# Development of analytical methods: Studies of the main degradation processes of pesticides in commodities during the extraction steps

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## 1. Aim and scope

This study reports different modifications in the extraction process, with the aim of reducing or eliminating pesticide degradation during the extraction step. The pesticides diafenthiuron, thiodicarb, dichlofluanid and tolyfluanid have been evaluated in different matrices such as tomato, broccoli, pepper, onion and rice. In the case of diafenthiuron, a compilation of the various modifications previously reported is made with some more recently studied modifications.

## 2. Short description

The analysis of pesticides that undergo degradation during the extraction process constitutes an analytical challenge. Some of the evaluated compounds are degraded in the extraction and cleaning process, which makes them very problematic for routine analytical laboratories, and may cause laboratories to report false negatives or incorrect quantitation. The extraction process is also complicated by the multiple factors that favour these degradation processes, such as temperature, light, water, and oxidation caused by the enzymes present in the matrices, among others. All this hinders the mission of its analysis and correct quantification.

During the extraction process of some matrices, thiodicarb is degraded to methomyl. In this study, the effect of extraction temperature has been evaluated in tomato, rice and onion. Subsequently the effect of antioxidant addition on rice has been studied. For dichlofluanid and tolylfluanid analysis, the effect of PSA on the extraction was evaluated with respect to the exposure time of the supernatant with PSA during the clean-up. The influence of temperature during the clean-up process with a cold centrifuge was also studied on pepper and broccoli. The evaluation of diafenthiuron is a compilation of modifications during the extraction process with the aim of improving recovery rates. These modifications include the addition of antioxidants (broccoli), testing how pH affects the degradation of diafenthiuron (broccoli, leek, orange, grape and pepper) and how extraction in cold conditions affect the recoveries (broccoli, pepper, and tomato).

## 3. Apparatus and consumables

- Automatic pipettes, suitable for handling volumes from 1 µL to 5000 µL
- Graduated 10 mL pipette.
- 50 mL and 15 mL PTFE centrifuge tubes
- Vortex Shaker IKATM 4 Basic
- Axial shaker Agytax SR1 CP57

- Centrifuge Orto Alresa Consul 21, suitable for the centrifuge tubes employed in the procedure and capable of achieving at least 4000 rpm
- Centrifuge Sigma 2-16KL
- Injection vials, 2 mL, suitable for LC and GC auto-sampler
- Amber vials, 4 mL
- Direct reading thermometer with thermocouple probe

## 4. Chemicals

- Acetonitrile ultra-gradient grade (AcN)
- Trisodium citrate dihydrate
- Disodium hydrogenocitrate sesquihydrate
- Sodium chloride
- Anhydrous magnesium sulphate
- Anhydrous calcium chloride
- Primary secondary amine (PSA)
- Ascorbic acid
- Tocopherol
- Ammonium formate
- Ultra-pure water
- Ethyl acetate
- Formic acid
- Pesticide analytical standards
- Dry ice

## 5. Procedure

### 5.1. Sample preparation

The evaluation has been made in tomato, pepper, broccoli, onion and rice. All parts of the sample are taken as specified in Annex I to COMMISSION Regulation (EU) No 752/2014 of 24 June 2014 (replacing Annex I to Regulation (EC) No 396/2005). In the case of rice after crushing, a completely homogeneous powder with a particle size ≤ 1 mm must be obtained.

### 5.2. Pesticide stock solutions and working mix solutions.

Individual pesticide stock solutions (1000–2000 mg/L) were prepared in acetonitrile and were stored in screw-capped glass vials in the dark at -20 °C.

For spiking, representative portions of the previously homogenized samples were spiked homogenously with the appropriate amount of the working standard

solution in acetonitrile. The validation methods were performed at a fortification level of 0.010 mg/kg. Three replicates were analysed at the spiked level.

5.3. Extraction procedure

*5.3.1 Study of thiodicarb in tomato and rice*

For the evaluation of thiodicarb, the QuEChERS citrate extraction method has been used with tomato, onion and rice at room temperature and using a cold bath at 2 °C during the whole extraction process.

