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Title: Capillary electrophoresis of human follicular fluid

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**Title page:****Capillary electrophoresis of human follicular fluid**

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**Abstract**

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4 Some of the major serum proteins that are also found in follicular fluid, including transferrin,  $\alpha$ -  
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6 macroglobulin and albumin, are thought to play a role in oocyte maturation. This study set out to  
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8 identify proteins in human follicular fluid by capillary zone electrophoresis and to investigate their  
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10 relationship to follicular/oocyte maturity and fertility outcome.

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13 176 individual follicular fluid samples, from 30 women undertaking *in vitro* fertilization/  
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15 intracytoplasmic sperm injection (IVF/ICSI), were run using an optimized capillary zone  
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17 electrophoresis method that gave a good separation of sixteen peaks in most samples. Nine of the  
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19 peaks were identified and quantified but seven remain unknown and require further proteomic  
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21 identification. Of the identified protein peaks, levels of each were corrected for follicular volume and  
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23 total content calculated. No significant difference in protein levels was found with regard to oocyte  
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25 recovery and fertilization. Protein concentrations tended to decrease as the follicular sphere increased  
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27 whilst total content in follicular fluid increased in proportion to size. This is consistent with simple  
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29 transudation across a sphere surface area which does not increase in proportion to the follicular fluid.  
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31 This is not true of the concentration and content pattern of other proteins/biomolecules which are  
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33 produced by follicular cells locally.  
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40 In conclusion, neither concentration nor absolute levels of nine major proteins identified in follicular  
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42 fluids correlated with oocyte presence and fertility outcome. Future work to remove more  
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44 concentrated proteins (*e.g.* albumin) would enhance separation of smaller peaks and identification of  
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46 the unknown molecules.  
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52 **Key words:** follicular proteins, capillary zone electrophoresis, follicular size, oocyte presence, and  
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## 1. Introduction:

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3 Follicular fluid (FF) is composed of serum transudates and granulosa cell secretions, and thus it has  
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5 similar components to serum with some additional proteins (600 spots by two-dimensional  
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7 polyacrylamide electrophoresis) [1]. The introduction and development of assisted reproductive  
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9 technology (ART) have urged researchers and clinicians to carry out numerous studies on FF in  
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11 order to better understand the dynamic changes of preovulatory follicles and search for a reliable  
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13 marker for oocyte maturation. Many potential factors have now been investigated using various  
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15 techniques but mostly specific immunoassays [2,3,4], but results to date have been inconsistent.  
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17 Currently determination of follicular diameter by ultrasound is still the most reliable indicator of  
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19 follicular development and oocyte maturity. Empirical studies for ART have established that 18mm  
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21 diameter follicles, along with a serum oestradiol concentration of 800pg/ml are optimum condition  
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23 for harvesting of an oocyte that has the greatest probability of being successfully fertilized [5].  
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31 Since its inception by Jorgenson and Lukacs in 1981 [6], capillary zone electrophoresis (CZE) has  
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33 become established as a reliable method of protein separation. Its application in protein analysis is  
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35 attractive due to the simplicity and automation of instruments, lack of organic waste, minimal sample  
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37 preparation and small volumes needed for analysis [7]. CZE of serum is now routine in clinical  
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39 laboratories [8]; however, data on the protein profiling of FF by capillary electrophoresis (CE) is  
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41 scarce. To the authors' knowledge, only two recent studies have been carried out using CE on pooled  
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43 follicular fluid, and this was also without knowledge of follicular diameter [9,10].  
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50 Based on our preliminary work on serum samples, this study examined proteins in individual FF  
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52 samples by CZE. Experimental conditions were varied and an optimum set of conditions was chosen  
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54 to give an efficient and rapid separation of FFs. Some proteins were identified and their correlation  
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56 with follicular diameter, oocyte presence and fertility outcome was examined.  
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## 2. Material and methods:

### 2.1 Patient Selection and sample collection

FF samples (n=176) were collected from 30 women (age 29-38 years) undergoing controlled ovarian hyperstimulation as part of IVF/ICSI treatment at St Bartholomew's Hospital's Infertility Unit, London U.K. The details of patients' selection and treatment are described elsewhere [11]. The procedure for FF aspiration involved random selection of individual follicles measured in two dimensions by transvaginal ultrasound (Hitachi, model EUB-525, Northants UK) in order to obtain a mean diameter. All measurements and aspirations were performed by the same operator. Following identification of a suitable follicle, the follicle was gently pierced using a double lumen needle and aspirated, allowing the follicle to collapse slowly around the needle. Each follicle was emptied completely to ensure the maximum oocyte recovery rate. The follicle was then flushed with 4.5 ml of heparinised saline (3 x 1.5 ml automated flushes) and aspirated again. If an oocyte was not obtained, three further flushes were carried out to retrieve it but these flushes were not added to the samples for analysis. Blood stained aspirates were discarded and a further follicle was measured and aspirated if appropriate. A detailed record was kept of the volume of fluid aspirated per individual follicle prior to washing. Following examination of the follicular fluid from the individual follicles by the embryologist, the fluid was placed into a sterile tube. Each tube was centrifuged at 220g for 10 minutes and the follicular fluid supernatant stored in aliquots at -20°C.

Research approval was obtained from the East London and The City Health Authority Research Ethics Committee (study number p/98/222). Written consent was received from all patients participating in this study.

