NONINVASIVE ASSESSMENT OF MENSTRUAL CYCLE HORMONE LEVELS AND OVULATION

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Noninvasive Assessment of Menstrual Cycle Hormone Levels and Ovulation

by

© Sarah J. Halfyard

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Abstract

Objective: The purpose of the study was to develop and validate a noninvasive, easy-to-use method of determining corpus luteum (CL) function.

Methods: This study documented progesterone levels obtained from tampons during menses from 18 women. The new method was compared to information obtained on analysis of progesterone concentrations from finger-prick bloodspots. The participants also kept daily temperature and Menstrual Cycle Diary© records for three cycles.

Results: CL functioning was evident in 28 cycles and nonexistent in 5 cycles as determined by bloodspot analysis. There was no statistically significant difference in progesterone concentrations extracted from tampons from functional and non-functional cycles ($p>0.05$). Diary assessment revealed functional cycles had significantly higher scores for the following experiences: side breast tenderness, cramps, fluid retention, frustration, sleep problems, depression, and breast size ($p<0.05$).

Conclusion: Progesterone in menstrual fluid may be an inappropriate marker for luteal function but select physical and emotional experiences observed during the menstrual cycle may indicate CL function.

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List of Abbreviations and Symbols

ANOVA – Analysis of variance

BBT – Basal body temperature

BMD – Bone mineral density

CaMos© - The Canadian Multicentre Osteoporosis Study

CL – Corpus luteum

CRH – Corticotropin-releasing hormone

EC – End of cycle

ERT – Estrogen replacement therapy

E_2 - Estradiol

FSH – Follicle-stimulating hormone

GABA - γ-Aminobutyric acid

GC-MS – Gas chromatography-mass spectrometry

GnRH – Gonadotropin-releasing hormone

ILP – Insufficient luteal phase

LH – Luteinizing hormone

LPD – Luteal phase defect

MC – Mid-cycle

M – Mean

ml – Milliliter

min – Minute

MMP's – Matrix metalloproteinases

NA – Not applicable

N_2 – Nitrogen

ng/ml – Nanograms per milliliter

P – Progesterone

PCOS – Polycystic ovary syndrome

QBT – Quantitative basal temperature

SLP – Short luteal phase

RIA – Radioimmunoassay

rpm – Revolutions per minute

3DA – The first three days of the following cycle

α – alpha

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1.0 Introduction

1.1 The Menstrual Cycle

The role of the ovaries in reproductive function has long been established. The human ovarian cycle involves cyclical changes which are induced by hormonal interactions among the hypothalamus, anterior pituitary, and the ovaries. Two phases divide the ovarian cycle. The first phase, called the follicular phase, typically involves the selective development of the dominant ovarian follicle in anticipation of ovulation. The luteal phase, the second phase of the cycle, begins immediately after ovulation. This phase is characterized by the formation of the hormone-secreting structure called the corpus luteum. At the level of the uterus the hormones secreted during the follicular phase promote endometrial regrowth and proliferation (Raine-Fenning *et al.*, 2004). Thus, this phase is often called the proliferative phase. During the luteal phase, the endometrium experiences higher glandular development and takes on more secretory properties (Franz III, 1988) in preparation for pregnancy. Fittingly referred to as the secretory phase, the end of this phase is signaled by the sloughing of the endometrium as menses. Collectively, the proliferative and secretory phases makeup what is generally called the menstrual cycle (Figure 1). For the purpose of this paper, the term menstrual cycle will be used.

1.1.1 The Follicular Phase

The menstrual cycle lasts an average of 28 days and ranges from 21-36 days (Prior, 1996). By definition the follicular phase starts on first day of menstruation and

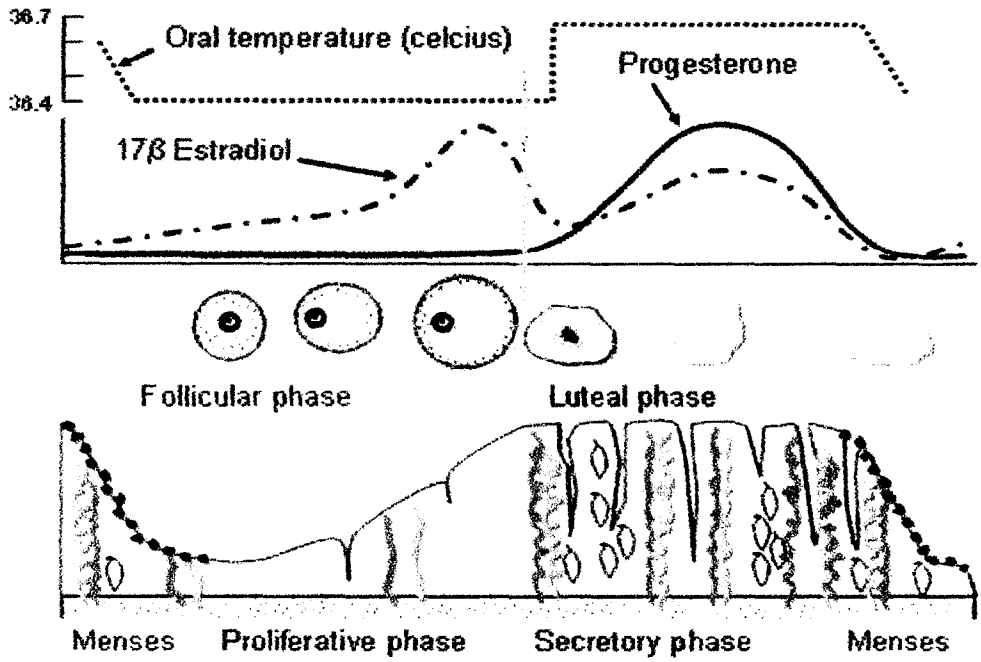


Figure 1. The menstrual cycle. The 17 β-estradiol (pg/ml) and progesterone (ng/ml) lines show the relative changes in these hormones over the menstrual cycle. The shaded arrow at mid-cycle indicates ovulation

lasts until ovulation (Chabbert Buffet, *et al.*, 1998). The follicular phase is characterized by the presence of a cohort of recruited maturing follicles within the ovaries. The hormonal environment during the follicular phase, principally, the presence of the anterior pituitary hormone, follicle-stimulating hormone (FSH), promotes the growth and development of the oocyte-containing follicles, which function to produce a mature oocyte ready for release by mid-cycle. Generally only one follicle becomes dominant and develops into a mature follicle while the others undergo atresia. During the follicular phase the granulosa cells that make up the follicle proliferate forming several layers which are surrounded by an outer layer of specialized thecal cells. These two cell-types work together, under the influence of FSH and another anterior pituitary hormone, luteinizing hormone (LH), to produce and secrete estradiol. The pulsatile release of LH and FSH from the anterior pituitary (Midgley & Jaffe, 1971) is controlled by gonadotropin-releasing hormones (GnRH) from the hypothalamus and modulated by ovarian steroids (Yen *et al.*, 1972). The levels of secreted FSH and LH are regulated by GnRH pulsatility elicited by external or internal stimuli (Leyendecker & Wildt, 1989). Typically, less frequent pulses lead to preferential FSH secretion whereas rapid recurrent pulses lead to LH secretion (McNeilly, 1988). FSH and LH leave the anterior pituitary, enter the circulation and travel to the granulosa and thecal cells in the ovary. During the follicular phase, LH stimulates androgen synthesis in the thecal cells and FSH promotes estrogen synthesis in the granulosa cells (Franz III, 1988) although it has been argued that preovulatory estradiol secretion may be independent of gonadotropin regulation, specifically by LH (Korenman & Sherman, 1973). The two-cell theory of estrogen

production states that the androgens synthesized in the thecal cells of the follicle are transported to the granulosa cells where they are converted to estrogens (Falck, 1959; Ryan & Petro, 1966; Bjersing, 1968). Increasing quantities of estradiol act on the hypothalamus and pituitary bringing about a surge in LH that triggers ovulation (Yussman & Taymor, 1970; Korenman & Sherman, 1973). Just prior to ovulation, the granulosa cells begin to transform into lutein cells with the capacity to produce progesterone (Yussman & Taylor, 1970; WHO, 1980). As a result of the changing follicle there is a dip in the level of circulating estradiol and a slight increase in the level of progesterone before the LH surge (Chabbert Buffet *et al.*, 1998). When the mature oocyte is expelled from the follicle and available for fertilization, the remaining ruptured follicle is transformed into a hormone-secreting structure called the corpus luteum (CL). This marks the beginning of the luteal phase.

1.1.2 The Luteal Phase

The luteal phase is often defined as the time period between the LH peak and the day preceding menses (Lenton *et al.*, 1984), generally lasting 12 to 16 days characterized by a dominant progesterone presence and a lower but still significant level of estradiol (Abraham *et al.*, 1972). The CL rapidly becomes highly vascularized within days of ovulation due to the guidance of angiogenic factors (Chabbert Buffet *et al.*, 1998). Under the influence of LH this structure secretes progesterone and estradiol (Filicori *et al*, 1984), however, some argue that the CL can function independent of LH (Asch *et al.*, 1982). Others report a slowing of LH pulsatility during the luteal phase due to

progesterone modulation (Soules *et al.*, 1984), therefore, progesterone likely acts directly on the hypothalamus and possibly the anterior pituitary preventing new follicular maturation and ovulation. The CL functions for about two weeks and in the absence of pregnancy will degenerate within 14 days. The deteriorating CL eventually forms a fibrous tissue mass called the corpus albicans. The decline in LH during the luteal phase may contribute to the demise of the CL (Soules *et al.*, 1984). The decline in GnRH pulsatility, as evident from decreased LH secretion, is known to result in an increase in FSH during the late luteal phase (Soules *et al.*, 1984) possibly allowing for new follicles to be recruited at that time. With the involution of the CL comes a decline in progesterone and estradiol levels with the resultant sloughing of the endometrium as menstrual flow. The first day of menstrual flow is considered the beginning of a new cycle.

1.2 Physiological and Behavioural Changes during the Menstrual Cycle

Associated with the cyclical hormonal fluctuations throughout the menstrual cycle are the dynamic, physiological and behavioural changes that occur within a woman. Changes in endometrial histology, cervical mucus, and temperature during the different phases of the menstrual cycle have been documented extensively and are commonly used to determine cycle phase. Behavioural changes in mood, sleeping patterns, and appetite are among numerous psychological parameters that have been studied.

1.2.1 Cyclic Changes in Endometrial Histology

The endometrium, the innermost lining of the uterus, is composed of a basilar layer and a functional layer. During the early follicular phase, the endometrium is thin (< 2 mm in thickness) due to the desquamation of the endometrial lining at menstruation (Strauss III & Coutifaris, 1999). Regeneration and desquamation coexist as proliferating epithelium has been seen within 36 hours from the onset of menstruation (Nogales-Ortiz *et al.*, 1978). As estradiol levels rise during the late follicular phase, the endometrium experiences glandular hyperplasia and an increase in stromal extracellular matrix that peaks around the time of ovulation (Clarke & Sutherland, 1990). Estradiol promotes the rapid proliferation of epithelial and stromal cells of the endometrium and by mid-cycle the endometrium has reached a thickness of 5 mm (Franz III, 1988). After ovulation, progesterone secreted by the CL initiates secretory development of the estrogen-primed endometrium and edema of the stroma cells and inhibits estrogen-induced cell proliferation (Clarke & Sutherland, 1990; Chabbert Buffet *et al.*, 1998). By the late luteal phase, the endometrium has reached a thickness of 5-6 mm, is highly vascularized, and has accumulated glycogen-rich vacuoles (Strauss III & Coutifaris, 1999). This nutrient-rich environment is necessary for preparation of possible implantation of an embryo. If pregnancy does not occur, the CL involutes resulting in a decline in the level of estradiol and progesterone. Although the mechanisms involved in menstruation are not clearly understood, the withdrawal of progesterone and estradiol lead to the infiltration of leukocytes, matrix metalloproteinases (MMPs), prostaglandins, and lysosomes (Fraser, 1999). These are associated with a hypoxic environment and the sloughing of the

endometrial lining of the uterus (Fraser, 1999). Desquamation of the endometrium occurs in the superficial layers of the endometrium, generally the compacta and spongiosa layers of the functional layer (Nogales-Ortiz *et al.*, 1978). Menstrual effluent consists of tissue fragments and blood and total fluid volume may vary from cycle to cycle (Fraser *et al.*, 2001). The normal amount of blood loss averages about 42 ml (range of 16.0-65.7 ml) with day 2 being the heaviest day of bleeding (Vasilenko *et al.*, 1988).

1.2.2 Cyclic Changes in Cervical Mucus

During the follicular phase, when estradiol is at its peak just prior to ovulation, the endometrial glands, notably the glands in the cervical area, secrete an abundance of stretchy, thin mucus (Pommerenke & Viergiver, 1947, Rarick *et al.*, 1990). This has biological importance in that the mucus strings align along the cervical canal creating a channel-like conduit for sperm motility in the direction of the to-be-fertilized egg (Lamar *et al.*, 1940). The day of peak mucus discharge is averaged to be day 15 (± 2.6) of the menstrual cycle (WHO, 1983) and has been documented to occur 1-2 days prior to the basal temperature shift (Pommerenke & Viergiver, 1947). Patterns of mucus discharge have been used to define the follicular and luteal phases and determine the fertile period of the cycle, which was defined as any day before the peak mucus discharge up to 3 days after the peak (WHO, 1983). The probability of pregnancy was highest on peak day (WHO, 1983; Stanford *et al.*, 2003). In the latter half of the cycle, under the influence of progesterone, the cervical mucus becomes thick and sticky and in effect acts as a barrier for sperm passage through the cervical canal (Lamar *et al.*, 1940; Niaraki *et al.*, 1981).

1.2.3 Cyclic Changes in Temperature

Basal body temperature is also influenced by the cyclical changes in sex hormones throughout the menstrual cycle. Body temperature is reported to be at its lowest during the late follicular phase when estrogen is at its highest (Rubenstein, 1938; Stachenfeld *et al.*, 2000). A small but significant rise in basal (resting) temperature by 0.2°C-0.5°C occurs during the luteal phase of the menstrual cycle and is not seen in anovulatory cycles (Vollman, 1977). This rise starts about 24 hours after ovulation (Franz III, 1988) and about 48 hours after the LH peak (Prior *et al.*, 1990a) due to the thermogenic properties of progesterone (Israel & Schneller, 1950). This temperature rise is typically maintained throughout the length of the luteal phase as long as progesterone levels are elevated. Temperature decreases to baseline values again during the follicular phase. It has been suggested that estrogen modifies the temperature effects of progesterone (Stachenfeld *et al.*, 2000). The mechanism for the thermogenic effect of progesterone is not clear, however, progesterone may act on areas within the central nervous system, such as the preoptic area of the hypothalamus, which control body temperature (Tsai *et al.*, 1988). Warm-sensitive neurons located in the preoptic area are inhibited by progesterone which may result in the blockage of heat-loss mechanisms and the facilitation of heat-production mechanisms (Tsai *et al.*, 1988). In rat medial preoptic area neurons, allopregnanolone, progesterone's primary metabolite, enhances γ-Aminobutyric acid (GABA)-mediated transmission, which plays an important role in the control of body temperature (Uchida *et al.*, 2002).

1.2.4 Cyclic Changes in Behaviour

Behavioral changes during the normal menstrual cycle have been widely documented and numerous studies have investigated the role of cycle phase in this phenomenon. Studies have reported cycle-phase related changes in affect showing a correlation of negative affect or mood change such as depression prior to and during menses (Beumont *et al.*, 1975; Johnson *et al.*, 1995). However, other reports fail to reveal cycle-phase related changes in mood (Little & Zahn, 1974; Laessle *et al.*, 1990; Sato *et al.*, 1995) thus challenging the commonly held assumption that mood change is related to menstrual phase.

Generally, women report poor sleep in the late luteal phase and during menstruation (Shaver, 2002; Baker & Driver, 2004). In contrast, earlier research reported no effects of cycle phase on sleep (Laessle *et al.*, 1990; Driver *et al.*, 1996). More recently however, sleep quality or duration was shown to be associated with FSH levels (Touzet *et al.*, 2002), and lowered estradiol levels in older menstruating women (Hollander *et al.*, 2001) suggesting that menstrual cycle hormones influence sleep patterns.

Appetite (food intake) and food craving have been documented to increase during the luteal phase of the menstrual cycle (Dalvit, 1981; Both-Orchman *et al.*, 1988; Bancroft *et al.*, 1988; Johnson *et al.*, 1995) and decrease around the time of ovulation (Lyons *et al.*, 1989). Energy expenditure increases during the luteal phase and it was postulated that this higher expenditure may be related to the increase in food intake during the latter half of the menstrual cycle perhaps due to the metabolic influence of

progesterone (Webb, 1986). Barr *et al.* (1995) provide confirmatory evidence of a higher energy intake in the luteal phase of ovulatory cycles. In that study, mean energy intake was 1259 KJ/d higher during the luteal phase than during the follicular phase (Barr *et al.*, 1995). However, some researchers have documented two significant peaks in appetite just prior to ovulation and menses (Laessle *et al.*, 1990), while others fail to detect a difference in energy intake throughout the menstrual cycle (Fong & Kretsch, 1993).

Changes in other physical and emotional experiences such as breast tenderness (Hale *et al.*, 2003), fluid retention (Feuerstein & Shaw, 2002), and irritability (Feuerstein & Shaw, 2002) have been documented throughout the menstrual cycle. According to Magyar *et al.* (1979), menstrual cycle experiences such as these identify ovulation in women with cycles of normal length. If this is indeed true, then documentation of ovulation may be obtained without the assistance of the invasive ovulation detection methods used today.

1.3 Menstrual Cycle Abnormalities

The normal menstrual cycle lasts an average of 28 days and ranges from 21-36 days (Prior, 1996). An ovulatory menstrual cycle can be described as one that is documented as having a normal cycle length, a mid-luteal serum progesterone threshold of 5 ng/ml and progesterone peak equal to or greater than 14 ng/ml (Abraham *et al.*, 1974; Marinho *et al.*, 1982; Petsos *et al.*, 1985), and a maximal cervical mucus stretchiness observation (Rarick *et al.*, 1990) 12 days or more before the onset of menses.

Deviations from this criterion would be considered abnormal. The absence of menstruation for 6 months or more is called amenorrhea and cycles of length greater than 36 days are considered oligomenorrheic (Prior, 1996). Luteal phase deficiency (LPD), also termed luteal insufficiency, is defined as inadequate CL function (McNeely & Soules, 1988) and can occur in cycles of any length. A short luteal phase, a variant of LPD, is associated with a luteal phase length of $\leq$11 days in duration as determined by LH and temperature analysis (Lenton *et al.*, 1984; Jordan *et al.*, 1994, respectively). Using quantitative basal temperature (QBT) analysis (Prior *et al.*, 1990a), a short luteal phase is less than ten days (Vollman, 1977). An anovulatory cycle, which can also occur in cycles of any length, is one in which there is no release of the oocyte at mid-cycle thus no formation of a CL (Prior, 1996). Although respecting the differences between these two concepts, for the purpose of this paper the terms ovulatory cycle and functional CL will be used interchangeably as will anovulatory cycle and non-functional CL.

