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Case Study To Illustrate an Approach for Detecting Contamination and Impurities in Pesticide Formulations

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ABSTRACT: Counterfeit pesticides threaten public health, food trade, and the environment. The present work draws attention to the importance of regular monitoring of impurities in formulated pesticide products. General screening revealed the presence of carbaryl as a contaminant in a copper oxychloride formulated product. In this paper, as a case study, a liquid chromatographic diode array−mass spectrometric method developed for general screening of pesticide products and quantitative determination of carbaryl together with its validation is presented. The proposed testing strategy is considered suitable for use as a general approach for testing organic contaminants and impurities in solid pesticide formulations.

KEYWORDS: *pesticides, impurities, counterfeit, quality control, monitoring, carbaryl, HPLC, LC-MS, copper oxychloride*

INTRODUCTION

The phenomenon of production and sale of counterfeit plant protection products (PPPs) is increasing worldwide, representing a serious risk for public health and the environment. Although precise and detailed data on counterfeit PPPs are difficult to obtain, estimates range from around 5−7% of sales in developed countries to over 20−30% in developing countries.[1] Counterfeiting has been reported in the form of absence of the active substance, wrong active ingredient, insufficient active ingredient, fake packaging, and contamination with unexpected active substances that might lead to intoxication incidents.

Impurities in PPPs and especially in generic products represent a special case. In most cases, the synthesis route of the active ingredient, the raw materials used, and the formulation of the generic product are different from the innovator's product. Consequently, the generic product might contain impurities that are not covered by the safety evaluation of the original pesticide or reported in the scientific literature. The unknown impurities could pose health threats to farmers through exposure during application and to residents as a result of spray drift. On the other hand, cross-contamination in pesticide production plants could be an important problem from environmental or legislative points of view. In a multipurpose nondedicated production line, pesticide products can be manufactured after each other to be commercialized in specific areas such as the European Union (EU) or the United States together with products to be exported worldwide to other countries where no specific restrictions are in place. Residues of unknown and untested substances could be carried into harvested food with potential adverse health effects or violation of legal limits. Producers who have used such products can have their products rejected by food companies.[2]

For instance, isomalathion, the impurity of technical grade malathion, caused poisoning of many spraymen (including five deaths).[3] It was recently reported that isomalathion substantiated malathion's cytotoxicity and genotoxicity in human HepaRG cells.[4] Dioxins in some herbicides resulted in serious health side effects, whereas DDT, the byproduct of captafol synthesis, was the source of illegal residues in treated commodities.[5] For the above reasons, the quality control of plant protection products including their impurities, especially in the case of generic ones entering the market based mostly on parallel import, is an important component of the plant protection policy aiming to reduce risks associated with pesticide use.[6,7]

Nevertheless, there is a lack of legislation regarding impurities in both technical and formulated pesticide products, and only the Food and Agricultural Organization (FAO) has published specifications on individual active ingredients. These specifications, which are freely available on the FAO Web site, provide information for the manufacturers, formulators, producers, and registrants of pesticide products regarding the maximum concentrations of significant impurities of pesticide active ingredients, which may be present in technical grade active ingredients as well as formulated products evaluated by the FAO/WHO Panel of Experts on Pesticide Specification.[8] It should be pointed out that these specifications do not cover the potential impurities in pesticides that are manufactured using different routes of synthesis or different qualities of starting materials. Identification and quantification of impurities are quite common in pharmaceutical products,[9,10] but still few studies have been carried out on pesticide products.[11]

Assessing impurities of a pesticide is quite a difficult task. Analytical techniques used for the detection of impurities or unexpected compounds can include gas or liquid chromatography coupled with mass spectrometry to achieve the selectivity and sensitivity required. In specific cases near-infrared imaging could also be applied.[11]

