BASELINE

SELECTION AND IMPROVING OF FIT-FOR-PURPOSE SAMPLING PROCEDURES FOR SPECIFIC FOODS AND RISKS

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SUMMARY

In this deliverable two analytical methodologies to detect pesticides in vegetables are described together with their adjustment and refinement to specific laboratory conditions. Both methods are based on a similar solvent extraction procedure of the sample, and a subsequent analytical determination either by gas chromatography and/or liquid chromatography. In both procedures, tandem mass spectrometry was the detection method used to quantify the amount of residues of active substances present in the samples.

The quality assurance (QA) and quality control (QC) procedures necessary to improve reliability (trueness and precision) of the results when applying an established standard method (such as QuEChERS), are also described as well as the practical requirements necessary to trustfully apply a standard method in a laboratory.

Slightly modifications on the QuEChERs method have been included and the reason for them explained and justified.

Along the document describing the entire protocol, the reader can find *tips in italics* including explanations, practical clarifications and highlighting differences with the established QuEChERs method.

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PESTICIDE DETERMINATION BY GC-MS/MS

AIM

To describe a method for the analytical determination of pesticides in plant products by gas chromatography coupled with tandem mass spectrometry detection and the quality requirements for its practical implementation in a laboratory.

SCOPE

The method can be used to determine pesticides in plant matrixes of the following characteristics:

Plant products with high water content and low fat content (i.e. vegetables and fruits).
Plant products with low water content and low fat content (i.e. dry fruits)
Plant products with low water content and high starch/protein content (i.e. cereals, legumes)
Spices and aromatic herbs.

REFERENCES

- ENAC Document: NT-19/Rev 2: Laboratorios de Ensayo: Acreditación de Análisis de Residuos de Plaguicidas en Productos Alimenticios.
- Document CEN/TC 275 EN 15662:2008: "Foods of plant origin- Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and cleanup by dispersive SPE-QuEChERS-method"
- Introduction to statistical quality control 4ª ed (2001). Douglas C. Montgomery. Ed. Wiley.
- Commission Recommendation of 3 March 1999 concerning a coordinated Community monitoring programme for 1999 to ensure compliance with maximum levels of pesticide residues in and on cereals and certain products of plant origin, including fruit and vegetables (notified under document number C(1999) 478) (Text with EEA relevance); Annex II: “Quality control procedures for pesticide residues analysis”.
- Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed. Document Nº SANCO/10684/2009 (01/01/2010).
- Regulation (EC) No 396/2005 of the European Parliament and of the Council on maximum residue levels (MRLs) of pesticides in products of plant and animal origin defines a new fully harmonised set of rules for pesticide residues. This Regulation simplifies the existing legislation by harmonising pesticide MRLs and making them directly applicable.
- Commission Regulation (EC) No 178/2006 of 1 February 2006 amending Regulation (EC) No 396/2005 of the European Parliament and of the Council to establish Annex I listing the food and feed products to which maximum levels for pesticide residues apply.

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- Commission Regulation (EC) No 149/2008 of 29 January 2008 amending Regulation (EC) No 396/2005 of the European Parliament and of the Council by establishing Annexes II, III and IV setting maximum residue levels for products covered by Annex I thereto. (Text with EEA relevance).
- International Regulations on Pesticides in Plant Products, published by Spanish Ministry of Economy (S.G. Inspección Certificación y Asistencia Técnica del Comercio Exterior). CD.
- OECD, Environment Directorate. Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology. Test Guidelines Programme. Approval of Draft Guidance Document on Residue Analytical Methods (19th Meeting of the Working Group of National Coordinators of the Test Guidelines Programme); 28-30 March 2007, Paris, France.
- Commission Directive 2002/63/EC of 11 July 2002 establishing Community methods of sampling for the official control of pesticide residues in and on products of plant and animal origin and repealing Directive 79/700/EEC.

BASIS AND DEFINITIONS

Analysis is based on difference of pesticides solubility between aqueous phase (vegetable matrix) and organic phase (solvent). Sample is extracted with acetonitrile and pesticides present in the vegetable are transferred to the organic phase. Salts are used together with the acetonitrile to avoid interferences and to improve extraction yield.

Magnesium sulphate: removes excess of water.
PSA: removes sugars, fatty acids, organic acids and pigments.
Buffer salts: stabilize the pH between 5 and 5.5.

Instrumental determination can be carried out either by gas chromatography or liquid chromatography coupled with tandem mass detection. A specific chapter for liquid chromatography method is described after the GC-MS/MS section.

Definitions:

Laboratory sample: refers to the sample intended to the laboratory. The sample sent to, or received by, the laboratory. A representative quantity of material removed from the bulk sample.
Analytical sample: The material prepared for analysis from the laboratory sample, by separation of the portion of the product to be analysed and then by mixing, grinding, fine chopping, etc., for the removal of analytical portions with minimal sampling error.
Analytical portion: A representative quantity of material, removed from the analytical sample, of proper size for measurement of the residue concentration.
Autotune: internal calibration of mass detector.
Batch and analysis sequence: For extraction, clean-up and similar processes, a batch is a series of samples dealt with by an analyst (or team of analysts) in parallel, usually in one day, and must incorporate at least one recovery determination. For the determination system, a batch is a series undertaken without a significant time break and which incorporates all relevant calibration determinations (also referred to as an “analysis sequence”, a “chromatography sequence”, etc.) [EU AQCL Gls 2010].