For rice matrix, the addition of ascorbic acid (3 %) as well as tocopherol (0.4 %) before water addition during the extraction process for dry matrices, has been evaluated.

**QuEChERS citrate:**

1. Weigh 10 g ± 0.05 g of sample in 50 mL PTFE centrifuge tube.
2. Add 10 mL of acetonitrile.
3. Shake the sample using an automatic axial shaker for 6 min.
4. Add 6.5 g ± 0.1 g of the prepared salt mixture or from the individual reagents (sodium chloride (1 g ± 0.1), magnesium sulphate (4 g ± 0.1), disodium hydrogen citrate 1 ½ hydrate (0.5 g ± 0.1), sodium citrate (1 g ± 0.1)).
5. Shake the samples again in the automatic shaker for 6 min.
6. Centrifuge the tubes at 3700 rpm for 10 min.
7. Transfer 5 mL of the supernatant to a 15 mL PTFE tube containing 750 mg magnesium sulphate and 125 mg PSA.
8. Vortex the tube for 30 sec.
9. Centrifuge the tubes at 3700 rpm for 5 min.
10. Transfer the supernatant to a 4 ml vial, add 10 µl per ml extract of 5% formic acid solution in acetonitrile.

**QuEChERS citrate for dry matrices:**

1. Weigh 5 g ± 0.05 g of sample in 50 mL PTFE centrifuge tube.
2. Add 10 mL of water for hydrate the sample.
3. Add 10 mL of acetonitrile.
4. Shake the sample using an automatic axial shaker for 30 min.
5. Add 6.5 g ± 0.1 g of the prepared salt mixture or from the individual reagents (sodium chloride (1 g ± 0.1), magnesium sulphate (4 g ± 0.1), disodium hydrogen citrate 1 ½ hydrate (0.5 g ± 0.1), sodium citrate (1 g ± 0.1)).
6. Shake the samples again in the automatic shaker for 6 min.
7. Centrifuge the tubes at 3700 rpm for 10 min.

8. Take 8 mL of the supernatant with a micropipette and transfer to a 15 mL Falcon tube. Place the tube on dry ice for 20 min and centrifuge for 1 min at 3700 rpm.
9. Transfer 5 mL of the supernatant to a 15 mL PTFE tube containing 750 mg magnesium sulphate and 125 mg PSA.
10. Vortex the tube for 30 sec.
11. Centrifuge the tubes at 3700 rpm for 5 min.
12. Transfer the supernatant to a 4 ml vial, add 10 µl per ml extract of 5% formic acid solution in acetonitrile.

*5.3.2 Study of dichlofluanid and tolylfluanid in pepper and broccoli:*

Dichlofluanid and tolylfluanid rapidly hydrolyse in the extract at basic pH. For the evaluation of the extraction of theses pesticides, the effect produced by the time elapsed after PSA addition in the clean-up stage was studied. The extraction procedure used was the QuEChERS citrate method. In the first experiment (A), the supernatant obtained from the samples was left in contact with the clean-up salts during the different established times. Three times were evaluated: T0, T30 and T60 min **(Figure 1)**.

