ABSTRACT
Humans are currently exposed to a large number of synthetic chemicals. Many of these, known as endocrine disruptors, have hormonal activity and/or interfere with hormonal regulation. Some make their way into our bodies through food, toiletries, lawn care products, and cleaners. Epidemiological studies show that exposure to endocrine disruptors increases the risk of disease. Those that mimic the effect of the female hormone oestrogen are the most numerous. Sporadically, reports in the scientific literature link cases of feminisation of boys and men with oestrogens found in particular toiletries and personal care products. In addition to these rare high dose exposure events, the presence of a variety of endocrine disruptors in the urine of sub-populations from the UK, US and elsewhere, suggests chronic exposure to low levels of hormonally active agents. Some endocrine disruptors are ingested, some may reach the body through air, and yet others are absorbed transdermally (through the skin). If skin care products contain endocrine disruptors, they can reach the body through this route, thus adding to the endocrine disruptor load taken into the body through food and drink. To explore this possibility we conducted a pilot study that assessed the oestrogenicity of five anti-ageing creams chosen among those widely used in the UK; all five creams had oestrogenic activity. Chemical analysis revealed the presence of oestrogenic substances, some of them not present in the ingredient list. In conclusion, the presence of oestrogenic activity in these creams raises concern about the additive effect that could arise from the sum of various small exposures.

INTRODUCTION
Research shows that toxic chemicals in our daily lives may play a role in the increase of the incidence of various diseases. Today, more than 80,000 chemicals are registered for use in the US, many without safety data. In contrast, the European Union takes a “precautionary approach” to chemicals regulation, which means if a chemical is suspected of causing harm steps should be taken to restrict its use. However, despite stringent regulations and the requirement to produce safety data, certain potentially harmful chemicals remain permitted and are used widely. Many of these chemicals make their way into our bodies through various means, such as food, toiletries, gardening products, and cleaners, to name a few. Exposure to these chemicals is putting millions of consumers at increased risk to contract diseases such as asthma, cancer, obesity and infertility. Among these chemicals those that interfere with the endocrine (hormone) system are of particularly high concern. We call them endocrine disruptors. According to the Endocrine Society definition, endocrine disruptors are exogenous chemicals, or a mixture of chemicals, that interfere with any aspect of hormone action. Among endocrine disruptors, those that mimic the effect of the female hormone oestrogen are most numerous.

Our group at Tufts University School of Medicine has demonstrated that environmentally relevant exposure levels of bisphenol-A, a ubiquitous chemical with endocrine disruptor activity, induce anatomical and functional deficits in animals exposed during fetal and neonatal life [1-8]. Once these animals become adult, they have an increased propensity to undergo behavioral [3] and metabolic alterations [9-11], mammary cancer [1;12] and decreased fertility and fecundity [13].

Drs. Soto and Sonnenschein also developed various in vitro tests to screen chemicals for their endocrine disrupting potential, such as the E-SCREEN and the A-SCREEN assay. These assays have allowed them and others to identify numerous endocrine disruptors among chemicals used in large volumes, such as pesticides, plasticizers, industrial chemicals, toiletries and disinfectants [14-16].

Oestrogenic endocrine disruptors, like natural oestrogens could be absorbed transdermally, and thus potentially increase the risk of users to develop diseases such as breast cancer. For this reason, Breast Cancer UK provided us with 5 widely used creams so that we could test their oestrogenic activity. In order to assess this activity the creams have to be treated with solvents that would preferentially extract oestrogens. An accredited independent US laboratory carried out the extractions and analyzed extracts to screen for the presence of chemicals known to have oestrogenic activity.

METHODS

Materials and extraction:

Five brands of European anti-ageing facial cream were selected by Breast Cancer UK and were shipped to the United States for sample preparation and two dimensional gas chromatography-mass spectrometry (GCxGC-MS) analysis. The country of origin listed on the packaging included France, Germany, Poland, United Kingdom and European Union. Three aliquots of 1 gram each of the cream sample were removed from the container and 10 mL of solvent was added. The three types of solvent included dichloromethane, n-hexane and methanol. Each sample aliquot was extracted in a sonicator bath with ice water for one hour. The samples were centrifuged and 5 mL of the supernatant was removed. The 5 mL supernatants were concentrated and reconstituted with 100 µL of ethanol. The ethanol extracts were sent to Tufts University for E-SCREEN analysis. One mL each of the dichloromethane extracts from the 10 mL volume was submitted for GCxGC-MS analysis.

