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Measurement of paraben concentrations in human breast tissue at serial locations across the breast from axilla to sternum

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ABSTRACT: The concentrations of five esters of *p*-hydroxybenzoic acid (parabens) were measured using HPLC-MS/MS at four serial locations across the human breast from axilla to sternum using human breast tissue collected from 40 mastectomies for primary breast cancer in England between 2005 and 2008. One or more paraben esters were quantifiable in 158/160 (99%) of the tissue samples and in 96/160 (60%) all five esters were measured. Variation was notable with respect to individual paraben esters, location within one breast and similar locations in different breasts. Overall median values in nanograms per gram tissue for the 160 tissue samples were highest for *n*-propylparaben [16.8 (range 0–2052.7)] and methylparaben [16.6 (range 0–5102.9)]; levels were lower for *n*-butylparaben [5.8 (range 0–95.4)], ethylparaben [3.4 (range 0–499.7)] and isobutylparaben 2.1 (range 0–802.9). The overall median value for total paraben was 85.5 ng g^{-1} tissue (range 0–5134.5). The source of the paraben cannot be identified, but paraben was measured in the 7/40 patients who reported never having used underarm cosmetics in their lifetime. No correlations were found between paraben concentrations and age of patient (37–91 years), length of breast feeding (0–23 months), tumour location or tumour oestrogen receptor content. In view of the disproportionate incidence of breast cancer in the upper outer quadrant, paraben concentrations were compared across the four regions of the breast: *n*-propylparaben was found at significantly higher levels in the axilla than mid ($P = 0.004$ Wilcoxon matched pairs) or medial ($P = 0.021$ Wilcoxon matched pairs) regions ($P = 0.010$ Friedman ANOVA). **Copyright © 2011 John Wiley & Sons, Ltd.**

Keywords: paraben; breast; mammary; oestrogen; cosmetics; breast cancer

INTRODUCTION

The alkyl esters of *p*-hydroxybenzoic acid (parabens) continue to be used widely as antimicrobial preservatives in consumer products to which the human population is exposed, including not only pharmaceuticals and foods but also cosmetics (Loretz *et al*., 2006; Andersen, 2008; Yazar *et al*., 2011). Measurement in 2004 of intact esters in human breast tumour tissue (Darbre *et al*., 2004) at a mean level of 20.6 ng g^{-1} tissue sparked international debate because, although parabens were thought to possess low toxicity (Golden *et al*., 2005; Soni *et al*., 2005), studies from 1998 onwards (Routledge *et al*., 1998) had begun to show parabens as possessing oestrogenic properties (reviewed in Darbre and Harvey, 2008), and oestrogen is known to play a central role in the development, growth and progression of breast cancer (Miller, 1996). Although the source of the paraben could not be identified in the human breast tissue, it was suggested that low-level dermal absorption from personal care products applied to the breast region over the long term might have contributed (Darbre *et al*., 2004; Harvey 2004; Harvey and Darbre, 2004).

Since then, other studies have confirmed that parabens can indeed be absorbed systemically in humans as intact esters from topical application of parabens in cosmetic creams (Janjua *et al*., 2007, 2008), with paraben esters measurable in blood after as little as 1 h after dermal application (Janjua *et al*., 2007). Later, a Norwegian study reported that paraben esters can be measured in over 60% of blood samples taken from the general population and that there was a significant association between blood paraben concentration and self-reported use of personal care products (Sandanger *et al*., 2011). As part of a national survey of exposure to environmental chemicals in the USA, parabens were measured in urine samples across the general US population (Ye *et al*., 2006a, 2006b; Calafat *et al*., 2010). Analysis of 2548 US urine samples showed methylparaben (median 63.5 ng ml^{-1}) and propylparaben (median 8.7 ng ml^{-1}) to be measurable at greatest levels, compared with other esters, and were detectable in 99.1% and 92.7% of samples, respectively (Calafat *et al*., 2010). These data were in agreement with methylparaben also being detectable at the highest levels in archival human breast tumour tissue from Scotland (Darbre *et al*., 2004) and in the survey of blood of the Norwegian general population (Sandanger *et al*., 2011). In the US urine survey, differences in paraben concentrations across demographic groups, especially sex and race/ethnicity, were suggested to reflect different patterns of cosmetic use (Calafat *et al*., 2010), as was also found

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for paraben levels in the blood of the Norwegian population (Sandanger *et al*., 2011). Studies in Denmark and Spain have confirmed the presence of paraben also in urine of the general European population, with parabens measurable in 98% of urine samples from 60 Danish men (Frederiksen *et al*., 2010) and in 100% of urine samples from pregnant women and children in Spain (Casas *et al*., 2011). Two publications report measurement of parabens in human milk samples (Ye *et al*., 2008; Schlumpf *et al*. 2010), and although parabens were reported as being mainly in a conjugated form in human urine (Ye *et al*., 2006b), parabens were detected in human milk in free unconjugated form (Ye *et al*., 2008; Schlumpf *et al*., 2010). Other studies have found parabens in human semen in Denmark (Frederiksen *et al*., 2010) and in the USA (Meeker *et al*., 2011), which has opened a debate on possible involvement in sperm DNA damage and male reproductive health (Meeker *et al*., 2011), especially in view of previously reported reproductive toxicity in male animal models (Oishi 2001, 2002a, 2002b, 2004).

Within the European Union, parabens have been permitted for use in cosmetic products with a maximum concentration of each one of 0.4% and a total maximum concentration of 0.8% (EU Cosmetics Directive 76/768/EEC). However, a recent review has recommended reduction in the levels of *n*-propylparaben and *n*-butylparaben in cosmetic products to a combined maximum concentration of 0.19% with recommendation still pending for isopropylparaben and isobutylparaben (SCCS/1348/10). Parabens are also registered for use in foods and recent regulatory reviews have resulted in the withdrawal of an acceptable daily intake (ADI) level for propylparaben and butylparaben on the grounds of reproductive and endocrine toxicity (EFSA, 2004; JECFA, 2007).

Since exposure of the human population to paraben continues to be widespread (Yazar *et al*., 2011) and parabens have now been detected in human blood, milk and urine as well as human breast tissue (see above), we have analysed a larger series of human breast tissue samples in order to further investigate levels and distribution of parabens in the human breast. Measurement of parabens in the 2004 study (Darbre *et al*., 2004) used a small number (20) of archival breast tumour tissue samples stored from material collected in the 1980s. In the current study, more recent breast tissue was collected from 40 mastectomies for primary breast cancer and measurement at four serial locations across the breast from axilla to sternum was performed to enable assessment of the distribution of parabens at different locations within the breast and in relation to the site of the tumour.

MATERIALS AND METHODS

Source of Human Breast Tissue Material

Ethical approval for the study was obtained from the South Manchester Research Ethics Committee. After patient consent, samples of human breast tissue were collected at the Manchester Genesis Breast Cancer Prevention Centre between 2005 and 2008, stored as fresh frozen aliquots of tissue at $-80\,^{\circ}$C and transported on dry ice. Following mastectomy for primary breast cancer, samples of breast tissue were collected from each of four serial locations linearly across the breast from axilla to sternum, at locations below the dermis and in linear order from axilla lateral, mid to medial. The tissue from each location was divided into four separate vials for storage and allocated two numbers by the clinical team. Patients were numbered firstly as 1–40 and tissues were numbered secondly as 1–4 according to serial location across the breast, with the location numbers 1–4 randomized for each patient. Samples were extracted and HPLC-MS/MS reports were released to the clinical team before release of any decoding of the randomized location numbering or patient data. All patients had primary breast cancer with no evidence of metastases and none had received prior neo-adjuvant treatment. In compliance with the ethical approval obtained, tumour tissue was not collected in order to avoid interfering with histological interpretation and patient management.

Chemical Standards

Methylparaben, ethylparaben, *n*-propylparaben, *n*-butylparaben and benzylparaben were purchased from Sigma (Poole, UK). Isobutylparaben was a gift from Nipa laboratories (Mid-Glamorgan, UK). $^{13}C_6$-*n*-butylparaben and $^{13}C_6$-methylparaben (Cambridge Isotope Laboratories Inc., MA, USA) were prepared in methanol and dilutions also prepared in methanol.

Extractions of Parabens from Human Breast Tissue

All glassware was soaked overnight in concentrated sulfuric acid, rinsed at least six times in HPLC-quality water, soaked overnight in 1 M aqueous NaOH, again rinsed at least six times in HPLC-quality water and left to air-dry. No plasticware was used for any extractions. Glass homogenizers, glass tubes and glass pipettes were used and all glassware went through the same treatment as above. For patients 1–19, the water was deionized and glass distilled. For patients 20–40, the water was purified by reverse osmosis polished to 18.2 mΩ (Triple Red system).

Weighed samples of human breast tissue (100–700 mg) were homogenized in 6.25 ml ethanol–acetone (1:1 v/v) in a glass homogenizer. This mixture was left with periodic shaking overnight at room temperature in a Corex glass tube. The next day, the mixture was centrifuged at 2500 rpm for 10 min in a bench centrifuge at room temperature. The supernatant was transferred to a clean Corex glass tube. The pellet was re-extracted with a further 1.5 ml ethanol–acetone (1:1 v/v), centrifuged at 2500 rpm for 10 min at room temperature and the resulting supernatant combined with the initial supernatant. The combined supernatants were evaporated to dryness under a stream of clean air at room temperature for 2–3 h in a fume hood.

The dried extract was taken up in 3 ml 70% (v/v) aqueous methanol, vortexed well and placed overnight at $-20\,^{\circ}$C. The next day, the mixture was centrifuged at 3200 rpm (rotor Sorvall SW50) for 20 min at $4\,^{\circ}$C in a precoooled rotor/centrifuge. The supernatant was collected and transferred to a clean Corex glass tube. The fat pellet was washed with a further 0.5 ml ice-cold 70% (v/v) aqueous methanol, recentrifuged under the same conditions and the supernatants combined. The combined supernatants were mixed and were divided into two equal samples in new screw-cap autosampler vials (Agilent technologies) and each dried down under centrifugal force and vacuum at room temperature in a SpeedVac concentrator system SPD1010 (ThermoScientific).

