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Elsevier Editorial System(tm) for Food and Chemical Toxicology
Manuscript Draft

Manuscript Number: FCT-D-13-00489R1

Title: The E-screen test and the MELN gene-reporter assay used for determination of estrogenic activity in fruits and vegetables in relation to pesticide residues.

Article Type: Full Length Article

Keywords: Estrogenic activity; E-screen; MELN; Vegetables; Pesticides; in vitro test.

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Order of Authors: Tiziana Schilirò; Arianna Porfido; Annalisa Longo; Sara Coluccia; Giorgio Gilli

Abstract: Endocrine-disrupting chemicals (EDCs) may lead to adverse systemic effects by interfering with normal hormone homeostasis, and diet is considered to be among the main routes of EDCs exposure. The present study investigated the total estrogenic activity of fruits and vegetables by calculating the 17-b-estradiol equivalent quantity (EEQ) using two in vitro tests: the human breast cancer cell line (MCF-7 BUS) proliferation assay (E-screen test) and the luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay.
Of the 24 analyzed fruits and vegetables, 14 contained from 1 to 4 pesticide residues in concentrations ranging from 0.02 to 1.19 ppm, whereas the other 10 did not contain any pesticide residues. The EEQ values for all positive samples ranged from 0.010 to 0.616 μg/100 g for the above in vitro tests. Our study demonstrates that estrogenic activity was present in fruits and vegetables and that the concentration of allowable pesticide residues and EEQ values were positively correlated; however, no correlation was found by comparing the estrogenic activity and the intrinsic content of phytoestrogens obtained from the available literature. A theoretical adult dietary intake of 0.7-0.9 ng EEQ/L/day from fruits and vegetables was calculated.

Response to Reviewers: Dear Editor,

please find enclosed the revised version of the manuscript entitled: " The E-screen test and the MELN gene-reporter assay used for determination of estrogenic activity in fruits and vegetables in relation to pesticide residues", hoping that it can now be accepted for publication on Food and Chemical Toxicology.

We have changed and clarified what the reviewers have recommended and the English language has been revised for grammatical errors by an AJE English expert (certificate in supplemental material section).

The notes detailing the changes to the paper and our replies to the reviewers' comments are also enclosed. We also attach a further copy of the manuscript where all changes are marked in red.

Best regards

Tiziana Schilirò

Response to Reviewer #1 comments:

Thank you for the suggested amendments. All the grammatical and stylistic minor corrections were solved over the text in the enclosed manuscript. All specific corrections are answered and listed in the original reviewer text below.

We have deleted the sentence in the Introduction and it is present at page 4, paragraph 2.3.
We have corrected at page 3, paragraph 2.1.
We have wrote ib full ARPA at page 3, paragraph 2.1
We have decided to not move the paragraph because "2.3 Preparation of vegetable samples for in vitro tests" is more understandable after "2.2 Detection of pesticide residues" in which it is described the homogenization of vegetables that is the first step of procedure.
We have re-wrote the equations at page 7, paragraph 2.5
We have replaced big L throughout the test.
We have decided to not change ppm for consistency with the previous published study and because they are results by A.R.P.A.
Ok we have replaced exponent (E) throughout the test.
Ok we have corrected at page 13, paragraph 3.3.2.

Response to Reviewer #2 comments:

Thank you for all the comments. The English language has been revised for grammatical errors all over the text by an AJE English expert. All specific corrections are answered and listed in the original reviewer text below.

Specific comments;
Ok we agreed and we have changed the title of the manuscript.

Introduction:
The Introduction section has been largely rewritten also considering the references as you pointed out in the end of your comments.

We have corrected the sentences at page 11, paragraph 3.2.2 and at page 13, paragraph 3.3.2.

Material and Methods
Thank you for your suggestion, for consistency we have used Tamoxifen as in previous works and as reported in literature; in future we will experience even its active metabolite, hydroxytamoxifen, which actually has a higher affinity than tamoxifen for binding to estrogen receptors.

Ok we have corrected the dilutions both at page 6, paragraph 2.5 and page 8, paragraph 2.6.

Results
Ok we have improved and corrected the sentence at page 10, paragraph 3.1 about pesticides residues.

Thank you for your comment. As you can see in Table 2, the twelve EEQ E-screen values are very variable being very different the samples themselves; moreover the last two values are outliers but they could not be omitted, otherwise the number of samples would be too small.

Throughout the manuscript was revised also considering the bibliography that has been reduced by eliminating the less closely related.

Response to Reviewer #3 comments:

Thank you for all the comments. All specific corrections are answered and listed in the original reviewer text below.

Thank you for your suggestion we have changed the title of the manuscript (also suggested by another reviewer).

Section 4 “Discussion and Conclusion has been improved, the English language has been revised for grammatical errors all over the text by an AJE English expert.

The vegetables were tested for pesticides listed in the Regulation no. 396/2005 of the European Parliament, see also Schilirò et al. 2011. The Regional Environmental Protection Agency (Piedmont A.R.P.A., Agenzia Regionale per la Protezione dell'Ambiente del Piemonte) collects commercial vegetal products destined for human consumption in order to perform analyses as part of the regular national monitoring programme for pesticide residues in foods.

Ok we have improved Table 1, we have included vegetables in which no pesticide residues were found and we have specified which samples were from organic agriculture.

The vegetables were tested for pesticides listed in the Regulation no. 396/2005 of the European Parliament. The list is too long to be quoted in the text or table (more than 100 residues of pesticides were routinely tested); however you can find the pesticide list in the supplemental material.

Thank you for your suggestion we have improved figure legends.

Thank you for your suggestion we delete figure 1 (positive controls) and added a new figure 1 in which is reported the proliferative effects of estrogenic vegetables for the two in vitro tests.

Ok we have corrected and improved the Astract.

All the grammatical and stylistic minor corrections were solved all over the text. The English language has been revised for grammatical errors all over the text by an AJE English expert.

Ok we have deleted the sample codes throughout the manuscript.

Ms. Ref. No.: FCT-D-13-00489

Dear Editor,

please find enclosed the revised version of the manuscript entitled: " **The *E-screen* test and the MELN gene-reporter assay used for determination of estrogenic activity in fruits and vegetables in relation to pesticide residues**", hoping that it can now be accepted for publication on ***Food and Chemical Toxicology***.

We have changed and clarified what the reviewers have recommended and the English language has been revised for grammatical errors by an AJE English expert (certificate in supplemental material section).

The notes detailing the changes to the paper and our replies to the reviewers' comments are also enclosed. We also attach a further copy of the manuscript where all changes are marked in red.

Best regards
Tiziana Schilirò

Response to Reviewer comments

Reviewer #1:

Thank you for the suggested amendments. All the grammatical and stylistic minor corrections were solved over the text in the enclosed manuscript. All specific corrections are answered and listed in the original reviewer text below.

Authors determined the estrogenic effects of pesticide residues in 24 different vegetables using two kinds of cancer cell lines. Authors have done lots of work and the data worth publishing in the journal. I recommend for the acceptance of the manuscript subject to a few (and minor) amendments below:
Grammar in some portions of the manuscript needs revision: page 1 line 5 -12; page 3 line 27; page 3 line 27; page 3 line 41; page 7 line 19-22.
Page 3 line 53-58: move to "Methods"
Ok we have deleted the sentence in the Introduction and it is present at page 4, paragraph 2.3.
Page 4 line 17: correct ".24 vegetables (17 fruits and 1 vegetables") –
Ok corrected at page 3, paragraph 2.1.
Page 4 line 19: ARPA: Write in full
Ok done at page 3, paragraph 2.1
Page 4 line 29: 2.2 "Detection of pesticide residues" must come only after 2.3
We have decided to not move the paragraph because "*2.3 Preparation of vegetable samples for in vitro tests"* is more understandable after "*2.2 Detection of pesticide residues*" in which it is described the homogenization of vegetables that is the first step of procedure.
Page 7: Re-write the equations and number them appropriately
Ok we have re-write the equations at page 7, paragraph 2.5
Page 8 line 34: µL (big L for litres). Page 9 line 4, line 53
Ok have replaced big L throughout the test.
Page 10 line 32: change ppm to the appropriate concentration unit
We have decided to not change ppm for consistency with the previous published study and because they are results by A.R.P.A.

Page 11 line 32: the use of exponent (E) for is confusing. Use x10 to the power. Page 12 and elsewhere.
Ok we have replaced exponent (E) throughout the test.
Page 13 line 19-25: This short sentence can't make a paragraph"
Ok we have corrected at page 13, paragraph 3.3.2.

Reviewer #2:

General comments:
The manuscript needs general English correction. The present style of language makes the potential reader unable to follow information contained in the paper. Although the essence of the manuscript may be useful for the readers, the English causes that the manuscript can not be accepted in the present form. As I am not a native speaker, I avoid to give any specific suggestion how to improve the language quality.

Thank you for all the comments. The English language has been revised for grammatical errors all over the text by an AJE English expert (certificate in supplemental material section). All specific corrections are answered and listed in the original reviewer text below.

Specific comments;
I propose change the title for "E-screen test and the MELN gene-reporter Luciferase assay used for determination of estrogenic activity in vegetables"
Ok we agreed and we have changed the title of the manuscript.

Introduction:
Introduction part is not sufficient to demonstrate the issues or hypothesis the authors want to investigate. Re-construction of "introduction" is recommended. Based on the available data from the manuscript, more targeted purpose is appropriate and make this manuscript easily accepted. Too much unnecessary information not related to the purpose of publication.
The Introduction section has been largely rewritten also considering the references as you pointed out in the end of your comments.
Page 11 Line 47 "In order to confirm that the cell proliferation induced by estrogenic activity was ER-mediated, we observed that co-incubation with Tam led to inhibition of the proliferative response (36 ? 15 %) while co-incubation with E2 led to a greater proliferative response (103 ? 29 %) in all cases; in both cases, the difference was statistically significant (t-test, $p < 0.05$)" - this is an example of one of many sentences confirming the the necessity of total rewording the manuscript.
Ok we have corrected the sentences at page 11, paragraph 3.2.2 and at page 13, paragraph 3.3.2.

Material and Methods
Page 7- line 10- not Tamoxifen but hydroxytamoxifen should be used.
Thank you for your suggestion, for consistency we have used Tamoxifen as in previous works and as reported in literature; in future we will experience even its active metabolite, hydroxytamoxifen, which actually has a higher affinity than tamoxifen for binding to estrogen receptors..
Line 12- please clarify sentence "five dilutions between 0.01 mM and 1 nM"- 0.01 mM (10 µM is a higher concentration than 10 nM
Line 15- the same - "five dilutions between 0.1 mM and 10 nM"
Ok we have corrected both at page 6, paragraph 2.5 and page 8, paragraph 2.6.

Results

Page 10 line 29- " Among the 24 vegetables analysed, 14 contained from 1 to 4 residues per sample, with concentrations ranging between 0.02 and 1.19 ppm. Two of these were samples from organic agriculture. The other 10 did not contain any pesticide residues. Table 1 shows the positive samples among those tested in this study, that contained residues above the detection limit (EC, 396/2005). Positive samples contained 12 different types of residues.
Underlined in red sentences seem to contradictory-
Ok we have improved and corrected the sentence at page 10, paragraph 3.1.
Page 11- line 39- "The average value of EEQ for all positive samples was 0.148 ± 0.205 <mu>g/100" I am not sure whether can be take into account the result when standard deviation is 2 times greater than the mean.
Thank you for your comment. As you can see in Table 2, the twelve EEQ *E-screen* values are very variable being very different the samples themselves; moreover the last two values are outliers but they could not be omitted, otherwise the number of samples would be too small.
69 cited references- indicates inability to the selection of that are closely related to the subject of studied. Has already pointed at the beginning of review.
Throughout the manuscript was revised also considering the bibliography that has been reduced by eliminating the less closely related.

Reviewer #3:

Highlights:
1. Name the 2 in vitro assays used.
2. good.
3. I find this to be the main conclusion of the paper. And I suggest you stress this throughout
4. Give the number for the theoretical dietary intake.

Graphical Abstract: good

Overall it is a well written and informative paper. Some general comments.

Thank you for all the comments. All specific corrections are answered and listed in the original reviewer text below.

The title seems not to tell the primary result, that estrogenic activity is correlated to pesticide concentration. Or is the primary result that MCF and Luciferase assays give comparable results? The most important conclusion should be decided and the title should reflect this.
Thank you for your suggestion we have changed the title of the manuscript (also suggested by another reviewer).

Section 4, Discussion and Conclusion, needs to be thoroughly corrected by a mother tongue English speaker. The rest of the paper has few and minor grammatical and/or word choice errors (some are corrected later in this review), but Section 4 has too many to correct here. After language correction, the content can be reviewed, not before.
Section 4 "Discussion and Conclusion has been improved, the English language has been revised for grammatical errors all over the text by an AJE English expert (certificate in supplemental material section).

It is not clear which pesticides were tested. Pg 4, lines 51 +, is the only mention. Since we can only find pesticides we test for, it is important to specify which pesticides were tested for and WHY those pesticides were chosen.

The vegetables were tested for pesticides listed in the Regulation no. 396/2005 of the European Parliament, see also Schilirò et al. 2011. The Regional Environmental Protection Agency (Piedmont A.R.P.A., Agenzia Regionale per la Protezione dell'Ambiente del Piemonte) collects commercial vegetal products destined for human consumption in order to perform analyses as part of the regular national monitoring programme for pesticide residues in foods.

Table 1 should present all fruits and vegetables tested, including those in which no pesticide residues were found. It is also of interest to know which samples were from organic agriculture.
Ok we have improved Table 1, we have included vegetables in which no pesticide residues were found and we have specified which samples were from organic agriculture.