## 2.2 Total protein analysis

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3 In order to develop a CE method for FF a previously established method [12] for the analysis of  
4 serum proteins was modified. Serum is normally diluted 1 in 5 prior to CE analysis. Sampling of FF  
5 automatically lead to dilution since follicles are flushed with saline in order to ensure the harvest of  
6 the oocyte. It was possible that this dilution could reduce the protein concentration below the limit of  
7 detection of the CE method. In order to develop the CE method a pooled FF (PFF) sample was  
8 prepared. Twelve randomly chosen FF samples, (200 µl each), were thawed at room temperature and  
9 centrifuged at 400g for 5 minutes. 100 µl of supernatant was taken from each and mixed to form the  
10 pool. Following method development the PFF was then used as the quality control sample in all  
11 subsequent analysis of the individual FF samples. The concentration of total protein in the PFF was  
12 determined and then used to calculate any further dilution required for the CE method. Total protein  
13 concentrations were analysed in serial dilutions (1:10; 1:20; 1:50; 1:100; 1:200; 1:500; 1:1000) using  
14 double distilled water (ddH<sub>2</sub>O) of this pool. A fluorescence method with o-phthaldialdehyde / N-  
15 acetyl cysteine as the derivatising reagent was employed to assay protein content against bovine  
16 serum albumin (BSA) standards [12,13]. The total protein concentration of the PFF was 56 g/l.  
17 Wise (1986) reported that FF total protein content ranged from 57 – 72 g/l whilst the total protein  
18 concentration of serum was 60-85g/L [14]. Therefore FF and serum total protein levels are closely  
19 comparable and CE would appear to have suitable sensitivity for use with the FF flushes.  
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## 2.3 CE equipment and preparation

### 2.3.1 Instrumentation:

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52 CZE was carried out on the H-P<sup>3D</sup> CE Instrument (Agilent, Bracknell UK) controlled by HP  
53 Chemstation software for instrument control, data acquisition and analysis. A UV-Vis diode array  
54 detector was employed.  
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### 2.3.2 Capillary preparation:

A fused silica polyimide coated capillary (Polymicro Technologies, UK) with 50  $\mu\text{m}$  i.d. and 375  $\mu\text{m}$  o.d. length 44 cm (36cm to the detection window) was used.

### 2.3.3 Sample Preparation:

Samples were thawed at room temperature and spun at 13,000g for 5 minutes in a microhaematocrit centrifuge. 10  $\mu\text{l}$  of supernatant was transferred to a vial with inbuilt inserts, diluted as necessary based on the measured total protein concentration and vortexed prior to use. Precautions were taken to ensure that no bubbles which might have obstructed the capillary and affected the separation were present.

### 2.3.4 Final method:

Each variable was studied individually and the experimental conditions optimized. The following conditions were chosen as the final optimized methodology: Buffer sodium tetraborate pH=9.2, 25mM, 1mM EDTA: run at 24kV, 30 $\mu\text{A}$  and 19°C: hydrodynamic injection was 2 seconds at 50mBar of FF diluted with ddH<sub>2</sub>O. The separation was monitored between 190 nm and 310 nm but quantitative measurements were made at 195nm. The run time was 15 minutes. Between runs the capillary was flushed with 0.1M NaOH for 1 minute followed by background electrolyte (BGE) for 3 minutes.

## 2.4 Linearity study/ Running of samples

Standard BSA solutions between 0-30 mg/ml were run under the optimal conditions and calibration curve was plotted to show the relationship between spatial peak area and protein concentration.

Samples were run in sets of twelve along with a standard and a quality control (PFF). The capillary was cleaned as stated above between runs and new BGE was prepared and used after every 4 sets (48 samples). The PFF could not be refreshed each time due to sample shortage.

## 2.5 Data analysis

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3 The Chemstation software was used to analyse the electropherograms by manually – since automated  
4 integration was found to be unreliable. Scatter plots were produced using *Microcal Origin 6.0*  
5 software (OriginLab Corporation, USA) and statistical analysis was performed on *StatsDirect 6.0*  
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10 (StatsDirect Ltd, Cheshire, UK). As the data were not normally distributed, the Mann-Whitney U-  
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12 test was chosen for non-parametric analysis. All p values < 0.05 were taken to be as significant.  
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### 3. Results:

#### 3.1 Method development

The starting conditions were based on initial work on serum separation (see figure 1).

##### 3.1.1 Sample dilution and injection time

Based upon peak sharpness and the number of peaks resolved, a 1+1 (PFF + dH<sub>2</sub>O) dilution with 2sec hydrodynamic injection was found to be optimal. Other variables were then optimized and chosen in a similar manner.

##### 3.1.2 Buffer concentration and pH

Sodium Tetraborate was chosen as the buffer based on our previous work with serum [12]. The optimum concentration was found to be 25mM since this gave the sharpest unsplit peaks. Various pH values (between 8.7 and 9.7) were studied and migration time was plotted for  $\gamma$ -Globulin, Complement, Transferrin,  $\alpha$ -Macroglobulin,  $\alpha$ -Antitrypsin,  $\alpha$ -Acid-Glycoprotein, Albumin and Prealbumin. All proteins studied migrated fastest and most efficient peaks at pH 9.2, and therefore pH 9.2 was chosen for this study.

##### 3.1.3 Use of additives

EDTA chelates metal ions and minimizes peak tailing to improve resolution of separation [15]. The effect of EDTA concentration was varied but the optimum was judged to be 1 mM based on the number of peaks and their shapes.

