Journal of Steroid Biochemistry & Molecular Biology 155 (2016) 120–125

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

# Quantitative determination of estrone by liquid chromatography–tandem mass spectrometry in subcutaneous adipose tissue from the breast in postmenopausal women

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ARTICLE INFO

*Article history:*
Received 11 June 2015
Received in revised form 30 September 2015
Accepted 7 October 2015
Available online 22 October 2015

*Keywords:*
Estrone
Estrogen
Adipose tissue
Menopause
Breast cancer
Liquid chromatography–tandem mass spectrometry

ABSTRACT

Estrone is the most abundant estrogen after the menopause. We developed a liquid chromatography-tandem mass spectrometric method (LC–MS/MS) for determination of estrone in adipose tissue. Subcutaneous adipose tissue from the breast was collected during elective surgery in postmenopausal women undergoing mastectomy for treatment of breast cancer ($n = 13$) or reduction mammoplasty (controls, $n = 11$). Homogenized adipose tissue was extracted with organic solvents and the estrone fraction was purified by LH-20 column chromatography from the excess of lipids. The concentration of estrone was analyzed by LC–MS/MS. The method was accurate with an intra-assay variation of 8% and an interassay variation of 10%. The median concentration of estrone in subcutaneous adipose tissue from the breast did not differ between breast cancer and control women, 920 pmol/kg and 890 pmol/kg, respectively. In breast cancer patients but not in the controls, breast adipose tissue estrone levels correlated positively with the serum estrone concentration. In conclusion, the new method provides a reliable means to measure estrone concentrations in adipose tissue in postmenopausal women.
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## 1. Introduction

In postmenopausal women, adipose tissue is the principal source of estrogen [1]. The most abundant estrogen after the menopause is estrone which is formed in peripheral tissues primarily from androstenedione by aromatase, a cytochrome p450 enzyme [2]. Activity of aromatase in adipose tissue is positively related to weight and age in women [3,4]. Moreover, there are relatively high concentrations of estrone sulfate in the circulation which may be taken up and hydrolyzed to free estrone in tissues by steroid sulfatase (STS) [5]. Estrogens are thought to play an important role in the maintenance and growth of estrogen receptor positive breast cancers [6,7].

The concentration of estrone in adipose tissue as reported in postmenopausal women is several times higher than its circulating levels [8,9]. Breast adipose tissue estrone levels have been shown to correlate with body mass index (BMI) in postmenopausal women [10]. Estrone is a precursor of 17β-estradiol in the biosynthesis of estrogens in adipose tissue and, accordingly, concentration of estrone is higher than that of 17β-estradiol in breast adipose tissue as studied in breast cancer patients [8–12].

Estrone is more lipophilic compared to 17β-estradiol. The previous reports on estrone concentrations in adipose tissue from the breast have used radioimmunoassay (RIA) as the analytical method [8–15]. When quantified in adipose tissue, the estrone fraction needs to be separated from great amounts of free cholesterol, triglycerides and other lipids that might otherwise interfere with the analytical method for example by causing signal suppression. In the present study, we describe a new quantitative method to determine estrone in adipose tissue by liquid chromatography-tandem mass spectrometry (LC–MS/MS), and

*Abbreviations:* BMI, body mass index; GC–MS, gas chromatography-mass spectrometry; IS, internal standard; LC–MS/MS, liquid chromatography–tandem mass spectrometry; RIA, radioimmunoassay; STS, steroid sulfatase.

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http://dx.doi.org/10.1016/j.jsbmb.2015.10.004
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applied the method for analyzing the estrone concentrations in subcutaneous adipose tissue from the breast in postmenopausal women with or without breast cancer.

## 2. Materials and methods

### 2.1. Subjects

Subcutaneous adipose tissue from the breast was obtained from postmenopausal women during mastectomy for treatment of breast cancer ($n = 13$) or reduction mammoplasty ($n = 11$) as described in [16]. Two distinct adipose tissue biopsies were taken from every subject. Blood samples were collected before the operation, and the tissue and blood samples were stored as described in [16]. The study was approved by the Ethics committee of Helsinki University Central Hospital and the subjects gave their written informed consent.

### 2.2. Estrogens and solvents

Estrone (vetranal) was purchased from Sigma–Aldrich, St. Louis, MO. [2,4,6,7-$^3$H(N)]Estrone (specific activity 94 Ci/mmol) was purchased from NEN, Boston, MA. Prior to use, [$^3$H]estrone was purified by Sephadex LH-20 column chromatography (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using 9% methanol in toluene as eluent [17]. $^{13}C_3$-estrone was purchased from Iso-Sciences, King of Prussia, PA.

