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ANTIMUTAGENIC AND ANTIGENOTOXIC EFFECTS OF VEGETABLE MATRICES ON THE ACTIVITY OF PESTICIDES

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3 1 **ANTIMUTAGENIC AND ANTIGENOTOXIC EFFECTS OF VEGETABLE MATRICES**
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5 2 **ON THE ACTIVITY OF PESTICIDES**
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10 5 **Abstract**
11 6

12 7 Two *in vitro* tests, one to detect bacterial mutagenicity (Ames test) on *Salmonella typhimurium*
13 8 TA98, TA100, TA1535 and the other the primary DNA damage (SOS Chromotest) on *E. coli*
14 9 PQ37, were applied to determine the overall genotoxic activity of twelve pesticides (Azinphos
15 10 methyl, chlorothalonil, chlorpyrifos ethyl, chlorpyrifos methyl, λ -cyhalothrin, cypermethrin,
16 11 cyprodinil, fenazaquin, fludioxonil, indoxacarb, iprodione and penconazol). These were detected
17 12 by GC analysis with electron capture (ECD) and nitrogen-phosphorus detection (NPD) in eighteen
18 13 samples of vegetables. Some extracts of vegetables, found positive for pesticides with GC, were
19 14 subjected to Ames test and SOS Chromotest to evaluate the possible antimutagenic and/or
20 15 antigenotoxic effects of vegetable matrices. The same bioassays were also performed on the
21 16 mixtures of pesticides found in these samples to evaluate whether interactions could occur between
22 17 pesticides and be responsible of the possible antimutagenic and/or antigenotoxic effects of the
23 18 contaminated matrices. Experiments were also carried out to compare the results found for
24 19 contaminated vegetables with their content of antioxidant components. Significant differences in
25 20 mutagenicity and genotoxicity were found among the pesticides selected for this study. Of the
26 21 twelve pesticides tested only azinphos methyl, cyprodinil, fludioxonil and iprodione were found to
27 22 be positive both for *S. typhimurium* and for *E. coli*. No mutagenic/genotoxic activity was found in
28 23 the extracts of vegetables contaminated by pesticides. The *S. typhimurium* TA1535 showed a
29 24 strong positive mutagenic effect for the mixtures of pesticides while they were not able to induce
30 25 the SOS system. The data concerning the content in polyphenols and the total reducing activity of
31 26 the contaminated vegetables indicated high amounts of antioxidants that could explain the
32 27 inhibitory effect on the activity of pesticides shown by vegetables.
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3 29 **Keywords:** *Pesticides, vegetables, Ames Test, SOS Chromotest, GC*
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8 31 **Introduction**
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10 32 Pesticides are peculiar compounds because, in addition to their intentional effects, they are toxic for
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12 33 several non-target organisms included humans and their toxicity has limited species selectivity. In
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15 34 several studies and reviews about the risk of occupational exposure to pesticides, they have been
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17 35 implicated in causing or promoting cancers, leukemias, and lymphomas due to their properties of
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20 36 inducing mutations, chromosomal alterations or DNA damage (Viel and Challier 1995; Maroni et
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22 37 al. 2006; Provost et al. 2007; Maele-Fabry et al. 2008). Some of them interfere with normal
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25 38 neurotransmitter function (Calamandrei et al. 2006; Eyer et al. 2008; Slotkin et al. 2008) and others
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27 39 act as enzymatic and/or endocrine disruptors (Petrelli and Figà-Talamanca 2001; Hoogduijn et al.
28
29 40 2006). But, exposure to pesticides may be a significant risk factor also for food consumers for their
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31 41 frequent occurrence as residues in fruit and vegetables even at very low concentrations (ng- μ g/g
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33 42 food). Many studies showed that chronic exposure to low levels of pesticides in fruits and
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35 43 vegetables is associated with genetic toxicity (De Marco et al, 2000; Feretti et al. 2007). Among the
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37 44 adverse effects of pesticides, genotoxicity is of particular concern because of the irreversibility of
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39 45 this process. Plant systems are able to bio-concentrate pesticides as well as other xenobiotics but
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41 46 also to modulate their activity. In recent years, many studies have been focused on the antioxidative
42
43 47 and antimutagenic activities of vitamins, polyphenols and other metabolites contained in vegetables
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45 48 and fruits (Toor and Savage, 2004; Makena and Chung, 2007). So that, if on the one hand the
46
47 49 consumption of vegetables is often reported as a risk for the human health due to the occurrence of
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49 50 pesticide residues, on the other hand, epidemiological studies have shown that cancer incidence in
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51 51 populations with high intake of vegetables and fruits is lower than in others with less intake of these
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53 52 foods (Weisburger, 1991, Lam et al. 2009). Lately, Yeh et al. (2009) observed significant inverse
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55 53 associations for women in the highest vs. lowest quartiles of intake of total vegetables, vitamin E,
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57 54 dietary fiber, beta-carotene and folate suggesting that vegetables and related nutrients are associated

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3 55 with decreased risk of endometrial cancer. For this reason, the mere detection of pesticides in
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5 56 vegetables could be not associated with their actual risk for human health for the presence of plant
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8 57 metabolites such as carotenoids, lycopene, vitamins and polyphenols acting as antioxidants,
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10 58 antimutagens and anticarcinogens.
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15 60 The objective of the present study was to evaluate the genotoxicity of different pesticides found in
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17 61 vegetables and compare these data with those of vegetable extracts containing those pesticides. For
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19 62 this reason, 18 samples of retail vegetables cultivated in South Italy were tested to detect the
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21 63 presence of pesticides by means of GC with electron capture (ECD) and nitrogen-phosphorus
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23 64 detection (NPD). Twelve pesticides from different chemical classes were found in the samples
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25 65 examined and the respective pure compounds were used in two different microbial *in vitro*
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27 66 bioassays, the Ames test and the SOS chromotest to determine their mutagenic and genotoxic
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29 67 activity. The Ames test (*Salmonella*/microsome mutagenicity assay), used to test mutagenic
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31 68 properties of a chemical compound through the increase of revertants, was performed on
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33 69 *Salmonella typhimurium* TA98, TA100 and TA1535 while the SOS Chromotest, based on the
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35 70 detection of damage to DNA measured through the SOS DNA repair system, was performed on
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37 71 *Escherichia coli* PQ37. Some extracts of vegetables, found positive for pesticides with GC, were
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39 72 then subjected to Ames test and SOS Chromotest to assess their possible mutagenic/genotoxic
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41 73 activity. The same tests were performed on the mixtures of pesticides in them detected to evaluate
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43 74 possible interactions among the pesticides.
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53 76 Furthermore, the reducing activity and content of polyphenols in the vegetables in this study were
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55 77 determined to estimate if the activity of pesticides was affected by the presence of natural products
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57 78 and metabolites. This latter activity was detected performing the Ames test and SOS Chromotest on
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59 79 pesticides previously co-incubated with standard antioxidants (ascorbic acid and gallic acid).
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81 **Methods and Materials**