##### 3.1.4 Capillary temperature and applied voltage

The effect of increasing the temperature (16°-26°) and increasing voltage (17kV-29kV) on the separation was investigated. Increasing the temperature and increasing voltage reduced the migration time of all the peaks but reduced the resolution of the early peaks. Optimum conditions were

1 deduced to be 23kV and 20°C. Final optimum conditions gave the electropherogram as shown in  
2 figure 2.  
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### 7 **3.2 Reproducibility and linearity**

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10 Reproducibility was evaluated by analyzing of repeated injections of PFF on the same day and on  
11 different days at optimal conditions.  $\gamma$ - Globulin and Albumin spatial peak areas and migration times  
12 were measured. Mean and standard deviation (SD) were calculated. Intra- coefficient variations (CV)  
13 were lower than 10%, but inter-CVs were higher up to 17% (see table 2).  
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20 The PFF was assayed for protein content using BSA. Increasing concentrations of BSA were run  
21 under optimum conditions to study the relationship between peak area and concentration. A linear  
22 relationship was found between 0 – 30 mg/ml BSA.  
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### 28 **3.3 Peak identification**

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31 Most peaks were identified by comparison to a human serum separation at the optimal conditions,  
32 and of the seven unknown peaks, Peak 2 and Peak 3 corresponded with serum proteins (figure 1 and  
33 2). Further analysis of an ultrafiltrate of PFF using a 5kD filter showed no peaks with similar  
34 migration time and spectrum, except peak 4 (see figure 3) indicating that they were almost certainly  
35 proteins.  
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45 Based on spectral evidence and spiking with standard solution, peak 4 was identified as uric acid (see  
46 figure 4). The other spectra of the other peaks were less distinct, other than that peak 2 and 3 had  
47 similar spectra to the known proteins - all were maximal around 195nm.  
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### 54 **3.4 Proteins concentration /total content and follicular size**

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57 176 FF samples were run under optimal conditions. Two samples were excluded due to excessive  
58 dilution (46-fold) during the original FF collection resulting in skewed results throughout (>3 Inter-  
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quartile range). Concentrations and total content were calculated using the standard BSA spatial peak areas (see table 1). The relationship between protein concentration and follicular diameter and total content of peak molecule and follicular diameter were shown in figure 5.

### 3.5 Statistical analysis

As the data were not normally distributed, the Mann-Whitney U test was applied to ascertain if there was any statistically significant difference with respect to two outcome parameters: oocyte presence and fertility outcome. No significant difference was found in any identified components or unidentified peaks with regard to oocyte retrieval and fertility outcome.

## 4. Discussion:

### 4.1 Protein content in follicular fluid

Most biochemical analysis of FF has focused on steroids, cytokines and growth factors measured by immunoassay, and a few have been conducted using 2-D gels to detect proteins [1]. CZE in this study, however, provided an efficient method for an initial comparative study on protein content for a large number of FF samples. There is considerable literature on CZE analysis of serum, plasma and amniotic fluid [16, 17], but little work, except for two recent studies [9, 10] has been undertaken on FF. However both of these studies examined pooled FFs and not individual follicular samples as in the present study.

Based on our previous work from serum [12, 13], we optimized experimental conditions to achieve an adequate and highly reproducible separation of follicular components. Compared to the levels found in serum, average concentrations of all proteins detected in this study were lower, which is in agreement with previous published work, using conventional techniques for the analysis for serum proteins present in FF [18]. In contrast uric acid concentrations were at the upper limit of that found in serum. However, due to the high sample dilution required to reduce the peak height of albumin, the less abundant molecular species were often difficult to recognize. In addition, the sample dilutions which were made during the initial sample collection increased the difficulty in finding the ideal conditions for identifying minor components on the electropherogram. Although small organic compounds would presumably also be present and separated by CZE, they were largely obscured by the dominant signals from proteins. As mentioned above the exception was uric acid which was dominant non protein component.

1 The concentrations of unknown peak 1 (Figure 5E<sub>1</sub>),  $\gamma$ -globulin (Figure 5A<sub>1</sub>) and prealbumin (Figure  
2 5C<sub>1</sub>) showed a clear negative correlation with follicular size. Complement (Figure 5B<sub>1</sub>), uric acid  
3 (Figure 5D<sub>1</sub>), unknown protein peaks 3, 5 and 6 exhibited an exponential decay in concentrations, *i.e.*  
4 their levels dropped significantly with the follicular size until follicular diameter reached ~18-20 mm,  
5 then remained at constant levels afterwards. However, there are indications that all protein  
6 concentrations are slightly higher at the lower extremes of follicular diameter.  
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16 The absolute amount of protein per follicle was calculated to correct for the variability in volume and  
17 a significant positive correlations with follicular diameter were observed in all analyses (figure 5A<sub>2</sub>-  
18 5D<sub>2</sub>) with the notable exception of unknown protein peak 1 where the total concentration remained  
19 constant as the follicular diameter increased (figure 5E<sub>2</sub>).  
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27 As FF is a mixture of serum transudate and granulosa cell secretions, some components may reflect  
28 this transfer across the ovarian blood-follicular barrier, and the influence of the granulosa cells. The  
29 blood-follicle barrier has previously been described as, “leaky” [19] but also as selective to size and  
30 charge [20]. The barrier is more permeable to relatively low molecular weight proteins such as  
31 albumin (66 kDa) compared to  $\alpha$ -macroglobulin (700 kDa) and shows a preference for cations ( $\gamma$ -  
32 Globulins) over anions (Inter-alpha-inhibitor) [20]. The molecular mechanism of follicular growth is  
33 unknown but the fluid filled cavity would appear to be due to an influx of water taken from the blood  
34 circulation accompanied by a barrier delay in serum solutes such as serum proteins drawn in by  
35 osmotic pressures. As the spherical fluid cavity grows, the surface area will not increase at the same  
36 rate as the total volume. Since the boundary is a barrier to protein transfer, the total content of  
37 proteins increases but the concentration of serum transudate proteins decreases. This explains the  
38 pattern of concentration for most of the proteins identified. However, follicular granulosa and thecal  
39 cells, which form the peripheral boundary tissue, stimulated by gonadotropins alter their permeability,  
40 allowing access to previously excluded anionic molecules [20]. A change in permeability causing an  
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influx of proteins from plasma could account for why declining protein concentrations in growing follicles would plateau. This could be an explanation for the profile seen in complement, uric acid, unknown protein peak 3, 5 and 6. Thus, large follicles contain more proteins due to their increased permeability and sensitivity to gonadotrophins. However, what is clear is that all but one resolved compound in the CZE profiles demonstrated a decrease in concentration but a significantly correlated increase in total content. This would be consistent with a growth resulting from a sudden influx of water (which drives the increase in follicular volume) but a delayed transudate influx of larger molecules such as serum proteins, which have to cross a boundary membrane with a restrictive rate(s) of permissive transport. These rates of transport are not increasing as much as the total volume, *i.e.* a mathematical relationship one would expect in respect to the increase of surface area versus the volume of an expanding balloon.

