Innovative water suspension of zoxamide nanocrystals based, preparation, characterization and accumulation / retention assessment in tomato plants

Running title: Develop of a new nanotechnological zoxamide formulation and assessment of its ability to improve ZO accumulatio / retention on different parts of tomato plants.

Francesco Corrias[a *], Anna Melis[a], Alessandro Atzei[a], Salvatore Marceddu[b], Fabrizio Dedola[c], Antonella Sirigu[c], Rosa Pireddu[a], Francesco Lai[a], Alberto Angioni[a]

[a] Department of Life and Environmental Science, Food Toxicology Unit, University of Cagliari, University Campus of Monserrato, SS 554, 09042 Cagliari, Italy,

[b] Istituto di Scienze delle Produzioni Alimentari (ISPA)-CNR, sez. di Sassari, 07040 Baldinca, Italy.

[c] Agricultural Research Agency of Sardinia (AGRIS), Service of Environmental Studies, Crop Protection and Production Quality, Viale Trieste 111, 09100 Cagliari, Italy.

*Corresponding author

Francesco Corrias

Food Toxicology Unit, University of Cagliari, University campus of Monserrato, SS 554, 09042 Cagliari, Italy. Tel. +390706758615, Fax +390706758612.

e-mail: francesco.corrias@unica.it

Abstract

BACKGROUND: Because of its poor water solubility, pesticide formulations require the use of high levels of stabilizers and organic solvents. Moreover, it has been established that only the 0.1% of the applied pesticides reaches the target pests, while the 99.9% is leaked in the surrounding environment. So, in the last years an intensive research to find more environmentally sustainable alternatives was carried on.

RESULTS: Zoxamide nanosuspension was prepared through a media milling technique by using polysorbate 80 as stabilizer. The thin and acicular crystals obtained showed a particle size and a polydispersion index of 227 nm and 0.247, respectively, moreover the zeta potential accounted for -28 mV. Dimensional data and morphology of zoxamide nanocrystals alone and both on tomato leaves and berries were confirmed by scanning electron microscopy. The reduction in size for zoxamide crystals obtained after milling process increased pesticide water solubility until 47.6 mg L^{-1}, about twice the solubility obtained with a commercial formulation. Finally, both in field and dip contamination trials performed on tomato plants disclosed the ability of the nanosuspension to increase zoxamide deposition and accumulation than a coarse zoxamide suspension and commercial formulation, respectively.

CONCLUSIONS: The nano-formulation proposed in this work resulted in low cost, easy to make and showing a lower environmental impact due to its solvent free and low surfactants composition. Moreover, the increase of fungicide retention and deposition reached by using nanocrystals technology provides the opportunity of reducing the amounts of zoxamide applied in tomatoes.

Keywords: Nanosuspensions, zoxamide, wet media milling, eco-friendly, fungicide.

1. Introduction

Tomato (*Solanum lycopersicum L.*) is one of the most important and widely grown vegetable food. It represents one of the basic components of the Mediterranean, American, and Asian diets, and is consumed daily raw, or processed as cooked, canned products, juice, or ketchup.[1] Tomatoes are susceptible to several abiotic disorders like sudden frost or drought, as well as attack by fungi, insects, nematodes and weeds that can significantly lessen yields or even destroy the entire crop production.[2] Late blight (*Phytophthora infestans*), tomato russet mite (*Aculops lycopersici*), and tomato moth (*Tuta absoluta*) represent the most dangerous fungal pests on tomato cultivars.[3] Nowadays, the use of fungicides still represents the major control strategy for this pathogens in tomatoes.