Methanol, hexane, diethyl ether and ethyl acetate were HPLC grade (Rathburn Chemicals Ltd., Walkerburn, Scotland). Chloroform and toluene were purchased from Merck (Kenilworth, NJ).

### 2.3. Sample preparation

Control serum pools containing two concentrations of exogenous estrone (180 and 540 pmol/l) were prepared from pooled human male sera (Sigma–Aldrich). The control sera were divided into 1 ml fractions and stored at −20 °C. In addition, subcutaneous adipose tissue from the breasts of 24 women (13 reduction plasty and 11 cancer patients) was homogenized as described below, pooled and used as control.

The flow chart of the method is shown in Fig. 1. Weighed adipose tissue (~200 mg) was homogenized in 1 ml of distilled water. Tissue and control serum samples (1 ml) were pipetted into disposable borosilicate extraction tubes. [$^3$H]Estrone (~49,000 dpm in 10 μl of ethanol) was added to adipose tissue samples obtained from the control pool and used as an internal standard to measure recovery. The samples were extracted four times with 3.5 volumes of diethyl ether–ethyl acetate (1:1 by volume). The combined organic phases were evaporated to dryness and weighed. A mean of 78% of the original tissue sample's ($n = 56$) weight was recovered in the organic phase extract, calculated as [the weight of fat extracted from adipose tissue (mg)/the weight of the original adipose tissue sample (mg)] × 100%. After extraction, the samples were subjected to hydrophobic chromatography on Sephadex LH-20 in hexane–chloroform (2:1 by volume) (chromatography I). The samples were applied to the columns in two 0.3-ml aliquots of hexane–chloroform (2:1 by volume). The interfering fatty substances were eluted in the first lipoidal fraction with 10 ml of the same solvent and discarded. The estrone fraction was eluted with 5 ml of methanol and evaporated to dryness. To further purify the samples, a second Sephadex LH-20 column chromatography (chromatography II) was carried out in 9% methanol in toluene as described in [17]. The sample was applied to the column in two 0.1 ml aliquots of 9% methanol in toluene and estrone was eluted from the column in the first fraction in 2.4 ml of the same solvent. The estrone fraction was evaporated and dissolved in 1 ml of methanol. An aliquot of 50 μl was taken from the control samples for liquid scintillation counting to determine the recovery of the [$^3$H]estrone internal standard (Rack-beta, Wallac Oy, Turku, Finland). An aliquot of 250 μl was taken for analysis by LC–MS/MS as described below.