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Blank matrix: Material (a sample, or a portion or extract of a sample) known not to contain detectable levels of the analyte(s) sought. Also known as a "matrix blank".
Calibration: amount of operations to establish, in specific conditions, the relationship between instrumental values and amount of pesticide obtained from a reference standard or material.
Chemical ionization (QI): ionization system based on a chemical reaction with reactive ions coming from methanol.
Electronic impact (EI): ionization system based on impact of electrons.
Fmatch: parameter that indicates the similarity degree between the standard mass-mass spectrum and sample spectrum. It can take values from 1 to 1000.
Full scan: working mode of mass detector in which spectra resultant from the fragmentation of a precursor ion is fully acquired.
HR (mg/kg): high working range for a specific pesticide that corresponds to the higher level of addition during method validation.
id: internal diameter.
Integration window: time interval selected in instrumental method in which a chromatographic peak is integrated and its central time matches up with the expected retention time.
Ion time: parameter of mass detector.
LCL (mg/l): lowest calibrated level.
LOQ (mg/kg): Limit of quantitation (quantification) (also known as limit of determination (LOD)) is the minimum concentration or mass of the analyte that can be quantified with acceptable accuracy and precision. It should apply to the complete analytical method. It must be a value greater than the limit of detection (LOD). LOQ is preferable to LOD because it avoids possible confusion with "limit of detection". However, in legislation MRLs that are set at the limit of quantification/determination are referred to as "LOD MRLs", not "LOQ MRLs".
Mass spectrometry: analytical technique to obtain qualitative and quantitative information of different compounds. Information about molecular mass and structural information of the analysed compounds can also be obtained.
Mixture: solution of several pesticides prepared from the stock solutions.
MRL (mg/kg): Maximum Residue Level. Upper legal level of a concentration for a pesticide residue in or on food or feed set in accordance with the Regulation, based on good agricultural practice and the lowest consumer exposure necessary to protect vulnerable consumers. In Regulation 396/2005 list MRLs for pesticide/commodity combinations, an asterisk indicates that the MRL* is set at or about the LOQ, with the LOQ being here a consensus figure rather than a measured value.
Peak reject: parameter that indicates the minimum area of that a chromatographic peak must have to be integrated.
Peak width: indicates, in seconds, the width of a chromatographic peak.
PRA: product with quality level for pesticide residue analyses.
QuEChERS: Quick, Easy, Cheap, Effective, Rugged and Safe, are the characteristics of the extraction method based on two steps: extraction with acetonitrile/partition after adding a mixture of clean up salts through solid phase dispersion (D-SPE) and injecting directly in the chromatograph.

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R. Match/Match threshold: parameter that indicates similarity between tandem mass sample spectra and spectra from active substance standard (found in reference standard). It can vary from 1 to 1000 and considering 700 as threshold for acceptance.
Reference material: material that has been characterised regarding its concentration in one specific pesticide.
Report threshold: minimum concentration of a pesticide to be included in the analysis report.
rpm: revolutions per minute.
Sampling of opposite quarters: refers to the process of dividing the laboratory sample into quarters and retaining the opposite ones.
Search window: time interval selected in instrumental method in which a chromatographic peak is searched and central time is similar to the expected retention time.
Slope sensitivity: minimum signal/noise rate needed to integrate chromatographic peaks.
Stock solution: solution of one pesticide prepared from a reference material of high concentration in the pesticide.
TIC: "total ion count", refers to the total amount of ion fragments in mass analyzer.

MATERIALS AND EQUIPMENT

Standard laboratory material (micropipettes, volumetric flasks, filters...)
Chromatographic system: gas chromatograph and mass detector.
Capillary-column chromatography 30 m x 0.25 mm (i.d) x 0.25 µm, stationary phase: 5% diphenyl- 95% dimethylpolysiloxane and low bleed.
Consumables (ferrules, insert lines, septum....)
Helium filters to capture oxygen and water.
Bead beating system.

REAGENTS

Helium (quality C-50)
Carbon dioxide (liquid)
Cyclohexane (PRA quality)
Acetone (PRA quality)
Acetone (synthesis grade)
Methanol (for liquid chromatography)
Ethyl acetate (PRA quality)
Acetonitrile (for liquid chromatography)
Formic acid 85% (PA quality or superior)
Formic acid solution 5% in acetone: in a 25 ml volumetric flask, pour 1.47 ml of formic acid (85%) and take to a final volume of 25 ml with acetone.
Sodium hydroxide (PA quality or superior)
Sodium hydroxide 5 N. Dissolve 2 g of sodium hydroxide and take to a final volume of 10 ml with MilliQ water.
Magnesium sulphate anhydrous (PA quality). Before use, introduce in the muffle furnace (550ºC) during 8 h to eliminate phthalates.

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Sodium chloride (PRS quality or superior)
Tri-sodium citrate anhydrous (PRS quality or superior)
Di-sodium hydrogencitrate sexquihydrate (PRS quality or superior)
Primary secondary amine (PSA)
Salt mixture (commercially available or prepared in laboratory):
Mixture 1: Buffer salts mixture:
4 g ± 0.2 g $MgSO_4$
1 g ± 0.05 g NaCl,
1 g ± 0.05 g Na_3Citrato dihydrate
0.5 g ± 0.03 Na_2HCitrato sesquihydrate
Mixture 2: Cleaning up mixture:
25 mg of PSA per ml of extract
150 mg of $MgSO_4$ pr ml of extract
Triphenyl phosphate (TPP) (synthesis grade) internal standard.
Internal standard solution 25 mg/L in acetonitrile: weigh 5 mg of triphenyl phosphate in acetonitrile and take to a final volume of 200 ml in a volumetric flask. Store in refrigeration.
Nitrogen of analytical quality (C-50 or superior).
Analytical standards of pesticides, pure and certified.
Standard solution of pesticides.

PROCEDURE

Sample processing

For fresh vegetable samples, analysis will be performed within 24 h after sample reception in the laboratory. If this is not possible, homogenised analytical samples will be frozen (-20ºC) and analysis will be performed within 24 h after defrost process.

For frozen samples, analysis will be performed within 24 h after defrost process.

For low-water content samples and canned vegetables, analysis will be performed within 48 h from sample reception.

The analytical sample will be constituted from the laboratory sample taking into account Regulation 396/2005/CE where the parts of the product to be analysed are specified in which MRL are applicable.

For specific samples or studies, analytical sample will be composed of the plant material specified in the study.