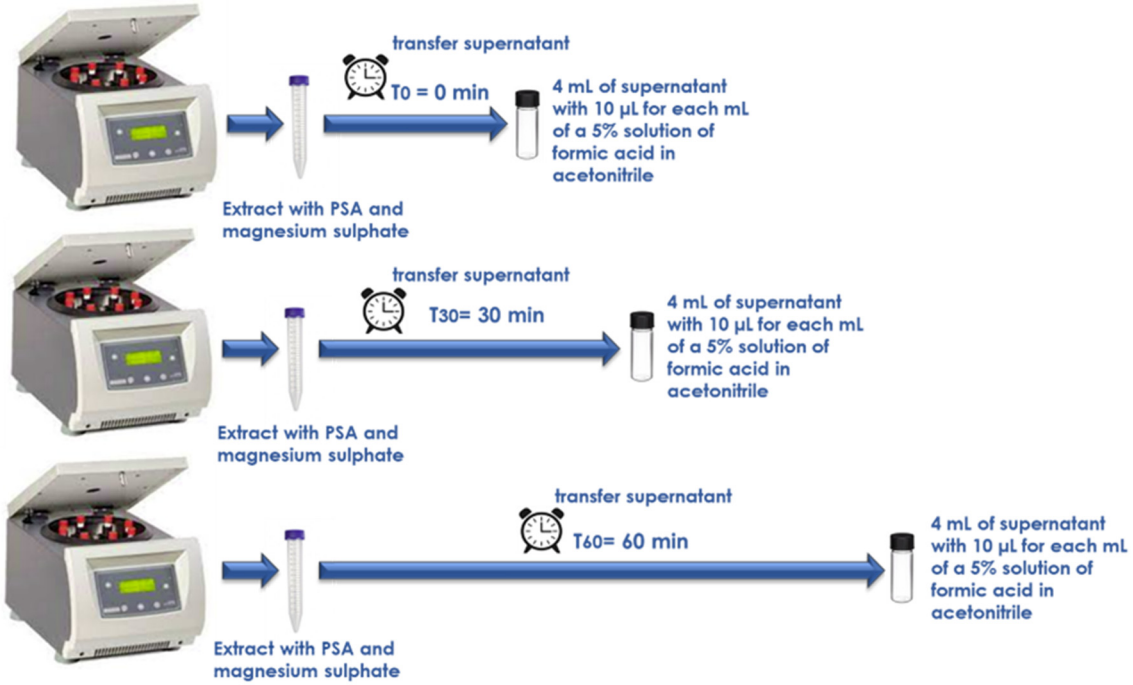



**Figure 1.** Scheme of experiment A

In the second experiment (B), the samples were centrifuged in the clean-up stage under cold conditions at -2 °C. In this case, only two exposure times were evaluated, T0 and T15 min **(Figure 2)**.

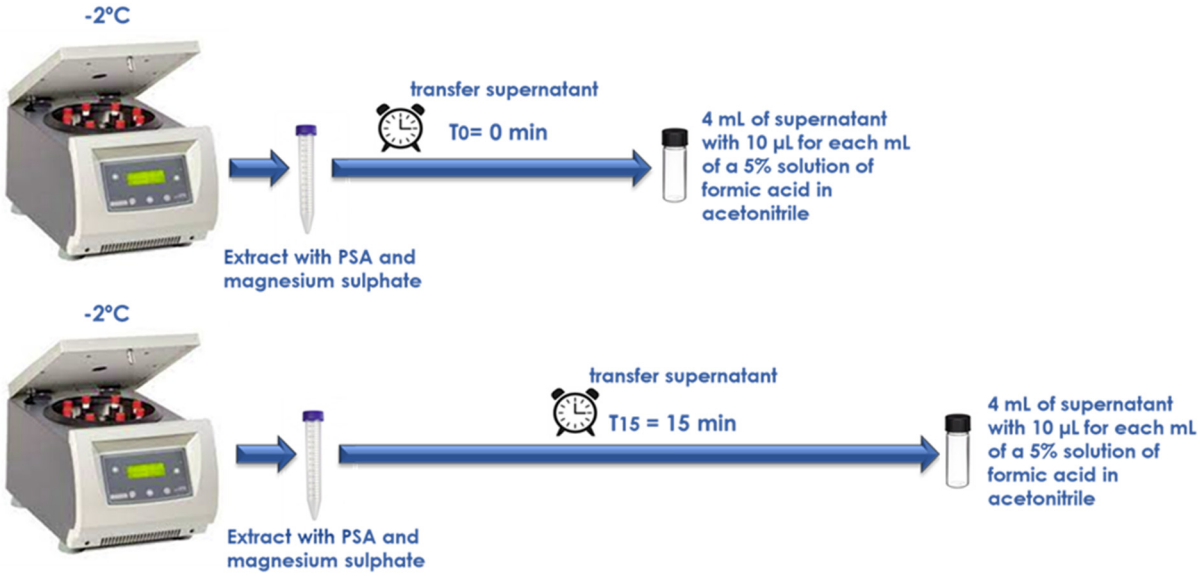



**Figure 2.** Scheme of experiment B

*5.3.3 Study of diafenthiuron in tomato, pepper and broccoli:*

For this test, QuEChERS citrate was used. Two different extraction conditions were studied, extraction at room temperature and an extraction in a cold bath at 2 °C.