Menstrual cycle abnormalities are common among women (Treloar *et al.*, 1967; Vollman, 1977). Intraindividual variability of cycle length is also found in regularly menstruating women (Treloar *et al.*, 1967; Prior *et al.*, 1996). Abnormalities in the menstrual cycle are more often observed in younger women within the first few years after menarche and in older women approaching menopause (Southam & Richardt, 1966; Treloar *et al.*, 1967; Doring, 1969; Metcalf, 1983). Many studies have documented anovulation and luteal phase defects including short luteal phase in regularly menstruating women using one or more ovulation detection methods (Table 1). Luteal

Table 1. A selection of studies revealing menstrual cycle abnormalities in women using various ovulation detection methods

Ovarian Disturbance	Methodology	Author(s)
Anovulation	BBT	Jones, 1949
		Marshall, 1963
		Döring, 1969
		Nagata *et al.*, 1986
	QBT	Prior *et al.*,1990b
		Barr *et al.*, 1994a
		Vollman, 1977
	Serum progesterone	Wathen *et al.*, 1984
	Urinary pregnanediol	Jones, 1949
		Metcalfe, 1983
		De Souza *et al.*, 1998
	Salivary progesterone	Vuorento *et al.*, 1989
	Endometrial biopsy	Jones, 1949
	Ultrasonography	Queenan *et al.*, 1980
		Petsos *et al.*, 1985
	Urinary LH	De Souza *et al.*, 1998
LPD	BBT	Jones, 1949
	Urinary pregnanediol	Jones, 1949
		De Souza *et al.*, 1997
		De Souza *et al.*, 1998
	Serum progesterone	Jordan *et al.*, 1994
		De Souza *et al.*, 1997
	Urinary LH	De Souza *et al.*, 1998
	Endometrial biopsy	Jones, 1949
		Davis *et al.*, 1989
SLP	BBT	Barr *et al.*, 1994a
		Jones, 1949
		Döring, 1969
		Prior *et al.*, 1982
	QBT	Prior *et al.*,1990b
	Endometrial biopsy	Annos *et al.*, 1980
	Plasma LH	Smith *et al.*, 1984
		Sherman & Korenman, 1974a
		Lenton *et al.*, 1984

BBT, Basal body temperature; LPD, Luteal phase defect; SLP, Short luteal phase; LH, Luteinizing hormone; QBT, Quantitative basal temperature

phase defects occur with similar incidence in both infertile women and those with normal fertility (Davis *et al.*, 1989). Smith *et al.* (1984) documented the incidence of short luteal phase as 9% for infertile women and 8% for women without fertility problems. In a study evaluating the prevention of bone loss by synthetic progesterone administration, 61% of the 73 recruited recreational athletes that reported regular cycles experienced either an anovulatory cycle or a cycle of short luteal phase length during two prescreening cycles (Prior *et al.*, 1994). A prospective one year study investigating bone loss in premenopausal women found only 20% of the 66 women participating in the study experienced normal menstrual cycles consistently throughout the study period (Prior et al., 1990b). The remaining 80% experienced one or more anovulatory cycles, short luteal phases, or cycles of abnormal length. Although some question the occurrence of anovulation in regularly menstruating women (Malcolm & Cumming, 2003), the abundance of literature on menstrual cycle function supports the view that menstrual cycle abnormalities in women are a real phenomenon.

1.4 Risk Factors for Menstrual Aberrations

What causes menstrual cycle abnormalities in healthy, menstruating women? The body's response to physical and psychological stressors may influence and be reflected through menstrual cycle patterns. Physical training, body weight or changes in body weight, and eating behaviour are among a multitude of possible factors that may

influence the hypothalamic-pituitary-gonadal axis. This influence may be expressed as alterations in menstrual cycle functioning.

1.4.1 Exercise

Research on the effects of exercise on reproductive function has recognized the occurrence of menstrual cycle abnormalities in female athletes (Feicht *et al.*, 1978; Dale *et al.*, 1979; Prior *et al.*, 1982). Teenage swimmers experienced anovulatory cycles and short luteal phases and had lower luteal phase progesterone levels compared to age-matched moderately active controls and adults (Bonen *et al.*, 1981). In another study, the sample prevalence of abnormal ovarian function in twenty-four exercising women was 48%, while the sample incidence of LPD and anovulation over a 3-month period was as high as 79% (De Souza *et al.*, 1998). The sedentary controls in this study ovulated 90% of the time and had zero inconsistencies between cycles (De Souza *et al.*, 1998). Blunted elevation of FSH during the follicular-luteal transition may have attributed to the high frequency of luteal phase defects and anovulation in this study (De Souza *et al.*, 1998). Alterations in levels of LH, FSH, progesterone, and estradiol resulting in menstrual dysfunction have been documented in athletes and untrained women with increasing training intensity (Shangold *et al.*, 1979; Bonen *et al.*, 1981; Bullen *et al.*, 1985; Baker & Demers, 1988). Amenorrhea, anovulation, and short luteal phases were more common in runners with longer training runs or more mileage per menstrual cycle (Feicht *et al.*, 1978; Shangold *et al.*, 1979; Prior *et al.*, 1982), which suggests a relationship between the incidence of menstrual cycle abnormalities and the intensity of training. However, Prior

et al. (1990b) found no difference in the number of ovulatory disturbances between women training for a marathon, moderately active women, and sedentary controls. In addition, De Souza and colleagues (1997) found no relationship between the amount of training and menstrual disturbances. Vigorous training is unlikely to be the sole cause of menstrual disturbances. Swartz *et al.* (1981) have shown that amenorrheic runners associate more stress with their physical training than do runners with regular menses. Stress can promote the release of cortisol from the adrenal gland via the hypothalamus-pituitary-adrenal axis. An association between cortisol and menstrual abnormalities may exist due to the inhibition of hypothalamic output by corticotrophin releasing hormone (CRH) which in turn may affect pituitary regulation of the menstrual cycle (Chrousos *et al.*, 1998). However, Loucks *et al.* (1998) found that altered LH pulsatility was due to low energy availability in exercising women and not the stress of exercise. Shangold & Levine (1982) concluded that the best predictor of menstrual dysfunction during training was pre-training menstrual pattern and that an individual's weight influenced menstrual cycle functioning irrespective of training, though Bullen *et al.* (1985) found that weight loss compounded the effects of exercise on reproductive function.

1.4.2 Weight

Body weight and weight change have long been associated with menstrual cycle functioning. The onset of menarche and the maintenance of menstrual cycles are hypothesized to depend on body weight (Frisch & Revelle, 1970; Frisch & McArthur, 1974), although it has been argued that the amount of body fat is more important to

ovulatory menstrual function (Wentz, 1980). In essence, adolescent girls must reach a critical or minimum weight for height to trigger the onset of menarche (Frisch & Revelle, 1970) and a minimum level of fatness is necessary for normal menstrual functioning (Frisch & McArthur, 1974). Menstrual cycle disturbances have been reported in healthy women who lose weight through dieting (Lev-Ran, 1974; Kapen *et al.*, 1981; Pirke *et al.*, 1985; Fichter & Pirke, 1986) and in obese women (Hartz *et al.*, 1979; Friedman & Kim, 1985). During a 6-week dieting experiment in which 6 to 8 kg of body weight was lost by the participants, three of the six women who showed pre-dieting ovulatory cycles experienced anovulatory cycles during the dieting period (Pirke *et al.*, 1985). In another study, young women experienced a loss of menses within 4 months on an uncontrolled weight-reducing diet (Lev-Ran, 1974). During a 6-month study investigating fat distribution and ovulation only nine of fifty-six overweight or obese women experienced regular cycles as determined by basal body temperature and serum progesterone (Morán *et al.*, 1999). Hartz *et al.* (1979) reported more irregular cycles of abnormal lengths in women who were significantly heavier or more obese than their regularly menstruating counterparts. Similarly, Sherman *et al.* (1981a) reported cycle irregularity in obese women during the 7 years after menarche however this was limited to those obese at age 18 who thereafter gained 5 lbs or more.

Dysfunction of the hypothalamic-pituitary-gonadal axis has been observed in women who suffer from eating disorders characterized by severe weight loss such as anorexia nervosa (Pirke *et al.*, 1979; Henley & Vaitukaitis, 1985). Simple weight loss, in the absence of pathology, has also been shown to cause disruptions in endocrine function.

During a period of weight loss resulting in amenorrhea, the sleep-wake pattern of LH secretion during sleep reverted back to pubertal secretory patterns where there was enhanced LH secretion (Kapen *et al.*, 1981). When the weight was regained LH secretion returned to adult patterns (no sleep-wake LH secretion difference). Similarly, low levels of LH as well as estrogen were reported during the starvation phase in five healthy women participating in a 3-week starvation experiment (Fichter & Pirke, 1986). Other reports, however, found no alteration of LH secretion in mild dieting, but, did observe a decrease in steroid production (Pirke *et al.*, 1985). In overweight or obese women abnormalities in endocrine function have also been reported. Obese women have been documented to have an excess of androgen levels produced by adipose tissue (Friedman & Kim, 1985; Unzer *et al.*, 1995). This hyperandrogenemia may indirectly cause the increase in LH through elevated levels of estrone, an estrogenic hormone. Estrone through its influence on the aromatizing system in adipose tissue causes shifts in LH/FSH and testosterone/estradiol ratios that may contribute to anovulation (Unzer *et al.*, 1995). Seemingly paradoxically, reduction of weight has resulted in the return of normal menstrual cyclicity and fertility in obese women (Bates & Whitworth, 1982; Harlass *et al.*, 1984).

1.4.3 Eating Behaviour

Women with a restrained eating style (i.e. conscious limitation of food intake) have experienced abnormalities in reproductive function without weight change (Schweiger *et al.*, 1992). Out of thirteen less restrained eaters, only 2 women had

abnormal menstrual cycles. Out of the highly restrained eaters only 2 of 9 women had normal cycles (Schweiger *et al.*, 1992). Low progesterone concentrations, fewer ovulatory cycles, and/or shorter luteal phase lengths have been documented in women scoring high for restrained eating (Schweiger *et al.*, 1992; Barr *et al.*, 1994a; Barr *et al.*, 1994b) providing evidence of an association between dietary restraint and ovulatory function. However, the reduction in caloric intake in restrained eaters compared to unrestrained eaters has not always been documented as significant (Barr *et al.*, 1994b) or accurate (Schoeller *et al.*, 1990).

Nonetheless, it has been postulated that the decrease in progesterone is a compensatory reaction to the reduction in caloric intake and energy expenditure in restrained eaters (Schweiger *et al.*, 1992). More recently it has been shown that low energy availability alters LH secretion (Loucks *et al.*, 1998) which in turn may shape the menstrual pattern. Mclean and colleagues (2001) found higher 24-h urinary cortisol excretions in women scoring high for food restraint. These authors speculate that women who score high for restraint may be experiencing higher stress than those scoring low for restraint (Mclean *et al.*, 2001). The heightened CRH release in these women may have an inhibitory impact on the hypothalamic-pituitary-gonadal axis thus interfering with the menstrual cycle (Chrousos *et al.*, 1998).

1.4.4 Summary

Exercise, absolute weight or modest fluctuations in body weight and eating behaviour may trigger changes in the hypothalamic-pituitary-gonadal axis resulting in

ovulatory disturbances. Not all obese, underweight, athletic, or dietary conscious women experience menstrual cycle abnormalities. Thus the sensitivity of the endocrine system to physical, hormonal, nutritional, and psychological factors may vary from individual to individual and perhaps from cycle to cycle.

1.5 Health and the Menstrual Cycle

Whatever may be the cause of menstrual cycle abnormalities in women, the more critical issue is the effect of these disturbances on the well-being of the individual. Irregularity of the cyclical hormonal milieu driving the menstrual cycle may play a significant role most obviously in reproductive function but also in other bodily systems. The interplay of steroid hormones throughout the body is essential for optimal bodily function. Therefore, abnormalities within the reproductive system stemming from peripheral or central origin may have effects in other areas of the body that are influenced either directly or indirectly by the hormonal changes related to the menstrual cycle.

1.5.1 Infertility

The most apparent outcome of menstrual disturbances is infertility - the inability to conceive a child or the inability to sustain a pregnancy. Although the etiologic factors of infertility may be multiple, menstrual cycle abnormalities are observed in infertile women. Anovulation is the most obvious cause of infertility; without the release of an oocyte there can be no fertilization. Anovulation along with hyperandrogenemia are the

most common causes of infertility in massively obese women (Friedman & Kim, 1985). Endometrial biopsy of 1022 patients with primary infertility showed anovulation in 12.6% of women (Rameshkumar & Thomas, 1991). However, in patients with either primary or secondary infertility inadequate CL function was the most frequently observed menstrual cycle abnormality. Follicular and ovulatory disturbances occurred less often (Jones, 1949). Thus, when ovulation did occur either progesterone secretion by the CL was not enough to prepare the endometrium for maintenance of a pregnancy or the endometrium was unable to respond to the secreted progesterone. For example, in some cases urinary pregnanediol levels were normal but endometrial biopsy showed a nonsecretory endometrium (Jones, 1949). Menstrual cycles of short luteal length are suggested to be a common form of infertility in women. This is possibly due to deficient FSH secretion resulting in insufficient follicle maturation leading to the derivation of an inadequate CL (Sherman & Korenman, 1974a). However, if short luteal phase is associated with infertility, then infertile women might be expected to have a higher incidence of this form of luteal phase defect. This was not observed in a study by Smith *et al.* (1984) who found the incidence of short luteal phase to be 8% and 9% respectively in a group of normal and infertile women. As cycles of short luteal length are also found in women without fertility problems (Smith *et al.*, 1984), and serum progesterone levels were greater than 2 SEM below normal in all infertile women with short luteal phase length studied by Sherman & Korenman (1974a), it is not necessarily the length of the luteal phase but ultimately the adequacy of steroidogenesis that is the major factor in infertility. In support of this, an infertile patient with consistent insufficient luteal

function was able to carry her pregnancy to term with progesterone therapy (Jones & Madrigal-Castro, 1970). Endometrial inadequacy is an entity associated with infertility that is usually caused by luteal abnormalities and can be treated with progesterone therapy (Moszkowski *et al.*, 1962).

1.5.2 Breast Cancer

Epidemiological studies have identified reproductive events as risk factors for the development of breast cancer (Kelsey, 1979; Sherman *et al.*, 1981b). Histological and morphological changes of the human breast correlate with menstrual phase (Vogel *et al.*, 1981; Battersby *et al.*, 1992; Ramakrishnan *et al.*, 2002) leaving little doubt that breast tissue is sensitive to fluctuations in hormones associated with the menstrual cycle. The action of progesterone on alveolar cells and lobular development within the breast has been generally described as proliferative causing a transient swelling of the breasts. Chang *et al.* (1995) describes this action as short-term, the long term (10-13 days) action of progesterone being anti-estrogenic, reducing estrogen-induced mitotic activity within the breast. Down-regulating estrogen receptors by progesterone (Battersby *et al.*, 1992) will influence the epithelial cell cycle and perhaps preventing breast epithelial hyperplasia, a risk factor for breast cancer (Page *et al.*, 1990). The presence of estrogen and progesterone receptors within the breast (Battersby *et al.*, 1992; Atalay *et al.*, 2002) indicates that this organ is a target of the hormonal action of these two steroids. Thus, it can be assumed that during altered hormonal conditions responsible for menstrual cycle abnormalities, the breast will be subject to the consequences of the same disorder. In a

prospective study investigating the incidence of breast cancer in infertile women, those with a history of progesterone deficiency had 5.4 times the risk of developing premenopausal breast cancer compared to women treated for non-hormonal-based infertility (Cowan *et al.*, 1981). As well, the women with persistent progesterone deficiency were 10 times more likely to die from malignant neoplasms compared to the hormone sufficient group (Cowan *et al.*, 1981). Bulbrook *et al.* (1978) found a correlation between sub-normal luteal phase plasma progesterone levels and increased risk of breast cancer in premenopausal women. Those with higher risk characteristics for breast cancer such as earlier age at menarche, a family history of breast cancer, later age at first parity, and lower urinary aetiocholanolone excretion (due to its reflection of progesterone levels) had a diminished level of progesterone in the luteal phase and more anovulatory cycles compared to those with lower or no risk factors (Bulbrook *et al.*, 1978). Early menarche and late menopause have been associated with increased risk of breast cancer (Staszewski, 1971; Trichopoulos *et al.*, 1972) possibly due to the exposure of a lengthened reproductive life time to the incidence of abnormal menstrual function (Sherman & Korenman, 1974b). Late age at first pregnancy and nulliparity have also been linked to an increase risk of breast cancer (Lowe & MacMahon, 1970). Sherman and Korenman (1974b) interpret these reproductive breast cancer risk factors as manifestations of an inadequate CL. In support of this, Grattarola (1964) found secretory endometrium during the luteal phase in 67.7% of normal premenopausal women but only in 17.2% of premenopausal breast cancer patients. Women with benign breast disease have lower than normal progesterone concentrations during the luteal phase (Sitruk-Ware

et al., 1977). Breast cancer patients show similar patterns of luteal deficiency (Swain *et al.*, 1974; Kodama *et al.*, 1977). These luteal phase abnormalities may reflect abnormal menstrual functioning during the earlier reproductive years in these women. Thus, these results are consistent with a role of altered ovarian hormone production in disease. Specifically, unopposed estrogen levels in the face of diminished progesterone concentrations have been implicated in the development of breast cancer and other malignancies.

Swain *et al.* (1974), however, found luteal deficiency only in women with advanced breast cancer and no increased incidence of ovulatory failure or decreased progesterone concentrations in early-stage breast cancer patients when compared to controls. Similarly, McFayden *et al.* (1976) found no evidence of abnormal hormone concentrations in women with breast cancer whereas other groups have found increased levels of estradiol and progesterone in premenopausal women with benign or malignant breast disease (England *et al.*, 1974; Skinner *et al.*, 1975). Coulom et al. (1983) reported that the risk for breast cancer in women with chronic anovulation equaled that of the general population. Thus, more research is needed to re-evaluate the relationship between menstrual cycle endocrine function and the risk for developing breast cancer.

1.5.3 Endometrial Cancer

Endometrial cancer has similar reproductive risk factors as breast cancer (Kaaks *et al.*, 2002). As Martel and Sommers (1957) observed, endometrial and breast adenoma often occur in the same individual. Associations among early menarche (Kaaks, *et al.*,

2002), late menopause, late age at first pregnancy (Salazar-Martinez *et al.*, 1999), nulliparity (Shu, *et al.*, 1991) and increased risk of endometrial cancer have been reported. However, some of these associations have not been corroborated (Henderson *et al.*, 1983; Salazar-Martinez *et al.*, 1999).

Infertility caused by chronic anovulation increases the risk of endometrial cancer 3-fold (Coulam *et al.*, 1983). Escobedo *et al.* (1991) investigated the relationship between endometrial cancer and infertility and concluded that risk for endometrial cancer may be limited to long-term infertility (>2 years) and women with infertility-associated conditions such as polycystic ovary syndrome (PCOS). Shu et al. (1991) has also reported an association between increased risk for endometrial cancer and PCOS as well as obesity. Women with endometrial cancer before the age of forty often have a past reproductive profile of menstrual irregularities, infertility and PCOS (Fox, 1976). In 16 endometrial cancer patients aged 19-35 years old, amenorrhea, sterility, obesity or menorrhagia (abnormally heavy or extended menstrual flow) were reported (Sommers *et al.*, 1949). Also these patients were more likely to have hyperplasia or fibrosis of stromal cells and luteinization of thecal cells. These findings are indicative of abnormal endocrine stimuli and responses which may potentially play a role in the pathogenesis of the disease.

The unopposed estrogen hypothesis is the principal theory used to explain the association between endocrine functioning and the development of endometrial cancer (Siiteri, 1978; Key & Pike, 1988). During the follicular phase, estrogens activate mitotic activity in endometrial cells which is counterbalanced by the anti-proliferative effects of

progesterone in the luteal phase (Key & Pike, 1988). Progesterone does this by reducing estrogen receptors within the endometrium and by inducing estradiol-metabolizing enzymes such as estradiol dehydrogenase and sulfotransferase which convert estradiol to the less active estrogen, estrone (Siiteri, 1978; Clarke, Adams, & Wren, 1982; Key & Pike, 1988). Thus, in progesterone deficient cycles, the mitogenic effects of estradiol are unopposed creating a continuous proliferative state within the endometrium which may lead to the development of cancerous tissue. In a prospective study, Modan *et al.* (1998) found a high rate of endometrial cancer in infertile women with unopposed estrogen status (i.e. progesterone deficient group). In support of this, women taking estrogen-only oral contraceptives or estrogen replacement therapy (ERT) have been reported to be at high risk for developing endometrial cancer whereas women taking a combination-type pill of estrogen and progestogen show decreased risk for developing the disease (Henderson *et al.*, 1983; Key & Pike, 1988; Shu *et al.*, 1991; Kaaks *et al.*, 2002).

Lifestyle factors such as obesity increase the risk for developing endometrial cancer (Shu *et al.*, 1991) and women with this disease are often of heavier stature (Sommers *et al.*, 1949; Austin *et al.*, 1991). Obesity has been associated with menstrual and hormonal abnormalities (Hartz *et al.*, 1979; Unzer *et al.*, 1995). Overweight premenopausal and menopausal women have lower than normal levels of sex hormone binding globulin (SHBG) and higher than normal levels of androstenedione and testosterone, and in menopausal women only, estradiol (Harlass *et al.*, 1984; Kaaks *et al.*, 2002). In premenopausal women this profile contributes to the increase in bioavailable ovarian androgens leading to ovulatory failure and progesterone deficiency (Barbieri *et*

al., 1988; Unzer *et al.*, 1995). It is not surprising then that obese women and women with PCOS, which is characterized by ovarian hyperandrogenism, reduced SHBG, chronic anovulation, and often obesity (Barbieri *et al.*, 1988), are at increased risk of developing endometrial cancer (Austin *et al.*, 1991; Shu *et al.*, 1991). Thus in premenopausal women, endometrial cancer risk is associated more with progesterone deficiency rather then higher estrogen levels. In menopausal women, however, excess weight is related to an increase in bioavailable estrone and estradiol due to the peripheral conversion of androstenedione to estrone and then estrone to estradiol (Kaaks *et al.*, 2002). Thus, in menopausal women increased risk of endometrial cancer is related to an elevation in bioavailable estrogens.