Extraction procedure

Weight the amount of sample required (according to Table 1) in a 50 ml falcon conical tube with a precision of 0.05 g and add cold distilled water if necessary.

Table 1. Sample type, usual weights and water addition.

Sample type	Weight	Water	Notes
Cereals, flours, legumes	5 g	10 g	-

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Dry fruits	5 g	7.5 g	Water can be added during homogenisation process (then use 12.5 g for the analysis)
Fruits and vegetables (>80 % water)	10 g	-	-
Fruits and vegetables (25-80 % water)	10 g	X g	X=10 g – water content in 10 g sample
Spices	2 g	10 g	-

When distilled water has been added, wait at least 15 minutes to continue with the next step.
Add internal standard (TPP) 25 mg/L in acetonitrile following the amounts indicated in Table 2 hereunder.

Table 2. Sample weight, final dilution and internal standard volume.

Sample weight (g)	Final Dilution Factor	Internal Standard volume (µL)
10	1	100
5	1	50
2	1	20
2	5	100

Add 10 ml of acetonitrile.
Close the falcon tube and shake vigorously manually or in the automatic bead beating system for a minute.
Add Mixture 1 (described above) in the falcon tube and shake vigorously for some seconds to avoid salt lumps. For acid matrixes of pH<3 (i.e. citrus), add 600 µL of sodium hydroxide 5 N.
Shake the tubes manually or in the automatic bead beating system for 2 minutes (800 rpm).
Centrifuge for 10 minutes at 4500 rpm or more.
Transfer X ml of the upper phase to a centrifuge tube of 15 ml containing Mixture 2 (cleaning up mixture: X*25 mg PSA and X*150 mg $MgSO_4$). *(Usually 6 ml of the upper phase will be transferred to a 15 ml falcon tube with 150 mg of PSA and 900 mg of $MgSO_4$).*
Shake during at least 30 seconds in orbital shaker.
Centrifuge during 10 minutes at 4500 rpm or more.
Filter the upper phase (filter of 0.22 µm) and pour Y ml *(usually 1 ml)* into a chromatographic vial and evaporate under N_2 until dryness.
Redissolve in Z ml of **cyclohexane** and add 10*Z µL of formic acid (5% in acetone). Usually, redissolve in 1 ml of cyclohexane and add 10 µL of formic acid (5% in acetone).

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This previous step differs from standard method CEN/TC 275 EN 15662:2008 standing that sample should be injected in the GC system dissolved in acetonitrile. This modification avoids damage in the chromatographic columns and enhances performance of the chromatographic peaks. Column life shortens if samples are injected in a medium polarity solvent such as acetonitrile, so an apolar solvent such as cyclohexane results more convenient to preserve columns and obtain good chromatographic performance.

It is important to take into account the dilution factor in the extraction process in relation to the sample weight and the final reconstitute volume. The following cases may happen:

Dilution factor 1: final concentration in extract is 1 g/ml.
Dilution factor 5: final concentration in extract is 0.2 g/ml.

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Table 3. Dilution factors.

Dilution Factor	Sample Weight (g)	ACN Vol. (ml)	Evaporation Vol. (ml)	Reconstruction Vol. (ml)	Examples
1	10	10	1	1	Fruit and vegetables
1	5	10	1.5	0.75	Cereals, legumes, dry fruits.
1	2	10	1.5	0.3	Spices
5	2	10	1	1	Spices, concentrated samples

Inject the sample in the GS-MS/MS system.

When instrumental analysis cannot be performed immediately after extraction, chromatographic vials will be stored at -20ºC until the analysis.

If pesticide amount in samples is above the upper calibration level (more than 10%), dilution of the samples will be performed. Dilutions will be performed using a volume of the corresponding sample extract and extract of a blank matrix following the formulas:

$$Vm = \frac{Vfinal}{Dilution}$$

$$Vext = Vfinal - Vm$$

During the dilution process, concentration of matrix (1 g/ml) keeps constant. Maximum dilution factor will be 50.