Two Dimensional Gas Chromatography Time-of-Flight Mass Spectrometry (GCxGC-TOF MS).

GCxGC-TOF MS is a high-resolution form of GC/MS wherein two analytical gas chromatography columns are connected in series affording two degrees of separation. Ideally, the two columns have different coating materials rendering the separations on the two columns orthogonal. In between the two columns is a modulator, which regulates the flow of sample between the two columns. The thermal modulator works by applying cryogenically cooled nitrogen gas to trap and focus chemicals eluting from the first column, then injects them onto the second column by the application of heated gas. The second column is typically very short with a separation time on the order of the modulator cycle time (4-6 seconds). As chemicals elute from the second column, they are routed into a Time-of-Flight Mass Spectrometer (TOF MS). A TOF mass spectrometer is utilized instead of a quadrupole because the cryogenically-sharpened peaks require a fast mass analyzer for optimum peak definition.

GCxGC/TOF analysis was performed using an Agilent 7890 gas chromatograph coupled to a LECO PEGASUS 4D -TOF (LECO, St. Joseph, MI). Injection volume was 1.0 µL. The inlet temperature was 260°C and the inlet mode was splitless with a 1 min purge. Separation was achieved using two columns. The primary column (first dimension) was a RXi-1MS (30 m × 0.25 mm × 0.25 µm; Restek, Bellefonte, PA) and the second column (second dimension) was a RXi-17SilMS, (1.5 m × 0.18 mm × 0.18 µm; Restek, Bellefonte, PA). The first column was held at 50 °C for 3 minutes, ramped to 290 °C at a rate of 8°C/min, ramped to 300°C at a rate of 20°C/min and held for 3 minutes. The second column and modulator were offset by 5 and 20 degrees Celsius, respectively. Helium carrier flow was set to constant flow at 1.0 mL/min. The modulation period was 4 seconds (1.0 s hot, 1.0 s cold with 2 cycles per modulation period) through entire run. The mass spectrometer was operated using electron ionization (EI) at 70eV. Spectra were collected from 45–600 m/z with a scan time of 100 spectra/sec.

Data was processed using LECO's Chromatof software (version 4.50.8.0) to deconvolute and integrate peaks and to match them against the NIST 2014 library. The deconvoluted Total Ion Chromatograms

(dTIC) was used to generate peak area. Compounds in the blanks were excluded unless the area was 10x greater in the measured sample. Peaks were reviewed and categorized. Reported concentrations are estimates, as most compounds were quantified against the internal standard, naphthalene-D8, response at a single concentration of 1.0 µg/mL. This does not take into account the response factor of the compounds. Furthermore, extraction efficiency was assumed to be 100% while in reality it is likely lower for many compounds. This assumption results in an under-estimation of concentration. Identifications by the NIST library without reference standard confirmation are tentative. A match factor of 650 was required for a name to be assigned to the peak. All peaks, both named and unknowns, were manually reviewed in which the measured spectrum was compared to the library match. In some cases named compounds did not match the library spectrum and were categorized as unknowns. Compounds were flagged in cases of non-specific mass spectra or when the chemical class but not the exact isomer or chain length could be identified. Compounds below an area count of 100,000 were generally excluded but categorized and archived for future review, if necessary. Following processing on the LECO Chromatof software system, sample data was exported to Excel.

E-SCREEN assay

Chemicals: 17β-E2 (>97% pure) was purchased from Calbiochem; stock solutions and dilutions were made in ethanol and stored at -20 °C.

E-SCREEN: Human breast cancer oestrogen-sensitive MCF-7 cells were used. The assay was described elsewhere [14]. Briefly 24 hours after seeding, cells were rinsed and the medium replaced with cream extracts diluted with 5% charcoal-dextran stripped FBS (CDFBS) in phenol red-free medium. Each experiment included a 15-point 17β-E2 standard dose-response curve (0.05 pM–10.0 nM) run simultaneously with the samples. Assays were repeated three times. Cytotoxicity was evaluated by adding 100 pM 17 β-E2 to each extract to induce maximal proliferation. Lack of toxicity is demonstrated by maximal proliferating activity in all E2-containing wells.

Cell counting and data analysis: Cell number was measured on day six post-treatment after fixing and staining the cells using sulforhodamine B dye; aliquots were transferred to 96-well plates and scanned using a microplate reader.18 The 17β-E2 dose-response curve was used as standard to quantify oestrogenic activity of test samples in oestradiol equivalents (E2Eq) (see figure 1).

