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Endocrine disrupting activity in fruits and vegetables evaluated with the *E-screen* assay in relation to pesticide residues☆

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ABSTRACT

Food is likely to be one of the most important routes of human exposure to endocrine disrupting compounds (EDCs). In the present study, we evaluated the total estrogenic activity of fruits and vegetables, which was calculated using the human breast cancer cell line (MCF-7 BUS) proliferation assay (*E-screen*), in relation to pesticide residues. We analysed 44 food samples, 30 fruits and 14 vegetables. Of these samples, 10 did not contain any pesticide residues. The other 34 samples contained from 1 to 7 pesticide residues in concentrations ranging from 0.03 to 1.91 ppm. Estrogenic activity was detected in the 59% of samples tested. The positive controls used were 17-β-estradiol (E2), the phytoestrogen genistein and the pesticide endosulfan. The average value of estradiol equivalency quantity (EEQ) for all positive samples was 0.15 ± 0.32 μg/100 g. A low correlation was found between the concentration of pesticide residues and the EEQ values (Spearman correlation $r = 0.376$ and $p = 0.012$). Using values obtained from the literature, we compared the estrogenic activity of food samples with the intrinsic content of phytoestrogens, but we found no correlations. Our results also suggested that the calculated intake of dietary EDCs might represent a concentration comparable to the normal endogenous estrogen concentration in human blood.

1. Introduction

A number of naturally occurring and synthetic chemicals have been shown to exert adverse effects upon the endocrine system across animal classes, including humans [1–3]. Recognition that chemicals in the environment possess the ability to interact with hormone receptors and mimic hormone activity is considered one of the top five most significant developments in endocrinology of the past century [4]. Endocrine disrupting compounds (EDCs) are defined as "exogenous substances or mixture that alter function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" [2]; at the European Union level, EDCs are included in the list of so-called emerging contaminants [5]. EDCs are ubiquitous in the environment because of their very frequent use in residential, industrial and agricultural applications. The major routes of humans exposure to these EDCs are assumed to involve a normal dietary regimen that includes food containing added antioxidants, compounds leaking from food-wrapping materials and residues of pesticides (i.e., vegetables, fruits and beef and dairy products) [6–8]. A normal diet exposes its consumer to a wide variety of ECDs. The sources of these compounds can be natural in part and anthropogenic. The natural contribution consists of phytoestrogens, nonsteroidal compounds that possess estrogen-like biological activity and that include some isoflavonoids, flavonoids, stilbenes and lignans [9]. Several commonly eaten fruits and vegetables contain phytoestrogens belongin to different classes and present in different quantities. Many literature databases describe food's phytoestrogens content; soy is the major dietary source of phytoestrogens isoflavones [10–12]. The Asian diet is rich in phytoestrogens because it includes large amount of soy products; compared with the Western diet, the Asian diet is associated with a lower incidence of hormone-related diseases including breast cancer and prostate cancer and postmenopausal symptoms (e.g., osteoporosis and hot flashes) [13,14]. The intake of phytoestrogens is estimated to vary from 0.15 to 3 mg/day for the US population to 25–50 mg/day for the population of Eastern and Southern Asia [15–18]. Estrogenic activity of phytoestrogens has

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; PE, proliferative effect; RPE, relative proliferative effect; rS, Spearman rank correlation; SPE, solid phase extraction; Tam, tamoxifen; WHO, World Health Organization.

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been demonstrated in cell culture. However the lack of consistency in epidemiological and experimental results puts these chemicals in category III. This category includes agents for which *in vitro* data exist but for which data from experimental animals concerning adverse effects on endocrine homeostasis are weak or lacking [19].

Anthropogenic endocrine disruptors present in food are substances having different origins. Examples include pesticide residues, as well as compounds leaking from food wrapping material, such as bisphenol A [20]. Some pesticides regularly used in Italian agriculture have shown weak estrogenic responses *in vitro*, for example tolclofos-methyl [21] and triadimenol [22]. Imazalil showed weak anti-estrogenic activity in an *in vitro* reporter gene assay [23] and a negligible proliferation response (not statistically significant) in MCF7 cell proliferation assay [22]. Endosulfan showed an estrogenic response in several *in vitro* tests [21,24,25].

A normal human diet therefore results in exposure to a complex mixture of xenoestrogens that enter the systemic circulation in the body [26]. Natural estrogens are often associated with beneficial effects on the organism; however, it is suspected that anthropogenic estrogens are linked to an increased prevalence of hormone-dependent diseases, including breast and endometrial cancer as well as endometriosis in women [27,28] and testicular dysgenesis syndrome in men [29], furthermore, they are also suspected in the decline in male fertility [30,31].