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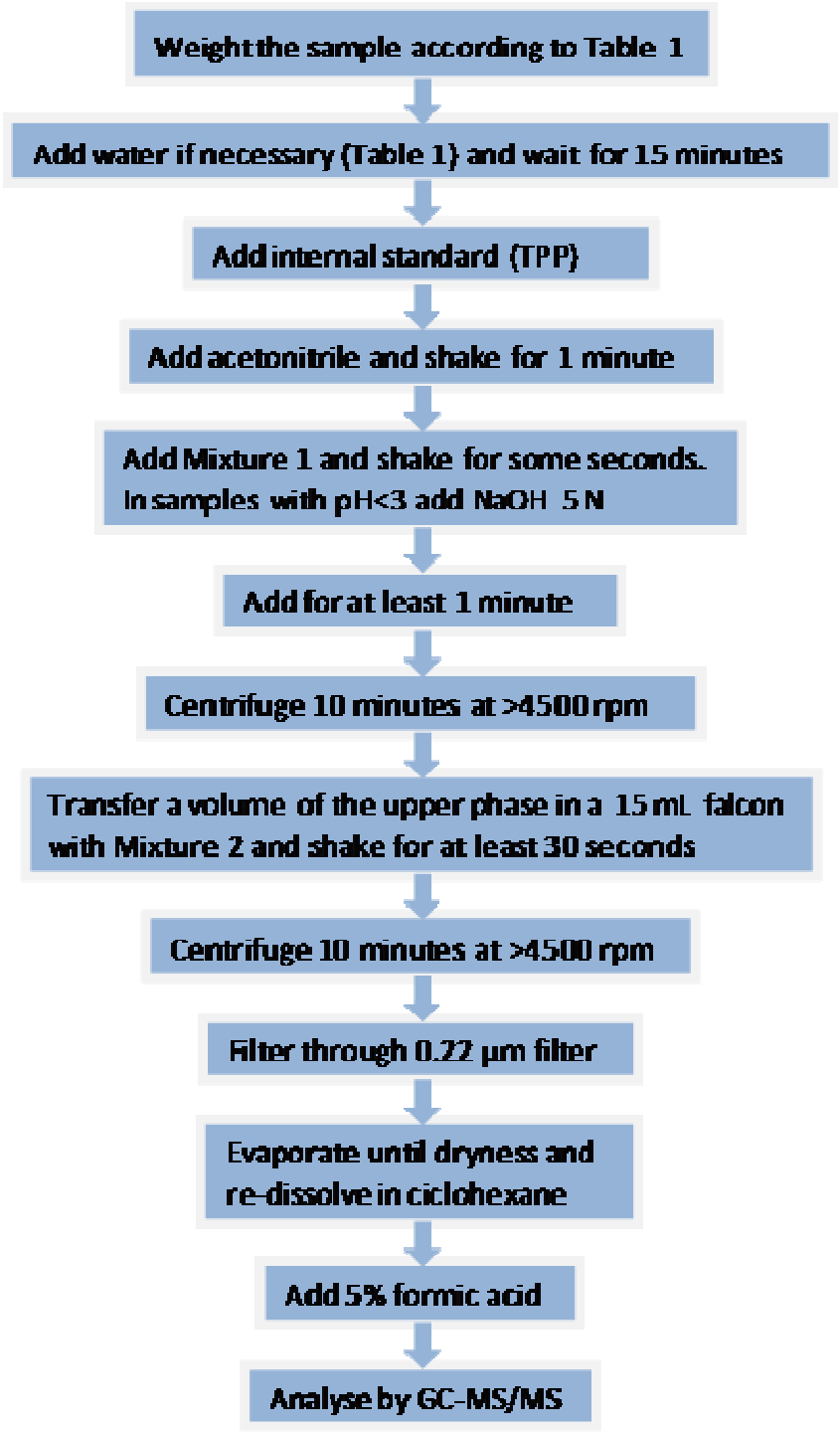
Flow chart of the extraction process for GC



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Preparation, control and storage of standards and reference material

Certified standard materials must be unequivocally identified at the laboratory reception. Control of lot, reception date, opening date, expiry/finish date must be recorded.

Volumetric flasks of A class must be used.

Stock solutions will be prepared from the certified pure reference materials and concentration values corrected by the purity of the standard materials used.

Stock solutions will generally have an expiry date of 1 year or less if one of the individual pure components has a reduced expiry date.

Stock solutions will be prepared in volumetric flasks and afterwards stored in a dark glass bottle in the freezer.

Stock solutions will be prepared in ethyl acetate.

Stock solution will be identified with the number of stock solution and date of preparation.

Concentration in stock solution will be computed as:

$$Concentration(mg/l) = \frac{mg}{vol} * \frac{R}{100}$$ where,

mg= mg of weighted pesticide

vol= final volume of solution (l)

R= purity of the certified material.

To control any loss of solvent or entry of water in stock solutions glass bottles containing stock solutions should be weighted in a 0.01 g precision balance. Weight values should be conveniently registered. Every time stock solutions have to be used, bottles will be weighted and if there is a difference of more than 0.5 g, solutions will be discarded.

Working mixtures will be prepared from stock solutions. Stock solutions will be at room temperature and out of light. Working mixtures will be prepared with micropipettes and corresponding solvents (cyclohexane for calibration mixture and acetonitrile for addition mixture).

Expiry date is defined by the shorter expiry date of stock solutions used.

Stock solution volume will be computed as follows:

$$V_1 = \frac{C_2 * V_2}{C_1}$$

V_1 = volume of stock solution (l).

C_1 = concentration of pesticide in stock solution (mg/l).

V_2 = final volume of the mixture (l).

C_2 = analyte concentration in the mixture (mg/l).

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Calibration standards will be prepared on a matrix blank of the corresponding family of plant products analysed to avoid a possible matrix effect in the quantification of the samples. If matrix-match standards are performed and method is fully validated, it is not necessary the use of analyte protectants during the extraction step.

Working mixtures will be codified indicating the type of matrix and instrumental technique used, date of preparation, use (calibration/addition) and calibration level.

Certified reference materials will be stored following manufacturer specifications. The three possibilities are:

Freezing: -18ºC
Refrigeration: 2-8ºC
Room temperature: 18-28ºC

Solvents used in the preparation of standards and extraction procedure will be stored at room temperature.

Blank matrixes and extracts thereof will be stored at -18ºC.
Stock pesticides solutions will be stored under refrigeration conditions.
Pesticide working mixtures will be stored at -18ºC.

When a stock solution must be replaced by a new one, both solutions may be compared. To check this, two solutions of the same concentration in matrix extract may be prepared and analyzed the same day and under the same conditions. Peak areas may be compared and do not differ more than 10%.

RESULTS

Identification

Tandem mass technology allows a high selectivity in the determination of analytes. Identification and confirmation of a positive result will be performed at the same time using the instrumental software.

Identification of each pesticide can be automatically performed. A pesticide is identified as positive when it fulfils specifications regarding *Retention Time, Match Threshold, Peak reject* and *Report threshold* established in the instrumental method.

Confirmation

In order to avoid a false positive, all the pesticides found automatically by the chromatographic software must be revised one by one. Comparing the spectrum of the pesticide in the samples and reference spectrum of the pesticide in the standard mixture the following parameters must be checked:

Rmatch ≥ 700.
Check the existence of characteristic m/z of each pesticide.
Check retention time for the daily method specifications.

Retention time, *Integration Window, Peak Width, Slope Sensitivity, Peak Reject* and *Search Window* are fixed for each pesticide in the base instrumental method.

Analyst must review one by one the pesticides that have not been identified automatically but *Rmatch* and *Fmatch* score are superior to 400. If some of them are confirmed, the

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analyst must integrate the peaks and add the pesticide to the confirmed list for that sample.