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The products selected to implement the pesticide impurities detection program in Greece include both solid and liquid formulations. Initial screening of formulated pesticide products revealed the presence of carbaryl in copper oxide formulation. Carbaryl (1-naphthyl-*N*-methylcarbamate) is a widely used highly effective carbamate insecticide, with both agricultural and residential/domestic uses. However, it poses a potential hazard for health due to its relatively high acute toxicity.[12]

For the quantitative determination of carbaryl residues in plant matrices, soil, and water, several sample preparation methods have been developed, depending on the matrix used. For pesticide formulations liquid−liquid extraction (LLE) is the easiest and most common procedure followed by many pesticide laboratories. Conventional high-performance liquid chromatographic methods (HPLC) using reversed phase C18 columns have been extensively used with aqueous−organic mobile phases containing various proportions of acetonitrile, methanol, and methanol/acetonitrile in isocratic or gradient elution and UV, electrochemical, and fluorescence detection. Liquid chromatography with ion trap mass spectrometry and GC-MS were also used in multiresidue methods for quantitation of carbaryl in fresh fruit and vegetable samples. The typical limits of detection achieved were 0.01−0.02 mg/kg in plant matrices,[13,14] 0.2−5 μg/L in water,[15−17] and 0.15−0.64 μg/kg in soil[18−20]

Nowadays, the online combination of high-performance liquid chromatography and mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) holds a significant position in the analysis of contaminants and pesticides, in particular, because these methods provide unambiguous identification of thermally labile and polar pesticides at trace levels. Interfaces for LC-MS such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have been important tools in environmental analytical chemistry, especially in the area of pesticide analyses in soil and water. Recent developments in instrumentation made more readily available the high-resolution mass spectrometer (e.g., QTOF-UHPLC systems) with ≥10000 resolution. These instruments are ideal for the detection and identification of unknown substances and will have wide application in pesticide formulation control in the future.

Thus, a logical strategy is first to inject a diluted solution of the pesticide formulation to the GC-MS system, because the number of peaks eluting from the system can be assessed and possibly identified with existing libraries and then ideally confirmed. The next step is to use HPLC analysis coupled with mass spectrometry to incorporate thermally labile compounds that are not stable under GC conditions.

The aim of this work is to illustrate our testing strategy using as an example the determination of a trace amount of carbaryl (organic contaminant) in copper oxychloride solid pesticide formulation including the development and full validation of the method based on HPLC-DAD and confirmation of the identity of the compound with HPLC-PDA-ESI-MS.

MATERIALS AND METHODS

Working Standard Solutions and Sample Preparation. An analytical standard of carbaryl (99.8%) was purchased from Sigma-Aldrich (Steinheim, Germany). HPLC grade water, acetone, and methanol were obtained from Fischer Scientific (USA) and LC-MS water and methanol from Merck (Germany). The stock solution of carbaryl (1000 μg/mL) was prepared in acetone. For GC-MS all solutions used for verification purposes were prepared in acetone as well. All working solutions used for method validation in the HPLC part were prepared in methanol (HPLC grade). Samples of two different inorganic copper oxychlorides formulated as wettable powder were obtained from the Greek market.

The GC-MS analysis was performed on a Chromtech Evolution MS/MS triple-quadrupole mass spectrometer built on an Agilent 5975 B inert XL EI/CI MSD system. Samples were injected with a Gerstel MPS-2 autosampler using a 10 μL syringe. Separations were performed on a DB-5MS 30 m × 0.25 mm × 1.0 μm column (J&W, Folsom, CA, USA). Helium was used as the carrier gas at a flow rate of 1.2 mL min^{-1}. Briefly, the oven temperature was initially programmed to 45 °C. The temperature was increased to 130 °C at 25 °C/min and held for 2 min. The temperature was further increased to 180 °C at 12 °C/min, to 240 °C at 7 °C/min, and finally to 320 °C at 10 °C/min, which was held for 2 min. The injector, MS transfer line, and MS heater temperatures were maintained at 250 °C. The mass spectrometer was operated in the full scan data acquisition mode. The transfer line, manifold, and source of ionization temperatures were 250, 40, and 230 °C, respectively. The electron multiplier voltage was set at 2000 V. The total GC analysis time was 44.85 min, and carbaryl eluted at 11.05 min.