Several *in vivo* and *in vitro* studies have identified single chemicals that can elicit estrogen like effects [26,32]. In view of the suggested adverse effects of estrogenic chemicals on human and animal health it is important for the risk assessment process to establish the effects of interactions that may result from mixture of these "dietary" chemicals [26,33]. The wide range of possible endocrine-disrupting pathways means that it is difficult to estimate the total sum of dietary (e.g., phytoestrogens-related) and environmental (e.g., pesticides-related) influences, particularly because compounds that do not act at the same point in a particular pathway do not necessarily have additive effects [34].

The aim of the present study is to evaluate the estrogenic properties of fruits and vegetables by using the *in vitro E-screen* assay, performed with human breast cancer cell line MCF7 BUS [32]. We used an unspecific (broad) extraction adapted by Charles et al. [35], in order to obtain crude aqueous preparations of whole foods. These preparations contained a complex mixtures of all the nutrients and the substances present in the foods. The estrogenic response should be the result of the interactions between the natural and the synthetic estrogens. This outcome represents the global estrogenic burden carried by certain plant-derived foods. A further aim of this study is to compare the estrogenic activity of food samples with the content of pesticide residues and also with the intrinsic content of phytoestrogens as found in the literature. Moreover, theoretical blood estrogen activity levels were derived from the dietary intake of EDCs.

2. Materials and methods

2.1. Food samples

We analysed 44 food samples (30 fruits and 14 vegetables) provided by the Regional Environmental Protection Agency (Piedmont A.R.P.A.) between January and June 2007. This agency 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.

2.2. Detection of pesticide residues

All procedures for 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 [36]. All fruit and vegetable samples (500 g each) were first homogenised with ultra turrax according to the provision of Italian Ministerial Decree 27/08/2004 [37] and to Regulation no. 396/2005 of the European Parliament [38]. For the determination of N-methylcarbamates, 20 g of sample was first added to a rate of diatomaceous earth sufficient to obtain the complete absorption of the sample, then analysed using solid phase extraction (SPE) on columns with 1 g polystyrene copolymer resin C18 and a 6 mL reservoir (Varian), eluted with 150 mL CH_2Cl_2, evaporated and dried with a gentle stream of nitrogen. Residues were resuspended in 4 mL cyclohexane:ethyl acetate (30:70), passed through 0.45-μm filters and then purified with gel permeation chromatography (GPC). The column was eluted using a flow rate of 1 mL/min. The solvent was evaporated and dried using a gentle stream of nitrogen. Sample residues were resuspended in 2 mL of methanol and passed trough 0.20-μm filters. This method is based on the ability of the single residue to release methylamine during hydrolysis. This reaction produces, highly fluorescent 1-methyl-2-alkythioisindolo, which can be measured using a fluorimeter. Determination was performed using reversed-phase high-pressure liquid chromatography (HPLC) with post-column reaction and fluorescence detection, SCL-10AVP (Shimadzu Corp, Japan). Analytical conditions were as follow: column temperature 42 °C, reactor temperature 100 °C, flow of each reagent 0.3 mL/min, total flow rate of mobile phase 0.8 mL/min, binary gradient: from 10 to 70% of acetonitrile in 40 min. The limit of quantification was 0.01 ppm [36,39]. Pesticides organophosphorus, organochlorine, pyrethroids, triazine herbicides, and other classes, were determined using a multi-residue analytical method. Briefly, the method consisted of a phase of pre-extraction in which 50 g of the sample were homogenised with 50 mL acetone, 50 mL methanol and 5 g celite; after 15 min of decantation, the sample was filtered and the liquid phase was collected and diluted with distilled water to obtain an acetone concentration ≤5%. Residues were then extracted in SPE on columns with 1 g polystyrene copolymer resin C18 (Varian), activated with 3 mL n-exane, 3 mL methanol and 3 mL of distilled water. Subsequently, residues were eluted with two parts of 3 mL n-exane/ether. The fractions collected were evaporated using a gentle stream of nitrogen. Residues were determined with GC–MS equipped with selective detectors, quadrupole ion trap, ITQ Series GC–Ion Trap MS (Thermo Scientific, Ohio, USA). Samples were resuspended in 1 mL of exane (the internal standard was fenclorofos, 1 ppm). GC conditions were as follow: initially isothermal 70 °C for 1 min, 10 °C/min up to 190 °C, isothermal for 5 min then 5 °C/min up to 250 °C with isothermal for 5 min and then 3 °C/min up to 285 °C with finally isothermal for 17 min with helium flow of approximately 1 mL/min. MS conditions were as follow: acquisition of total ion current in the range 50–450 amu, the electron impact source (E+) by applying a potential of 70 eV. The spectrometer was calibrated by performing the tuning procedure and optimizing the *m/z* ratio at 69 amu (intensity ~100), 131 amu (intensity ~48), 264 amu (intensity ~13) and 502 amu (intensity ~2). From the acquired total ion current, the chromatogram was checked for the simultaneous presence of specific fragments characteristic of the individual chemical species to be analysed. Finally the compounds present in the sample were identified by comparing these results with the spectra obtained from the appropriate libraries (PEST, WILEY, NIST). The quantification procedure took into account the concentration factor of 0.05 associated with the samples. The limit of quantification was 0.01 ppm [36,40].

