MULTI-ACTIVE METHOD FOR THE ANALYSIS OF ACTIVE SUBSTANCES IN FORMULATED PRODUCTS TO SUPPORT QUALITY CONTROL

SCOPE

The method is designed for use by Quality Control laboratories and is suitable for determining a range of active substances in a range of formulated product types.

The current GC scope is given in **Appendix 1** and the current HPLC scope is given in **Appendix 2** and the current UHPLC scope is given in **Appendix 3**.

Revision history:

Rev 1 – updated to include use of internal standard and comments from ESPAC 2019
Rev 2 – updated to include UHPLC.

1 Sampling Ideally an intact product should be taken from the market as intended for use with an end user.

2 Identity tests

2.1 DAD, UV, GC-MS, LC-MS or FTIR or any other suitable technique. depending on the product being analysed.

3.1 Active substances

OUTLINE OF METHOD.

The sample preparation is different depending on the formulation type being analysed. Procedures are given for solid and liquid formulations. Once the samples are prepared the active substances are analysed by either gas chromatography with flame ionization detection (GC-FID) or high performance liquid chromatography with UV detection (HPLC-DAD) or ultra high-performance liquid chromatography (UHPLC) with internal or external standard calibration.

REAGENTS

Analytical s*tandards* of known purity, stored in refrigerator or as per laboratory protocol.

Dicyclohexyl Phthalate (DCHP) > 97% pure – internal standard

Ethyl Acetate: HPLC grade

Acetone HPLC grade

Acetonitrile : HPLC grade

Water: HPLC grade

***Phosphoric Acid or Formic** Acid*: HPLC grade

Internal standard solution where appropriate.

HPLC and UHPLC Mobile Phase: **A = 0.1% o-Phosphoric acid (or 0.1% Formic acid). pH 2.0 – 2.2**

B = Acetonitrile

Calibration solution. Weigh in duplicate (to the nearest 0.1 mg) about 10 mg of the required analytical standard (s mg) into a volumetric flask (100 ml). Dissolve and fill to mark with acetonitrile for LC analysis or ethyl acetate / internal standard solution (GC analysis). This gives a standard concentration of approximately **100 mg/l** (calibration solutions C_A and C_B).

Internal standard stock solution: Dissolve 80 mg (to the nearest 0,1 mg) Dicyclohexyl phtalate in a volumetric flask (1000 ml) and make up to the mark with the acetone. Internal standard solution concentration is approx. 0.08 mg/ml.

Sample preparation.

Liquid or suspension formulations: Thoroughly shake the sample container to homogenize the sample before use. Weigh in duplicate (to the nearest 0.1 mg) sufficient sample to contain 9 to 11 mg (*w* mg) of the active substance into a volumetric flask (100 ml). For suspension formulations add 5 ml of water to disperse – **LC analysis only**. For **GC analysis** add 70ml of ethyl acetate or internal standard solution. Allow the solution to cool to ambient temperature and fill to the mark with ethyl acetate (Solutions S_A and S_B). For **LC analysis** add 70ml of acetonitrile. If it is necessary, sonicate for dissolution of active ingredient. Allow the solution to cool to ambient temperature and fill to the mark with acetonitrile (Solutions S_A and S_B). This gives an active ingredient concentration of approximately 100mg/l (Sample solutions C_A and C_B). For UHPLC filter samples through 0.22μm filter.

Solid formulations: For solid formulations dispersion with 10% water prior to solvent addition is recommended. Weigh **15 - 20**g of the sample into a mortar and pestle **or grinder/pulverizer** and homogenise thoroughly. Subsample by weighing in duplicate (to the nearest 0.1 mg) sufficient sample to contain 9 to 11 mg (*w* mg) of the active substance into a volumetric flask (100 ml). Add 70ml of the internal standard solution. If it is necessary, sonicate for dissolution of active ingredient. Allow the solution to cool to ambient temperature and fill to the mark with ethyl acetate **OR** internal standard solution **(for GC analysis) or acetonitrile (for LC analysis) and** mix well (Solutions S_A and S_B). This gives an a sample concentration of approximately 100mg/l (Sample solutions C_A and $C_{B)}$. For UHPLC folter samples through 0.22μm filter.

