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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.

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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].

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