Table 1
Fruit and vegetable pesticide concentrations, above the detection limit (the detection limit for pesticides residues was 0.01 ppm).

Fruits and vegetables (sample code)	Pesticides residues (MW)	Pesticides residues (ppm)
Apple (284)	Bromopropylate (428.10)	0.05
	Chlorpyrifos-methyl (350.59)	0.05
	Diphenylamine (169.23)	0.73
Apple (39)	Captan (300.59)	0.28
Apple (994)	Captan (300.59)	0.05
	Chlorpyrifos-methyl (350.59)	0.05
Banane (368)	Imazalil (297.18)	0.13
	Thiabendazole (201.25)	0.33
Banane (666)	Phosalone (367.81)	0.05
	Thiabendazole (201.25)	0.18
Banane (692)	Imazalil (297.18)	0.16
	Thiabendazole(201.25)	0.24
Carrots (415)	Pyrimethanil (199.25)	0.40
	Procymidon (284.14)	0.05
	Tolclofos-methyl	0.05
Courgette *(634)*	Procymidon (284.14)	0.06
Grape (1782)	Procymidon (284.14)	0.03
Grapefruit (398)	Chlorpyrifos-methyl (350.59)	0.14
	Ortho-phenylphenol (170.21)	0.38
Grapefruit (706)	Chlorpyrifos-methyl (350.59)	0.09
	Imazalil (297.18)	0.39
	Ortho-phenylphenol (170.21)	0.72
	Thiabendazole (201.25)	0.14
Kiwi (404)	Azinphos-methyl (317.32)	0.05
	Chlorpyrifos-methyl (350.59)	0.05
	Diphenylamine (169.23)	0.05
	Fenhexamide (302.20)	1.33
	Fludioxonil (248.19)	0.05
	Vinclozolin (286.11)	0.05
Kiwi (664)	Fenhexamide (302.20)	1.91
Lattuce (733)	Azoxystrobin (403.39)	0.33
Orange (295)	Chlorpyrifos-methyl (350.59)	0.05
	Malathion (330.36)	0.23
Orange (3297)	Chlorpyrifos-methyl (350.59)	0.05
	Imazalil (297.18)	0.54
	Ortho-phenylphenol (170.21)	0.05
Orange (544)	Chlorpyrifos-methyl (350.59)	0.12
	Imazalil (297.18)	0.78
	Ortho-phenylphenol (170.21)	0.04
Pear (1739)	Chlorpyrifos-methyl (350.59)	0.06
	Etofenprox (376.49)	0.03
Pear (789)	Tolylfluanide (347.26)	0.09
Pear (967)	Chlorpyrifos-methyl (350.59)	0.05
	Etofenprox (376.49)	0.05
	Fenitrotion (277.23)	0.12
	Phosmet (317.32)	0.06
	Procymidon (284.14)	0.25
	Thiabendazole (201.25)	0.70
	Tolylfluanide (347.26)	0.23
Pepper (730)	Cyprodinil (225.29)	0.05
	Triadimenol (295.76)	0.05
Pepper (400)	Cyprodinil (225.29)	0.06
	Fludioxonil (248.19)	0.03
	Pyrimethanil (199.25)	0.05
Pineapple (382)	Triamidefon (293.75)	0.15
	Triadimenol (295.76)	0.19
Pineapple (729)	Piperonyl butoxide (338.44)	0.76
	Triamidefon (293.75)	0.14
	Triadimenol (295.76)	0.24
Pineapple (759)	Piperonyl butoxide (338.44)	1.10
	Triamidefon (293.75)	0.10
	Triadimenol (295.76)	0.29
Potato (297)	Chlorpropham (213.66)	0.19
Potato (507)	Chlorpropham (213.66)	0.63
Potato (830)	Chlorpropham (213.66)	0.15
Raspberry (1553)	Etofenprox (376.49)	0.08
Strawberry (684)	Cyprodinil (225.29)	0.16
	Endosulfan sulphate (422.92)	0.09
	Fludioxonil (248.19)	0.35
Strawberry (783)	Pyrimethanil (199.25)	0.15
	Procymidon (284.14)	0.66
Tangerine (88)	Imazalil (297.18)	0.58
	Orto-phenylphenol (170.21)	0.05
Tomato (369)	Fenhexamide (302.20)	0.26
	Iprodione (330.17)	0.50
	Pyrimethanil (199.25)	0.05
	Procymidon (284.14)	0.15
	Tolylphluanid (170.21)	0.05
Tomato (399)	Cyprodinil (225.29)	0.11
	Fenhexamide (320.20)	0.28
	Fludioxonil (248.19)	0.05
	Procymidon (284.14)	0.20
	Tolylphluanid (170.21)	0.13

If not otherwise specified, all chemicals were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). All chemical standards had a degree of purity ≥99%.