For the HPLC-DAD method 10 matrix-matched solutions were freshly prepared by spiking copper formulation product (with no organic impurities present at the retention time of carbaryl) performed in such way to obtain concentrations of 0.2, 0.4, 1, 2, 4, 8, 12, 16, 20, and 200 μg/g formulated product. A similar 10 solutions were prepared for the HPLC-MS method, at a concentration range from 0.1 to 20 μg/g formulated product.

A representative pesticide formulation subsample of 5 g was placed into a 100 mL glass volumetric flask with methanol. The sample was pretreated in an ultrasonic bath, to ensure complete dissolution of organic components, for 15 min. The sample solution was filtered through a 0.45 μm nylon filter membrane and/or centrifuged (Heraeus Labofuge 400R Thermo Electron Corp., 5 min, 2000 rpm) prior to injection of the solution into the HPLC column.

High-Performance Liquid Chromatography. The HPLC system used was a Shimadzu UFLC instrument, equipped with a column oven (CTO-20A), a diode array detection system (SPD-M20A), a degasser (DGU-20As), and an autosampler (Sil-20AC) and connected to a reverse-phase Nucleosil C18 column (250 mm × 4 mm × 5 μm, Macherey-Nagel). The analysis was carried out using, as mobile phase, an isocratic mixture of methanol/water 70:30. The mobile phase was filtered and degassed prior to use. The detection wavelength was set at 220 nm. The flow rate of the mobile phase was maintained at 1.0 mL min^{-1}, which provided a pressure of 148 bar. System control and data analysis were carried out using LC LabSolution software (Shimadzu). The temperature of the column was maintained at 40 °C during all runs, and the injection volume was 10 μL. The total run time was 10 min. The retention time of carbaryl was 4.9 min.

Liquid Chromatography Coupled with Diode Array and Mass Spectrometry. Carbaryl presence was confirmed using HPLC-DAD-ESI-MS. A Shimadzu LCMS-2010 EV liquid chromatograph−mass spectrometer together with a SIL-20A prominence autosampler and an SPD-M20A diode array detector was used with LC-MS solution version 3.0 software. The latter was coupled in series with a mass selective detector equipped with an atmospheric pressure ionization source usable as either ESI interface. The LC separation was achieved on a Shim-Pack XR-ODS 2.2 μm, 100 × 4.6 mm i.d.. chromatographic column using the isocratic mobile phase of methanol/water 70:30 with the addition of formic acid (0.1%) in the aqueous phase to promote ionization of carbaryl molecule. The flow rate was 0.5 mL min^{-1}. The identification of carbaryl (4.20 min, retention time) was achieved by the HPLC-ESI-MS system functioning in the positive ionization mode. Full scan and selected ion monitoring mode (SIM mode) were applied, and the characteristic ions at 145 and 202 amu were observed (Figure 2).

RESULTS AND DISCUSSION

In the presented work, the first step of the screening procedure revealed the presence of carbaryl in the copper oxychloride formulation (see full scan MS chromatogram, Figure 1).

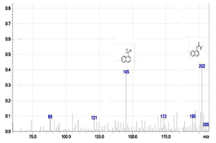

Figure 1. Full scan mass spectrum peak eluting at 4.2 min attributed to carbaryl (diluted pesticide formulation).

Because carbaryl is thermally labile[21] and decomposes to α-naphthol either in the injector or additionally during elution, it was decided to determine its concentration in the formulations by HPLC under the conditions described above (Figures 2−4). Additionally, maximum information was gained through the use of HPLC coupled with mass spectrometry (Figures 5 and 6) and PDA (Figure 7).