Among the pesticide formulations for preventive and curative use, zoxamide (ZO)(Figure 1), developed in 1998 by Dow AgroSciences LIC (Indianapolis, IN) and commercialized since 2001, is the only fungicide belonging to the benzamide family on the market.[4] This pesticide is highly effective against oomycetes and is used on potato, tomato, vine crops, and other vegetables to control several diseases.[5] ZO MRL has been set in tomato at 0.5 mg Kg^{-1}. ZO is a lipophilic pesticide with high octanol/water partition coefficient value (LogP=3.76), therefore it easily penetrates through the cuticular waxes and binds to pathogens β-tubulin inhibiting their polymerization and cell division.[6] According to previous toxicity studies, ZO does not show severe harmful effects in humans, however, it may lead to toxicity of several marine organisms such as river invertebrates and fishes.[7]

Water-based formulations of ZO require the use of high levels of stabilizers, co-emulsifiers and solubility promoters, as a consequence of its poor water solubility (about 0.681 mg/L at 20°C), moreover, the use of common pesticide formulations, such as emulsifiable concentrate (EC), wettable powders (WP), microemulsion (ME) and suspension concentrate (SC), can be subjected to drift and rainfastness in field during treatment, requiring higher concentrations or repetition of treatments and negatively affecting environmental safety.[8] Nowadays, it has been established that only the 0.1% of the applied pesticides reaches the target pests, while the 99.9% is leaked in the surrounding environment.[9] Increased consumer awareness on food safety and environmental quality has led in the

last years, to an intensive research devoted to assessing the impact of the spread of agrochemical residues in the environment and to find more environmentally sustainable alternatives.[10]

In the early 90', a new nanosuspension technology was developed to overcome low bioavailability of drugs showing poor water solubility. Nanosuspensions are sub-micron colloidal dispersions of pure active particles (nanocrystals) stabilized by surfactants, polymers or a mixture of both.[11] Nanosuspensions can be prepared using two different approaches: the bottom up and the top down techniques or a combination of the two.[12,13] Between top down techniques, wet media milling is more reproducible, low cost and scalable method.

Nowadays it is well known that the increased surface-to-volume ratio of the nanocrystals, especially for particle size below 1 µm, can lead to an increase in both dissolution rate and saturation solubility.[14-16] Moreover, nanosuspensions show high adhesion capacity to the surface of both targeted insects and vegetables and the ability to promote the penetration of active substance molecules.[17,11]

As reported by Muller and Peters (1998), this technology can produce water-based formulations starting from totally insoluble active ingredients.[15] Therefore, nanosuspension formulations has been adopted in the pharmaceutical field, for oral, parenteral, dermal, pulmonary and ocular administration.[18-25]

Recently the nanotechnological approaches have been used also in the agrochemical field for the development of crop protection products.[26-28] The attention of the researchers has been focused mostly on pesticide-loaded nano-particles and / or micro-emulsions stabilized with different polymer or surfactant blends,[29-41] while the number of published articles dealing with the use of the nanosuspension technology in the agrochemical field is limited. Nanosuspension formulations of beta-cypermethrin, lambda-cyhalothrin, permethrin, pyraclostrobin, emamectin benzoate and abamectin have been prepared in the last decade,[42-49] however, no zoxamide nanosuspensions were prepared and studied before. Moreover, to the best of our knowledge, in literature there is a lack of

studies concerning the behavior of nanocrystals pesticide formulations on vegetables, regarding pesticide residues deposition and bioavailability.

The aim of this study was to develop a solvent free, water-based and low surfactant nanocrystals formulation able to improve zoxamide adhesion and accumulation in tomato berries and leaves. Therefore, different nanosuspension formulations were prepared by a top down – wet media milling method and characterized by photon correlation spectroscopy for mean size and size distribution. Scanning electron microscopy was used for morphological studies, and in vitro dissolution and retention tests were performed. Finally, the formulations have been tested performing open field treatments on tomato plants and dip treatments on tomato berries. The residue levels of zoxamide in tomato berries and leaves after pollution have been quantified by HPLC-DAD, and the deposition rate of the formulation has been evaluated vs a ZO coarse suspension and a commercial suspension concentrate (SC) formulation.