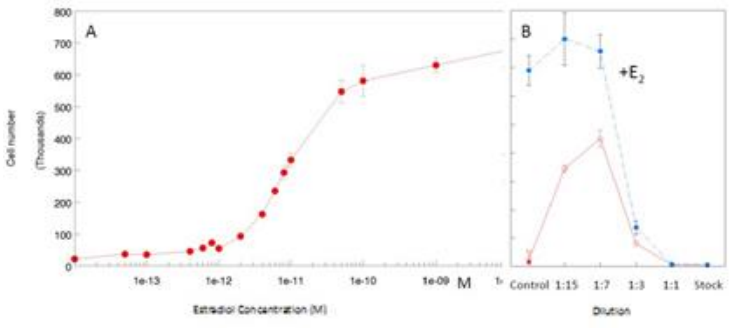

Figure 1. Measuring estrogenicity. A. Dose-response curve to estradiol (E_2). Cell proliferation increases in a dose-dependent manner as the concentration of E_2 increases. B. Dilution curve of a product extract showing estrogenicity. The extract was solvent-exchanged and tested at 5 serial dilutions alone or with 100pM E_2 (blue line). Cell toxicity is observed in a more concentrated dilution of the extract is added (red line, from the stock to 1:3). Amount of estrogenicity is calculated as E_2 equivalents by interpolation to the estrogen dose response curve. Only non-toxic dilutions are used for this determination (1:7 and 1:15). The estrogenic activity of the product tested is 13.23 pM/gram.

RESULTS AND DISCUSSION

Using the ESCREEN assay we have found oestrogenic activity in all the creams. We do not put the creams directly in contact with the cells as this would kill the cells. We try instead to extract oestrogenic activity while aiming to get rid of toxicity. The toxic activity (i.e., killing cells) of creams for cell in culture conditions does not in any way represent true toxic activity to human organisms. If this seems

counterintuitive, consider a common drink such as orange juice; although not toxic, it can kill cells growing in culture, as a result of its acidity. Oestrogenic activity, instead, is a specific activity that can only manifest in cells that contain oestrogen receptors.

Since oestrogenic activity is carried by a variety of compounds of diverse chemical structure that have different physicochemical properties, we treated separate aliquots of each cream with 3 different solvents in the hope that the solvents would preferentially extract different oestrogenic compounds in each extract. These three solvents were Methanol, Dichloromethane and Hexane.

Each of these solvents extracts a different pool of chemicals and among these extracted mixtures there may be some chemicals with oestrogenic activity. This gives us the opportunity to find that one solvent may not extract oestrogenic chemicals but another may extract oestrogenic compounds with different polarity. However, while in general the molecular profile of each extract is different from the others, some components may be extracted by more than one solvent, and so could be present in 2 or even the 3 extracts. For this reason, we cannot add up the oestrogenic activity of each extract at this time. In order to do so, we should do serial extractions (this is, extract the same aliquot of cream with one solvent after the other.

In cream A, the Methanol extraction yielded oestrogenic activity but also demonstrated toxic effects so while the assay showed an oestrogenic equivalent of 3.5 pmoles /g of cream, we expect that the actual level would be higher if there were not toxic compounds present. The oestrogenic equivalents in the Dichloromethane extraction was below detection and the Hexane extraction had an oestrogenic activity of 0.28 pmoles/g of cream.

In cream B, the Methanol and Dichloromethane extractions yielded oestrogenic activity but also demonstrated toxic effects. So while the methanol showed an oestrogenic equivalent of 4.8 pmoles /g of cream and the oestrogenic equivalents in the Dichloromethane extraction was 3.76 pmoles/g, we expect that the actual levels would be higher if there were not toxic compounds present. The Hexane extraction had an oestrogenic activity of 1.58 pmoles/g of cream.

In cream C, the Methanol extraction yielded oestrogenic activity but also demonstrated toxic effects so while the assay showed an oestrogenic equivalent of 3.4 pmoles /g of cream, we expect that the actual level would be higher if there were not toxic compounds present. The oestrogenic equivalents in the Dichloromethane extraction was 0.73 pmoles/g of cream and the Hexane extraction had an oestrogenic activity of 0.68 pmoles/g of cream.