If a potential carbaryl peak was detected in the copper oxide pesticide formulations analyzed with the optimum chromatographic conditions described under High-Performance Liquid Chromatography (Figure 1), that peak was then identified by full scan mass spectrometry with HPLC-PDA-ESI-MS (Figures 5 and 7) and further quantified with the use of both techniques.

The only peak that was assessed with regard to its mass spectrometry profile was the peak eluting at 4.9 min in the HPLC (or at 4.2 min in LC-MS method) because the other two eluting peaks (1.6 and 2.3 min) are lower than the 2.91 min void time of the column.

With regard to the sample preparation step and its impact on analyte retention, the critical step is purification of the sample prior to injection. In this context both nylon filtering and centrifugation showed similar GC-MS chromatograms, indicating that for GC-amenable compounds no significant alteration of their composition was evidenced.

Validation of the HPLC LC-PDA-ESI-MS Procedure. For the validation of the method, the following parameters were determined: linearity, repeatability, reproducibility, limit of detection (LOD) and limit of quantification (LOQ), recoveries, and matrix-dependent variations as is established by the EU guidelines for HPLC-UV and LC-MS methods.[22,23] Linearity and matrix effect were assessed by analyzing standard solutions and matrix-matched standards to cover the expected range of carbaryl concentrations as an impurity in commercial formulations. The results are presented in Table 1. Both HPLC and HPLC-MS provided linear responses over the mentioned concentration ranges with excellent correlation coefficient (r^2) values. No matrix effect was observed.

Accuracy was assessed after performing a recovery study with both methods. Recoveries and repeatability relative standard deviations (RSD_r) with the HPLC method were, respectively, in the range of 89.2−102.1% and 5.10, 3.13, and 2.45% at the three concentration levels of 2, 16, and 200 μg/kg. In the case of LC-MS the recoveries and RSD values were in the range of 91.1−102.9% and 3.12, 4.11, and 4.77%, respectively, at 0.4, 4, and 16 μg/g spike levels. The Dixon test revealed that there was no outlier in the recovery values obtained by both methods.

For reproducibility studies with both methods, five replicate determinations were performed on five different days at three standard concentration levels (2, 16, and 200 μg/g, HPLC method and 0.4, 4, and 16 μg/g, LC-MS method) on one pesticide lot. The RSD_r values for reproducibility were 2.38, 4.1, and 4.34%, respectively. In the LC-MS method, the RSD_r values for reproducibility were 4.05, 3.65, and 2.48%, respectively. To calculate the homogeneity of the variances of the repeatability and reproducibility RSDs, the Cochran test was applied, which indicated no outliers. The LOD and LOQ were determined as 3 times the standard deviation of the blank (matrix without analyte) and 10 times the standard deviation of the blank (formulation sample not containing carbaryl). LOD and LOQ values are presented in Table 1, and the respective chromatograms at LOQ level are shown in Figures 4 and 6.

The above results demonstrate the reliability and accuracy of the measurement of carbaryl in commercial pesticide formulations and indicate an absence of systematic error.

Chromatographic Peak Confirmation. It is important to point out that the same sample solutions were used for both HPLC-DAD and LC-PDA-MS systems, simultaneously, to ensure the presence of carbaryl in the samples analyzed.