1.5.4 Bone Health/Osteoporosis

Bone loss and menopause are often linked. Estrogen deficiency due to the loss of ovarian function contributes to osteoporosis in menopausal women (Albright *et al.*, 1940; Albright *et al.*, 1941; Slemenda *et al.*, 1987). Estrogen therapy decreases bone loss by preventing or slowing bone resorption (Snow & Anderson, 1986). Furthermore, bone turnover is increased when estrogen levels fall (Carr *et al.*, 2003). Circulating progesterone levels also decrease at menopause. Progesterone action promotes bone formation and regulates bone turnover (Prior, 1990). Menopausal women treated with combination estrogen and progestin therapy showed increased bone mineral density (BMD) and reduced risk of fracture at the hip, vertebrae, and wrist (Cauley *et al.*, 2003). One year of cyclic medroxyprogesterone treatment increased spinal bone density in

active premenopausal women who experienced menstrual disturbances (Prior *et al.*, 1994).

In vitro studies have shown that progesterone competes with synthetic glucocorticoid binding sites on osteoblasts (bone forming cells) antagonizing the resorptive effects of glucocorticoids (Yoshioka *et al.*, 1980). Other studies have demonstrated direct anabolic effects of progesterone on the stimulation of bone cell proliferation in human osteoblast cell cultures (Tremollieres *et al.*, 1992). Ovariectomy in rats resulted in a decrease in progesterone-dependent osteoprogenitors in vertebral and femoral cell populations while treatment with estrogen and progesterone increased progesterone receptors in vertebral cell populations (Pei *et al.*, 1999, Pei *et al.*, 2003). Receptors specific for estrogen and progesterone have been identified in human osteoblast-like cells (Eriksen *et al.*, 1988; MacNamara *et al.*, 1995) suggesting a direct role of these hormones in bone metabolism. Therefore, a reduction in estrogen and progesterone levels at menopause may be involved in the uncoupling of bone formation to bone resorption resulting in net bone loss leading to osteoporosis in menopausal women.

Studies have shown that bone loss occurs in menstruating women of premenopausal age (Prior, 1990) and that reproductive status or alterations in the hormonal milieu of the menstrual cycle may be a determinant of current and future bone health (Drinkwater *et al.*, 1990; Prior *et al.*, 1990b; Prior *et al.*, 1994, Prior *et al.*, 1996; Cooper and Sandler, 1997, Sowers *et al.*, 2003). In a cross-sectional study of 70 healthy women, bone loss of the lumbar spine was determined to decrease by 44% from the age

34 throughout life (Krølner & Pors Nielsen, 1982). The rate of bone loss accelerates immediately after menopause. Prior *et al.* (1990b) correlated decreased bone density with subclinical ovulatory disturbances in 66 premenopausal women, aged 20-42 years, studied over a 12-month period. These authors found a significant decrease in bone density in women experiencing short luteal phases and anovulatory cycles in which progesterone was deficient. Bone density was maintained in women who experienced normal ovulatory cycles consistently throughout the year however, as a whole, the mean decrease in bone density in all participating women was 2.0% per year (Prior *et al.*, 1990b). A follow-up study showed an association between ovulatory disturbances initially documented in the first year and the rate of bone loss over a five year assessment (Prior *et al.*, 1996). In the same group of women, those in the lower tertile of luteal length tended to lose bone whereas those in the upper tertile of luteal length were protected from any loss in bone density (Petit *et al.*, 1999). In a group of progesterone deficient women treated for 1-year with medroxyprogesterone or placebo, spinal bone density of those in the placebo group significantly decreased while women treated with medroxyprogesterone showed increased bone density (Prior *et al.*, 1994). These results underscore the importance of progesterone in preventing bone loss and improving bone integrity.

Despite this evidence, some studies dispute an association between luteal abnormalities and changes in bone density (Waller *et al.*, 1996; De Souza, *et al.*, 1997; Bemben *et al.*, 2004). Bemben and colleagues (2004) found no significant effect of menstrual status on bone mineral density in young women athletes, however,

eumenorrheic women tended to have higher bone density than those who experienced oligomenorrhea or amenorrhea. In this study, gymnasts reported more menstrual disturbances but had significantly higher bone density compared to cross-country runners indicating that the type of mechanical loading can mitigate the effects of the hormonal environment. De Souza *et al.* (1997) argued that a reduction in estradiol and not progesterone was the cause of bone loss in female runners and that bone density is unaffected by progesterone deficient luteal phases as long as estradiol levels are adequate. Other studies, however, have failed to associate estradiol with changes in bone mineral density (Sowers *et al.*, 2003; Bemben *et al.*, 2004). Waller *et al.* (1996) did not find an association between bone density and either luteal phase length or progesterone (urinary pregnanediol). A slightly significant decrease in whole body bone density was seen with increased cycle length, but no significant decrease in bone density was noted with decreasing luteal function (Waller *et al.*, 1996). Petit *et al.* (1996, 1998) have questioned several methodological aspects of the studies by De Souza *et al.* (1997) and Waller *et al.* (1996). Adequacy of the detection methods of bone measurement, type of bone assessed, adequacy of ovulatory assessment, and timing of ovulatory assessment relative to bone assessment are all critical factors. Indeed, more research and standard research methods are needed to confirm potential relationships between menstrual and ovulatory disturbances and bone health.

1.6 Ovulation Detection Methods

The incidence of anovulation and short luteal phases differs among the many studies investigating menstrual cycle abnormalities. For example, Nagata *et al.* (1986) and Prior *et al.* (1990b) have documented the incidence of menstrual cycle aberrations as high as 64.9% and 80.3%, respectively. Waller *et al.* (1996) and Malcolm & Cumming (2003), on the other hand, have reported the prevalence of reproductive abnormalities to be much fewer among eumenorrheic women (5.1% and 3.7%, respectively). More than likely this disparity lies with the difference in study methods as well as design, i.e. longitudinal versus cross-sectional studies. Several methods are used to predict or detect ovulation in women. Methods include LH testing, basal body temperature recordings, serum progesterone measurement, endometrial biopsy, and ovarian ultrasonography.

1.6.1 LH Testing

Ovulation occurs between 8-40 hrs after the plasma LH peak (WHO, 1980), therefore, increased LH concentrations are often measured to predict ovulation. The failure to detect a significant rise in LH levels has been used as a criterion for anovulation and cycle abnormalities (Table 1). Colorimetric home kits have been made available whereby LH is measured in the urine by monoclonal anti-body-based enzyme immunoassay dipstick test kits in which color intensity estimates LH concentration (Elkind-Hirsch *et al.*, 1986). These kits have been deemed clinically reliable by some (Elkind-Hirsch *et al.*, 1986; Vermesh *et al.*, 1987) but questionable by others (Lloyd & Coulam, 1989). In the study by Elkind-Hirsch *et al.* (1986) the LH surge was associated

with basal temperature and serum progesterone in all cycles assessed, however, when compared with ultrasound the LH test was less reliable. Lloyd and Coulam (1989) documented the rise of LH after follicle rupture in 9% of cases as measured by ultrasonography. Anovulation has been documented in cycles in spite of the presence of a LH surge (McNeely & Soules, 1988). In this case, additional means of assessing luteal function were warranted. LH testing whether measured in serum or urine requires several days of testing and often more than once daily (Elkind-Hirsch *et al.*, 1986) which can make the process tedious and inconvenient.

1.6.2 Basal Body Temperature Recordings

Progesterone elicits thermogenic changes during the luteal phase (Israel & Schneller, 1950). As the rise in temperature during the second half of the cycle is indicative of luteal function, it is commonly used as an easy and inexpensive surrogate method of detecting ovulation in women. Jones (1949) described basal temperature charts as the most sensitive indicator of CL function. A biphasic temperature pattern during the menstrual cycle is correlated with ovulation (i.e. luteal function) whereas a monophasic pattern is associated with anovulation or no evidence of luteal function. Temperature recordings have been used for years as a method of determining menstrual status (Table 1).

Some consider basal body temperature recordings to be unreliable and inaccurate (Lenton *et al.*, 1977). In a study by Moghissi (1976) where ovulation was documented by hormonal analysis of E_2, LH, and progesterone, 20% of women participants had

monophasic temperature patterns indicative of anovulation. Studies by Johansson *et al.* (1972) and Hilgers and Bailey (1980) have found that basal body temperature curves have led to the unreliability of analyses in 12% and 6.8% of cycles examined, respectively. Some researchers have concluded that the presence of a monophasic temperature patterns is not sufficient to determine anovulation (Johansson *et al.*, 1972). Thus, other determinants of ovulation are required in addition to temperature.

In the past, many experimental subjects were required to plot and chart their own temperature (Marshall, 1963). Charts have been analyzed visually giving a nonquantitative interpretation of temperature data (Morris *et al.*, 1976; Hilgers & Bailey, 1980). These as well as a number of other issues such as the time of temperature taking and errors in reading thermometers have been considered problems that may compromise the reliability of temperature methods in determining menstrual status (Prior *et al.*, 1990a). To resolve these problems, Prior *et al.* (1990a) validated a quantitative basal temperature (QBT) analysis method against the midcycle LH peak in which ovulation and luteal phase lengths can be determined using a least mean square method of analysis (Maximina® Quantitative Basal Temperature Analysis Program). The participants were instructed how and when to take their temperature and to note any situations out of the ordinary such as sleeping in that may have affected their temperature. Using the least mean square criteria, a significant increase in temperature is used to determine the onset of the luteal phase, and this is suggestive of ovulation (Prior *et al.*, 1990a). This computer program has been used in the present study. Similar accuracy and reliability

were obtained with the mean temperature method (Vollman, 1977) and QBT (Prior *et al.*, 1990a).

1.6.3 Serum Progesterone Measurements

The CL secretes progesterone during the luteal phase of the menstrual cycle thus progesterone measurements in serum can be and are often used as the "gold standard" when evaluating CL function in women (Table 1). Thus, low luteal phase progesterone would suggest menstrual cycle abnormality (van Zonneveld, te Velde, & Koppeschaar, 1994) whether it is anovulation or CL dysfunction. Jordan *et al.* (1994) recommended a single midluteal serum value greater than 10 ng/ml or the sum of three serum values greater than 30 ng/ml as the best test for normal luteal phase function. However, progesterone concentrations are pulsatile and highly variable during the luteal phase (Soules *et al.*, 1988) thus the timing of progesterone measurements still remains problematic. Therefore, several serum or urine samples would be necessary to make a proper assessment of luteal function. Progesterone measurements obtained via venipuncture or through urine collection can be considered by some to be invasive, uncomfortable, and inconvenient (Elkind-Hirsch *et al.*, 1986; McLoughlin *et al.*, 1994). Furthermore, ascertaining their timing requires knowing the dates of onset for both the preceding and subsequent menstrual cycles.

An alternative to venipuncture is finger-prick blood sampling (McLoughlin *et al.*, 1994). This method requires only a few drops of blood pricked from the finger and collected on filter paper cards. Progesterone concentrations measured from finger-prick

blood samples has been highly correlated with progesterone concentrations from serum samples (r = 0.98, Petsos *et al.*, 1985; r = 0.98, Petsos *et al.*, 1986; r = 0.997, McLoughlin *et al.*, 1994; r = 0.98, Shirtcliff *et al.*, 2001). Bloodspot progesterone concentrations can thus be converted to serum equivalents using linear regression coefficients. There are many advantages to using bloodspot sampling. Firstly, they are useful for diverse study designs such as cross-sectional surveys and longitudinal and epidemiological studies that often require serial sampling over long periods of time and/or sampling of large populations (Worthman & Stallings, 1997). Bloodspot filter cards yield easy and long-time storage. Bloodspot cards can be stored up to 9 and 15 weeks at 25°C and 37°C, respectively, and nine months at moderate temperatures with little degradation in steroid hormones (Howe & Handelsman, 1997). When frozen, bloodspot samples are stable for at least a year (Worthman & Stallings, 1997). Lastly, bloodspot sampling is minimally invasive, convenient, and can be performed by the subjects themselves; bloodspot cards are easily transportable and because the blood sample is dried on the card, viruses such as hepatitis and HIV are killed which lowers a potential biohazard risk (Worthman & Stallings, 1997). Bloodspot sampling was used in the present study.

1.6.4 Endometrial Biopsy

Not withstanding Jones' (1949) view that temperature change is the most sensitive test of CL function, he maintains that endometrial biopsy analysis is the most sensitive test of luteal adequacy. When assessing CL function, endometrial biopsies are often obtained during the late luteal phase and timed by temperature recordings, LH

surge, or ultrasound scanning. Endometrial biopsy can be useful for detecting the presence (or absence) of secretory endometrium indicating a functional CL (or CL dysfunction) (Table 1). A luteal phase defect is predicted when the endometrium is out-of-phase by at least two days such that there is a discrepancy between the observed endometrium and the expected endometrial pattern (Annos, Thompson, & Taymor, 1980). Endometrial biopsy results have been correlated with luteal phase progesterone levels (Nadji *et al.*, 1975). Annos, Thompson, & Taymor, (1980) however, have shown a 50% discrepancy between these two parameters suggesting that one does not obviate the need for the other. Disparities among progesterone levels, endometrial biopsy assessments and temperature data analysis may be due to the failure of endometrium to be stimulated by progesterone action (Jones, 1949; Shangold *et al.*, 1983). Jordan *et al.* (1994) concluded that the sensitivity and specificity of endometrial biopsy was marginal in the diagnosis of luteal phase defects. In addition, Li *et al.* (1987) reported a low correlation between the interpretations of two experts ($r = 0.70$) making observer subjectivity problematic. Endometrial biopsy may also precipitate early menses which may shorten luteal phase length (Annos *et al.*, 1980). This procedure is invasive and the problem of proper timing of the biopsy remains unsolved.

1.6.5 Ultrasonography

Ultrasonography is a relatively direct method used for the prediction and detection of ovulation. The prediction of ovulation is based on follicle size and detection is based on the abrupt disappearance or collapse of the follicle (Marinho *et al.*, 1982).

Ultrasound scanning has been used to visualize follicle growth (O'Herlihy *et al.*, 1980) and the development of the CL indicating ovulation (Queenan *et al.*, 1980). This method is also useful in revealing menstrual cycle abnormalities (Table 1). In a study by Vermesh *et al.* (1987) ovulation as determined by ultrasonography followed LH peak for every cycle. However, the development and collapse of a dominant follicle (18-25 mm in size) as visualized by ultrasonography does not always prove ovulation (Queenan *et al.*, 1980; Petsos *et al.*, 1985). Used alone, ultrasonography may not be sufficient. Other studies have observed premature ovulation during the late follicular phase in women after the ultrasonography procedure (Testart *et al.*, 1982). Ultrasound scanning also requires a trained operator, several days of testing, is invasive (vaginal penetration) and expensive (Marinho *et al.*, 1982). Thus, ultrasonography is not practical for large-scale population studies.

1.7 Purpose of Present Study

The purpose of the present study is to develop and validate a noninvasive, easy-to-use method of determining CL function in women. The lack of standardized tests to detect menstrual cycle abnormalities makes it difficult to document the variability of menstrual cycle function among women. These ovarian disturbances can be a result of stress, weight loss or gain, illness and intense exercise or a combination of these factors (Prior, 1985). Previous research has associated ovarian disturbances with bone loss with attending risk for osteoporosis (Prior *et al.*, 1990b; Prior *et al.*, 1994). Women with a

history of ovulatory defects, specifically progesterone deficiency, are more at risk for breast cancer (Cowan *et al.*, 1981) and women experiencing chronic anovulation may be at higher risk for certain cancers such as breast cancer and endometrial carcinoma (Coulam *et al.*, 1983). However, more research and better research methods to document menstrual cycle hormonal characteristics are necessary to confirm these findings in prospective studies. The proposed study aims to develop such a method.

Current ovulation detection methods are variably reliable, tedious, invasive, and/or expensive (See section 1.6). Timing of tests for documentation of appropriate hormonal changes is difficult due to varying cycle lengths and the absence of a clear clinical marker of the appropriate timing within the cycle. This study proposes to document the progesterone levels obtained from tampons during menses to overcome the major problem, which is timing the sample appropriately. Measurement of sex hormones such as progesterone is useful in determining luteal function. Receptors for estradiol and progesterone in the endometrium (Haukkamaa & Luukkainen, 1974; Rao *et al.*, 1974; Jänne *et al.*, 1975; Flickinger *et al.*, 1977) concentrate these hormones. During menses the endometrium is shed and the shed tissue should contain these hormones.

Hormones can be extracted from used sanitary products (Hofer *et al.*, 1993; Bieglmayer *et al.*, 1995; Peddle [Hons Diss], 1996). Several studies have measured prostaglandin levels in menstrual fluid collected on tampons to examine its relationship to dysmenorrhia (painful menstruation) in women (Chan & Hill, 1978; Powell *et al.*, 1985; Hofer *et al.*, 1993; Bieglmayer *et al.*, 1995). Zhou *et al.* (1989) measured reproductive hormones in peripheral blood samples and menstrual fluid samples collected in silicone

rubber menstrual cups. Peripheral blood and menstrual fluid collected on days 1-3 of menses were centrifuged and the plasma assayed. Mean progesterone concentrations were less than 1 ng/ml for both peripheral and menstrual samples and no correlation was found between the progesterone measurements (Zhou *et al.*, 1989). These authors did not demonstrate the ovulatory status of the women participants.

Studies that have examined menses as an indicator of ovulation or luteal function are limited. Peddle (1996) developed a method to extract progesterone from doped and participant used tampons using a Sep-Pak C_{18} Classic cartridge. The results were variable and the amount of progesterone recovered from participant used tampons was low (<2 ng/tampon). Neither serum hormone levels nor basal temperature were measured at any time prior to menses thus menstrual status was not assessed in the participants.

The method in the present study will be compared to information obtained on quantitative assessment of serial daily basal temperature measurements (Prior *et al.*, 1990a), and analysis of progesterone concentrations from blood spots obtained after finger prick, which will act as the "gold standard" (Petsos *et al.*, 1986). Potentially this new method could be used cycle by cycle over many years to advance our understanding of menstrual cycle variability.

2.0 Materials and Methods

2.1 Participants

Women responded to an advertisement to participate in a study about ovarian function and menstrual experiences. Healthy women volunteers ages 21 to 37 were recruited on the basis of the following eligibility criteria: refrained from taking oral contraceptives for at least six months prior to starting the study, experienced regular cycles of normal length (21-36 days), and had previously used tampons. The initial meeting between the investigator and participant took about an hour. During that time, the participant first learned about the Menstrual Cycle Diary© (Prior, 1990) and answered questions regarding their health, diet, exercise habits, and history of menstruation and reproduction from a subset of the Baseline Questionnaire for CaMos©, the Canadian Multicentre Osteoporosis Study. Each woman signed a consent form outlining the protocol approved by the Human Investigation Committee, Faculty of Medicine, Memorial University of Newfoundland. The participants were given a copy of their signed consent form, 3 copies of the Menstrual Cycle Diary© (one for each cycle), and an explanation sheet describing briefly the menstrual cycle, the study protocol, and the Menstrual Cycle Diary©. See Appendix A for a copy of the consent form, Menstrual Cycle Diary©, and explanation sheet.

As participants, the women agreed to the following: keep a daily Menstrual Cycle Diary© for three menstrual cycles; record morning oral temperature for a three cycle period; monitor vaginal stretchiness of mucus daily for a three cycle period; have finger-prick blood samples taken; and collect all used tampons during the first two full days of

flow for three consecutive menstrual periods (Figure 2). Each woman began the study on the first day of her period.