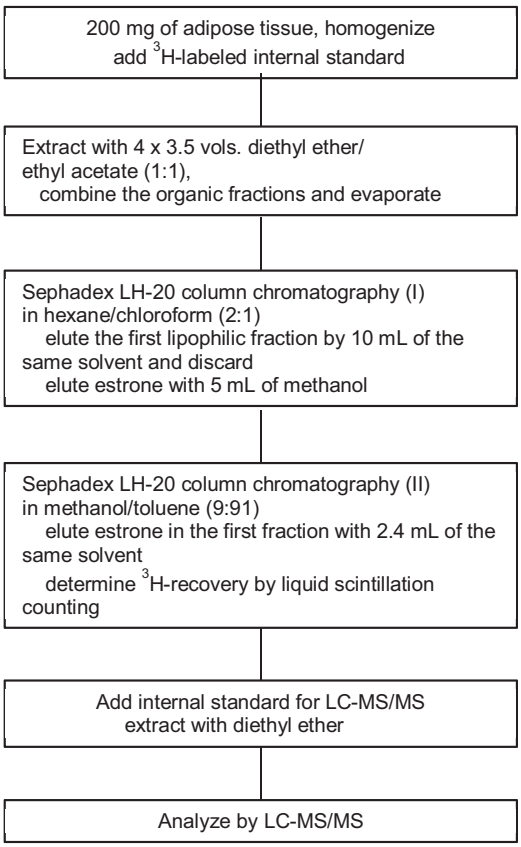


**Fig. 1.** Outline of the method. Flowchart for quantitative determination of estrone in adipose tissue by LC–MS/MS.

### 2.4. LC–MS/MS method for determining estrone concentration in adipose tissue

After evaporation to dryness, 30 μl of $^{13}C_3$-estrone (internal standard (IS), 2.5 nM) was added followed by 250 μl of water and 500 μl of 50 mM ammonium acetate/$NH_3$ (pH 9). The samples were extracted with 2 ml of diethyl ether. After mixing for 3 min, the organic layer was collected and evaporated to dryness under nitrogen. The residue was dissolved in 125 μl of 50% methanol. Calibrators containing 25–1000 pmol/l of estrone were prepared in 50% methanol. 25 μl was injected on an LC–MS/MS system equipped with an API 4000 triple quadrupole mass spectrometer (AB Sciex, Concord, Canada). Peripherals included an Agilent series 1200 HPLC system with a binary pump (Waldbronn, Germany). Separation was performed on a SunFire C18 column (2.1 × 100 mm; Waters, Milford, MA). The mobile phase was a linear gradient consisting of methanol (B) and water (A), at a flow rate of 300 μl/min. The gradient was: 0 min, 50% B; between 0 and 5 min linearly increased to 100% B; 5–8.5 min 100% B; between 8.5–9 min linearly decreased to 50% B; and 9–15 min 50% B. The column was directly connected to the electrospray ionisation probe. Estrone was detected with the following transitions: $m/z$ 269.1 to $m/z$ 145.0 (quantification) and $m/z$ 269.1 to $m/z$ 142.9 (confirmation) and IS, $m/z$ 272.1 to $m/z$ 148.0. Data were acquired and processed with the Analyst Software (version 1.4.2; Sciex). All results were generated in negative-ion mode: with the entrance potential at −8 V, the declustering potential at −100 V, and the collision cell exit