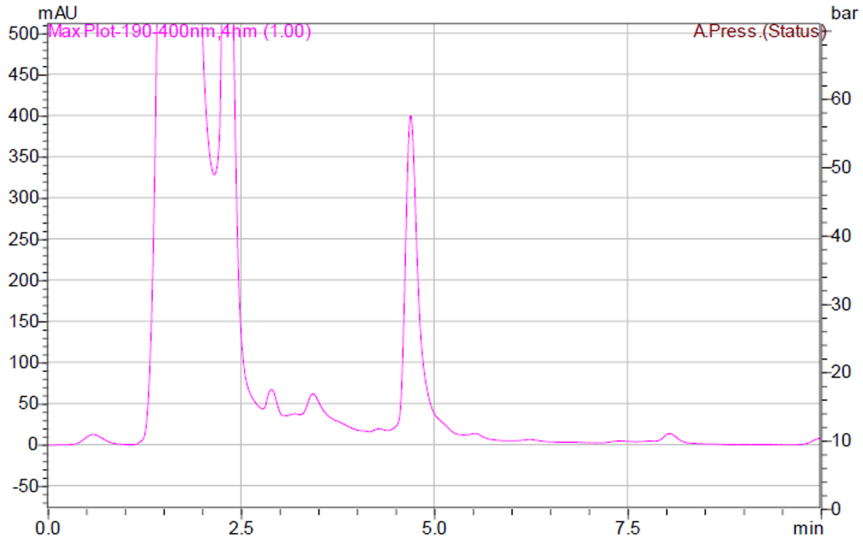

Figure 2. HPLC-PDA maximum plot chromatogram of the diluted pesticide formulation.

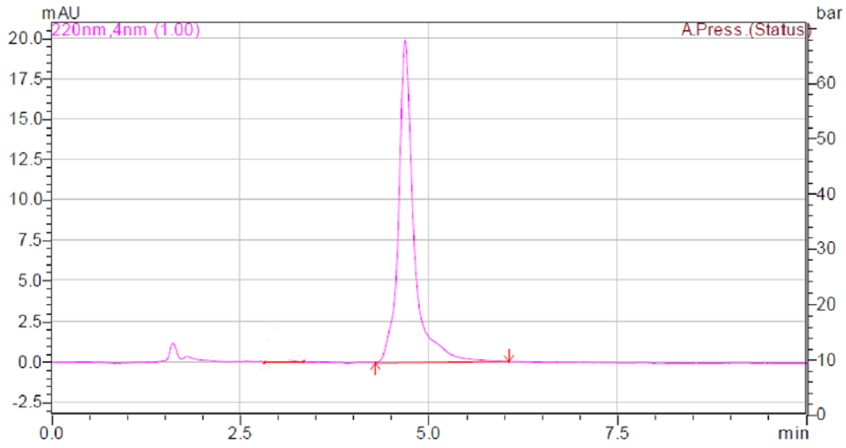

Figure 3. HPLC-UV chromatogram of carbaryl analytical standard.

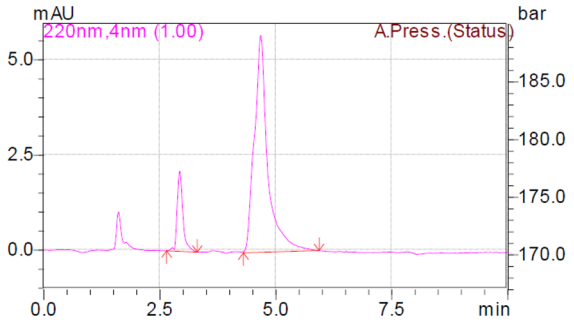

Figure 4. HPLC-UV chromatogram of carbaryl in a sample extract spiked at LOQ level.

Specificity is a measurement of the degree of interference in the analysis of pesticide formulation. The chromatographic peak was identified/confirmed according to molecular masses obtained by LC-ESI-MS in the positive ionization mode. The SIM chromatogram at 4.2 min verified the presence of two specific anions that characterize the molecule.

The ions obtained by this method are typical and previously reported by other research teams.[13,24] Thus, m/z 202 corresponds to $[M + H]^+$, whereas m/z 145 corresponds to $[M - 56]^+$.

Comparison with Other Analytical Studies. The advantage of the proposed methodology is the quantitative and sensitive determination of carbaryl in pesticide formulations. The extraction of the analyte from a formulation product assisted with ultrasonication resulted in repeatable and reproducible results. Considering the need to test for organic impurities in inorganic formulations, the presented method is an efficient solution for the analysis of carbaryl in such formulations. The principle of the stepwise procedure developed can be applied for testing other formulations as well.