2. Materials and Methods

2.1 Chemicals and reagents

Polysorbate 80 (Tween 80) was purchased from Galeno (Milan, Italy), Poloxamer 188 (Lutrol F68) and Poloxamer 407 (Kolliphor p 407) were purchased respectively from BASF (Rome, Italy) and Sigma – Aldrich (Milan, Italy). Zoxamide analytical standard (> 98%) was purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile was LC/MS grade (Sigma Aldrich, Milan, Italy). Double-deionized water with a conductivity less than 18.2 MΩ was obtained with a Milli-Q apparatus (Millipore, Bedford, MA, USA). QuEChERS reagents were: Part No: 5982 – 6650, 4 g MgSO, 4.1 g NaCl, 1 g trisodium citrate dihydrate, 0.5 g disodium hydrogen citrate sesquihydrate (En Method 15662, Agilent Technologies, Milan, Italy); Part No: 5982 - 5056, 150 mg PSA, 900 mg MgSO4 (EN Method, fruit and vegetable, Agilent Technologies, Milan, Italy).

Stock standard solution of zoxamide (1000 mg L^{-1}) was prepared in acetonitrile and stored at 4C° before use. Working standard solutions were prepared before analysis from the stock solution by dilution with the eluent mixture.

Tomatoes, purchased from a local bio-market in Sardinia, were sorted to eliminate those with defects and selected for uniform size (medium weight 166 ± 4.33 g ± RSD%).

The commercial SC ZO formulation at 21.8% active ingredient (CF) was purchased in a local market and used as control.

ZO coarse powder was kindly donated by the producer.

2.2 Zoxamide nanosuspensions preparation

Nanosuspensions were prepared using the Wet-media-milling technique.[50] Six dispersions were prepared as follows: a properly amount of ZO coarse powder to reach 0.2% w/v, was dispersed in an aqueous solution plus one of the three stabilizers selected (poloxamer 188, poloxamer 407 and polysorbate 80) at two different concentrations (0.02 and 0.1%, w/v) using an Ultra Turrax basic for 5 minutes at 6500 rpm (Table 1). These ZO coarse suspensions were divided into 1.5 mL conical tubes containing approximately 0.4 g of Silibeads® Typ ZY type (ranging 0.1-0.2 mm diameter) beads made of zirconium oxide and stabilized with yttrium (Sigmund Lindner, Germany). The microtubes were oscillated at 3000 rpm for five cycles of ten minutes each using a beads-milling cell disruptor equipment (Disruptor Genie®, Scientific Industries, USA). The obtained nanosuspensions of each microtubes were separated from the milling beads by sieving and stored at room temperature. The control coarse suspension (CS P-80) was prepared by dispersing ZO powder (0.2% w/v) in a 0.1% w/v bi-distilled aqueous solution of polysorbate 80 using an Ultra Turrax T25 basic (IKA, Werke) for 1 minutes at 6500 rpm, avoiding the shift from microcrystals to nanocrystals.

2.3 Physical characterization of nanosuspensions

The average diameter and polydispersity index (PI) of the samples were determined by Dynamic Light Scattering (DLS) using a Zetasizer nano-ZS (Malvern Instrument, UK). Samples were backscattered by a helium–neon laser (633 nm) at an angle of 173° and a constant temperature of 25°C. Zeta potential (ZP) was estimated using the Zetasizer nano-ZS by means of the M3-PALS (Phase Analysis Light Scattering) technique. All samples were suitably diluted with deionized water during the whole measurement process. The data were measured in triplicate for each sample.

159 **2.4 Storage stability**

160 The best ZO nanosuspension (Nano P-80B) was subjected to shelf life stability test as follows: the
161 suspension was stored in a closed dark glass bottle at room temperature for 60 days. Samples were
162 withdrawn at T = 0 day, 1 days, 7 days, 15 days, 30 days and 60 days to determine the physical
163 stability by analyzing the nanosuspension average diameter and PI as reported in section 2.3.

164 **2.5 Morphological study**

165 CS P-80, Nano P-80B, and CF were placed on a glass support and dried at room temperature.
166 Subsequently all the samples were subjected to metallization with gold in an Edwards S150A Sputter
167 Coater unit (England). Finally, the morphological structure was analyzed using a Zeiss ESEM EVO
168 LS 10 (Germany) environmental scanning electron microscope, operating at 20 KV in high vacuum
169 mode with secondary electron detector. For the visualization of nanocrystals on the surface of tomato
170 berries and leaves, nanosuspension was deposed both on a tomato peel and leaf specimen and the
171 water was left to evaporate at room temperature. The sample were then dried in an Edwards freeze
172 tissue dryer, Model EPD3 (England), for 48 h, and mounted onto glass stubs. Tomato samples were
173 then analyzed in the same conditions described above.