Extractions were performed in small groups such that each group had at least one blank extraction performed with all procedures identical except for the omission of any breast tissue. An internal standard was used to estimate recovery through the extraction procedure: for samples 1–19, benzylparaben (50 ng) was

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used in each extraction as internal standard on the basis that this paraben was not previously detected in human breast tissue (Darbre *et al*., 2004). However, since the benzylparaben was not well recovered (see later), for samples 20–40, $^{13}C_6$ *n*-butylparaben (12.5 ng) was used as internal standard.

Analysis by HPLC MS/MS

Analysis was performed in the laboratories of SGS M-Scan in Geneva, Switzerland. Samples 1–19 were analysed in 2008 using an Agilent 1100 HPLC system and ABI/Sciex API3000 triple quadrupole mass spectrometer and samples 20–40 in 2009 using an Agilent 1200 HPLC system and ABI/Sciex API4000 QTrap mass spectrometer, both operating in the on-line liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry–collisionally activated dissociation–mass spectrometry (LC/APCI-MS-CAD-MS) mode. A second internal standard was added to all samples to estimate recovery through the HPLC MS/MS independent of the extraction procedure, and for this $^{13}C_6$-methylparaben was used for all samples analysed in 2008 and 2009. The contents of one autosampler vial (equivalent to half the total extract) were taken up in 250 μl of a solution of the internal standard $^{13}C_6$-methylparaben (25 ng ml^{-1}) in 15 mM ammonium acetate pH 4.5, vortexed for 10 s, sonicated for 1 min and centrifuged for 5 min at 16 000 RCF. Resulting supernatants (10 μl) were chromatographed on a reversed-phase YMC-UltraHT Pro C_{18} column (50 × 2.0 mm) at a flow rate of 400 μl min^{-1} at 25 °C and eluted with a linear binary gradient of 15 mM ammonium acetate pH 4.5 (solvent A) and acetonitrile (solvent B) ($t = 0$ B 30%; $t = 4$ B 70%; $t = 5$ B 70%; $t = 5.1$ B 30%; $t = 11.0$ B 30%). A set of 12 calibration standards were prepared in solvent A at concentrations of 0, 0.25, 0.50, 0.75, 1.0, 2.5, 5.0, 10, 25, 50, 100 and 250 ng ml^{-1} for each paraben ester (methylparaben, ethylparaben, *n*-propylparaben, *n*-butylparaben, isobutylparaben, benzylparaben) and 25 ng ml^{-1} of $^{13}C_6$-methylparaben. Five quality control (QC) standards were also prepared at 0.5, 1.0, 5.0, 10 and 50 ng ml^{-1} of each paraben ester and 25 ng ml^{-1} of the $^{13}C_6$-methylparaben. Negative ion MS-MS detection of the compounds used the transitions as previously published (Darbre *et al*., 2004) each with a dwell time of 75 ms (2008) or 150 ms (2009). The calibration curves all showed linearity with R^2 values >0.95, suggesting the method was linear for all of the analytes over the concentration range used. Calibration and QC standards gave accuracy values in the range of 90.2–112.9 and 81–134% for methylparaben, 90.4–110.8 and 81–135% for ethylparaben, 93.8–106.9 and 94–112% for *n*-propylparaben, 83.1–117.1 and 87–156% for *n*-butylparaben, 81.7–118.2 and 78–144% for isobutylparaben, 84.6–124.6 and 89–141% for benzylparaben for the analyses in 2008 and 2009, respectively. Benzylparaben was not well recovered as an internal standard in the extractions and in no analysis was it recovered above 16%. For this reason, $^{13}C_6$-butylparaben was prepared and used as internal standard to estimate recovery through the extraction process for samples 20–40. The recovery on $^{13}C_6$-butylparaben from the QC standards was within the range 96–195%. In the blanks and extracts, the recovery of $^{13}C_6$-butylparaben gave a median value of 58% with a range from 18 to 102% [average 58 ± 15% (SD)]. The estimated detection limit was 0.25 ng ml^{-1} (equivalent to 0.06 ng per autosampler vial) for the 2008 analyses, and 1.0 ng ml^{-1} (equivalent to 0.25 ng per autosampler vial) for the 2009 analyses.

Data Analysis

Calculation of the amount of paraben measured was carried out by subtraction of the relevant blank from the amount measured per vial and this was then normalized to nanograms per gram tissue. In view of the variability in recovery of internal standards, no corrections were made for recovery to the values per g of tissue presented. Since the measured levels of parabens did not give a normal linear distribution, results are presented as medians, statistical comparisons made with nonparametric tests using the software package for PC Statistica, and graphs are presented using a logarithmic scale. In line with the exploratory nature of this study, no direct, formal adjustment to the significance levels of the multiple Friedman ANOVA analyses has been made. Consistent significant findings at the 5% level are interpreted as giving an indication of a true difference in paraben concentrations.

RESULTS

Paraben Levels in Human Breast Tissue

Parabens were extracted by a method analogous to that used to extract oestradiol from human breast tissue (Van Landegham et al., 1984) and as used for the earlier measurements of parabens in breast tissue (Darbre *et al*., 2004). Extracts were analysed by HPLC-MS/MS against paraben standards as described in the Materials and Methods section. At a practical level, extractions were performed in 23 separate small groupings, each with at least one blank extraction which was performed under identical conditions but simply lacked the addition of any breast tissue. Samples from all four serial locations of one breast were extracted at the same time. Patient numbers 1–19 were extracted in random order and assayed in 2008. Patient numbers 20–40 were collected later, extracted again in random order and assayed in 2009. Table 1 shows the raw data for levels of each of five parabens as measured in one autosampler vial for all blanks and tissue extracts with the relevant amount of tissue used in each case and the randomized patient/location numbers given.

Table 2 shows the concentrations of each of the five parabens with the relevant blank values subtracted and normalized to nanograms per gram tissue. The data are sorted in Table 2 according to linear patient number and serial location across the breast. Values are shown for each of the five parabens individually and also for the total of the five parabens summed together (total). Where blank values were below the detection limit of 0.06 ng per vial (in 2008) or 0.25 ng per vial (in 2009), the upper value of 0.06 or 0.25 was used for subtraction purposes, respectively. Where blank values were higher than levels measured in the tissue, the final number was presented as zero.

Paraben was detected in 158/160 (99%) of the tissue samples: in only two tissue samples were none of the five parabens detectable (patient 9, mid region; patient 34, lateral region) and therefore there was no patient without detectable paraben in their breast tissue. In 96/160 (60%) of the samples all five parabens were measurable. Methylparaben was detected in 152/160 samples (95%), ethylparaben was detected in 147/160 samples (92%), *n*-propylparaben was detected in 138/160 samples (86%), *n*-butylparaben was detected in 120/160 samples (75%), and isobutylparaben was detected in 136/160 samples (85%). The overall median values in nanograms per gram tissue for the 160 samples were for methylparaben 16.6 (range 0–5102.9), for ethylparaben

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Table 1. The HPLC-MS/MS analysis of parabens in four serial locations across the breast (axilla, lateral, mid, medial) from 40 women. Tissue was from radical mastectomy for primary breast cancer but none of the tumour tissue itself was used. Paraben extractions were performed in 23 separate small groupings (Gp) each with at least one blank extraction. For randomized numbering of samples, see Materials and Methods section. Results are shown in nanograms per vial: blank extractions (blank) contained no tissue; amounts of tissue used for each extract in one vial are shown (milligrams tissue)