Another sensitive and selective way to detect carbaryl would be using a fluorescence detector. An indicative work in this field was published in 2011.[18] However, the fluorescence method

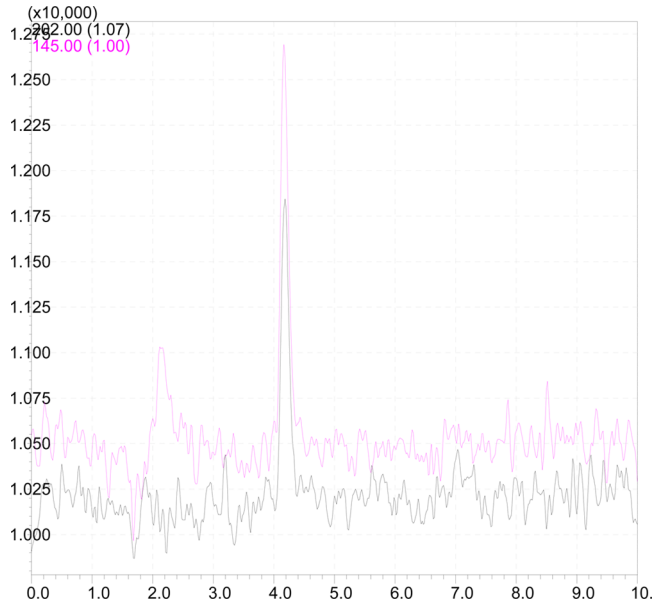

Figure 6. SIM chromatogram of carbaryl in sample extract spiked at LOQ level.

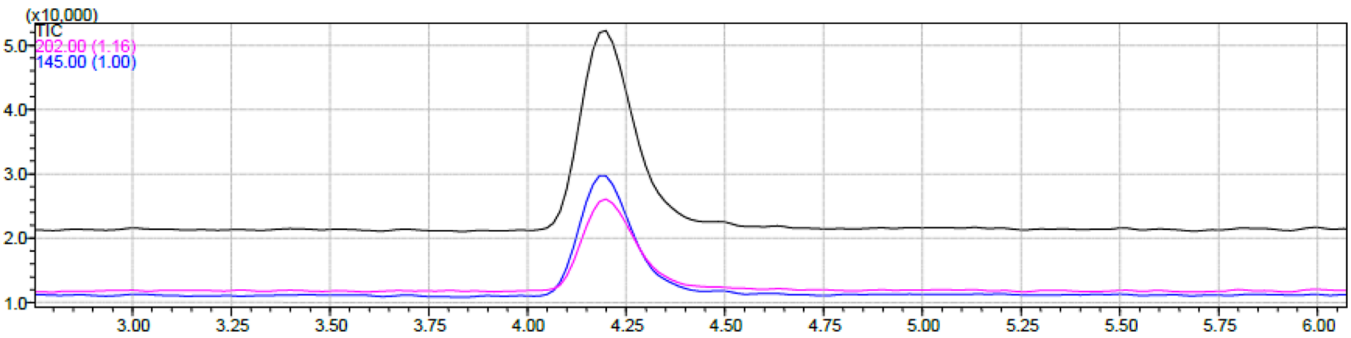

Figure 5. SIM chromatogram of carbaryl standard solution at 1 μg mL^{-1}.