174 **2.6 Retention test**

175 The retention test was performed according to Cui et al.[48] Briefly, Nano P-80B, CS P-80, and CF,
176 respectively, were diluted to a ZO final concentration of 0.02% (w/w). Water was used as control.
177 Each leaf was weighed using an electronic balance (ABT 220-5DM, Kern, Balingen, Germany) and
178 its surface area was measured using a leaf area meter (LI-COR LI-3100C, Ecosearch, Perugia, Italy).
179 Leaves were then completely immersed in the above dispersions and pure water for 10 s, removed
180 and allowed to dry at room temperature. Finally, each leaf was weighed again. Retention (R_m) was
181 calculated:

182 $$R_m = W_1 – W_0 / S$$

Where, W_0 (mg) and W_1 (mg) represent leaf weight before and after the dip treatment in ZO dispersions, respectively, while S (cm^2) is the leaf area. Retention tests were performed in triplicate for each formulation.

2.7 Saturation solubility test

The water saturation solubility of ZO was determined for the starting raw ZO powder, CS P-80, Nano P-80B, and CF. For this purpose, 5 mL of the suspensions were incubated at room temperature for 72 hours under magnetic stirring. An appropriate approach for phase separation is sedimentation. Thus, 1 mL of sample was withdrawn from each suspension after 24, 48h and at the end of the study and centrifuged at 13,000 rpm for two cycles of 60 minutes each. In addition, membrane filtering was performed with 0.1 μm pore sized poly-ether sulfonate syringe mountable filters (Millipore Corporation) after centrifugation from supernatants to completely remove undissolved ZO crystals. Finally, samples were suitably diluted and analyzed by HPLC. All the samples were analyzed in triplicate.

2.8 Sample processing and zoxamide extraction

Tomato berry and leaf samples were processed for the analysis according to an inhouse validated method. Briefly, tomato samples were homogenized with a blender (Electrolux K552V, Italy) for 1 min at room temperature, while leaves were processed whole. 10 g of homogenized tomato sample and 5 g of leaves (about 10 leaves) were weighed in a 50 ml test tube plus 10 mL and 20 mL of ACN, respectively, and agitated in vortex (Reax Top, Heidolph, Germany) for 1 minute. Thereafter, 6.5 g of QuECheRS salts (Part No: 5982 – 6650) were added and the test tube was agitated 2 minutes in vortex and 15 minutes in rotatory shaker. The sample was centrifuged for 5 minutes at 4000 rpms and 10°C (Centrifuge 5810 R, Eppendorf AG 22331 Hamburg). 5 mL of the supernatants were recovered and transferred to a 15 mL test tubes containing 1 g of the second QuECheRS salts (Part No: 5982 – 5056, Agilent, Milan, Italy). The tubes was agitated in vortex for 2 minutes and in rotatory shaker for

15 minutes, the solution was centrifuged for 5 minutes at 4000 rpms at 10°C and the organic solution was filtered at 0.45 µm (PTFE, Thermo Scientific) and transferred in a 1.8 mL vial for HPLC analysis.

2.9 Recovery test

10 g of homogenous samples and 5 g of leaves from untreated tomato plants were added with appropriate volumes of stock standard solution to reach ZO concentrations of 0.5, 1 and 5 mg kg^{-1}, respectively, left to rest for 30 min and treated in accordance with the reported extraction method (section 2.8). All recovery trials were performed in triplicate. The matrix effect was assessed by comparing the analytical response of the pesticide dissolved in acetonitrile / H_2O, and in blank tomato and leaf extracts, respectively.