Group	Tissue no.	Site	mg tissue	Methyl	Ethyl	*n*-Propyl	Isobutyl	*n*-Butyl
1	Blank			0.14	<0.06	0.08	0.10	<0.06
	Blank			0.13	<0.06	0.07	0.09	0.08
	17,1	mid	161	2.50	0.48	0.98	1.00	2.50
	17,2	ax	140	2.75	0.40	0.90	112.50	8.75
	17,3	lat	106	2.13	0.45	0.90	12.75	3.75
	17,4	med	198	2.08	0.43	0.98	4.25	3.00
2	Blank			0.09	<0.06	<0.06	<0.06	0.07
	Blank			0.15	<0.06	<0.06	0.08	0.07
	2,1	mid	168	4.00	0.63	1.85	1.05	3.00
	2,2	ax	198	3.50	0.73	1.83	0.95	3.00
	2,3	med	210	3.50	0.83	1.83	1.00	3.50
	2,4	lat	281	6.25	1.25	2.23	1.23	2.75
3	Blank			<0.06	<0.06	<0.06	<0.06	<0.06
	Blank			<0.06	<0.06	<0.06	<0.06	<0.06
	3,1	lat	173	3.50	0.48	1.73	0.83	2.75
	3,2	med	235	5.00	0.50	1.40	0.63	3.25
	3,3	mid	193	217.50	0.50	1.20	0.40	2.15
	3,4	ax	111	3.50	0.60	1.40	0.85	3.50
	8,1	lat	180	147.50	90.00	2.38	0.88	5.00
	8,2	mid	133	6.50	1.18	2.50	0.73	3.50
	8,3	ax	225	12.50	0.70	3.75	1.08	4.75
	8,4	med	176	10.75	5.50	4.25	0.68	4.00
4	Blank			2.08	0.33	2.00	0.70	3.00
	Blank			0.48	0.18	0.83	0.38	2.00
	14,1	ax	187	4.25	0.58	2.50	0.65	3.50
	14,2	mid	201	3.50	0.58	1.78	0.43	2.50
	14,3	med	103	3.75	0.63	3.00	0.68	3.25
	14,4	lat	146	4.75	0.63	2.00	2.50	3.75
	15,1	mid	147	4.75	0.60	2.50	1.15	3.25
	15,2	med	207	4.00	0.60	2.50	0.78	3.00
	15,3	lat	215	4.25	0.55	2.30	0.65	2.75
	15,4	ax	174	4.50	0.50	2.28	0.65	2.75
	19,1	med	129	5.25	0.73	3.75	0.80	3.50
	19,2	mid	134	3.50	0.65	2.40	0.60	2.75
	19,3	lat	167	3.25	0.70	2.05	0.55	2.75
	19,4	ax	163	4.00	0.85	2.13	0.83	3.75
5	Blank			<0.06	<0.06	<0.06	<0.06	<0.06
	Blank			<0.06	<0.06	<0.06	<0.06	<0.06
	4,1	mid	89	5.25	0.75	3.25	0.55	1.70
	4,2	ax	63	4.00	10.00	3.00	37.50	<0.06
	4,3	lat	66	4.25	0.88	2.75	0.75	3.50
	4,4	med	113	6.25	1.13	6.50	1.65	4.25
	5,1	med	183	4.75	1.08	4.00	2.03	4.00
	5,2	ax	81	4.25	1.18	3.00	1.00	4.25
	5,3	lat	210	3.75	0.80	2.23	0.75	2.50
	5,4	mid	115	3.00	0.65	1.63	1.23	2.20
6	Blank			2.00	0.50	2.40	0.88	1.80
	Blank			0.95	0.28	0.68	0.21	1.40
	9,1	mid	227	0.13	0.15	0.13	0.11	0.10
	9,2	lat	189	3.00	0.63	1.78	0.65	2.75
	9,3	med	151	3.25	0.55	1.40	0.48	2.75
	9,4	ax	261.5	5.50	1.00	5.50	0.90	4.25
	11,1	med	259	4.25	0.88	2.10	0.53	3.00
	11,2	ax	173	3.75	0.75	2.35	0.45	2.15
	11,3	lat	159	3.00	0.58	1.80	0.48	2.75
	11,4	mid	189	3.50	0.83	2.75	0.58	2.75
	10,1	ax	268	3.75	0.83	2.35	0.53	2.38
	10,2	lat	226	4.50	0.98	2.75	0.65	22.50
	10,3	mid	136	2.43	0.60	1.50	0.48	2.33
	10,4	med	114	2.75	0.58	1.45	0.38	2.28
7	Blank			0.50	0.23	0.95	0.30	1.80
	Blank			0.63	0.30	1.20	0.33	1.80
	16,1	med	321	4.25	0.85	3.75	0.35	2.75
	16,2	mid	244	4.25	0.93	1.75	0.73	3.75
	16,3	ax	190	4.25	1.40	2.75	0.55	2.40
	16,4	lat	168	3.25	1.03	2.13	0.43	2.43
	13,1	med	323	4.25	1.35	4.50	0.68	3.75
	13,2	mid	274	4.25	1.18	3.50	0.68	3.25
	13,3	lat	171	7.00	1.90	3.75	0.80	5.00
	13,4	ax	218	5.25	1.73	3.75	0.55	3.75
8	Blank			0.28	0.11	0.73	0.25	1.40
	Blank			0.50	0.14	0.80	0.40	2.30
	1,1	ax	137	3.75	0.60	27.50	0.35	2.75
	1,2	lat	200	3.75	0.60	4.25	11.00	7.25
	1,3	mid	142	725.00	0.65	4.25	0.38	2.30
	1,4	med	190	8.75	1.00	30.00	0.75	3.75
	6,1	ax	231	6.25	2.30	20.50	0.73	3.25
	6,2	mid	152	5.25	1.95	7.75	0.45	2.75
	6,3	med	214	5.75	2.35	15.50	0.70	3.00
	6,4	lat	149	6.00	1.80	18.75	0.78	3.75
9	Blank			2.20	0.35	10.00	0.35	2.80
	Blank			2.40	0.43	2.20	0.40	2.80
	7,1	mid	246	8.25	3.50	47.50	0.80	4.50
	7,2	ax	171	5.25	2.13	85.00	0.58	3.00
	7,3	med	255	8.25	3.50	24.75	0.68	3.25
	7,4	lat	228	6.75	4.00	100.00	0.93	3.75
	12,1	med	232	5.50	1.13	9.25	0.43	2.35
	12,2	ax	226	4.50	0.90	4.50	0.40	2.25
	12,3	lat	176	5.50	1.30	7.75	0.63	2.50
	12,4	mid	198	4.25	0.95	7.00	0.28	1.45
10	Blank			1.93	0.38	1.10	0.30	1.80
	Blank			1.60	0.33	1.50	0.40	2.10
	18,1	mid	86	6.00	1.75	5.50	0.63	3.75
	18,2	ax	106	3.50	0.90	3.00	0.48	2.20
	18,3	lat	104	4.25	1.18	40.00	0.60	3.00
	18,4	med	122	3.75	0.93	3.25	0.58	2.48

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Table 1. (Continued)

Group	Tissue no.	Site	mg tissue	Methyl	Ethyl	*n*-Propyl	Isobutyl	*n*-Butyl
11	Blank			12.39	<0.25	7.81	1.91	4.63
	31,1	med	167	20.37	1.89	10.57	2.05	5.10
	31,2	mid	149	18.79	<0.25	9.90	1.98	6.75
	31,3	lat	249	18.18	1.38	9.62	2.15	4.99
	31,4	ax	151	23.55	2.36	10.37	2.21	6.16
	32,1	med	198	15.78	1.31	8.17	1.25	3.98
	32,2	lat	198	15.00	1.37	11.66	1.36	5.32
	32,3	ax	204	15.30	<0.25	8.63	1.27	4.72
	32,4	mid	191	15.53	1.27	10.15	1.52	17.91
12	Blank			<0.25	<0.25	<0.25	<0.25	14.35
	40,1	med	146	16.96	1.27	7.24	1.39	11.34
	40,2	mid	161	14.17	1.32	7.14	1.27	14.20
	40,3	lat	159	17.14	1.86	9.88	1.89	13.63
	40,4	ax	151	13.18	<0.25	7.20	1.50	13.96
13	Blank			2.35	0.25	3.72	1.13	16.08
	28,1	mid	149	18.57	1.51	8.72	1.69	17.84
	28,2	lat	111	14.57	<0.25	8.40	1.27	22.29
	28,3	med	97	16.44	1.53	8.18	1.51	19.04
	28,4	ax	113	17.09	1.53	8.83	1.87	19.83
14	Blank			4.64	<0.25	6.51	1.45	27.50
	39,1	ax	162	16.25	1.53	9.43	1.63	18.28
	39,2	lat	132	17.79	1.52	8.19	1.39	17.19
	39,3	mid	144	16.89	1.63	9.28	2.04	26.16
	39,4	med	145	17.90	1.83	9.08	1.19	21.37
	35,1	mid	137	16.43	1.27	8.16	1.62	9.63
	35,2	med	128	16.51	1.73	8.56	1.92	11.65
	35,3	lat	152	14.50	1.78	9.80	2.33	11.92
	35,4	ax	139	15.82	1.84	9.75	1.80	13.11
15	Blank			2.27	0.22	4.50	1.11	9.37
	29,1	ax	129	19.32	8.16	10.44	1.74	9.37
	29,2	lat	127	16.98	1.85	8.24	2.11	6.95
	29,3	mid	129	14.98	1.73	9.04	45.25	17.01
	29,4	med	175	15.31	2.11	10.92	2.36	11.24
16	Blank			<0.25	<0.25	<0.25	<0.25	7.02
	30,1	med	160	28.94	2.21	14.98	2.55	16.99
	30,2	ax	119	27.19	2.21	14.82	2.25	16.57
	30,3	lat	200	27.69	2.67	13.87	2.26	12.60
	30,4	mid	149	332.96	1.54	9.99	1.90	12.95
	36,1	med	180	24.10	2.34	152.64	2.51	16.79
	36,2	mid	110	26.83	2.46	19.40	3.36	1.16
	36,3	ax	174	18.02	1.70	14.20	1.73	8.02
	36,4	lat	120	19.16	1.75	12.21	2.44	8.72
17	Blank			<0.25	<0.25	37.50	<0.25	7.25
	33,1	ax	152	2.52	0.75	98.99	0.90	9.94
	33,2	mid	113	2.03	0.64	49.29	0.93	6.70
	33,3	med	142	2.17	0.72	58.37	0.60	5.21
	33,4	lat	143	2.35	0.73	60.06	1.12	8.31
18	Blank			<0.25	<0.25	13.26	<0.25	2.42
	25,1	ax	160	2.65	0.78	17.70	0.57	2.71
	25,2	mid	106	1.36	0.98	17.21	0.59	2.35
	25,3	med	163	0.83	0.38	17.06	0.43	2.38
	25,4	lat	143	0.84	0.34	21.21	0.30	2.07
19	Blank			<0.25	<0.25	27.50	<0.25	3.28
	38,1	ax	152	1.43	0.67	34.74	0.62	3.64
	38,2	lat	125	1.37	0.64	32.30	0.44	2.92
	38,3	mid	150	1.09	0.35	29.84	0.37	3.17
	38,4	med	197	0.61	0.30	31.92	0.34	2.47
	37,1	mid	172	0.62	0.34	41.91	0.42	2.31
	37,2	ax	149	0.63	0.99	73.07	0.67	3.45
	37,3	lat	155	0.40	0.32	83.26	0.73	4.57
	37,4	med	123	0.49	0.36	119.63	1.04	6.02
	26,1	mid	181	0.93	0.45	114.29	0.83	6.43
	26,2	ax	103	1.41	0.54	127.04	0.77	6.54
	26,3	med	137	0.81	0.47	109.41	0.82	7.10
	26,4	lat	200	1.01	0.40	45.90	0.67	6.37
20	Blank			<0.25	<0.25	45.00	<0.25	3.00
	Blank			<0.25	<0.25	55.00	0.30	2.69
	Blank			<0.25	<0.25	67.50	<0.25	3.73
	27,1	lat	195	1.18	0.37	74.57	0.65	2.94
	27,2	mid	185	0.38	0.25	62.74	0.76	3.50
	27,3	ax	146	0.72	0.41	74.55	0.41	2.27
	27,4	med	191	1.90	0.38	6.49	0.62	3.16
21	Blank			<0.25	<0.25	14.53	<0.25	0.47
	Blank			<0.25	<0.25	14.36	<0.25	0.87
	Blank			<0.25	<0.25	115.00	<0.25	1.10
	20,1	ax	46	7.69	0.44	142.39	0.63	5.21
	20,2	lat	60	0.65	0.43	123.26	0.78	6.02
	20,3	mid	57	0.76	0.50	119.18	0.60	5.36
	20,4	med	74	0.74	0.32	138.04	0.60	4.62
	21,1	mid	118	0.59	0.50	137.72	0.67	5.67
	21,2	ax	73	0.69	0.50	135.49	0.83	5.49
	21,3	med	196	0.88	0.49	137.47	0.39	5.51
	21,4	lat	108	0.63	0.45	7.82	0.45	4.65
22	Blank			<0.25	<0.25	15.80	0.37	0.67
	22,1	lat	112	3.36	0.58	12.47	0.53	1.07
	22,2	med	88	0.76	0.49	10.21	0.42	1.00
	22,3	mid	106	0.59	0.29	10.48	5.77	1.09
	22,4	ax	117	<0.25	<0.25	12.88	<0.25	4.63
	23,1	mid	77	0.27	0.34	41.06	0.76	2.09
	23,2	ax	56	0.29	0.42	10.78	1.04	0.93
	23,3	lat	67	<0.25	0.33	8.06	1.11	0.74
	23,4	med	76	0.59	0.57	9.70	37.02	<0.25
23	Blank			<0.25	<0.25	9.88	3.39	1.06
	24,1	med	80	<0.25	<0.25	14.92	0.87	1.03
	24,2	ax	80	<0.25	<0.25	19.79	<0.25	1.29
	24,3	lat	92	<0.25	<0.25	14.17	0.89	1.51
	24,4	mid	68	<0.25	<0.25	14.16	7.36	1.40
	34,1	med	156	0.70	0.59	7.93	<0.25	0.74
	34,2	ax	108	0.33	0.40	4.75	<0.25	<0.25
	34,3	mid	181	0.31	0.26	4.99	2.21	<0.25
	34,4	lat	133	0.25	<0.25	7.86	<0.25	<0.25