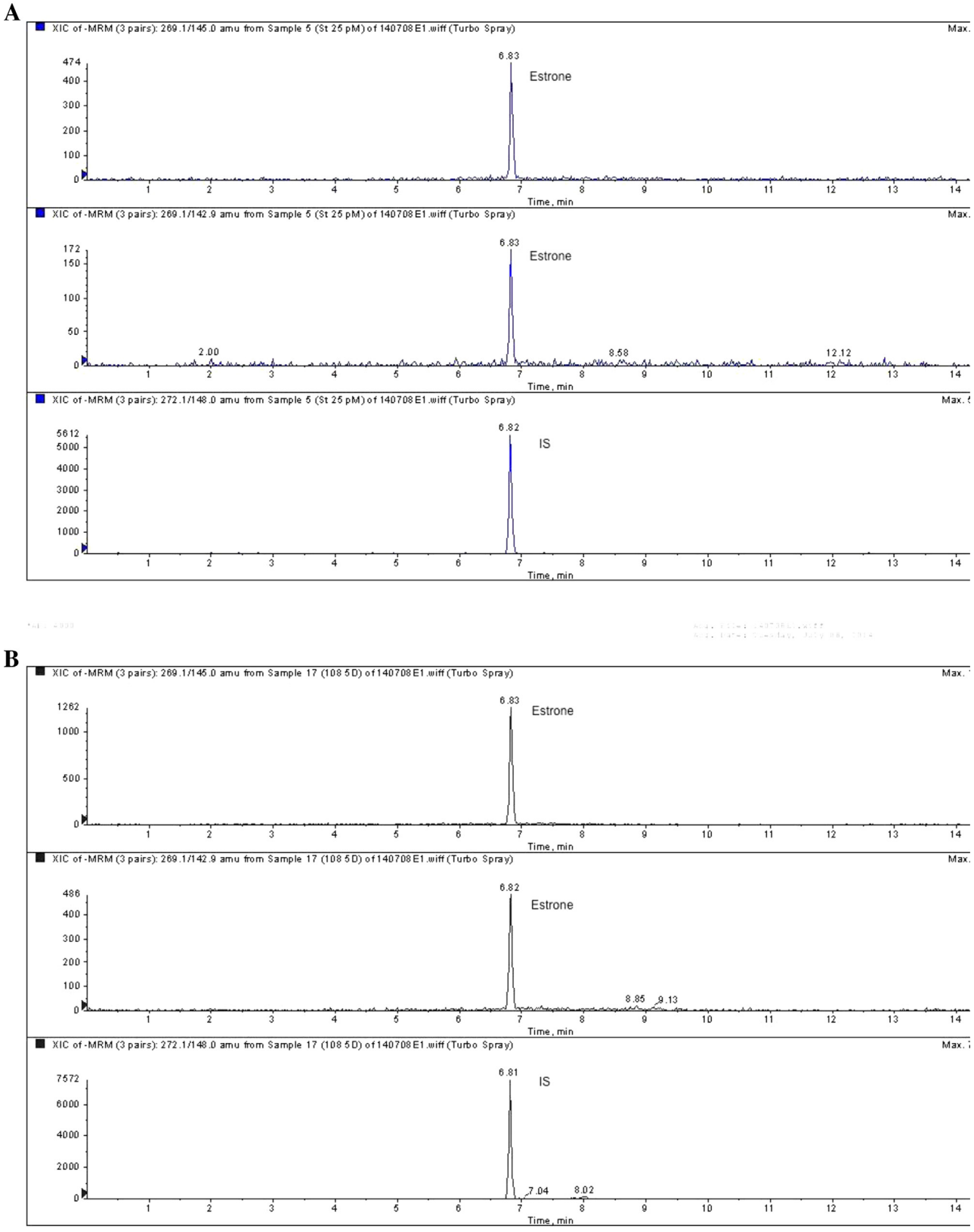


**Fig. 2.** LC–MS/MS chromatograms of the lowest estrone calibrator and an adipose tissue sample showing no interference. LC–MS/MS chromatograms of (A) the 25 pmol/l estrone calibrator and (B) a subcutaneous adipose tissue sample from the breast of a postmenopausal breast cancer patient containing 68 pmol/l estrone (480 pmol/kg as calculated per tissue weight of 202 mg and corrected by the recovery of [$^{3}$H]estrone of 71%). Upper panel: ion chromatogram following the fragmentation 269.1/145.0. Middle panel: ion chromatogram following the fragmentation 269.1/142.9. Lower panel: ion chromatogram following the fragmentation of 272.1/148.0 of IS ($^{13}C_3$-estrone internal standard). *Y*-axis: Intensity, cps.

potential at −8 V. The collision energy potentials were set at −55 V for $m/z$ 145 and −70 V for $m/z$ 142.9, as determined by manual tuning. Front-end electrospray settings for the MS/MS ionization source were as follows: curtain gas, 20; GS1, 20; GS2, 30; CAD, 8; temperature 525 °C; and ion source at −4500 V. The mass calibration and resolution adjustments for the resolving quadrupoles (at 0.7 atomic mass units at full width and half height) were optimised using an infusion pump with polypropylene glycol solution.

## 3. Results

### 3.1. Assay characteristics

Fig. 2 A shows LC–MS/MS chromatograms for the lowest estrone calibrator of 25 pmol/l and IS. A typical chromatogram of estrone in subcutaneous adipose tissue from the breast of a breast cancer patient is shown in Fig. 2 B with no interference from the tissue matrix components.

The intra-assay imprecision was 7% (180 pmol/l, $n = 11$) for the low and 8% (540 pmol/l, $n = 11$) for the high control serum sample (section 2.3). The intra-assay variation of endogenous estrone in adipose tissue (four parallel determinations of control adipose tissue pool, section 2.3) ranged from 9.9% to 20% in four separate assays (mean, 16%).

The interassay imprecision of the control adipose tissue pool was 10% in four consequent assays (mean concentration of estrone in pooled adipose tissue, 1962 pmol/kg). The interassay coefficient of variation of LC–MS/MS was 3.5% at 115 pmol/l concentration of the estrone calibrator (number of assays, $n = 14$).

For analytical recovery, pooled adipose tissue from 16 breast cancer and reduction plasty patients was spiked with 300 pmol/l or 600 pmol/l estrone. The mean (SD) analytical recoveries from three parallel samples were 124% (6) and 117% (2), respectively. The results were calculated by subtracting the value of adipose tissue without added estrogen and correcting according to the recovery of the tritiated internal standard. The mean recovery of [$^3$H]estrone added to control adipose tissue was 74% (SD 8.9; range, 53–85%; number of determinations $n = 26$ in six assays).

The limit of quantification of estrone by LC–MS/MS was 10 pmol/l (signal to noise-ratio S/N = 10). If calculated for the 200 mg starting weight of adipose tissue, the limit of quantification of estrone was approximately 70 pmol/kg, corrected for the mean recovery of 74% of the method.