Additionally, the table should indicate which pesticides were tested for not only which were found.
The vegetables were tested for pesticides listed in the Regulation no. 396/2005 of the European Parliament. The list is too long to be quoted in the text or table (more than 100 residues of pesticides were routinely tested); however you can find the pesticide list in the supplemental material.

Figures should indicate statistical significance in the figure and/or the figure legend. Figure legends overall are too brief and don't allow an understanding of the primary point of the figure without referring to the text.
Thank you for your suggestion we have improved figure legends.

Figure 1 shows only positive controls. This does not warrant a figure. Instead show the samples E2 compared to controls as that is the point of the paper - that fruits and vegetables have estrogenic activity which is correlated with their pesticide content. Pg 11, line 24 on and Pg 13 line 1 on is the data I'd like to see as a graph. ok
Thank you for your suggestion we delete figure 1 (positive controls) and added a new figure 1 in which is reported the proliferative effects of estrogenic vegetables for the two *in vitro* tests.

More Specific comments:
The abstract should be a single paragraph. No "-" in the abstract. The EEQ wasn't measured, it was calculated. State what the 2 assays measured. The abstract conclusion is weak. Don't say what can't be concluded, say what can be concluded - not only that estrogens are in vegetables but that they come from pesticides and not from phytoestrogens
Ok we have corrected and improved the Astract.

Pg 3 line 24, that should be which
Pg 3, lines 22 - 27. The sentence is too long and should be made into 2
Pg4, line 17. Tell us what the fruits and vegetables were or refer to the figure here.
Pg 8, line 36. Vegetable not vegetables. Line 41, concentrations not concentration
There are other small grammatical mistakes as above, the entire paper should be checked by a native English speaker.
All the grammatical and stylistic minor corrections were solved all over the text. The English language has been revised for grammatical errors all over the text by an AJE English expert.

Results, Tables:
Not necessary to tell the Sample Codes, they are your codes. For the reader it confuses the text and the figures.
Ok we have deleted the sample codes throughout the manuscript.

*Conflict of Interest Statement

ELSEVIER

Food and Chemical Toxicology

Conflict of Interest Policy

Supplement:
Article Title: *Estrogenic activity in vegetables by means of the E-screen test and the MELN gene-reporter luciferase assay*

Author name: Tiziana Schilirò, Arianna Porfido, Annalisa Longo, Sara Coluccia, Giorgio Gilli.

Declarations

Food and Chemical Toxicology requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest
A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

No conflict of interest

Funding Source
All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

Please state any sources of funding for your research

This study was financed by a Research Projects of National Interest grant (PRIN, 2008) and by a Piedmont Regional grant (Ricerca Sanitaria Finalizzata, 2008).

Signature (a scanned signature is acceptable, but each author must sign)

Print name

Tiziana Schilirò,
Arianna Porfido,
Annalisa Longo,
Sara Coluccia,
Giorgio Gilli.

The *E-screen* test and the MELN gene-reporter assay used for determination of estrogenic activity in fruits and vegetables in relation to pesticide residues.

Schilirò Tiziana[1*], Porfido Arianna[1], Longo Annalisa[2], Coluccia Sara[2], Gilli Giorgio[1]

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[2]*Regional Environmental Protection Agency (Piedmont A.R.P.A., SS0204 Polo Chimico Regionale Alimenti), La Loggia (Torino), Italy*.

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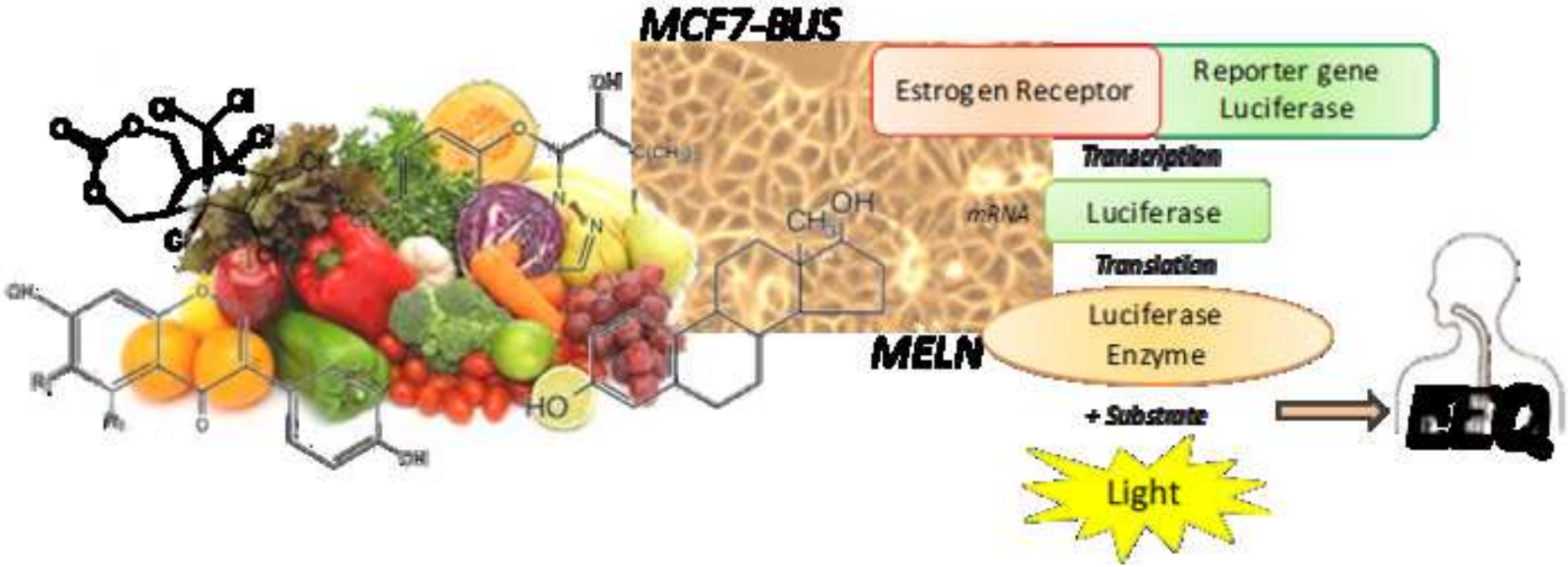
Abbreviations: COU, coumestrol; DMEM, Dulbecco's modified eagle medium; E2, 17b-estradiol; EC50, effective concentration 50; EDCs, endocrine disruptor compounds; EEF, estradiol equivalency factor; EEQ, estradiol equivalency quantity; EFSA, European food safety authority; EU, European; FCS, fetal calf serum; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography ISO, isoflavones; LIG, lignans; MRLs, maximum residue levels; PE, proliferative effect; RPE, relative proliferative effect; rS, spearman rank correlation; SPE, solid phase extraction; Tam, tamoxifen; TRANS, increased rate of luciferase gene expression; WHO, world health organization.

Highlights

1. The two *in vitro* tests are suited for estrogenic activity assessment in extracts from whole vegetables.
2. Estrogenic activity of soluble compounds is present in vegetables.
3. The estrogenic activity of vegetables and the total concentration of pesticide residues are positively correlated.
4. A theoretical dietary intake of 17b-estradiol equivalent quantity from vegetables for adults was calculated.

Graphical Abstracts (for review)



Abstract

Endocrine-disrupting chemicals (EDCs) may lead to adverse systemic effects by interfering with normal hormone homeostasis, and diet is considered to be among the main routes of EDCs exposure. The present study investigated the total estrogenic activity of fruits and vegetables by calculating the 17-β-estradiol equivalent quantity (EEQ) using two *in vitro* tests: the human breast cancer cell line (MCF-7 BUS) proliferation assay (*E-screen* test) and the luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay.

Of the 24 analyzed fruits and vegetables, 14 contained from 1 to 4 pesticide residues in concentrations ranging from 0.02 to 1.19 ppm, whereas the other 10 did not contain any pesticide residues. The EEQ values for all positive samples ranged from 0.010 to 0.616 µg/100 g for the above *in vitro* tests. Our study demonstrates that estrogenic activity was present in fruits and vegetables and that the concentration of allowable pesticide residues and EEQ values were positively correlated; however, no correlation was found by comparing the estrogenic activity and the intrinsic content of phytoestrogens obtained from the available literature. A theoretical adult dietary intake of 0.7-0.9 ng EEQ/L/day from fruits and vegetables was calculated.

The *E-screen* test and the MELN gene-reporter assay used for determination of estrogenic activity in fruits and vegetables in relation to pesticide residues.

Schilirò Tiziana[1*], Porfido Arianna[1], Longo Annalisa[2], Coluccia Sara[2], Gilli Giorgio[1]

[1]*Department of Public Health and Pediatrics, Piazza Polonia, 94 - 10126, Torino, Italy.*

[2]*Regional Environmental Protection Agency (Piedmont A.R.P.A., SS0204 Polo Chimico Regionale Alimenti), La Loggia (Torino), Italy*.

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Abbreviations: COU, coumestrol; DMEM, Dulbecco's modified eagle medium; E2, 17b-estradiol; EC50, effective concentration 50; EDCs, endocrine disruptor compounds; EEF, estradiol equivalency factor; EEQ, estradiol equivalency quantity; EFSA, European food safety authority; EU, European; FCS, fetal calf serum; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography ISO, isoflavones; LIG, lignans; MRLs, maximum residue levels; PE, proliferative effect; RPE, relative proliferative effect; rS, spearman rank correlation; SPE, solid phase extraction; Tam, tamoxifen; TRANS, increased rate of luciferase gene expression; WHO, world health organization.

Abstract

Endocrine-disrupting chemicals (EDCs) may lead to adverse systemic effects by interfering with normal hormone homeostasis, and diet is considered to be among the main routes of EDCs exposure. The present study investigated the total estrogenic activity of fruits and vegetables by calculating the 17-β-estradiol equivalent quantity (EEQ) using two *in vitro* tests: the human breast cancer cell line (MCF-7 BUS) proliferation assay (*E-screen* test) and the luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay.

Of the 24 analyzed fruits and vegetables, 14 contained from 1 to 4 pesticide residues in concentrations ranging from 0.02 to 1.19 ppm, whereas the other 10 did not contain any pesticide residues. The EEQ values for all positive samples ranged from 0.010 to 0.616 µg/100 g for the above *in vitro* tests. Our study demonstrates that estrogenic activity was present in fruits and vegetables and that the concentration of allowable pesticide residues and EEQ values were positively correlated; however, no correlation was found by comparing the estrogenic activity and the intrinsic content of phytoestrogens obtained from the available literature. A theoretical adult dietary intake of 0.7-0.9 ng EEQ/L/day from fruits and vegetables was calculated.

1. Introduction

The study of the chemical disruption of the endocrine system has been an active area of research during the last decade and has captured the attention of governments, policy makers, and media (De Rosa et al., 1998; Choi et al., 2005). Endocrine-disrupting compounds (EDCs) are defined as "exogenous substances or mixture that alters the function of the endocrine system and generate noxious effects on the health of a safe body, its descendants, or its sub-population" (WHO, 2002); at the European Union level, EDCs are included in the list of so-called emerging contaminants (EU, 2001). In terms of adverse health effects, there is concern that substances with endocrine-disrupting properties may be causally involved in a number of diseases or conditions, such as hormone-dependent cancer, reproductive disorders, a decline in fertility, or obesity (Diamanti-Kandarakis et al., 2009; Shaw, 2009; Hotchkiss et al., 2008). However, causality between EDCs and effects on human health remains controversially debated in both science and the public (Wagner and Oehlmann, 2009).

EDCs are ubiquitous in the environment because of their very frequent use in residential, industrial, and agricultural applications; in particular, the origin and fate of these contaminants can lead to their transmission in the food chain (Schwartz, 2001). It is widely accepted that food and diet are among the most important exposure routes for EDCs. Therefore, a normal human diet results in exposure to a complex mixture of xenoestrogens that enter systemic circulation in the body. There are many types of EDCs in food, ranging from natural compounds (e.g., hormones, phytoestrogens, and mycotoxins) to synthetic compounds (e.g., pesticides, pharmaceuticals, and industrial or process chemicals). The natural contribution of phytoestrogens includes some isoflavonoids, flavonoids, stilbenes, and lignans; however, their role in endocrine disruption remains highly controversial (Patisaul et al., 2010). Indeed, the lack of consistency in epidemiological and experimental results places these chemicals in EDC category III, a category that includes compounds for which *in vitro*

data exist but for which data from experimental animals concerning adverse effects on endocrine homeostasis are weak or lacking (Foster and Agzarian, 2008). Among synthetic endocrine compounds some pesticides regularly used in agriculture have shown weak estrogenic responses *in vitro*, for example, tolclofos-methyl (Andersen et al., 2002) and triadimenol (Vinggaard., 1999). Imazalil showed weak anti-estrogenic activity in an *in vitro* gene-reporter assay (Kojima et al., 2005) and a negligible proliferation response in an MCF7 cell proliferation assay (Soto et al., 1994). Endosulfan also showed an estrogenic response in several *in vitro* tests (Soto et al., 1994; Andersen et al., 2002). New pesticide regulations were recently introduced by the European Parliament and contain, for the first time, specific reference to endocrine-disrupting properties (on 21 October 2009, regulation (EC) No 1107/2009 replaced Council Directive 91/414/EEC). Although it is clear that substances with endocrine-disrupting properties should be avoided, there is no clear consensus of how to identify and evaluate endocrine-disrupting properties, and no guidance is yet provided in the new European Regulation (Flynn, 2011).