Peak 1 may be a molecule that is present initially but no longer being produced or transferred from serum, hence it showed a continuous decline in concentration (figure 5E<sub>1</sub>).

#### 4.2 Identifying peaks on electropherogram

Seven unknown peaks were found. Peak 4 was identified as uric acid based on migration time and a spectrum that corresponded with standard uric acid (figure 4). The migration order of molecules in serum suggests that peak 2 could be a haptoglobin as it was placed between  $\alpha_2$ -macroglobulin and  $\alpha_1$ -antitrypsin [22]. Apolipoproteins appear near albumin and  $\alpha_1$ -acid-glycoproteins, thus it could possibly be peak 3. Nothing similar to peaks 5-7 was seen in serum and it is possible that these three molecules are novel molecules, locally produced by granulosa-lutein cells. Further work is required to identify these molecules.

### 4.3 Role of proteins in follicular development

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3 Previous work using 2-D gel analysis showed differences in the protein patterns between small (10-  
4 14 mm) and large follicles (18-20 mm) [18]. These changes included  $\gamma$ -globulin, transferrin,  $\alpha_1$ -  
5 antitrypsin, haptoglobins, apolipoproteins and albumin, which all increased in concentration with  
6 increasing follicular size. This present study has shown that the total quantitative amounts of these  
7 proteins increase with increasing follicular diameter; however, their concentrations in FF either  
8 decreased ( $\gamma$ -globulin) or remained constant / randomly distributed (transferrin,  $\alpha_1$ -antitrypsin,  
9 albumin).

10  
11 Transferrin is a vital iron chelator that transports and protects tissues from the toxic effects of free  
12 iron. Evidence of transferrin secretion by granulosa cells emphasises its possible role in oocyte  
13 maturation [19]. Its antioxidant properties could be protective against tissue damage and follicular  
14 atresia. Another possibility is that transferrin may be present to meet the iron demands of the  
15 expanding oocyte; granulosa cells could be compensating for low levels of transferrin being filtered  
16 across in the early follicle.  $\alpha$ -Macroglobulin had a similar pattern to that of transferrin. It was shown  
17 previously that it can bind to inhibins and activins, and has a role in their delivery and clearance [23].  
18 Inhibins and activin A are involved in follicular development and oocyte maturation, so it is possible  
19 that  $\alpha$  Macroglobulin also has a role to play in this process indirectly. Albumin acts as binding  
20 protein, and is the major protein in FF (median 21.5 mg/ml). It also has antioxidant properties as  
21 does uric acid [24]. Thus, albumin could be another important protein that has the potential role  
22 during follicular development. As the second most abundant component in serum,  $\gamma$ -globulin is also  
23 present in FF at high concentrations (median 3.42 mg/ml). Other proteins detected in this study, such  
24 as Prealbumin,  $\alpha$ -Macroglobulin,  $\alpha$ -Antitrypsin,  $\alpha$ -Acid Glycoprotein, are essential for maintaining  
25 osmotic pressures within the follicle and provision of nutrients [25].  
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1 All of the changes in protein concentration and total content could simply reflect changes in  
2 transudation of serum proteins across the follicle-blood barrier. The follicular surface area increases  
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4 with follicle maturity and thus will facilitate further transudation (with rate dependant on the  
5 permeability of the individual protein across the tissue barrier). However, the increase in FF volume  
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7 is greater than that of the follicle surface area and thus a dilutional effect will simultaneously occur.  
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9 The net result is a modest increase in total serum proteins but a decrease of individual serum protein  
10 concentrations within FF. Therefore, although various functional roles for these serum proteins in  
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12 follicular-oocyte maturity can be postulated, it is highly likely that they are simple transudates, and  
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14 levels are secondary to maturity events dictated by follicular size.  
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## 5. Conclusion:

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3 CZE is ideal for studies on a large number of samples due to its simplicity. All but one resolved  
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5 compound in the CZE profiles demonstrated a decrease in concentration as the diameter of the  
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7 follicle increased but a significantly correlated increase in total content. This would be consistent  
8  
9 with a growth resulting from a sudden influx of water that drives the increase in follicular volume  
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11 and a delayed transudate in flux of larger molecules such as serum proteins; which have to cross a  
12  
13 boundary membrane with a restrictive rate(s) of permissive transport. However, there are suggestions  
14  
15 of more complex interactions with granulosa/thecal cells and changing permeability of their barrier.  
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17 Some serum proteins, including transferrin,  $\alpha$ -Macroglobulin and albumin, are thought to have a role  
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19 in oocyte maturity, but no significant difference in protein levels was found with regard to oocyte  
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21 recovery and fertilisation.  
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29 Identification of the unknown peak involving mass spectrometry analysis is under way and selective  
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31 removal of more concentrated protein (*e.g.* albumin) will enhance separation of smaller peaks and  
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33 explore their correlation with oocyte maturation and fertility outcome.  
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**Figure legends:**

**Figure 1:** Typical CZE electropherogram of pooled serum diluted 1:5 with ddH<sub>2</sub>O.

Conditions: 25mM NaTB with 1mM EDTA; pH 9.2; Voltage 23kV; Temperature 20°C; Injection 2sec hydrodynamic; Detection: 195nm.