5.4. Measurement

All samples were analysed by LC-MS/MS and GC-MS/MS system operated in multiple reaction monitoring mode (MRM). Selected reaction monitoring (SRM) experiments were carried out to obtain the maximum sensitivity for the detection of the target molecules. Two SRM transitions and a correct ratio between the abundance of the two optimised SRM transitions (SRM2/SRM1) were used for confirmation of the studied compounds, along with retention time matching. The mass transitions used are presented in **Appendix I (Table A1)**.

5.5. Instrumentation and analytical conditions for the LC- MS/MS system

*5.5.1. 1290 UHPLC (Agilent)*

- Column: Zorbax Eclipse Plus C8 2.1x100 mm and 1.8 µm particle size (Agilent)
- Mobile phase A: Water (0.1 % formic acid, 5 mM ammonium formate, 2 % MeOH)
- Mobile phase B: Methanol (0.1 % formic acid, 5 mM ammonium formate, 2 % water)
- Column temperature: 35 °C
- Flow rate: 0.3 ml/min
- Injection volume: 5 µL (10 µL in the case of rice)
- Autosampler temperature: 12 °C

Mobile phase gradient for pesticides analysis:

| Time [min] | Mobile phase A% | Mobile phase B % |
|---|---|---|
| 0 | 100 | 0 |
| 2 | 80 | 20 |
| 15 | 0 | 100 |
| 18 | 0 | 100 |

*5.5.2. 6490A triple quadrupole system (Agilent)*

- Ionisation mode : positive mode and negative mode
- Capillary (positive and negative): 3000 V
- Nebulizer: 45 psi
- Nozzle: 400 V
- Drying gas Flow: 13 L/min
- Drying gas temperature: 120°C
- Sheath gas Flow: 10 L/min
- Sheath gas temperature: 375 °C
- High Pressure RF (positive): 150 V
- High Pressure RF (negative): 110 V
- Low Pressure RF (positive): 60 V
- Low Pressure RF (negative): 60 V

5.6. Instrumentation and analytical conditions for the GC- MS/MS system

*5.6.1 Intuvo 9000 GC system (Agilent)*

- Column: 2 planar columns HP-5MS UI (15 m long x 0.25 mm i.d. x 0.25 µm film thickness)
- Injection mode: splitless
- Ultra-inert inlet liner with a glass wool frit from Agilent
- Sample Injection volume: 1 µL
- Inlet temperature: 80°C hold for 0.1 min, then up to 300°C at 600°C/min, hold for 5 min and then to 250°C at 100°C/min
- Carrier gas: Helium at constant flow=1.28 mL/min column 1, 1.48 mL/min column 2
- Carrier gas purity: 99.999%
- Oven temperature: 60°C for 0.5 min, up to 170°C at 80°C/min, and up to 310°C at 20°C/min (hold for 3.5 min)

*5.6.2 7410 triple quadrupole system (Agilent)*

- Ionisation mode: electron impact ionisation
- Temperature of the transfer line: 280°C
- Temperature of ion source: 280°C

- Collision gas: nitrogen
- Collision gas purity: 99.999 %
- Solvent delay: 2.6 min

## 6. Results

6.1 Thiodicarb study:

Thiodicarb is a carbamate insecticide used for the control of lepidopterans, coleopterans, slugs and other pests of fruits and vegetables. Thiodicarb comprises two methomyl molecules linked by a sulphur atom and it is metabolised to methomyl in animals and plants.