82 83 *Test substances*

84 The characteristics and properties of the pure pesticides utilized in the present study are given in
85 Table 1. All pesticides, 4-nitroquinoline-N-oxide (4-NQNO), 2-nitrophenyl- β -galacto-pyranoside
86 (ONPG), *p*-nitrophenyl phosphate disodium (PNPP), citric acid, gallic acid, ascorbic acid, Folin
87 Ciocalteu's phenol reagent, iron chloride, trichloroacetic acid and sodium carbonate were purchased
88 from Sigma-Aldrich Chemicals (St.Louis, MO, USA). Sodium azide was obtained from Acros,
89 distributed by Carlo Erba Reagenti (Milano, Italy) and 2-Nitrofluorene (2-NF) from Merck & Co.
90 Inc. (Whitehouse Station, NJ, USA). Diatomaceous earth fine basic pH 10 was supplied by
91 Phenomenex Inc., (Torrance, CA, USA). Dichloromethane was supplied by ROMIL
92 Ltd.(Cambridge, UK). Potassium ferricyanide, acetone and n-Hexane were purchased from Carlo
93 Erba Reagenti. Multiple internal standard (Series M) were supplied by LabService Analytica S.r.l.
94 (Bologna, Italy). Dimethylsulfoxide (DMSO) (Sigma-Aldrich Chemicals) and distilled water were
95 used to solubilize the pesticides.

96 *Vegetables sampling*

97 Samples of vegetables (tomatoes, cherry tomatoes, yellow, green and red peppers, aubergines and
98 lettuces), subjected to analysis, were cultivated in Campania and Sicily, two Southern Italy regions.
99 Unwashed parts of vegetables (500-1000 g) were cut and homogenized to obtain a sample ready to
100 be extracted for residue analysis.

101 *Pesticide residues extraction*

102 An aliquot of 10 g of homogenized sample was mixed with about 12 g of diatomaceous earth and 1
103 mL of citric acid 20% (w/v) was added. The mixture was transferred in a cartridge of 60 mL
104 equipped with frit (Phenomenex Inc.). The cartridge was, then, eluted with 100 mL of
105 dichloromethane in a flask of 250 mL. The eluates were dried by rotary evaporation at 40 – 45°C
106 and re-suspended in 2 mL acetone/n-hexane solution (1:1) for gas-chromatographic analysis
107 (Decreto Ministeriale 20 dicembre 1980; Dagna et al. 1993).

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3 108 *GC Analysis*
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5 109 A bi-channel system Shimadzu GC-17A (Shimadzu Corporation, Tokyo, Japan) was utilized for the
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8 110 residue analysis. Each eluate was passed through a filter of 0.45 μm and 1 μL was injected at the
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10 111 same time into two columns with different polarities (ZB-5 30 m x 0.32 mm i.d. 0.25 μm film and
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12 112 ZB-35 30 m x 0.32 mm i.d. 0.25 μm film) (Phenomenex, Torrance, CA, USA) associated with
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15 113 electron capture detector (ECD) and nitrogen-phosphorus detector (NPD). In this way every
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17 114 pesticide was recorded simultaneously to avoid overlapping of different pesticides.

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20 115 Phosphorates, triazoles and acylalanines were identified using a NPD detector while organochlorine
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22 116 pesticides, pyrethroids, dicarboximides and thiophthalimides were identified using an ECD
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24
25 117 detector. The temperature programme for the column connected to NPD detector consisted of an
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27 118 initial temperature of 50°C held for 2 min, followed by an increase to 150°C at a rate of 25°C min⁻¹
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29 119 and to 270°C at a rate of 4°C min⁻¹ held for 15 min; for ECD detector the temperature programme
30
31 120 of the column consisted of an initial temperature of 50°C held for 2 min, followed by an increase to
32
33 121 150°C at a rate of 25°C min⁻¹ and to 270°C at 2°C min⁻¹ held for 30 min. For both detectors the
34
35 122 temperature of injection is at 250°C, for NPD detector the working temperature is fixed at 280°C,
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37 123 while for ECD detector is fixed at 300°C. All these analysis were performed using pesticide
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39 124 standards. Quantitative gas-chromatography was performed using a multiple internal standard
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41 125 (Series M).
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46 126 *Sample preparation for Mutagenesis / genotoxicity testing*
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48 127 The pure pesticides, vegetables, some mixtures of pesticides and the same pesticides co-incubated
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50 128 with antioxidants were subjected to Ames test and SOS Chromotest. Pure pesticides were initially
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52 129 dissolved in DMSO, if necessary, and then diluted in water to obtain the final stock solutions. The
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54 130 homogenized vegetable samples (aliquot of 10 g) were processed as the extraction of pesticide
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56 131 residues reported above, but the eluates, dried by rotary evaporation at 40 – 45°C, were then re-
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58 132 suspended in DMSO. The mixtures of the pesticides, concurrently detected in the vegetables
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60 133 examined, were analyzed starting from the maximum concentration found in food and then diluted

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3 134 in distilled water. Finally, the mixtures were co-incubated with ascorbic acid (3000 μM) and gallic
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6 135 acid (400 mg/L) at room temperature for 1h on mechanical shaker before mutagenesis/genotoxicity
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8 136 testing to evaluate if the activity of pesticides could be influenced by the presence of those
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10 137 metabolites.

12 13 138 *Mutagenesis / genotoxicity testing*

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15 139 Ames test was performed on *Salmonella typhimurium* strain TA98 for frame-shift mutations and
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17 140 TA100 and TA 1535 for base-pair substitution to detect direct mutagenic compounds. The plate
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20 141 incorporation assay was conducted without metabolic activation (S9) to detect direct mutagenic
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22 142 compounds (Maron and Ames, 1983). Preliminary tests of mutagenesis/genotoxicity were carried
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24
25 143 out to select proper test doses independently for each sample tested. Sodium azide (2, 1 and 0.5
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27 144 $\mu\text{g}/\text{plate}$) and 2-nitrofluoren (1, 0.5 and 0.25 $\mu\text{g}/\text{plate}$) were used as positive controls for TA100,
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29 145 TA1535 and TA98, respectively. Results obtained showed that these positive controls behaved as
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32 146 expected and that consequently all tests could be considered valid. The number of His⁺ revertants
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34 147 was counted (colony counter Cardinal, Perceptive Instruments, Suffolk, UK). Results were
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36 148 expressed as mutation ratios (MRs) between the number of colonies on test plates and the number
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39 149 of colonies on solvent control plates. A compound was considered mutagenic when the MR was
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41 150 more than double and a dose-response effect was clear (US.EPA, 1983).