$\gamma$ G=  $\gamma$ -Globulin; C= Complement; T= Transferrin;  $\alpha$ M=  $\alpha$ -Macroglobulin;  $\alpha$ AT=  $\alpha$ -Antitrypsin;  $\alpha$ AG=  $\alpha$ -Acid Glycoprotein; A= Albumin; P= Prealbumin. 1 and 2 mean that the two unidentified peaks on this graph.

mAU: milli arbitrary units; NaTB: Sodium tetraborate.

**Figure 2:** Typical electropherograms of PFF at final optimal conditions (a) and with magnification of the smaller peaks post albumin (b).

15 peaks were elicited.  $\gamma$ G =  $\gamma$ -Globulin, C = Complement, T = Transferrin,  $\alpha$ M =  $\alpha$ -Macroglobulin,  $\alpha$ AT =  $\alpha$ -Antitrypsin,  $\alpha$ AG =  $\alpha$ -Acid-Glycoprotein, A = Albumin, P = Prealbumin. 1-7 labelled unknown peaks 1 to 7.

**Figure 3:** CZE separation of both PFF and 5 kD ultra filtrate at optimal conditions.

Peak 4 was present in both electropherograms.

**Figure 4:** Identification of peak 4 as uric acid (overlay not to scale).

Migration times and spectrums of the peak in PFF matched with standard 10mM uric acid at optimal conditions. UV absorption maxima are at 214, 234 and 290 nm.

**Figure 5:** Scatter plots demonstrating the relationship between follicular diameter with follicular concentrations and total follicular fluid content of three serum proteins, uric acid and unknown peak

1:

$5A_1$ - $5E_1$ : Scatter plots of concentrations for  $\gamma$ - globulin ( $A_1$ ), complement ( $B_1$ ), prealbumin ( $C_1$ ), peak 4 ( $D_1$ : uric acid) and unknown protein peak 1 ( $E_1$ ) against follicular diameter. Linear or non-linear

correlation fit were displayed for each graph accordingly.

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5A<sub>2</sub>-5E<sub>2</sub>: Scatter plots of total amount for  $\gamma$ - globulin (A<sub>2</sub>), complement (B<sub>2</sub>), prealbumin (C<sub>2</sub>), peak 4 (D<sub>2</sub>: uric acid) and unknown protein peak 1 (E<sub>2</sub>) against follicular diameter. Linear correlation line was fitted for each graph accordingly.

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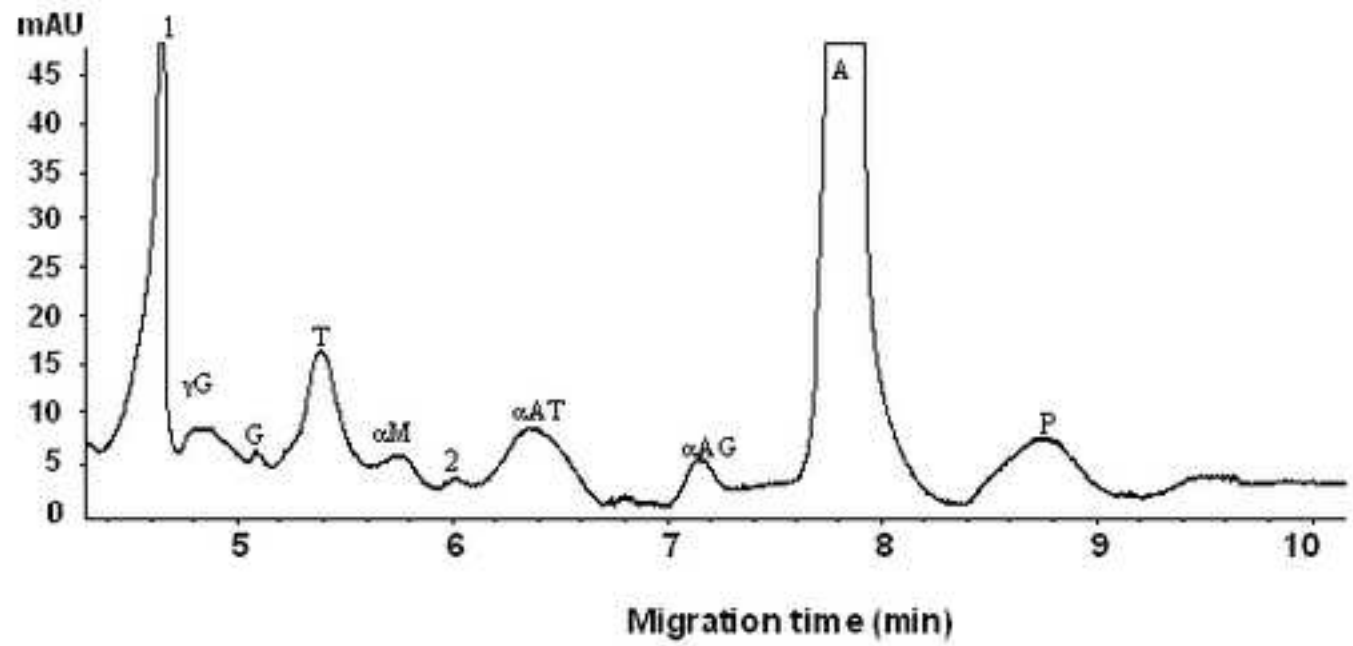