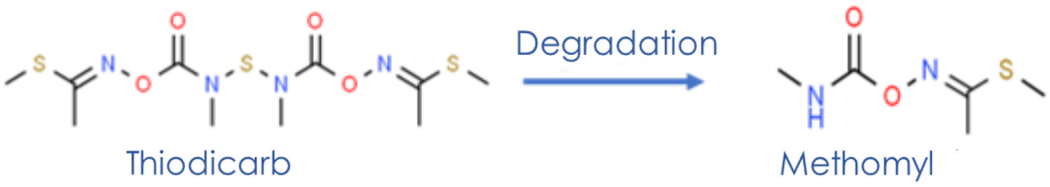



**Figure 3.** Chemical structure of thiodicarb and its degradation product

Two different tests have been carried out in this study. First, the effect of temperature control during extraction was evaluated. For that, the QuEChERS extraction was carried out at room temperature and in a cold bath at 2ºC. The matrices of the study were tomato, onion, and rice. The samples were spiked at 10 µg/kg in triplicate.

**Figure 4** shows that tomato and onion have good recovery values, between 70-120 % with extraction at room temperature and cold conditions. In the case of the rice matrix, the recoveries obtained are less than 70 %, the cold applied during the extraction slightly improves the recoveries up to 30 %.

The second test, in rice matrix, consists of adding two antioxidants, ascorbic acid and tocopherol during the extraction process. Ascorbic acid (3%) or tocopherol (0.4 %) were added before the sample hydration step and the QuEChERS extraction. The results obtained are shown in **Figure 5**.

**Figure 5** shows that addition of ascorbic acid decrease degradation of thiodicarb during the extraction, improving recovery by up to 76 %.

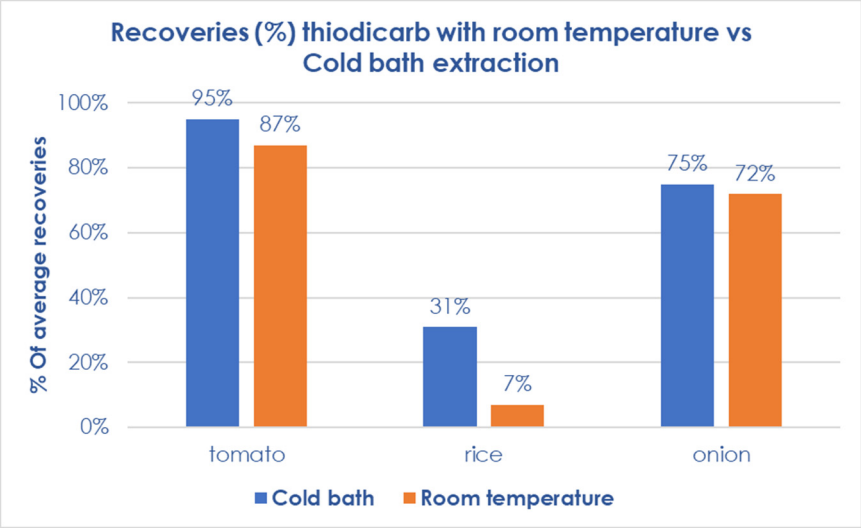



**Figure 4.** Recoveries (%) of thiodicarb at room temperature vs cold bath

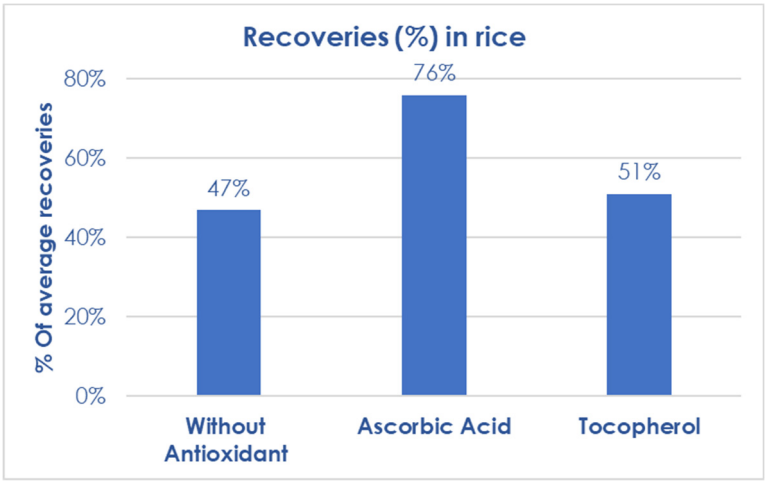
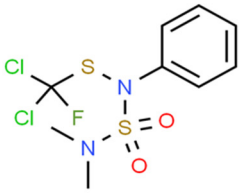