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Table 2. Concentrations of parabens in human breast tissue from four serial locations across the breast (axilla, lateral, mid, medial) from 40 women. Tissue was from radical mastectomy for primary breast cancer but none of the tumour tissue itself was used. Results are as in Table 1, but now shown with the corresponding blank values subtracted, values normalized to nanograms per gram tissue and samples sorted according to patient number and location within the breast

	Axilla						Lateral						Mid						Medial					
Patient	Methyl	Ethyl	*n*-Propyl	Isobutyl	*n*-Butyl	Total	Methyl	Ethyl	*n*-Propyl	Isobutyl	*n*-Butyl	Total	Methyl	Ethyl	*n*-Propyl	Isobutyl	*n*-Butyl	Total	Methyl	Ethyl	*n*-Propyl	Isobutyl	*n*-Butyl	Total
1	24.5	3.4	195.2	0.1	6.5	229.8	16.8	2.4	17.5	53.4	27.0	116.9	5102.9	3.7	24.6	0.3	3.1	5134.5	44.0	4.6	153.9	2.2	9.9	214.6
2	17.1	3.4	8.9	4.4	14.8	48.6	21.8	4.2	7.7	4.1	9.5	47.4	23.1	3.4	10.7	5.8	17.4	60.4	16.1	3.6	8.4	4.4	16.3	48.9
3	31.0	4.9	12.1	7.1	31.0	86.0	19.9	2.4	9.6	4.4	15.5	51.9	1126.6	2.3	5.9	1.8	10.8	1147.4	21.0	1.9	5.7	2.4	13.6	44.6
4	62.5	157.8	46.7	594.3	0.0	861.3	63.5	12.3	40.8	10.5	52.1	179.2	58.3	7.8	35.8	5.5	18.4	125.8	54.8	9.4	57.0	14.1	37.1	172.3
5	51.7	13.8	36.3	11.6	51.7	165.1	17.6	3.5	10.3	3.3	11.6	46.3	25.6	5.1	13.6	10.1	18.6	73.0	25.6	5.5	21.5	10.7	21.5	85.0
6	25.4	9.4	85.5	1.7	6.0	127.9	37.7	11.2	120.7	3.0	12.7	185.3	32.0	12.0	46.0	0.8	5.9	96.6	25.0	10.4	68.9	1.7	5.3	111.4
7	17.3	10.1	461.4	1.1	1.5	491.5	19.6	15.8	411.8	2.4	4.4	454.0	24.2	12.6	168.3	1.7	7.1	214.0	23.4	12.2	73.1	1.2	2.0	111.8
8	55.3	2.8	16.4	4.5	20.8	99.9	819.1	499.7	12.9	4.5	27.4	1363.6	48.4	8.4	18.3	5.0	25.9	106.0	60.7	30.9	23.8	3.5	22.4	141.3
9	15.4	2.3	15.1	1.4	10.2	44.5	8.1	1.2	1.2	0.6	6.2	17.4	0.0	0.0	0.0	0.0	0.0	0.0	11.9	1.1	0.0	0.0	7.7	20.7
10	8.5	1.6	3.0	0.0	3.0	16.1	13.5	2.6	5.3	0.5	92.6	114.4	7.1	1.5	0.0	0.0	5.5	14.1	11.3	1.6	0.0	0.0	6.1	19.0
11	13.2	2.1	4.6	0.0	3.3	23.2	9.7	1.2	1.6	0.0	7.4	19.8	10.8	2.3	6.3	0.2	6.2	25.8	10.8	1.9	2.1	0.0	5.5	20.3
12	9.8	2.3	0.0	0.1	0.0	12.1	18.2	5.2	9.4	1.4	0.0	34.2	9.9	2.8	4.5	0.0	0.0	17.3	13.8	3.2	13.6	0.2	0.0	30.8
13	21.5	6.7	12.2	1.1	8.9	50.5	37.7	9.5	15.6	2.9	18.7	84.4	13.5	3.3	8.8	1.3	5.3	32.2	11.4	3.3	10.6	1.1	6.0	32.5
14	15.9	1.7	5.8	0.6	5.3	29.4	23.8	2.6	4.0	13.4	8.6	52.4	11.0	1.6	1.8	0.0	0.0	14.5	24.0	3.6	15.4	1.3	7.3	51.7
15	18.5	1.4	5.0	0.6	1.4	27.0	13.8	1.4	4.1	0.5	1.2	21.0	23.6	2.4	7.4	4.1	5.1	42.7	13.1	1.7	5.3	1.1	2.4	23.6
16	19.4	5.9	8.8	1.3	3.2	38.6	16.0	4.5	6.2	0.7	3.7	31.1	15.1	2.7	2.7	1.7	8.0	30.2	11.5	1.8	8.3	0.1	3.0	24.7
17	18.6	2.4	5.9	802.9	62.0	891.9	18.7	3.7	7.8	119.3	34.7	184.3	14.7	2.6	5.6	5.6	15.1	43.5	9.8	1.8	4.6	21.0	14.8	51.9
18	16.6	5.2	16.2	1.2	2.1	41.3	24.1	7.9	372.3	2.4	9.8	416.6	49.5	16.3	49.1	3.2	20.6	138.7	16.5	4.7	16.1	1.8	4.1	43.2
19	16.7	3.7	4.4	1.7	7.7	34.2	11.8	2.7	3.8	0.1	1.5	19.9	16.6	3.0	7.4	0.4	1.9	29.3	30.8	3.7	18.1	2.0	7.8	62.4
20	161.8	4.1	2052.7	8.3	95.4	2322.3	6.7	3.0	1255.0	8.8	86.8	1360.2	9.0	4.4	1249.5	6.2	79.7	1348.8	6.6	1.0	1217.2	4.7	51.4	1280.9
21	6.1	3.4	1199.0	7.9	64.1	1280.5	3.5	1.9	0.0	1.8	35.5	42.7	2.9	2.2	760.7	3.6	41.2	810.5	3.2	1.2	456.7	0.7	23.9	485.8
22	0.0	0.0	0.0	0.0	33.8	33.8	27.8	2.9	0.0	1.4	3.6	35.7	3.2	0.4	0.0	50.9	4.0	58.6	5.8	2.8	0.0	0.5	3.8	12.8
23	0.7	3.1	0.0	11.9	4.6	20.3	0.0	1.2	0.0	11.1	1.1	13.3	0.2	1.2	328.1	5.1	18.5	353.1	4.5	4.3	0.0	482.2	0.0	491.0
24	0.0	0.0	123.9	0.0	2.9	126.8	0.0	0.0	46.6	0.0	4.9	51.6	0.0	0.0	62.9	58.4	5.1	126.3	0.0	0.0	62.9	0.0	0.0	62.9
25	15.0	3.3	27.7	2.0	1.8	49.9	4.1	0.6	55.6	0.3	0.0	60.7	10.4	6.9	37.2	3.2	0.0	57.7	3.5	0.8	23.3	1.1	0.0	28.7
26	11.2	2.8	881.5	5.0	33.0	933.6	3.8	0.8	48.3	2.1	16.2	71.1	3.8	1.1	431.2	3.2	18.2	457.5	4.1	1.6	534.0	4.1	29.0	572.8
27	3.3	1.1	91.1	0.9	0.0	96.3	4.8	0.6	68.3	1.9	0.0	75.6	0.7	0.0	8.0	2.6	1.6	12.9	8.7	0.7	0.0	1.8	0.0	11.2
28	130.5	11.4	45.3	6.6	33.2	226.8	110.0	0.0	42.2	1.3	55.9	209.5	108.9	8.5	33.6	3.8	11.8	166.5	145.2	13.2	46.0	4.0	30.5	239.0
29	132.2	61.6	46.0	4.8	0.0	244.6	115.9	12.8	29.5	7.9	0.0	166.0	98.6	11.7	35.2	342.1	59.2	546.8	74.5	10.8	36.7	7.1	10.7	139.8
30	226.4	16.5	122.4	16.8	80.3	462.5	137.2	12.1	68.1	10.0	27.9	255.4	2232.9	8.7	65.4	11.1	39.8	2357.9	179.3	12.3	92.1	14.3	62.3	360.4
31	73.9	14.0	17.0	2.0	10.2	117.0	23.2	4.5	7.3	1.0	1.5	37.4	42.9	0.0	14.0	0.5	14.3	71.7	47.8	9.8	16.6	0.8	2.8	77.8
32	14.3	0.0	4.0	0.0	0.5	18.7	13.2	5.7	19.4	0.0	3.5	41.8	16.4	5.3	12.2	0.0	69.6	103.6	17.1	5.4	1.8	0.0	0.0	24.3
33	14.9	3.3	404.5	4.3	17.7	444.7	14.7	3.4	157.8	6.1	7.4	189.3	15.7	3.4	104.3	6.0	0.0	129.5	13.5	3.3	147.0	2.4	0.0	166.3
34	0.7	1.4	0.0	0.0	0.0	2.1	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.4	2.9	2.2	0.0	0.0	0.0	5.1
35	80.4	11.4	23.3	2.5	0.0	117.7	64.9	10.1	21.7	5.8	0.0	102.5	86.1	7.5	12.0	1.3	0.0	106.9	92.7	11.6	16.0	3.7	0.0	124.0
36	102.1	8.3	80.1	8.5	5.8	204.9	157.5	12.5	99.6	18.3	14.2	302.2	241.6	20.1	174.1	28.3	0.0	464.2	132.5	11.6	846.6	12.6	54.3	1057.6
37	2.5	4.9	247.1	2.8	2.1	259.5	1.0	0.4	303.3	3.1	9.3	317.0	2.2	0.5	32.9	1.0	0.0	36.6	2.0	0.9	677.9	6.4	23.5	710.6
38	7.7	2.8	0.0	2.5	3.3	16.2	9.0	3.1	0.0	1.5	0.0	13.6	5.6	0.7	0.0	0.8	0.2	7.3	1.9	0.3	0.0	0.5	0.0	2.6
39	71.7	7.9	18.0	1.1	0.0	98.8	99.7	9.6	12.7	0.0	0.0	122.0	85.1	9.6	19.2	4.1	0.0	118.0	91.5	10.9	17.7	0.0	0.0	120.2
40	85.6	0.0	46.0	8.2	0.0	139.9	106.2	10.1	60.6	10.3	0.0	187.2	86.5	6.6	42.8	6.4	0.0	142.3	114.5	7.0	47.9	7.8	0.0	177.2
Median	**17.2**	**3.4**	**17.5**	**2.0**	**5.0**	**99.3**	**17.9**	**3.2**	**14.2**	**2.4**	**7.4**	**73.3**	**16.1**	**3.1**	**16.2**	**3.2**	**5.7**	**100.1**	**15.0**	**3.5**	**17.1**	**1.8**	**5.8**	**70.4**