2.10 Open field treatments

Open field trial was carried out in a tomato cultivation at fruiting stage located in Serramanna (Cagliari, Sardinia, Italy). A single treatment was carried out on September 2, 2020, by using a pressure sprayer 2 L pump (Pamex, Castlebar, Ireland) on a cloudy and windy day, with a temperature of 24 °C. Plant spacing was set at 120 cm between pairs of rows, 80 cm between rows in the pairs, and 40 cm between plants in a row. Four blocks of about 4 m^2 each and consisting of about 10 - 12 tomato plants were sprayed with CS P-80, CF and Nano P-80B at a suggested ZO concentration of 0.75 L/ha. Control plants were treated with deionized water. Each block was separated from the others by a space of about two meters to avoid drift contaminations. A single randomized sampling (4 kg of tomatoes and 2 kg of leaves for each block, respectively) on dry plants was carried out about 7 h after treatment. Samples were transported to the laboratory and processed immediately for analysis.

2.11 Dip treatments

6 tomatoes (1 kg), were spiked one at a time by dip treatment to ensure a homogeneous application of the pesticide. Each tomato was dipped at room temperature in a beaker under magnetic stirring for 1, 3 and 5 min. Three concentrations of zoxamide nanosuspension (Nano P-80B) were selected: 200,

231 400 and 1000 mg L^{-1}. Tomatoes were then left to dry at room temperature for 3 hours in the dark, and
232 finally processed as reported in section 2.2.9. The experiment was replicated under the same
233 conditions with CS P-80 and CF.
234 Before contamination study, untreated tomato samples were analyzed to confirm the total absence of
235 zoxamide. All tests were performed in triplicate.

236 **2.12 Determination of zoxamide content**

237 ZO residues were analyzed by HPLC-DAD according to Borahan et al. (2019),[51] with some little
238 changes. An Agilent 1100 series chromatograph equipped with a photodiode detector (G1315B DAD)
239 and a computerized data integration system (ChemStation- Agilent), was used. The column was a
240 Phenomenex C18 (5 µm - 150 x 4.6 mm) working at room temperature. The DAD conditions were
241 set ranging from 200 nm to 450 nm and fixed wavelength at 254 nm, the analysis was carried out in
242 isocratic condition and the mobile phase consists of a binary solvent A (ACN) at 70% and B (MilliQ
243 water) at 30%, the flow was set at 1 ml min^{-1}. The linearity range (r^2), evaluated from a 6-points
244 calibration curve (50 – 0.05 mg L^{-1}) performed in triplicate, showed a determination coefficient (r^2)
245 of 0.9991 ± 2.3% resulting appropriate for the present study.

246 **2.13 Statistical analysis**

247 All data are presented as the mean ± standard deviation (SD), and significant differences were
248 evaluated by the Fisher's least significant difference test at $p \leq 0.05$.

249 **3 Result and Discussion**

250 **3.1 Nanosuspensions preparation**

251 For both nano and coarse suspensions, the concentration of ZO was kept constant (0.2%), while three
252 surfactants well known also for their application in the cosmetic and food industry (Poloxamer 188,
253 Poloxamer 407 and polysorbate 80) were tested as stabilizer at two different concentrations (0.02%
254 and 0.1%) (Table 1). All nanosuspension formulations were prepared using the highly reproducible,
255 low cost and scalable wet media milling process. Milling parameters as milling time, rotor speed

(rpm) and number of milling cycles have a significant impact on the size and PI of the resultant nanocrystals and so different combinations were tested to optimize the process. At the end, milling conditions of 5 milling cycles, 10 minutes of milling time and 3000 rpm showed the best results and were used to prepare all the zoxamide nanosuspensions.