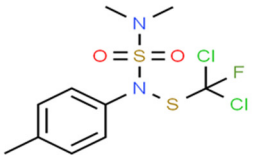

**Figure 5.** Recoveries (%) in rice without antioxidant, ascorbic acid, and tocopherol

6.2 Dichlofluanid and tolylfluanid study:

Dichlofluanid is a fungicide member of the class of sulfamides, introduced in 1965 and no longer approved for use in the European Union. Tolylfluanid is a fungicide which has a similar molecular structure than dichlofluanid, in which the hydrogen at the *para*- position of the phenyl group is replaced by a methyl group. The fungicide, first marketed in 1971 and used in the cultivation of fruit and vegetables, is no longer approved for use in the European Union.




Dichlofluanid




Tolylfluanid

**Figure 6.** Chemical structure of dichlofluanid and tolylfluanid

The matrices of the study were broccoli and pepper. The samples were spiked at 10 µg/kg in triplicate for GC-MS/MS analysis. Two different tests have been carried out in this study:

- Test (A): The effect of the time of exposure of the compounds to PSA in the clean-up stage was evaluated. The supernatant obtained from the samples was left in contact with the clean-up salts during different times T0, T30 and T60 minutes.

- Test (B): The effect produced by centrifugation at cold conditions (-2 °C) after PSA addition and the time of exposure of the compounds to PSA in the cleaning stage were evaluated. In this test, the supernatant obtained from the samples was left in contact with the cleaning salts for different times T0 y T15 minutes.

As can be seen in **Figure 7**, a longer exposure time of dichlofluanid and tolylfluanid to PSA translates into lower recoveries. At 0 min the recovery values are higher than at 30 and 60 min; from 30 minutes onwards the recovery values remain constant below 70-120 %. This shows that time is a critical factor for these compounds in the clean-up stage.

The results obtained in test (B) with cold centrifugation (-2 °C) are shown in **Figure 8**. Centrifugation at 2 °C after PSA addition improve recoveries of dichlofluanid and tolylfluanid in all cases. Good recoveries between 73-94 % are obtained at 0 min for both compounds in broccoli and pepper.