APPARATUS – Use as required.

High performance liquid chromatography (HPLC) equipped with detector suitable for operation at variable wavelengths, a constant temperature column compartment and an auto-sampler capable of delivering 10 μL.

Ultra high performance liquid chromatography (UHPLC) equipped with detector suitable for operation at variable wavelengths, a constant temperature column compartment and an auto-sampler capable of delivering 0,5μl.

Gas chromatograph equipped with a split/splitless injection and a flame ionization detector.

HPLC column, Kinetex C18, 100 mm x 4.6 mm (id) x.2.6 μm / 100 **or equivalent**.

UHPLC column, Kinetex C18, 100 mm x 2,1 mm (id) x.2.6 μm **or equivalent (Recommendation:** for UHPLC use precolumn Phenomenex C 18, 2,1 mm ID or equivalent)

GC Columns – J&W HP5, 30 m x 250 μm x 0.25 μm **or equivalent.**
TG-5MS 30 x 0.25mm x 0.25μm **or equivalent.**

Electronic integrator or data system

Ultrasonic bath capable of being heated up to 50°C

Water bath**Analytical Balance**

Grinder/Pulverizer

Mechanical shaker

Calibrated pH meter

Organic PTFE filter, 0.22 or 0.45μm

PROCEDURE 1 – GC Method

(a) Chromatographic conditions

Columns HP5MS 30 x 0.25mm x 0.25µm
TG-5MS 30 x 0.25mm x 0.25µm

Injection system

Injector Split injection
Injection volume 1 µl
Split ratio 5:1

Carrier gas Helium or Hydrogen

Detector Flame ionisation detector
Temperatures:
Injection port 250ºC
Detector 310ºC

Temperature program

Temp ºC	Rate ºC/min	Time
65	---	0.5
280	50	0
280	0	6

Total Run Time = 10.80 mins

(b) **Equilibration of the system.** Prepare two calibration solutions. Inject 0.2µl portions of the first one until the response factors obtained for two consecutive injections differ by less than 1.0 %. Then inject a 0.2µl portion of the second solution. The response factor for this solution should not deviate by more than 1.0 % from that for the first calibration solution, otherwise prepare new calibration solutions.

(c) Determination: Inject in duplicate 0.2µl portions of each sample solution bracketing them by injections of the calibration solutions as follows; calibration solution C_A, sample solution $S1_A$, sample solution $S1_B$, calibration solution C_B, sample solution $S2_A$, sample solution $S2_B$, calibration solution C_A, and so on. Measure the relevant peak areas.
(d) Calculation. Calculate the mean value of each pair of response factors bracketing the two injections of a sample and use this value for calculating the active substance contents of the bracketed sample injections.

(1) Without internal standard

$$fi = \frac{s \times P}{Hs}$$

Content of active substance $= \frac{f \times Hw}{w}$ g/kg

(2) With internal standard

$$fi = \frac{s \times Ir}{Hs}$$

Content of active ingredient $= \frac{f \times Hw \times P}{w \times Iq}$ g/kg

where:

fi = individual response factor
f = mean response factor
H_s = peak area of active substance in the calibration solution
H_w = peak area of the active substance in the sample solution
I_r = area of the internal standard peak in the calibration solution
I_q = area of the internal standard peak in the sample solution
s = mass of the active substance working standard in the calibration solution (mg)
w = mass of sample taken (mg)
P = purity of the active substance working standard (g/kg)