2.2 Temperature Recording

Each woman was provided with a digital thermometer (Becton Dickinson). Oral temperature was taken in the morning before rising from bed. This was required every morning for the entire study period. Temperature was recorded in the designated section of the Menstrual Cycle Diary©. Missed days were left blank and the participants were asked to make note of illness, poor sleep, and late or early rising. Two types of Becton Dickinson digital thermometers were used during the study, one which read to one decimal place and the other which read to two decimal places. One of each thermometer type was returned upon request by two study participants in order to test the accuracy and precision of the thermometers. The thermometers were simultaneously tested twenty times at twenty different temperatures. The readings from the two thermometers were compared to a calibrated reference thermometer.

2.3 Menstrual Cycle Diary©

The Menstrual Cycle Diary© consists of a 21-item list of emotional and physical experiences with a column for each day of the cycle and was completed in the evening before going to bed (Prior, 1996). The experiences were recorded using either one of two

coding systems. The upper section of the diary consisted of experiences that a woman may/may not observe on different days of her cycle such as breast tenderness, cramps, headache, and depression and were rated for intensity on a 0-4 scale. The lower section of the diary consisted of experiences that occur daily but that may fluctuate from its usual state which were letter-rated: M (much less), L (a little less), U (usual), Y (a little increased), and Z (much increased). These experiences included appetite, breast size, feeling of self-worth and outside stresses. At the base of the diary was a designated area for temperature recordings and comments such as illness, poor sleep, late/early rise.

2.4 Monitoring Vaginal Mucus Stretchiness

Each woman monitored her vaginal mucus stretchiness each day she was not observing menstrual flow. An observation of 6-8 cm in mucus stretchiness was deemed maximal. The women were requested to call the investigator when maximal mucus stretchiness was observed. To monitor the stretchiness of cervical mucus, a woman would insert her index and middle fingers into her vagina, remove them and spread her fingers apart observing the abundance and stretchiness of the mucus. Ratings of mucus stretchiness were recorded on the Menstrual Cycle Diary© under the heading 'Mucus Secretions'. A scant or dry mucus observation received a reading of zero; increasing abundance and stretchiness of mucus received a rating between one and three and an observation of maximal mucus stretchiness (i.e. 6-8 cm in stretchiness) a rating of four.

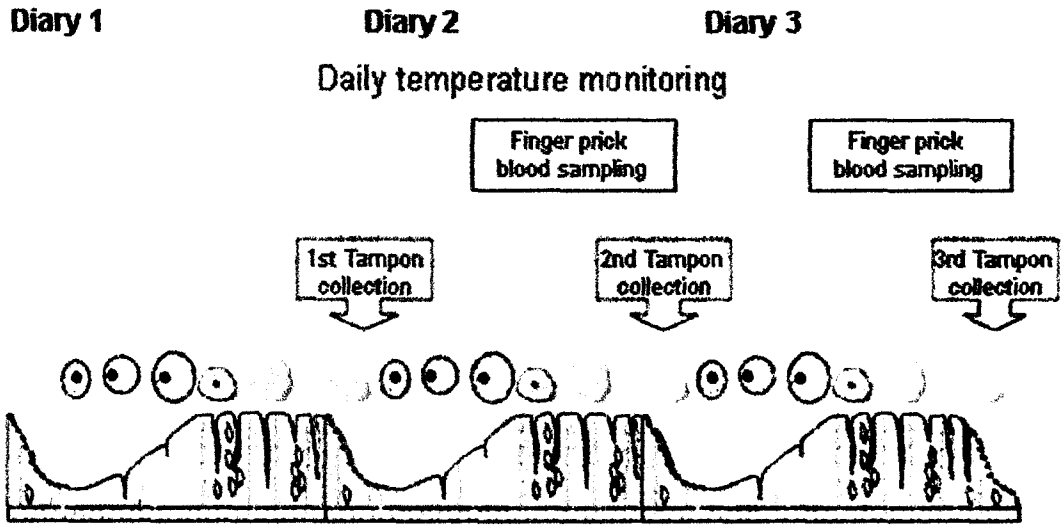


Figure 2. Timeline of the study protocol

2.5 Finger-prick Blood Sampling

Finger-prick blood sampling took place at the participant's home, her work place or at the investigator's laboratory located at the Health Sciences Centre. The tip of the finger was first swabbed with alcohol and wiped dry with gauze (patient ready, 2x2). Blood was drawn at the tip of the finger using a lancet (Becton Dickinson), a device with a retractable blade used to make a small incision. By gently squeezing the finger, eight free-flowing drops of blood were collected onto two filter paper cards (Mandel, Blood Collection Card #903). Each blood collection card was labeled with the participant's code, the date and time of sampling, and the sample number. The blood spots were air-dried at the laboratory, placed in plastic bags and stored in a -20°C freezer until radioimmunoassay (RIA). An additional drop of blood was collected in a capillary tube and capped at one end with a crito-cap (Monoject Scientific, Sherwood Medical) then centrifuged (5 min) and hematocrit measured using a Damon/IEC Division micro-capillary reader.

No finger-prick blood samples were taken during the first cycle but were taken during the second and third cycles. A series of seven finger-prick blood samples were obtained during each of the two cycles: three during the follicular phase, three during the luteal phase and one on the first day of menstrual flow. The timing of finger-prick blood samples relied on the observation of maximal stretchiness of vaginal mucus, a rating of four on the Menstrual Cycle Diary©. The participants contacted the investigator once maximal stretchiness was observed and a meeting was arranged within 24 hours after contact. Finger-prick blood samples were then taken for three consecutive days and

again for another three consecutive days about 5-7 days later. The women were also asked to contact the investigator once her period began. Finger-prick blood samples were collected within 24 hours of that call.

2.6 Collection of Used Tampons

The participants were provided with a box of tampons (Kotex Regular); screw-top, opaque plastic bottles for storing the used tampons; two Ziploc plastic bags; a permanent black marker; and coded labels. Tampons were generously donated by Kimberly-Clark (USA). Used tampons were collected by the participants for the first two full days of menstrual flow. Once a used tampon was placed in a bottle, a label was applied and the date and time recorded. The bottles were placed in a plastic bag and stored in the participant's freezer until a time for sample exchange was arranged. The samples were then stored at -20°C in a freezer in the Health Sciences Centre.

2.7 Extraction of Tampons

All glassware used in the extraction was silanized with 1% v/v dichlorodimethylsilane (Aldrich Chemical Company Inc.) in toluene (HPLC Grade, Fisher Scientific), air-dried, then rinsed twice with methanol and air-dried. Before silanization, re-used glassware were first washed in detergent, rinsed with tap water then twice with deionized water, baked under high heat for 1-2 hours, cooled, rinsed with methanol, and re-baked under high heat for another 1-2 hours.

Used tampons were removed from storage and individually weighed. Each tampon was submerged in 100 ml methanol (Optima, Fisher Scientific) in separate 250 ml Erlenmeyer flasks (Peddle, 1996). Methanol was used to extract progesterone from menstrual fluid on the tampon. The flasks were capped with aluminum foil, vortexed (speed 3 for 5 min, SMI® Multi-Tube Vortexer), stored in a 4°C refrigerator overnight, and revortexed again (speed 3 for 5 min) the following day (Peddle, 1996). The tampons were squeezed dry and discarded. About 60 ml of the methanol extract was transferred into two, 50-ml glass tubes (30 ml each tube) and centrifuged (3000 rpm for 10 min, Sorvall RT6000). About 25 ml of extract from each tube was evaporated under nitrogen (N_2) and heat (in fume hood, N-Evap Organomations Assoc.) in 5 ml increments into a third 50-ml glass tube. These tubes were capped and refrigerated until the following morning. Ether (5 ml, PRA Grade, Aldrich Chemical Company Inc.) was then added to each tube to precipitate out possible protein and non-lipid components from the extracts. The tubes were vortexed vigorously (Max Min™ Thermolyne Vortexer), and centrifuged (3000 rpm for 10 min). From each tube, 4 ml's of extract was evaporated under nitrogen (N_2) and heat (in fume hood) in 1 ml increments into 16x100 glass culture tubes. Acetonitrile (3 ml, Optima, Fisher Scientific), into which the progesterone dissolved, was added to the tubes, which were then vortexed vigorously. Hexane (3 ml, Optima, Fisher Scientific) was then added to dissolve lipid components of the extract. The tubes were revortexed and centrifuged (15 min at 3000 rpm). The immiscible layers (hexane on top) were then separated into two 16x100 culture tubes. Acetonitrile (2 ml) was added to the hexane extract and hexane (2 ml) added to the acetonitrile extract. The samples were

vortexed and centrifuged (15 min at 300 rpm) and placed in a -20°C freezer (30 min). Once removed from the freezer, the layers were again separated into two 16x100 tubes. Acetonitrile (2 ml) was added to the hexane extract and hexane (2 ml) to the acetonitrile extract. The samples were vortexed, centrifuged (15 at 3000 rpm), and placed in the freezer (-20°C for 30 min). Afterward, the hexane layers were discarded and the acteonitrile layers combined and placed in the freezer (-20°C for 30 min). Remaining hexane was removed and discarded. The acetonitrile extract was evaporated under nitrogen (N_2) and heat and stored in a -20°C freezer until the radioimmunoassays (RIAs) were performed.

2.8 Validation of Methodology

The process of extracting hormone from both the bloodspot filter cards and the tampons first had to be validated. Below are the steps taken to validate these two methods.

2.8.1 Finger-prick Bloodspot Assay

To validate the bloodspot assay of progesterone against the gold standard of a serum assay, low, medium and high concentrations of progesterone were needed from contemporaneous samples. This range of progesterone was obtained by collecting samples from women during the follicular phase, the luteal phase and during pregnancy, respectively. One pregnant woman and nine women with regular cycles were recruited. Each woman signed a consent form outlining the protocol approved by the Human

Investigation Committee (See Appendix A). The volunteers gave sequential finger-prick blood and serum samples once during the follicular phase and once during the luteal phase. One tube of serum (4 ml) by venous sampling and 4-8 drops of blood by finger-prick sampling were collected each time. The pregnant volunteer only needed to provide serum and finger-prick blood samples one time. Venous sampling was performed by a trained phlebotomist. Serum was centrifuged (20 min, 3000rpm), portioned into storage tubes, and frozen until radioimmunoassays were performed.

Serum and finger-prick blood samples were measured in duplicate using a commercially available radioimmunoassay kit (DSL-3900, Pro-lab Diagnostics). Standards and controls for the finger-prick blood sample assay were prepared by pipetting several 25 µl drops of each standard and control from the kit onto filter cards. The filter cards were air-dried and stored at -20°C. Twelve 1/8th inch diameter discs of standards, controls, and unknowns were punched from the filter cards and added to the assay tubes.

The efficacy of recovery of hormone was determined by spiking a sample of known low progesterone (follicular phase finger-prick blood sample) with elevated amounts of progesterone prepared on filter cards using progesterone standards from the RIA kit. To the assay tubes were added twelve 1/8th inch diameter discs: eight discs of the follicular phase sample and four discs of standard A (0 ng/ml) for the control, standard D (4.5 ng/ml), standard E (20 ng/ml), or standard F (70 ng/ml). Assuming that 25 µL of progesterone standard fills the entire 1.19 cm^2 circle on the filter cards, the expected progesterone concentrations were calculated for progesterone standards D (4.5

ng/ml), E (20 ng/ml), and F (70 ng/ml). As it takes 15 times the amount of progesterone in one 1/8th disc to be equivalent to the amount of one full circle, the expected progesterone concentrations in the four 1/8th discs added to the follicular phase sample for standards D, E, and F are 1.2 ng/ml, 5.3 ng/ml, and 18.7 ng/ml, respectively.

Linearity was determined by diluting a finger-prick blood sample of known high progesterone concentration (pregnancy sample) with a finger-prick blood sample of known low progesterone concentration (menopausal sample) by a factor of a half (1/2), a quarter (1/4), and an eighth (1/8). To the assay tubes, for the half dilution, were added six discs of the pregnancy sample and six discs of the menopausal sample; for the quarter dilution, three discs of the pregnancy sample and nine discs of the menopausal sample; and for the eighth dilution, 1.5 discs of the pregnancy sample and 10.5 discs of the menopausal sample.

Intra-assay precision of the finger-prick blood assay was determined by the mean of ten replicates of a low progesterone sample (follicular phase sample). Inter-assay variability was determined by assaying (in duplicate) the same sample on eight different occasions.

2.8.2 Validation of Extraction Method

The efficacy of the extraction of progesterone from used sanitary products was validated two ways:

In the first validation procedure un-used tampons were removed from the applicator and doped with 1 ml of 8 ng/ml, 16 ng/ml, 32 ng/ml, 64 ng/ml, or 128 ng/ml

progesterone in ethanol. The tampons were stored in labeled, opaque, plastic bottles, and stored in a -20°C freezer until extracted.

The second validation procedure involved extracting progesterone of known amount from tampons that mimicked actual used tampons during menstrual flow. To simulate used tampons, un-used tampons were doused with progesterone-doped menstrual fluid. A correspondent from the University of British Columbia was recruited to collect menstrual flow for one full period. Recruitment fell under the same guidelines as in the original study. Menstrual fluid was collected with The Keeper, a rubber receptacle for menstrual fluid. The volunteer was a current user of The Keeper. Collected menstrual flow was emptied into several sterile plastic containers and stored at freezer temperature until shipped (under dry ice) to Newfoundland. The containers were then stored at -20°C. In preparation for extraction, the samples were thawed and pooled. Phosphate buffered saline (15 ml) was added to thin the fluid, which was then divided into four equal portions (~11 ml). Each portion received 220 µl ethanol alone, 200 ng/ml, 600 ng/ml, or 1200 ng/ml progesterone in ethanol to yield a final concentration of at least 0 ng/ml, 4 ng/ml, 12 ng/ml, and 24 ng/ml, respectively. To maintain the integrity of the menstrual fluid, the addition of ethanol alone or progesterone in ethanol equaled only 2% of the total volume. Un-used tampons were removed from the applicator and doped with 5 ml of menstrual fluid. The tampons were placed in labeled, opaque, plastic bottles and stored at -20°C until extracted.

Samples were measured in duplicate using a radioimmunoassay kit (DSL 3900, Pro-lab Diagnostics). Before assay, the samples were reconstituted with 1 ml Standard A

buffer (0 ng/ml, DSL 3900-100, Pro-lab Diagnostics), vortexed vigorously and sonicated for 30 min (Bransonic 3510 ultrasonic cleaner) and then gently vortexed immediately before addition to the assay tube. Hexane extracts were also retained and assayed for the validation.

Linearity of the assay was determined by diluting a high progesterone sample (un-used tampon doped with 5 ml of 24 ng/ml P in menstrual fluid) with progesterone Standard A (0 ng/ml) by a dilution factor of a half (1/2), a quarter (1/4), and an eighth (1/8).

Intra-assay precision was determined from the mean of ten replicates of a low (un-used tampon doped with 5 ml of 4 ng/ml P in menstrual fluid), medium (un-used tampon doped with 5 ml of 12 ng/ml P in menstrual fluid), or high progesterone sample (un-used tampon doped with 5 ml of 24 ng/ml P in menstrual fluid). Inter-assay variability was determined by assaying (in duplicate) a high progesterone sample (un-used tampon doped with 5 ml of 24 ng/ml P in menstrual fluid) on four different occasions.

2.9 Gas Chromatography-Mass Spectrometry (GC-MS)

Initial extractions of blank un-used tampons showed a measurable amount of progesterone in the RIA print-out. Several un-used tampons were extracted, reconstituted with 500 µl methanol, and analyzed for progesterone using gas chromatography-mass

spectrometry (GC-MS). Taking into consideration the sensitivity of the procedure a sample concentrated 10X was also analyzed for progesterone.

2.10 Determination of Menstrual Cycle Characteristics

Progesterone measured from the bloodspots was used as the gold standard when determining menstrual function. Luteal phase lengths were considered short if they were less then 12 days in length from the onset of progesterone greater than 5 ng/ml or from the time of maximal mucus observation. Cycles having normal progesterone secretion (bloodspot progesterone > 5 ng/ml and mid-luteal peak ≥ 14 ng/ml) with adequate cycle and luteal phase lengths (21-36, ≥ 12 days, respectively) were considered to have normal luteal function and likely ovulatory. Cycles were considered not to have a functional CL and were likely anovulatory (non-functional CL) when progesterone values were less than 5 ng/ml. Progesterone concentrations were charted against cycle day. Three experts in the field of reproductive endocrinology used the graphs to determine the normalcy of each cycle. The cycle was so categorized when 2 of 3 experts were in agreement.

2.11 Statistical Analysis

Regression analysis was used to assess the validity of the bloodspot assay and extraction method. Temperature data from each cycle were analyzed using the Maximina® Quantitative Basal Temperature Analysis Program (See section 1.6.2 for

further explanation). The Maximina® results were then analyzed by three independent experts. The cycles were categorized as 'ovulatory' or 'anovulatory' when 2 of 3 experts were in agreement. Specificity, sensitivity, and the predictive ability of the finger-prick blood sampling and temperature method in determining CL function were assessed using computations from Sackett *et al.* (2000).

A repeated measures analysis of variance was used to determine effect of sample number of the extracted tampons (i.e. sample number 1, 3, 5, 7). That is, whether there is a significant difference in progesterone concentrations measured from any of the four extracted tampons. All other statistical comparisons were done using nonparametric tests. The Kruskal-Wallis test was used to determine a difference in extracted samples from cycles with varying degrees of functional CL (i.e. Ovulatory/Normal vs. SLP vs. Insufficient). Otherwise, the Mann-Whitney U test was used for all other extracted sample comparisons. SPSS for Windows (Version 11.5) was used for these analyses and values of $p < 0.05$ were considered significant.

The purpose of the Menstrual Cycle Diary© in this study is to determine whether physical and emotional experiences observed during the menstrual cycle differ for ovulatory and anovulatory cycles. Varying menstrual cycle lengths among the participants makes it difficult to analyze scores from the daily Menstrual Cycle Diary©. To maximize the data for cycles with corresponding bloodspot data, scores were examined within one 5-day mid-cycle window (MC, 11-15) and one 5-day end of cycle window (EC, last 5 days of the cycle). These days were chosen to assess physical and emotional changes during the follicular and luteal phases. Each participant experienced

at least three days of menses, therefore, a 3-day menstrual window was examined. The first few days of a menstrual cycle could be influenced by ovulatory function of the previous cycle; therefore, scores from the 3-day window were taken from the proceeding cycle (3DA, first 3 days of onset of the following cycle). Scores for each of the three windows were summed and averaged to account for missing data. For the analysis, the letter scores were given numerical values (M = 0, L = 1, U = 2, Y = 3, Z = 4). Depending on the experience analyzed it is possible that scores from some days of the menstrual cycle are less informative than other days. Single factor analysis of variance (ANOVA) was used (Microsoft Excel 10.2) to determine cyclicity among the scores from the three windows. For those experiences with a significant cyclic pattern, scores from a particular window were not included in subsequent analyses if the average score was close to the 'usual' score ("0"for those diary items with number-scores or "2" for diary items with letter-scores). Otherwise, scores from all three windows were used. The Mann-Whitney U test (SPSS for Windows 11.5) was used to assess whether experiences differed between cycles with and without a functional CL (i.e. ovulatory vs. anovulatory). Alpha was set at 0.05 and only significant results are reported.

3.0 Results

3.1 Menstrual Cycle Characteristics of the Study Participants

A total of 52 completed menstrual cycle records were collected from 18 women giving an average of 2.9 cycles per participant. Table 2 shows the menstrual cycle characteristics of the study participants. The average cycle length was 29.3 ± 6.7 days with a range 21-72 days. Two participants had oligomenorrheic cycles during the study period (cycle lengths: 39 and 72). Out of 52 cycles, 33 had corresponding finger-prick bloodspot data. Among the 18 women, 16 % (3 of 18) showed no evidence of CL function and were termed "anovulatory". The remaining women showed varying degrees of CL function, sufficient that they were deemed "ovulatory". However, of those with "ovulatory" cycles, only in 53% (8 of 15) did they appear normal. One or more short luteal phase lengths were observed in 66% (10 of 15) of the women and 26% (4 of 15) of the women had at least one insufficient luteal phase.

Twenty-eight women were initially recruited for the study but only eighteen women provided at least two full cycles worth of data. Reasons for withdrawing from the study were pregnancy (one woman), starting birth control (three), discomfort with aspects of the study protocol (two), an occupational move (one), or personal reasons (three).