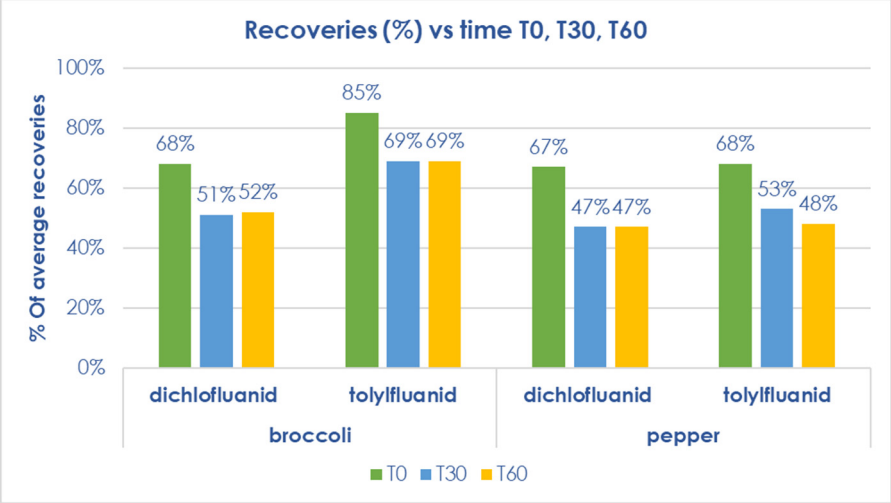



**Figure 7.** Recoveries (%) vs time T0, T30, T60 for broccoli and pepper

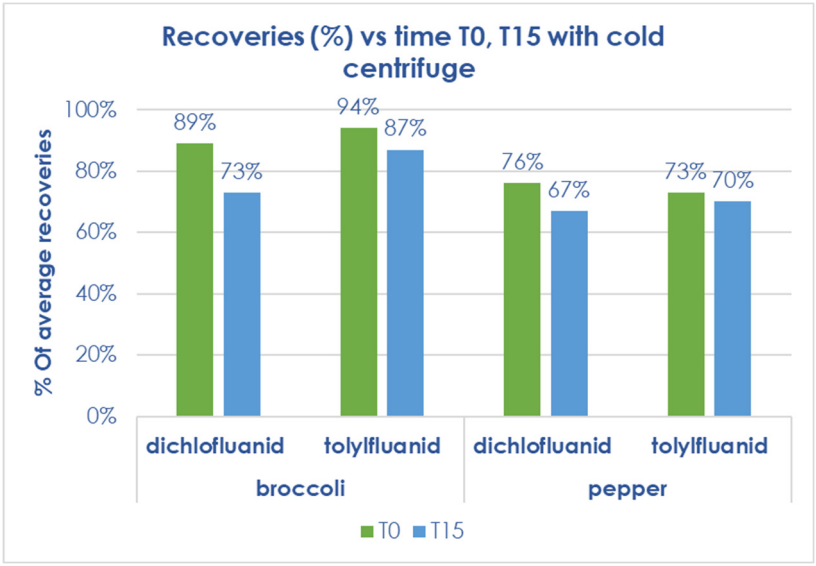



**Figure 8.** Recoveries (%) vs different time with cold centrifugation

6.3 Diafenthiuron study:

Diafenthiuron is a thiourea derivative, which has been widely used for the control of phytophagous mites, whiteflies, and aphids resistant to other classes of pesticides. In the EU, diafenthiuron is a pesticide without regulatory approval for use. This compound easily degrades during the extraction in some matrices such as broccoli, and as consequence, very low recoveries can be obtained.

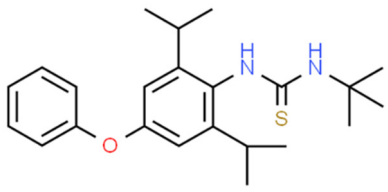



**Figure 9:** Chemical structure of diafenthiuron

In previous studies, to improve the recovery values of diafenthiuron in the QuEChERS method in broccoli, leek, orange, lettuce, and grape, different modifications were reported [2]. The first attempt to increase the extractability efficiency of diafenthiuron was to evaluate the recoveries at different pH values. The pH of the matrix was modified prior to QuEChERS extraction by using hydrochloric acid (HCl) to acidify the samples or sodium hydroxide (NaOH) to increase the pH of the matrix. Low recoveries of diafenthiuron were observed at all pH values tested, demonstrating that low recoveries are not dependent on pH values. Three antioxidants were evaluated: ascorbic acid (3 %), citric acid (3 %) and tocopherol 0.4 %. The recovery values obtained at 50 µg/kg in broccoli, with the addition of tocopherol 60 % were the closest value to 70-120%.

Finally, data were also provided on the addition of tocopherol in matrices with different characteristics, such as grape (high sugar content), leek (high content of sulphur compounds), orange (high acid content) and lettuce, which may influence the extractability of diafenthiuron. In all cases with tocopherol addition an improvement in recovery value, with good RSD%, was achieved.

In a new study, a QuEChERS citrate extraction using a cold bath during extraction has shown to improve recoveries of diafenthiuron. However, the results in broccoli are far from the accepted 70-120 %. The same has been observed for pepper (62 %). In tomato matrix, the average recovery at room temperature is 77 %, improving with the cold bath up to 102 %.