2.3. Preparation of fruit and vegetable samples

Homogenised raw fruits and vegetables were subjected to unspecific extraction in order to obtain whole food extracts. The extraction of food samples was carried out according to the method proposed by Charles et al. [35] and modified for this application: 50 g of homogenised fruit or vegetable was added to 50 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 × *g* for 10 min and the supernatant collected in 50 mL brown glass tubes to obtain a 1 g/mL food extract. Whole food preparations were made ahead of time, frozen and stored at −20 °C. To test the food samples in the *E-screen* assay, they were first thawed at 4 °C overnight, kept at room temperature, filter-sterilised using a 0.22-μm filters and then diluted in steroid-free experimental DMEM (four dilutions from 1 to 0.001 mg/mL).

2.4. Cell culture

The simple and sensitive *E-screen* cell proliferation assay was performed with human MCF7 BUS breast cancer cell line. These cells yield high ERα and are considered the most sensitive line in existence. They are particularly suitable for this assay [41]. 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 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.

2.5. E-screen assay

The *E-screen* assay was carried out according to the method of Korner et al. [42], modified by Schilirò et al. [43] and adapted to food samples. Briefly, subconfluent MCF-7 BUS cells were trypsinised and resuspended in steroid-free experimental medium. The steroid-free experimental medium consisted 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. Cells were seeded into 24-well plates at a density of 40,000 cells/well. The endogenous estrogen 17-β-estradiol (E2), the phytoestrogens genistein and the pesticides endosulfan were used as positive controls. Stock solutions of 10 mM E2 and endosulfan (α and β, 2:1) were prepared with ethanol and a stock solution of 10 mM genistein was prepared with

Table 2
Mean fruit and vegetable pesticide concentrations, positive samples (mean ± standard deviation).

	Positive samples	Fruits	Vegetables
Pesticides residues, ppm	0.571 ± 0.504 (no. 34)	0.655 ± 0.545 (no. 24)	0.372 ± 0.324 (no. 10)
EEQ, μg/100 g	0.250 ± 0.285 (no. 26)	0.279 ± 0.336 (no. 20)	0.146 ± 0.139 (no. 6)

DMSO. All the stocks were stored in brown glass tubes at −20 °C and then diluted to the desired concentrations with steroid-free experimental medium.

After 24 h, the medium was replaced with experimental medium containing one of four different dilutions of food samples. Each dilution was tested in six replicates per assay. One dilution of each food sample found to induce a significant proliferative effect was tested together with 5 nM antiestrogen tamoxifen (Tam) and 0.1 nM E2. Six wells without hormones comprised the negative control. E2 in five concentrations between 1 pM and 10 nM, endosulfan and genistein in five concentrations between 10 μM and 1 nM, formed the positive controls in each assay. The maximum solvent concentration in the culture medium did not exceed 0.1%, a concentration known to have no effect on cell viability. The assays were stopped after six days by determining the absorbance (595 nm) in each well after crystal violet staining.

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:

$$\mathrm{PE} = \frac{(\text{max cell number})\text{sample}}{(\text{cell number})\text{negative control}} \quad (1)$$

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

$$\mathrm{RPE}\,\% = \frac{(\mathrm{PE}-1)\text{sample}}{(\mathrm{PE}-1)\mathrm{E2}} \times 100 \quad (2)$$

Full agonistic activity, RPE > 100%, can be distinguished from partial agonistic activity when RPE is less than 100% [32].

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

$$\mathrm{EEQ} = \frac{(\mathrm{EC50})\mathrm{E2}}{(\mathrm{EC50})\text{sample}} \quad (3)$$

$$\mathrm{EEF} = \frac{(\mathrm{EC50})\mathrm{E2}}{(\mathrm{EC50})\text{compound, positive control}} \quad (4)$$

The EC50 value for the E-screen test (concentration at which 50% of PE is achieved) was calculated with a probit regression (SPSS, Chicago, IL). PE and EC50 values of each sample were calculated from mean dose–response curves established from each experiment. The EEQ, expressed in ng/L, is the total concentration of estrogenic active compounds in a food sample normalised to the natural estrogen E2. The EEF is the quotient of the EC50 values of E2 and of the test compound relative to the natural estrogen E2. If not otherwise specified, all chemicals were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA).