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46 152 *E. coli* PQ37 was used in the colorimetric assay SOS-Chromotest. This strain exhibits *sfiA::lacZ*
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48 153 fusion and has a deletion of the normal *lac* region, so that β -galactosidase activity is strictly
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50 154 dependent on *sfiA* expression. The assay is quantitative (Quillardet and Hofnung 1993).

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55 156 An overnight culture of *E. coli* PQ37 was diluted and incubated for 2h at 37°C to a bacterial density
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57 157 of 2×10^8 bacteria/mL. Then, 1mL of the culture was diluted in 9 mL of fresh medium. Fractions
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60 158 (600 μL) were distributed to test tubes containing 20 μL of the sample and negative (solvent) and
159 positive controls (4-nitroquinoline-N-oxide). After shaking for 2h at 37°C, two aliquots (300 μL)

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3 160 were withdrawn from each tube for β -galactosidase and alkaline phosphatase assay with 4-
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6 161 nitrophenyl- β -D-galactopyranoside (ONPG) and 4-nitrophenyl phosphate (PNPP) solutions,
7
8 162 respectively. The β -galactosidase and alkaline phosphatase activity were measured as o-nitrophenol
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11 163 and 4-nitrophenyl concentration, respectively, by photometric measurement at 405 nm. The
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13 164 genotoxic activity was identified as the ratio $R = \beta/\rho$ where β represents β -galactosidase activity and
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15 165 ρ , phosphatase alkaline activity. The results were expressed as induction factor (IF) for each
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18 166 dilution of compound, defined as $IF = R/R_0$, in which R_0 is the spontaneous ratio measured in the
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20 167 solvent control. A sample was considered genotoxic when the IF was more than double with a clear
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22 168 dose-effect relationship (Ruiz and Marzin 1997). Also SOS Chromotest was carried out in the
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25 169 absence of S9 fractions of liver homogenate from rat.

26 27 170 *Determination of Polyphenols*

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29 171 The phenolic content of the selected vegetables was determined using the Folin-Ciocalteu method
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32 172 as described by Rekha and Vijayalakshmi (2008). The vegetable sample was homogenized, mixed
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34 173 with distilled water (1:10) and shaken at 26°C for 2 h. The extract was centrifuged at 3000 rpm for
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36 174 15 min, 0.25 mL of Folin-Ciocalteu (1:2) and 1 mL of 10% Na_2CO_3 were added to supernatant
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39 175 solution. The mixture was incubated at room temperature for 2 h and the absorbance was measured
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41 176 at 760 nm using a Unicam, Helios Alfa spectrophotometer. Gallic acid was used as a standard. A
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44 177 solvent control was prepared for each dilution of sample and positive control. Each sample was
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46 178 analyzed three times. Results were expressed as microgram gallic acid equivalent per g of vegetable
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48 179 ($\mu\text{gGAE/g}$).

49 50 51 180 *Measurement of Reducing Activity*

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53 181 The method of Oyaizu described by Rekha and Vijayalakshmi (2008) was used to determine the
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55 182 reducing activity. The sample was homogenized and mixed with distilled water (1:100) and
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58 183 centrifuged at 1500 rpm for 20 min. The extract (0.5 mL) was supplemented with 0.5 mL
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60 184 potassium ferricyanide (1%) and 0.5 mL of sodium phosphate buffer (0.02 M, pH 7) and the
185 mixture was incubated at 50°C for 20 min. Then, 0.5 mL of trichloroacetic acid (10 %) was added

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3 186 and this solution was left at room temperature for 15 min and then was centrifuged at 1000 rpm for
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6 187 5 min. The supernatant (1.5 mL) was mixed with 0.2 mL ferrichloride (0.1 %) and incubated at
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8 188 room temperature for 5 min. Vitamin C was used as standard. The absorbance was measured at 700
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10 189 nm (Unicam, Helios Alfa spectrophotometer). The results were expressed as an equivalent amount
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13 190 of ascorbic acid (μM).

15 191 *Data analysis*

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17 192 All tests were performed three time (three independent assays). The results of all assays were
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20 193 analyzed using analysis of variance (one-way ANOVA) and difference among means were
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22 194 compared to negative control for critical F at $p \leq 0.05$ or $p \leq 0.01$. The level of significance is given
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25 195 in the respective tables. Furthermore, the coefficient correlation (R) was estimated with linear
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27 196 regression statistic method and compared to Student's *t* distribution for $p \leq 0.05$ or $p \leq 0.01$.

31 198 **Results and Discussion**

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33
34 199 Many pesticides of different classes were detected in the samples of vegetables examined and all
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36 200 results are reported in Table 2. Seven samples (38.8%) did not contain any pesticide but five out of
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39 201 eighteen (27.7%) showed two or three pesticides simultaneously present. Five vegetables were not
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41 202 in compliance with Italian directive on Maximum Residue Limits (MRL). Among the pesticides
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44 203 found at higher concentrations, azynphos methyl, was detected only into aubergine and indoxacarb
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46 204 in a tomato sample was found at concentrations three fold higher than the allowed limit.
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48 205 Cypermethrin, chlorpyrifos methyl and indoxacarb were found, concurrently, into a sample of
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51 206 cherry tomato at concentrations higher than the respective MRLs, as well as λ -cyhalothrin in yellow
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53 207 pepper and fenazaquin in cherry tomato. One sample of green pepper contained penconazol (0.02
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55 208 mg/kg), a compound not allowed in Italy. Looking at these findings we chose the twelve pesticides,
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58 209 representative of different chemical classes, to be subjected to mutagenesis/genotoxicity testing to
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60 210 determine whether compounds commonly used in South Italy against pests can damage DNA.