The assessment of EDC food contaminants is a continual challenge and has traditionally been performed mainly through analytical chemistry with respect to the detection of a few, specific chemicals (De Brabander et al., 2009). However, despite rapid improvements in analytical chemistry, merely evaluating single chemicals ignores the potential mixture effects between different compounds and the effects caused by as-yet-unidentified compounds (Connolly et al., 2011; US EPA, 2012). Another concern is that, although some of these EDCs have been deemed to be relatively safe at low individual levels of consumption, they may combine with other low-level EDCs to create low-level cocktail or mixture effects (Kjaerstad et al., 2010; Kortenkamp, 2007; Payne, 2010). Considering these points, the most appropriate way of detecting and studying the effects of EDCs and their mixtures may be through the use of bioassay systems that utilize the natural ligands and pathways. This can be achieved through,

preferably, *in vitro* bioassays, which, although they cannot assess behavioral effects, have the benefit of closely related natural systems without the use of animal testing. Such bioassays can detect compounds based on their effects, enable the detection of the effects caused by currently unidentified compounds, and integrate the effect of complex chemical mixtures (Wagner et al., 2011). Moreover, quantification of estrogenic activity as the 17-β-estradiol equivalent quantity (EEQ) facilitates the estimation of the total dietary intake of estrogenicity. Although such assessments provide valuable information on the human exposure to estrogen-like compounds, they are rare in the literature (Safe 1995; Shaw and McCully, 2002; Behr et al., 2011; Schilirò et al., 2011).

The aim of the present study is to evaluate the estrogenic properties of fruits and vegetables by calculating the EEQ using two *in vitro* tests: the human breast cancer cell line (MCF-7 BUS) proliferation test or *E-screen* test (Soto et al, 1995) and the luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay (Balaguer et al., 1999). A further aim of this study is to compare the estrogenic activity of food samples to the pesticide residue content and the reported intrinsic content of phytoestrogens, as found in the literature. The results of the two *in vitro* tests were compared to each other. Finally, we combined the results to assess the theoretical blood EEQ levels for adults.

2. Materials and Methods

2.1 Fruit and vegetable samples

We analyzed 24 fruits and 7 vegetables (Table 1) supplied by the Regional Environmental Protection Agency (Piedmont A.R.P.A., *Agenzia Regionale per la Protezione dell'Ambiente del Piemonte*) between April 2010 and March 2011. This agency collects commercial plant products destined for human consumption for analyses as part of the regular national monitoring program for pesticide residues in food.

2.2 Detection of pesticide residues

All the fruit and vegetable samples (500 g each) were first homogenized using an Ultra-Turrax according to the provision of Italian Ministerial Decree 27/08/2004 and Regulation n° 396/2005 of the European Parliament. All the procedures for the analysis of pesticide residues in food samples were conducted according to the quality control procedures of the European Commission for pesticide residue analysis in food and feed (EC, 2009) and using the same methods as Schilirò et al. 2011. The determination of N-methylcarbamates was performed using reverse-phase high-pressure liquid chromatography (HPLC) with post-column reaction and fluorescence detection (SCL-10AVP, Shimadzu Corp, Japan) (EC, 2009; Branca and Longo, 2002). Organophosphorus, organochlorine, pyrethroids, triazine herbicides, and other classes of pesticides were determined by Gas Chromatography with Mass Spectrometry (GC-MS) equipped with selective detectors, a quadrupole ion trap, and ITQ Series GC-Ion Trap MS (Thermo Scientific, Ohio, USA) (EC, 2009; Branca and Sacchero, 1997).

2.3 Preparation of samples for in vitro *tests*

After homogenization, the raw fruits and vegetables were subjected to nonspecific extraction to obtain whole-food extracts. The extraction of food samples was performed according to the method proposed by Charles and colleagues (Charles et al., 2002) and modified for this application (Schilirò et al. 2011): 25 g of homogenized sample was added to 25 mL of incomplete cell culture medium, (phenol-red-free Dulbecco's modified Eagle's medium, DMEM) in brown glass beakers protected from direct light. The sample was incubated overnight while being agitated at 4°C. The sample was then centrifuged at 9000 x g for 15 minutes, and the supernatant was collected in 50-mL brown glass tubes to obtain a 1 g/mL food extract. Whole-food preparations were processed ahead of time, frozen, and stored at -20°C. Prior to testing by the *E-screen* assay and the MELN gene-reporter assay, the samples were first thawed at 4°C overnight, kept at room temperature, filter-sterilized using a 0.22-µm

filter, and then diluted in steroid-free experimental DMEM at five dilutions (from 0.001 to 10 mg/mL).

2.4 Cell lines, culture conditions, and chemicals

Estrogen-sensitive human MCF-7 BUS breast cancer cells were kindly provided by Drs. A.M. Soto and Dr. C. Sonnenschein (Tufts University School of Medicine, Boston, Massachusetts, USA) and were cultivated in Dulbecco's modified Eagle's medium (DMEM) with 15 mg/L phenol red, 10% fetal calf serum (FCS), 2% L-glutamine 200 mM, 2% HEPES buffer 1 M, 1% sodium pyruvate 100 mM and 1% penicillin/streptomycin 10 mg/mL at 37°C in an atmosphere of 5% carbon dioxide and 95% air under saturating humidity.

MELN cells, provided by Dr. P. Balaguer (INSERM, Montpellier - France), are MCF-7 cells stably transfected with an estrogen-responsive gene (ERE-βGlob-Luc-SVNeo) carried by integrated plasmids. These plasmids contain both an antibiotic resistance selection gene (SVNeo) and the estrogen-responsive elements to which the estrogen receptor-ligand complex can bind, thereby inducing the transcription of the luciferase reporter gene (Berckmans et al., 2007). Therefore, the luciferase activity measured is proportional to the concentration of estrogenic compounds (Hernandez-Raquet et al., 2007). MELN cells were cultured in Dulbecco's Modified Eagle Medium Nutrient Mixture F12 Ham (DMEM-F12) with phenol red supplemented with 5% fetal calf serum (FCS), 2% L-glutamine 200 mM, 1% penicillin/streptomycin, and 1 mg/ml G418 sulfate. The cell line was maintained in an incubator at 37°C, a relative humidity of 95%, and a CO_2 concentration of 5%. The cells were subcultured once a week, with medium refreshment between subculturing steps. For the experiments, we used cells from passage number 4 to passage number 15. The cells were regularly examined for mycoplasm infection to guarantee experimental work with mycoplasm-free cells and to comply with good cell culture practice (GCCP) guidelines.

A stock solution of 10 mM 17-β-estradiol (E2) was prepared with ethanol. The stock solutions of endosulfan and triadimenol were prepared with ethanol, and a stock solution of 10 mM daidzein was prepared with DMSO. All the stock solutions were stored in brown glass tubes at -20°C and then diluted to the desired concentration with steroid-free experimental medium. Unless otherwise specified, all the chemicals and materials for cell culture were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

2.5 E-screen *test*

The *E-screen* test was performed according to the method of Körner (Körner et al., 1999) and modified by Schilirò (Schilirò et al., 2009). Briefly, subconfluent MCF-7 BUS cells were trypsinized and resuspended in the steroid-free experimental medium, consisting of phenol red-free DMEM supplemented with 5% stripped-FCS, 2% L-glutamine 200 mM, 2% HEPES buffer 1 M, 1% sodium pyruvate 100 mM, and 1% penicillin/streptomycin 10 mg/mL. The cells were seeded into 24-well plates at a density of 40000 cells/well. After 24 hours, the medium was replaced with the experimental medium containing one of five different dilutions of the food extracts. Each dilution was tested in six replicates per assay. Six wells without hormones comprised the negative control. The endogenous estrogen 17-β-estradiol (E2), in five concentrations between 1 pM and 10 nM, was used as a positive control. One dilution of each food sample found to induce a significant proliferative effect was tested together with 5 nM anti-estrogen tamoxifen (Tam) and 0.1 nM E2. Other controls tested included the following: the phytoestrogen daidzein (five dilutions between 1 nM and 10 µM) and the pesticides endosulfan and triadimenol, both individually and in combination (five dilutions between 10 nM and 0.1 mM). The assays were stopped after six days by crystal violet staining, and the absorbance (595 nm) in each well was determined.

The proliferative effect (PE) of a sample is the ratio between the highest cell number achieved with the sample or E2 and the cell number of the negative control:

(1) PE = [(max cell number)sample/(cell number)negative control]

The estrogenic activity of a sample is evaluated by determining the relative efficacy, called the relative PE (RPE%). RPE compares the maximum proliferation induced by a sample with that induced by E2:

(2) RPE % = [(PE-1)sample/(PE-1)E2] x 100.

Full agonistic activity, RPE ≥ 100%, can be distinguished from partial agonistic activity when RPE is less than 100% (18).

Relative potency, called the estradiol equivalency quantity or factor (EEQ or EEF) is thus calculated as follows:

(3) EEQ = [(EC50)E2/(EC50)sample]

(4) EEF = [(EC50)E2/(EC50)compound or positive control]

The EC50 value for the *E-screen* test (the concentration at which 50% of PE is achieved) was calculated using a Probit regression (SPSS, Chicago, IL). The PE and EC50 values of each sample were calculated from the mean dose-response curves established for each experiment. EEQ, expressed in ng/L, is the total concentration of estrogenic-active compounds in an environmental sample normalized to the natural estrogen E2.

2.6 MELN gene-reporter assay

The MELN gene-reporter assay has been widely used for the detection of estrogenic activity in complex environmental samples (Fenet et al., 2003; Cargouet et al., 2007; Jugan et al., 2009; Cambalbert et al., 2012). In the present study, the test was performed according to the method of Balaguer (Balaguer et al., 1999).

Due to the phenol red and FCS estrogenic activity, the *in vitro* experiments were performed in DMEM F12 without phenol red and supplemented with 5% dextran-coated charcoal-treated

fetal calf serum (DCC-FCS), 2% L-glutamine 200 mM, and 1% antibiotics (penicillin/streptomycin). To adapt the cells to DCC-FCS, the growth medium was replaced with fresh test medium three days prior to the experiment. The cells were then harvested and seeded in 96-well plates with a flat, clear bottom (Corning) at a density of 40000 cells/well in 100 µL of DCC-FCS per well. After 24 hours, the test medium was removed, and 100 µL of each sample concentration was added to three replica wells. The cells were treated with the samples for 16 h. A negative control, without hormones, and a positive control, E2 (between 1 pM and 10 nM), were included in each assay. One dilution of each food sample found to induce a degree of significant luciferase activity was tested together with 5 µM of the anti-estrogen tamoxifen (Tam) and 0.1 nM E2. As in the *E-screen* test, daidzein (in five dilutions between 1 nM and 10 µM) and endosulfan and triadimenol (individually and in combination, in five concentrations between 10 nM and 0.1 mM) were tested. All experiments were performed in triplicate.

2.6.1 Determination of luciferase activity in MELN cells

We used One Glo Luciferase Assay System (Promega, USA) according to manufacturer's instructions to determine the luciferase activity. Briefly, at the end of the incubation, 100 µL of One Glo Reagent (containing fluoroluciferin) was added to each well and mixed for optimal consistency; luminescence was measured with a luminometer after at least 3 minutes to allow complete cell lysis and within 30 minutes of reagent addition (Tecan, Infinite M200 PRO). The proliferative effect on the MELN cells relative to the positive control (E2) is represented as transactivation % (TRANS %): the increased rate of luciferase gene expression triggered by the total estrogenic compounds present in the samples. The induction of luciferase activity is expressed as a percentage; the 100% value is the maximum value obtained in the presence of E2. The estrogenic activity was expressed as the 17-β-estradiol

equivalent quantity or factor (EEQ or EEF). EEQ was thus calculated as (EC50)E2/(EC50)sample in ng/L and EEF as (EC50)E2/(EC50)compound or positive control.

2.6.2 Cytotoxicity assessment in MELN cells

The Multitox-Fluor Multiplex Cytotoxicity Assay (Promega, cat. nr 9200) is a fluorescent assay that utilizes the changes membrane integrity to measure cell viability or cytotoxicity after luciferase measurements using the same test plates (Berckmans et al., 2007). This assay technology simultaneously measures two distinct protease activities, with rapid catalytic cleavage rates, as markers for cell viability or cytotoxicity.

The cytotoxicity test was applied to the more concentrated 10 and 1 mg/mL dilutions of the samples. Measurements of cytotoxicity are essential in all bioassays, as extracts can be cytotoxic due to compounds co-extracted during processing (Brabander, 2009).

At the end of incubation, 100 µL of the assay reagent was added to all wells and mixed; the plates were then incubated for at least 30 minutes at 37°C. Fluorescence was determined at an excitation/emission wavelength of 360 nm/460 nm for cell viability and 485 nm/528 nm for cytotoxicity using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). The mean values and standard deviation of replicate wells and the mean fold increase in cytotoxicity in relation to the negative control were calculated.

2.7 Statistical analyses

The statistical analyses were performed using the SPSS Package, version 18.0, for Windows (SPSS for Windows, Chicago, IL, USA). The EC50 data were analyzed by means of a Probit regression analysis, the means were compared with the Mann-Whitney test, and the Spearman rank correlation coefficient (rS) was used to assess relationships between the variables. The mean difference and correlation were considered significant at $p < 0.05$.