Figure 2

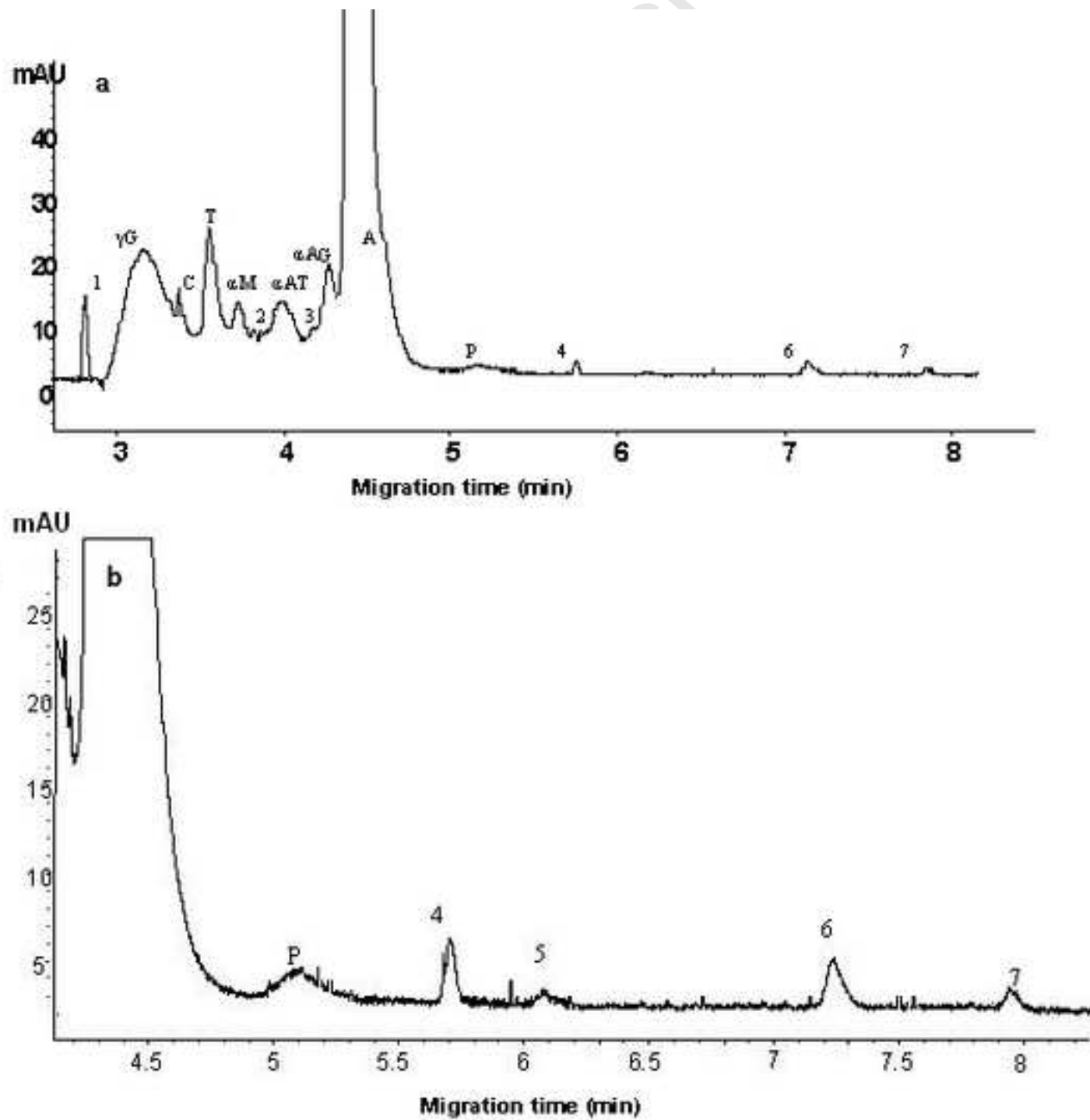


Figure 3

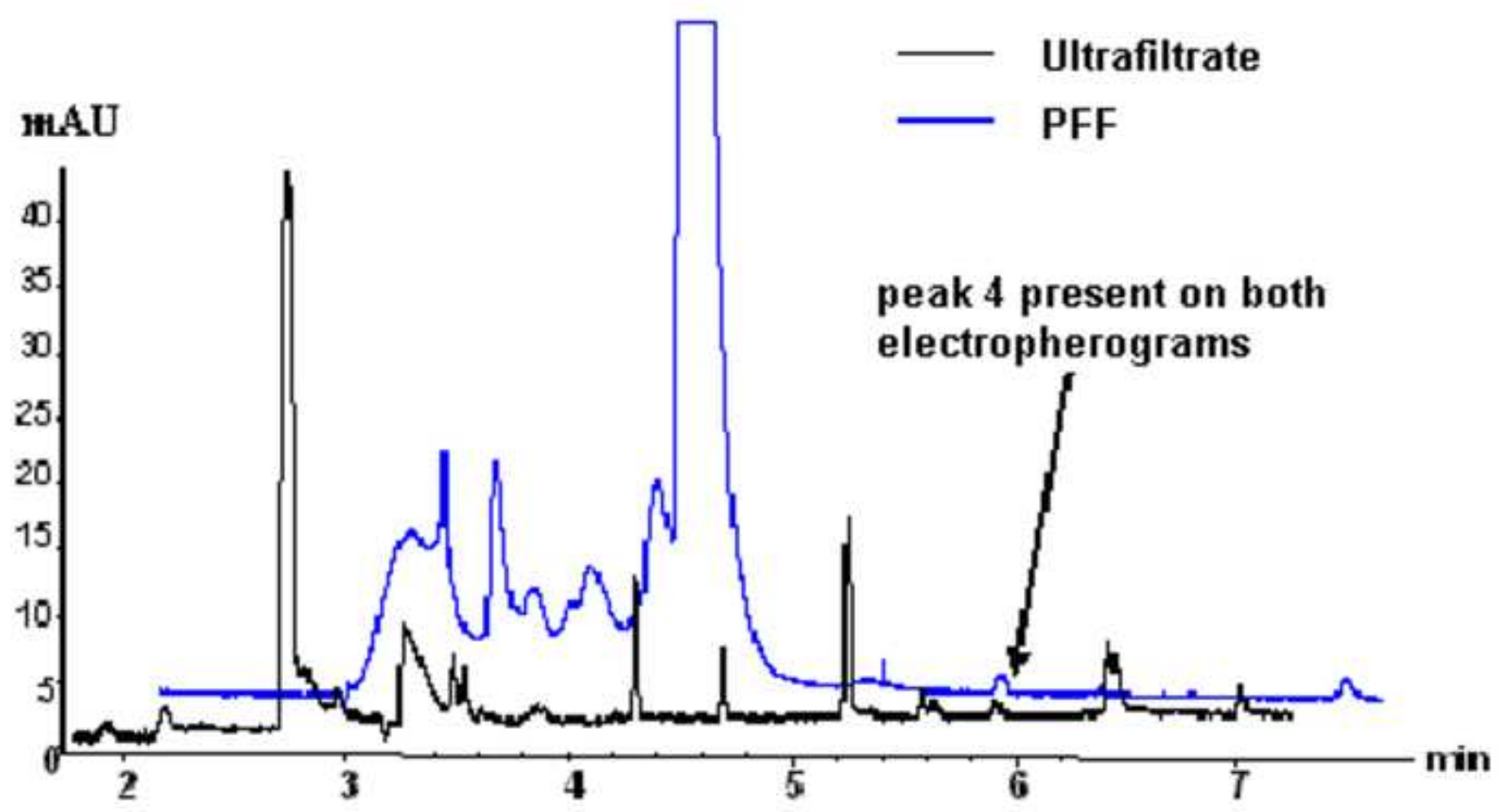