Quantification: calibration table

Injection of calibration standards will be daily performed. Calibration of "representative pesticides" (described below) will be injected with every series of samples.

"Non representative pesticides" will be injected once a year and when a positive appears in a sample series. However, the analyst must check in each series that peak of LCL of each pesticide is correctly visible.

Calibration method will be daily edited introducing the new standard areas for each of the pesticides.

Integration of each pesticide must be carefully checked.

Peak Reject for each pesticide is set as half value of its LCL area.

Once calibration is performed, all parameters *Retention Time, Integration Window, Peak Width* and *Slope Sensitivity* must be edited in the daily copy method.

Analyst must check the following parameters for each of the calibration curves of each pesticide:

Check all calibration curves and confirm that $r^2 \geq 0.990$.
Check that RSD(%) ≤ 30. RSD corresponds to the relative standard deviation of the response factors for each point of the calibration curve.
RSD and r^2 only applies positive pesticides due to linearity only affects quantification quality.
Check that residual deviations between standards and predicted values for each individual calibration level do not differ more than ±20% in the relevant region (±10% when MRL is approached or exceeded).
Check that recuperation factor for internal standard TPP obtained in the analysed samples is above the established interval (60-140%). If not, extraction process must be repeated.
Check that signal/noise ratio in the LCL for each pesticide should be at least 3:1.

Quantification will be performed by external calibration by interpolation of the area of the quantification ion peak in the calibration curve obtained for a pesticide in a series of samples.

$$Ap = Cp * m + b$$ where:

$Ap =$ area of the quantification ion peak area of the standard.

$Cp =$ standard concentration.

$m =$ slope of the calibration curve.

$b =$ y-intercept.

Then, concentration in the sample (Cm) is obtained substituting the area value in the following equation:

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$$Cm(mg/kg) = \frac{Am - b}{m}$$

where $Am =$ is the area of the quantification ion peak of the sample.

Each of the calibration curves will be fitted by Weighted Least Squares Regression with the objective of weighting the lower part of the calibration curve and correctly quantify the pesticides in the whole calibration range minimising the prediction errors in the LCL.

Recuperation factor for internal standard TTP must be within the established range:

- Added concentration: 0.25 mg/kg
- Acceptance interval: 0.150-0.350 mg/kg

Calibration control

In each series of analyses (sequence), chromatographic signal will be checked by comparison of the areas of the lowest calibration level (LCL) at the beginning of the sequence with the similar standard injected at the end of the sequence.

Four pesticides should be selected to perform this verification. Pesticides must belong to different chemical families and have different retention times. Reference pesticides must be periodically rotated each time that one cycle of control additions of representative pesticides is performed.

Acceptance criteria will be that areas of both standards differ less than 30%. If not, samples must be repeated except from the following cases:

negative samples, if peak areas of the LCL injected at the end of the sequence have acceptable values for all pesticides,
positive samples, if criteria of 30% fulfils for positive pesticides and peak areas of LCL injected at the end of the sequence have acceptable areas.

Reporting results

The following criteria will be applied:

if result is under the LCL, it will be reported as “<LCL” indicating the value with a significant figure (i.e. <0.01 mg/kg),
if result is above the LCL and lower than 10 mg/kg, it will be expressed with two significant figures (i.e. 0.052, 0.25 or 9.5 mg/kg).
if result is equal or superior to 10 mg/kg, it will be expressed with three significant figures (i.e. 11.2 mg/kg).
if result is out of the upper working range for each pesticide (HR), it is expressed as “above high working range” (>HR) indicating the value of HR for each pesticide. As set before, maximum dilution factor will be 50.

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METHOD CONTROL AND PERFORMANCE VERIFICATION

It is necessary to verify the correct performance of the method. The following controls must be carried out:

Analysis of blank reagents

A whole analysis without matrix and internal standard must be carried out when a change in the lot of some of the extraction reagent occurs (acetonitrile, cyclohexane, salts, etc...).

It must be checked that no pesticide peak is detected with an area 20% larger than the LCL peak area. All results must be recorded.

Control series

Analysis of instrumental blank matrixes

It represents the negative control of the instrumental part of the analytical method.

For each sequence of analyses, an extract of a blank matrix must be injected. This blank extract must have been previously analysed and the absence of the analysed pesticides confirmed.

If some of the blank extracts present a peak (>20% LCL) for some of the pesticides, the cause must be investigated (possible causes: contaminated insert line, defective syringe washing, etc...).

Results must be recorded.

Recovery control through internal standard addition

Before the sample is extracted, a known amount of internal standard is added to the sample, and after the chromatographic analysis the recovered value must be within the acceptance range.

Added concentration: 0.25 mg/kg
Acceptance interval: 0.150-0.350 mg/kg

Results must be recorded.

Pesticides control addition and performance of routine recoveries

In each sequence, a series control is performed by the addition of some pesticides included in the method at a level of addition of the LCL or other interesting level (i.e. MRL or HR) (spiked sample). The spiked sample will be one of the selected representative matrixes.

Recovery data obtained, must be reported in a control chart with the aim of monitoring trueness and precision for each pesticide and matrix group. The following criteria must be fulfilled:

Acceptance criteria for not fully validated matrixes:
Recovery interval for validity of control series: 60-140 %

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Recovery out of the former interval and within interval 40-160%. Control series can be considered correct but further monitoring of the following control series must be performed to check that the same thing does not happen two consecutive times with the same pesticide. If so, control series is not valid and sequence of analyses must be repeated.
Recovery out of interval: 40-160 %. Control series is not valid. Sequence of analyses must be repeated.
Acceptance criteria for fully validated matrixes:
For a validated matrix, the mean recovery and RDS of the recovery values are calculated using within-laboratory reproducibility data (routine on-going recovery) or repeatability data from initial validation.
Recovery must be within the interval ± 2 RSD to accept the control series.
Recovery out of the former interval and within interval ± 3 RSD. Control series can be considered correct but further monitoring of the following control series must be performed to check that the same thing does not happen two consecutive times with the same pesticide. If so, control series is not valid and sequence of analyses must be repeated.
Recovery out of interval ± 3 RSD. Control series is not valid. Sequence of analyses must be repeated.