3. Results

3.1 Pesticide concentrations in fruits and vegetables

Among the 24 fruit and vegetable samples analyzed, 14 contained pesticide residues with concentrations ranging between 0.02 and 1.19 ppm; these samples contained a total of 12 different types of residues. Nevertheless all these positive samples complied with the legal maximum residue levels (MRLs), according to regulation (EC, 396/2005). The other 10 samples did not contain any pesticide residues, and two of these were samples from organic agriculture. Table 1 shows the content of pesticide residues in the 24 fruit and vegetable samples. Almost all of the positive samples had a single pesticide residue, with the exception of banana (samples 1 and 2) and parsley (sample 2), which had two, and tomato (sample 2), which had four. Those most frequently detected compounds were imazalil (in six samples) and thiabendazole, fenhexamid, and chlorpyrifos (in two samples). Some of the positive samples contained residues reported to exert estrogenic activities in *in vitro* tests: chlorpyrifos-methyl (Kang et al., 2004) in tangerine, imazalil (Kojima et al., 2005) in grapefruit (yellow and pink) and lemon (samples 1 and 2), iprodione (Andersen et al., 2002) in tomato (sample 2), and thiabendazole (Manabea et al., 2006) in banana (samples 1 and 2).

3.2 Estrogenic activity by the E-screen *test*

3.2.1 Positive controls

The mean EC50 value of E2 for the *E-screen* was 115.01 ± 42.40 pmol/L (31.05 ± 42.39 ng/L), and maximum cell proliferation was generally induced by 0.1 nM E2. The EC50 values of the positive controls were as follows: daidzein, 8.95×10^4 pmol/L; endosulfan, 1.30×10^6 pmol/L; triadimenol, 6.90×10^8 pmol/L; and endosulfan with triadimenol, 1.04×10^6 pmol/L.

3.2.2 Fruit and vegetable samples

Among the 24 samples analyzed, 12 produced an increase in MCF-7 BUS proliferation when compared to the control (Figure 1), whereas the other 12 samples did not induce significant

cell proliferative activity. The mean EC50 value of the samples for the *E-screen* test was 4.7 ± 3.6 g/L, with an EEF of 6.6 x 10^{-9}. The proliferative effect of the positive samples on the MCF-7 BUS cells relative to the positive control (E2) is shown in terms of RPE % and EEQ in Table 2. The mean EEQ value for all the samples analyzed was 0.070 ± 0.016 µg/100 g; the average EEQ value for all the positive samples was 0.148 ± 0.205 µg/100. RPE of the fruits and vegetables generally showed partial agonist activity (RPE < 100%), with only one sample exhibiting full agonist activity (RPE ≥ 100%).

We observed that co-incubation with Tam led to an inhibition of the proliferative response (36% ± 15%), whereas co-incubation with E2 led to a greater proliferative response (103% ± 29%) in all cases, confirming that the observed cell proliferation was ER mediated. In both cases, the difference was statistically significant (t-test, $p < 0.05$).

The eight samples containing the residue of pesticides having known estrogenic activity, banana (samples 1 and 2), grapefruit (yellow and pink), lemon (samples 1 and 2), peach, and tangerine, induced MCF-7 BUS cell proliferation and showed significant EEQ values, with the exception of peach and banana sample 2.

The correlation analysis between the total concentration of pesticide residues and EEQ values, including all the samples, was positive and significant ($rS = 0.431$ and $p < 0.05$). The EEQ values increased with increasing residue concentration (Figure 2).

The estrogenic activity of all the food samples analyzed was compared to the natural content of phytoestrogens, as based on values obtained from the literature. This study used the database compiled by Thompson (Thompson et al., 2006), which is one of the most frequently updated food phytoestrogen databases in the literature; the database includes most of the foods used in our study (67%) and describes 3 classes of phytoestrogens (isoflavones, lignans, and coumestans). We also used the database compiled by Kuhnle (Kuhnle et al., 2009), which lists the content of isoflavones, lignans, and coumestans for 240 fruits and vegetables. The

total content of phytoestrogens in the 24 samples in our study, representing 14 different types of foods, is presented in Table 3. The analysis of all of the samples showed no correlation between the total concentration of phytoestrogens and the EEQ values obtained by the *E-screen* test ($p < 0.05$).

3.3 Estrogenic activity by MELN gene-reporter assay

3.3.1 Positive controls

The mean EC50 value of E2 for the MELN gene-reporter assay was 30.93 ± 23.50 pmol/L (8.35 ± 6.34 ng/L); the EC50 values were calculated from the control curves obtained from each of the bioassays performed. Maximum luciferase activity was generally induced by 1 nM E2. The EC50 values of the positive controls were as follows: daidzein, 2.61×10^4 pmol/L; endosulfan, 1.97×10^6 pmol/L; triadimenol, 1.19×10^7 pmol/L; and endosulfan with triadimenol, 1.27×10^6 pmol/L.

3.3.2 Fruit and vegetable samples

A total of 14 of the 24 samples analyzed produced an increase in MELN luciferase activity when compared to the control (Figure 1); the other 10 samples did not induce significant luciferase activity. The MELN cell luciferase activity of the positive samples relative to the positive control (E2) is shown in terms of TRANS % and EEQ in Table 2. The mean EC50 value of the samples for the MELN gene-reporter luciferase assay was 1.4 ± 1.9 g/L, with an EEF of 5.9×10^{-9}. The mean value of EEQ for all the samples analyzed was 0.090 ± 0.017 µg/100 g, and the average value of EEQ for all positive samples was 0.153 ± 0.206 µg/100. The eight samples containing the residue of pesticides having known estrogenic activity all induced MELN luciferase activity and showed a significant EEQ value, with the sole exception of the peach sample.

We noted that co-incubation with Tam led to an inhibition of luciferase activity (30% ± 5%), whereas co-incubation with E2 led to greater activity (112% ± 19%) in all cases, confirming

that luciferase activity was effectively ER mediated; the difference was statistically significant (t-test, $p < 0.05$) in both cases.

The correlation analysis between the total concentration of pesticide residues and EEQ values, including all the samples, was positive and significant ($rS = 0.602$ and $p < 0.01$); the EEQ values increased with increasing residue concentration (Figure 2).

Again, the estrogenic activity of all the food samples analyzed was compared to their natural content of phytoestrogens; as for the *E-screen*, this comparison was based on values obtained from the literature. The analysis of all these samples showed no correlation between the total concentration of phytoestrogens and the EEQ values obtained by means of the MELN gene-reporter assay ($p > 0.05$).

3.3.3 Cytotoxicity of fruit and vegetable samples in MELN cells

To examine whether there was a toxic effect on the cells in our assays, the 10 and 1 mg/mL sample dilutions were tested for cytotoxicity; being less diluted, these concentrations could potentially mask the real estrogenic activity of the compounds present.

The mean fold cytotoxicity increase of the 1 mg/mL dilution in relation to the negative control was 0.57 ± 0.18 ($p > 0.05$), and that of the 10 mg/mL dilution was 0.61 ± 0.16 ($p > 0.05$). However, the absence of estrogenicity cannot be ascribed to the toxicity of the sample itself.

3.4 Comparison of estrogenic activity by the E-screen *test and the MELN gene-reporter assay*

The EC50 values for E2 by both the *E-screen* and MELN gene-reporter assay were not significantly different ($p > 0.05$). With regard to the positive controls, the phytoestrogen daidzein was found to be the most estrogenic by both tests. The mixture of the two pesticides, endosulfan and triadimenol, showed an EEF value slightly higher than each single EEF in both tests, highlighting the additive action of these residues. A comparison of the results

obtained in the two assays highlights the non-significance of the differences in average EEQs ($p > 0.05$): both assays revealed that the sample containing the greatest number of pesticide residues was also the most estrogenic (tomato 2), emphasizing the additive action of some residues. The correlation between all the EEQ values obtained with the MELN gene-reporter assay and with *E-screen* (Figure 3) suggests a positive correlation between the two tests ($rS = 0.581$ and $p < 0.01$). However, some of the test result differences could be partially explained by the end-points of the two tests: the *E-screen* is based on a binding mechanism that causes proliferation as a cellular response, which could be affected by other external factors; in contrast, the MELN gene-reporter assay is specific for the receptor. Another important difference between the two tests is represented by the execution times in terms of stimulation with the test compounds: 16 hours for the MELN gene-reporter assay and 120 hours for *E-screen* (Soto et al., 2006; Witters et al., 2010).

3.5 Estimating dietary EEQs

To obtain rough estimates of the dietary intake of EEQs (Shaw and McCully, 2002; Thomson et al., 2003), the following assumptions were made: the total absorption of dietary EEQs is given by the values calculated in this study (0.070 ± 0.016 µg/100 g and 0.090 ± 0.017 µg/100 g by the *E-screen* test and MELN gene-reporter assay, respectively); the mean intake of fruits and vegetables in the European population is 335 g/day (Boffetta et al., 2010); a human blood volume of 5 L; the EEQ half-life was not considered; and the body can be represented by a single-compartment pharmacokinetic model (although this assumption is clearly not correct). Given these assumptions, the adult human EEQ (dietary intake) would be 47-60 ng EEQ/L/day. These values were comparable to the normal serum level of E2 in humans (10-50 ng/L for males, 20-350 ng/L for women).

4. Discussion and Conclusion

Over the last 10 years, the pesticide concentrations found in fruits and vegetables have decreased and the samples comply with the legal MRLs of pesticides have increased (Borrello, 2008; EFSA, 2009). Moreover, the total quantity of utilized pesticides has been reduced; however, specific data for EDC pesticides use for all Europe are unavailable (McKinlay et al., 2008). Regardless, evidence that many pesticides are active *in vivo* at extremely low doses suggests that the permitted residue levels in food may be too high (Hayes et al., 2002; Storrs and Kiesecker, 2004; Weltje et al., 2005). In the present study, all the fruit and vegetable samples that were positive for pesticide residues showed a concentration that was below the MRLs, according to the European Regulation in force (EC 396/2005). However, we also showed that such concentrations might have an estrogenic activity. To date, there is no information in the literature on the estrogenicity of fruits and vegetables in relation to the content of allowable pesticide residues.

In the present study, the *E-screen test* and MELN gene-reporter assay proved to be suitable for the determination of estrogenic activity in extracts from whole fruits and vegetables. The fruit and vegetable samples exhibited an estrogenic potency that was approximately nine orders of magnitude lower than that of E2 and was also lower than either daidzein or endosulfan and triadimenol. Our results noted that the pesticides endosulfan and triadimenol were approximately six and eight orders of magnitude, respectively, less potent than E2, whereas the phytoestrogen daidzein was three to four orders of magnitude less potent than E2. In literature has previously been reported that pesticides and phytoestrogens have different degrees of estrogenicity (Soto et al., 1995; Van Meeuwen et al., 2007), and this observation should be taken into account when determining exposure levels because estrogenicity, and not the concentration, is an important determinant of the biological effect (Shaw and McCully, 2002). It has been shown that the levels of phytoestrogens in human diets tend to be much

higher than the levels of synthetic endocrine-active chemicals (Franke et al., 1998; Irvine et al., 1998; Behr et al. 2011; Waring et al., 2008). However, despite the small number of samples analyzed, we found a correlation between the estrogenicity of fruits and vegetables and the total concentration of pesticide residues, with no correlation between EEQs and the endogenous phytoestrogen concentration. These findings partially confirm the results obtained in a previous work in which only the *E-screen* test was employed (Schilirò et al., 2011). It should be observed that the extracts used in the present study had no specificity, thus they did not reflect the activity of any particular chemical component of the foods (Charles, 2002); furthermore, it should be noted that *in vitro* analyses greatly depend on the preparation of the sample. Many EDCs can be found in fruits and vegetable at very low concentrations, and these kind of produce contain interfering matrix elements that can mask or interfere with the analysis of the sample of interest. There is therefore a requirement for the development of a sample preparation method that can extract, concentrate, and purify samples of interest (Connolly et a., 2011). The results presented here indicate that, most likely for some foods and for low doses, non-additive effects might occur due to different combinations of EDCs. This suggestion is in agreement with other studies (Suzuki et a., 2001; Charles et al., 2007).

The human EEQ values that we calculated from dietary data (47-60 ng EEQ/L/day) most likely represent a significant overestimate because of the underlying assumptions of 100% absorption and no metabolism or excretion. Applying the factor used by Thomson (Thomson et al., 2003), the EEQ plasma levels obtained would result in an estimate of 0.7-0.9 ng EEQ/L, a level that is not likely to be of health significance for the population, confirming the results of our previous study (Schilirò et al., 2011). However, it is also feasible that total daily intake of EDCs might exceed our estimate due to possible contributions from as-yet-undetermined EDCs in foods and beverages (Guenter et al., 2002; Muncke et al., 2009; Stanford et al., 2010; Wagner et al., 2011) and from other environmental sources (Takamura-

Enya et al., 2003). For example, Behr and colleagues (Behr et al., 2011) conclude that pesticide residues exhibit only marginal impacts on the estrogenic activity in the diet.

Our study demonstrates that estrogenicity is present in fruits and vegetables, even in samples that comply with the legal MRLs. However, it is not currently possible to draw conclusions on the potential implications for human health based on the presented *in vitro* data. Further studies are necessary to identify the compounds accounting for the activity and to evaluate their relevance to human health.

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Table 1. Pesticide residue concentrations in 24 fruit and vegetable samples (17 fruits and 7 vegetables) supplied by the Regional Environmental Protection Agency. All the positive samples complied with the legal maximum residue levels (MRLs), according to regulation (EC, 396/2005).

