRL
Thiabendazole	12	4	6	14	9	9	89	91	94
Acetamiprid	8	5	4	13	8	9	97	91	94
Dimethoate	11	9	7	17	8	13	101	104	102
Tricyclazole	15	7	9	19	11	12	85	98	101
Pirimicarb	8	8	5	10	13	9	97	99	102
Aldicarb	8	6	7	15	11	10	87	90	95
Carbofuran	8	5	6	13	10	12	89	107	110
Dichlorvos	10	8	8	17	10	11	91	97	95
Propoxur	10	9	7	14	12	4	94	88	98
Carbaryl	6	2	5	12	10	8	101	103	109
Fosthiazate	7	12	10	11	14	15	100	110	98
Methacrifos	10	13	9	15	11	10	88	92	111
Malathion	11	4	9	13	10	11	103	107	111
Pirimiphos Methyl	9	12	8	13	15	11	94	87	102
Chlorpyrifos Methyl	15	13	10	20	15	12	86	88	91
Chlorpyrifos Ethyl	12	7	11	15	10	13	97	100	99

VALIDATION DATA



The recovery was calculated as the ratio of the peak area of the spiked (A) vs the area of the same sample spiked after elution of the microextraction (B).

Matrix effect was evaluated for each analyte by comparing the peak area of the quantifier ion current obtained from blank samples fortified after the extraction process (B) with the peak area of a standard at the same concentration in MeOH (C).

Analyte	Matrix effect B/C
Thiabendazole	1,00
Acetamiprid	0,98
Dimethoate	1,02
Tricyclazole	1,00
Pirimicarb	0,95
Aldicarb	0,89
Carbofuran	0,87
Dichlorvos	0,94
Propoxur	0,94
Carbaryl	0,80
Fosthiazate	0,91
Methacrifos	0,88
Malathion	0,93
Pirimiphos Methyl	0,81
Chlorpyrifos Methyl	0,78
Chlorpyrifos Ethyl	0,85

ANALYSIS ON REAL SAMPLES

ANALYTE	Flour 00 (mg/kg)	Flour 00 for pizza (mg/kg)	Flour 0 (mg/kg)	Organic flour (mg/kg)	LOQ (mg/kg)	LMR (mg/kg)
Aldicarb	0,002	0,002	< LOQ	< LOQ	$1x10^{-3}$	0,02
Chlorpyrifos methyl	< LOQ	< LOQ	0,04	< LOQ	0,03	3
Chlorpyrifos ethyl	0,003	0,003	0,002	< LOQ	0,01	0,05
Dichlorvos	0,009	0,008	0,008	< LOQ	0,005	0,01
Fosthiazate	0,006	0,006	0,006	< LOQ	$1x10^{-3}$	0,02
Malathion	0,003	0,007	0,003	< LOQ	$1x10^{-3}$	8
Methacrifos	0,007	0,005	0,007	< LOQ	$1x10^{-3}$	0,05
Pirimicarb	0,006	0,006	0,006	< LOQ	0,002	0,5
Pirimiphos methyl	0,104	0,116	0,160	< LOQ	$1x10^{-3}$	5