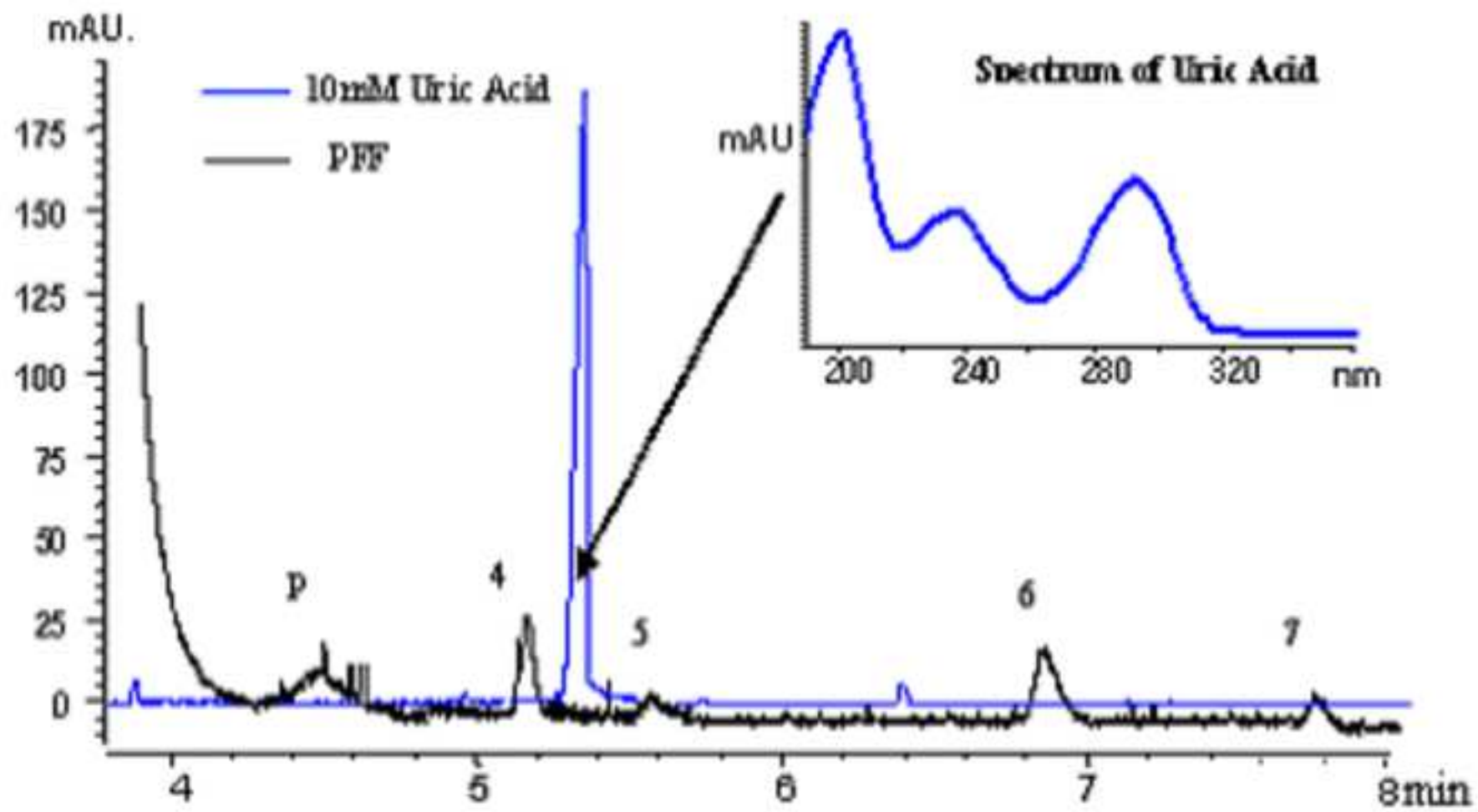
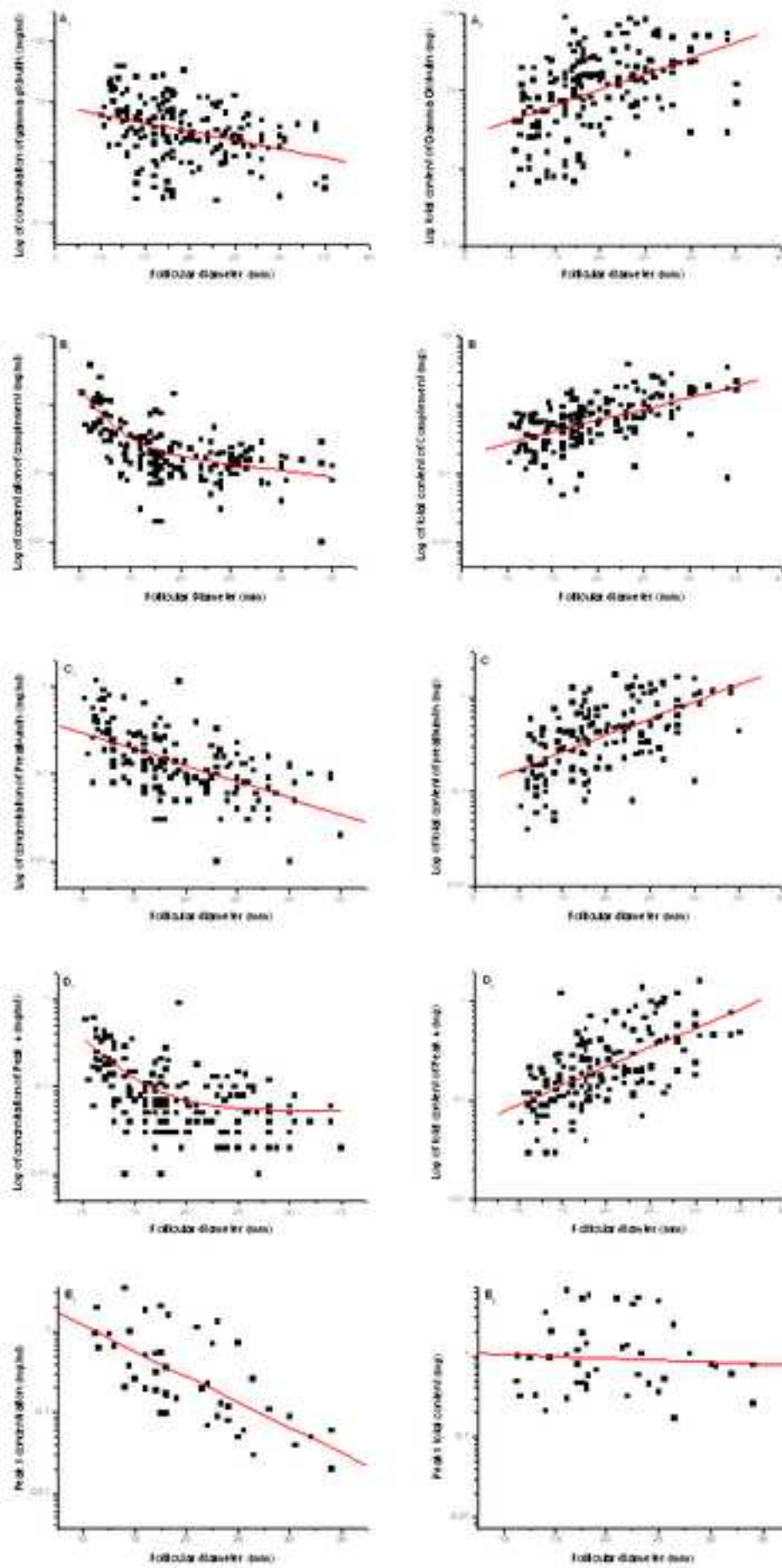


