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ORGANOPHOSPHORUS PESTICIDE RESIDUES IN GREEK VIRGIN OLIVE OIL: LEVELS, DIETARY INTAKE AND RISK ASSESSMENT

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Manuscripts

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4 **Organophosphorus pesticide residues in Greek virgin olive oil: levels,**
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6 **dietary intake and risk assessment**
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3 **Abstract**
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5 The occurrence of organophosphorus pesticide (OPs) and their metabolite residues
6 was investigated in 167 samples of Greek virgin olive oil during a two-year (2004–
7 2005) sampling campaign. A total of 30.5% of samples contained detectable residues
8 although only one sample contained dimethoate residues above the maximum residue
9 limit. Among the seven detected OPs, fenthion and fenthion sulfoxide residues were
10 detected in 10.8% and 14.4% of the samples respectively at 0.003 to 0.61 mg kg⁻¹
11 followed by dimethoate that was detected in 10.2% of the samples, at 0.003 to 0.057
12 mg kg⁻¹. The acute dietary risk assessment was undertaken by determining the
13 National Estimated Short-Term Intake (NESTI) while for the chronic dietary risk
14 assessment the National Theoretical Maximum Daily Intake (NTMDI) and National
15 Estimated Daily Intake (NEDI) were calculated. The estimated intakes (NESTI and
16 NEDI) of each pesticide were <7% and <0.86% of the corresponding acute reference
17 doses (ARfDs) and acceptable daily intakes (ADIs), respectively. A cumulative risk
18 assessment was performed using the Hazard Index (HI) and the Toxicity Equivalence
19 Factor (TEF) approaches taking into account that OPs share the same toxicological
20 mechanism. The determined HI and TEFs were found to represent only a small
21 portion of the respective ADIs or ARfDs. These results indicate that there is neither
acute nor chronic risk for the Greek population through olive oil consumption.

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51 **Keywords:** Olive oil; pesticides; monitoring; dietary intake; risk assessment
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22 Introduction

23 Olive oil production is heavily concentrated in the Mediterranean countries, which
24 have 99 % of acreage and produce 98% of the world's olive oil. The European Union
25 (EU) has the lead role in the world production with a share of about 75%. The
26 consumption of olive oil is also practically concentrated in the producing regions,
27 with 71% centered in the EU and 92% of this figure corresponds to the three
28 leading countries, Italy, Spain and Greece (Luchetti 2000). Olive groves occupy more
29 than 60% of cultivated land in Greece. Spread throughout the mainland and the
30 islands, 132 million olive trees produce approximately 350.000 tons of olive oil
31 annually, of which 75% is extra – virgin olive oil (International Olive Oil Council
32 2007a).

34 The pesticides authorised for the treatment of olives in Greece belong to different
35 chemical families such as organophosphates, pyrethrins, carbamates, benzoylureas
36 and spinosad for use as insecticides, dithiocarbamates as fungicides while substituted
37 ureas, bipyridinium salts, simazine and glyphosate are mainly applied as herbicides.
38 However, organophosphorus pesticides (OPs) are used in the largest quantities in
39 olive groves (Lentza-Rizos et al. 2001, Skouras et al. 2007) for controlling the olive
40 fruit fly (*Dacus oleae*), the key insect pest of olives in the Mediterranean area and
41 they are consequently the main target compounds for olive oil residue analysis (Hiskia
42 et al. 1998; Lentza-Rizos et al. 2001; Botitsi et al. 2004). Among OPs, fenthion and
43 dimethoate are the compounds that are mostly frequently applied (Hiskia et al. 1998;
44 Tsatsakis et al. 2003).

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3 46 Taking into account that pesticides residues in food represent a potential health risk
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5 47 and the high consumption rate (30-70 g/day) of olive products in many countries,
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8 48 especially those surrounding the Mediterranean Sea, attention has been drawn to the
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10 49 possible presence of residues in olives and consequently in olive oil. Therefore, both
11
12 50 European Union (EC 1976) and the *Codex Alimentarius* Commission of the Food and
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14 51 Agriculture Organization (FAO) of the United Nations (Codex Alimentarius
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16 52 Commission 1996) have established maximum pesticide residues limits (MRLs) for
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18 53 olives and olive oil.
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26 55 Although pesticide residues in foodstuffs seldom exceed the MRLs when these
27 56 compounds are applied according to good agricultural practices (GAP), consumer
28 57 awareness of health issues due to pesticide residues is continuously increasing. In this
29 58 direction, monitoring of pesticide residues is one of the most important aspects for
30 59 ensuring that consumers are not exposed to unacceptable pesticide residue levels
31 60 minimising thus, potential hazards to human health. The presence of pesticide
32 61 residues in olive oil has been reported in several studies (Hiskia et al. 1998; Tsatsakis
33 62 et al. 2003; Rastrelli et al. 2002; Cabras et al. 1997a; EC 2003). Because of the high
34 63 olive oil consumption among people of the producing countries, the continuous
35 64 control of pesticide residues in olive oil is of great importance.
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51 66 Problems due to the complexity of the olive oil matrix, along with the presence of
52 67 interferences, makes the analysis of pesticide residues in olive oil samples difficult.
53 68 Many multi-residue procedures have been reported for the determination of OP
54 69 residues in olive oil (Rastrelli et al. 2002; Vreuls et al. 1996; Cabras et al. 1997b).
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60 70 Clean-up steps are usually needed to eliminate interfering compounds from the

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3 71 matrix, which increases the amount of high purity solvents used, the number of
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5 72 manual operations required, the uncertainty in the determination, the cost of the
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8 73 method and can result in the loss of the analyte. Headspace solid phase
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10 74 microextraction (HS-SPME) has proven its ability to analyze trace amounts of
11
12 75 pesticides in olive oil (Tsoutsi et al. 2004; Tsoutsi et al 2006) as well in other matrices
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15 76 (Lambropoulou et al 2007; Lambropoulou et al. 2001). It simplifies the process of
16
17 77 sample treatment before quantitative determination by GC or HPLC and avoids the
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20 78 use of organic solvents.
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24 80 The assessment of dietary intake can be performed using either deterministic methods
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26 81 that compare point estimates of intake with a toxicologically acceptable level or the
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28 82 probabilistic method (e.g. Monte Carlo) that takes into account the variation in
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30 83 pesticide residue levels, in food consumption and body weight levels of the population
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32 84 addressed (Caldas et al. 2006). Furthermore, the outcomes give information on the
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34 85 likelihood at which a certain exposure level will occur, as well as the uncertainties
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36 86 associated with it.
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43 88 The purpose of this study was to apply a HS-SPME method for the determination of
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45 89 OPs in virgin olive oil samples, within the context of a pesticide-monitoring program
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47 90 for the Mediterranean diet in order to estimate the dietary risk exposure to OPs in the
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49 91 Greek population which has not been reported so far in previous studies. The present
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51 92 study has focused on OPs that present a common mechanism of action in order to
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53 93 perform a cumulative risk assessment. Both chronic and acute exposure was
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55 94 considered for the risk assessment that was made based on individual pesticides as
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57 95 well as on cumulative approaches. Two deterministic approaches, the Hazard Index
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3 96 (HI) and the Toxicity Equivalence Factor (TEF), were used for the cumulative risk
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5 97 assessment of the intake of OPs and to assess potential health concerns.
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99 **Materials and Methods**