3.2 Physical characterization of the nanocrystals

DLS analysis of the ZO nanocrystal formulations showed an average diameter between 227.3 ± 7.3 (Nano P-80B) and 563.6 ± 3.1 nm (Nano P-407A), and PI values always lower than 0.45 (Table 1). An increase in the concentration of the stabilizer from 0.02% to 0.1% led to a decrease in the average size and an improvement in the PI in all cases (Table 1). Moreover, all formulations showed a negative zeta potential values ranging from -20.9 ± 1.1 mV (Nano P-188B) to -29.3 ± 1.5 mV (Nano P-80A). A ZP value greater than ± 30 mV generally indicates a good repulsive activity among the various crystals with a consequent decrease in flocculation and precipitation phenomena of the active ingredient dispersed in water.[52]

Among the formulations subjected to DLS analysis, the nanosuspension prepared with polysorbate 80 at 0.1% (Nano P-80B) was the most suitable formulation with a mean diameter of 227 ± 7.3 nm, almost half those obtained with the other surfactants and a PI of 0.247 ± 0.028, 1.75 times lower than the other formulation. This difference was probably due to a different interaction between stabilizers and the ZO nanocrystals during the milling process. Both the Poloxamer 188 and 407 were unable to stabilize the nanocrystals after their formation leading to a subsequent aggregation in bigger particles. In general, Nanotechnology deal with the application of materials with a size ranging from 1 to 100 nm.[53] On the contrary, a wider concept of nano-pesticide formulations is basically accepted because systems with dimensions smaller than 500 nm exhibit novel properties associated with their small size.[54-57]

However, thanks to its small size and as predicted by Stokes' law of resistance, Nano P-80B was a homogeneous and milky suspension with no visible particles or precipitation, being a relatively stable system. Thus, Nano P-80B, stored at room temperature (~25°C) in the dark, was selected for the

following trials. DLS data from CF and the CS P-80, showed an average diameter in the micron range together with a very high PI, indicating a poor homogeneity among the crystal's populations (Table 1). In particular, the mean diameter of ZO crystals in CF (2022 ±1070 nm) and in CS-P80 (2771 ± 1044 nm) were about 9 and 12-fold greater than Nano P-80B, respectively, with PI values more than double (Table 1). Without the milling process, the presence of surfactants or emulsifiers was not enough to bring zoxamide crystals into the nanometric range. This data confirms that, choosing the correct stabilizer and its concentration is possible to obtain zoxamide nanocrystals using the simple and replicable milling process.

3.3 Long-term stability

Long-term stability test showed high stability for Nano P-80–B, with constant values of average size and PI for the first 30 days ranging from 227 nm to 422 nm and from 0.243 to 0.247, respectively. Among day 30 and day 60 the two parameters increased till 573 nm and 0.315, respectively (Figure 2). The suspension is a thermodynamically unstable system and the a.i. crystals suspended in it may suffer Ostwald ripening leading to crystals aggregation, flocculation and finally precipitation. However, the steric stabilization due to polysorbate 80 coupled with the obtained negative ZP allowed to avoid nanocrystals aggregation phenomena ensuring the stability of the system for the whole 60 days.

Moreover, the formulation has been stored and kept stable in liquid form thus avoiding the freeze-drying process and resulting in a ready-to-use formulation.

3.4 Retention test

The deposition rate and adhesive strength of pesticides on leaves surface play an essential role in decreasing pesticide loss and improving application efficiency.[40] Retention of the Nano P-80B was 2.8, 1.6 and 3.9 times that of CS P-80, CF and water, respectively (Table 2). The nonionic surfactant Polysorbate 80 can reduce the surface tension and act as wetting agents in increasing the distribution and diffusion of the solution on the leaf surface significantly.[49] Moreover, crystals size reduction leads to an enlarged specific surface area, thus contributing to increased adhesion, retention, and

consequently pesticide efficacy. Cui et al. (2018) investigated a 230 nm abamectin nanosuspension retention on cabbage (B. oleracea L.) leaves.[48] Our data showed a lower scale of values probably due to the different nature of the leaf sample. However, the trend was the same with nanosuspension retention > commercial formulation > water.