3.2 Validation - Finger-prick Bloodspot

3.2.1 Serum vs. Finger-prick Bloodspot

Contemporaneous serum and bloodspot samples were assayed for progesterone. Regression analysis gave the relationship of y (serum) = 0.4338x + 0.4328 (R^2 = 0.9963,

n = 10) allowing for the conversion of bloodspot samples to serum equivalents (Figure 3). Figure 4 shows the profiles of the ten contemporaneous serum and finger-prick blood progesterone concentrations.

3.2.2 Recovery

Converting the progesterone standards (serum) expected values to bloodspot values using the regression equation y (bloodspot) = 2.2965x – 0.925, the expected progesterone concentrations in the four $1/8^{th}$ discs added to the follicular phase sample for standards D, E, and F are 1.83 ng/ml, 11.32 ng/ml, and 41.94 ng/ml, respectively. Observed results were adjusted subtracting the baseline RIA progesterone concentration 2.73 ng/ml (follicular phase sample + Standard A (0 ng/ml)). Regression analysis of the observed and expected progesterone recovery provided a squared correlation coefficient $R^2 = 0.9997$ (n = 3). Figure 5 shows the correlation between expected progesterone recovery and observed recovery of progesterone concentrations.

3.2.3 Linearity

Dilution (1/2, 1/4, 1/8) of a pregnancy bloodspot sample (high progesterone) with a menopausal bloodspot sample (low progesterone), reported as a linear regression between expected and observed progesterone concentrations was $R^2 = 0.9579$ (n = 3). Figure 6 shows the correlation between expected and observed dilution results.

Table 2. Menstrual cycle characteristics based on finger-prick blood progesterone analysis and mucus determinations of 18 premenopausal women during the study period

Cycle length (days)	29.3 ± 6.7
Range (days)	21-72
	*No. of women**
Menstrual cycle analysis	
Normal cycles	8
Short luteal phases (SLP)	
1	8
>1	2
Insufficient luteal phases (ILP)	4
Anovulatory cycles	3
Cycle length (days)	
>36 days	2
<21 days	0

* Some women had both SLP and ILP thus were counted in more then one category

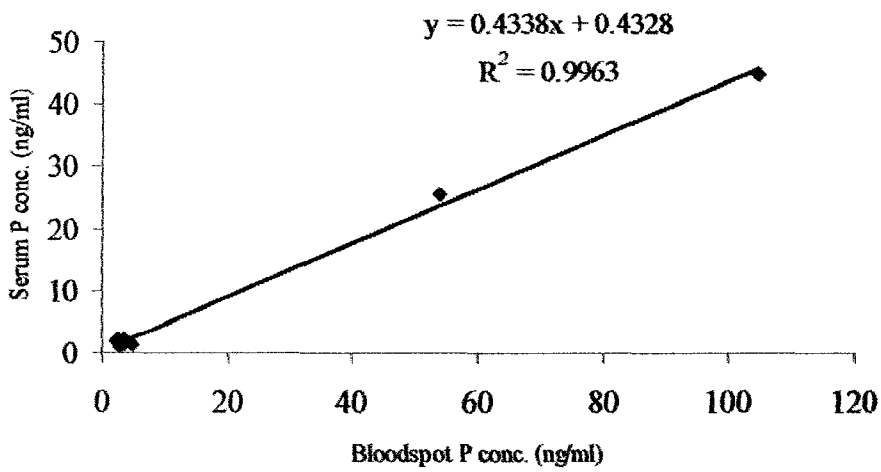


Figure 3. Correlation between serum and finger-prick bloodspot progesterone concentrations

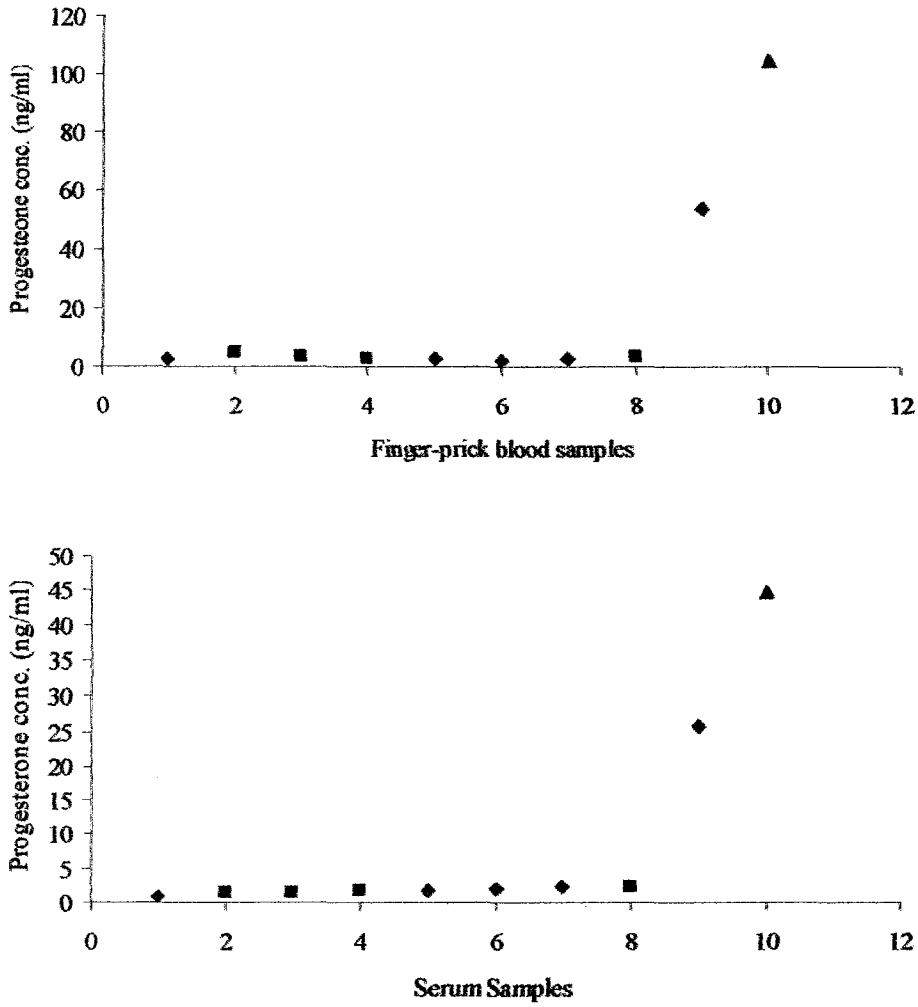


Figure 4. Profiles of contemporaneous serum and finger-prick progesterone concentrations from six women during the follicular phase (■), luteal phase (♦), or first trimester of pregnancy (▲)

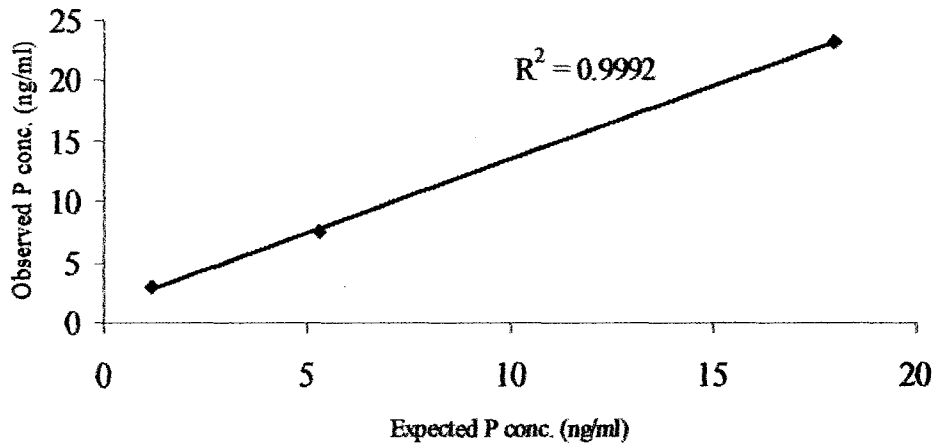


Figure 5. Correlation between expected and observed recovery of added progesterone (P) from finger-prick blood samples

3.2.4 Assay Variability

The intra-assay %CV based on 10 determinations from a single finger-prick blood sample was 9.66. The inter-assay %CV based on eight determinations measured in duplicate from the same sample was 23.04.

3.3 Hematocrit

Hematocrit was measured using the microcapillary method. The population mean (± SD) hematocrit measurement was 33.7 ± 3.22. Thus, for every 100 μL, there is about 66.3 μL of serum. Table 3 shows a selection of extreme hematocrit values and the adjustment of the corresponding finger-prick blood raw progesterone concentrations based on hematocrit values and intra- and inter-assay variability. The adjustment of progesterone concentrations for hematocrit was based on the percentage difference between an individual serum value (converted from the hematocrit value) and the population mean serum value (i.e. 66.3). For example, a hematocrit value of 26.9 would have a serum value 73.1 which is 9.3% more then the population mean serum value. The

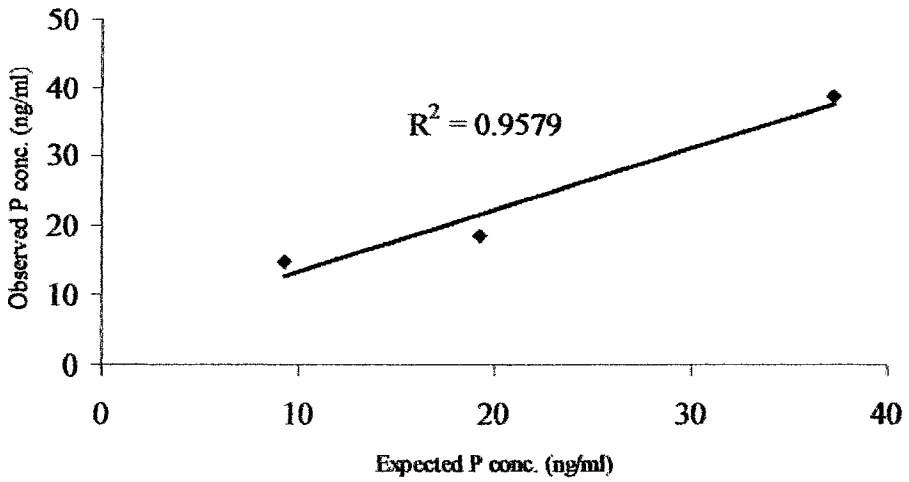


Figure 6. Correlation between expected and observed results of the dilution (1/2, 1/4, 1/8) of a high progesterone (P) finger-prick blood sample with a low progesterone finger-prick blood sample

corresponding progesterone value would be adjusted for that difference. The adjusted progesterone values from Table 3 show that the hematocrit contributed uncertainty similar to that of bloodspot intra-assay %CV. That is, when taking into account hematocrit and intra-assay variability, the adjustment of progesterone was similar for both. Inter-assay %CV contributed uncertainty of a slightly greater magnitude than that of hematocrit. That is, the change in progesterone concentration was greater for inter-assay variability than for hematocrit. Therefore, it was not necessary to adjust the progesterone concentrations on the basis of the hematocrit values.

3.4 Gas Chromatography-Mass Spectrometry (GC-MS)

Assays of extracts from blank un-used tampons showed measurable progesterone concentrations. The mean (± SD) progesterone concentration for 10 un-used tampons was 1.203 ng/ml ± 0.245. Extractions of un-used tampons including a sample concentrated 10X were measured for progesterone using GC-MS. The GC-MS results showed no indication of actual progesterone in either the acetonitrile or hexane extracts. The concentrated sample (10X) also showed no progesterone. Appendix B shows the results for the 10X samples (both acetonitrile and hexane extracts) only.

Table 3. From finger-prick blood samples, the comparison of the adjustment of raw progesterone (P) based on extreme hematocrit values, intra- and inter-assay variability

			Adjusted P (ng/ml) based on:		
Hematocrit %	Δ difference % *	Raw P (ng/ml)	Hematocrit	Intra-assay 9.66 %CV range	Inter-assay 23.04 %CV range
26.9	-9.3	2.6	2.4	2.4-2.9	2.0-3.3
26.9	-9.3	12.5	11.4	11.3-13.7	9.6-15.4
40.9	+12.1	1.7	1.9	1.5-1.9	1.3-2.1
27.4	-8.7	5.0	4.6	4.6-5.5	3.9-6.2
42.1	+14.5	14.4	16.4	13.0-15.8	11.1-17.7
43.2	+16.7	2.8	3.2	2.5-3.0	2.1-3.4

* Population mean hematocrit was 33.7. Thus, for every 100 µl there were 66.3 µl of serum. The Δ difference % was calculated by converting individual hematocrit values to serum values (e.g. 26.9 = 73.1) then determining its percentage difference from the population serum value (i.e. 66.3).

3.5 Validation - Extraction Method

Both the acetonitrile and hexane extracts were assayed during the validation process. As anticipated the acetonitrile layer contained the bulk of the expected progesterone concentration while the hexane layer contained a negligible amount of hormone. Thus and thereafter the hexane layer was discarded at the final step of the extraction procedure.

Un-used tampons were doped with 1 ml of 8 ng/ml, 16 ng/ml, 32 ng/ml, 64 ng/ml, or 128 ng/ml of progesterone in ethanol. The validity of the extraction method was measured through recovery and dilution of progesterone in the samples. The expected recovery of progesterone was calculated taking into account only half of the initial 100 ml methanol extract was further extracted and the 20 % loss of extract during the ether purification step. Regression analysis gave a high squared correlation coefficient for recovery and dilution ($R^2 = 0.9887$, $R^2 = 0.9767$, respectively). The intra-assay variability based on 10 determinations of 3 samples doped with 1 ml of 16 ng/ml, 32 ng/ml, or 128 ng/ml progesterone in ethanol averaged 14.07 (range 5.5 – 19.03). Repetition of this validation procedure gave similar results. As a result, continuation with the second validation procedure ensued.

3.5.1 Recovery

Un-used tampons were doped with 5 ml of 0 ng/ml, 4 ng/ml, 12 ng/ml, or 24 ng/ml of progesterone in menstrual flow resulting in the expected progesterone concentration 0 ng/ml, 20 ng/ml, 60 ng/ml, or 120 ng/ml per tampon. Six tampon

samples were extracted for this validation, two for each progesterone concentration above. The RIA results of two samples (0 ng/ml and 20 ng/ml) were not included in the overall analysis due to pipetting errors during the doping process. In addition to taking into account procedural extraction losses (explained above) the baseline value (1.8752 ng/ml) measured from an un-used tampon doped with 5 ml of menstrual flow without added progesterone (i.e. the 0 ng/ml sample) was calculated into the expected recovery results. The values obtained for expected and observed recovery of added progesterone were highly correlated in a linear regression ($R^2 = 0.991$, regression equation $y = 0.9319x + 0.826$, $n = 4$, Figure 7).

3.5.2 Linearity

The dilution (1/2, 1/4, 1/8) of an un-used tampon doped with 120 ng/ml progesterone (in menstrual fluid) with Standard A buffer (0 ng/ml) reported as a linear regression between expected and observed results was $R^2 = 0.9969$ ($y = 1.1088x - 6.3604$, $n = 3$). Figure 8 shows the correlation between the expected and observed results from the dilution of the extracted sample.

3.5.3 Assay Variability

Intra-assay %CV based on 10 determinations of 4 samples (0 ng/ml, 20 ng/ml, 60 ng/ml, 120 ng/ml) averaged 6.25 with a range 4.12 – 8.16. The inter-assay %CV based on 4 determinations of a single sample (120 ng/ml progesterone-doped sample) measured in duplicate was 13.36.

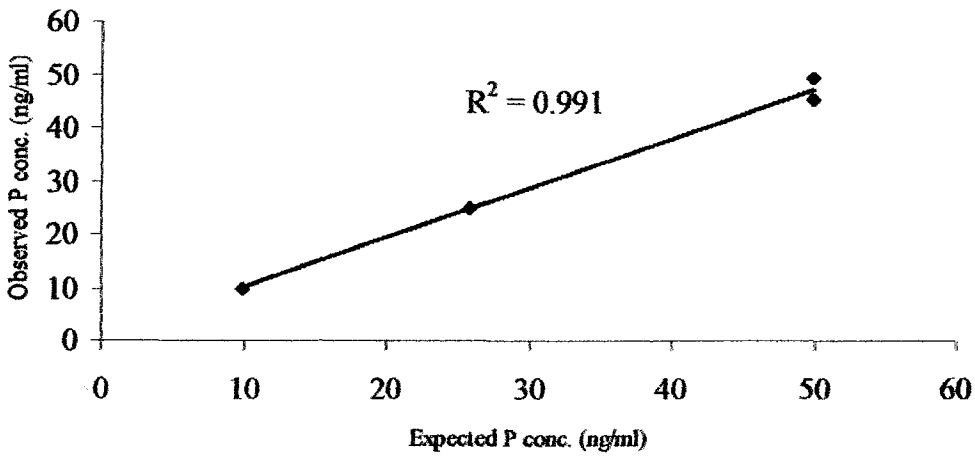


Figure 7. Correlation between expected and observed recovery of extracted progesterone (P) from un-used tampons doped with increasing concentrations of progesterone-doped menstrual fluid

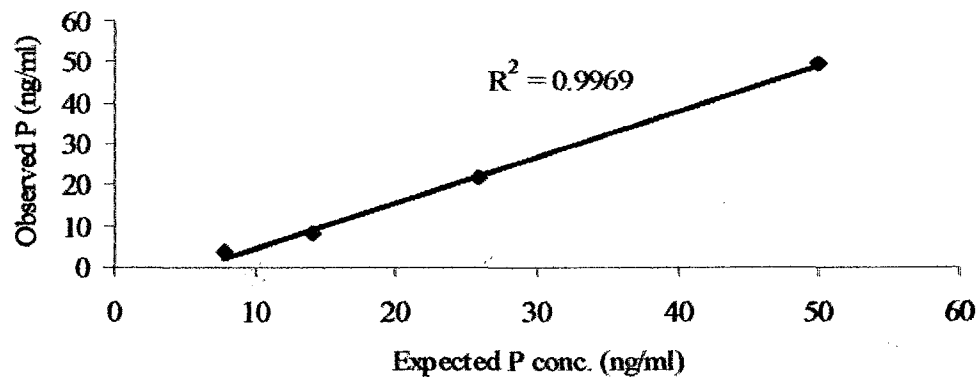


Figure 8. Correlation between expected and observed dilution (1/2, 1/4, 1/8) of an extracted sample doped with 120 ng/ml of progesterone (P) in menstrual fluid and diluted with Standard A (0 ng/ml)

3.6 Corpus Luteum (CL) Functioning

3.6.1 Finger-prick Bloodspot

Finger-prick blood samples were requested from each subject at seven different occasions during her menstrual cycle. Seven bloodspot samples could not be collected for every participant but each woman gave at least two finger-prick blood samples per cycle. The finger-prick blood samples were assayed for progesterone and those bloodspot values converted to serum values (ng/ml) using the regression equation y (serum) = 0.4338x + 0.4328. Progesterone concentrations were charted against cycle day (Figure 9).

Using progesterone concentration as the gold standard, 33 cycles were grouped into one of two categories: functional CL and non-functional CL. Out of those 33 cycles, 28 cycles (85%) were determined to have a functional CL and 5 (15%) showed no signs of CL functioning. Out of those cycles determined to have a functional CL, 12 of 28 (43%) had adequate cycle and luteal phase lengths (21-36 days, ≥12 days, respectively); 12 of 28 (43%) were categorized as having SLP (≤ 11 days); and 4 cycles (14%) categorized as ILP (P < 14 ng/ml) but having adequate luteal phase lengths (Table 4).