### 3.2. Concentration of estrone in subcutaneous adipose tissue from the breast

The LC/MS–MS method for quantitative determination of estrone was applied to adipose tissue samples previously obtained from breasts of postmenopausal women with or without breast cancer [16]. The clinical characteristics of the study subjects as well as the concentrations of estrone in subcutaneous adipose tissue from the breast and serum are shown in Table 1. The concentration of estrone in adipose tissue did not significantly differ between cancer and control women. The median concentration of estrone was slightly but not significantly higher in N1 compared to N2 in breast cancer patients as well as in reduction plasty control subjects ($P = 0.463$, N1 vs. N2 in breast cancer patients; Wilcoxon signed rank test). Instead, the estrone concentrations measured in N1 and N2 correlated positively with each other in both groups ($r = 0.857$, $P < 0.001$, $n = 13$, breast cancer patients; and $r = 0.764$, $P = 0.006$, $n = 11$, reduction plasty control subjects; Spearman's correlation) (Fig. 3).

In all subjects, adipose tissue estrone concentration correlated positively with the body mass index (mean estrone concentration in adipose tissue vs. BMI; $r = 0.575$, $P = 0.003$, $n = 24$; Spearman's correlation).

**Table 1**
Clinical characteristics as well as estrone concentrations in subcutaneous adipose tissue from the breast and in serum in postmenopausal women with breast cancer and reduction mammoplasty control women.

| | Breast cancer | Control | *P*-value |
|---|---|---|---|
| | $n = 13$ | $n = 11$ | |
| Age, years | 63 (54–70) | 60 (54–75) | 0.228 |
| Years from menopause | 11 (3–28) | 11 (0.5–23) | 0.628 |
| Body mass index, kg/m$^2$ | 25 (20–34) | 29 (22–38) | 0.150 |
| Waist to hip ratio | 0.87 (0.76–1.0) | 0.93 (0.82–0.99) | **0.035** |
| Adipose tissue E1, pmol/kg | | | |
| the mean of N1 and N2 | 922 (238-1 911) | 893 (333-3 130) | 0.494 |
| N1 | 1 009 (245-1 672) | 1 005 (353-2 426) | 0.691 |
| N2 | 781 (231-2 150) | 816 (312-4 152) | 0.392 |
| Serum E1, pmol/l[a] | 111 (39-141) | 72 (19-285) | 0.132 |

The data are expressed as median (range). Statistically significant *P*-values are bolded ($P < 0.05$, Mann–Whitney *U*-test). E1, estrone; N1, adipose tissue proximal to the tumor; N2, adipose tissue distal to the tumor (breast cancer patients).
[a] Serum estrone as previously reported in Ref. [16].

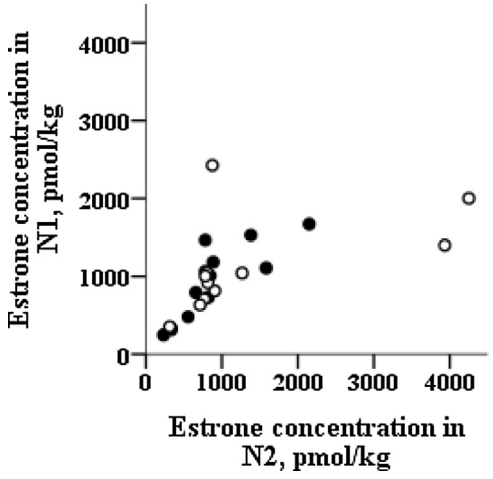


**Fig. 3.** A positive correlation between estrone concentrations in subcutaneous adipose tissue from the breast measured proximal and distal to the tumor. The concentration of estrone in subcutaneous adipose tissue correlated positively between two samples obtained from different parts of the breast ($r = 0.818$, $P < 0.001$, $n = 24$; Spearman's correlation). In postmenopausal women with breast cancer, the adipose tissue concentration of estrone was determined proximal to the tumor (N1) and distal to the tumor (N2) as described in the methods. ●, Women with breast cancer; ○, control women.

A significant positive correlation between adipose tissue estrone concentration and serum estrone was observed in breast cancer patients only (Fig. 4). In reduction plasty control subjects, there was no significant correlation between adipose tissue and circulating estrone concentrations ($r = -0.176$, $P = 0.627$, $n = 10$).