100 *Chemicals, reagents and materials*

101 Pesticide standards (dimethoate, diazinon, fenitrothion, malathion, fenthion, parathion
102 ethyl, ethyl bromophos, methyl bromophos, methidathion, ethion) and metabolites
103 (omethoate, malaoxon, fenthion sulfoxide and fenthion sulfone) were purchased from
104 Riedel-de Haën (Seelze, Germany). All pesticide standards were 98-99% purity.
105 Bromophos ethyl was used as the internal standard (I.S.) at a concentration of 0.10 mg
106 Kg⁻¹. Stock standard solutions of 1000 mg L⁻¹ of each compound were prepared in
107 pure acetone and stored at -18 °C. Working standard solutions of analyzed insecticides
108 at concentration levels 50, 100 and 250 mg L⁻¹ were prepared by diluting the stock
109 solutions with acetone. Acetone (Labscan, Ltd., Dublin, Ireland) was pesticide residue
110 analysis grade. SPME holder and fibre assemblies for manual sampling were provided
111 by Supelco (Bellefonte, PA, USA) and used without modification.
112 Polydimethylsiloxane (PDMS, 100 mm) fibre coating was used for the extraction of
113 OPs from oil samples. Before measurements the PDMS fibre was conditioned in the
114 injector of the gas chromatograph (GC) for 3 h at 240 °C, with the split vent open to
115 fully remove any contaminant that might have caused very high baseline noise and
116 large ghost peaks. The fibre was then repeatedly injected into the GC system until
117 interfering peaks disappeared.

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119 *Sampling*

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3 120 One hundred and sixty-seven (167) samples of virgin olive oil samples were collected
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6 121 from some of the major olive oil production areas of Greece, namely Crete (CRE;
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8 122 n=75), Peloponnese (PEL; n=48), Central Greece (CGR; n=21), Chalkidiki (CHA;
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10 123 n=6), Aegean islands (AEI; n=6) and Ionian islands (IOI; n=11), during 2004 and
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12 124 2005. The samples consisted of bulk samples from large volume storing tanks of olive
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14 125 oil merchants thus, representing about 10000 tonnes of olive oil. According to our
15
16 126 knowledge this study is amongst those with the highest number of samples and the
17
18 127 highest percentage of total production monitored for olive oil in Greece and other
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20 128 Mediterranean countries. Virgin olive oil samples (0.5 L) were collected in dark glass
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22 129 bottles. The samples were transported to the laboratory and kept for a short time at
23
24 130 room temperature until analysis.
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132 *Headspace Solid Phase Microextraction procedure*

133 Olive oil samples used to determine the limits of detection (LODs), limits of
134 quantification (LOQs), recovery and precision were “pesticide free” and different
135 from the samples studied. A “pesticide-free” virgin olive oil was obtained from
136 organically grown olive tree cultivation with known spray history and environmental
137 background. To certify as residue free, the sample was analyzed for
138 organophosphorus pesticides. The olive oil sample (known as a virgin olive oil blank)
139 did not contain detectable levels of any of the analytes. To obtain spiked olive oil
140 samples, the appropriate volume of standard working solutions was added to 5 g of
141 olive oil blank at spiking levels ranging from 0.025 to 0.50 mg kg⁻¹. The I.S. solution
142 was also added to each sample at 0.10 mg kg⁻¹ concentration level.
143 Extraction/concentration of pesticides was carried out according to previous HS-
144 SPME extraction protocol (Tsoutsi et al. 2006). Briefly, 5 g of olive oil sample were

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3 145 placed into 10 mL crimp-top headspace vials, capped with PTFE- grey butyl-coated
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6 146 septa. The samples were heated at $75\text{ }^{\circ}\text{C} \pm 1$ and after 10 min, the fiber was immersed
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8 147 to the headspace of the sample for 60 min. Magnetic stirring with a PTFE-coated stir
9
10 148 bar was used to agitate the sample at 960 rpm. After extraction, the thermal desorption
11
12 149 of the analytes was achieved by inserting the fiber into the injection port (held at 250
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14 150 $^{\circ}\text{C}$) for 7 min. No carry-over effect was detected after that time.
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20 152 *Analysis of pesticide residues*

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22 153 Chromatographic analysis was performed using a Shimadzu 14A capillary gas
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24 154 chromatograph equipped with flame thermionic detector (FTD) operated at $250\text{ }^{\circ}\text{C}$.
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26 155 The DB-1 column, $30\text{ m} \times 0.32\text{ mm i.d.}$, used contained dimethylpolysiloxane (J & W
27
28 156 Scientific, Folsom, CA). The temperature was programmed as follows: initial
29
30 157 temperature was kept at $150\text{ }^{\circ}\text{C}$ for 2 min, which was increased to $200\text{ }^{\circ}\text{C}$, at $5\text{ }^{\circ}\text{C min}^{-1}$
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32 158 $^{\circ}\text{C min}^{-1}$, held for 8 min, then raised to $210\text{ }^{\circ}\text{C}$ at $1\text{ }^{\circ}\text{C min}^{-1}$ and kept for 2 min. The
33
34 159 temperature was finally increased to $270\text{ }^{\circ}\text{C}$ at $20\text{ }^{\circ}\text{C min}^{-1}$ and held for 4 min. The
35
36 160 injection temperature was $250\text{ }^{\circ}\text{C}$. Helium was used as the carrier (1.5 mL min^{-1}) and
37
38 161 make-up gases (40 mL min^{-1}). The detector gases were hydrogen and air, and their
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40 162 flow rates were regulated at 4 mL min^{-1} and 120 mL min^{-1} , respectively. After
41
42 163 sampling, the SPME fiber was placed into the injection port of the GC system,
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44 164 equipped with an SPME liner from Supelco (Bellefonte, PA), where it was desorbed
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46 165 for 7 min at $250\text{ }^{\circ}\text{C}$ (Safe 1990). The injection port was in splitless mode, the splitter
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48 166 opening after 2 min.
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59 168 Confirmatory analysis was performed on selected positive olive oil samples using a
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169 Shimadzu QP 2010 GC/MS system equipped with DB-5-ms capillary column (length,

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3 170 30 m; column i.d., 0.25 mm; film thickness, 0.25 μm) following the previous
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6 171 temperature program. Helium with a flow rate of 1.0 ml/min was used as carrier gas.
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9 172 The interface was kept at 200 $^{\circ}\text{C}$ and the spectra were obtained at 70 eV in the
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11 173 selected ion monitoring (SIM) mode. For each of the substances analysed, three
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13 174 characteristic ions were monitored during the analysis.
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176 *Method performance and Quality Control*

177 During the in-house validation process, the sensitivity, linearity, accuracy and
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22 178 precision were examined in blank matrix samples. Recovery assays and quantification
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25 179 of pesticide residues were accomplished by the internal standard method. The
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27 180 calibration curves constructed were linear over the range of 0.025 to 0.50 mg kg^{-1} .
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29
30 181 The correlation coefficient were >0.985 . LODs calculated as three times the noise
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32 182 levels ranged between 0.006-0.010 mg kg^{-1} , thus, the maximum residue limits (MRLs)
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34 183 required by European and international regulations for the selected analytes can be
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36 184 verified without difficulty (Codex Alimentarius Commission 1996).
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42 186 Concerning the accuracy of the method, the relative recoveries of the extraction
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44 187 procedure at three fortification levels (0.080, 0.16 and 0.32 mg kg^{-1}) ranged between
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46 188 80 % and 106 % for all the analytes studied. The relative recovery R (%) was
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48 189 calculated as follows:

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$$50$$
$$51 190 R(\%) = C_{\text{obs}}/C_{\text{spike}} \times 100$$
$$52$$

53 191 where C_{obs} is the mean concentration of the fortified sample and C_{spike} is the spiked
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55 192 concentration as previously described in Tsoutsis et al. 2006. Repeatability (n=5, intra-
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57 193 day precision), expressed as RSD (%) values, were below 5 % while the
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59 194 reproducibility (n=5, inter-day precision) never exceeded 10 %. In all cases the results
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4 195 of accuracy and precision fulfilled the criteria of the European guidelines with
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6 196 accuracy data comprised between 70 and 110% with RSD<15% indicating that a
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8 197 method can be considered efficient for analysing the target compounds in a given
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10 198 matrix (EC, 2006).

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15 200 Equilibrium methods like SPME are more selective and sampling in the headspace
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17 201 reduces the matrix effects and interferences present in the liquid phase while poses
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19 202 little risks of cross contamination between samples (Pawliszyn, 1997). Nevertheless
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21 203 relative retention times have been checked for each pesticide and only a maximum
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23 204 deviation of 1% from the expected RRT was considered acceptable in order to prevent
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25 205 misidentification of analytes due to interferences. In addition, confirmation of selected
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27 206 positive olive oil samples was followed using a GC-MS instrument in SIM mode.

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32 33 34 208 *Dietary intake and risk assessment*

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36 209 The results of the monitoring program were not only used to check the compliance of
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38 210 olive oil samples with MRLs, but also for the assessment of the consumers' risk
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40 211 resulting from their exposure to pesticide residues via olive oil consumption. The
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42 212 dietary risk was estimated at both acute and chronic level using the determined
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44 213 pesticide residue levels (mean, median and 90th percentile values) and mean
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46 214 consumption data of olive oil in Greece (69.7 g day⁻¹) extracted from the International
47
48 215 Olive Oil Council reports (International Olive Oil Council 2007b). An important issue
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50 216 in the exposure assessment was how to treat samples that were reported to contain no
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52 217 residues (<LOD). All intake estimations were performed assuming that non-detected
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54 218 residue concentrations were equal to half of the respective limit of detection (ND=1/2
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56 219 LOD). A person body weight of 60 kg was assumed in the calculation according to

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3 220 FAO guidelines for adults (WHO 1997) for comparison with other studies while 75kg
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5 221 of adult body weight was also assumed for being closer to the nowadays mean body
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7 222 weight of an adult in developed countries. Acceptable daily intakes (ADI), Maximum
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9 223 Residue Limits (MRLs), and Acute Reference Doses (ARfDs) given by the Food and
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11 224 Agriculture Organization (FAO) are used for the determination of dietary exposure
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13 225 and the estimation of cumulative risk.
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20 227 *(a) Chronic dietary exposure*

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22 228 In a first tier, the chronic dietary risk assessment was conducted by comparing the
23
24 229 National Theoretical Maximum Daily Intake (NTMDI) of the studied OPs with the
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26 230 ADI ($\text{mg kg}^{-1} \text{bw day}^{-1}$). The NTMDI was defined as the Maximum Residue Limits of
27
28 231 each pesticide (MRL_i , mg kg^{-1}) multiplied by the average consumption data (F , kg
29
30 232 day^{-1}) of olive oil per person in Greece divided by the mean body weight (b.w., kg)
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32 233 according to the following equation:
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36 234
$$\text{NTMDI} = \text{MRL}_i \times F / \text{b.w.} \quad (1)$$

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38
39 235 This methodology that uses MRL as a parameter of residue concentration in the intake
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41 236 calculation is very conservative as it assumes situations that are unlikely always to
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43 237 occur. The assumptions include a life daily consumption of food items that: 100% of
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45 238 the daily food consumption will be derived from treated crops; 100% of the treated
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47 239 crop will contain pesticide residues at the MRL level; no dissipation or degradation of
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49 240 the pesticide will occur during storage, transport, preparation, commercial processing
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51 241 and cooking. Thus, a refinement of the intake calculation with more realistic residue
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53 242 data is a better assessment of the risks to consumers.
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3 244 The refinement of the NTMDI was conducted by estimating the National Estimated
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5 245 Daily Intake (NEDI) that is based on the most realistic estimate of residue levels. The
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8 246 NEDI was calculated replacing the MRL in the calculation for the residue data from
9
10 247 the monitoring program (mean or 90th percentile value of pesticide residue levels; LR_i,
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12 248 mg kg⁻¹) and the average consumption data (F, kg day⁻¹) according to the following
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14
15 249 equation:

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18 250
$$\text{NEDI} = \text{LR}_i * \text{F} / \text{b.w.} \quad (2)$$

19
20 251 The values of NTMDI and NEDI are then compared to ADI of each pesticide. The
21
22 252 estimate of the total intake is expressed as percentage of the ADI (%ADI). Risk might
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24
25 253 exist when NTMDI and NEDI exceed ADI.

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30 255 *(b) Acute dietary exposure*

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32 256 An acute dietary exposure assessment is the exposure resulting from a single eating
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34 257 occasion or short-term (e. g. single day) exposure including the maximum residue
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36 258 level expected in food as consumed. The intake has to be compared with the
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39 259 toxicological effects that are of concern from short-term exposures, i.e. an Acute
40
41 260 Reference Dose (ARfD) has to be used instead of the ADI. The National Estimated
42
43 261 Short-Term Intake (NESTI) is used to estimate acute dietary exposure. NESTI is
44
45 262 calculated as the highest residue (HR, mg kg⁻¹) level detected multiplied by the
46
47
48 263 national consumption data (F, kgday⁻¹) for the commodity divided by the body weight
49
50
51 264 (kg) as described below:

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53 265
$$\text{NESTI} = \text{HR} * \text{F} / \text{b.w.} \quad (3)$$

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55
56 266 In cases where the estimated exposure is at levels below the ARfD, the exposure is
57
58 267 considered to be too low for any toxicity to occur, and the risk is deemed to be
59
60 268 insignificant or negligible.

269

270 *(c) Cumulative risk assessment of the intake*

271 When humans are exposed to multiple pesticides with a common mechanism of
 272 toxicological action, the risk associated with exposure can be characterized by a
 273 cumulative risk assessment (EPA 2002). Organophosphorus insecticides share the
 274 same mechanism of toxicological action by inhibition of acetylcholinesterase activity
 275 in the nervous system by phosphorylation, thus, in the present work, two deterministic
 276 (point estimate) methods was used to perform the cumulative risk assessment of the
 277 intake from olive oil. The Hazard Index (HI) and Toxicity Equivalence Factor (TEF)
 278 approaches (Teuschler and Hertzberg 1995; Wilkinson et al. 2000; Boon and Van
 279 Claveren 2000; Jensen et al 2003) were used in both the acute and chronic exposure
 280 assessment of the Greek population.