PROCEDURE 2 – HPLC Method

(a) Chromatographic conditions

Column	HPLC column, Kinetex C18, 150 mm x 4.6 mm (id) x.2.6 µm **or equivalent**
Flow rate	1.0 ml/min
Injection volume	**5** µL
Detector wavelength	**220 nm – 340 nm. The optimum wavelength can be established by analyzing the analytical standard on PDA detector prior to analysis**
Run Time	**18** min
Mobile Phase	**A = 65% - 0.1% (pH 2.0 – 2.2) o-Phosphoric or Formic Acid acid**
	B = 35% - Acetonitrile

Pump Gradient:

Time (min)	% A	%B
0.01	65	35
10.0	15	85
16.0	15	85
16.4	65	35
18.0	65	35

Column temperature **25** °C

(b) Equilibration of the system. Pump sufficient mobile phase through the column to equilibrate the system. Inject 5µl portion of the standard solution until the response obtained from two consecutive injections deviate by less than 1.0 %.

(c) Determination: Inject in duplicate 5µl portions of each sample solution bracketing them by injections of the calibration solutions as follows; calibration solution C_A, sample solution $S1_A$, sample solution $S1_B$, calibration solution C_B, sample solution $S2_A$, sample solution $S2_B$, calibration solution C_A, and so on. Measure the relevant peak areas.

(d) Calculation. Calculate the mean value of each pair of response factors bracketing the two injections of a sample and use this value for calculating the active substance contents of the bracketed sample injections.

$$fi = \frac{s \times P}{Hs}$$

Content of active substance $= \frac{f \times Hw}{w}$ g/kg

where:

fi = individual response factor
f = mean response factor
H_s = peak area of active substance in the calibration solution
H_w = peak area of the active substance in the sample solution
s = mass of the active substance working standard in the calibration solution (mg)
w = mass of sample taken (mg)
P = purity of the active substance working standard (g/kg)

PROCEDURE 3 – UHPLC Method

(a) Chromatographic conditions

Precolumn	Phenomenex C 18, 2,1 mm ID **or equivalent**
Column	Kinetex C18, 2,6µm, 100 x 2,1 mm **or equivalent**
Flow rate	0,4 ml/min
Injection volume	0,**5** µl
Detector wavelength	**210 nm – 330 nm. The optimum wavelength can be established by analyzing the analytical standard on DAD detector prior to analysis**
Run Time	**8.5**min
Mobile Phase	**A = 65% - 0.1% Phosphoric acid (pH 2.0-2.2)** **B = 35% - Acetonitrile**

Pump Gradient:

Time [min]	A%	B%
0.00	65	35
4.00	15	85
6.00	15	85
6.01	65	35
7.00	65	35

Column temperature **25**°C

(b) Equilibration of the system. Pump sufficient mobile phase through the column to equilibrate the system. Inject 0.5µl portion of the standard solution until the response obtained from two consecutive injections deviate by less than 1.0 % for retention times and deviate by less than 2.0% for peak areas.

(c) Determination: Inject in duplicate 0.5µl portions of each sample solution bracketing them by injections of the calibration solutions as follows; calibration solution C_A, sample solution $S1_A$, sample solution $S1_B$, calibration solution C_B, sample solution $S2_A$, sample solution $S2_B$, calibration solution C_A, and so on. Measure the relevant peak areas.

(d) Calculation. Calculate the mean value of each pair of response factors bracketing the two injections of a sample and use this value for calculating the active substance contents of the bracketed sample injections.

$$fi = \frac{s \times P}{Hs}$$

$$\text{Content of active substance} = \frac{f \times Hw}{w} \text{ g/kg}$$

where:

fi = individual response factor
f = mean response factor
H_s = peak area of active substance in the calibration solution
H_w = peak area of the active substance in the sample solution
s = mass of the active substance working standard in the calibration solution (mg)
w = mass of sample taken (mg)
P = purity of the active substance working standard (g/kg)

Addition of new active substances to the scope:

To add a new active substance to the scope of this method the following validation data must be provided:

1. An unequivocal identity test
2. Injection repeatability data for standards – RSD should be $\leq 1\%$
3. Linearity should be demonstrated between 50 and 150mg/kg
4. The repeatability for sample analysis should be less than 5% - usually for three subsamples of the product and two injections of each sub-sample.
5. Proficiency Test or collaborative trial data would also be beneficial

Appendix 1a GC-FID scope – data without internal standard

	Active substance	Form. Type	unit	Declared content	Tol (±)
1	Azoxystrobin	EC	g/l	62.5	6.3
2	b-Cyfluthrin	WP	g/kg	10	1
3	Chlorothalonil	SC	g/l	500	25
4	Chlorpyrifos	EC	g/l	480	24
5	Chlorpyrifos methyl	EC	g/l	225	13.5
6	Clodinafop propargyl ester	EC	g/l	100	10
7	Cypermethrin	EC	g/l	100	10
8	Cyprodinil	EC	g/l	187.5	11.3
9	Deltamethrin	DP	g/kg	0.05	0.013
10	Difenoconazole	SC	g/l	250	15
11	Dimethoate	EC	g/l	400	20
12	Epoxiconazole	SC	g/l	125	7.5
13	Esfenvalerate	EC	g/l	25	3.8
14	Fenoxaprop p ethyl	EW	g/l	69	6.9
15	Fenpropidin	EC	g/l	750	25
16	Fenpropimorph	SC	g/l	250	15
17	Fluazifop-P-Butyl	EC	g/l	125	7.5
18	Fluroxypyr 1 MHE	EC	g/l	144	8.6
19	Folpet	SC	g/l	500	25
20	Ioxynil Octanoate	EC	g/l	268.1	13.4
21	Isopyrazam	EC	g/l	62.5	6.3
22	l-Cyhalothrin	CS	g/l	100	10
23	Metconazole	EC	g/l	25	3.8
24	Metribuzin	WG	g/kg	70	2.5
25	Myclobutanil	EW	g/l	200	12
26	Paclobutrazol	SC	g/l	125	7.5
27	Permethrin	DP	g/kg	0.488	0.12
28	Picoxystrobin	SC	g/kg	17.9	1.07
29	Piperonyl butoxide	XX	g/kg	1.0	0.15
30	Pirimicarb	WG	g/kg	50	2.5
31	Procloraz	EC	g/l	300	15
32	Prodiamine	XX	g/kg	2.73	0.27
33	Prometron	SC	g/kg	14.3	0.86
34	Propiconazole	EC	g/l	104	6.2
35	Propyzamide	WG	g/kg	4.0	1.0
36	Prosulfocarb	EC	g/l	800	25
37	Pyrethrins	XX	g/kg	0.1	0.02
38	Spiroxamine	EC	g/l	250	15
39	Tebuconazole	SC	g/l	250	15
40	Terbutylazine	SC	g/l	330	16.5
41	Triclopyr butoxy ethyl ester	EO	g/l	334	16.7

42	Trifluralin	WG	g/kg	1.47	0.37

Appendix 1b GC-FID scope – data with internal standard

	Active Substance	Form. Type	Unit	Declared content	Tol (±)
1	Ametryn	WG	g/kg	800	25
2	Ametryn	SC	g/kg	457	22,85
3	Azoxystrobin	SL	g/l	15	1,95
4	Azoxystrobin	SC	g/l	120	7,2
5	Bixafen	EC	g/l	75,0	7,5
6	Cyflufenamid	DC	g/l	30	3
7	Difenoconazole	DC	g/l	60	6
8	Epoxiconazole	SE	g/kg	60	6
9	Fludioxonil	SL	g/l	37,5	3,75
10	Metalaxyl-M	SL	g/l	29	2,9
11	Metazachlor	SC	g/l	500	25
12	Metribuzin	WG	g/kg	700	25
13	Metribuzin	WP	g/kg	700	25
14	Metribuzin	SC	g/l	600	25
15	Metribuzin	SC	g/l	480	24
16	Myclobutanil	EW	g/kg	195	9,75
17	Pendimethalin	EC	g/l	330	16,5
18	Pethoxamide	EC	g/l	600	25
19	Pirimiphos-methyl	EC	g/kg	50	5
20	Pyraclostrobin	SE	g/kg	82	8,2
21	Spiroxamine	EC	g/l	107	6,4
22	Spiroxamine	EC	g/l	150	9
23	Tebuconazole	EW	g/l	250	15
24	Tebuconazole	WG	g/kg	500	25
25	Thiabendazole	SL	g/l	300	15
26	Trifloxystrobin	EC	g/l	100	10