2.6. Statistical analyses

Statistical analyses were performed using the SPSS Package, version 14.0 for Windows (Chicago, IL, USA). EC50 data were analysed by means of a probit regression analysis, means were compared with the *t*-test, and the Spearman rank correlation coefficient (rS) was used to assess relationships between 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 44 food samples analysed, 10 did not contain any pesticide residues. Five of these were samples from organic agriculture. The other 34 contained from 1 to 7 residues per sample, with concentration from 0.03 to 1.91 ppm. Table 1 shows the positive samples in this study among those tested [38], that contained residues above the detection limit. Overall, positive samples contained 28 different types of residues. Those most frequently detected were chlorpyrifos-methyl (in 10 fruits), procymidon (in 3 fruits and 4 vegetables), thiabendazole (in 7 fruits) and imazalil (in 6 fruits). Some positive samples contained residues known to exert estrogenic activities in *in vitro* tests in literature: endosulfan sulphate [21] in strawberry (sample code 684), tolclofos-methyl [23] in carrot (sample code 315), triadimefon [22] in pineapple (sample codes 729, 759, 382) and triadimenol [22] in pineapple (sample codes 729, 759, 382) and peppers (sample code 730).

Based on the total content of pesticide residues in each food, the average concentration of pesticide residues found by the study was greater in fruits than in vegetables, but the difference was not statistically significant ($p > 0.05$) (Table 2).

3.2. Estrogenic activity in positive controls

The mean EC50 value of E2 for the *E-screen* was 1.03 ± 1.72 ng/L (3.82 ± 6.37 pM), EC50 values were calculated from the control curves obtained from each of the bioassays performed. Maximum cell proliferation was generally induced by 0.1 nM E2. Genistein showed a maximum increase in cell proliferation at 1 μM, with an EC50 of 4300 ng/L and endosulfan a maximum increase in cell proliferation at 1 μM and an EC50 of 371,000 ng/L. The isoflavone genistein and the pesticide endosulfan had EEF values of 2.4E−4 and 2.8E−6 respectively. Fig. 1 represents proliferative effects of the three positive controls, expressed using dose–response curves and compared with the negative control.

3.3. Estrogenic activity in fruits and vegetables

Among the 44 food samples analysed, 26 produced an increase in MCF-7 BUS proliferation compared with the control. Maximum PE values are shown in Fig. 2. The other 18 samples did not induced significant cell proliferative activity. 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, EC50 and EEQ in Table 3. The mean EC50 value of fruits and vegetables for the *E-screen* was 1.60 ± 3.63 g/L, with an EEF of 6.4E−10. The mean value of EEQ for all the samples analysed was 0.147 ± 0.21 μg/100 g. The average value of EEQ for all positive samples was 0.250 ± 0.385 μg/100 g and ranged from a minimum of 0.001 to a maximum of 1.587 μg/100 g. The average EEQ concentration of fruits was greater than that of vegetables, but the difference was not statistically significant ($p > 0.05$) (Table 2). The RPE of the fruits and vegetables generally showed partial agonist activity (RPE < 100%), however seven samples exhibited full agonist activity (RPE ≥ 100%).

The five samples containing residue of pesticides having known estrogenic activity (strawberry, 684; carrot, 315; pineapple 729,

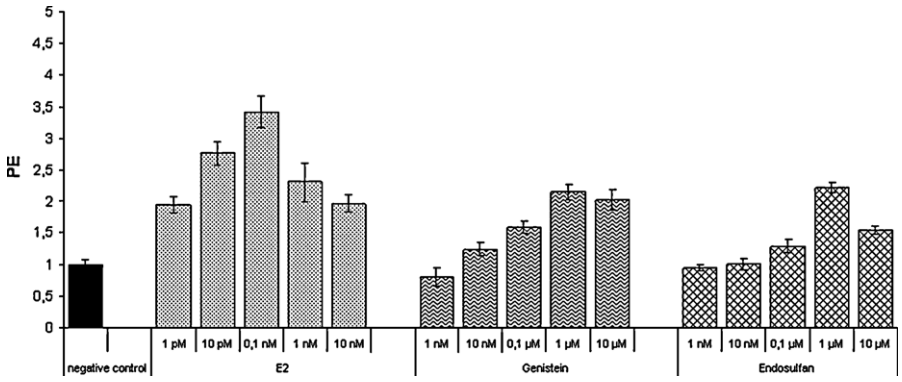

Fig. 1. Proliferative effect (PE) induced in MCF-7 BUS breast cancer cells by 17-β-estradiol (E2), genistein and endosulfan expressed using dose–response curves and compared with the negative control. Values represent means ± standard deviations.

759, 382 and peppers, 730) all induced MCF-7 BUS cell proliferation and showed a significant EEQ value.

A correlation analysis, including all the samples, found a low but significant correlation between the total concentration of pesticide residues and the EEQ values (Fig. 3). EEQ values increased with increasing residue concentration (rS = 0.376 and $p = 0.012$).