Vegetables	**Pesticides residues (MW)**	***Pesticides residues* (ppm)***
Apple 1	-	<0.01
Apple 2	Boscalid (343.21)	0.02
Banane 1	Imazalil (297.18) Thiabendazole (201.25)	0.09 0.06
Banane 2	Imazalil (297.18) Thiabendazole (201.25)	0.12 1.19
Carrot	-	<0.01
Grape	Quinoxifen (308.13)	0.04
Grapefruit yellow	Imazalil (297.18)	0.21
Grapefruit pink	Imazalil (297.18)	0.18
Kiwi	-	<0.01
Lemon 1	Imazalil (297.18)	0.03
Lemon 2	Imazalil (297.18)	0.07
Parsley 1	-	<0.01
Parsley 2	Chlorthal-dimethyl (331.97) Piperonyl butoxide (338.44)	0.16 0.15
Peaches	Chlorpyrifos-methyl (350.59)	0.20
Pear 1	-	<0.01
Pear (organic)	-	<0.01
Potato 1	-	<0.01
Potato 2	Chlorpropham (213.66)	0.02
Strawberry 1	Fenhexamide (302.20)	0.06
Strawberry 2	-	<0.01
Strawberry (organic)	-	<0.01
Tangerine	Chlorpyrifos-methyl (350.59)	0.10
Tomato 1	-	<0.01
Tomato 2	Cyprodinil (225.29) Fenhexamide (320.20) Iprodione (330.17) Mepanipyrim (223.27)	0.11 0.17 0.17 0.07

* detection limit for pesticides residues 0.01 ppm.

Table 2. Estrogenic activity of fruit and vegetable samples in MCF-7 BUS breast cancer cells, as represented by RPE % (relative proliferative effect) and EEQ (estradiol equivalency quantity, µg/100 g), and in MELN gene-reporter luciferase cells, as represented by TRANS % (increased rate of luciferase gene expression) and EEQ (estradiol equivalency quantity, µg/100 g).

	E-screen		***MELN luciferase assay***	
Vegetables (sample code)	**RPE %**	**EEQ (µg/100g)**	**TRANS %**	**EEQ (µg/100g)**
Banana 1*	8	0.100	51	0.607
Banana 2*	nd	nd	30	0.107
Carrots	nd	nd	40	0.034
Grape	26	0.062	40	0.017
Grapefruit yellow*	17	0.034	34	0.203
Grapefruit pink *	>100	0.077	29	0.250
Lemon 1*	84	0.123	55	0.010
Lemon 2*	75	0.076	43	0.019
Parsley 2	nd	nd	49	0.014
Pear, organic	nd	nd	43	0.093
Potato 1	37	0.011	nd	nd
Potato 2	22	0.035	nd	nd
Strawberry 1	28	0.037	42	0.071
Strawberry organic	19	0.062	49	0.041
Tangerine *	17	0.548	29	0.064
Tomato 2*	6	0.616	42	0.610

nd: not detected; * vegetables which contain pesticide's residue with known estrogenic activity.

Table 3. Phytoestrogen concentrations in fruits and vegetables from the databases of [a]Thomson, 2006 and [b]Kuhnle, 2009. ISO, isoflavones; LIG, lignans; COU, coumestrol.

	Phytoestrogens µg/100g			
Vegetables (sample code)	**ISO**	**LIG**	**COU**	**tot**
Apple[a] (1, 2)	2.1	2.9	0	5.0
Banana[a] (1, 2)	0.8	1.8	0	2.6
Carrot[a]	0.2	6.4	0	6.6
Grape[a]	0.8	8.7	0.1	9.6
Grapefruit[a] (yellow, pink)	0.4	5.6	0.2	6.2
Kiwi[b]	0.5	110	0	110.5
Lemon[b] (1, 2)	2.5	13.5	1.0	17.0
Parsley[b] (1, 2)	59.0	137.0	<1.0	197
Peaches [b]	<1.0	42.0	0	43
Pear[b] (1, organic)	2.3	5.6	0.3	8.2
Potato[a] (1, 2)	0.2	1.2	0	1.4
Strawberry[a] (1, 2, organic)	2.4	48.9	0.3	51.6
Tangerine [b]	2.0	2.0	1.0	5.0
Tomato [a] (1,2)	0.5	9.1	0	9.6

Figure Captions

Figure 1. Maximum proliferative effects induced by fruit and vegetable samples in MCF-7 BUS breast cancer cells, *E-screen*, (A) and maximum luciferase activity induced by fruit and vegetable samples in MELN cells, the MELN gene-reporter assay (B). The sample concentration that produced the maximum effect is reported in brackets: (1) = 1 g/L, (2) = 0.1 g/L, (3) = 0.01 g/L, (4) = 0.001 g/L, and (5) = 0.0001 g/L. The values represent the means ± standard deviations.

Figure 2. Relationship between the calculated *E-screen* test EEQs (open circles), MELN gene-reporter assay EEQs (filled circles), and total pesticide residues in fruit and vegetable samples ($rS = 0.431$, $p < 0.05$, and $rS = 0.602$, $p < 0.01$, respectively).

Figure 3. Relationship between EEQ values (µg/100 g) of fruit and vegetable samples obtained with the *E-screen* test and with the MELN gene-reporter assay ($rS = 0.581$, $p < 0.01$).

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1. Introduction

The study of the chemical disruption of the endocrine system has been an active area of research during the last decade and has captured the attention of governments, policy makers, and media (De Rosa et al., 1998; Choi et al., 2005). Endocrine-disrupting compounds (EDCs) are defined as "exogenous substances or mixture that alters the function of the endocrine system and generate noxious effects on the health of a safe body, its descendants, or its sub-population" (WHO, 2002); at the European Union level, EDCs are included in the list of so-called emerging contaminants (EU, 2001). In terms of adverse health effects, there is concern that substances with endocrine-disrupting properties may be causally involved in a number of diseases or conditions, such as hormone-dependent cancer, reproductive disorders, a decline in fertility, or obesity (Diamanti-Kandarakis et al., 2009; Shaw, 2009; Hotchkiss et al., 2008). However, causality between EDCs and effects on human health remains controversially debated in both science and the public (Wagner and Oehlmann, 2009).

EDCs are ubiquitous in the environment because of their very frequent use in residential, industrial, and agricultural applications; in particular, the origin and fate of these contaminants can lead to their transmission in the food chain (Schwartz, 2001). It is widely accepted that food and diet are among the most important exposure routes for EDCs. Therefore, a normal human diet results in exposure to a complex mixture of xenoestrogens that enter systemic circulation in the body. There are many types of EDCs in food, ranging from natural compounds (e.g., hormones, phytoestrogens, and mycotoxins) to synthetic compounds (e.g., pesticides, pharmaceuticals, and industrial or process chemicals). The natural contribution of phytoestrogens includes some isoflavonoids, flavonoids, stilbenes, and lignans; however, their role in endocrine disruption remains highly controversial (Patisaul et al., 2010). Indeed, the lack of consistency in epidemiological and experimental results places these chemicals in EDC category III, a category that includes compounds for which *in vitro*

data exist but for which data from experimental animals concerning adverse effects on endocrine homeostasis are weak or lacking (Foster and Agzarian, 2008). Among synthetic endocrine compounds some pesticides regularly used in agriculture have shown weak estrogenic responses *in vitro*, for example, tolclofos-methyl (Andersen et al., 2002) and triadimenol (Vinggaard., 1999). Imazalil showed weak anti-estrogenic activity in an *in vitro* gene-reporter assay (Kojima et al., 2005) and a negligible proliferation response in an MCF7 cell proliferation assay (Soto et al., 1994). Endosulfan also showed an estrogenic response in several *in vitro* tests (Soto et al., 1994; Andersen et al., 2002). New pesticide regulations were recently introduced by the European Parliament and contain, for the first time, specific reference to endocrine-disrupting properties (on 21 October 2009, regulation (EC) No 1107/2009 replaced Council Directive 91/414/EEC). Although it is clear that substances with endocrine-disrupting properties should be avoided, there is no clear consensus of how to identify and evaluate endocrine-disrupting properties, and no guidance is yet provided in the new European Regulation (Flynn, 2011).

The assessment of EDC food contaminants is a continual challenge and has traditionally been performed mainly through analytical chemistry with respect to the detection of a few, specific chemicals (De Brabander et al., 2009). However, despite rapid improvements in analytical chemistry, merely evaluating single chemicals ignores the potential mixture effects between different compounds and the effects caused by as-yet-unidentified compounds (Connolly et al., 2011; US EPA, 2012). Another concern is that, although some of these EDCs have been deemed to be relatively safe at low individual levels of consumption, they may combine with other low-level EDCs to create low-level cocktail or mixture effects (Kjaerstad et al., 2010; Kortenkamp, 2007; Payne, 2010). Considering these points, the most appropriate way of detecting and studying the effects of EDCs and their mixtures may be through the use of bioassay systems that utilize the natural ligands and pathways. This can be achieved through,

preferably, *in vitro* bioassays, which, although they cannot assess behavioral effects, have the benefit of closely related natural systems without the use of animal testing. Such bioassays can detect compounds based on their effects, enable the detection of the effects caused by currently unidentified compounds, and integrate the effect of complex chemical mixtures (Wagner et al., 2011). Moreover, quantification of estrogenic activity as the 17-β-estradiol equivalent quantity (EEQ) facilitates the estimation of the total dietary intake of estrogenicity. Although such assessments provide valuable information on the human exposure to estrogen-like compounds, they are rare in the literature (Safe 1995; Shaw and McCully, 2002; Behr et al., 2011; Schilirò et al., 2011).

The aim of the present study is to evaluate the estrogenic properties of fruits and vegetables by calculating the EEQ using two *in vitro* tests: the human breast cancer cell line (MCF-7 BUS) proliferation test or *E-screen* test (Soto et al, 1995) and the luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay (Balaguer et al., 1999). A further aim of this study is to compare the estrogenic activity of food samples to the pesticide residue content and the reported intrinsic content of phytoestrogens, as found in the literature. The results of the two *in vitro* tests were compared to each other. Finally, we combined the results to assess the theoretical blood EEQ levels for adults.

2. Materials and Methods

2.1 Fruit and vegetable samples

We analyzed 24 fruits and 7 vegetables (Table 1) supplied by the Regional Environmental Protection Agency (Piedmont A.R.P.A., *Agenzia Regionale per la Protezione dell'Ambiente del Piemonte*) between April 2010 and March 2011. This agency collects commercial plant products destined for human consumption for analyses as part of the regular national monitoring program for pesticide residues in food.

2.2 Detection of pesticide residues

All the fruit and vegetable samples (500 g each) were first homogenized using an Ultra-Turrax according to the provision of Italian Ministerial Decree 27/08/2004 and Regulation n° 396/2005 of the European Parliament. All the procedures for the analysis of pesticide residues in food samples were conducted according to the quality control procedures of the European Commission for pesticide residue analysis in food and feed (EC, 2009) and using the same methods as Schilirò et al. 2011. The determination of N-methylcarbamates was performed using reverse-phase high-pressure liquid chromatography (HPLC) with post-column reaction and fluorescence detection (SCL-10AVP, Shimadzu Corp, Japan) (EC, 2009; Branca and Longo, 2002). Organophosphorus, organochlorine, pyrethroids, triazine herbicides, and other classes of pesticides were determined by Gas Chromatography with Mass Spectrometry (GC-MS) equipped with selective detectors, a quadrupole ion trap, and ITQ Series GC-Ion Trap MS (Thermo Scientific, Ohio, USA) (EC, 2009; Branca and Sacchero, 1997).

2.3 Preparation of samples for in vitro *tests*

After homogenization, the raw fruits and vegetables were subjected to nonspecific extraction to obtain whole-food extracts. The extraction of food samples was performed according to the method proposed by Charles and colleagues (Charles et al., 2002) and modified for this application (Schilirò et al. 2011): 25 g of homogenized sample was added to 25 mL of incomplete cell culture medium, (phenol-red-free Dulbecco's modified Eagle's medium, DMEM) in brown glass beakers protected from direct light. The sample was incubated overnight while being agitated at 4°C. The sample was then centrifuged at 9000 x g for 15 minutes, and the supernatant was collected in 50-mL brown glass tubes to obtain a 1 g/mL food extract. Whole-food preparations were processed ahead of time, frozen, and stored at -20°C. Prior to testing by the *E-screen* assay and the MELN gene-reporter assay, the samples were first thawed at 4°C overnight, kept at room temperature, filter-sterilized using a 0.22-µm

filter, and then diluted in steroid-free experimental DMEM at five dilutions (from 0.001 to 10 mg/mL).

2.4 Cell lines, culture conditions, and chemicals

Estrogen-sensitive human MCF-7 BUS breast cancer cells were kindly provided by Drs. A.M. Soto and Dr. C. Sonnenschein (Tufts University School of Medicine, Boston, Massachusetts, USA) and were cultivated in Dulbecco's modified Eagle's medium (DMEM) with 15 mg/L phenol red, 10% fetal calf serum (FCS), 2% L-glutamine 200 mM, 2% HEPES buffer 1 M, 1% sodium pyruvate 100 mM and 1% penicillin/streptomycin 10 mg/mL at 37°C in an atmosphere of 5% carbon dioxide and 95% air under saturating humidity.

MELN cells, provided by Dr. P. Balaguer (INSERM, Montpellier - France), are MCF-7 cells stably transfected with an estrogen-responsive gene (ERE-βGlob-Luc-SVNeo) carried by integrated plasmids. These plasmids contain both an antibiotic resistance selection gene (SVNeo) and the estrogen-responsive elements to which the estrogen receptor-ligand complex can bind, thereby inducing the transcription of the luciferase reporter gene (Berckmans et al., 2007). Therefore, the luciferase activity measured is proportional to the concentration of estrogenic compounds (Hernandez-Raquet et al., 2007). MELN cells were cultured in Dulbecco's Modified Eagle Medium Nutrient Mixture F12 Ham (DMEM-F12) with phenol red supplemented with 5% fetal calf serum (FCS), 2% L-glutamine 200 mM, 1% penicillin/streptomycin, and 1 mg/ml G418 sulfate. The cell line was maintained in an incubator at 37°C, a relative humidity of 95%, and a CO_2 concentration of 5%. The cells were subcultured once a week, with medium refreshment between subculturing steps. For the experiments, we used cells from passage number 4 to passage number 15. The cells were regularly examined for mycoplasm infection to guarantee experimental work with mycoplasm-free cells and to comply with good cell culture practice (GCCP) guidelines.