Figure 4





**Table 1:** Calculations for corrected peak areas, concentrations and total content for CZE.

$CP_{sam}$ - corrected peak area of samples;  $CP_{std}$  = corrected peak area for the standards;  $Conc_{.std}$  = standard concentration of BSA;  $Conc.$ = concentration of the samples;  $DF$  = dilution factor;  $TC$  = total content;  $Conc_{.PFF}$  = total protein concentration in PFF;  $DF_{PFF}$ = further dilution of PFF;  $DF_{ave}$ = average dilution factor of PFF (during FF aspiration).

Variables	Calculations
Corrected peak area ( $CP_{sam}$ , $CP_{std}$ )	$CP = \text{Peak area}/\text{Migration time}$
Concentration of substance ( $Conc.$ ) (mg/ml)	$Conc. = (CP_{sam}/CP_{std}) \times Conc_{.std} \times DF$
Total content of substance ( $TC$ ) (mg)	$TC = Conc. \times \text{FF volume}$
Total protein concentration in PFF (mg/ml)	$Conc_{.PFF} = Conc. (\text{derived from BSA standard curve}) \times DF_{PFF} \times DF_{ave}$

Table 2: Reproducibility of PFF separation by CZE using optimum condition. n= number of runs;  
SD= standard deviation; CV= coefficient of variation.

	$\gamma$ Globulin			Albumin		
	Mean	SD	CV	Mean	SD	CV
Intra-assay Reproducibility	n=10					
Spatial Peak Area	173.6	8.8	5.1	1560.4	123.9	7.9
Migration Time (min)	3.2	0.05	1.5	4.5	0.1	2.4
Inter-assay Reproducibility	n=4					
Spatial Peak Area	298.2	32.8	11.0	2870.0	492.3	17.2
Migration Time (min)	3.2	0.1	4.6	4.6	0.3	6.4