The estrogenic activity of all the food samples analysed was compared with their natural content of phytoestrogens. This comparison was based on values obtained from the literature. Many databases describe the phytoestrogen content of foods. Phytoestrogens content differ according to the type of food examined, the class of phytoestrogens investigated and the methods utilised for the analysis. This study used the database compiled by Thomson et al. [44]. This database gives the content of isoflavones, lignans and cumestan for 121 foods habitually consumed in Canada. This database is one of the most frequently updated databases in the literature on food phytoestrogens. It includes most of the foods used in our study (67%) and describes 3 classes of phytoestrogens. We also used the database compiled by Kuhnle et al. [45]. This database gives the content of isoflavones, lignans and cumestan for 240 fruits and vegetables commonly consumed in the UK. The total content of phytoestrogens in 42 food samples in our study, representing 18 different kinds of foods (only the phytoestrogens content of artichokes remained unknown), is presented in Table 4. Analysis of all these samples shows no correlation between the total concentration of phytoestrogens and the EEQ values (rS = 0.246 and $p > 0.05$).

3.4. Estimating the dietary intakes of EDCs

In order to obtain rough estimates of the dietary intake of EDCs [46], the following assumptions were made. We assumed that the total absorption of dietary ECDs was given by the value calculated in this study (0.147 ± 0.321 μg/100 g), we assumed that the

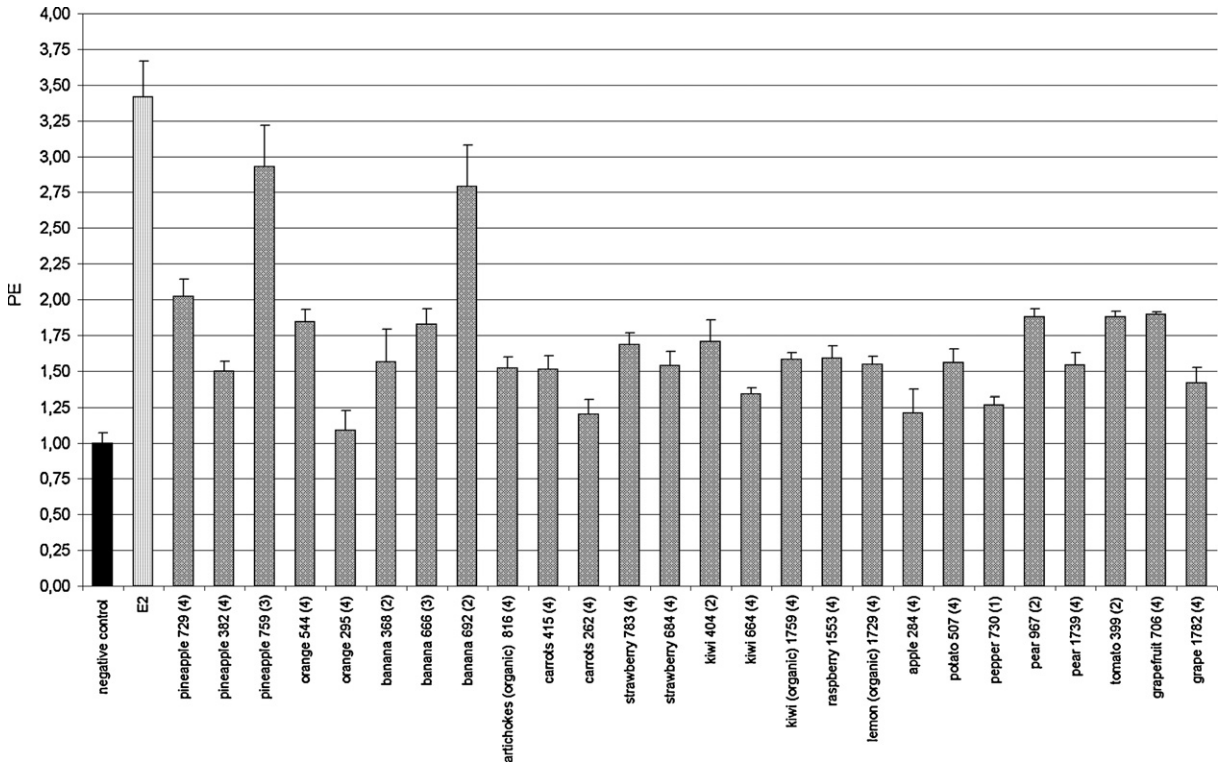

Fig. 2. Maximum Proliferative Effects (PE) induced by fruits and vegetables samples in MCF-7 BUS breast cancer cell. For each sample the sample code is reported along with the concentration that produced the maximum PE: (1) = 1 g/L, (2) = 0.1 g/L, (3) = 0.01 g/L, (4) = 0.001 g/L. E2 concentration is 0.1 nM. Values represent means ± standard deviations.