3.6.2 Temperature

Basal body temperature was recorded daily by each of the participants (Figure 9). The temperature data were analyzed using the Maximina® Quantitative Basal Body

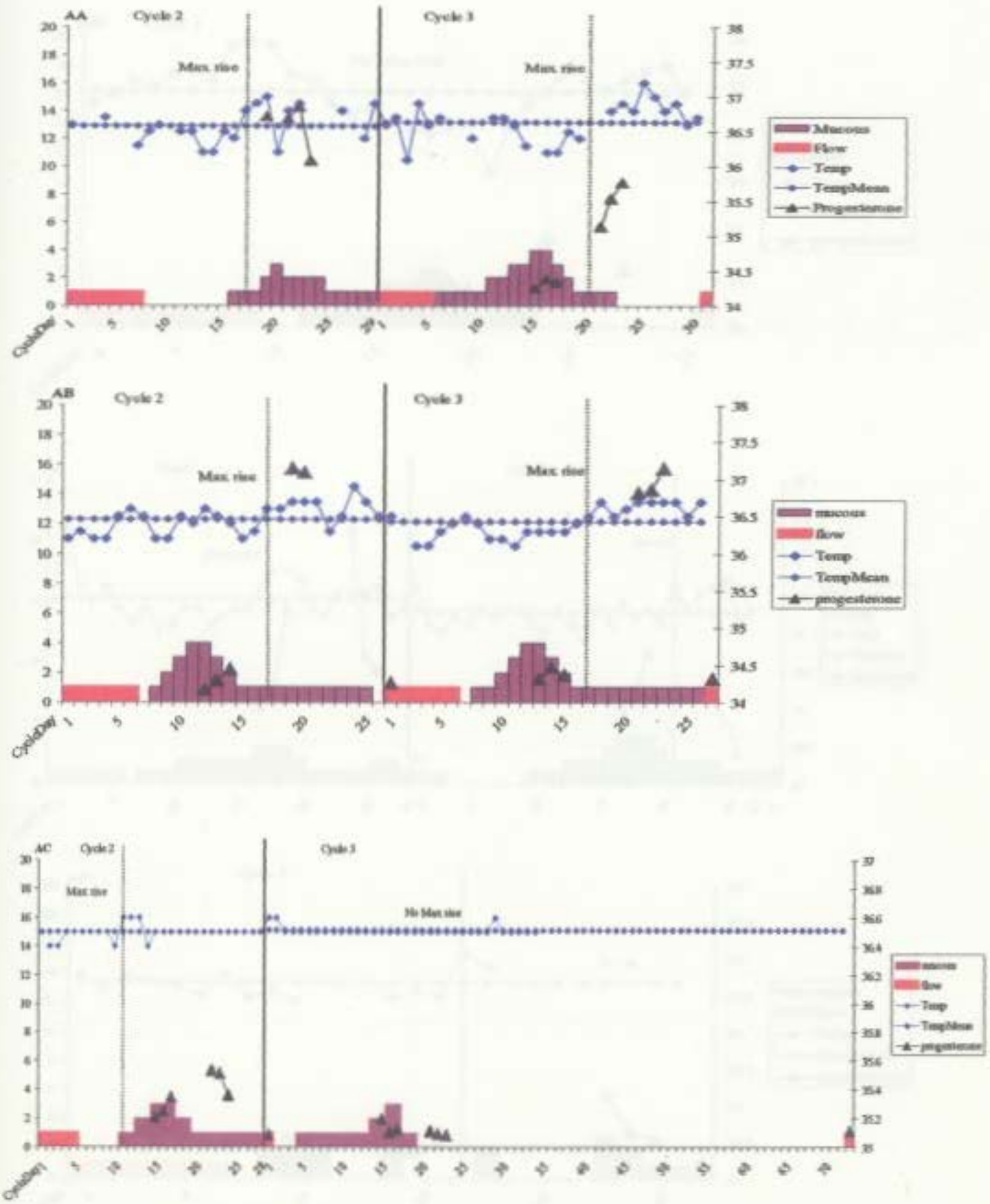


Figure 9. Charted menstrual cycle finger-prick progesterone concentrations and daily temperatures of study participants

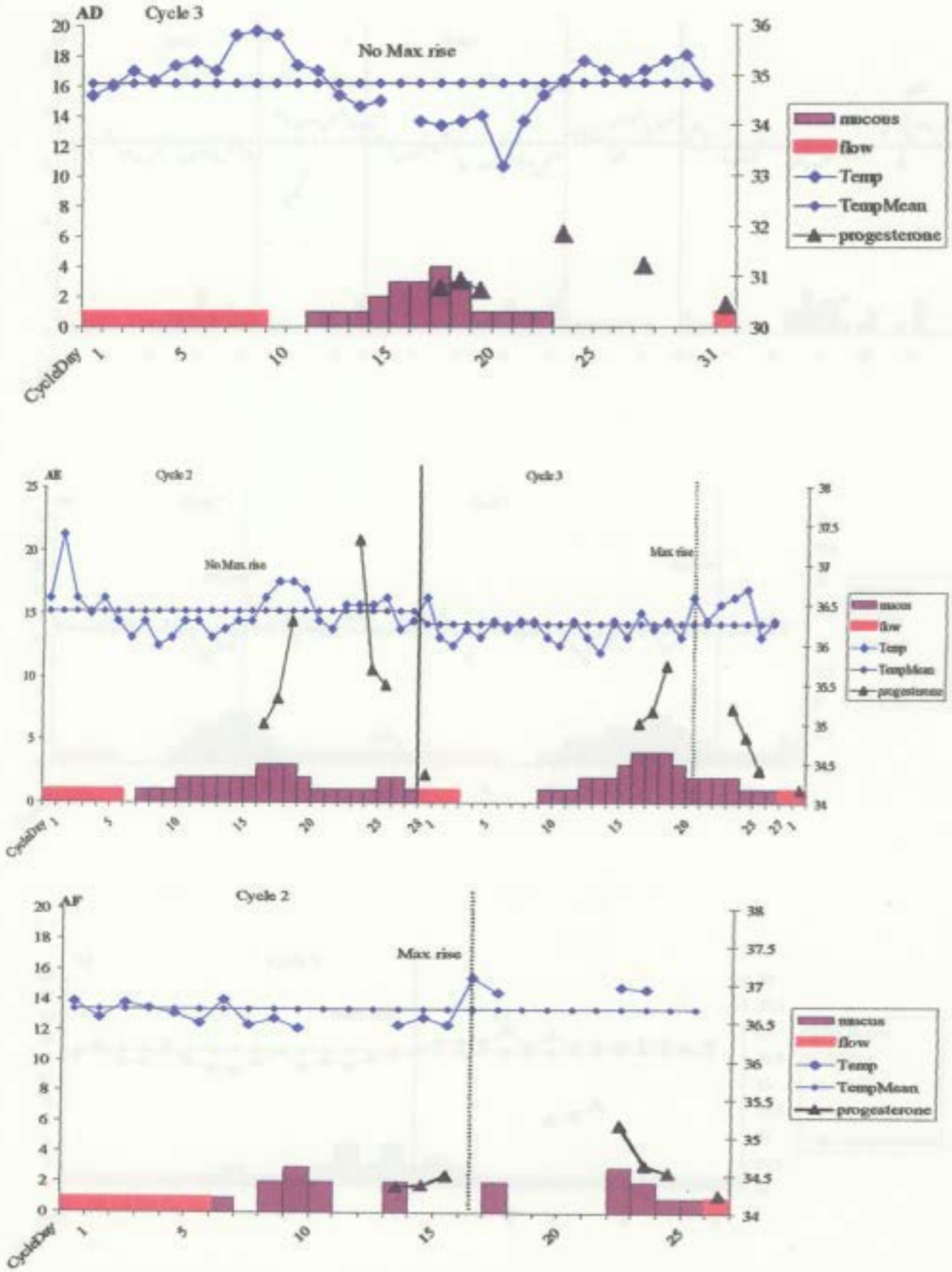


Figure 9. Charted menstrual cycle finger-prick progesterone concentrations and daily temperatures of study participants

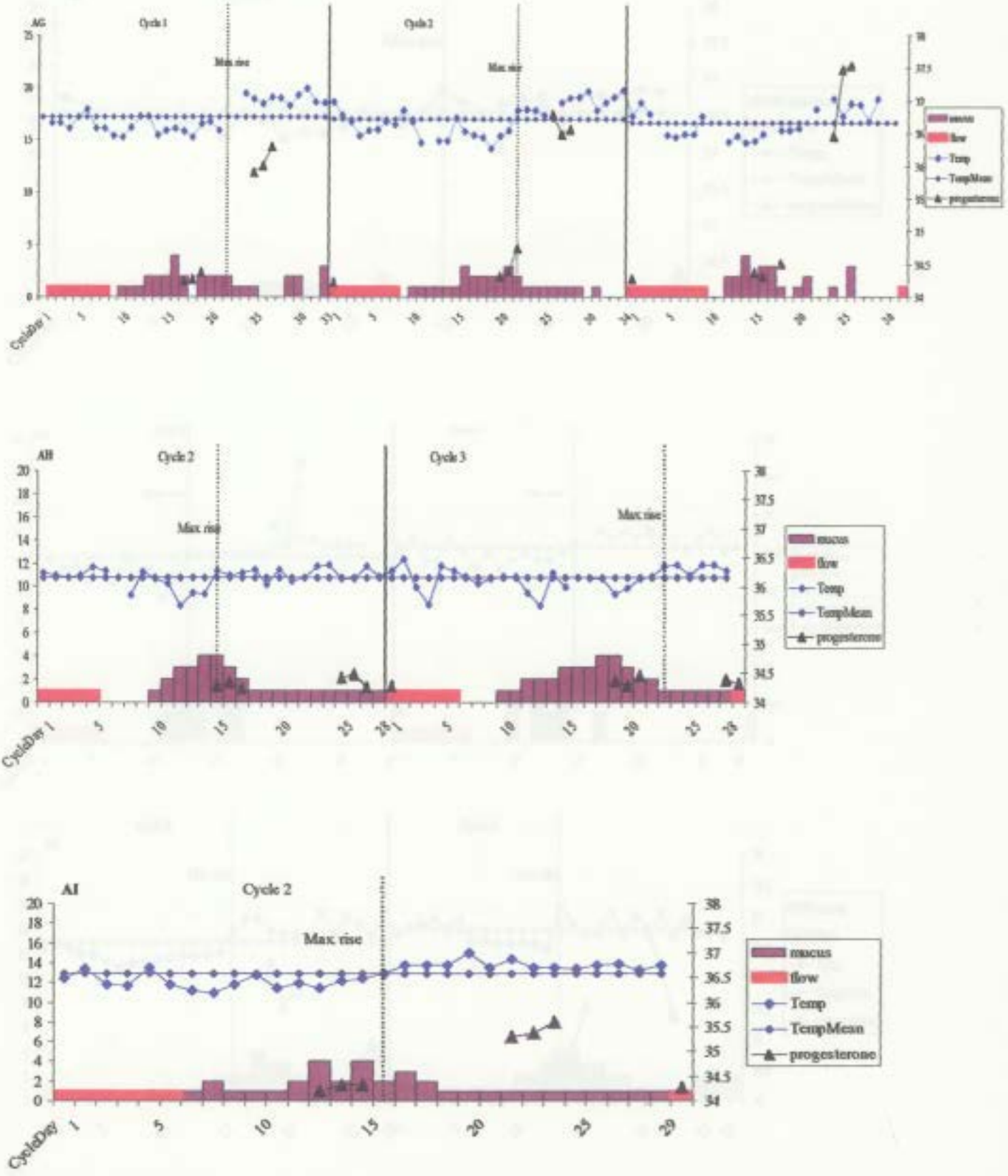


Figure 9. Charted menstrual cycle finger-prick progesterone concentrations and daily temperatures of study participants

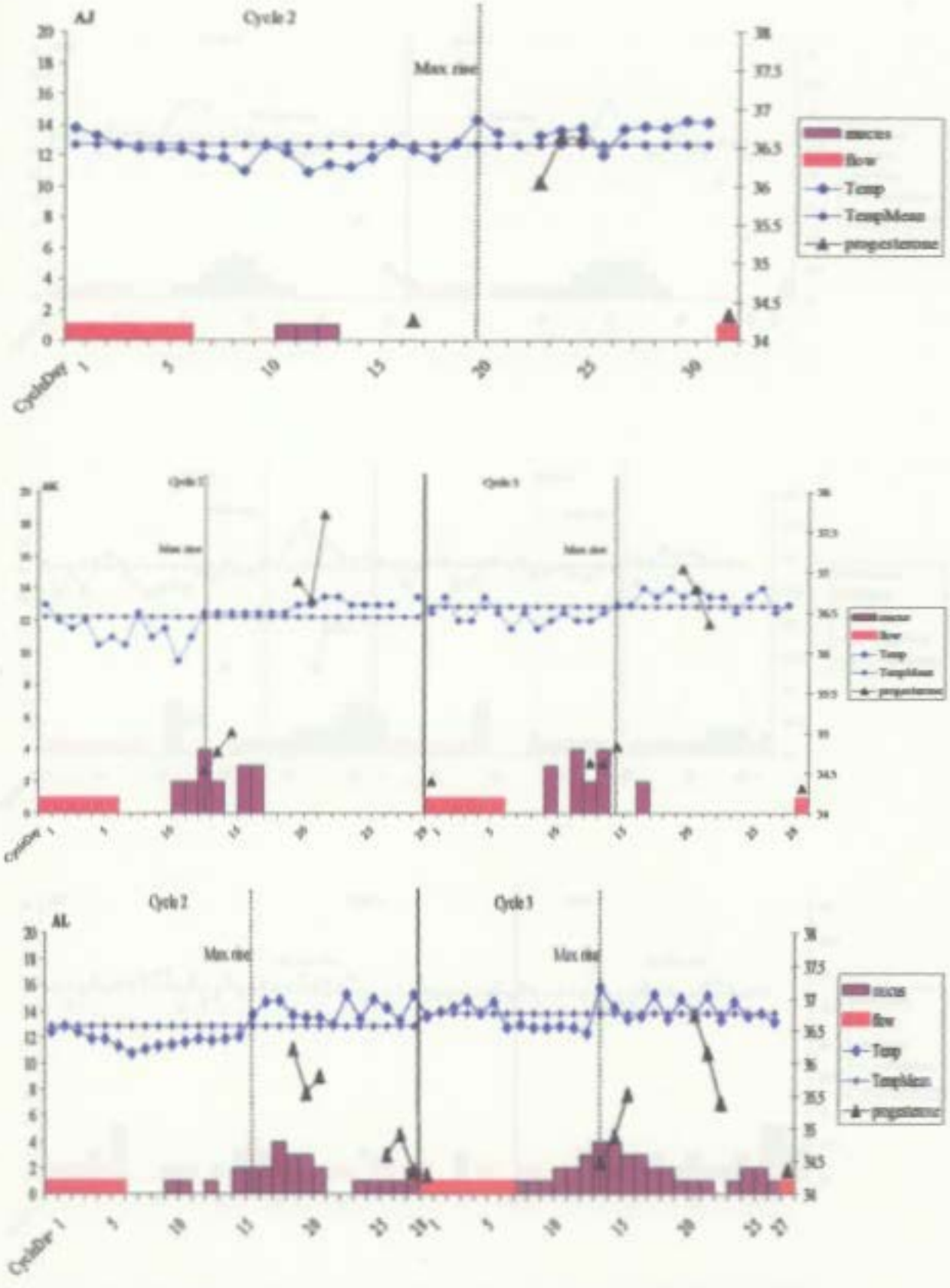


Figure 9. Charted menstrual cycle finger-prick progesterone concentrations and daily temperatures of study participants

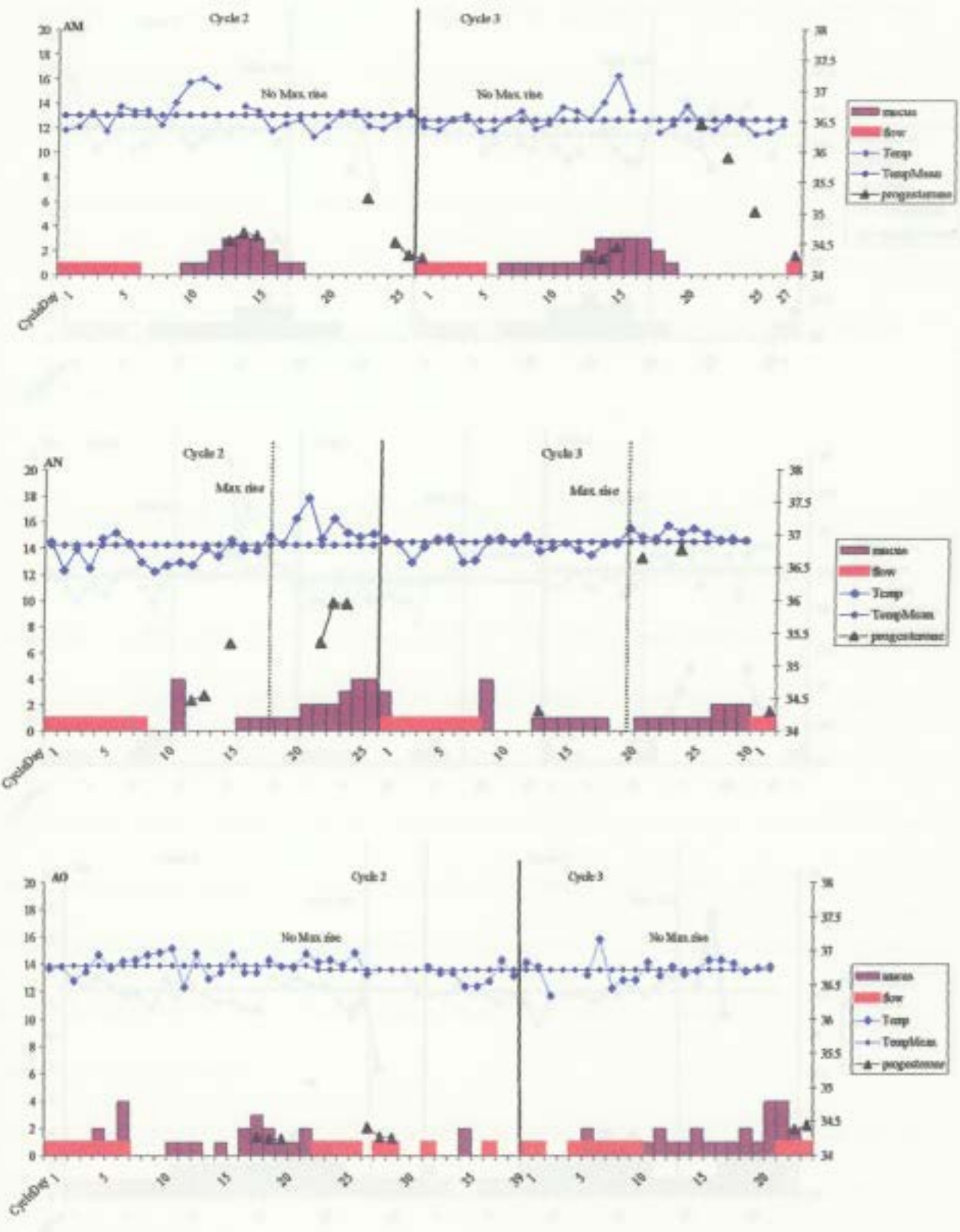


Figure 9. Charted menstrual cycle finger-prick progesterone concentrations and daily temperatures of study participants

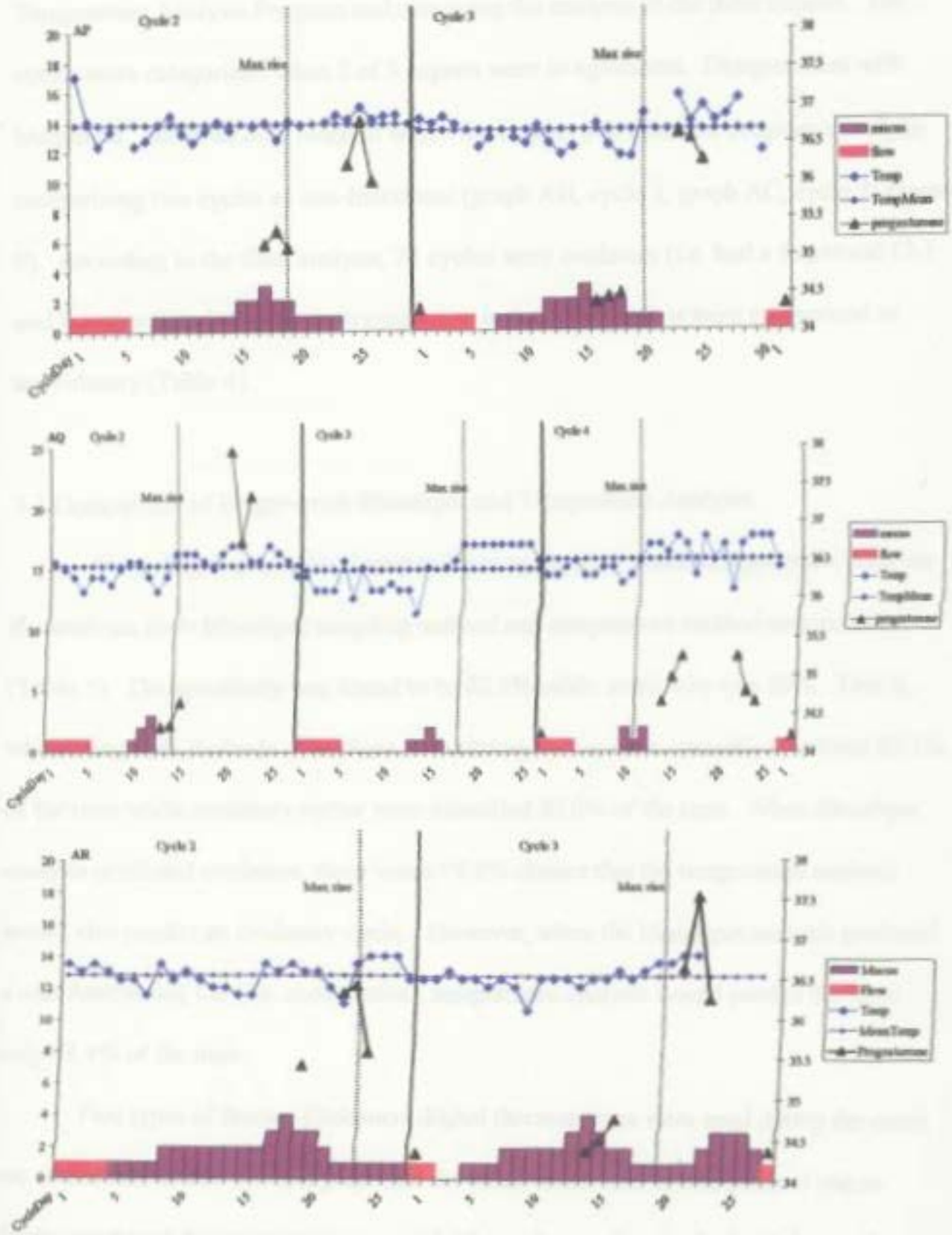


Figure 9. Charted menstrual cycle finger-prick progesterone concentrations and daily temperatures of study participants

Temperature Analysis Program and also using the analysis of the three experts. The cycles were categorized when 2 of 3 experts were in agreement. Disagreement with Maximina® occurred only twice in which the experts overruled the program's analysis categorizing two cycles as non-functional (graph AH, cycle 2; graph AC, cycle 2; Figure 9). According to the final analysis, 24 cycles were ovulatory (i.e. had a functional CL) and 9 cycles failed to show a persistent rise in temperature thus were categorized as anovulatory (Table 4).