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3.4 (range 0–499.7), for *n*-propylparaben 16.8 (range 0–2052.7), for *n*-butylparaben 5.8 (range 0–95.4), for isobutylparaben 2.1 (range 0–802.9), and for the total of the five parabens 85.5 (range 0–5134.5).

Correlation of Paraben Levels with Serial Location Across the Breast

Table 2 gives the values of each paraben as measured in nanograms per gram tissue in each of the four serial locations across the breast from axilla to lateral to mid to medial region. Since variation was substantial between both rows and columns, Table 2 gives the median values, rather than means, for measurement of each of the parabens in each of the locations. The median values were notably similar for each of the parabens in the four serial locations across the breast with methylparaben (15.0–17.9 ng g^{-1} tissue) and *n*-propylparaben (14.2–17.5 ng g^{-1} tissue) being at highest levels. The next highest was *n*-butylparaben (5.0–7.4 ng g^{-1} tissue). The two parabens with lowest levels were ethylparaben (3.1–3.5 ng g^{-1} tissue) and isobutylparaben (1.8–3.2 ng g^{-1} tissue).

Table 2 shows that the distribution of individual parabens was markedly varied. For this reason, all statistical analyses were performed using nonparametric testing. The Wilcoxon matched pairs test was used to compare levels of each of the five individual parabens and the total paraben between every paired combination of the four breast regions. A statistically significant difference was found between levels of propylparaben in the axilla region vs the mid region ($P = 0.004$) and vs the medial region ($P = 0.021$); the correlation with the lateral region was just below the level of significance ($P = 0.064$). Total paraben in the axilla region was significantly different from total paraben in the medial region ($P = 0.048$). None of the other total paraben levels or individual paraben levels showed any significant difference between two sites and all *P*-values were in excess of 0.1 (range 0.116–0.994). The Wilcoxon matched pairs test was also carried out comparing the sums of individual parabens and total paraben in outer (axilla + lateral) vs inner (mid + medial) regions of the breast but no *P*-values below 0.05 were found (range 0.115–0.780).

The Friedman test was used to compare differences between the four serial locations in the 40 patients for each of the individual parabens and for total paraben. A statistically significant value was found for *n*-propylparaben ($P = 0.010$). Values for other tests did not reach significance ($P = 0.677$ for methylparaben; $P = 0.642$ for ethylparaben; $P = 0.512$ for *n*-butylparaben; $P = 0.537$ for isobutylparaben; $P = 0.488$ for total paraben). The Friedman test ranks the level of each paraben as measured in nanograms per gram tissue across the four serial locations of the breast and the average rank values for the 40 patients are shown in Fig. 1 for each paraben across the four breast regions.

Correlation of Paraben Levels with Tumour Location

Owing to the small amount of tissue, it was not considered ethical to use primary breast cancer tissue for nondiagnostic purposes, but since the location of the cancer was known, correlations could be made between the level of paraben measured and whether a tumour was present in that region or not. No tumours were located in the axillary region; 50% of patients had tumours in the lateral region (20/40); four patients had tumours in more than one region. The Mann–Whitney *U*-test showed that there was no significant difference in the percentage of total paraben in a region and whether the tumour was located there or not ($P = 0.742$). Mann–Whitney *U*-tests showed also that there was no significant difference in the amount of methylparaben, ethylparaben, *n*-propylparaben, *n*-butylparaben or isobutylparaben individually in lateral, mid or medial regions and whether the tumour was located there or not ($P > 0.05$ in every case).

Correlation of Paraben Levels with Age of Patient

Spearman correlations were used to investigate any correlation with measured paraben level and age of patient. The mean age of the 40 patients was 65 years with a range from 37 to 91 years. Figure 2 shows graphs of the total paraben in each of the breast regions (axilla, lateral, mid, medial) with age of the patient and Spearman ρ values are given. No statistically significant correlations with $P < 0.05$ were found. Spearman correlations were also calculated for each of the five individual parabens in each of the four locations with age but no statistically significant correlations were found ($P > 0.05$ in every analysis).

Correlation of Paraben Levels with ER Status of the Tumour

Since the presence of oestrogen receptor (ER+) or its absence (ER−) impacts on cancer biology and treatment (Miller, 1996; Lonning 2004) and, since parabens possess oestrogenic activity (Darbre and Harvey, 2008), levels of parabens were correlated between breast tissue samples from patients with ER + and ER − tumours. Information on whether the primary tumour was ER + or ER − was available for 37/40 of the patients. For 27 of the patients, the tumour was ER + and for 10 patients the tumour was ER−. Mann–Whitney *U*-tests were used to investigate whether paraben levels differed between tissue samples taken from breasts containing ER + and ER − primary tumours. Comparisons were made for total parabens and for each of the five individual parabens in each of the four breast locations but none of the *P*-values were statistically significant ($P > 0.05$ in all cases) for differences between ER + and ER−. The Friedman test was used to compare differences for ER + patients and separately for ER − patients between the four serial locations in the 40 patients for each of the individual parabens. The Friedman test ranks the level of each paraben as measured in nanograms per gram tissue across the four serial locations of the breast and is shown in Fig. 3 as performed separately for the ER + and ER − groups with the *P*-values shown. Statistically significant values were found for *n*-propylparaben in both the ER + grouping ($P = 0.035$) and ER − grouping ($P = 0.013$), and also for methylparaben in the ER − grouping ($P = 0.035$).

On the basis that oestrogenic properties of paraben would only influence growth of ER + tumours, the correlation of paraben level with tumour location was repeated using only ER + patients ($n = 27$) rather than all 40 patients, but Mann–Whitney *U*-tests did not reveal any significant ($P < 0.05$) difference in the percentage of total paraben or individual paraben in that region and whether the tumour was located there or not.