Table 3
Estrogenic activity of the fruit and vegetable samples in MCF-7 BUS breast cancer cells represented as RPE % (relative proliferative effect), EC50 (concentration at which 50% of the proliferative effect is achieved) and EEQ (estradiol equivalency quantity).

Fruits and vegetables, sample code.	RPE%	EC50 (g/L)	EEQ (μg/100g)
Apple, 284	46	8.241	0.001
Artichokes (organic), 816	115	0.121	0.036
Banana, 368	74	0.380	0.400
Banana, 666	41	0.170	0.735
Banana, 692	50	0.038	0.089
Carrots, 262	22	0.045	0.080
Carrots, 415[a]	24	9.902	0.053
Grape, 1782	11	0.038	0.095
Grapefruit, 706	198	0.040	0.109
Kiwi (organic), 1759	16	0.119	0.030
Kiwi, 404	93	0.489	1.077
Kiwi, 664	36	3.627	0.145
Lemon (organic), 1729	15	0.079	0.046
Orange, 295	3	1.474	0.002
Orange, 544	110	14.666	0.036
Pear, 1739	15	0.039	0.092
Pear, 967	41	0.150	0.029
Pepper, 730[a]	59	0.035	0.124
Pineapple, 382[a]	18	1.150	0.003
Pineapple, 729 [a]	50	0.152	0.822
Pineapple, 759[a]	145	0.002	1.587
Potato, 507	124	0.026	0.167
Raspberry, 1553	21	0.015	0.233
Strawberry, 684[a]	20	0.146	0.025
Strawberry, 783	151	0.143	0.030
Tomato, 399	115	0.302	0.413

[a] Fruits and vegetables which contain pesticide's residue with known estrogenic activity.

mean intake of fruits and vegetables in the European population was 335 g/day [47], we assumed a human blood volume of 5 L, and we assumed that the body can be represented by a single-compartment pharmacokinetic model (although this assumption is clearly not correct). Given these assumptions, the human EEQ (dietary intake) would be 98.5 ng EEQ/L, a value comparable to the normal serum level of E2 in humans. The normal values of E2 serum levels in human are 10–50 ng/L for males, whereas the range of values for nonpregnant premenopausal women is 20–350 ng/L.

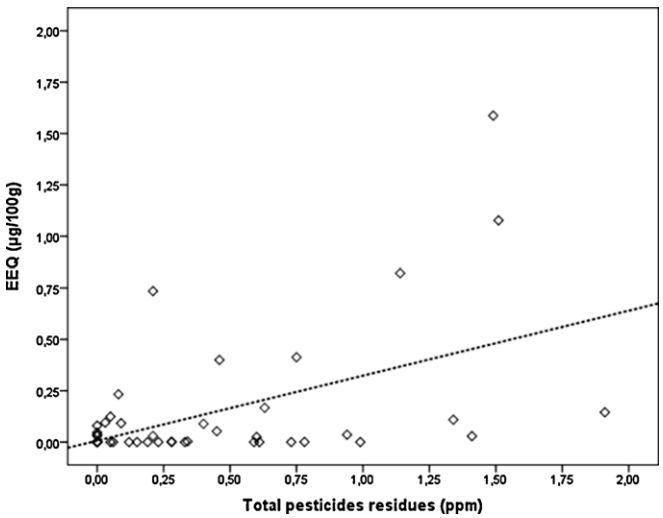

Fig. 3. Relation between the measured *E-Screen* assay EEQ (μg/100 g) and the total pesticide residues (ppm) in all fruit and vegetable samples.

4. Discussion

Several *in vivo* and *in vitro* studies have demonstrated the endocrine-disrupting potential of certain compounds present in human food, in which low-affinity ER ligands such as phytoestrogens and pesticides, can be found [48]. Many attempts have been made to estimate the estrogenic load of food samples by quantifying known ECDs and extrapolating the results based on the individual estrogenic potency of each detected contaminant [49–51]. In the present study, we investigated the estrogenic effect of extracts from whole fruits and vegetables. We analysed these results to find possible correlations with the content of synthetic EDCs (e.g., pesticides) and also of natural EDCs (e.g., phytoestrogens). In general pesticides and phytoestrogens have different degrees of estrogenicity [26,32,46]. Our investigation found that the pesticide endosulfan and the phytoestrogens genistein were 6- and 4-fold less potent than E2 respectively. This consideration must be taken into account when determining exposure levels, because estrogenicity, not concentration, is important the determinant of pharmacological effect [52]. It has been argued that dietary phytoestrogens would overwhelm any activity caused by synthetic EDCs [53] and that the levels of phytoestrogens in human diets and biological fluids tend to be much higher than the levels of synthetic endocrine-active chemicals [54–58]. However, the wide range of possible endocrine-disrupting pathways means that it is difficult to estimate the overall sum of natural (e.g., phytoestrogens-caused) and anthropogenic (e.g., pesticides-caused) influences. In particular, compounds that do not act at the same point in these pathways do not necessarily have additive effects [34]. Indeed, we found little or no correlation between the estrogenicity of food samples and either the total concentration of pesticide residues or phytoestrogen concentration. Furthermore, it should be noted that the fruit and vegetable extracts used in this study did not reflect the activity of any particular chemical components of foods [35]. The study only highlighted the activity of physiologically soluble active compounds.