Tol – Refers to the FAO tolerances

Appendix 2 HPLC-DAD scope

	Active substance	Form. Type	λ (nm)	unit	Declared content	Tol (±)
1	Acetamiprid	ME	260	g/kg	0.05	0.008
2	Amidosulfuron	WG	240	g/kg	75	2.5
3	Asulam	SL	260	g/l	400	20
4	Atrazine	SL	280	g/l	4	0.6
5	Benzovindiflupyr	EC	260	g/l	75	7.5
6	Bifenthrin	ME	260	g/l	0.02	0.003
7	Bixafen	EC	260	g/l	60	6
8	Carfentrazone-et	WG	260	g/kg	33.3	1.67
9	Chloridazon	WP	260	g/kg	650	25
10	Chloroantriniliprole	FS	280	g/kg	50	25
11	Chlorothalonil	SC	260	g/l	500	25
12	Chlorotoluron	SC	240	g/l	250	15
13	Cloquintocet-mexyl	EC	340	g/l	25	2.5
14	Cymoxanil	WG	260	g/kg	500	25
15	Daminozide	WG	220	g/kg	85	8.5
16	Difenacoum	RB	260	g/kg	0.005	0.0013
17	Diflufenican	SC	280	g/kg	200	12
18	Dimethomorph	WP	260	g/kg	500	25
19	Epoxiconazole	SC	260	g/l	50	5
20	Flazasulfuron	WG	260	g/kg	250	12.5
21	Flonicamid	WG	260	g/kg	500	25
22	Florasulam	SC	260	g/l	50	5
23	Fluazinam	EC	260	g/l	400	20
24	Flufenacet	SC	245	g/l	400	20
25	Flumioxazin	SC	280	g/l	300	15
26	Fluopicolide	SC	260	g/kg	5.53	0.55
27	Fluopyram	EC	260	g/l	65	6.5
28	Fluoxastrobin	EC	260	g/l	75	7.5
29	Flurtamone	SC	280	g/l	120	7.2
30	Fluxapyroxad	EC	260	g/l	62.5	6.25
31	Halauxifen-methyl	XX	260	g/l	6.3	0.9
32	Imidacloprid	SL	260	g/l	0.125	0.0188
33	Iodosulfuron-Me-Na	SC	240	g/l	0.3	0.05
34	Iprodione	SC	240	g/l	256	12.8
35	Isoproturon	SC	260	g/l	500	25
36	Isoxaflutole	WG	260	g/kg	100	10
37	Linuron	SC	260	g/l	450	22.5
38	Mesosulfuron-Me	WG	240	g/kg	0.9	0.14
39	Mesotrione	SC	260	g/l	70	7
40	Metamitron	SC	260	g/l	700	70
41	Metazachlor	SC	260	g/l	375	18.8
42	Metconazole	EC	260	g/l	60	6