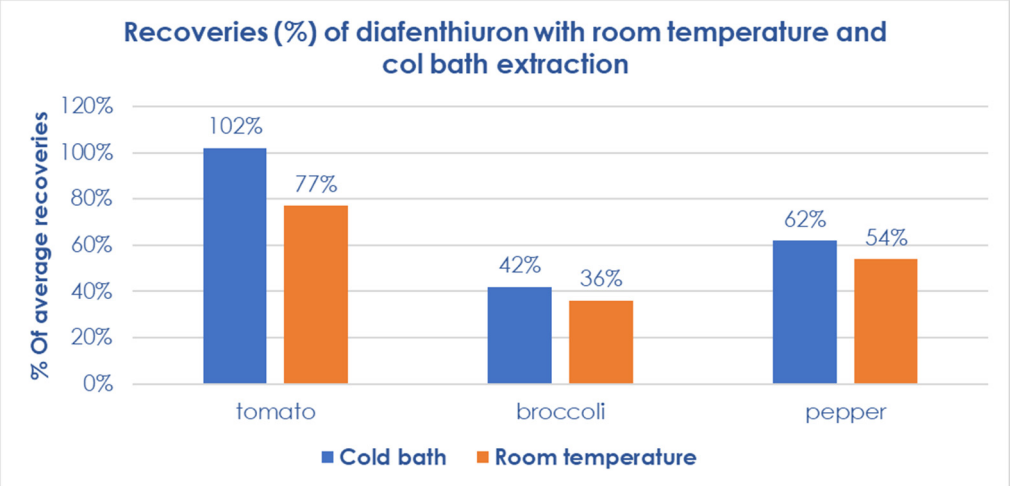



**Figure 10.** Recoveries of diafenthiuron (%) at room temperature and cold bath extraction

## 7. Conclusions

After the modifications evaluated, we can conclude that a decrease in temperature during the QuEChERS by applying a cold bath, slightly increases the

recoveries obtained for thiodicarb without causing a significant impact. In the case of the rice matrix, this impact does not achieve a recovery between 70-120 %. However, in rice matrix, there is an important improvement when adding ascorbic acid before QuEChERS, obtaining a recovery of 76 %.

For dichlofluanid and tolylfluanid extraction, the study has shown that the exposure time of the compounds to PSA is a critical factor during the clean-up. Recovery values decrease as clean-up time increase. The application of a cold centrifugation (-2 °C) after the addition of PSA improves the recoveries up to the range 70-120. There is also evidence that at T15 min these recovery values already begin to decrease.

In the diafenthiuron study, it has been observed that the recovery values improve when the extraction is carried out on a cold bath, improving extraction efficiency in tomato from 77 to 102 %. However, this improvement is not enough for broccoli and pepper, where values below 70 % were obtained.

## 8. References

[1]EU Pesticides Database | Food Safety. https://ec.europa.eu/food/plant/pesticides/eu-pesticides-db_en

[2] Influence of the antioxidant tocopherol on diafenthiuron recoveries using QuEChERS protocol. Sonia Herrera, Ana Lozano, Ana Mª Aguilera del Real, Amadeo R. Fernández-Alba. Agrifood Campus of International Excellence (ceiA3), European Union Reference Laboratory for Pesticide Residues in Fruits and Vegetables. University of Almería (Spain).

## APPENDIX I: MASS TRANSITIONS AND VALIDATION RESULTS

**Table A1.** Detection parameters for the selected compounds analysed by LC-MS/MS.

| Name | Retention time (min) | Precursor Ion (*m/z*) | Product ion (*m/z*) | Fragmentor | Colision Energy | Cell Accelerator Voltage | Polarity |
|---|---|---|---|---|---|---|---|
| Thiodicarb | 8.3 | 355.06 | 108.1 | 380 | 10 | 2 | Positive |
| Thiodicarb | 8.3 | 355.06 | 88.1 | 380 | 8 | 2 | Positive |
| Diafenthiuron | 14.3 | 385.2 | 329.2 | 380 | 20 | 3 | Positive |
| Diafenthiuron | 14.3 | 385.2 | 287.1 | 380 | 20 | 3 | Positive |

**Table A2.** Detection parameters for the selected compounds analysed by GC-MS/MS.

| Name | Retention time (min) | Precursor Ion (*m/z*) | Product ion (*m/z*) | Colision Energy |
|---|---|---|---|---|
| Dichlofluanid | 6.5 | 224.0 | 123.0 | 8 |
| Dichlofluanid | 6.5 | 167.0 | 124.0 | 8 |
| Tolylfluanid | 7.0 | 238.0 | 137.0 | 10 |
| Tolylfluanid | 7.0 | 137.0 | 91.0 | 10 |