All the food samples that tested positive for pesticides showed a pesticide concentration that was below the allowable maximum level of residues, according to the European Regulation in force [38]. In general, fruits contained more pesticides than vegetables. This trend reflects the Italian situation described by the Minister of Health. In Italy, fruit trees are probably treated with more pesticides than are vegetables because fruit trees have a longer vegetative cycle and a greater number of pests. Furthermore, fruit trees are subjected to several treatments during bloom, fructification and post-harvesting. In recent years, both the number of irregular samples and the pesticide concentrations found in fruit or vegetable samples have decreased [59,60]. We showed that the pesticide concentrations of the fruits and vegetables in our study fell within the ranges anticipated. However, such concentrations might also have pharmacological effects. Fruits and vegetables had an estrogenic potency approximately 10 orders of magnitude lower than that of E2. However, the values of estrogenic potency that we found for fruits and vegetables were also lower than those of either genistein or endosulfan. These results suggest that for some foods and for low doses, nonadditive effects might occur for combinations of different EDCs. This suggestion is in agreement with the findings of other studies [33,61,62]. The human intake of EEQ that we calculated from dietary data is nevertheless comparable to the normal serum level of E2. This finding is likely to represent a significant overestimate because of the underlying assumptions of 100% absorption, no metabolism, excretion and intake that were made in order to determine the plasma levels of the individual EDCs. Thompson et al. [52] have hypothesised a total theoretical EEQ plasma levels of 467 ng/L for an adult male. They also include a factor in their calculation to account for the difference between theoretical and actual human

Table 4
Fruit and vegetable phytoestrogen concentrations (isoflavones, ISO, lignans, LIG, and coumestrol, COU) from the databases of [a]Thomson et al. [44] and [b]Kuhnle et al. [45].

Fruits and vegetables (sample code)	Phytoestrogens, μg/100 g			
	ISO	LIG	COU	Tot
Apple (39, 284, 319, 683, 994[a])	2.1	2.9	0	5.0
Banana (398, 666, 692[a])	0.8	1.8	0	2.6
Carrot (262, 415[a])	0.2	6.4	0	6.6
Courgette (634, 797[a])	0.1	5.1	0	5.2
Grape (1782[a])	0.8	8.7	0.1	9.6
Grapefruit (398, 706[a])	0.4	5.6	0.2	6.2
Kiwi (404, 664, 1759 [b])	0.5	110	0	110.5
Lattuce (733[a])	0.7	9	0.1	9.8
Lemon organic (1729[b])	2.5	13.5	1	17.0
Orange (295, 315, 544, 3297[a])	2.4	16.5	0.2	19.1
Pear (789, 967, 1739[b])	2.3	5.6	0.3	8.2
Pepper (400, 730[b])	3.9	9	0.3	13.2
Pineapple (382, 729, 759[b])	21	17	0	38.0
Potato (297, 507, 830[a])	0.2	1.2	0	1.4
Raspberry (1553[a])	9.3	37.7	0.5	47.5
Strawberry (684, 758, 783[a])	2.4	48.9	0.3	51.6
Tangerine (88[b])	2	2	1	5.0
Tomato (369, 399[a])	0.5	9.1	0	9.6

plasma levels. The EEQ blood levels obtained using this factor for an adult male were significantly lower 7 ng/L. In terms of our calculations, this factor would result in an estimate of approximately 1.5 ng/L, a level that is not likely to be of health significance for the population.

A final consideration is that the MCF-7 BUS *in vitro* system does not include features other than estrogenic activity. In particular, this system does not account for interactions with ERb, possible nongenomic pathways resulting in estrogenic effects, steroidogenesis, metabolism or kinetics of these compounds [26].

It is therefore theoretically possible that total daily intake of estrogenic compounds might exceed our estimate, owing to possible contributions from as-yet unassessed EDCs in food, EDCs in drinking water and from other sources (e.g., environmental sources). Adding these exposure routes to the intake model might mean that the resulting estimate of total intake would be greater. It is important to estimate EEQ from diet because some susceptible subgroups (e.g., pregnant women and infants) may be more at risk owing to variations in genetic responses to diet and the environment.

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