Representative pesticides: quantification of a spiked sample is performed on a series of representative pesticides selected according to the following criteria:

Higher prevalence in the analysed product.
Different physico-chemical properties.
Different retention times.

The number of representative pesticides to be spiked will be at least 15 + 25% of the public pesticide list of the analytical method of the laboratory.

At least 10% of the spiked representative pesticides will be quantified in each sequence of analyses (at least 5 pesticides by sequence). The selected pesticides will rotate every 5 additions (approximately once a week) within a rolling programme.

If some of the representative pesticides appears (>LCL) in one of the samples of a sequence, and it is not one of the quantified spiked pesticides, a correct quantification of its control addition is necessary, before the analytical report is emitted.

If calibration curve for some of the representative pesticides does not fulfil RSD and r^2 conditions (previously described), control series will not be quantified for this pesticide. However, it must be verified that the chromatographic peak in the spiked sample can be correctly observed.

Not-representative pesticides: these pesticides will be controlled at least once a year performing similar additions. However, if one of these pesticides resulted positive in some of the samples, quantification will include a positive control series of the corresponding pesticide.

Matrixes used for the control series will also rotate periodically once analysed the whole list of representative pesticides. Some of the current seasonal matrices will be used for the control series.

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Analysis of blank matrixes

Laboratory must look for blank matrices without any detectable level of pesticide residue (i.e. organic production, own production, etc...). All blank matrixes will be analysed to assess they fulfil the characteristics of a blank matrix (see definitions section at the beginning of the document).

The matrix is considered to be a "blank" for a specific pesticide if the concentration found in the sample for that pesticide is < 20% LCL.

VALIDATION

The method must be tested to assess for:

sensitivity,
mean recovery (as a measure of trueness or bias),
precision, and
limit of quantification (LOQ).

A minimum of 5 replicates is required (to check the precision) at two levels: the LCL (to check the sensitivity of the method), and, at least, another higher level (usually the MRL). According to NT-19/Rev 2, at least 3 representative matrixes from each of the commodities groups must be validated to consider the pesticides analysis by a specific technique valid.

Method must be validated for the most representative commodities and when it is used in routine for several types of matrixes, on-going quality controls and validation data should be acquired during the routine analysis.

Average recoveries for each representative commodity must be in the range 70-120%, with a RSDr ≤ 20%
Other approaches to demonstrate that the analytical method complies with the performance criteria may be used, provided that they achieve the same level and quality of information.
Where the residue definition incorporates two or more analytes, if possible, the method should be validated for all the analytes included in the residue definition.

Validation parameters to be studied

Linearity should be studied through calibration curve with the criterion of residuals <±20%
Matrix effect should be studied through comparison of response from solvent standards and matrix-matched standards.
LOQ: lowest level for which it has been demonstrated that criteria for accuracy and precision have been met.
Specificity: response in reagent blank and control samples (<30% LOQ).
Trueness: computed as average recovery for both spike levels (70-120%).
Precision (RSD_r%): repeatability for both spike levels (<20%).
Precision (RSD_{wR}%) (within-laboratory reproducibility) to be computed from on-going quality control in laboratory.
Robustness: it can be derived from on-going method validation or verification through establishing average recovery and RSD_{wR}%.

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Linearity

Standard mixtures of 1 µg/ml and 0.10 µg/ml can be used to prepare 7 different calibration levels within calibration range and a blank. Standards' concentrations are 1, 5, 10, 25, 50, 100 and 300 µg/l.

Standards are added on 1 ml of plant extract obtained from a blank representative matrix, the volume is evaporated under N_2 and redissolved in 1 ml final volume. The final matrix concentration is 1 g/ml similar to routine samples.

Blank level is used to verify absence of pesticides.

At least three linearity tests at different sessions should be performed in the selected representative matrixes. Slope (m) and intercept (b) are calculated as follows:

$$y = mx + b$$ where,

y: response area

x: pesticide concentration

Linearity can be computed using the formula:

$$C_m = \left[1 - \frac{S_m}{m}\right] * 100$$ where,

S_m: standard deviation of the slope

m: slope

C_m: linearity coefficient.

Linearity function must be ≥95% in a chromatographic method. Criterion for residuals in each calibration level must be ±20% for the calibration curves.

Selectivity and specificity

Using the linearity studies and, once determined the retention times and the characteristic mass for each pesticide, it must be checked that no interferences in the mixture are found.

For specificity, response in reagents blank and control samples must be <30% the limit of quantification.

Matrix effect should be studied through comparison of response from solvent standards and matrix-matched standards.

Limit of Quantification (LOQ)

LOQ corresponds to the LCL for each pesticide. Signal to noise ratio must be at least 3:1.

LOQ should be verified through additions at the LCL in the samples. The recoveries should meet the requirements of trueness and precision described next.

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Trueness

It is defined as the closeness of agreement between the average value obtained from a series of test results (i.e. the mean recovery) an accepted reference or true value (ISO 5725-1). The measure of trueness is normally expressed as 'bias'.

Trueness must be determined through the study of recoveries at different concentration levels within working range for each pesticide.

Additions should be performed at the LCL and the MRL for each pesticide.

Five different extractions for each of the spike levels and each of the representative matrixes selected, should be performed in repeatability and reproducibility conditions.