281

282 The HI is the sum of the hazard quotients (HQ) of the individual OPs (Eq. 4) i.e., it is
 283 the sum of the exposures to each of the pesticides in the group expressed as a fraction
 284 of its respective ARfD (in case of acute hazard index), or its ADI (in case of chronic
 285 hazard index) (Teuschler et al. 1995). The function of this first profiling step is to
 286 identify the potentially critical samples. If a sample has a value of HI that exceeds 1
 287 equal to 100% this could indicate an unacceptable risk. The calculations of “acute HI”
 288 and “chronic HI” are described below

$$289 \quad HI = \sum HQ_i \quad (4)$$

290 Where $HQ_i = \left(\frac{\text{Exposure}}{\text{ADI}} \right)_i = \left(\frac{\text{NEDI}}{\text{ADI}} \right)_i$ in the calculation of “chronic HI”

291 and $HQ_i = \left(\frac{\text{Exposure}}{\text{ARfD}} \right)_i = \left(\frac{\text{NESTI}}{\text{ARfD}} \right)_i$ in the calculation of “acute HI”

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4 292 Using the TEF approach OPs with different potencies were normalized to yield a total
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6 293 equivalent exposure related to one of the pesticides, the so-called index compound
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8 294 (IC) (Wilkinson et al 2000). The TEF approach was first developed to estimate the
9
10 295 toxicity of mixtures of polychlorinated dibenzo-p-dioxins, dibenzofurans and
11
12 296 biphenyls (Safe et al 1990) but since then the TEF approach have also been used in a
13
14 297 number of studies for OPs (Jensen et al. 2003; Boon et al. 2000). As index compounds
15
16 298 chlorpyrifos or methamidophos were used and TEFs for nine OPs were extracted from
17
18 299 previously published studies (Table 1) (Boon et al. 2000; EPA 2002; Caldas et al.
19
20 300 2006). The exposure ($\text{mg kg}^{-1} \text{ day}^{-1}$) of each pesticide is multiplied by its TEF and the
21
22 301 resulting products are summed (Wilkinson et al. 2000) according to equations 5 and 6
23
24 302 for the chronic (TEF_c) and acute (TEF_a) effect level respectively. The resulting sum is
25
26 303 then compared with the ADI (0.01 and $0.04 \text{ mg kg}^{-1} \text{ bw day}^{-1}$ for chlorpyrifos and
27
28 304 methamidophos respectively) for the chronic exposure assessment and with the ARfD
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30 305 ($0.1 \text{ mg kg}^{-1} \text{ bw day}^{-1}$) for the acute exposure assessment. If it is lower than the trigger
31
32 306 effect values the cumulative risk is assumed to be negligible.

$$307 \quad \text{TEF}_c = \sum (\text{NEDI} \times \text{TEF})_i \quad (5)$$

$$308 \quad \text{TEF}_a = \sum (\text{NESTI} \times \text{TEF})_i \quad (6)$$

309

310 **Results and Discussion**

311 *Monitoring data*

312 Of the 167 samples analyzed, pesticide residues were detected in 51 samples ($\approx 30\%$),
313 while the rest contained no detectable residues. Of the thirteen organophosphorus
314 pesticide residues investigated in this study, only seven, omethoate, dimethoate,
315 malaoxon, fenthion, ethion, fenthion sulfoxide and fenthion sulfone, were found
316 above their detection limits. Fig. 1 shows the pesticides which were detected in the

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3 317 samples at levels > LOD. The percentage of detection, average, median and 90th
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5 318 percentile concentration as well as the range of organophosphorus residues in olive oil
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8 319 samples are summarized in Table 2.

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12 321 The co-occurrence of pesticide residues is shown in Figure 2. A total of 35 samples
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14 322 (20.96 %) had at least one organophosphorus pesticide at detectable levels, 12
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16 323 samples (7.78 %) contained residues of two pesticides and only 4 samples (2.40 %)
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18 324 were contaminated with three or four pesticide residues. The results of our monitoring
19
20 325 of organophosphorus pesticides indicate that, among 167 samples of virgin olive oil
21
22 326 that were examined, only one sample was found to have dimethoate (0.057 mg kg⁻¹)
23
24 327 in concentration above the MRL of *Codex Alimentarius* for olive oil (0.05 mg kg⁻¹).
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29 328 Contamination status of OPs varied on the sampling area (Fig. 3). Pesticide residues
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31 329 from different cultivation areas, along with the detection rates are shown in Table 3.
32
33 330 The highest percentage of detection (%) was found in samples from the region of
34
35 331 Crete (≈55%) one of the most important olive oil producing areas of Greece
36
37 332 (Tsatsakis et al. 2003). Olive oil samples from the region of Peloponnese and
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39 333 Chalkidiki followed with ≈15% of pesticide detection.
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44
45 335 The most frequently detected pesticides were fenthion and its metabolite fenthion
46
47 336 sulfoxide and dimethoate. Over 27 different formulations for dimethoate and two for
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49 337 fenthion are registered in Greece (Tsatsakis et al. 2003). Fenthion was detected in 18
50
51 338 samples (10.8%) at levels ranging from 0.003 to 0.61 mg kg⁻¹ and its metabolite
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53 339 fenthion sulfoxide was found in 24 samples (14.4 %) at levels ranging from 0.005 to
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55 340 0.15 mg kg⁻¹. Dimethoate was found in 17 samples (10.2%) in the concentration range
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57 341 of 0.003-0.057 mg kg⁻¹.
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5 343 The occurrence of pesticide residues in olive oil is related also on the fate of
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8 344 pesticides during olive oil extraction that is closely depended to the fat and water
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10 345 solubility of the compounds under consideration. Residues of fat soluble fenthion
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12 346 were generally higher than those of water-soluble insecticide dimethoate. This is
13
14 347 probably due to the higher water solubility of dimethoate (25 g L^{-1}). Because of this a
15
16 348 large fraction of the active ingredient draws away with the water phase during the
17
18 349 olive oil production. On the contrary, fenthion is a lipophilic compound, which is only
19
20 350 slightly soluble in water and has an octanol-water partition coefficient ($\log K_{ow}$) of 4.1
21
22 351 in contrast to dimethoate which has a $\log K_{ow}$ of 0.3. In the study of Cabras et al.
23
24 352 (Cabras et al. 1993) it was observed that ca. 70% of fenthion is transferred from the
25
26 353 olives to the oil while the remaining part is split between the cake (20%) and
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28 354 vegetation water (10%). Accordingly, the transfer factors of dimethoate and fenthion
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30 355 from olives to olive oil have been as 0.03 and either 3.3 or 5.20 respectively (Farris et
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32 356 al. 1992). Finally, Leone et al. have reached the same conclusion regarding the
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34 357 importance of the fat- or water-solubility of these two insecticides (Leone et al. 1990).
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36 358 Furthermore, the persistence of pesticides after their application in olives is also a key
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38 359 factor for the occurrence of their residues. The fate of fenthion and dimethoate in field
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40 360 trials have been studied by several researchers (Cabras et al. 1997a; Cabras et al.
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42 361 1993; Rotunno et al. 1997), where low degradation rates have been observed,
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44 362 resulting to the presence of residues in olive oil. The occurrence of fenthion is
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46 363 consistent with the study of Cabras et al. (1993), that considered a rather slow
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48 364 metabolic breakdown of this insecticide with a half life of ca. 38 days and that there is
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50 365 a good chance to observe their residues in olive oil. On the other hand, dimethoate
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3 366 degrades more rapidly (half-life 4.3 days) (Cabras et al. 1997a) thus, there is a much
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5 367 lower probability to detect residues of dimethoate in higher percentages and levels.
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10 369 Earlier pesticide monitoring data conducted in olive oil samples from the Greek
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12 370 territory are similar to the levels and frequencies reported here in. Generally, results
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14 371 from previous studies that indicated a considerable decrease in residue concentrations
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16 372 (Hiskia et al. 1998; Tsatsakis et al. 2003; Lentza-Rizos et al. 1994), was also
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18 373 confirmed by the present study.
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24 375 Surveillance work on OPs insecticides in 131 olive oil samples produced in Corfu
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26 376 (Ionian island) has shown the presence of fenthion and dimethoate with
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28 377 concentrations ranging between 0.01 – 0.83 mg kg⁻¹ and 0.016 – 0.03 mg kg⁻¹
29
30 378 respectively. The insecticides detected, in this study, were mainly fenthion, either as
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32 379 the parent molecule or as its oxidative metabolites (especially fenthion sulfoxide and
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34 380 to a lesser extent fenthion sulfone). Dimethoate was detected less frequently. The
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36 381 detected pesticide concentrations in most samples were bellowing the Maximum
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38 382 Residue Limits. The significant reductions in the use of fenthion for bate spraying and
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40 383 its replacement by spinosad, has resulted in a significant decrease in the OPs residues
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42 384 in olive oil produced in Corfu (Kantas et al. 2006).
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51 386 Contamination by fenthion and dimethoate was observed in more than forty-six virgin
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53 387 olive oil samples analyzed in Greece during 1994-1995 but always below legal limits
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55 388 (Hiskia et al. 1998). Additionally residues of fenthion and its metabolites fenthion-
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57 389 sulfoxide and fenthion sulfone with levels of 0.007–1.57 mg kg⁻¹ have been reported
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59 390 in virgin olive oil samples collected directly from olive mills at various locations on
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3 391 the Ionian island of Corfu during 1999-2002 (Botitsi et al. 2004). The analysis of
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5 392 organic and conventional olive oil samples from Crete during 1997-1999 showed the
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8 393 presence of fenthion and dimethoate with mean concentrations ranging between 0.001
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10 394 and 0.0271 mg kg⁻¹ for dimethoate and between 0.0035 and 0.1702 mg kg⁻¹ for
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12 395 fenthion (Tsatsakis et al. 2003).