A stock solution of 10 mM 17-β-estradiol (E2) was prepared with ethanol. The stock solutions of endosulfan and triadimenol were prepared with ethanol, and a stock solution of 10 mM daidzein was prepared with DMSO. All the stock solutions were stored in brown glass tubes at -20°C and then diluted to the desired concentration with steroid-free experimental medium. Unless otherwise specified, all the chemicals and materials for cell culture were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

2.5 E-screen *test*

The *E-screen* test was performed according to the method of Körner (Körner et al., 1999) and modified by Schilirò (Schilirò et al., 2009). Briefly, subconfluent MCF-7 BUS cells were trypsinized and resuspended in the steroid-free experimental medium, consisting of phenol red-free DMEM supplemented with 5% stripped-FCS, 2% L-glutamine 200 mM, 2% HEPES buffer 1 M, 1% sodium pyruvate 100 mM, and 1% penicillin/streptomycin 10 mg/mL. The cells were seeded into 24-well plates at a density of 40000 cells/well. After 24 hours, the medium was replaced with the experimental medium containing one of five different dilutions of the food extracts. Each dilution was tested in six replicates per assay. Six wells without hormones comprised the negative control. The endogenous estrogen 17-β-estradiol (E2), in five concentrations between 1 pM and 10 nM, was used as a positive control. One dilution of each food sample found to induce a significant proliferative effect was tested together with 5 nM anti-estrogen tamoxifen (Tam) and 0.1 nM E2. Other controls tested included the following: the phytoestrogen daidzein (five dilutions between 1 nM and 10 μM) and the pesticides endosulfan and triadimenol, both individually and in combination (five dilutions between 10 nM and 0.1 mM). The assays were stopped after six days by crystal violet staining, and the absorbance (595 nm) in each well was determined.

The proliferative effect (PE) of a sample is the ratio between the highest cell number achieved with the sample or E2 and the cell number of the negative control:

(1) PE = [(max cell number)sample/(cell number)negative control]

The estrogenic activity of a sample is evaluated by determining the relative efficacy, called the relative PE (RPE%). RPE compares the maximum proliferation induced by a sample with that induced by E2:

(2) RPE % = [(PE-1)sample/(PE-1)E2] x 100.

Full agonistic activity, RPE ≥ 100%, can be distinguished from partial agonistic activity when RPE is less than 100% (18).

Relative potency, called the estradiol equivalency quantity or factor (EEQ or EEF) is thus calculated as follows:

(3) EEQ = [(EC50)E2/(EC50)sample]

(4) EEF = [(EC50)E2/(EC50)compound or positive control]

The EC50 value for the *E-screen* test (the concentration at which 50% of PE is achieved) was calculated using a Probit regression (SPSS, Chicago, IL). The PE and EC50 values of each sample were calculated from the mean dose-response curves established for each experiment. EEQ, expressed in ng/L, is the total concentration of estrogenic-active compounds in an environmental sample normalized to the natural estrogen E2.

2.6 MELN gene-reporter assay

The MELN gene-reporter assay has been widely used for the detection of estrogenic activity in complex environmental samples (Fenet et al., 2003; Cargouet et al., 2007; Jugan et al., 2009; Cambalbert et al., 2012). In the present study, the test was performed according to the method of Balaguer (Balaguer et al., 1999).

Due to the phenol red and FCS estrogenic activity, the *in vitro* experiments were performed in DMEM F12 without phenol red and supplemented with 5% dextran-coated charcoal-treated

fetal calf serum (DCC-FCS), 2% L-glutamine 200 mM, and 1% antibiotics (penicillin/streptomycin). To adapt the cells to DCC-FCS, the growth medium was replaced with fresh test medium three days prior to the experiment. The cells were then harvested and seeded in 96-well plates with a flat, clear bottom (Corning) at a density of 40000 cells/well in 100 µL of DCC-FCS per well. After 24 hours, the test medium was removed, and 100 µL of each sample concentration was added to three replica wells. The cells were treated with the samples for 16 h. A negative control, without hormones, and a positive control, E2 (between 1 pM and 10 nM), were included in each assay. One dilution of each food sample found to induce a degree of significant luciferase activity was tested together with 5 µM of the anti-estrogen tamoxifen (Tam) and 0.1 nM E2. As in the *E-screen* test, daidzein (in five dilutions between 1 nM and 10 µM) and endosulfan and triadimenol (individually and in combination, in five concentrations between 10 nM and 0.1 mM) were tested. All experiments were performed in triplicate.

2.6.1 Determination of luciferase activity in MELN cells

We used One Glo Luciferase Assay System (Promega, USA) according to manufacturer's instructions to determine the luciferase activity. Briefly, at the end of the incubation, 100 µL of One Glo Reagent (containing fluoroluciferin) was added to each well and mixed for optimal consistency; luminescence was measured with a luminometer after at least 3 minutes to allow complete cell lysis and within 30 minutes of reagent addition (Tecan, Infinite M200 PRO). The proliferative effect on the MELN cells relative to the positive control (E2) is represented as transactivation % (TRANS %): the increased rate of luciferase gene expression triggered by the total estrogenic compounds present in the samples. The induction of luciferase activity is expressed as a percentage; the 100% value is the maximum value obtained in the presence of E2. The estrogenic activity was expressed as the 17-β-estradiol

equivalent quantity or factor (EEQ or EEF). EEQ was thus calculated as (EC50)E2/(EC50)sample in ng/L and EEF as (EC50)E2/(EC50)compound or positive control.

2.6.2 Cytotoxicity assessment in MELN cells

The Multitox-Fluor Multiplex Cytotoxicity Assay (Promega, cat. nr 9200) is a fluorescent assay that utilizes the changes membrane integrity to measure cell viability or cytotoxicity after luciferase measurements using the same test plates (Berckmans et al., 2007). This assay technology simultaneously measures two distinct protease activities, with rapid catalytic cleavage rates, as markers for cell viability or cytotoxicity.

The cytotoxicity test was applied to the more concentrated 10 and 1 mg/mL dilutions of the samples. Measurements of cytotoxicity are essential in all bioassays, as extracts can be cytotoxic due to compounds co-extracted during processing (Brabander, 2009).

At the end of incubation, 100 µL of the assay reagent was added to all wells and mixed; the plates were then incubated for at least 30 minutes at 37°C. Fluorescence was determined at an excitation/emission wavelength of 360 nm/460 nm for cell viability and 485 nm/528 nm for cytotoxicity using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). The mean values and standard deviation of replicate wells and the mean fold increase in cytotoxicity in relation to the negative control were calculated.

2.7 Statistical analyses

The statistical analyses were performed using the SPSS Package, version 18.0, for Windows (SPSS for Windows, Chicago, IL, USA). The EC50 data were analyzed by means of a Probit regression analysis, the means were compared with the Mann-Whitney test, and the Spearman rank correlation coefficient (rS) was used to assess relationships between the variables. The mean difference and correlation were considered significant at $p < 0.05$.

3. Results

3.1 Pesticide concentrations in fruits and vegetables

Among the 24 fruit and vegetable samples analyzed, 14 contained pesticide residues with concentrations ranging between 0.02 and 1.19 ppm; these samples contained a total of 12 different types of residues. Nevertheless all these positive samples complied with the legal maximum residue levels (MRLs), according to regulation (EC, 396/2005). The other 10 samples did not contain any pesticide residues, and two of these were samples from organic agriculture. Table 1 shows the content of pesticide residues in the 24 fruit and vegetable samples. Almost all of the positive samples had a single pesticide residue, with the exception of banana (samples 1 and 2) and parsley (sample 2), which had two, and tomato (sample 2), which had four. Those most frequently detected compounds were imazalil (in six samples) and thiabendazole, fenhexamid, and chlorpyrifos (in two samples). Some of the positive samples contained residues reported to exert estrogenic activities in *in vitro* tests: chlorpyrifos-methyl (Kang et al., 2004) in tangerine, imazalil (Kojima et al., 2005) in grapefruit (yellow and pink) and lemon (samples 1 and 2), iprodione (Andersen et al., 2002) in tomato (sample 2), and thiabendazole (Manabea et al., 2006) in banana (samples 1 and 2).

3.2 Estrogenic activity by the E-screen *test*

3.2.1 Positive controls

The mean EC50 value of E2 for the *E-screen* was 115.01 ± 42.40 pmol/L (31.05 ± 42.39 ng/L), and maximum cell proliferation was generally induced by 0.1 nM E2. The EC50 values of the positive controls were as follows: daidzein, 8.95×10^4 pmol/L; endosulfan, 1.30×10^6 pmol/L; triadimenol, 6.90×10^8 pmol/L; and endosulfan with triadimenol, 1.04×10^6 pmol/L.

3.2.2 Fruit and vegetable samples

Among the 24 samples analyzed, 12 produced an increase in MCF-7 BUS proliferation when compared to the control (Figure 1), whereas the other 12 samples did not induce significant

cell proliferative activity. The mean EC50 value of the samples for the *E-screen* test was 4.7 ± 3.6 g/L, with an EEF of 6.6 x 10^{-9}. The proliferative effect of the positive samples on the MCF-7 BUS cells relative to the positive control (E2) is shown in terms of RPE % and EEQ in Table 2. The mean EEQ value for all the samples analyzed was 0.070 ± 0.016 µg/100 g; the average EEQ value for all the positive samples was 0.148 ± 0.205 µg/100. RPE of the fruits and vegetables generally showed partial agonist activity (RPE < 100%), with only one sample exhibiting full agonist activity (RPE ≥ 100%).

We observed that co-incubation with Tam led to an inhibition of the proliferative response (36% ± 15%), whereas co-incubation with E2 led to a greater proliferative response (103% ± 29%) in all cases, confirming that the observed cell proliferation was ER mediated. In both cases, the difference was statistically significant (t-test, $p < 0.05$).

The eight samples containing the residue of pesticides having known estrogenic activity, banana (samples 1 and 2), grapefruit (yellow and pink), lemon (samples 1 and 2), peach, and tangerine, induced MCF-7 BUS cell proliferation and showed significant EEQ values, with the exception of peach and banana sample 2.

The correlation analysis between the total concentration of pesticide residues and EEQ values, including all the samples, was positive and significant ($rS = 0.431$ and $p < 0.05$). The EEQ values increased with increasing residue concentration (Figure 2).

The estrogenic activity of all the food samples analyzed was compared to the natural content of phytoestrogens, as based on values obtained from the literature. This study used the database compiled by Thompson (Thompson et al., 2006), which is one of the most frequently updated food phytoestrogen databases in the literature; the database includes most of the foods used in our study (67%) and describes 3 classes of phytoestrogens (isoflavones, lignans, and coumestans). We also used the database compiled by Kuhnle (Kuhnle et al., 2009), which lists the content of isoflavones, lignans, and coumestans for 240 fruits and vegetables. The

total content of phytoestrogens in the 24 samples in our study, representing 14 different types of foods, is presented in Table 3. The analysis of all of the samples showed no correlation between the total concentration of phytoestrogens and the EEQ values obtained by the *E-screen* test ($p < 0.05$).

3.3 Estrogenic activity by MELN gene-reporter assay

3.3.1 Positive controls

The mean EC50 value of E2 for the MELN gene-reporter assay was 30.93 ± 23.50 pmol/L (8.35 ± 6.34 ng/L); the EC50 values were calculated from the control curves obtained from each of the bioassays performed. Maximum luciferase activity was generally induced by 1 nM E2. The EC50 values of the positive controls were as follows: daidzein, 2.61×10^4 pmol/L; endosulfan, 1.97×10^6 pmol/L; triadimenol, 1.19×10^7 pmol/L; and endosulfan with triadimenol, 1.27×10^6 pmol/L.

3.3.2 Fruit and vegetable samples

A total of 14 of the 24 samples analyzed produced an increase in MELN luciferase activity when compared to the control (Figure 1); the other 10 samples did not induce significant luciferase activity. The MELN cell luciferase activity of the positive samples relative to the positive control (E2) is shown in terms of TRANS % and EEQ in Table 2. The mean EC50 value of the samples for the MELN gene-reporter luciferase assay was 1.4 ± 1.9 g/L, with an EEF of 5.9×10^{-9}. The mean value of EEQ for all the samples analyzed was 0.090 ± 0.017 µg/100 g, and the average value of EEQ for all positive samples was 0.153 ± 0.206 µg/100. The eight samples containing the residue of pesticides having known estrogenic activity all induced MELN luciferase activity and showed a significant EEQ value, with the sole exception of the peach sample.

We noted that co-incubation with Tam led to an inhibition of luciferase activity (30% ± 5%), whereas co-incubation with E2 led to greater activity (112% ± 19%) in all cases, confirming

that luciferase activity was effectively ER mediated; the difference was statistically significant (t-test, $p < 0.05$) in both cases.

The correlation analysis between the total concentration of pesticide residues and EEQ values, including all the samples, was positive and significant ($rS = 0.602$ and $p < 0.01$); the EEQ values increased with increasing residue concentration (Figure 2).

Again, the estrogenic activity of all the food samples analyzed was compared to their natural content of phytoestrogens; as for the *E-screen*, this comparison was based on values obtained from the literature. The analysis of all these samples showed no correlation between the total concentration of phytoestrogens and the EEQ values obtained by means of the MELN gene-reporter assay ($p > 0.05$).