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3 211 The results for the pesticides inducing or not direct mutagenic activity in the Ames test are shown
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6 212 in Table 3. All pesticides were examined at concentrations around values detected in the
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8 213 contaminated vegetables. Chlorothalonil, chlorpyrifos ethyl and penconazol were negative for all
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10 214 three tester strains while cyprodinil had mutagenic effect only for TA98, inducing frame-shift
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12 215 mutations at concentrations ranging from 0.5 µg/mL to the highest concentration tested (2 µg/mL),
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14
15 216 with a clear dose-response effect. Azinphos methyl showed activity for TA100 and TA 1535 strains,
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17 217 inducing base-pair substitution at concentrations starting from 0.25 and 0.5 µg/mL, respectively.
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19
20 218 This result does not agree with the finding of Gómez-Arroyo et al. (2007) for whom this pesticide
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22 219 was only an indirect mutagen. Fludioxonil and iprodione were positive for TA 98 and 1535, while
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24
25 220 fenazaquin showed positive effect for TA 98 and TA 100 strains. It is worth to note the strong
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27 221 mutagenic effect of fludioxonil for TA 1535 that showed activity starting from 0.1 µg/mL with a
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29 222 number of revertants/µg equal to 1740±27 while its MRL in lettuce is 10µg/g. Only λ-cyhalothrin
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31
32 223 and indoxacarb had mutagenic effect for all tester strains. As example of the statistical analysis of
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34 224 data performed for all pesticides, a detailed table with each tested dose, mean of revertants/plate
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36 225 ±SD (standard deviation), the mutagenic ratios for each concentration, the respective positive and
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39 226 negative controls of the three strains tested is reported in Table 4 for λ-cyhalothrin. cypermethrin
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41 227 was negative for all tester strains in agreement with Pluijmen et al (1984) in a study on TA98 and
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43
44 228 TA100. Chlorpyrifos methyl induced only frame-shift mutations (TA98) and these results are
45
46 229 different from those achieved by Ruiz and Marzin (1997) who tested this pesticide at higher
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48
49 230 concentrations, toxic in our experiments.
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51 231
52
53 232 The SOS chromotest results (Table 5) indicated the genotoxic effect of azinphos methyl, cyprodinil,
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55 233 fludioxonil, iprodione with Induction Factors (IFs) higher than 2 that increased with the doses
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57
58 234 assayed even if a decline of IF due to the growing toxic effects was found for azinphos methyl at
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60 235 higher concentrations (1.5 and 2 µg/mL). Based on the IFs obtained, cyprodinil and fludioxonil
236 demonstrated the highest genotoxicity activating SOS DNA repair system at concentrations starting

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3 237 from 0.004 µg/mL. The other pesticides were not able to determine a significant SOS system
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6 238 induction.

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10 240 Among the twelve pesticides tested, azinphos methyl, cyprodinil, fludioxonil and iprodione were
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12 241 positive to Ames test as well as stimulated SOS DNA repair system in *E. Coli* PQ37. chlorothalonil,
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15 242 chlorpyrifos ethyl, cypermethrin and penconazol were not mutagenic for Salmonella neither
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17 243 genotoxic for *E. coli*.

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22 245 Our main objective was to investigate whether the contaminated vegetables maintained the same
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24 246 genotoxic activity detected for pure pesticides or they had any antimutagenic/antigenotoxic effect.

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27 247 For this reason bioassays were performed on food extracts. Of 18 vegetables analyzed by GC,

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29 248 sample number 3 (green pepper), sample number 7 (aubergine) and sample number 13 (cherry
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31 249 tomato) (see Table 2) were subjected to Ames Test and SOS Chromotest. Sample 3 was chosen

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33 250 because it contained penconazol, a pesticide not allowed in Italy that, even though it was found

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35
36 251 neither mutagenic nor genotoxic (Table 3 and Table 5) it could determine any effect when in
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38 252 presence of other pesticides. Sample 7 was chosen because azynphos methyl residue exceeded

39
40 253 three fold the maximum concentration allowed. The sample number 13 was selected because all

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42
43 254 residues in this cherry tomato were at concentrations higher than the respective limits. The results of

44
45 255 genotoxicity tests on vegetables are shown in Table 6. Neither mutagenic nor genotoxic effects

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47
48 256 were found in the vegetable extracts tested. The mutagenic/genotoxic activity found in the previous

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50 257 tests for the pure pesticides, some of them highly active such as iprodione and indoxacarb, was

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53 258 completely suppressed. The MRs (ratio between the number of revertants of the test plates and the

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55 259 number of revertants of the solvent control plates) was often less than 1, indicating that the food

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57 260 extracts reduced the number of revertants by comparison with the negative control, acting as

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59 261 protective factors for mutagenesis. The same findings were achieved for the SOS Chromotest.

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262 These results differ from those of Feretti et al (2007) that in a study on the extracts from pesticide-

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3 263 treated vegetables using the *Allium cepa* assay, found genotoxicity in the most extracts except in
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6 264 those not contaminated by pesticides. The Ames test and SOS Chromotest were also performed on
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8 265 the mixtures of pesticides found in sample number 3 (green pepper) (mixture A) and in sample
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10 266 number 13 (cherry tomato) (mixture B), to evaluate whether interactions could occur between
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13 267 pesticides. For this reason, the mixtures were analyzed starting from the respective maximum
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15 268 concentration of each pesticide found in that vegetable and maintaining the same concentration ratio
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17 269 between the pesticides (only dilutions of the mixture were done) to compare their effects with those
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20 270 of all constituents without testing all possible combinations of the individual pesticides. The
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22 271 genotoxicity of the mixtures could not be described with respect to synergistic, antagonistic or
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25 272 potentiating effects of pesticides because no dose-effect curves for each of the single pesticide were
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27 273 done. This approach just allowed us to establish if the effects of the combined pesticides could
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29 274 justify the decrease in genotoxicity showed by vegetables contaminated in the Ames test and SOS
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31
32 275 Chromotest. The analysis of data from Ames test on *S. typhimurium* TA98 and TA100 for pesticide
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34 276 mixtures (Table 7) showed mutagenic ratios > 2 only at 100% for mixture B. The results of
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36 277 mixture A indicated a MR >2 only at 100% for TA98 while for TA100 at 100% a toxic effects of
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39 278 the pesticides was found. A clear dose related effect was shown but the mixtures were not
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41 279 considered positive for those two strains because there were not two consecutive dose levels with
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44 280 responses at least twice that of the negative control. On the contrary, data obtained from the strain
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46 281 1535 showed a strong positive mutagenic effect also according to the statistical analysis for both the
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48 282 mixtures indicating that they induced direct mutations for pair base substitution. The mixture B was
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51 283 the most active with a MR = 2.33 already at 25% of the mixture dilution. Finally, the pure
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53 284 pesticides, found positive for mutagenesis, when in mixtures showed some interactions determining
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55 285 the decrease of activity on the strain TA98 while the mutagenic effect was maintained for TA1535.
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58 286 As reported in Table 8, the mixtures were not able to induce the SOS system, they exhibited a clear
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60 287 dose-response relationship and a toxic effect was shown for not diluted mixtures of pesticides
288 (100%). On the basis of these results, to demonstrate that the high decrease in activity of the