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17 397 Finally, some pesticide monitoring data in olive oil produced in Italy are presented
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19 398 below for comparison purposes. In the Campanian region, it was reported that
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21 399 dimethoate was one of the most important residues of Italian olive oil as it was
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23 400 detected in 44% of the samples with a mean value of 0.061 mg kg⁻¹ and fenthion was
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25 401 detected in 27% of olive oil samples with a mean value of 0.073 mg kg⁻¹ (Rastrelli et
26
27 402 al. 2002). In addition, different OPs were also reported like diazinon, methidathion
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29 403 and parathion with mean values 0.083 mg kg⁻¹, 0.063 mg kg⁻¹ and 0.080 mg kg⁻¹
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31 404 respectively. Finally, only fenthion residues were detected in Sicilian and Apulian
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33 405 samples at concentrations ranging from 0.09 to 0.73 mg kg⁻¹ (Dugo et al. 2005).

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35 406

407 *Assessment of dietary exposure*

408 Although the residue levels of these pesticides are below the MRLs, there was no zero
409 risk because there were pesticides present in the olive oil samples. In order to take
410 account of the maximum risk of consumer's exposure in olive oil, the National
411 Theoretical Maximum Daily Intakes (NTMDIs) was calculated. Table 4 shows the
412 NTMDIs of the detected OPs according to European MRLs and the dietary intakes
413 expressed as % of ADI. The NTMDIs for the detected insecticides ranged from 2.86
414 % ADI to 16.3 % ADI for a 60-kg person and from 2.29 % ADI to 13.1 % ADI for a
415 75-kg person.

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7 417 The refinement of the intake values was performed by determining the NEDI using
8 418 the average or the 90th percentile residues found in the samples analyzed (Table 5).
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10 419 Refinement of the residue levels resulted in a significant decrease in the % of ADI
11
12 420 percentages for all the compounds. Even for the more conservative assumption, i.e.
13
14 421 90th percentile, the calculated NEDIs for all detected pesticides represent only a small
15
16 422 portion of the respective ADIs (<0.86% of ADI). The highest NEDI obtained for
17
18 423 fenthion and fenthion sulfoxide were 0.78% and 0.86% of the ADI.
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24 425 The NESTI is used to estimate acute dietary exposure. All NESTI values (Table 6) of
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26 426 the detected OPs were <7% of the respective ARfDs recommended by the
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28 427 FAO/WHO, indicating minimum acute risk caused by these pollutants. The
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30 428 cumulative dietary risk exposure to OPs of the Greek consumers is shown in Fig 4 and
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32 429 Table 7 using the HI and TEFs approaches respectively for the samples with detected
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34 430 multiresidues. As shown in Fig. 4, both cumulative chronic and acute hazard indexes
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36 431 did not exceed 12 and 8% respectively of the risk cup. It should be noted that the
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38 432 chronic hazard index was higher than the acute hazard index in all cases.
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44 434 The percentage contribution of the pesticides detected in multi-residue samples is also
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46 435 depicted in Fig. 4. Fenthion, fenthion sulfoxide and dimethoate contributes more to
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48 436 the exposure than the other pesticides. The results for the chronic cumulative
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50 437 exposure based on TEFs are shown in Table 7. The total cumulative intake in
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52 438 chlorpyrifos equivalent for an adult Greek with an average weight of 75 kg varied
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54 439 from $7.7 \cdot 10^{-5}$ to $1.0 \cdot 10^{-3}$ mg kg⁻¹ bw day⁻¹, which corresponds to 0.77–10.3% of the
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56 440 ADI. In methamidophos equivalents, the results obtained were $9.1 \cdot 10^{-7}$ – $2.0 \cdot 10^{-4}$ mg
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3 441 $\text{kg}^{-1} \text{bw day}^{-1}$ that is <0.01–0.49% of the ADI. The results from the acute exposure
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5 442 risk assessment in chlorpyrifos equivalents were also shown in Table 7. The % ARfD
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7 443 exhaustion for an adult never exceeded 1% for an adult with 60 kg of body weight.
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9 444 From the results of both approaches it can be concluded that the cumulative risk i.e.
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11 445 the acetylcholinesterase inhibiting effect from exposure to OPs through olive oil
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13 446 consumption, was assumed to be very low. Thus, the contribution of olive oil in the
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15 447 cumulative dietary intake of these pesticide residues from the total diet for the Greek
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17 448 adult consumer is expected to be low.
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450 **Conclusions**

451 The overall findings of this study demonstrated the occurrence and the dietary risk of
452 organophosphorus pesticide residues in olive oil samples produced in the years 2004-
453 2005 in Greece. The results show that the great majority of the pesticide residues
454 detected were below the MRLs established by the EU. Fenthion and dimethoate were
455 the pesticides more frequently detected while their metabolites were also frequently
456 detected. Moreover, the exposure assessment even when using the more conservative
457 assumptions (90th percentile values) for each pesticide showed that the contribution of
458 olive oil to the dietary intake represents only a small portion of the respective ADIs or
459 ARfDs. The cumulative chronic exposure assessment for an adult Greek was <13% of
460 the ADI in chlorpyrifos equivalents and 0.61% of the ADI in methamidophos
461 equivalents. In conclusion, the study showed that the dietary intake of
462 organophosphorus pesticide residues detected through olive oil consumption for the
463 Greek consumers does not pose a relevant risk.