In cream D, all three extracts had oestrogenic effects without toxicity. The range of oestradiol equivalent in the methanol extracts was 13.23-14.63 pmoles /g of cream. The oestrogenic equivalents in the Dichloromethane extraction ranged from 5.99-6.36 pmoles/g and the Hexane extraction had an oestrogenic activity range of 0.89-0.91 pmoles/g of cream.

In cream E, the Methanol extraction yielded an oestrogenic equivalent of 2.46 pmoles /g of cream. The oestrogenic equivalents in the Dichloromethane extraction was 2.24 pmoles/g but also had toxicity present so we expect that the actual level would be higher if there were no toxic compounds. The Hexane extraction had an oestrogenic activity of 0.69 poles/g of cream but also demonstrated toxicity.

The initial results submitted in December 2016 have been confirmed using fresh extracts from the same creams.

Table 1

	Cream A (52)	**Cream B (53)**	**Cream C(54)**	**Cream D (55)**	**Cream E (56)**
MeOH extract	Slightly toxic ≥ 3.5 pmoles/g of cream (Logit b/b0 25%)	Toxic > 4.8 pmoles/g of cream (Logit b/b0 31%)	Toxic > 3.4 pmoles/g of cream (Logit b/b0 24%)	14.63-13.23 pmoles/g of cream (Logit b/b0 73-55%)	2.46 pmoles/g of cream (Logit b/b0 48%)
DCM extract	Below detection	Toxic >3.76 pmoles/g of cream (Logit b/b0 46%)	0.73 pmoles/g of cream (Logit b/b0 18%)	5.99 – 6.36 pmoles/g of cream (Logit b/b0 55-64%)	Toxic >2.24pmoles/g of cream (Logit b/b0 36%)
Hexane extract	Slightly toxic; 0.28 pmoles/g of cream (Logit b/b0 16%)	1.58 pmoles/g of cream (Logit b/b0 30%)	0.68 pmoles/g of cream (Logit b/b0 33%)	0.89-0.91 pmoles/g of cream (Logit b/b0 32-19%)	Toxic >0.69 pmoles/g of cream (Logit b/b0 34%)

Although there is no direct way to find which are the chemicals responsible for the oestrogenic activity just by looking at the analytical data obtained, it is possible to infer which are likely candidates. Table 2 identifies these candidates using different resources such as the Environmental Working Group (EWG) database (http://www.ewg.org/skindeep), the TEDEX database (http://endocrinedisruption.org/interactivetools/tedx-list-of-potential-endocrine-disruptors/search-the-tedx-list created by Theo Colborn), both based on scientific reports, and of course, our own search on the scientific literature.

Table 2: Compounds with reported endocrine disruptor activity found in the extracts.

Name	CAS	Expected Analyte R.T. (s)	EWG rating	in TEDX	591358 (BLK) ug/g	590152 (Cream A) ug/g	590153 (Cream B) ug/g	590154 (Cream C) ug/g	590155 (Cream D) ug/g	590156 (Cream E) ug/g
Styrene	100-42-5	1306 , 2.390	N/A	yes	0.067	0.196	0.181	1.058	1.619	2.839
Benzyl Alcohol	100-51-6	582 , 2.320	5	no				0.509	2.365	0.317
Octanal, 2-(phenylmethylene)-	101-86-0	1302 , 2.070	5	no					6.208	
Hexanedioic acid, bis(2-ethylhexyl) ester	103-23-1	2070 , 3.760	4	Yes						
1-Hexanol, 2-ethyl-	104-76-7	590 , 1.560	N/A	yes	0.557	2.045	3.637	9.647	1.794	8.866
Octanal, 7-hydroxy-3,7-dimethyl-	107-75-5	870 , 1.950	7	no					2.212	
Phenol	108-95-2	522 , 2.070	7	yes			0.367	0.792	0.361	0.239
Ethanol, 2,2'-oxybis-	111-46-6	534 , 2.070	5	no				0.250	0.635	
Benzoic acid, 2-hydroxy-, phenylmethyl ester	118-58-1	1394 , 2.570	7	no					9.009	4.861
2-Ethylhexyl salicylate	118-60-5	1358 , 1.880	4	no				267.666		
Methyl salicylate	119-36-8	778 , 2.150	3	yes			0.260	5.719		6.739
Benzophenone	119-61-9	1194 , 2.760	3	yes			9.992	1.331	0.214	1.066
Ethylparaben	120-47-8	1106 , 2.460	4	Yes			30.817		36.138	
Benzyl Benzoate	120-51-4	1310 , 2.630	6	no					21.093	
Heptanal, 2-(phenylmethylene)-	122-40-7	1218 , 2.110	7	no					0.961	
Ethanol, 2-phenoxy-	122-99-6	798 , 2.420	4	no		195.277	182.970	275.827	2.769	242.356
Tributyl phosphate	126-73-8	1218 , 1.850	N/A	yes		13.043	21.553	0.468	0.489	0.650
Butylated Hydroxytoluene	128-37-0	1110 , 1.760	6	Yes					0.201	48.662
Aspirin	50-78-2	1454 , 1.530	6	no			2.274	3.435		