## 4. Discussion

We developed a reproducible novel method for the quantitative determination of estrone in adipose tissue. To the best of our knowledge, this is the first study to report estrone levels in subcutaneous adipose tissue from the breast by LC–MS/MS. The sensitivity of the analytical method was adequate to determine estrone levels in postmenopausal women. Moreover, no interfering matrix effects were recognized after a careful purification of the adipose tissue samples from the excess of lipoidal substances.

We determined the concentration of estrone in subcutaneous adipose tissue from the breast in 24 postmenopausal women.

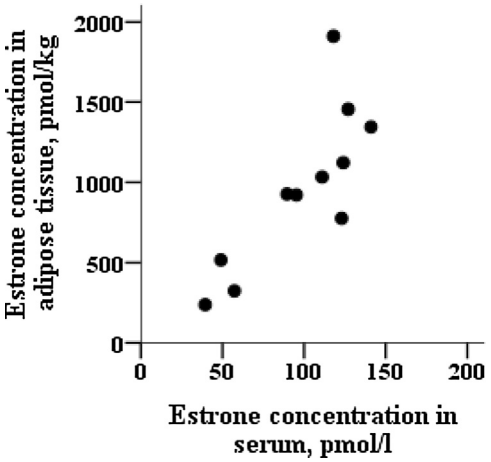


**Fig. 4.** Adipose tissue and serum estrone concentrations were positively related in women with breast cancer. The concentration of estrone in subcutaneous adipose tissue from the breast correlated positively with serum estrone concentration in postmenopausal women with breast cancer ($r = 0.800$, $P = 0.003$, $n = 11$; Spearman's correlation). Adipose tissue estrone concentration is expressed as the mean of two determinations (proximal and distal to the tumor).

The adipose tissue concentration of estrone was approximately ten times higher compared with the serum levels, in agreement with a previous study in abdominal subcutaneous adipose tissue by LC–MS/MS as studied in four postmenopausal women [18]. Another study in abdominal subcutaneous adipose tissue reported a slightly lower mean concentration of estrone by LC–MS/MS in eight postmenopausal subjects including women with gynecological cancer [19]. Altogether, these data support the concept that subcutaneous adipose tissue is a significant source of estrone in postmenopausal women. This has also been demonstrated in obese men with a ten-fold subcutaneous adipose tissue/plasma gradient for estrone as analyzed by gas chromatography–mass spectrometry, GC–MS [20].

The estrone concentrations presented here are in agreement with previous determinations by RIA in fatty tissues from the breast [9,10,13]. We observed a positive correlation between adipose tissue and serum estrone concentrations in breast cancer patients, in line with previous studies [9,15], but not in control women. As estrone levels in adipose tissue exceed those in serum, we assume that they could, in theory, influence serum estrone levels. However, it is not clear how increased production of estrone in breast cancer patients [21–23] could have an effect on serum levels, as the adipose tissue estrone concentration was not higher in women with breast cancer compared to control women.

As mentioned above, the concentrations of estrone in subcutaneous adipose tissue from the breast were comparable in subjects with breast cancer and control women. Moreover, we did not find any significant differences in estrone concentrations in adipose tissue located distally or proximally to the tumor, in line with two previous studies [13,14]. Instead, we detected a strong positive correlation between estrone concentrations analyzed from different adipose tissue samples from the same subject that adds to the reliability of the analytical method. Falk et al. used different methodology and reported estrone levels by RIA in nine pre- and postmenopausal women together in the oil harvested from breast adipocytes [14]. In addition to adipocytes, adipose tissue contains varying amounts of connective and other tissues. We studied subcutaneous adipose tissue from the breast and found that approximately 80% of the sample's weight was recovered in the lipophilic fraction after extraction with organic solvents. Therefore we also calculated the concentrations of estrone relative to the amount of fat extracted from adipose tissue samples, however, this did not change the results to any significant extent (data not shown).

In conclusion, we describe a reproducible and reliable LC–MS/MS method to measure estrone in postmenopausal adipose tissue. Subcutaneous adipose tissue from the breast contained relatively high concentrations of estrone compared to serum levels in postmenopausal women.

## Acknowledgements

We thank Adile Samaletdin and Päivi Ihamuotila for expert technical assistance. This work was financially supported by Folkhälsan Research Center, Helsinki University Central Hospital, the Sigrid Jusélius Foundation, the Päivikki and Sakari Sohlberg Foundation, the Finnish Foundation for Cardiovascular Research, Jane and Aatos Erkko Foundation, Finska Läkaresällskapet, and State funding for university-level health research.

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