3.3.3 Cytotoxicity of fruit and vegetable samples in MELN cells

To examine whether there was a toxic effect on the cells in our assays, the 10 and 1 mg/mL sample dilutions were tested for cytotoxicity; being less diluted, these concentrations could potentially mask the real estrogenic activity of the compounds present.

The mean fold cytotoxicity increase of the 1 mg/mL dilution in relation to the negative control was 0.57 ± 0.18 ($p > 0.05$), and that of the 10 mg/mL dilution was 0.61 ± 0.16 ($p > 0.05$). However, the absence of estrogenicity cannot be ascribed to the toxicity of the sample itself.

3.4 Comparison of estrogenic activity by the E-screen *test and the MELN gene-reporter assay*

The EC50 values for E2 by both the *E-screen* and MELN gene-reporter assay were not significantly different ($p > 0.05$). With regard to the positive controls, the phytoestrogen daidzein was found to be the most estrogenic by both tests. The mixture of the two pesticides, endosulfan and triadimenol, showed an EEF value slightly higher than each single EEF in both tests, highlighting the additive action of these residues. A comparison of the results

obtained in the two assays highlights the non-significance of the differences in average EEQs ($p > 0.05$): both assays revealed that the sample containing the greatest number of pesticide residues was also the most estrogenic (tomato 2), emphasizing the additive action of some residues. The correlation between all the EEQ values obtained with the MELN gene-reporter assay and with *E-screen* (Figure 3) suggests a positive correlation between the two tests ($rS = 0.581$ and $p < 0.01$). However, some of the test result differences could be partially explained by the end-points of the two tests: the *E-screen* is based on a binding mechanism that causes proliferation as a cellular response, which could be affected by other external factors; in contrast, the MELN gene-reporter assay is specific for the receptor. Another important difference between the two tests is represented by the execution times in terms of stimulation with the test compounds: 16 hours for the MELN gene-reporter assay and 120 hours for *E-screen* (Soto et al., 2006; Witters et al., 2010).

3.5 Estimating dietary EEQs

To obtain rough estimates of the dietary intake of EEQs (Shaw and McCully, 2002; Thomson et al., 2003), the following assumptions were made: the total absorption of dietary EEQs is given by the values calculated in this study (0.070 ± 0.016 µg/100 g and 0.090 ± 0.017 µg/100 g by the *E-screen* test and MELN gene-reporter assay, respectively); the mean intake of fruits and vegetables in the European population is 335 g/day (Boffetta et al., 2010); a human blood volume of 5 L; the EEQ half-life was not considered; and the body can be represented by a single-compartment pharmacokinetic model (although this assumption is clearly not correct). Given these assumptions, the adult human EEQ (dietary intake) would be 47-60 ng EEQ/L/day. These values were comparable to the normal serum level of E2 in humans (10-50 ng/L for males, 20-350 ng/L for women).

4. Discussion and Conclusion

Over the last 10 years, the pesticide concentrations found in fruits and vegetables have decreased and the samples comply with the legal MRLs of pesticides have increased (Borrello, 2008; EFSA, 2009). Moreover, the total quantity of utilized pesticides has been reduced; however, specific data for EDC pesticides use for all Europe are unavailable (McKinlay et al., 2008). Regardless, evidence that many pesticides are active *in vivo* at extremely low doses suggests that the permitted residue levels in food may be too high (Hayes et al., 2002; Storrs and Kiesecker, 2004; Weltje et al., 2005). In the present study, all the fruit and vegetable samples that were positive for pesticide residues showed a concentration that was below the MRLs, according to the European Regulation in force (EC 396/2005). However, we also showed that such concentrations might have an estrogenic activity. To date, there is no information in the literature on the estrogenicity of fruits and vegetables in relation to the content of allowable pesticide residues.

In the present study, the *E-screen test* and MELN gene-reporter assay proved to be suitable for the determination of estrogenic activity in extracts from whole fruits and vegetables. The fruit and vegetable samples exhibited an estrogenic potency that was approximately nine orders of magnitude lower than that of E2 and was also lower than either daidzein or endosulfan and triadimenol. Our results noted that the pesticides endosulfan and triadimenol were approximately six and eight orders of magnitude, respectively, less potent than E2, whereas the phytoestrogen daidzein was three to four orders of magnitude less potent than E2. In literature has previously been reported that pesticides and phytoestrogens have different degrees of estrogenicity (Soto et al., 1995; Van Meeuwen et al., 2007), and this observation should be taken into account when determining exposure levels because estrogenicity, and not the concentration, is an important determinant of the biological effect (Shaw and McCully, 2002). It has been shown that the levels of phytoestrogens in human diets tend to be much

higher than the levels of synthetic endocrine-active chemicals (Franke et al., 1998; Irvine et al., 1998; Behr et al. 2011; Waring et al., 2008). However, despite the small number of samples analyzed, we found a correlation between the estrogenicity of fruits and vegetables and the total concentration of pesticide residues, with no correlation between EEQs and the endogenous phytoestrogen concentration. These findings partially confirm the results obtained in a previous work in which only the *E-screen* test was employed (Schilirò et al., 2011). It should be observed that the extracts used in the present study had no specificity, thus they did not reflect the activity of any particular chemical component of the foods (Charles, 2002); furthermore, it should be noted that *in vitro* analyses greatly depend on the preparation of the sample. Many EDCs can be found in fruits and vegetable at very low concentrations, and these kind of produce contain interfering matrix elements that can mask or interfere with the analysis of the sample of interest. There is therefore a requirement for the development of a sample preparation method that can extract, concentrate, and purify samples of interest (Connolly et a., 2011). The results presented here indicate that, most likely for some foods and for low doses, non-additive effects might occur due to different combinations of EDCs. This suggestion is in agreement with other studies (Suzuki et a., 2001; Charles et al., 2007).

The human EEQ values that we calculated from dietary data (47-60 ng EEQ/L/day) most likely represent a significant overestimate because of the underlying assumptions of 100% absorption and no metabolism or excretion. Applying the factor used by Thomson (Thomson et al., 2003), the EEQ plasma levels obtained would result in an estimate of 0.7-0.9 ng EEQ/L, a level that is not likely to be of health significance for the population, confirming the results of our previous study (Schilirò et al., 2011). However, it is also feasible that total daily intake of EDCs might exceed our estimate due to possible contributions from as-yet-undetermined EDCs in foods and beverages (Guenter et al., 2002; Muncke et al., 2009; Stanford et al., 2010; Wagner et al., 2011) and from other environmental sources (Takamura-

Enya et al., 2003). For example, Behr and colleagues (Behr et al., 2011) conclude that pesticide residues exhibit only marginal impacts on the estrogenic activity in the diet.

Our study demonstrates that estrogenicity is present in fruits and vegetables, even in samples that comply with the legal MRLs. However, it is not currently possible to draw conclusions on the potential implications for human health based on the presented *in vitro* data. Further studies are necessary to identify the compounds accounting for the activity and to evaluate their relevance to human health.

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Figure Captions

Figure 1. Maximum proliferative effects induced by fruit and vegetable samples in MCF-7 BUS breast cancer cells, *E-screen*, (A) and maximum luciferase activity induced by fruit and vegetable samples in MELN cells, the MELN gene-reporter assay (B). The sample concentration that produced the maximum effect is reported in brackets: (1) = 1 g/L, (2) = 0.1 g/L, (3) = 0.01 g/L, (4) = 0.001 g/L, and (5) = 0.0001 g/L. The values represent the means ± standard deviations.

Figure 2. Relationship between the calculated *E-screen* test EEQs (open circles), MELN gene-reporter assay EEQs (filled circles), and total pesticide residues in fruit and vegetable samples ($rS = 0.431$, $p < 0.05$, and $rS = 0.602$, $p < 0.01$, respectively).

Figure 3. Relationship between EEQ values (µg/100 g) of fruit and vegetable samples obtained with the *E-screen* test and with the MELN gene-reporter assay ($rS = 0.581$, $p < 0.01$).

Table 1. Pesticide residue concentrations in 24 fruit and vegetable samples (17 fruits and 7 vegetables) supplied by the Regional Environmental Protection Agency. All the positive samples complied with the legal maximum residue levels (MRLs), according to regulation (EC, 396/2005).

Vegetables	**Pesticides residues (MW)**	***Pesticides residues* (ppm)***
Apple 1	-	<0.01
Apple 2	Boscalid (343.21)	0.02
Banane 1	Imazalil (297.18) Thiabendazole (201.25)	0.09 0.06
Banane 2	Imazalil (297.18) Thiabendazole (201.25)	0.12 1.19
Carrot	-	<0.01
Grape	Quinoxifen (308.13)	0.04
Grapefruit yellow	Imazalil (297.18)	0.21
Grapefruit pink	Imazalil (297.18)	0.18
Kiwi	-	<0.01
Lemon 1	Imazalil (297.18)	0.03
Lemon 2	Imazalil (297.18)	0.07
Parsley 1	-	<0.01
Parsley 2	Chlorthal-dimethyl (331.97) Piperonyl butoxide (338.44)	0.16 0.15
Peaches	Chlorpyrifos-methyl (350.59)	0.20
Pear 1	-	<0.01
Pear (organic)	-	<0.01
Potato 1	-	<0.01
Potato 2	Chlorpropham (213.66)	0.02
Strawberry 1	Fenhexamide (302.20)	0.06
Strawberry 2	-	<0.01
Strawberry (organic)	-	<0.01
Tangerine	Chlorpyrifos-methyl (350.59)	0.10
Tomato 1	-	<0.01
Tomato 2	Cyprodinil (225.29) Fenhexamide (320.20) Iprodione (330.17) Mepanipyrim (223.27)	0.11 0.17 0.17 0.07

* detection limit for pesticides residues 0.01 ppm.

Table 2. Estrogenic activity of fruit and vegetable samples in MCF-7 BUS breast cancer cells, as represented by RPE % (relative proliferative effect) and EEQ (estradiol equivalency quantity, µg/100 g), and in MELN gene-reporter luciferase cells, as represented by TRANS % (increased rate of luciferase gene expression) and EEQ (estradiol equivalency quantity, µg/100 g).

	E-screen		***MELN luciferase assay***	
Vegetables (sample code)	**RPE %**	**EEQ (µg/100g)**	**TRANS %**	**EEQ (µg/100g)**
Banana 1*	8	0.100	51	0.607
Banana 2*	nd	nd	30	0.107
Carrots	nd	nd	40	0.034
Grape	26	0.062	40	0.017
Grapefruit yellow*	17	0.034	34	0.203
Grapefruit pink *	>100	0.077	29	0.250
Lemon 1*	84	0.123	55	0.010
Lemon 2*	75	0.076	43	0.019
Parsley 2	nd	nd	49	0.014
Pear, organic	nd	nd	43	0.093
Potato 1	37	0.011	nd	nd
Potato 2	22	0.035	nd	nd
Strawberry 1	28	0.037	42	0.071
Strawberry organic	19	0.062	49	0.041
Tangerine *	17	0.548	29	0.064
Tomato 2*	6	0.616	42	0.610

nd: not detected; * vegetables which contain pesticide's residue with known estrogenic activity.

27 **Table 3**. Phytoestrogen concentrations in fruits and vegetables from the databases of

28 [a]Thomson, 2006 and [b]Kuhnle, 2009. ISO, isoflavones; LIG, lignans; COU, coumestrol.

	Phytoestrogens µg/100g			
Vegetables (sample code)	**ISO**	**LIG**	**COU**	**tot**
Apple[a] (1, 2)	2.1	2.9	0	5.0
Banana[a] (1, 2)	0.8	1.8	0	2.6
Carrot[a]	0.2	6.4	0	6.6
Grape[a]	0.8	8.7	0.1	9.6
Grapefruit[a] (yellow, pink)	0.4	5.6	0.2	6.2
Kiwi[b]	0.5	110	0	110.5
Lemon[b] (1, 2)	2.5	13.5	1.0	17.0
Parsley[b] (1, 2)	59.0	137.0	<1.0	197
Peaches [b]	<1.0	42.0	0	43
Pear[b] (1, organic)	2.3	5.6	0.3	8.2
Potato[a] (1, 2)	0.2	1.2	0	1.4
Strawberry[a] (1, 2, organic)	2.4	48.9	0.3	51.6
Tangerine [b]	2.0	2.0	1.0	5.0
Tomato [a] (1,2)	0.5	9.1	0	9.6

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Figure(s)