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3 289 pesticides contained in the vegetables, showed in Table 6, could depend on the presence of natural
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6 290 products such as vitamins, or secondary metabolites as flavonoids, we determined the content of
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8 291 polyphenols and the reducing activity of the vegetables because these may contribute to overall
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10 292 antioxidant activities of food. Subsequently, the mixtures A and B and azynphos methyl, the only
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12 293 pesticide found in the sample of aubergine (7), were co-incubated with standard antioxidants
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15 294 (ascorbic acid and gallic acid) and then subjected to Ames test and SOS Chromotest, for the strains
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17 295 for which were previously found positive. The data presented in Table 9, concerning the hydrophilic
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20 296 portion of the content in polyphenols and the total reducing activity of the contaminated vegetables
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22 297 indicated that the presence of antioxidants was high. The content in polyphenols expressed as μg
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25 298 GAE/g (gallic acid equivalents/vegetable) fell into the range of values reported for cherry tomatoes
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27 299 in previous studies (Toor and Savage, 2005; Brat et al. 2006) while our results were higher for
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29 300 green pepper when compared to those of Brat et al. (2006). The green pepper exhibited the highest
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31 301 antioxidative activity followed by aubergines and cherry tomatoes but no further data were found
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34 302 for aubergines in the literature. Figures 1 and 2 compare the mutagenic activity of azinphos methyl
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36 303 (for *S. typhimurium* TA100 and TA1535) and the mixtures A and B (for *S. typhimurium* TA1535) to
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38
39 304 the same activity when the pesticides were pre-incubated with gallic acid or ascorbic acid. The
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41 305 inhibitory effect of the antioxidants on the activity of pesticides was high and significantly different
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44 306 indicating that antioxidants positively influence the decrease in mutagenesis of pesticides. The same
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46 307 trend was shown for the genotoxic activity of azinphos methyl (Figure 3) that was suppressed going
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48 308 from $\text{IF} = 2.71$ to 1.14 and 1.12 with ascorbic acid and gallic acid, respectively.
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52 310 **Conclusions**

53 311 The present study has shown that vegetables cultivated and distributed in South Italy may contain
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56 312 mixtures of pesticides at concentrations higher than the respective MRLs. It is to be considered that
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59 313 the MRL expressed in mg kg^{-1} of food, is the amount that may be taken daily without evident health
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314 risk but it is referred to adults whose weight is comprised between 50 and 80 kg. The results of this

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3 315 study also show that, although some residues are found in food, the antioxidant components of
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6 316 vegetables could be responsible for the observed inhibitory effects of the vegetable matrices on the
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8 317 mutagenesis and genotoxicity found for pure compounds. Furthermore, other undetermined
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10 318 components of aubergines, tomatoes and green peppers could contribute to the overall inhibitory
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12 effects of vegetables and this requires further investigations.
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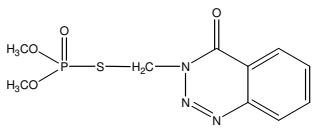
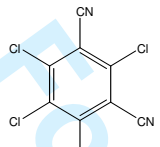
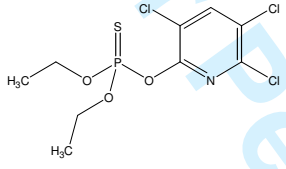
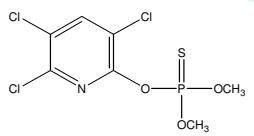
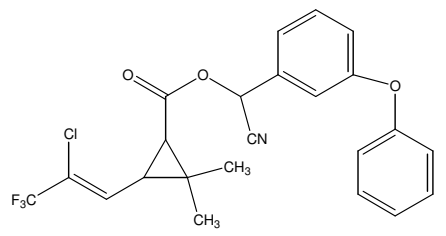
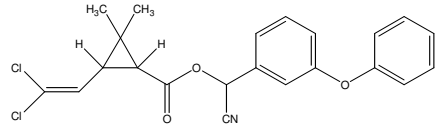
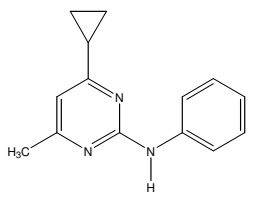
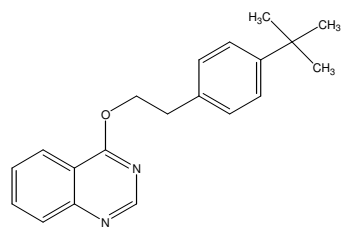
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Pesticide	Chemical structure	Chemical characteristics			Chemical class	Application
		Molecular weight	Melting point (°C)	Purity (%)		
Azinphos methyl		317.32	69.7-70.4	98,9	Organophosphorus	Acaricide
Chlorothalonil		265.91	252.8-253.9	99.2	Chloronitrile	Fungicide
Chlorphyriphos ethyl		350.59	42.4-42.6	99.9	Organophosphorus	Acaricide
Chlorphyriphos methyl		322.53	45.5-46.5	99.9	Phosphorothioate	Insecticide, acaricide
λ-Cyhalothrin		449.85	49.2-50.9	99.3	Pyrethroid	Acaricide
Cypermethrin		416.30	20-60	95.1	Pyrethroid	Acaricide
Cyprodinil		225.29	76.4-77	99.9	Pyrimidine	Fungicide
Fenazaquin		306.4	80.1-80.7	99.9	Quinazoline	Acaricide

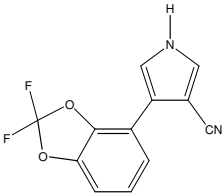
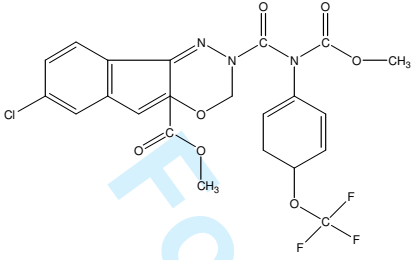
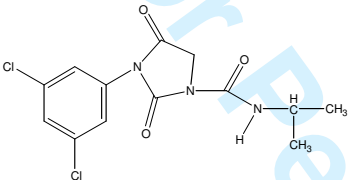
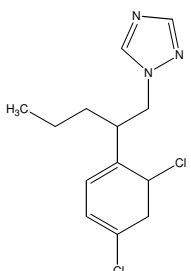
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6	Fludioxonil		248.2	199.4-200.7	99.9	Pyrole	Fungicide
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8							
9							
10							
11							
12	Indoxacarb		527.83	nd	93.9	Oxadiazine	Insecticide
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14							
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22	Iprodione		330.17	131.7-132.9	99.3	Imidazole	Fungicide
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30	Penconazol		284.18	60.7-61.8	99.1	Conazole	Fungicide
31							
32							
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35							

Table 1: Characteristics and properties of the tested pesticides.