3.7 Comparison of Finger-prick Bloodspot and Temperature Analysis

Using finger-prick blood progesterone as the gold standard, agreement between the analyses from bloodspot sampling method and temperature method was assessed (Table 5). The specificity was found to be 82.1% while sensitivity was 80%. That is, when using both methods of analysis anovulatory cycles were correctly identified 82.1% of the time while ovulatory cycles were identified 80.0% of the time. When bloodspot analysis predicted ovulation, there was a 95.8% chance that the temperature analysis would also predict an ovulatory cycle. However, when the bloodspot analysis predicted a non-functioning CL (i.e. anovulation), temperature analysis would predict the same only 44.4% of the time.

Two types of Becton Dickinson digital thermometers were used during the study, one which read to one decimal place and the other which read to two decimal places. Newly purchased thermometers were available reading to the one decimal place only. The mean difference of the two digit thermometer from the reference thermometer was

Table 4. Menstrual cycle characteristics of thirty-three menstrual cycles based on finger-prick bloodspot and temperature analysis

	Bloodspot	QBT
	no. of cycles	
Menstrual cycle analysis		
Ovulatory cycles	28	24
Normal cycles	12	19
Short luteal phases	12	5
Insufficient luteal phases	4	NA
Anovulatory cycles	5	9

QBT, Quantitative basal temperature; NA, not applicable

Table 5. Sensitivity, specificity, positive and negative predictive value of the finger-prick blood analysis of corpus luteum (CL) function of 33 cycles in comparison to the temperature analysis

		Finger-prick Blood Analysis	
		Functional CL No. of cycles	**Non-functional CL** No. of cycles
Temperature Analysis	**Functional CL** No. of cycles	23	1
	Non-functional CL No. of cycles	5	4
Sensitivity	80.0%	**Positive Predictive Value**	95.8%
Specificity	82.1%	**Negative Predictive Value**	44.4%

0.30 (range 0.18-0.44) and for the one digit thermometer, the mean difference was 0.25 (range 0.1-0.4). Regression analysis demonstrated that both thermometers were consistently reading about a 0.20°C higher than the reference thermometer at each test temperature ($y = 1.0134x + 0.2335$, for two digit readout; $y = 1.0113x + 0.1952$, for one digit readout).

3.8 Extraction Results

A total of 110 used tampons were collected and extracted. Samples 1, 3, 5, and 7 were extracted for each cycle. Tampons from 32 menstrual cycles were measured for progesterone (used tampons from one of the 33 cycles with corresponding bloodspot data were not collected). Each participant collected and stored at least three tampons per cycle. Two values were clearly outliers and omitted from statistical analysis (33.35 ng/ml, 134.27 ng/ml). Progesterone values obtained from the assay print-out were adjusted to take into account the weight of the sample and procedural extraction losses. The weight of each tampon was subtracted by the average weight from ten un-used tampons to give us the weight of menstrual effluent, assuming 1 ml for each gram. The mean adjusted progesterone value (± SD) was 1.98 ng/g ± 0.85 (range 0.28 ng/g – 7.85 ng/g). The adjusted progesterone values were used for statistical analyses (Table 6).

Table 6. Actual and mean (M) raw and adjusted extracted progesterone concentrations (ng/ml) from used tampons (1, 3, 5, and 7) of 32 menstrual periods.

Corpus Luteum (CL) Status		Progesterone (P) concentration from assay print-out (ng/ml) *					Adjusted P (ng/g) *				
		1	**3**	**5**	**7**	**M**	**1**	**3**	**5**	**7**	**M**
Functional CL (ovulatory cycles)	Normal	1.9	2.42	1.55	2.32	**2.05**	2.94	1.94	0.91	1.93	**1.93**
		1.91	3.74	1.78	1.91	**2.33**	7.85	3.31	0.88	1.18	**3.31**
		2.29	4.53	2.37	2.04	**2.81**	5.05	6.23	1.08	0.64	**3.25**
		3.46	2.19	1.57	1.93	**2.29**	3.29	3.58	3.07	1.37	**2.83**
		2.59	0.85	2.74	1.86	**2.01**	1.24	2.29	1.42	1.01	**1.49**
		1.98	1.70	2.33		**2.00**	3.99	0.55	1.19		**1.91**
		1.60	2.57	3.00	1.91	**2.27**	1.23	1.23	1.17	3.28	**1.73**
		1.99	2.09	2.52	2.57	**2.29**	1.45	2.27	2.46	3.31	**2.37**
		2.72	2.62	2.53		**2.62**	0.82	1.00	0.90		**0.91**
		6.02	2.50		2.25	**3.59**	1.55	0.62		0.79	**0.99**
		1.47	3.34	1.14	1.06	**1.75**	4.29	4.81	2.76	4.30	**4.04**
	SLP	2.89	4.71	3.06		**3.55**	1.48	4.14	2.62		**2.75**
		2.33	1.81	2.32		**2.15**	6.56	2.83	2.20		**3.86**
		1.87	2.31	2.66	1.91	**2.18**	2.08	1.18	1.11	1.15	**1.38**
		1.51	1.61			**1.56**	1.69	2.25			**1.97**
		6.38	3.13	1.80	2.55	**3.47**	1.20	0.77	0.28	0.88	**0.78**
		5.64	2.60	1.51		**3.25**	2.21	1.28	1.12		**1.54**
		1.79	2.34	2.56	2.22	**2.23**	1.09	0.66	1.17	2.22	**1.29**
		2.05	1.96	2.75	2.82	**2.39**	1.81	4.42	2.62	1.27	**2.53**
		1.33	1.17	1.84	2.65	**1.75**	2.04	1.00	1.87	0.79	**1.42**
			1.96	1.24		**1.60**		1.76	1.80		**1.78**
		1.40	2.71	1.14		**1.75**	1.31	1.08	1.13		**1.17**
		3.49	2.20	3.92		**3.20**	1.79	1.44	3.15		**2.13**
	ILP	0.93	1.18	2.12		**1.41**	2.65	2.31	1.07		**2.01**
		3.11	2.30	1.62		**2.34**	1.59	2.76	0.92		**1.76**
		1.57	1.08	2.31	1.46	**1.60**	1.31	0.41	3.36	4.94	**2.50**
		0.86	1.78	2.46		**1.70**	0.73	1.66	0.83		**1.07**
Non-functional CL (anovulatory cycles)		2.50	3.76			**3.13**	2.13	1.79			**1.96**
		2.18	2.85	2.66	3.74	**2.86**	2.65	2.83	3.00	3.20	**2.92**
		1.27	1.19			**1.23**	1.57	2.27			**1.92**
		3.91	2.08	2.35	2.31	**2.66**	1.57	0.73	0.64	0.94	**0.97**
		1.41	1.91	1.07		**1.46**	0.51	0.71	1.81		**1.01**

SLP, Short luteal phase; ILP, Insufficient luteal phase

*Blank spaces are missing data from used tampon samples not collected by participants.

3.8.1 Comparison of Extraction and Bloodspot

A repeated measures within-subjects test found no significant effect of sample number ($F = 1.147$, $p = 0.340$). That is, there was no significant difference in progesterone within samples 1, 3, 5, and 7. The progesterone values for those samples were combined and averaged for further analysis.

No statistically significant difference was found between the level of progesterone extracted from tampons of ovulatory cycles and anovulatory cycles ($U = 57.00$, $p = 0.586$). Comparing extracts from anovulatory cycles to extracts of ovulatory cycles of normal cycle and luteal lengths also showed no statistical significance ($U = 23.00$, $p = 0.461$).

A three-way comparison of progesterone values from ovulatory cycles of normal length with cycles of short luteal phases and insufficient luteal phases showed no statistical significant difference in progesterone concentration, ($p = 0.429$, $df = 2$).

As well, there was no significant difference in progesterone extracted from samples of normal cycles and cycles with ILP ($U = 19.00$, $p = 0.544$) nor in cycles with SLP when compared to ovulatory cycles of normal length ($U = 45.00$, $p = 0.196$).

There was also no statistical difference in extracted progesterone concentrations from cycles with SLP and ILP cycles ($U = 20.00$, $p = 0.794$).

3.9 Menstrual Cycle Diary© Data

As a result of the ANOVA, scores from the MC window were not used in further analysis for the following menstrual experiences: cramps, front and side breast

tenderness, breast size, fluid retention, feeling of energy, and interest in sex. Scores from the EC window were not used in further analysis for the following menstrual experiences: cramps, mucous secretions and interest in sex. Scores from the 3DA window were not used in further analyses in the following menstrual experience: mucus secretions. For all other menstrual experiences listed on the Menstrual Cycle Diary©, scores from the 3 windows (MC, EC, and 3DA) were used in the analysis.

The Mann-Whitney U test found a significant difference in scores of seven of seventeen experiences between cycles with or without a functional CL (Figure 10). Ovulatory cycles had significantly higher scores for side breast tenderness ($U = 15.5$, $p = 0.020$), cramps ($U = 17.0$, $p = 0.035$), fluid retention ($U = 8.0$, $p = 0.006$), frustration ($U = 23.0$, $p = 0.017$), sleep problems ($U = 15.0$, $p = 0.006$), depression ($U = 27.5$, $p = 0.029$), and breast size ($U = 18.0$, $p = 0.028$). Comparisons between ovulatory and anovulatory cycle scores of the ten other menstrual experiences from the Menstrual Cycle Diary© were not significant ($p > 0.05$, data not shown).

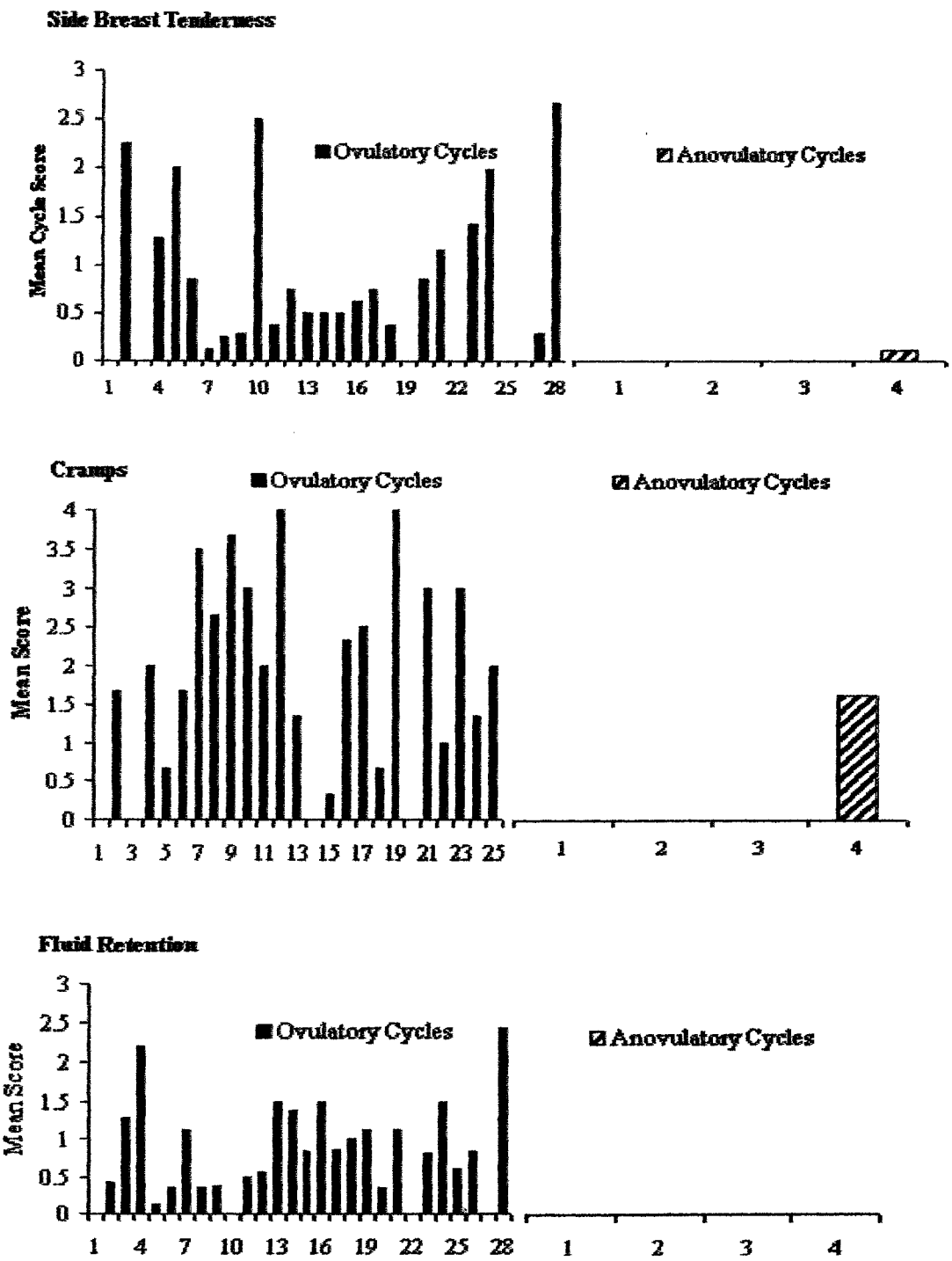
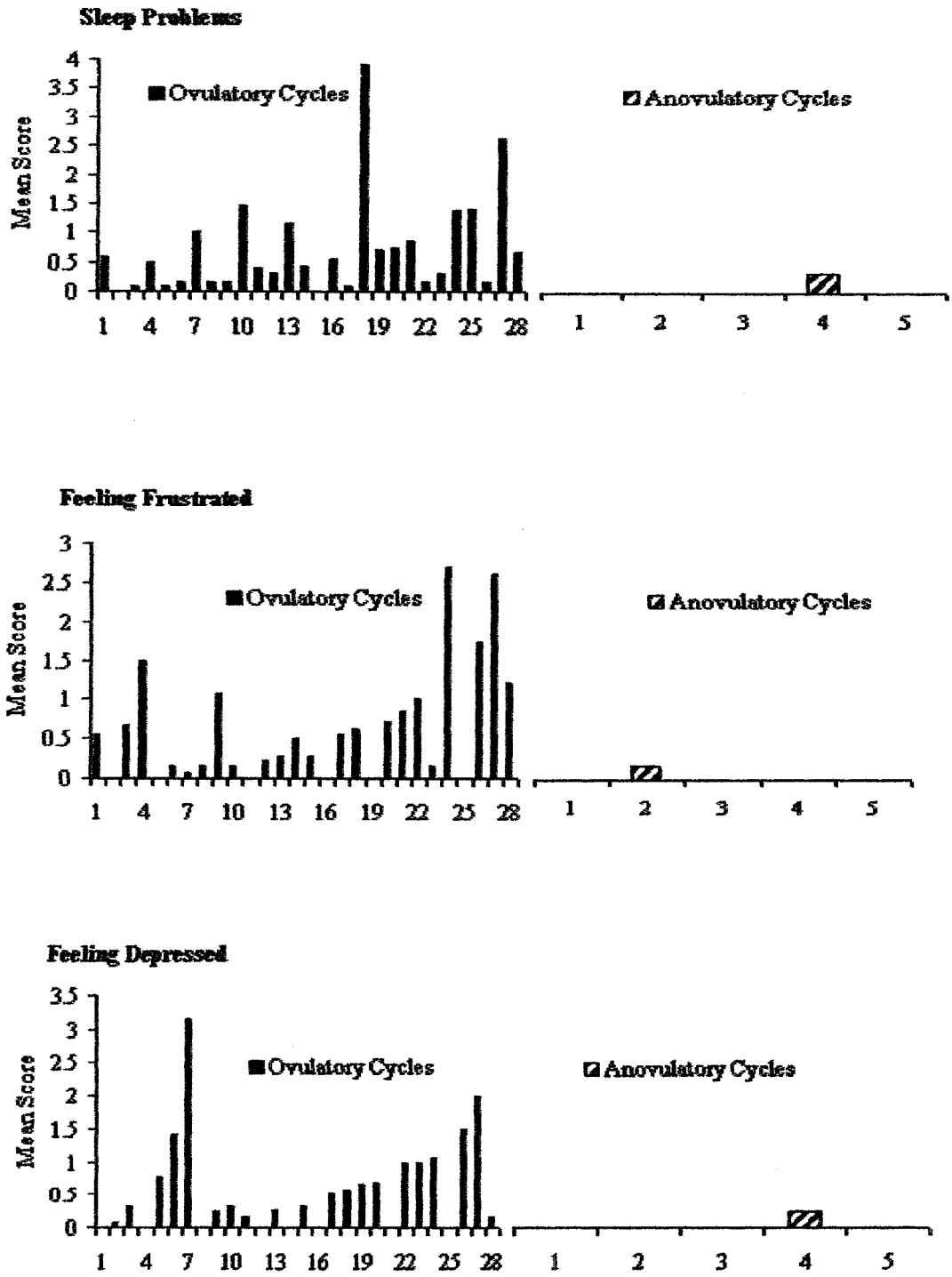


Figure 10. Representation of seven menstrual cycle experiences from the Daily Menstrual Cycle Diary© comparing the mean scores from ovulatory and anovulatory cycles in 18 women. [Full figure legend on page 83]



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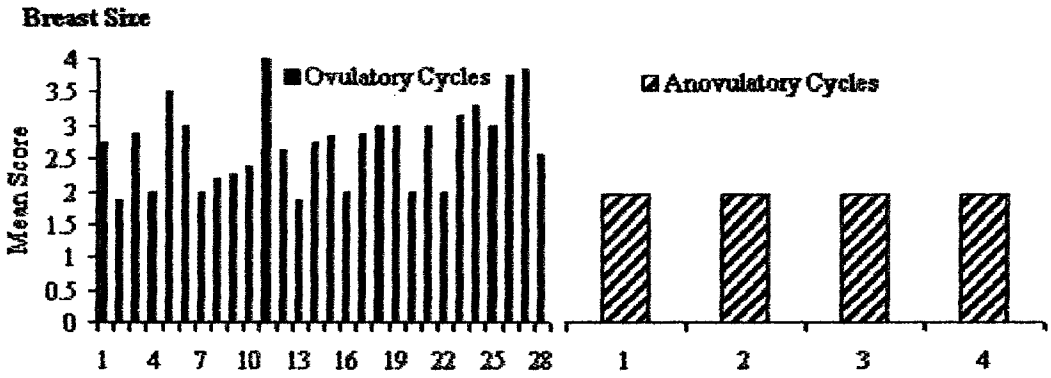

Figure 10. Representation of seven menstrual cycle experiences from the Daily Menstrual Cycle Diary© comparing the mean scores from ovulatory and anovulatory cycles in 18 women. Scores were examined within two 5-day windows and one 3-day window for all cycles with corresponding bloodspot data. Analysis of variance (ANOVA) was used to determine cyclicity among the scores from the three windows. For those experiences with a significant cyclic pattern, scores from mid-cycle (MC), end of cycle (EC), or the first 3 days of the following cycle (3DA) were not included in subsequent analyses if the average score was close to the 'usual' score ('0' for number-scored experiences or '2' for letter-scored experiences). See parenthesis below for windows included in analysis for each experience. The Mann-Whitney U test was then used to compare scores from ovulatory and anovulatory cycles. Side breast tenderness (EC, 3DA), cramps (3DA), fluid retention (EC, 3DA), sleep problems (MC, EC, 3DA), feeling frustrated (MC, EC, 3DA), and feeling depressed (MC, EC, 3DA) were scored on a 0-4 intensity scale; breast size (EC, 3DA) was scored on a letter scale (M = much less, L = a little less, U = usual, Y = a little increased, Z = much increased), which was changed to numerical form for analysis (M = 0, L = 1, U = 2, Y = 3, Z = 4). Scores for ovulatory cycles were statistically higher than anovulatory cycle scores ($\alpha = 0.05$).