Correlation of Paraben Levels with Breast Feeding

Since levels of some lipophilic organochlorine compounds in human milk may be influenced by breast feeding (Harris *et al*., 2001; Solomon and Weiss, 2002; Massart *et al*., 2005; Eskenazi *et al*., 2009; LaKind *et al*., 2009), Spearman correlations were used

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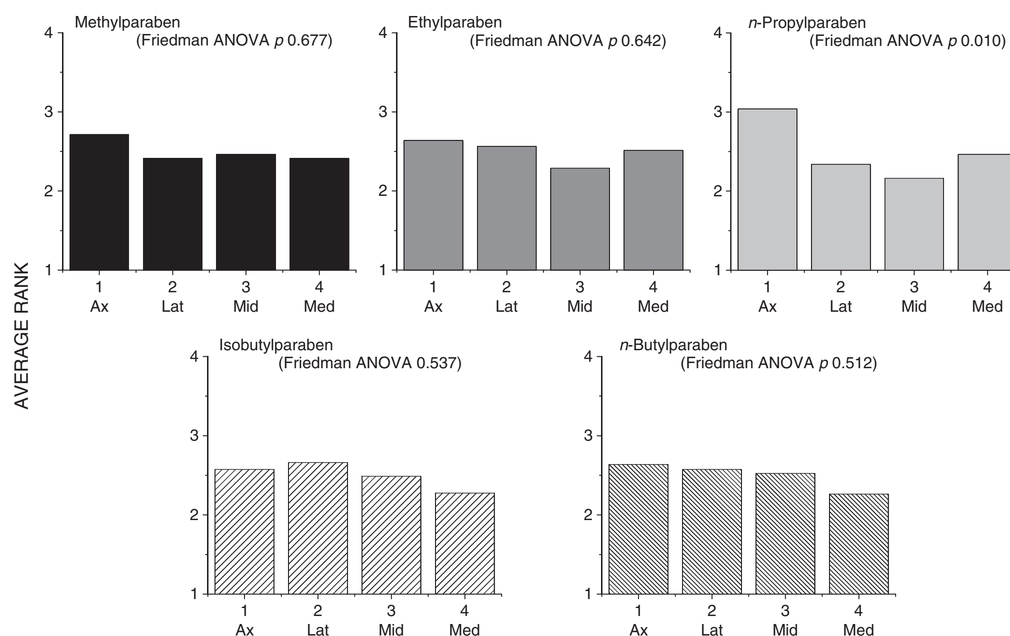

Figure 1. Average rank values according to the levels in nanograms per gram tissue of methylparaben, ethylparaben, *n*-propylparaben, isobutylparaben and *n*-butylparaben in four serial locations across the breast from axilla (Ax) to lateral (Lat) to mid (Mid) to medial (Med) region in breast tissue from 40 women ($n = 40$). Each paraben was analysed individually using Friedman ANOVA to rank the level as measured in nanograms per gram tissue across the four serial locations and the values plotted are the average rank values for the 40 patients in each breast region: the Friedman *P*-value is shown.

to investigate any correlation with measured paraben level and length of breast feeding. Information was available from a questionnaire for 35/40 of the patients as to whether they had breast-fed any children during their lifetime. Seventeen patients (17/35, 49%) reported that they had breast-fed one or more children, but 18 patients (18/35, 51%) reported that they had never breast-fed any child (six of these had not had any children, 12 had had children but had decided not to breast feed). The median length of breast feeding was 5 months (range 2–23 months). No statistically significant correlations with $P < 0.05$ were found. Spearman correlations were also calculated for each of the five individual parabens in each of the four locations with length of breast feeding, but no statistically significant correlations with *P* 0.05 were found. Figure 4 shows the levels of total paraben in each of the breast regions (axilla, lateral, mid, medial) according to length of breast feeding and Spearman ρ values are shown.

Correlation of Paraben Levels with Usage of Underarm Cosmetics

Information was available from a questionnaire for 35/40 of the patients as to whether they had used underarm cosmetics during their lifetime. Twenty-eight patients (28/35, 80%) reported that they had used underarm cosmetics at some time in their lives (user) but seven patients (7/35, 20%) reported that they had never used underarm cosmetic products at any time in their lives (nonuser). Information was not available for total cosmetics of any variety: this relates specifically only to use of underarm antiperspirant/deodorant products. The mean age of the 28 users was 61.8 years (range 37–91 years) and of the seven nonusers was 75.7 years (range 55–85 years).

Mann–Whitney *U*-tests were used to investigate whether paraben levels differed between tissue samples taken from breasts of users vs nonusers. Values of total paraben were significantly greater ($P = 0.043$) in users [median 115.7 ng g^{-1} tissue (range 0–1364)] compared with nonusers [median 41.8 ng g^{-1} tissue (range 13–166)] in the lateral region. However, no significant ($P < 0.05$) differences were found for total paraben in other regions. Analyses of individual parabens showed that values for *n*-butylparaben were significantly different ($P = 0.041$) between users and nonusers in the mid region, but no other significant differences were found for any of the five individual parabens in any of the four breast regions. The Friedman test was used to compare differences separately for users and nonusers between the four serial locations in the 35 patients for each of the individual parabens. The Friedman test ranks the level of each paraben as measured in nanograms per gram tissue across the four serial locations of the breast and average rank values for each paraben in each of the regions are shown in Fig. 5 as performed separately for the user and nonuser groups with the *P*-values shown.

Within the questionnaire, five patients reported that they had used underarm cosmetics during their lifetime but were no longer currently using these products. Reassessment of the Mann–Whitney *U*-test results was carried out as above but now based on 23 current users and 12 not currently using underarm cosmetics. When these five patients were switched in the groupings, no significant differences were found for any of the five individual parabens or the total parabens in any of the four breast regions (range of *P*-values 0.102–0.808).

On the basis that paraben in nonusers of underarm cosmetics could not have originated from the underarm, the correlation of paraben levels with serial location across the breast was reanalysed for the 28 users. Wilcoxon matched pairs testing was repeated for comparisons of the sums of individual parabens

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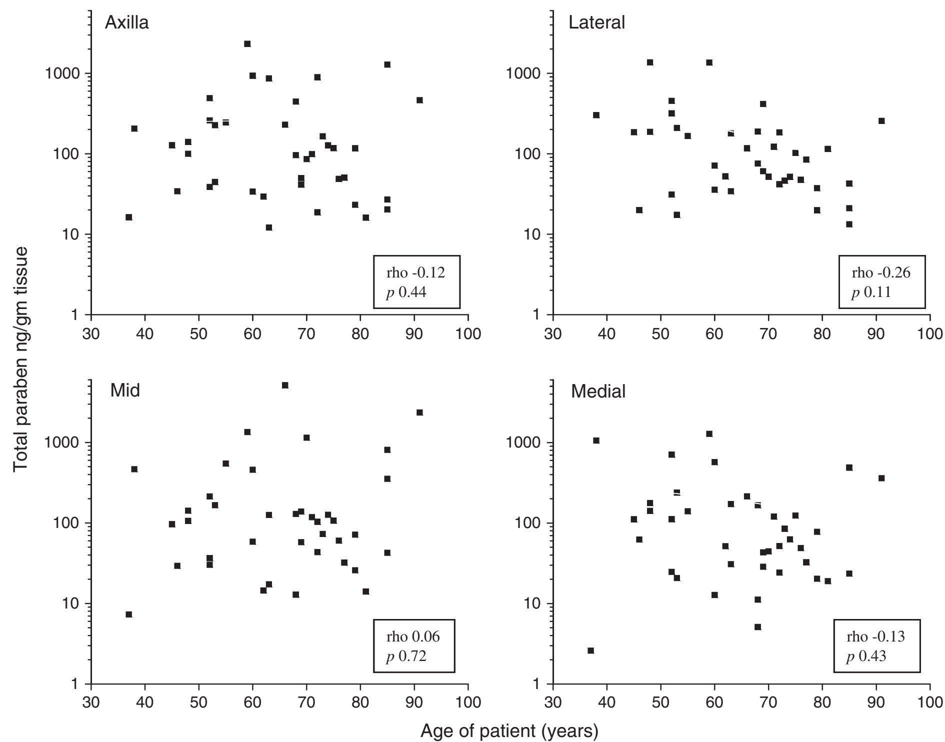

Figure 2. Relation of age of patient with total paraben in each of the four serial locations across the breast ($n = 40$). Results of Spearman correlations are shown in the boxes as ρ values and P-values.

and total paraben in outer (axilla + lateral) vs inner (mid + medial) regions of the breast and this time, significantly higher levels were found in the outer regions than the inner regions for *n*-propylparaben ($P = 0.040$) and for *n*-butylparaben ($P = 0.006$). The Friedman test comparing differences between the four serial locations in the 28 users gave a statistically significant value for *n*-propylparaben ($P < 0.001$). The value for n-butylparaben was $P = 0.073$, but values for the other parabens

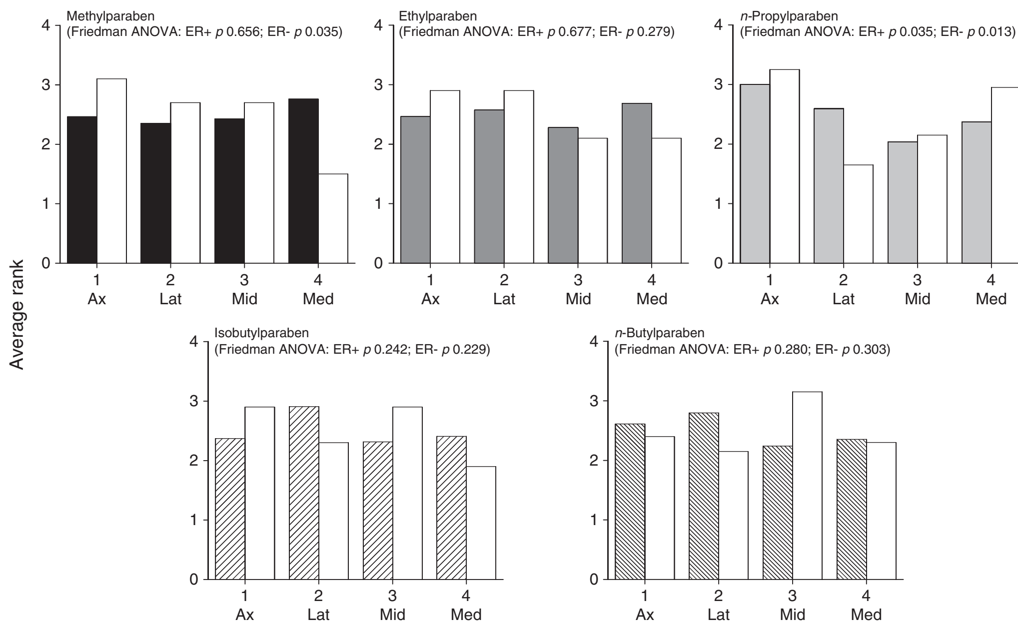

Figure 3. Relation of oestrogen receptor (ER) status of the tumour with average rank values according to the levels in nanograms per gram tissue of methylparaben, ethylparaben, *n*-propylparaben, isobutylparaben and *n*-butylparaben in four serial locations across the breast from axilla (Ax) to lateral (Lat) to mid (Mid) to medial (Med) region in breast tissue separated into ER − (open bars; $n = 10$) and ER + (shaded bars; $n = 27$) patients. Each paraben was analysed individually using Friedman ANOVA to rank the level as measured in nanograms per gram tissue across the four serial locations and the values plotted are the average rank values for each group: the Friedman P-value is shown.