3.5 Morphology of the nanocrystals

Dimensional data on Nano P-80B, CS P-80 and CF, obtained by DLS analysis were confirmed by morphological studies performed through scanning electron microscopy ESEM in high vacuum mode (Figure 3). ZO crystals in Nano P-80B, after grinding process, showed nanometric and homogeneous dimensional range coupled to a characteristic acicular shape long and fine in thickness (Fig. 3C and 3D). On the other hand, the two control formulations showed irregular crystals both in shape and in size with a heterogeneous distribution (Fig. 3A and 3B). ESEM analysis highlighted no morphological differences among Nano P-80B placed on glass stub and after deposition on tomato samples, confirming that no modifications in the biological environment occurred (Figure 4). The massive fill up by nanocrystals of the natural depressions present in the tomato skin, allowed to cover uniformly the surface (Figure 4C). Another perspective and magnification of the tomato specimen treated with Nano P-80B is showed in Figure 4D. Besides, Figure 4E and 4F showed two different magnifications of CF applied on tomato samples; Large and irregular crystals seem to rest muddled on the sample without filling the natural depressions uniformly. Finally, figure 5 displayed different magnifications of a tomato leaf sample after deposition of Nano P-80B. ZO nanocrystals are clearly visible both outside and inside the stomata (Figure 5C – 5F). When a nano-pesticide formulation is applied on a micro-roughness surface like that of the tomato (Figure 4C) can easily increase the coverage of nanoparticles and create deposits on the surface of foliage and vegetables leading to an increased pesticide retention rate,[10] and confirming once again the possibility to regulate the adhesion strength through size controlling.[48, 49]

3.6 Saturation solubility evaluation

The saturation solubility of Nano P-80B, CS P-80 and CF were determined ensuring the equilibrium was reached and compared with that of pure zoxamide. Figure 5 shows as after 48h all the systems tested were in equilibrium. Water solubility of pure ZO was approximately 1.21 mg L^{-1} according to literature, meanwhile the presence of Polysorbate 80 in coarse suspension lead to an increase of approximately 10 times of the solubility (13.8 mg L^{-1}), on the other hand the reduction in size after the grinding process of coarse crystals down to nanocrystals increased pesticide water solubility until 47.6 mg L^{-1}, about twice the solubility obtained with commercial formulation (29.3 mg L^{-1}) (Figure 5). Solubility rate of commercial formulation can be explained with the possible presence of different emulsifiers and or surfactants blends. However, our data were in accordance with several studies dealing with a high improve of saturation solubility (up to even 400 times)[58] when particle size of the selected active ingredient falls below micron range.[15, 59 - 63]

3.7 Recovery

Recovery test showed mean recovery at the three concentration levels ranging from 88.3 ± 5.70% (spiking at 0.5 mg kg^{-1}) to 103.6 ± 1.16% (spiking at 5 mg kg^{-1}) and from 78.0 ± 7.69% and 85.2 ± 8.63% for tomatoes and tomato leaves, respectively (Table 3). The proposed method was accurate and appropriate for the purposes of this study.

3.8 Open field treatment

Data reported in the commercial product data sheet indicate that after 1 hour from the treatment ZO rapidly and massively accumulates in the cuticular waxes of leaves. Thus, in this study we decide to perform a one-shot experiment to assess the capability of nanosuspension formulation to release zoxamide residue in tomato berries and tomato leaves compared with control formulations. ZO residue values obtained after field treatment in tomato leaves accounted for 4.94 ± 1.59 mg kg^{1}, 1.73 ± 1.36 mg kg^{-1}, and 3.07 ± 2.76 mg kg^{-1} for Nano P-80B, CS P-80 and CF, respectively (Table 4). On the contrary, ZO residues in tomato berries were below the LOQ for all formulations. This fact was probably due to a minor interaction ability among nanocrystals with the spherical shape of tomato berries, and to a possible dilution effect during zoxamide extraction process from tomato samples.

However, when spray drops impact on the leaf surface they crush, while particles recoil, and subsequently are retained or rejected. The physical properties of the hitting drops and particles and different plant factors as macro and microroughness of the leaf surface can strongly influence both the fate of the particles and therefore the outcome of the treatment.[64] In accordance with the retention test and ESEM images, the increased surface area of ZO nanocrystals grows their ability to adhere and create deposits on the surface of foliage and vegetables. Moreover, improved ZO nano-crystals solubility coupled with the high ZO lipophilicity ensure a concentration gradient between the formulation and the cuticular waxes increasing zoxamide systemic delivery and accumulation. Finally, given that the nanocrystals are far smaller than the stomata, Nano P-80B might easily enter the leaf via a "stomata pathway", and subsequently be transported inside the foliage (Figure 5C – 5F).[40]