464

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For Peer Review Only

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4 **Legends of figures**
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9 Figure 1: Distribution of OPs residues in the positive olive oil samples
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13 Figure 2: Distribution of OP residues in the analyzed olive oil samples and co-occurrence of
14 pesticide residues.
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19 Figure 3: Distribution of positive samples (%) in the major olive oil production regions of
20 Greece.
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25 Figure 4: Acute (A) and Chronic (C) Hazard Indexes (HI) of pesticide residues in olive oil
26 samples with detected pesticide multiresidues
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Table 1. Toxicity equivalence factors (TEFs) used for the calculation of cumulative dietary intake

Pesticide	Semi-chronic	Acute	Chronic	Chronic
	TEF ^a (NL)	TEF ^a (NL)	TEF ^b (USA)	TEF ^c
Omethoate	1.2	4	-	-
Dimethoate	25	0.5	0.33	0.32
Diazinon	50	5	0.024	0.01
Fenitrothion	0.2	0.02	-	0.03
Malathion	2	0.02	0.0003	0.0003
Fenthion	0.71	1.43	0.35	0.33
Methidathion	0.2	5	0.37	0.32
Ethion	0.6	2	-	1.1
Chlorpyrifos	1	1	0.1	0.05
Methamidophos	66.7	3.33	1	1

^a From Boon and van Klaveren 2000 (Boon et al. 2000)

^b From EPA 2002 (EPA 2002b)

^c From Caldas et al. 2006 (Caldas et al. 2006)

Table 2

Occurrence of pesticide residues in olive oil samples during 2004-2005

Pesticide	Minimum (mg kg⁻¹)	Maximum (mg kg⁻¹)	Average* (mg kg⁻¹)	Median* (mg kg⁻¹)	90th Percentile* (mgkg⁻¹)	No of positive samples	Detection rate (%)
Omethoate	<0.005	0.044	0.005	0.005	0.005	2	1.20
Dimethoate	<0.003	0.057	0.006	0.003	0.012	17	10.2
Malaoxon	<0.005	0.25	0.006	0.005	0.005	3	1.80
Fenthion	<0.003	0.61	0.026	0.003	0.048	18	10.8
Ethion	<0.005	0.093	0.006	0.005	0.005	6	3.59
Fenthion sulfoxide	<0.005	0.15	0.015	0.005	0.052	24	14.4
Fenthion sulfone	<0.005	0.13	0.007	0.005	0.005	3	1.80

* A default value of ½ LOD were used for samples with values below the LOD

Table 3

Distribution of positive samples in 2004-2005 pesticide residue monitoring program

Cultivation Area	Surveyed samples	Positive samples	Detection Rate (%)	No of samples with residue						No of multipesticide residues	
				Omethoate	Dimethoate	Malaoxon	Fenthion	Ethion	Fenthion sulfoxide		Fenthion sulfone
Crete	75	41	54.7	2	12	3	15	5	23	2	16
Peloponnese	48	7	14.6		4		3		1		1
Central Greece	21	1	4.8		1						0
Chalkidiki	6	1	16.7							1	0
Aegean Islands	6	0	0								0
Ionian Islands	11	1	9.1					1			0

Table 4

National Theoretical Maximum Daily Intakes (NTMDIs) of pesticide residues by adults in Greece and acceptable daily intake (ADIs)

Pesticide	NTMDI ^a	NTMDI ^a	NTMDI ^b	NTMDI ^b	ADI ^c	MRL ^d
	(mg kg ⁻¹ b.w. ⁻¹ day ⁻¹)	(% of ADI)	(mg kg ⁻¹ b.w. ⁻¹ day ⁻¹)	(% of ADI)	(mg kg ⁻¹ b.w. ⁻¹ day ⁻¹)	(mg kg ⁻¹)
Omethoate	5.7*10 ⁻⁵	2.86	4.6*10 ⁻⁵	2.29	0.002	0.05
Dimethoate	5.7*10 ⁻⁵	2.86	4.6*10 ⁻⁵	2.29	0.002	0.05
Malaoxon	- ^e	-	-	-	0.300	-
Fenthion	1.1*10 ⁻³	16.3	0.00	13.1	0.007	1.00
Ethion	- ^e	-	-	-	0.002	-
Fenthion sulfoxide	1.1*10 ⁻³	16.3	0.00	13.1	0.007	1.00
Fenthion sulfone	1.1*10 ⁻³	16.3	0.00	13.1	0.007	1.00

^a NTMDI calculated for an adult of 60 kg body weight^b NTMDI calculated for an adult of 75 kg body weight^c values established by the World Health Organization^d values established by the Codex Alimentarius Commission (Codex Alimentarius Commission, 1996)^e NTMDI was not determined since MRL in olive oil was not set for these pesticides

Table 5

National Estimated Daily Intakes (NEDIs) ($\text{mg kg}^{-1} \text{bw}^{-1} \text{day}^{-1}$ or % of ADI) of pesticide residues (average and 90th percentile) by adults in Greece.

Pesticide	NEDI ^a	NEDI ^a	NEDI ^b	NEDI ^b	NEDI ^a	NEDI ^a	NEDI ^b	NEDI ^b
	Average ($\text{mg kg}^{-1} \text{bw}^{-1} \text{day}^{-1}$)	(% of ADI)	Average ($\text{mg kg}^{-1} \text{bw}^{-1} \text{day}^{-1}$)	(% of ADI)	90 th Percentile ($\text{mg kg}^{-1} \text{bw}^{-1} \text{day}^{-1}$)	(% of ADI)	90 th Percentile ($\text{mg kg}^{-1} \text{bw}^{-1} \text{day}^{-1}$)	(% of ADI)
Omethoate	6.1×10^{-6}	0.30	4.8×10^{-6}	0.24	5.7×10^{-6}	0.29	4.6×10^{-6}	0.29
Dimethoate	7.0×10^{-6}	0.35	5.6×10^{-6}	0.28	1.3×10^{-5}	0.66	1.1×10^{-5}	0.53
Malaoxon	7.1×10^{-6}	<0.01	5.7×10^{-6}	<0.01	5.7×10^{-6}	<0.01	4.6×10^{-6}	<0.01
Fenthion	2.9×10^{-5}	0.42	2.3×10^{-5}	0.33	5.5×10^{-5}	0.78	4.4×10^{-5}	0.63
Ethion	7.0×10^{-6}	0.35	5.6×10^{-6}	0.28	5.7×10^{-6}	0.29	4.6×10^{-6}	0.23
Fenthion sulfoxide	1.8×10^{-5}	0.25	1.4×10^{-5}	0.20	6.0×10^{-5}	0.86	4.8×10^{-5}	0.68
Fenthion sulfone	7.9×10^{-6}	0.11	6.3×10^{-6}	0.09	5.7×10^{-6}	0.08	4.6×10^{-6}	0.07