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were much higher ($P = 0.769$ for methylparaben, $P = 0.532$ for ethylparaben, $P = 0.269$ for isobutylparaben; Fig. 5).

DISCUSSION

The results of the current study confirm and expand our previous work (Darbre *et al*., 2004) and indicate that intact paraben esters can be measured in human breast tissue. In our previous study we reported an average of 20.6 ng g^{-1} of paraben in 20 samples of breast cancer tissue collected in Scotland in the 1980s (Darbre *et al*., 2004). Now we report a median value, which is higher, of 85.5 ng g^{-1} of paraben using a larger sample size of 160 breast tissue samples collected in England between 2005 and 2008. There were several variables between these studies, most notably the chronological date, the country of origin and the fact that the 160 samples were of unaffected tissue from a cancerous breast compared with the 20 samples of cancer tissue itself. Whilst it was not considered ethical to use cancer tissue from primary breast cancers owing to the small tissue sample size, the current study has the advantage of enabling measurements at the earliest time point possible after cancer diagnosis and prior to any cancer therapy. The current study has provided a larger sample size (160 rather than 20), and multiple sampling at four serial locations across the breast (axilla, lateral, mid, medial) has enabled study of the distribution of parabens across a single breast for the first time. Since paraben esters were measured in 99% of the samples, this demonstrates that, within the population studied, paraben was widely distributed both within and between breasts. The distribution, however, was notably nonhomogenous and concentrations of individual paraben esters were very varied between the 160 samples. High concentration of one ester was not followed by high concentrations of all the other esters in the same tissue sample and high levels of one ester in one region of the breast was not always followed by high levels of that same ester in other regions from the same breast. Concentrations of individual paraben esters were also varied when compared between similar regions in different breasts.

In contrast to the previous study where 62% of the paraben measured was methylparaben (Darbre *et al*., 2004), this study showed *n*-propylparaben to be present at relatively higher levels and at equivalent levels to the methylparaben. The published measurements of individual parabens in different human tissues have been collated for comparison in Table 3, and assuming 1 g of tissue has a volume of 1 ml, all the units reported are comparable. All studies report methylparaben and *n*-propylparaben to be the two esters measured at highest levels, although in contrast to the present study, all previous studies give lower values for the *n*-propylparaben than for the methylparaben. The relative differences in Table 3 may reflect the different tissues used, the time period of assay and/or exposure in that country. It is also noteworthy that parabens measured in milk were unconjugated (Ye *et al*., 2008; Schlumpf *et al*., 2010), whereas parabens in urine were mainly conjugated (Ye *et al*., 2006b). The issue of conjugation is important to considerations of biological availability but in this study we measured only total paraben (free + conjugated).

The source of the paraben measured in any of these studies cannot be identified and, furthermore, it remains unknown as to whether the measured paraben results from long-term accumulation, current exposure or a combination of both (Darbre and Harvey, 2008). However, in view of previous suggestions concerning potential involvement of chemical components of underarm cosmetics in breast cancer development (Darbre,

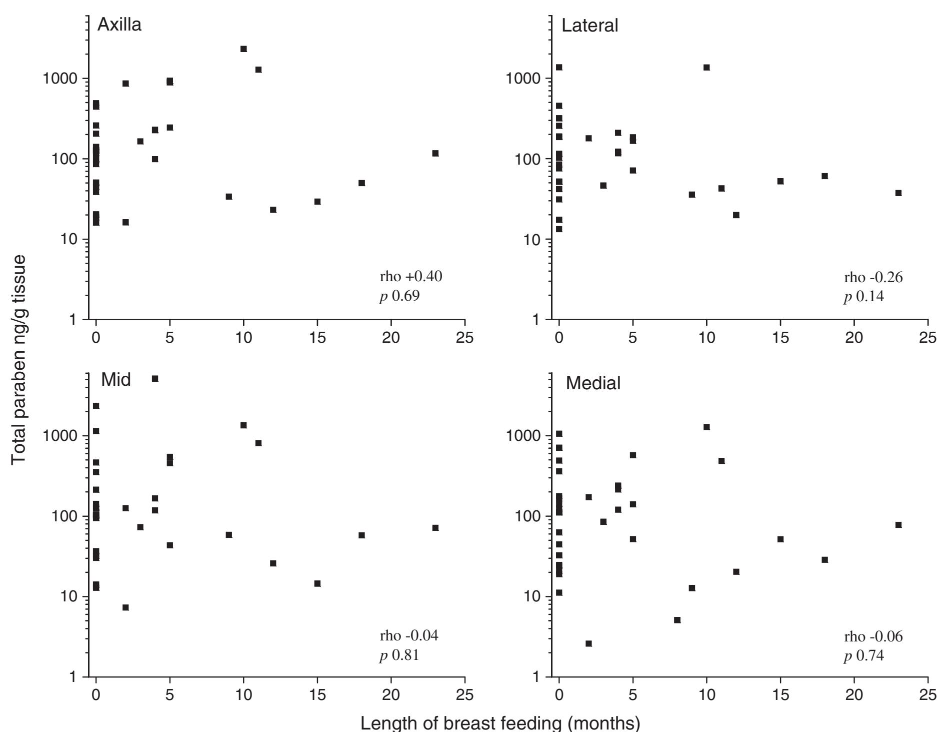

Figure 4. Correlation of total paraben level measured with length of breast feeding in each of the four serial locations across the breast ($n = 35$). Results of Spearman correlations are shown in the boxes as ρ values and P-values.

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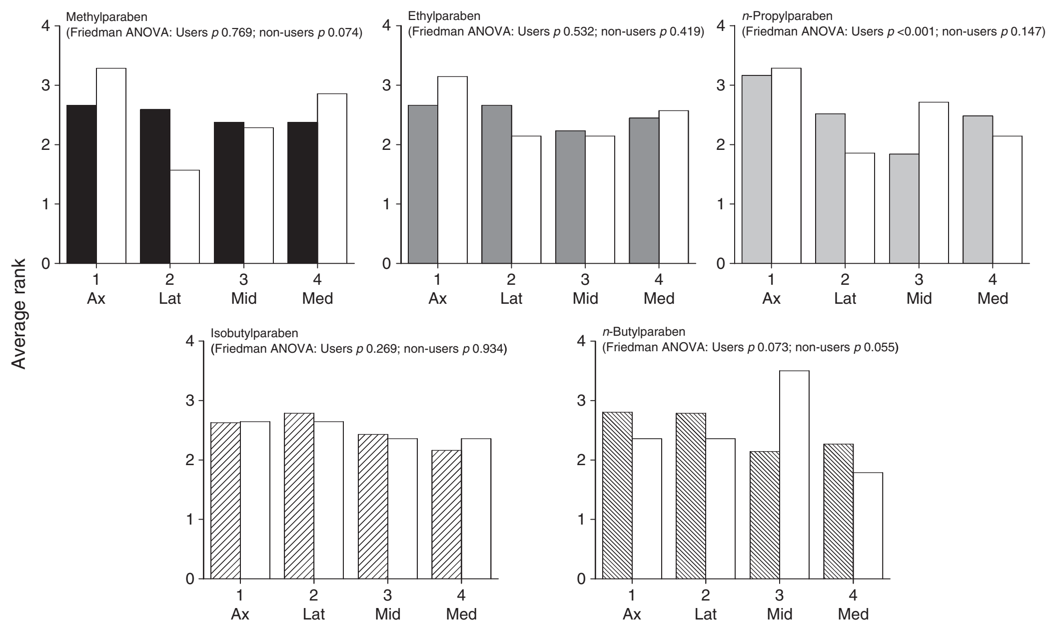

Figure 5. Relation of usage of underarm cosmetics with average rank values according to the levels in nanograms per gram tissue of methylparaben, ethylparaben, *n*-propylparaben, isobutylparaben and *n*-butylparaben in four serial locations across the breast from axilla (Ax) to lateral (Lat) to mid (Mid) to medial (Med) region in breast tissue. Self-reported data were used to present the data in two groupings according to use of underarm deodorant/antiperspirant products and were separated into those women who reported as never having used any of this type of product in their lifetime (open bars; $n = 7$; nonuser) and those who had used varied levels and types of these products (shaded bars; $n = 28$; user). Each paraben was analysed individually using Friedman ANOVA to rank the level as measured in nanograms per gram tissue across the four serial locations and the values plotted here are the average rank values for each group: the Friedman *P*-value is shown.