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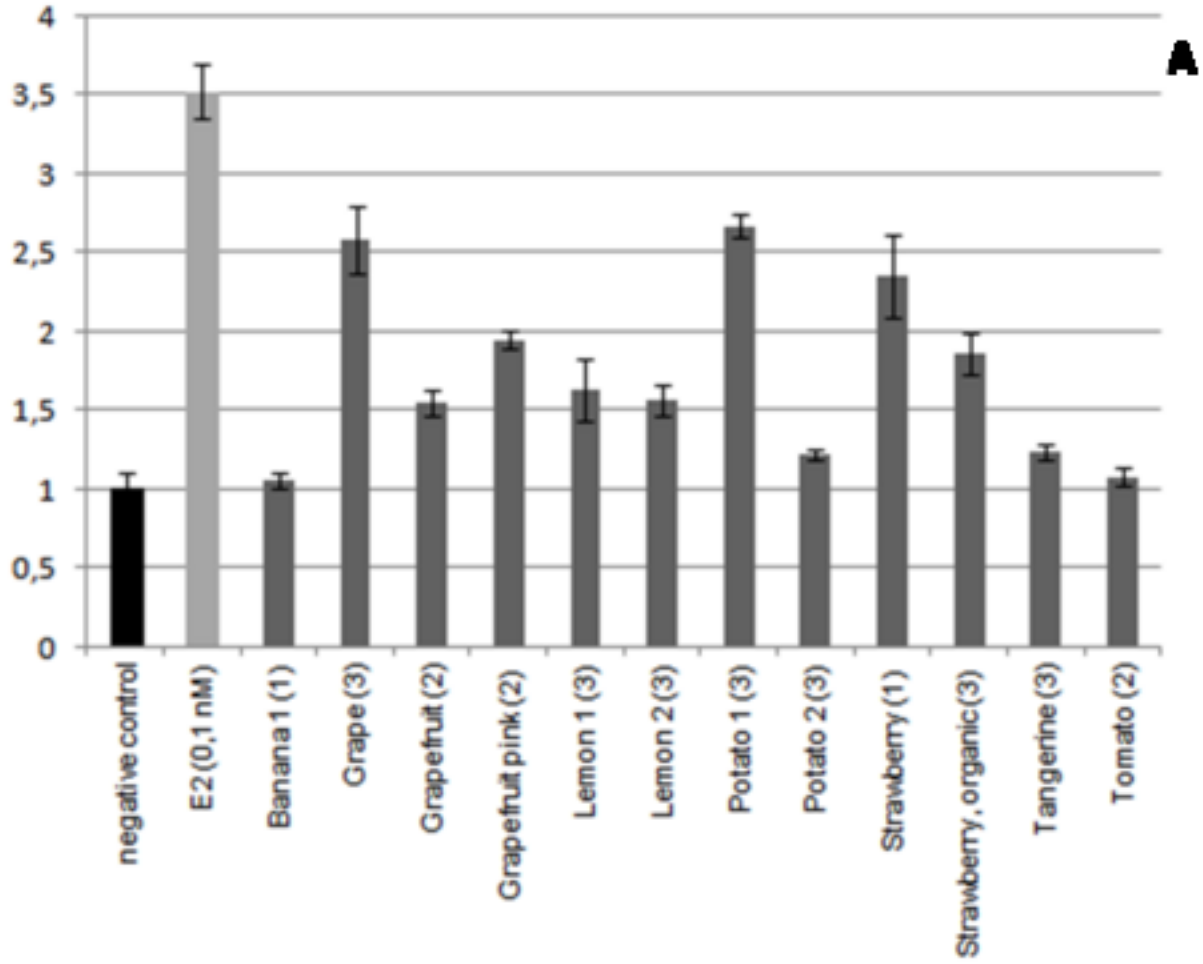

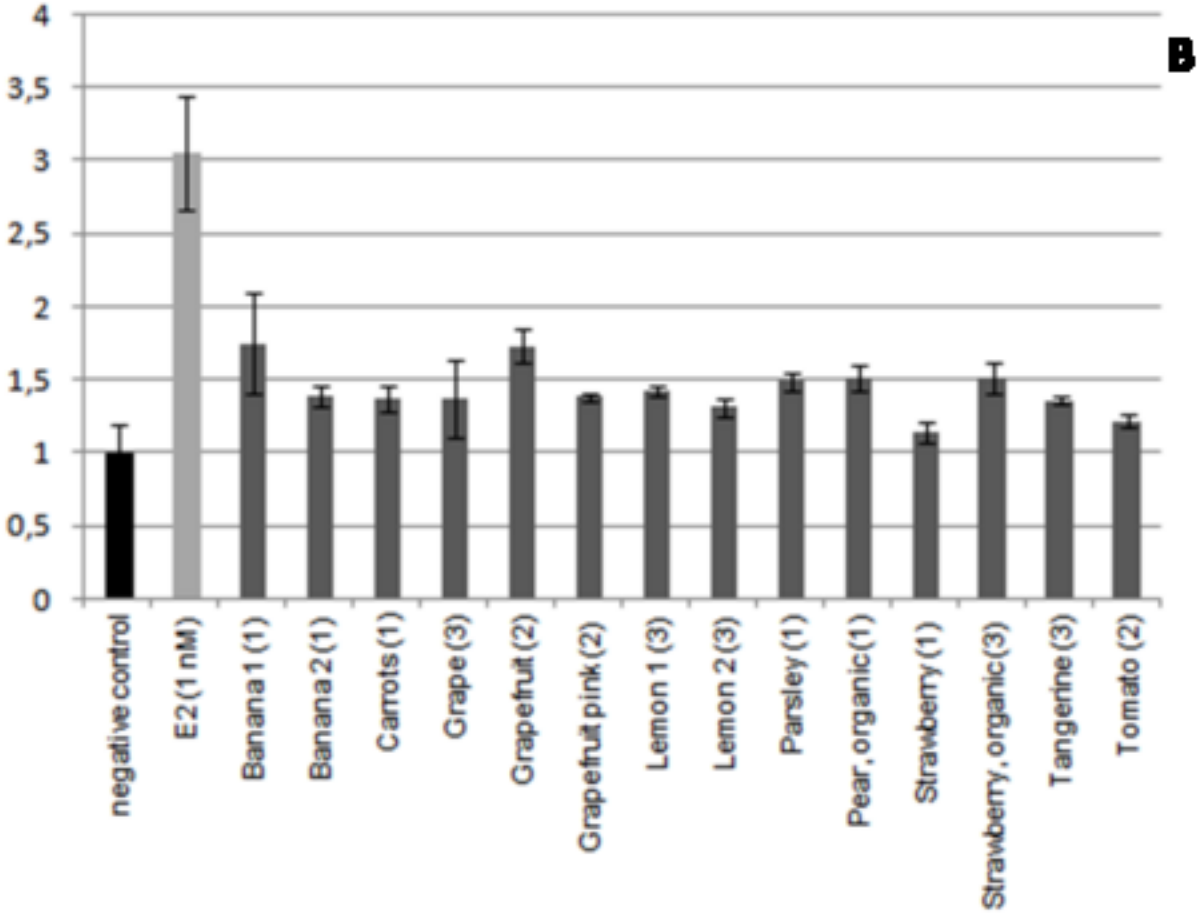

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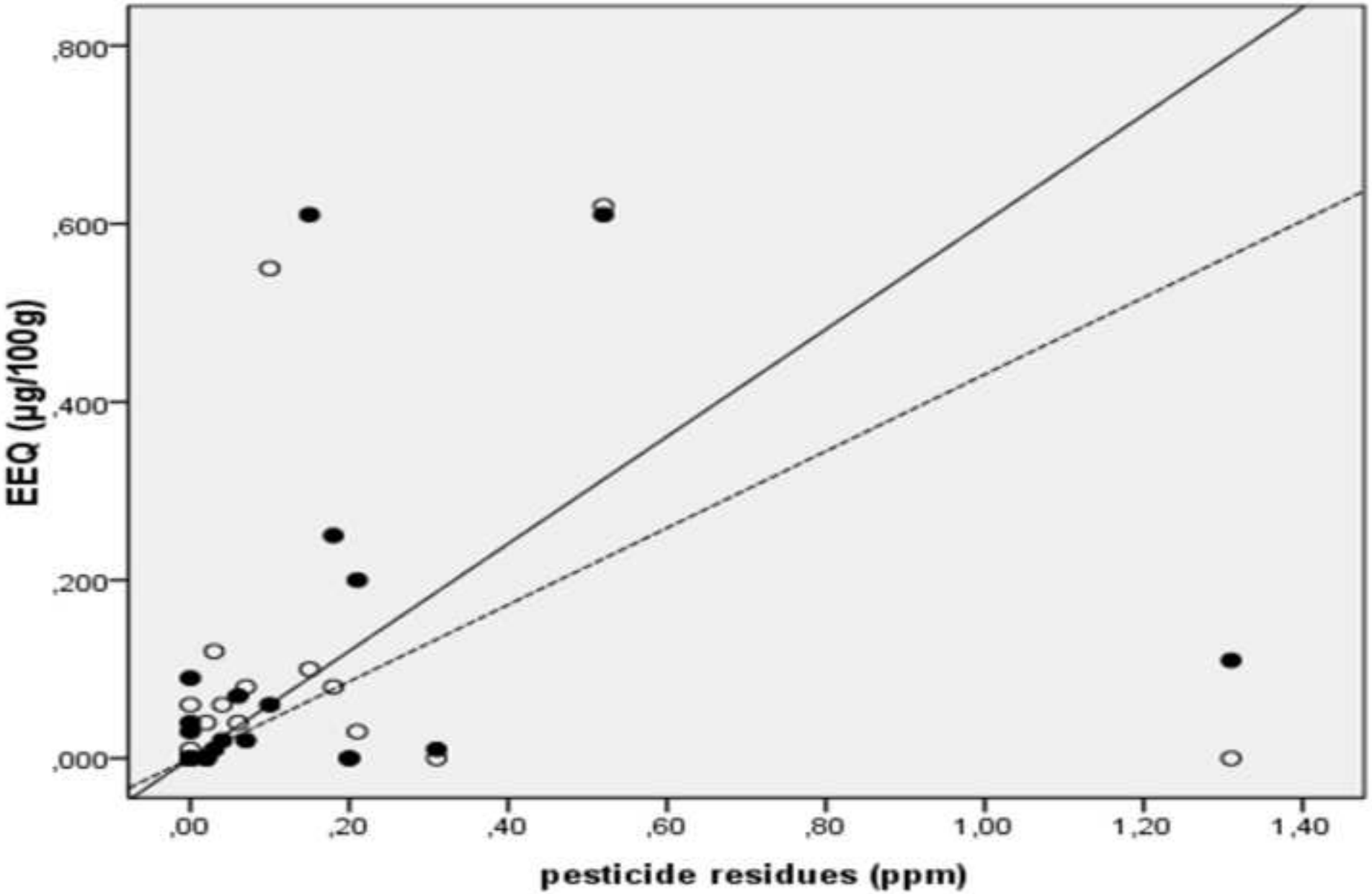

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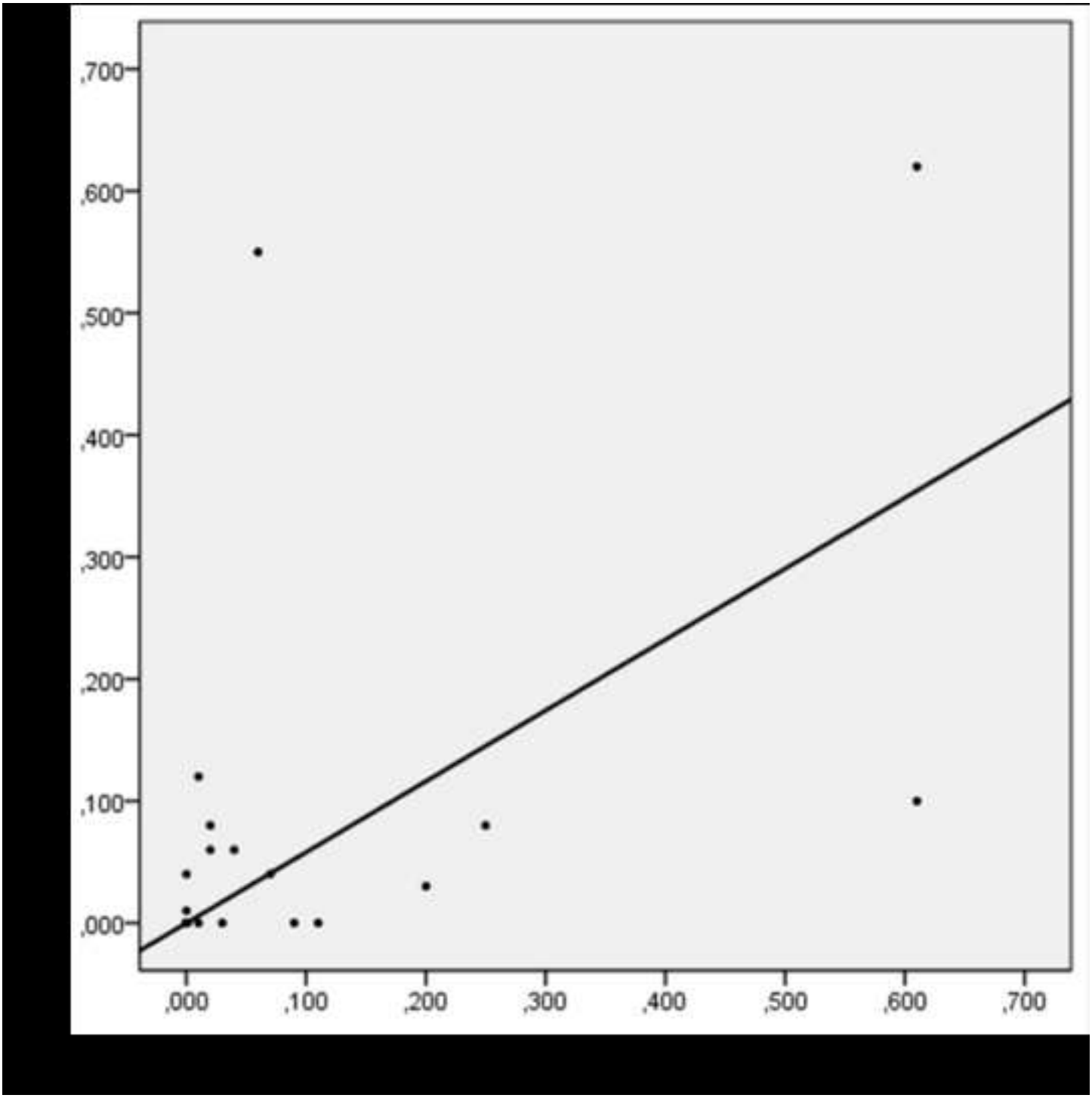

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