Sample	Vegetables contaminated	Pesticides in mgKg ⁻¹	MRL (mgKg ⁻¹)
1	Red pepper	Iprodione (0.55)	5.0
2	Red pepper	n.r.	
3	Green pepper	Chlorpyriphos ethyl (0.40) Iprodione (0.73) Penconazol (0.02)	0.50 5.0 not allowed in Italy
4	Green pepper	n.r.	
5	Yellow pepper	Chlorpyriphos ethyl (0.35) Indoxacarb (0.03) λ-cyhalotrin (0.15)	0.50 0.20 0.10
6	Yellow pepper	n.r.	
7	Aubergine	Azynphos methyl (1.52)	0.50
8	Aubergine	n.r.	
9	Aubergine	n.r.	
10	Tomato	Indoxacarb (0.33)	0.10
11	Tomato	Chlorothalonil (0.05)	2.0
12	Tomato	Chlorothalonil (0.03)	2.0
13	Cherry Tomato	Cypermethrin (0.84) Chlorpyriphos methyl (0.67) Indoxacarb (0.34)	0.50 0.50 0.10
14	Cherry Tomato	n.r.	
15	Cherry Tomato	Fenazaquin (0.78)	0.50
16	Lettuce	Iprodione (2.31) Cyprodinil (1.14) Fludioxonil (0.85)	10.0 10.0 10.0
17	Lettuce	n.r.	
18	Lettuce	Fludioxonil (0.13) Cyprodinil (0.47)	10.0 10.0

Table 2: Concentrations and Maximum Residue Limits of pesticides occurred in the tested vegetables. In bold the pesticides found at concentrations higher than the respective MRL.

n.r. = no residues

Compounds	MUTAGENICITY (AMES TEST)								
	TA98			TA100			TA1535		
	Mutag. concentr. range (tested concentr. range) in µg/mL	Revert./µg ±SD	Maximum ^a MR	Mutag. concentr. range (tested concentr. range) in µg/mL	Revert./µg ±SD	Maximum ^a MR	Mutag. concentr. range (tested concentr. range) in µg/mL	Revert./µg ±SD	Maximum ^a MR
Azinphos methyl	nm (0.25-2.00)	ns	1.00	0.25-2.00 (0.12-1.00)	4480 ± 30	2.60	0.50-2.00 (0.25-2.00)	785 ± 31	4.60
Chlorothalonil	nm (0.25-2.00)	ns	1.03	nm (0.25-2.00)	ns	1.53	nm (0.25-2.00)	ns	1.57
Chlorpyrifos ethyl	nm (0.25-2.00)	ns	0.80	nm (0.25-2.00)	ns	1.16	nm (0.25-2.00)	ns	1.50
Chlorpyrifos methyl	0.25-1 (0.06-1.00)	2220 ± 13	3.70	nm (0.06-1.00)	ns	1.08	nm (0.06-1.00)	ns	1.10
Cypermethrin	nm (0.06-1.00)	ns	1.74	nm (0.06-1.00)	ns	1.10	nm (0.06-1.00)	ns	1.30
Cyprodinil	0.50-2.00 (0.25-2.00)	386.5 ± 5	2.45	nm (0.25-2.00)	ns	1.53	nm (0.06-0.50)	ns	1.20
Fenazaquin	0.50-2.00 (0.25-2.00)	1155 ± 18	7.32	1.00-2.00 (0.25-2.00)	2420 ± 33	2.54	nm (0.12-1.00)	ns	1.18
Fludioxonil	0.50-2.00 (0.25-2.00)	705 ± 20	4.46	nm (0.25-2.00)	ns	1.17	0.10-0.50 (0.04-0.50)	1740 ± 27	5.12
Indoxacarb	0.25-1.00 (0.06-1.00)	3380 ± 2	5.45	0.25-1.00 (0.06-1.00)	1620 ± 66	5.60	0.25-0.50 (0.06-1.00)	1260 ± 28	4.60
Iprodione	0.50-1.00 (0.25-2.00)	760 ± 23	2.20	nm (0.25-2.00)	ns	1.13	0.50-2.00 (0.25-2.00)	421 ± 47	5.60
Penconazol	nm (0.25-2.00)	ns	1.06	nm (0.25-2.00)	ns	1.20	nm (0.12-1.00)	ns	1.10
λ-Cyhalothrin	0.12-1.00 (0.06-1.00)	3220 ± 41	7.49	0.50-1.00 (0.06-1.00)	5510 ± 26	3.26	0.50-2.00 (0.25-2.00)	1040 ± 5	6.10

Table 3: Mutagenic activity of the tested pesticides in Salmonella strains TA98, TA100 and TA1535. In bold the positive MRs.

± SD= standard deviation obtained from three independent experiments.

nm= no mutagenic effect.

ns= no significant number of revertants compared to the control.

^a MR (Mutagenic ratio): number of revertants/plate compared to the negative control.

	MUTAGENICITY (AMES TEST)								
	TA98			TA100			TA1535		
	Dose ($\mu\text{g/mL}$)	Mean Revert./plate \pm SD	^a MR	Dose ($\mu\text{g/mL}$)	Mean Revert./plate \pm SD	^a MR	Dose ($\mu\text{g/mL}$)	Mean Revert./plate \pm SD	^a MR
Negative Control		43 \pm 8	-	-	169 \pm 11	-	-	17 \pm 3	-
Sodium azide	-	-	-	5	385 \pm 13**	2.28	5	156 \pm 25**	4.89
	-	-	-	10	556 \pm 25**	3.29	10	320 \pm 19**	10.00
	-	-	-	20	809 \pm 36**	4.79	20	496 \pm 39**	15.50
2-Nitrofluoren	2.5	163 \pm 16**	3.80	-	-	-	-	-	-
	5	387 \pm 13**	9.00	-	-	-	-	-	-
	10	516 \pm 31**	12.00	-	-	-	-	-	-
λ -Cyhalothrin	0.6 x 10 ⁻¹	67 \pm 4**	1.56	0.6 x 10 ⁻¹	225 \pm 21**	1.33	2.5 x 10 ⁻¹	20 \pm 4	1.20
	1.2 x 10 ⁻¹	88 \pm 12**	2.05	1.2 x 10 ⁻¹	268 \pm 24**	1.58	5 x 10 ⁻¹	29 \pm 6*	1.70
	2.5 x 10 ⁻¹	103 \pm 7**	2.39	2.5 x 10 ⁻¹	304 \pm 7**	1.80	1.00	104 \pm 8**	6.10
	5 x 10 ⁻¹	201 \pm 8**	4.67	5 x 10 ⁻¹	375 \pm 4**	2.21	2.00	71 \pm 13**	4.20
	1.00	322 \pm 41**	7.49	1.00	551 \pm 26**	3.26			
^b R		0.99**			0.99**			1.00**	

Table 4 : Mutagenic activity of λ -Cyhalothrin in Salmonella strains TA 100, TA 98 and TA 1535. In bold the positive MRs.