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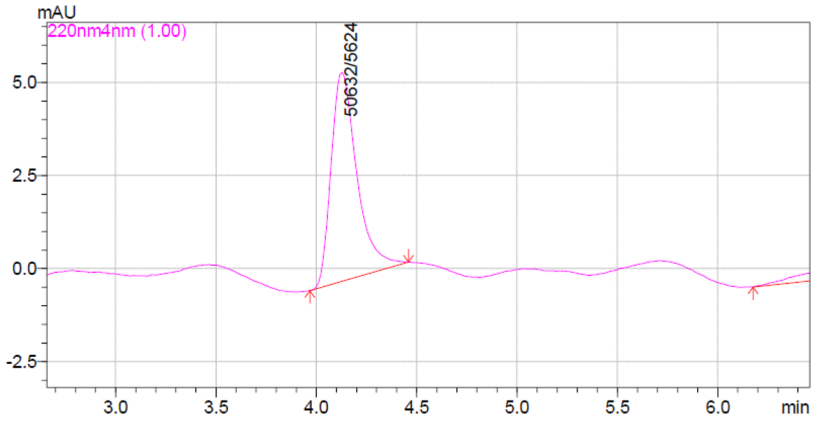

Figure 7. UV chromatogram of carbaryl of sample extract spiked at 220 nm using LC-PDA-ESI-MS.

Table 1. Analytical Performance of HPLC and HPLC-MS Methods

method	linear range ($\mu g/g_{form}$)	LOD ($\mu g/g_{form}$)	LOQ ($\mu g/g_{form}$)	calibration equation	correlation coefficient, r^2
HPLC-DAD	0.2−200	0.13	0.42	$y = 211948x + 47423$	0.9974
HPLC-ESI-MS	0.1−20	0.05	0.18	$y = 371278x - 1921.1$	0.9995

has some drawbacks related to (1) time-consuming postcolumn derivatization, (2) time-consuming sample preparation, and (3) the lack of confirmatory information from the analytical results.

The testing strategy proposed in this paper is a novel and logical combination of existing chromatographic techniques, which were fully validated for this particular purpose. Therefore, it could be used to monitor the purity profile of pesticide formulations.

Contamination of Real Samples and Preliminary Risk Assessment. Our procedure was applied for the determination of the concentration of carbaryl in formulations. Specifically, two formulations of copper oxychloride from the Greek market were received and analyzed for the presence of carbaryl, and one of them contained carbaryl at a concentration of 12.4 mg/kg formulation. This concentration clearly demonstrates that cross-contamination occurred probably in the production−assembly line, because it is far below the usual concentration of carbaryl in formulations with copper oxychloride.[25]

In the presented case carbaryl's concentration was determined at 12.4 mg/kg formulation. The MRL of carbaryl varies between 0.01 and 0.8 mg/kg.[26] Assuming the worst scenario of copper oxychloride being applied at double the suggested rate (8 kg/ha) and all pesticide being deposited on grape bunches harvested with a yield of 10 tons/ha on the day of treatment, the resulting carbaryl residue would be 0.0012 mg/kg. It would not be detectable and would not cause any risk for consumers or result in MRL violation. However, there might be some adverse health effects for the pesticide applicators. The latter scenario is of importance considering that users of plant protection products tend to be less careful with inorganic pesticides, and thus unintended exposure to an acute toxic substance (carbaryl in this case) could take place.

In this case study the presence of carbaryl was verified in an inorganic pesticide formulation, indicating cross-contamination in the production. In this regard, it is evident that the combination of various chromatographic techniques and detection methods should be employed in the portfolio of analytical laboratories aiming to detect impurities and ensure the quality of pesticide products.

Unexpected impurities or contaminations in pesticides might constitute a hazardous threat to the environment and human health. In this regard authorities should encompass regular monitoring applying specific techniques to detect the presence of harmful impurities. It is expected that the presented procedure will be used as a routine approach in laboratories involved in pesticide formulation analyses. This strategy will be strengthened by a complementary LC-MS screening approach that is currently being pursued in our laboratories. Regular monitoring of contaminants in pesticide formulations followed by appropriate regulatory actions could ideally prevent or substantially reduce counterfeiting and would ensure proper quality of plant protection products, minimize the risk for pesticide applicators and residents, and protect human health and the environment.

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Author Contributions

‖H.K. and K.M.K made equal contributions to this work.

Notes

The authors declare no competing financial interest.

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