Once obtained the concentration values, average, standard deviation, recovery and compatibility index will be computed. Compatibility index is a parameter to show if there are significant differences between the theoretical concentration of the added standard and the recovery value obtained. The following formulas should be applied:

$\bar{x} = \frac{\sum x_i}{n}$	$SD \cdot (standard \cdot deviation) = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n}}$	$CompatibilityIndex = \frac{\lvert V_{Ri} - \overline{x_i} \rvert}{\sqrt{U_{V_{Ri}^p}^2 + \left(t \cdot \frac{S_i}{\sqrt{n}} \right)^2}} \leq$
$Trueness = \frac{V_{Ri} - \overline{x_i}}{V_{Ri}} \cdot 100$	$100 - \text{Trueness} = \text{Recovery}$	

x: obtained concentration values

n: number of repetitions

V_R: reference value (real concentration added)

$U_{V_{Ri}^p}$ =uncertainty of the reference value with a confidence interval of 95%

t: students value for n=10 measures and α=0.05.

It must be checked that for each addition level, average recovery fulfils the requirements included in SANCO/10684/2009, 60-140% for the LCL and 70-120% for the MRL.

Precision

The precision is defined as the closeness of agreement between independent test results obtained under stipulated conditions. The measure of precision usually is expressed in terms of imprecision and computed as standard deviation of the test results (ISO 5725-1). It is usually obtained from recovery or analysis of reference materials, obtained using the same method on the same sample(s) in a single laboratory over a short period of time, during which differences in the materials and equipment used and/or the analysts involved will not occur.

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Data obtained from recovery studies can be used to obtain the mean value and standard deviation for each concentration and coefficients of variation according to the formula:

$$CV\,(\%) = \frac{S}{\bar{x}} * 100$$

It must be checked that repeatability for both spike levels is fewer than 20% (CV≤20%).

Uncertainty

It is defined as a range around the reported result within which the true value can be expected to lie with a specified probability (confidence level, usually 95%). Uncertainty data should encompass trueness (bias) and reproducibility.

As the process includes multiple independent steps (weighting of standards, standards mixture preparation, volume addition, repetitions, etc...), uncertainty could be computed out from the validation process data. Uncertainty of each step should be calculated and global uncertainty is then computed as square root of the sum of individual quadratic uncertainties:

$$U = \sqrt{U_{VR}^2 + \left(W_R \cdot \frac{S_R}{\sqrt{n_R}}\right)^2 + \left(\frac{W_R \cdot S_M}{\sqrt{n_M}}\right)^2}$$

where,

U_{VR}: reference value for uncertainty

S_R: standard deviation of reference samples (obtained in precision tests)

S_M: standard deviation of ordinary samples

W_R: W factor applying depending on the number of repetitions performed to obtain S_R.

n_R: number of repetitions to compute S_R.

n_M: number of repetitions to be computed according to method.

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PESTICIDE DETERMINATION BY LC-MS/MS

Some of the sections of the description of the LC method are similar to the GC-MS/MS method and they are not going to be repeated.

The two main differences compared to the previous method are the following:

- extraction process ends up in acetonitrile:water solvent to be injected in the liquid chromatograph system and
- confirmation of pesticides is made from the ion ratios instead of from the comparison of spectra as in the GC method.

AIM

To describe a method for the analytical determination of pesticides in plant products by liquid chromatography coupled with tandem mass spectrometry detection.

SCOPE

The method can be used to determine pesticides in plant matrixes of the following characteristics:

- Plant products with high water content and low fat content (i.e. vegetables and fruits).
- Plant products with low water content and low fat content (i.e. dry fruits)
- Plant products with low water content and high starch/protein content (i.e. cereals, legumes)
- Spices and aromatic herbs.

REFERENCES

Same references as in the GC-MS/MS method.

BASIS AND DEFINITIONS

Analysis is based on difference of pesticides solubility between aqueous phase (vegetable matrix) and organic phase (solvent). Sample is extracted with acetonitrile and pesticides present in the vegetable are transferred to the organic phase. Salts are used together with the acetonitrile to avoid interferences and improve extraction yield.

Magnesium sulphate: eliminates excess of water.
PSA: eliminates sugars, fatty acids, organic acids and pigments.
Buffer salts: stabilize the pH between 5 and 5.5.

Instrumental determination can be carried out by liquid chromatography coupled to tandem mass spectrometry detection.

Definitions (in addition to definitions already described):

API, Atmospheric pressure ionisation (for LC-MS): A generic term including electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI).
Ion ratio: for a chromatographic method coupled with tandem mass spectrometry detector is the ratio between diagnostic quantification ion and confirmation ion. It is

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used to establish recommended tolerances for residue confirmation according to SANCO/10684/2009.

Table 4. Tolerances for residue confirmation in LC-MS/MS

Relative Intensity (% of base peak)	LC-MS/MS
>50%	±20%
>20% to 50%	±25%
>10% to 20%	±30%
<10%	±50%

MATERIALS AND EQUIPMENT

Standard laboratory material (micropipettes, volumetric flasks, filters...)
Chromatographic system: HPLC liquid chromatograph and mass detector.
Column Atlantis T3® 3µm, 2.1x 100 mm from Waters or similar.
HPLC consumables.
Bead beating system.

REAGENTS *(only differences with regards to the previous method are described)*

Formic acid 85% (PA quality or superior)
Formic acid solution 5% in water: in a 25 ml volumetric flask, pour 1.47 ml of formic acid (85%) and take to a final volume of 25 ml with water.

PROCEDURE

Sample processing: extraction procedure for HPLC

Similar to GC-MS/MS procedure except from step 12 onwards:

12. Filter the upper phase (filter of 0.22 µm) and pour Y ml *(usually 0.5 ml)* into a chromatographic vial and *another 0.5 ml* of MilliQ water. Add 10 µL of formic acid (5% in water). At this point, a concentration step can be included by evaporating a volume of acetonitrile and redissolving in a smaller volume of acetonitrile.

It is important to take into account the dilution factor in the extraction process in relation to the sample weight and the final volume.