2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	5466-77-3	1702 , 2.200	6	yes					230.470	0.733
2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester:2		1602 , 2.210	6	yes					0.189	
2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester:3		1670 , 2.180	6	yes					0.267	
Propylene Glycol	57-55-6	226 , 1.770	3	no			32.109	0.123		
1,6-Octadien-3-ol, 3,7-dimethyl-	78-70-6	678 , 1.590	5	no					11.773	
Lilial	80-54-6	1114 , 2.050	7	yes					1.247	
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	84-69-5	1386 , 2.180	1	yes		0.042	0.048	0.322	0.178	
1,2-Benzenedicarboxylic acid, dihexyl ester	84-75-3	1458 , 2.240	1	yes		0.052	0.105	0.223	0.176	0.262
7-Acetyl-6-ethyl-1,1,4,4tetramethyltetralin	88-29-9	1390 , 2.050	N/A	yes						19.330
7-Acetyl-6-ethyl-1,1,4,4tetramethyltetralin:2		1426 , 2.200	N/A	yes						0.687
7-Acetyl-6-ethyl-1,1,4,4tetramethyltetralin:3		1434 , 2.200	N/A	yes						0.544
Methylparaben	99-76-3	1042 , 2.540	4	Yes			82.157		95.348	

The EWG column above is color coded by EWG to indicate the hazard level: green being low hazard and red high. These may be due to carcinogenicity, allergens, or endocrine or developmental toxicity. The TEDX column indicates whether these chemicals have been suspected to disrupt the endocrine system. This is not necessarily due to oestrogenic action but could also be androgenic, anti-oestrogenic or anti-androgenic or affecting the thyroid, or other systems in either male or female reproductive health and development.

In further reviewing the compounds above: styrene, [1-Hexanol, 2-ethyl], Phenol are suspected antagonists. Di-isobutyl phthalate and dihexyl phthalate are also found in some of the creams but these are most commonly reported to affect male health and development. While some salicylate esters are oestrogenic, there is no information available for 2-ethylhexyl salicylate and methyl salicylate is a weak oestrogen mimic [17]. Benzophenone and Butylated Hydroxytoluene are not oestrogenic but may have other endocrine disrupting characteristics [18]. Some benzophenone metabolites are oestrogenic. 7Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin is interacting with the oestrogen receptor [19]. 2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester (also called octylmethoxycinnamate or octinoxate) [20-24] is reported as an oestrogen in vivo in rats and medaka but as these assays can be less sensitive, it is unclear if this compound is active at the levels we have here however we are cautiously classifying this compound as oestrogenically active. Ethyl [25-28] and Methyl Paraben [25;26;29;30], Benzyl Benzoate, Lilial [31] are oestrogenic and are likely contributing to the oestrogenic effects obtained in the ESCREENs of cream extracts.

Because this table results from the DCM fractionation of the creams, the activity obtained in the Hexane and MeOH fractions may be stemming from different sources.

Regarding the compounds present in the analytical data (2D GC-MS) which are not listed in the ingredient list of the products tested, it is likely that they represent contaminants of the main ingredients, or chemical that leach during processing from containers, tubing or factory machinery, or are produced like chemical reactions between ingredients, or leach from packaging used in final marketing.

Finally, although these results are interesting and raise caution regarding product use, more work is necessary to make these data publishable in a scientific journal.

CONCLUSIONS

The most striking conclusion is that all five creams had oestrogenic activity. The second conclusion is that the chemical analysis revealed compounds that are oestrogenic. However, in order to attribute chemical identity to the biological activity revealed by the ESCREEN assay it would be necessary to fractionate the extract by means of liquid chromatography and further analyze the fractions that are oestrogenic.

The results of this pilot study highlight the possibility that long-term use of anti-ageing creams may be linked to an increased risk of breast cancer. Further research is necessary to determine whether this is true and which compounds might be responsible

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