\pm SD= standard deviation obtained from three independent experiments.

^aMR (Mutagenic ratio): number of revertants/plate compared to the control.

^bR : dose/response relationship evaluated for non-toxic concentrations

One-Way ANOVA used to determine differences among means in negative control and sample. *p \leq 0.05 **p \leq 0.01

GENOTOXICITY (SOS CHROMOTEST)			
Compounds	Dose ($\mu\text{g/mL}$)	^a I.F. \pm SD	^b R
Azinphos methyl	2.5×10^{-1}	0.50 ± 0.12	0.96*
	5.0×10^{-1}	1.43 ± 0.22	
	7.5×10^{-1}	2.64 ± 0.53	
	1.0	2.71 ± 0.27	
	1.5	1.66 ± 0.32	
Chlorothalonil	2.0	0.97 ± 0.34	-
	1.0×10^{-1}	0.94 ± 0.20	
	2.0×10^{-1}	1.04 ± 0.11	
	4.0×10^{-1}	1.18 ± 0.09	
	8.0×10^{-1}	1.52 ± 0.17	
Chlorpyriphos ethyl	1.0	1.61 ± 0.18	-
	5.0×10^{-1}	0.84 ± 0.28	
	1.0	0.93 ± 0.18	
Chlorpyriphos methyl	2.0	1.20 ± 0.14	-
	6.0×10^{-2}	0.75 ± 0.11	
	1.2×10^{-1}	0.76 ± 0.08	
	2.5×10^{-1}	1.00 ± 0.15	
	5.0×10^{-1}	0.75 ± 0.20	
Cypermethrin	1.0	0.50 ± 0.06	-
	6.0×10^{-2}	0.60 ± 0.15	
	1.2×10^{-1}	0.80 ± 0.25	
	2.5×10^{-1}	0.70 ± 0.23	
	5.0×10^{-1}	0.50 ± 0.18	
Cyprodinil	1.0	0.80 ± 0.16	0.94*
	1.0×10^{-3}	1.30 ± 0.05	
	2.0×10^{-3}	1.50 ± 0.28	
	4.0×10^{-3}	1.85 ± 0.21	
	2.0×10^{-2}	2.67 ± 0.22	
Fenazaquin	1.0×10^{-1}	3.90 ± 0.13	-
	4.0×10^{-3}	0.88 ± 0.22	
	2.0×10^{-2}	1.27 ± 0.15	
	1.0×10^{-1}	1.37 ± 0.22	
	2.0×10^{-1}	1.16 ± 0.24	
Fludioxonil	4.0×10^{-1}	0.83 ± 0.21	0.90*
	1.0×10^{-3}	1.39 ± 0.34	
	2.0×10^{-3}	1.77 ± 0.33	
	4.0×10^{-3}	2.40 ± 0.32	
	2.0×10^{-2}	2.95 ± 0.15	
	1.0×10^{-1}	4.08 ± 0.23	

		6.0×10^{-2}	0.50 ± 0.18	
		1.2×10^{-1}	0.73 ± 0.19	
	Indoxacarb	2.5×10^{-1}	0.64 ± 0.08	-
		5.0×10^{-1}	0.45 ± 0.11	
		1.0	0.73 ± 0.11	
		2.0×10^{-3}	1.10 ± 0.15	
	Iprodione	4.0×10^{-3}	1.51 ± 0.19	0.97*
		2.0×10^{-2}	2.22 ± 0.07	
		1.0×10^{-1}	3.90 ± 0.26	
		6.0×10^{-2}	0.56 ± 0.16	
		1.2×10^{-1}	0.69 ± 0.27	
	Penconazol	2.5×10^{-1}	0.80 ± 0.15	-
		5.0×10^{-1}	0.72 ± 0.11	
		1.0	0.25 ± 0.13	
		6.0×10^{-2}	0.67 ± 0.21	
	λ -Cyhalothrin	1.2×10^{-1}	0.33 ± 0.13	
		2.5×10^{-1}	0.43 ± 0.11	-
		5.0×10^{-1}	0.50 ± 0.09	
		1.0	0.83 ± 0.15	

Table 5: Genotoxic activity for *E. coli* PQ37 of the tested pesticides. In bold the positive IFs.

\pm SD= standard deviation obtained from three independent experiments.

* Significant at $p \leq 0.05$.

^aIF (induction factor) = R/R₀.

^bR: dose/response relationship evaluated for non-toxic concentrations.

CONTAMINATED VEGETABLES	MUTAGENIC RATIO for <i>S. typhimurium</i>			INDUCTION FACTOR
	TA 98	TA 100	TA 1535	<i>E. coli</i> PQ 37
Green pepper (3)	0.90	0.94	0.86	0.90
Aubergine (7)	1.30	1.02	0.50	0.98
Cherry tomato (13)	0.92	1.07	0.78	0.82

Table 6: Mutagenic and genotoxic activity of three contaminated vegetables expressed as Mutagenic Ratio and Induction Factor.

(3): N° sample contaminated by Chlorpyrifos ethyl; Iprodione; Penconazol

(7): N° sample contaminated by Azynphos methyl

(13): N° sample contaminated by Cypermetrin; Chlorpyrifos methyl; Indoxacarb

	MUTAGENICITY (AMES TEST)								
	TA98			TA100			TA1535		
	Mixture dilutions in %	Mean Revert./plate \pm SD	^a MR	Mixture dilutions in %	Mean Revert./plate \pm SD	^a MR	Mixture dilutions in %	Mean Revert./plate \pm SD	^a MR
Negative control	-	29 \pm 2	-	-	197 \pm 8	-	-	18 \pm 6	-
Mixture A	100	76 \pm 10**	2.62	100	276 \pm 42**	1.14	100	54 \pm 11**	3.00
	50	54 \pm 6**	1.86	50	386 \pm 13**	1.96	50	38 \pm 6*	2.11
	25	41 \pm 3**	1.41	25	286 \pm 4**	1.45	25	28 \pm 2	1.55
	12.5	30 \pm 9	1.03	12.5	264 \pm 13**	1.34	12.5	19 \pm 4	1.06
^b R		0.99*			0.99*			0.99*	
Mixture B	100	88 \pm 2**	3.03	100	401 \pm 6**	2.03	100	82 \pm 9**	4.56
	50	52 \pm 10**	1.79	50	366 \pm 16**	1.85	50	61 \pm 11**	3.39
	25	36 \pm 5*	1.24	25	305 \pm 15**	1.54	25	42 \pm 3**	2.33
	12.5	28 \pm 2	0.96	12.5	282 \pm 4**	1.43	12.5	33 \pm 4*	1.83
^b R		1.00*			0.96*			0.99*	

Table 7: Mutagenic activity of pesticide mixtures found in contaminated vegetables.