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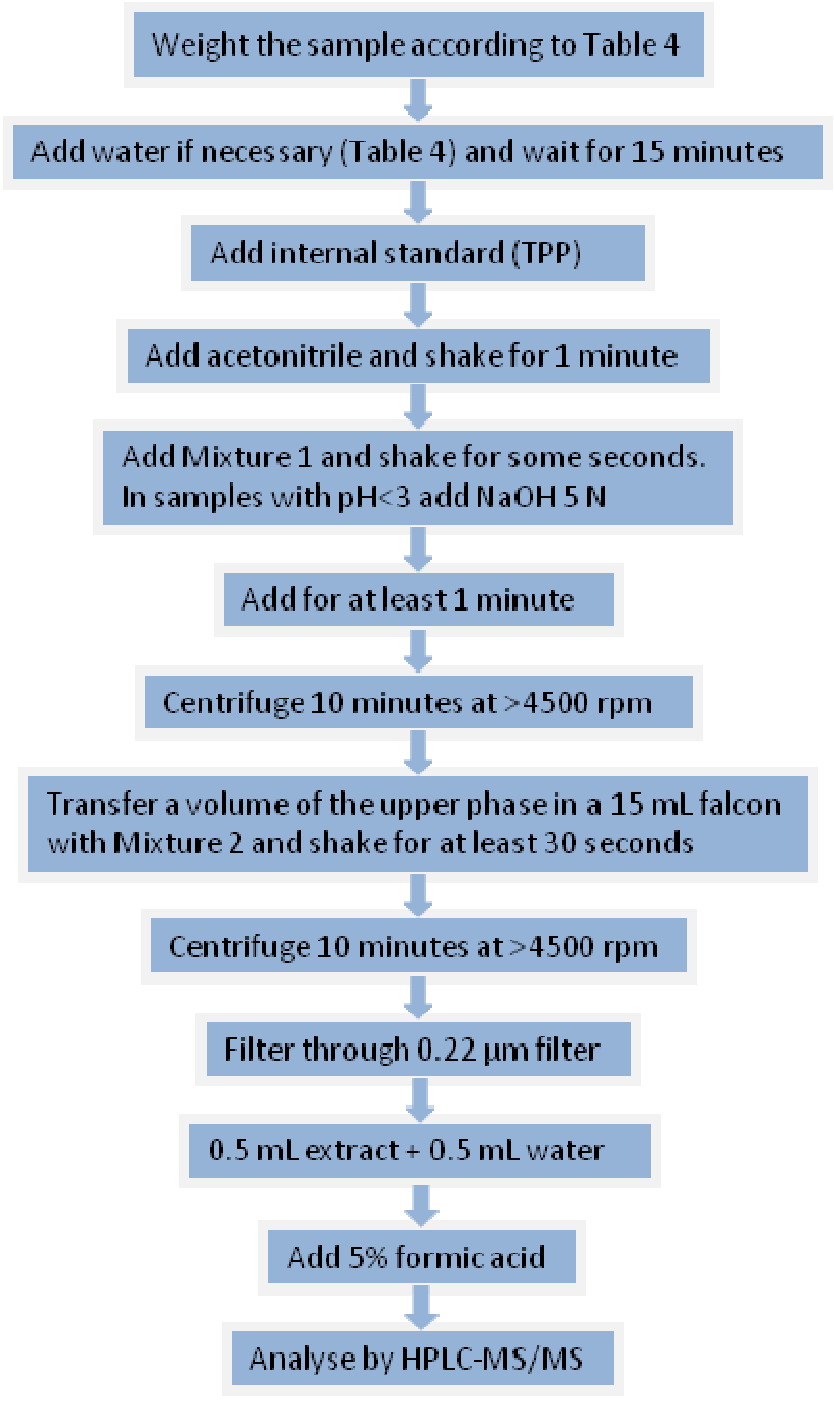
Flow chart for the extraction process for HPLC



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Chromatographic conditions

Gradient method at 0.300 ml/min (Table 5).

Solvent A: Ammonium formiate 5 mM acidified with formic acid 5%.
Solvent B: methanol
Solvent C: acetonitrile (clean up solvent)

Flush solvent: 50% methanol: 50% water.

Injection volume: 5 µl.

Ionization mode: Electrospray positive and negative mode.

Table 5. Method details for gradient elution in HPLC separation.

Time (min)	Total flow (µl/min)	A(%)	B(%)
0	300	95	5
1	300	95	5
1.10	300	70	30
10	300	0	100
17	300	0	100
17.10	400	95	5
20	400	95	5
20.10	300	95	5
22	300	95	5

Preparation and storage of standards and reference material

Same as in CG-MS/MS method except for:

Working mixtures will be prepared from stock solutions. Stock solutions will be at room temperature and out of light. Working mixtures will be prepared with micropipettes and corresponding solvents.

In this case, methanol is used for calibration and addition mixtures.

RESULTS

Confirmation

In order to avoid a false positive, all the pesticides found automatically by the software must be revised one by one. In the previous method, GC-MS/MS confirmation was performed by comparison between the pesticide spectrum in the sample and corresponding pesticide spectrum in standards. On the contrary, LC-MS/MS confirmation is performed through the comparison between ion ratios in the sample and standard for each individual pesticide. Tolerances for acceptance are described before (Table 4).

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All QA/QC procedures must be also performed as in GC-MS/MS method.

VALIDATION

The same validation programme should be performed as described before for GC-MS/MS method.

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CONCLUSIONS

Multi-residue methods based on chromatography tandem mass detection allow determining (identifying and quantifying) different pesticide residues present in a sample in one unique analysis.

In this document, two methods are described. In both, a common extraction method (QhEChERS extraction) and a posterior chromatographic analysis are included with the corresponding detailed description of the different steps, solvents and equipment used, controls established from the entrance on the sample in the laboratory until the emission of the results report.

The main contribution of this detailed description is that it joins all the guidelines included in the reference documents (SANCO/10684/2009, CEN/TC 275 EN 15662:2008, Commission Recommendation of 3 March 1999) and make them implementable from a practical point of view.

A modification from the QhEChERS standard method has been introduced in the GC protocol regarding an evaporation-redissolution step changing the solvent. It has been explained and justified to provide the basis for a possible future modification in the standard method.

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