^a: NEDI calculated for adult 60kg body weight

^b: NEDI calculated for adult 75kg body weight

^c: values established by the World Health Organization

Table 6

National Estimated Short-Term Intakes (NESTIs) ($\text{mg kg}^{-1}\text{bw}^{-1}\text{day}^{-1}$ or % of ARfD) of pesticide residues by adults in Greece and ARfDs

Pesticide	NESTI ^a	NESTI ^a	NESTI ^b	NESTI ^b	ARfD ^c
	($\text{mg kg}^{-1}\text{bw}^{-1}\text{day}^{-1}$)	(% of ARfD)	($\text{mg kg}^{-1}\text{bw}^{-1}\text{day}^{-1}$)	(% of ARfD)	($\text{mg kg}^{-1}\text{ b.w.}^{-1}$)
Omethoate	5.0×10^{-5}	0.25	4.0×10^{-5}	0.20	0.02
Dimethoate	6.5×10^{-5}	0.33	5.2×10^{-5}	0.26	0.02
Malaoxon	1.4×10^{-4}	0.01	1.1×10^{-4}	0.006	2.00
Fenthion	7.0×10^{-4}	7.00	5.6×10^{-4}	5.6	0.01
Ethion	1.1×10^{-4}	-	8.5×10^{-5}	-	-
Fenthion sulfoxide	1.7×10^{-4}	1.7	1.3×10^{-4}	1.3	0.01
Fenthion sulfone	1.5×10^{-4}	1.5	1.2×10^{-4}	1.2	0.01

^a: NESTI calculated for adult 60kg body weight

^b: NESTI calculated for adult 75kg body weight

^c: values established by the World Health Organization

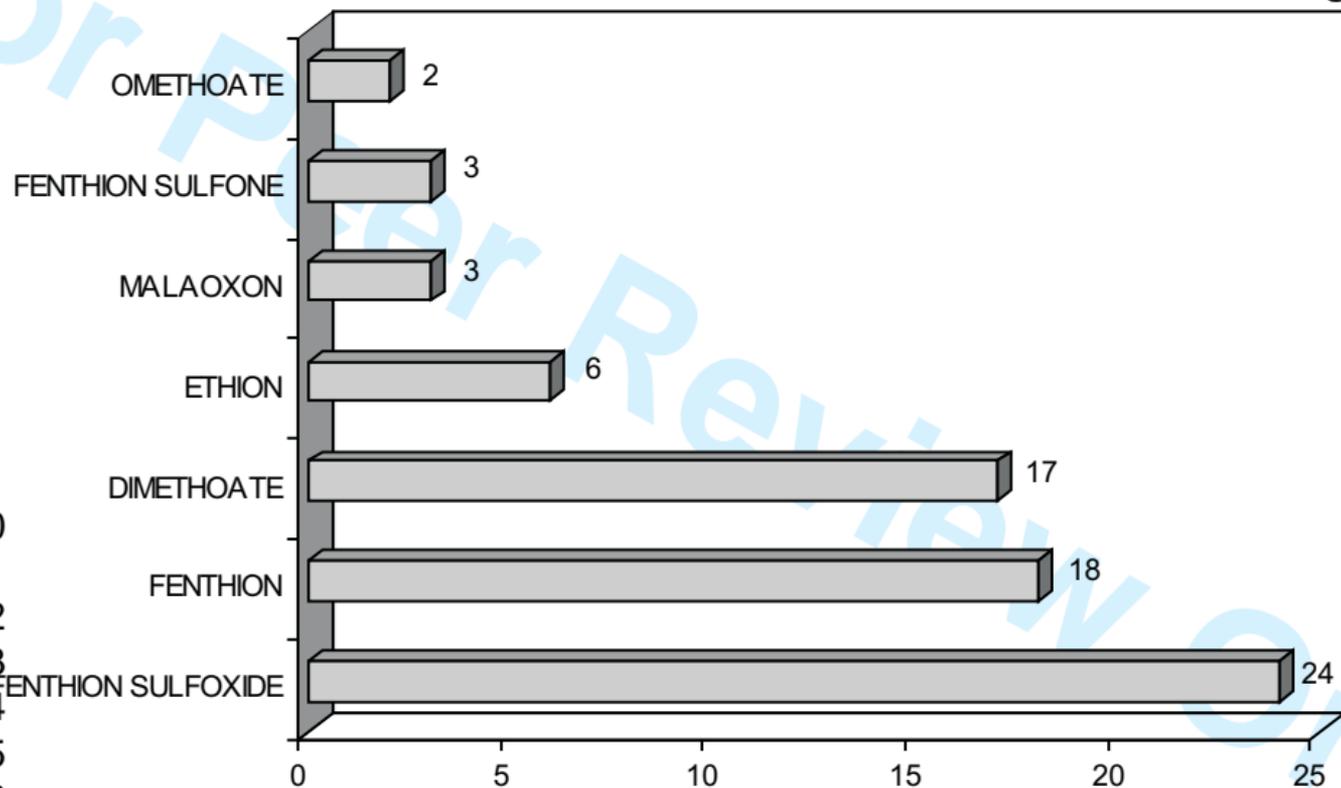
^d: values established by the Codex Alimentarius Commission (Codex Alimentarius Commission, 1996)

Table 7. Cumulative intake of organophosphorus pesticides as percentage part of ADI for the index compounds chlorpyrifos and methamidophos.