On the other hand, large and irregular dimensions of coarse crystals related with CS P-80 and CF coupled with a lower aqueous solubility than nanosuspension, reduce ZO accumulation. After treatment, probably, residual crystals resulting too large are not able to adhere on leaves surface and easily fall off (Figure 4E and 4F).[10]

In literature there are neither previous works relating the study of pesticide residue after nanosuspension technology application in field nor others zoxamide nano-formulations. Saini et al. (2015) evaluated pirydalyl bioavailability in tomato after spray application of a nano-capsule based formulation.[35]

3.9 Dip treatment

Finally, to fully understand how exposure time and pesticide concentration can affect ZO residue levels, dip treatment experiments were performed for the three formulations on tomato berries. ZO residue in tomato obtained after treatment at 200 mg L^{-1} for 1 minute showed residues of 3.49 ± 0.14, 0.47 ± 0.01, and 2.03 ± 0.27 mg kg^{-1} for Nano P-80B, CS P-80 and CF, respectively (Table 5). Treatments at 400 mg L^{-1} showed an increase of the residue in all test with different rates 30.3%, 11.3%, and 10.2% for Nano P-80B, CS P-80 and CF, respectively, while the further increase to 1000

385 mg L^{-1} did not lead to significant statistical increase (Table 5). Raising the dipping time to 3 min. did
386 not show any increase for Nano P-80 B, while showed an enhancement of the residues of ZO in CS
387 P-80 of almost 30% when the solution 2x and 5x were used. Moreover, the increase with CF were
388 more contained ranging from 20% and 28%. After 5 minute any different was detected versus the
389 dipping time for 3 min. for each formulate. Results showed that the nanocrystals application of ZO
390 Nano P-80B left residues 8.16 ± 0.84 times higher than CS P-80, and 1.96 ± 0.15 times higher of CF
391 applying the same suspension concentrations.
392 Contact time showed a not statistically significant influence on ZO residue in tomato for all three
393 formulations. On the other hand, the ZO residues obtained with the dip treatment are concentration
394 dependent, especially for the nano-crystals formulation (Figure 6). Compared to open field treatment,
395 this experiment allowed to ensure a homogeneous application of ZO on tomatoes minimizing external
396 variables and highlighting the physical properties of the different formulations involved in the trial.
397 Tomato is a commodity that can be consumed raw as a salad and so the use of a synthetic pesticide
398 for post-harvest dip treatment is dangerous and therefore excluded. However, these results seem to
399 disclose a second purpose for this nanotechnology, providing the opportunity to apply it on poor
400 aqueous soluble pesticide involved in post-harvest applications.

401 ## 4. Conclusion

402 This work describes the preparation and the characterization of a nanocrystal suspension of zoxamide
403 and its behavior when applied both in an in vitro model system and in open field on tomato plants.
404 Wet media milling method used for nanosuspension preparation allowed to obtain fine and acicular
405 crystals with a particle size and PI of 227 nm and 0.247, respectively, moreover the obtained
406 formulation showed a very good stability after 60 days storage at room temperature. Dimensional
407 data of the suspension of ZO alone and on tomatoes were confirmed using microscopy techniques.
408 The reduction in size for ZO crystals obtained after the grinding process increased pesticide water
409 solubility (47.6 mg L^{-1}) about twice the solubility obtained with commercial formulation.

Data obtained showed that the increase in specific surface area, solubility and target adhesion of ZO nanocrystals lead to an increased ZO residue accumulation in tomato leaves in comparison with a ZO coarse suspension and a ZO based commercial formulation.

Final formulation results low cost, easy to make, long-term stable and showing a lower environmental impact due to its solvent free and low surfactants composition. Moreover, the increase of fungicide residue and bioavailability provides the opportunity of reducing the amounts of zoxamide applied according to actual indications from the new regulations on pesticide use. Results clearly suggest that nanosuspensions could represent an alternative and very promising strategy for agro-chemical application of poorly soluble pesticides.

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