43	Methomyl	SC	240	g/kg	20	1.2
44	Metsulfuron-methyl	WG	260	g/kg	68	10
45	Myclobutanil	ME	260	g/l	0.075	0.0113
46	Nicosulfuron	WG	260	g/kg	750	25
47	Oxadiazon	SC	260	g/l	4.8	0.72
48	Oxamyl	GR	240	g/kg	5	0.5
49	Pendimethalin	SC	260	g/l	455	22.8
50	Penoxsulam	WG	280	g/kg	0.04	0.01
51	Penthiopyrad	SC	260	g/l	100	10
52	Picloram	SL	260	g/l	67	6.7
53	Pinoxaden	EC	260	g/l	100	10
54	Propaquizafop	EC	340	g/l	100	10
55	Proquinazid	EC	260	g/l	200	12
56	Prothioconazole	EC	260	g/l	200	12
57	Pyraclostrobin	SC	260	g/l	133	8
58	Pyraflufen-ethyl	SL	260	g/l	0.33	0.05
59	Pyridate	WP	260	g/kg	45	2.25
60	Pyrimethanil	SC	260	g/l	400	20
61	Pyroxsulam	WG	310	g/kg	7.1	0.71
62	Quizalofop-p-tefuryl	EC	240	g/l	40	4
63	Tebuthiuron	WG	260	g/kg	20	1.2
64	Tepraloxydim	EC	260	g/l	50	5
65	Thiacloprid	SC	240	g/l	40.4	2.02
66	Thifensulfuron-methyl	WG	260	g/kg	682	25
67	Tribenuron-methyl	SG	260	g/kg	40	2
68	Trifloxystrobin	SC	260	g/l	16	1.6
69	Triflusulfuron-methyl	WG	260	g/kg	500	25
70	Trinexapac-ethyl	EC	260	g/l	250	12.5
71	Triticonazole	ME	260	g/l	7.5	1.1

Tol – Refers to the FAO tolerances

Appendix 3 UHPLC-DAD scope

	Active substance	Form. Type	λ (nm)	unit	Declared content	Tol (±)
1	Acetamiprid	SP	240	g/kg	200	12
	Acetamiprid	AL	240	g/l	0.05	0.0075
2	Azoxystrobin	SC	220	g/l	120	7.2
3	Clomazone	CS	210	g/l	360	21.6
4	Deltamethrin	OD	210	g/l	10	1.5
5	Difenoconazole	FS	210	g/l	25	3.75
6	Diflufenican	SC	230	g/l	40	4
7	Dimethomorph	WP	240	g/kg	60	6
8	Epoxiconazole	SC	210	g/l	125	7.5
9	Fenhexamid	SC	240	g/l	500	25
10	Fipronil	WG	220	g/kg	0.143	0.036
11	Fludioxonil	FS	210	g/l	25	3.75
12	Flufenacet	SC	210	g/l	80	8
13	Flurprimidol	EC	240	g/kg	130	7.8
14	Giberelin GA4/GA7	SL	210	g/l	10	1.5
15	Chlortoluron	SC	240	g/l	280	14
16	Metamitron	SC	220	g/l	700	25
17	Pendimethalin	CS	240	g/l	455	22.75
18	Pethoxamid	EC	240	g/l	600	25
19	Prothioconazole	EC	254	g/l	250	15
20	Quizalofop-p-ethyl	EC	330	g/l	50	5
21	Pyraclostrobin	SE	260	g/l	85	8.5
22	Sedaxane	FS	220	g/l	25	3.75
23	Silthiofam	FS	210	g/l	125	7,5
24	S-metolachlor	EC	220	g/l	960	25
25	Spinetoram	SC	254	g/kg	117	7
26	Tebuconazole	WG	220	g/kg	500	25
	Tebuconazole	SC	220	g/l	200	12
27	Tefluthrin	GR	220	g/kg	15	3.75
28	Terbuthylazine	SE	220	g/l	187.5	11.22
29	Thiacloprid	OD	210	g/l	100	10
30	Thiophanate-methyl	*	260	g/kg	23	5,75
31	Triclopyr	AL	295	g/kg	0.7	0,105
32	Trifloxystrobin	WG	220	g/kg	250	15
33	Triflusulfuron-methyl	OD	240	g/l	150	9
34	Trinexapac-ethyl	EC	240	g/kg	50	5
35	Triticonazole	FS	260	g/l	25	3.75

*ground corn cob, which is effective as a carrier for various pesticides
Tol – Refers to the FAO tolerances