SAMPLE	Chlorpyrifos equivalents, TEF ^a , NL						Methamidophos equivalents, TEF ^b , USA					
	Semi-chronic			Acute			Chronic					
	Average intake (mg kg ⁻¹ bw ⁻¹ day ⁻¹)		% ADI	Average intake (mg kg ⁻¹ bw ⁻¹ day ⁻¹)		% ARfD	Average intake (mg kg ⁻¹ bw ⁻¹ day ⁻¹)		% ADI			
	60kg	75kg	60kg	75kg	60kg	75kg	60kg	75kg	60kg	75kg	60kg	75kg
CRE1	9.1*10 ⁻⁴	7.3*10 ⁻⁴	9.1	7.3	8.6*10 ⁻⁵	6.9*10 ⁻⁵	0.09	0.07	1.2*10 ⁻⁵	9.4*10 ⁻⁶	0.03	0.02
CRE2	9.6*10 ⁻⁴	7.7*10 ⁻⁴	9.6	7.7	2.2*10 ⁻⁴	1.7*10 ⁻⁴	0.22	0.17	1.2*10 ⁻⁵	9.5*10 ⁻⁶	0.03	0.02
CRE3	8.7*10 ⁻⁴	6.9*10 ⁻⁴	8.7	6.9	1.7*10 ⁻⁵	1.4*10 ⁻⁵	0.02	0.01	1.1*10 ⁻⁵	9.2*10 ⁻⁶	0.03	0.02
CRE4	8.6*10 ⁻⁴	6.9*10 ⁻⁵	0.86	0.69	1.7*10 ⁻⁶	1.4*10 ⁻⁶	<0.01	<0.01	1.1*10 ⁻⁶	9.1*10 ⁻⁷	<0.01	<0.01
CRE5	1.1*10 ⁻³	8.5*10 ⁻⁴	10.7	8.5	2.1*10 ⁻⁵	1.7*10 ⁻⁵	0.02	0.02	1.4*10 ⁻⁵	1.1*10 ⁻⁵	0.04	0.03
CRE6	5.0*10 ⁻⁴	4.0*10 ⁻⁴	5.0	4.0	1.0*10 ⁻³	8.0*10 ⁻⁴	1.00	0.80	2.5*10 ⁻⁴	2.0*10 ⁻⁴	0.61	0.49
CRE7	4.3*10 ⁻⁴	3.4*10 ⁻⁴	4.3	3.4	8.6*10 ⁻⁴	6.9*10 ⁻⁴	0.87	0.69	2.1*10 ⁻⁴	1.7*10 ⁻⁴	0.53	0.42
CRE8	7.4*10 ⁻⁴	5.9*10 ⁻⁴	7.4	5.9	1.0*10 ⁻⁴	8.4*10 ⁻⁵	0.11	0.08	3.2*10 ⁻⁵	2.5*10 ⁻⁵	0.08	0.06
CRE9	3.2*10 ⁻⁴	2.5*10 ⁻⁴	3.2	2.5	6.3*10 ⁻⁴	5.1*10 ⁻⁴	0.64	0.51	1.6*10 ⁻⁴	1.3*10 ⁻⁴	0.39	0.31
CRE10	1.1*10 ⁻³	8.6*10 ⁻⁴	10.7	8.6	3.3*10 ⁻⁴	2.7*10 ⁻⁴	0.33	0.27	8.9*10 ⁻⁵	7.1*10 ⁻⁵	0.22	0.18
CRE11	8.2*10 ⁻⁴	6.6*10 ⁻⁴	8.2	6.6	1.0*10 ⁻⁴	8.6*10 ⁻⁵	0.11	0.09	3.3*10 ⁻⁵	2.6*10 ⁻⁵	0.08	0.07
CRE12	3.9*10 ⁻⁴	3.2*10 ⁻⁴	3.9	3.2	7.9*10 ⁻⁴	6.4*10 ⁻⁴	0.80	0.64	1.9*10 ⁻⁴	1.6*10 ⁻⁴	0.49	0.39
CRE13	8.8*10 ⁻⁴	7.1*10 ⁻⁴	8.8	7.1	1.8*10 ⁻⁵	1.4*10 ⁻⁵	0.02	0.01	1.2*10 ⁻⁵	9.3*10 ⁻⁶	0.03	0.02
CRE14	1.3*10 ⁻³	1.0*10 ⁻³	12.9	10.3	2.6*10 ⁻⁵	2.1*10 ⁻⁵	0.03	0.02	1.7*10 ⁻⁵	1.4*10 ⁻⁵	0.04	0.03
CRE15	1.1*10 ⁻³	8.6*10 ⁻⁴	10.8	8.6	2.7*10 ⁻⁴	2.2*10 ⁻⁴	0.28	0.22	7.6*10 ⁻⁵	6.1*10 ⁻⁵	0.19	0.15
CRE16	2.6*10 ⁻⁴	2.1*10 ⁻⁴	2.6	2.1	5.2*10 ⁻⁴	4.2*10 ⁻⁴	0.52	0.42	1.3*10 ⁻⁴	1.0*10 ⁻⁴	0.32	0.26
PEL1	9.6*10 ⁻⁵	7.7*10 ⁻⁵	0.96	0.77	1.9*10 ⁻⁴	1.5*10 ⁻⁴	0.19	0.15	4.7*10 ⁻⁵	3.8*10 ⁻⁵	0.12	0.09

^a values established by Boon and van Klaveren et al 2000 (Boon et al. 2000)

^b values established by EPA 2002 (EPA 2002b)

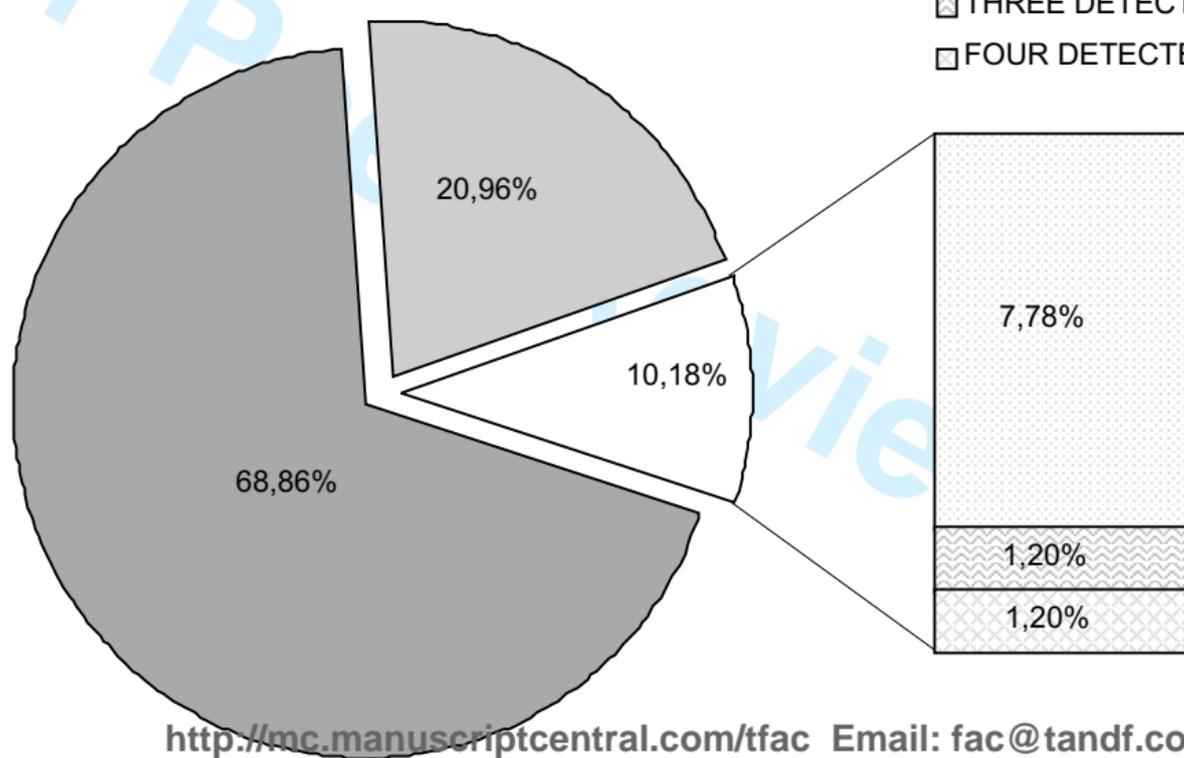
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SAMPLES at >LOD

Food Additives and Contaminants

- NON DETECTED
- ONE DETECTED
- TWO DETECTED
- THREE DETECTED
- FOUR DETECTED



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CENTRAL GREECE

PELOPONNESE

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