2001, 2009; Harvey and Darbre, 2004; Darbre, 2006, 2009; Darbre and Charles, 2010), we managed to identify seven patients in this study who could confirm that they had never used underarm antiperspirant/deodorant cosmetic products at any time in their life. Whilst self-reported questionnaire information is beset with issues of recall accuracy, patients who have been lifelong nonusers provide a more certain control group. The finding of similar concentrations of parabens in the breast tissue of women who reported to be current, past or nonusers of underarm cosmetics suggests the parabens to have originated from a source other than underarm cosmetic application. However, full analysis of their personal care product usage was not available and given that parabens continue to be used in a wide range of personal care products (Loretz *et al*., 2006; Andersen, 2008; Yazar *et al*., 2011) and given the greater capacity of liver compared with skin to metabolize esters, intact esters in body tissues might still be expected to arise from dermal rather than oral exposure (Harvey and Darbre, 2004; Darbre and Harvey 2008). This is supported by other published studies showing differences in measurements of methylparaben and propylparaben in urine to reflect personal care product use (Calafat *et al*., 2010), and showing a direct correlation between self-reported use of personal care products in terms of percentage skin area creamed per day and amount of methylparaben measured in the blood (Sandanger *et al*., 2011).

Whatever the source of the paraben, the ultimate distribution across the breast is an important question in relation to identifying environmental chemicals that might contribute to explaining the disproportionate incidence of breast cancer in the upper outer quadrant of the breast, which now exceeds 50% in the UK (Darbre, 2005; Darbre and Charles, 2010). This disproportionality has long been assumed to result from the presence of a greater amount of epithelial tissue in that region, with epithelial tissue being the major target tissue for breast cancer (Haagensen, 1971). However, this explanation has been questioned from studies showing an even distribution of cancer between quadrants in large and small breasts despite the less marked quadrant distribution of epithelial tissue in the smaller breasts (Rimsten, 1976) and from the reported annually rising incidence in the upper outer quadrant (Darbre, 2005; Darbre and Charles, 2010). If environmental chemicals were to contribute to this disproportionality, then levels of chemicals might be expected to be measurable at higher levels in the upper outer quadrant than in other regions of the breast. This study was designed to address this question in the context of parabens, by measuring levels of parabens, not in quadrants specifically, but in four serial locations linearly across the breast from axilla to sternum (axilla, lateral, mid, medial). Consistent differences were found across the breast only for *n*-propylparaben, which over all 40 patients was at higher levels in the axilla region compared with the mid or medial regions (Wilcoxon matched pairs test, $P = 0.004$ and $P = 0.021$, respectively) and showed differences between the four positions (Friedman test, $P = 0.010$). However, over the multiple statistical tests for the individual parabens across different regions, there were no overall consistent differences and median values were notably similar for each paraben across the four regions (see Table 2). The mechanism by which any one chemical might be present at different concentrations in different regions of the breast remains to be investigated. It is possible that systemically absorbed chemicals might accumulate in that region through some local circulatory and/or lymphatic mechanisms as yet to be identified. However, it has been suggested also that dermal application of personal care products to the underarm and adjacent upper breast region might result in long-term low-dose absorption and local diffusion of chemical

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Table 3. Comparison of published values for the levels of parabens in different human tissues. The units are given as published: assuming that 1 g of tissue has a volume of 1 ml, then all units are directly comparable. *N* is the sample number in each study

Human tissue	Collection	Country	*n*	Units	Mean/median	Methylparaben	Ethylparaben	*n*-Propylparaben	*n*-Butylparaben	Isobutylparaben	Reference
Breast (unaffected tissue adjacent to cancer)	2005-2008	England	160	ng/g	Median	16.6	3.4	16.8	2.1	5.8	This study
Breast (cancer)	1980s	Scotland	20	ng/g	Mean	12.8	2.0	2.6	0.9	2.3	Darbre *et al.*, 2004
Urine (general population)	2005-2006	USA	2548	µg/L	Median	63.5	0	8.7	0	0	Calafat *et al.*, 2010
Urine (general population)	2006	Denmark	60	ng/ml	Median	17.7	1.98	3.6	0.19		Frederiksen *et al.*, 2010
Urine (pregnant women)	2004-2008	Spain	120	ng/ml	Median	191.0	8.8	29.8	2.4		Casas *et al.*, 2011
Urine (4-year-old boys)	2004-2008	Spain	30	ng/ml	Median	150.0	8.1	21.5	1.2		Casas *et al.*, 2011
Blood (general population)	2005	Norway	332	ng/ml	Median	9.4	<3	<2	0	0	Sandanger *et al.*, 2011
Milk (general population)	2007	USA	4	ng/ml	Range	0.5-3.0		0-0.3			Ye *et al.*, 2008
Milk (general population)	2004-2006	Switzerland	54	ng/ml	Mean	2.18	1.26	1.42	0	0	Schlumpf *et al.*, 2010

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components to that breast region (Darbre, 2001, 2003; Harvey and Darbre, 2004; Darbre, 2006, 2009; Darbre and Charles, 2010). In this latter context, paraben in the seven nonusers of underarm cosmetics must have originated from a source other than dermal application to the underarm and reassessment of the distribution of parabens in the 28 users of underarm cosmetics confirmed not only the results with *n*-propylparaben, giving now $P < 0.001$ for the Friedman test and significantly higher levels in outer (axilla + lateral) vs inner (mid + medial) regions (Wilcoxon matched pairs test, $P = 0.040$), but also significantly higher levels in outer than inner regions for *n*-butylparaben (Wilcoxon matched pairs test, $P = 0.006$; although the Friedman test reduced only to $P = 0.073$). Interestingly, methylparaben also showed significantly decreasing levels across the breast from axilla to sternum, but only in nonusers of underarm products (Friedman ANOVA $P = 0.035$). Whatever the mechanism, there was a small trend towards significantly higher levels of at least some parabens in the axilla region than inner regions of the breast, most notably for *n*-propylparaben, which was the paraben present at greatest concentrations overall in the breast. However, although significance was deemed to be $P < 0.05$, some values were only slightly higher than this accepted cutoff, and in general it must also be noted that the gradient was only small even when significance was $P < 0.05$.

Although parabens have been detected in human milk (Ye *et al*., 2008; Schlumpf *et al*., 2010) and breast feeding has been reported to affect levels of lipophilic compounds in the breast (Harris *et al*., 2001; Massart *et al*., 2005; LaKind *et al*., 2009), we did not find any correlation between level of any paraben in the breast tissue in any location and length of breast feeding. Previously published data showed higher levels of methylparaben and propylparaben in urine of older than younger women, which was suggested to reflect some degree of bioaccumulation during life as for other environmental lipophilic compounds (Calafat *et al*., 2010). In this study, we did not find any correlation between level of paraben in the breast tissue and age of the patient. However, the age range in this study was 37–91 years, limited by the age at which breast cancers develop, and the previous correlation using urine sampling suggested a difference only between those above and below the age of 20 (Calafat *et al*., 2010).

Numerous studies have addressed the issue of whether measured levels of environmental chemicals are higher in women with breast cancer, and therefore whether raised levels of any specific chemical might be associated with breast cancer development, but to date this remains an unresolved issue (Brody *et al*., 2007). It was not considered ethical to use the breast cancer tissue for nondiagnostic purposes in these cases owing to the small amount of tissue available, but from knowledge of the tumour location, we were able to compare the percentage of total paraben in the region of the breast with the cancer with other regions without cancer. No correlation was found for total or individual parabens. One reason for this lack of correlation may relate to the fact that breast cancer could start many years before a breast lump is found (Russo and Russo, 1987), resulting in measurement of the paraben long after the time of carcinogenesis. However, a lack of correlation could also relate to variations in individual susceptibility, meaning that absolute levels are not the main determinant of risk. Since parabens are known to possess oestrogenic properties (Darbre and Harvey, 2008), and if parabens act by an oestrogen receptor-mediated mechanism *in vivo*, then it might be expected that only ER + tumours would be influenced by the presence of the paraben in the breast. We found no significant difference between the levels of parabens in the breast tissue from patients with ER + and ER − breast cancers, but it might still be that the paraben is widely distributed across all samples but the oestrogenic activity of the paraben only influences the ER + cancers. Given that increase in linear length and branching in the alkyl chain increases the relative binding affinity of the paraben to ER reducing by 100-fold the concentration needed for a proliferative effect on human breast cancer cells in culture (Darbre 2006), functionally relevant threshold concentrations will differ for each of the parabens. Furthermore, given that multiple parabens can have additive effects on cell proliferation (Darbre, 2009; Darbre and Charles, 2010), further complexity may arise when considering varied mixtures of the five parabens, and this now needs experimental investigation.

The presence of a chemical in the breast cannot be taken to imply causality *per se*, but it is nevertheless a prerequisite for consideration of any functional involvement in disease processes. With complex epidemiology (Key *et al*., 2001) and varied clinical manifestations (Haagensen, 1971), breast diseases, benign and malignant, must be considered multi-factorial in origin and it would seem unlikely that any one chemical would play a dominant overall role. However, measurement of individual chemicals in different locations across the breast in cases where a primary cancer has appeared does offer an approach that could eventually build up to provide a more detailed picture of the mechanisms by which environmental chemicals may enter and distribute across the breast. Although oestrogen is an acknowledged component in the development of breast cancer (Miller, 1996), it remains to be established as to whether environmental chemicals with oestrogenic properties contribute a functional component to the disease process (Darbre and Charles, 2010). Many xenoestrogens have been measured in the human breast (Darbre, 2006), but so far only in single samples and never all in one breast, and a new approach to investigating functionality would be to build a profile of the distribution of these many different chemicals within and across single breasts from different locations and lifestyles. This would enable comparison, not just between a single sample of cancerous vs noncancerous tissue at a time which may anyway be removed from the timing of carcinogenesis, but to investigate likely distributions across the breast in relation to clinical parameters such as tumour location.

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