Mixture A: chlorpyrifos ethyl (0.40 μ g/mL), iprodione (0.73 μ g/mL), penconazol (0.02 μ g/mL).

Mixture B: cypermethrin (0.84 μ g/mL), chlorpyrifos methyl (0.67 μ g/mL), indoxacarb (0.34 μ g/mL).

\pm SD= standard deviation obtained from three independent experiments.

^bR : Sample's dose/response relationship evaluated for non-toxic concentrations.

^aMR (Mutagenic ratio): number of revertants/plate compared to the control.

One-Way ANOVA used to determine differences among means in negative control and sample * $p \leq 0.05$ ** $p \leq 0.01$

GENOTOXICITY (SOS CHROMOTEST)		
	Mixture dilutions in %	^a I.F.
Mixture A	100	1.31 ± 0.16
	50	1.83 ± 0.11
	25	1.11 ± 0.15
	12.5	1.04 ± 0.10
Mixture B	100	1.31 ± 0.18
	50	1.99 ± 0.20
	25	1.47 ± 0.22
	12.5	0.41 ± 0.12

Table 8: Genotoxic activity of the pesticide mixtures found in contaminated vegetables.

Mixture A: Chlorpyrifos ethyl (0.40 µg/mL); Iprodione (0.73 µg/mL); Penconazol (0.02 µg/mL)

Mixture B: Cypermethrin (0.84 µg/mL); Chlorpyrifos methyl (0.67 µg/mL);

Indoxacarb (0.34 µg/mL)

^a IF (induction factor) = R/R₀

	POLYPHENOL CONTENT ($\mu\text{g/g}$)	REDUCING ACTIVITY (μM)
Aubergine	430.1 ± 48.2	3017.0 ± 103.1
Green pepper	668.5 ± 48.3	4150.3 ± 337.3
Cherry tomato	225.4 ± 8.6	1480.3 ± 140.3

Table 9: Polyphenol content and reducing activity of three contaminated vegetables.

Polyphenol content is expressed as microgram gallic acid equivalent per g of vegetable

Reducing activity is expressed as an equivalent amount of ascorbic acid (μM).

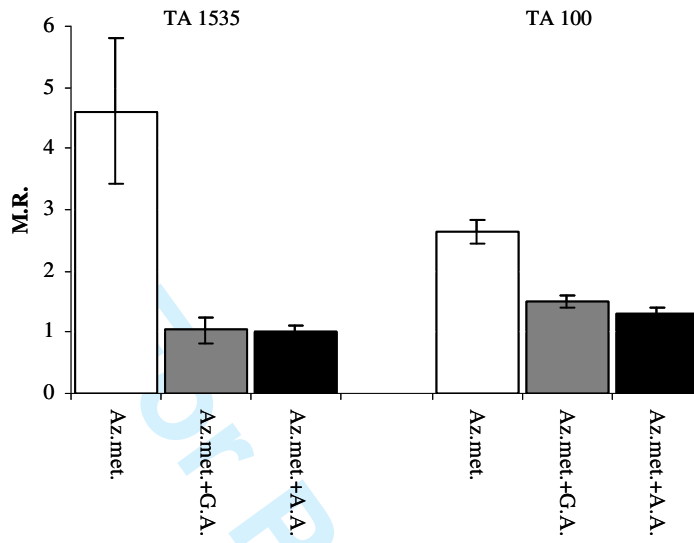


Figure 1

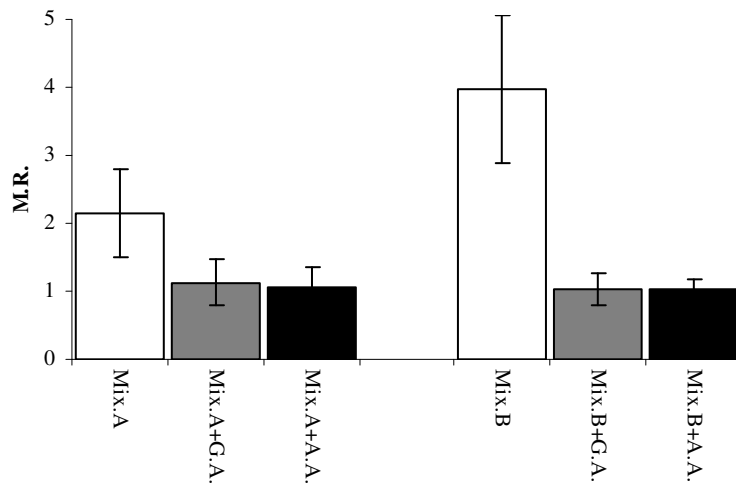


Figure 2

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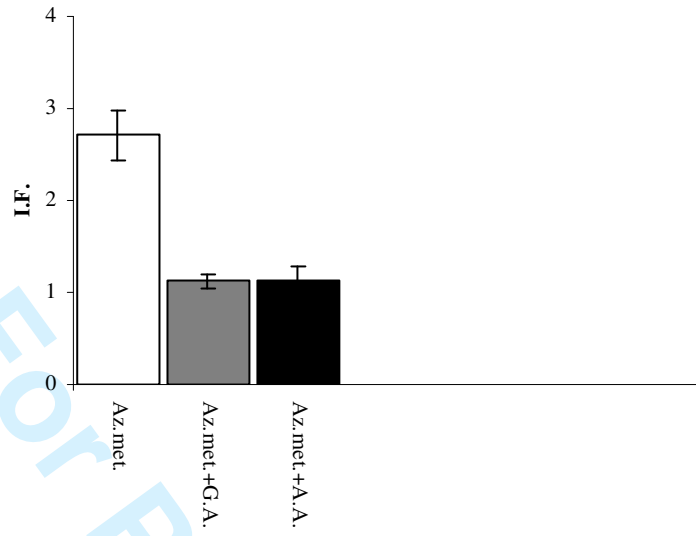


Figure 3

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