4.0 Discussion

4.1 Tampon Extraction Results and Method Validation

The purpose of this paper was to validate a novel non-invasive method of determining luteal function in women. This method of measuring progesterone from menstrual fluid collected on tampons failed to distinguish menstrual cycles with a functional CL from cycles with a non-functional CL. The overall mean adjusted progesterone concentration was quite low measuring just below 2 ng/ml (1.98 ± 0.85 ng/ml). Peddle (1996) also measured low progesterone content in menstrual fluid (<2 ng/tampon). Bieglmayer *et al.* (1995) measured progesterone in used tampons collected on days 1-3 of menses which averaged 4 ± 1 ng/g (median of 2 ng/g). Zhou *et al.* (1989) reported low progesterone levels (average of 1.6 – 2.6 nmol/L) in menstrual fluid collected in rubber receptacles and concluded that menstrual progesterone probably arose entirely from peripheral circulation. Neither Peddle (1996), Bieglmayer *et al.* (1995), nor Zhou *et al.* (1989) assessed the ovarian status of their participants.

Progesterone receptors are located in the endometrium (Haukkamaa & Luukkainen, 1974; Rao *et al.*, 1974; Jänne *et al.*, 1975; Flickinger *et al.*, 1977) which is shed during menses. The regulation of estrogen and progesterone receptor expression in the endometrium is menstrual cycle-dependent. Estrogen, predominant during the follicular phase, stimulates progesterone receptor synthesis in the endometrium whereas progesterone, predominant during the luteal phase, likely contributes to the degradation of its own receptor (Jänne *et al.*, 1975). The regulation of receptor content by steroid hormones is evident by the finding that progesterone receptor expression increases during

the follicular phase and decreases to low levels during the luteal phase (Punyadeera *et al.*, 2003). As serum levels of progesterone also decrease towards late luteal phase, the low levels of progesterone in menstrual fluid reported in the present study are not surprising. Despite the expected low progesterone concentrations it was hypothesized that progesterone concentrations would be higher in menstrual fluid of ovulatory versus anovulatory cycles, however, this study failed to support this hypothesis. In the present study, samples 1, 3, 5, and 7 were extracted from the first two days of menses. If the source of menstrual fluid progesterone is the blood, then it is unlikely that tampons extracted from day 3 onward would have significant progesterone concentrations as the majority of menstrual tissue is normally discarded within the first two days of menses (Vasilenko *et al.*, 1988).

In this study, extracts from used tampons of twenty-seven functional cycles were compared to extracts from tampons of only five non-functional cycles. The small sample size of five anovulatory cycles presents the possibility of creating a type II error; that is, finding no difference in progesterone levels in used tampons between functional and non-functional cycles when a difference actually exists. This would lower the power of the experiment. Thus, comparisons with a larger number of extracts from anovulatory cycles (as well as extracts from functional cycles) may be necessary to uncover a difference in menstrual fluid progesterone between ovulatory and anovulatory cycles.

Although progesterone from menstrual fluid was not presented in the present study as an appropriate marker for the determination of luteal function, the method of extracting progesterone from menstrual fluid collected on tampons was validated. The

regression analysis shows a high correlation between expected and observed progesterone concentrations from both the recovery and dilution experiments with satisfactory intra- and inter-assay variability. The results from the validation experiments show that this method of extraction can be used to measure progesterone in menstrual fluid collected on tampons.

Extracted blank tampons gave measurable but false assay readings. The extracted blank extracts contain uncharacterized compounds that appear to interact with or bind to the antibodies coating the tube giving false progesterone readings. As the extraction method was validated it does not appear that this affect is additive, however, the possibility that these compounds are interfering with the assay cannot be dismissed. Hofer *et al.* (1993) also observed a variety of unknown compounds extracted from blank tampons. They suggested that these compounds may be derived from fatty acids bound to the tampon material. Blank tampon extracts showed peaks at 237 and 280nm and the area of these peaks decreased with the increase in blood volume (Hofer *et al.*, 1993). They too were unable to remove these possible interferences.

4.2 Bloodspot Validation

Finger-prick blood sampling was a method used in the present study as an alternative to venous sampling. This method provided a minimally invasive, quick, and convenient way of collecting blood samples outside the laboratory. Another positive result from the present study was the validation of the finger-prick blood assay. In

agreement with results from Petsos *et al.* (1985, 1986), McLoughlin *et al.* (1994), and Shirtcliff *et al.* (2001) the correlation of capillary blood and serum progesterone was very high using regression analysis ($R^2 = 0.9963$). Recovery and dilution experiments also showed a high correlations ($R^2 = 0.9963$, $R^2 = 0.9579$, respectively) and the intra-assay variability was satisfactory (<10%). The inter-assay variability was relatively higher at 23.04% which suggests that for reasons unknown something is affecting the binding of progesterone to the antibodies coating the tube differentially for each assay. Hematocrit was not taken into account in the present study as it was determined not to affect changes greater than the variability of the assay. Overall, the validation of the finger-prick blood assay confirms the usefulness of finger-prick blood sampling in the measurement of progesterone from blood.

4.3 Menstrual Cycle Characteristics

The present study investigated a total of 33 menstrual cycles of 18 women volunteers. This study confirms the presence of menstrual cycle abnormalities reported in numerous studies (Table 1). Three of the eighteen women (16%) experienced cycles with a non-functional CL as determined by bloodspot analysis. This is similar to that reported by Prior *et al.* (1990b) who documented at least one anovulatory cycle in 19.6% of participating women. Prior *et al.* (1982) reported the incidence of anovulation in 33% of 48 cycles in 14 women as determined by temperature analysis. In the present study 15% (5 of 33) of cycles were anovulatory. This is less than half that reported by Prior *et*

al. (1982) however the present study used bloodspot data as the gold standard, and participants were on average 6 years younger. Smith *et al.* (1984) reported the incidence of SLP in fertile women to be 8% however in the present study SLP occurred in 66% (10 of 15) of ovulating participants and in 43% of the ovulatory cycles (36% of all cycles), a percentage similar to Prior *et al.* (1982) who reported SLP in 50% of ovulatory cycles (33% of all cycles). Only about 36% of all cycles in the present study fit the criteria of a normal cycle with a functional CL. With the possible connection of menstrual cycle abnormalities to health problems such as infertility (Sherman & Korenman, 1974a), bone loss (Prior *et al.*, 1990b), and breast and endometrial cancer (Cowan *et al.*, 1981; Coulam *et al.*, 1983) this finding reinforces the need to develop an easy-to-use and inexpensive method of determining menstrual functioning in women. The measurement of appropriate markers of luteal function in menstrual fluid is an idea that should be investigated further (See 4.6. Future research (Glycodelin)).

4.4 Bloodspot and Temperature Analysis

Bloodspot progesterone was used as the gold standard in the present study. When comparing the results obtained from bloodspot and temperature analysis some discrepancies were apparent. The sensitivity and specificity of the two methods were satisfactory however the negative predictive value for anovulation was low. That is, the prediction of a non-functional cycle by both bloodspot and temperature was asynchronous. Prior (1997) has previously shown lack of molimina is sensitive and

specific in predicting anovulation. Prior *et al.* (1990a) validated the temperature method (Maximina®) against the LH peak, however, it was not validated against serum progesterone. As only three finger-prick blood samples were taken during the luteal phase this disparity may be due to improper timing of bloodspot sampling. This is a common problem and one of the motivations for the present study. The release of progesterone is pulsatile and variable (Soules *et al.*, 1988) which makes the proper determination of luteal function difficult. It is quite possible that the finger-prick blood sampling strategy was insufficient to obtain samples with elevated progesterone concentrations in cycles with biphasic temperature patterns. The timing of the bloodspots relied on the participants observations of vaginal mucus changes. There was no difference in the scores for mucous secretion between ovulatory or anovulatory cycles, however the subjectivity of this parameter may have affected appropriate timing of blood sampling. Gaps in the luteal phase where no bloodspot sampling occurred may have hidden rises in progesterone where the samples taken showed low levels. Some samples were obtained very close to the first day of menses when progesterone values are expected to be lower. In future studies, bloodspot samples may have to be taken daily from mid-cycle (or maximal mucous secretion) onward.

Unlike the bloodspot sampling, temperature readings were recorded daily. Other researchers have documented disparities in temperature and other detection methods of ovulation such as plasma progesterone measurements (Johansson *et al.*, 1972). It is possible that the temperature analysis gave false results as problems can arise when technical errors occur or when temperature readings are improperly recorded. Accuracy

and precision of the two types of thermometers used were tested. Both were consistently precise and consistently less accurate (by ~0.20°C) when compared to a reference thermometer. Thus it is probably unlikely that the thermometers were the source of the disparity between the temperature and bloodspot analysis since the Maximina® analysis is programmed to detect significant shifts in temperature. Each participant in the present study was instructed on how to take their temperature. This lessens the chances of inaccurate readings but it doesn't abolish the possibility that errors could occur in temperature taking. When relying on data recorded by the participants this study assumed that the reported data were accurate. However, false data, missing data, or data recorded during an unrecorded illness or unusual awakening may affect the overall analysis. It is also possible that for some monophasic temperature cycles, ovulatory levels of progesterone for unknown reasons did not elicit measurable changes in temperature. Any of these situations may have contributed to the disparity between bloodspot and temperature analysis.

4.5 Diary Results

In the present study, the scores of seven diary experiences were reported as significantly greater for cycles with a functional CL than those with a non-functional CL. Use of the ANOVA provided a functional tool in eliminating seemingly less useful information from the Menstrual Cycle Diary©. Results from the ANOVA indicated significant cyclic changes occurring within the cycle (comparing the 3 windows) and

which particular window (e.g. MC, EC, or 3DA) provided the least distinguishing data. For example, side breast tenderness scores were cyclical as determined by ANOVA. Mid-cycle scores for side breast tenderness were discarded from subsequent analyses comparing experiences between ovulatory versus anovulatory cycles because the scores from the EC and 3DA windows were significantly greater than scores from the MC window, which was close to the usual score of zero, meaning none (or not observed). It is evident then that when comparing side breast tenderness, scores from only those two windows (EC, 3DA) are necessary to analyze. This can be utilized for all items of the diary providing a simple and objective way of looking at and comparing diary data.

The experience of pain manifested as cramps was greater for ovulatory cycles than anovulatory cycles. Studies show that extracts from used tampons of dysmenorrheic women have a greater content of prostaglandins, which have been linked to menstrual pain, than do women without painful menstruation (Chan & Hill, 1978; Powell *et al.*, 1985). Pain may be caused by the formation of arachidonic acid derivatives (prostaglandins, thromboxanes, and leukotrienes) in the uterus (Bieglmayer *et al.*, 1995). It may be that the significant difference in scores may be due to higher prostaglandin levels in ovulatory cycles. Prostaglandin levels are up-regulated by way of progesterone withdrawal during the late luteal phase (Fraser, 1999) and are synthesized by luteal cells of the corpus luteum. Interestingly, Laessle *et al.* (1990) found that pain symptoms increased with decreasing progesterone levels. Studies show that the use of oral contraceptives which prevent ovulation (i.e. no formation of CL) reduces prostaglandin levels (Bieglmayer *et al.*, 1995) which proposes the possibility that perhaps anovulatory

cycles may have lower prostaglandin levels thus explaining the lesser menstrual pain observed by women with anovulatory cycles.

Side breast tenderness was greater for those experiencing cycles with a functional CL. Scores for breast tenderness tend to be maximal in the mid-to-late luteal phase of the menstrual cycle and with ovulatory women (Beumont *et al.*, 1975; Laessle *et al.*, 1990; Hale *et al.*, 2003). Hale *et al.* (2003) found cyclicity in front breast tenderness in mid-life women. The present study found tenderness of the side breast to be greater in ovulatory cycles and did not find the same for front breast tenderness. Higher levels of urinary estrone and lower luteal phase progesterone excretion have been documented in perimenopausal women (Santoro *et al.*, 1996). This has been suggested to be associated with front breast tenderness in perimenopausal women (Hale *et al.*, 2003). Wang et al. (1996) suggested higher estradiol and lower progesterone levels were associated with breast tenderness in women with premenstrual syndrome when compared to controls. The relationship between breast tenderness and estrogen cannot be determined from the present study.

Change in breast size was also perceived to change more for ovulatory cycles than anovulatory cycles. Morphological changes of the human breast correlate with menstrual phase (Vogel *et al.*, 1981; Battersby *et al.*, 1992; Ramakrishnan *et al.*, 2002). Progesterone's action on the breast is, at least in the short term (Chang *et al.*, 1995), proliferative causing a transient swelling of the breasts. Feuerstein & Shaw (2002) describe the experiences of breast swelling and tenderness as suggestive of fluid retention which they found to score highest in the luteal phase. In the present study, fluid retention

was found to score higher for those with ovulatory cycles. It has been suggested that water-retaining hormones such as estrogen, angiotensin/aldosterone, and prolactin among others may mediate retention of fluid (Feuerstein & Shaw, 2002). Whether cycles with a non-functional CL lack the hormonal environment to influence changes in fluid retention, and/or breast swelling and tenderness needs to be investigated further. The results from the present study, however, do suggest differences to this effect.

Feelings of anxiety, depression, and frustration are often grouped under the category of mood. The results of investigations into the so-called menstrual cycle phase-dependent alterations of these behaviours have been mixed; some reports document cyclicity while others do not (Little & Zahn, 1974; Beumont *et al.*, 1875; Both-Orthman *et al.*, 1988; Laessle *et al.*, 1990). In the present study, feelings of depression and frustration scored significantly higher for those with ovulatory cycles than those with anovulatory cycles suggesting that the hormonal fluctuations that occur during a normal cycle contribute to these psychological changes.

Research on the relationship between sleep and the menstrual cycle has also been contradictory (Laessle *et al.*, 1990; Driver *et al.*, 1996; Baker & Driver, 2004). More sleep cycles have been documented in the luteal phase of the menstrual cycle (Le Bon *et al*, 2002). This increase may heighten a woman's sensitivity to dysphoric experiences during the luteal phase (Le Bon *et al*, 2002). In the present study, those with ovulatory cycles observed more sleep problems than those with anovulatory cycles. There was no significant change, however, in scores for sleep problems for any of the three windows in the present set of ovulatory cycles (i.e. all windows were used in the analysis). Hale *et*

al. (2003) also did not find any statistical difference in scores across the three cycle windows they investigated. An increase in sleep problems for women experiencing ovulatory cycles may be real. However, more research is needed to corroborate these findings.

One purpose of the Menstrual Cycle Diary© is to discover whether emotional or physical experiences observed throughout the menstrual cycle differ for women with functional or non-functional cycles. This information may be useful in determining luteal function in the absence of other detection methods. Magyar *et al.* (1978) has concluded that women presenting a history of regular cycles (as determined by length) in the company of premenstrual molimina can be identified as ovulatory. This may in fact be correct, however, due to the complexity of the menstrual cycle more research is warranted. In the present study seven experiences scored statistically higher for women with ovulatory cycles. The comparison of functional cycle scores with the small sample of non-functional cycle scores decreases the power of the findings and it may be that differences not found may actually exist. Therefore, it would be inappropriate to assume that the insignificant findings are absolute.

4.6 Future Research (Glycodelin)

The results from the present study suggest that progesterone may not be an appropriate marker to determine luteal function from menstrual fluid. The measurement

of additional markers that reflect progesterone's action may be a more suitable avenue of investigation. One such marker is glycodelin.

Glycodelin, also called PP14, is a 28 kDa glycoprotein belonging to the lipocalin superfamily expressed in the uterus, fallopian tubes, ovary, and breast among other tissues (Seppälä *et al.*, 2002). The expression of glycodelin in the endometrium appears to be menstrual phase-dependent, with near absent levels during the proliferative phase and rising levels during the secretory phase peaking during late luteal phase (Julkunen *et al.*, 1986a; Li *et al.*, 1993). Even though progesterone and glycodelin lack correlation in circulating levels (glycodelin's rise is later than progesterone's and is maintained until the first few days of the following cycle) glycodelin's synthesis is said to be regulated by progesterone (Rutanen *et al.*, 1987; Seppälä *et al.*, 2002). This is suggested due to the observation that anovulatory cycles show low levels of both progesterone and glycodelin throughout the menstrual cycle (Rutanen *et al.*, 1987; Seppälä *et al.*, 2002). Glycodelin is measurable in serum and uterine flushings; however, serum levels of this protein do not accurately reflect luteal function (Batista *et al.*, 1993a; Batista *et al.*, 1993b). The idea of measuring glycodelin to determine luteal function is not a new one (Batista *et al.*, 1993b; Julkunen *et al.*, 1986b; Fay *et al.*, 1990; Dalton *et al.*, 1998), however, its measurement in menstrual fluid has not been, to the knowledge of the authors of the present study, investigated for this purpose.

Preliminary work from the laboratory of the present study showed that glycodelin may be measurable in menstrual fluid extracted from used tampons. Two used tampons were extracted and analyzed for glycodelin using the western blot technique (See

Appendix C). Unfortunately, the luteal status of the cycle for which these samples were collected was not determined. The extraction procedure involved soaking the used tampons overnight in phosphate buffer saline (0.89%, PBS) at 4°C, centrifuging the extract for 10 min (3000 rpm), centrifuging ~20 ml through a filter device (Centricon Plus-20, Millipore) for about an hour until all the fluid has been centrifuged through the filter (3200 x g, 25°C), inverting the filter cup and centrifuging again for one minute (1000 x g, 25°C), and saving and storing the concentrated sample at -20°C. Protein separation and transfer was carried out using SDS-PAGE and western blot. Glycodelin was generously donated by Dr. Glenn Braunstein's laboratory (USA) with the permission of Dr. Børge Teisner (Denmark). Impurities in the long-stored protein samples, however, made for an unsuccessful control (not shown). The use of a purified protein sample, preferably from amniotic fluid as it is an excellent source of this protein, would be necessary for control purposes. Further research is needed to confirm the usability of glycodelin from menstrual fluid as a marker of luteal function.

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APPENDIX A

Consent Forms, Menstrual Cycle Diary©, Explanation Sheet

Menstrual Cycle Diary

Name: _____ Month: _____ Year: _____

Cycle Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Date																															
Tampons/pads/day																															

Record 0 = none, 1 = minimal, 2 = moderate, 3 = moderately intense, 4 = very intense

Amount Flow																															
Cramps																															
Breast Sore: Front																															
Breast Sore: Side																															
Fluid Retention																															
Mucous secretions																															
Constipation																															
Headache																															
Sleep Problems																															
Feeling Frustrated																															
Feeling Depressed																															
Feeling Anxious																															

Record M = much less, L = a little less, U = usual, Y = a little increased, Z = much increased

Appetite																															
Breast Size																															
Interest In Sex																															
Feeling Of Energy																															
Feeling Of Self-Worth																															
Outside Stresses																															
Basal Temperature																															
Comments (temperature taken late, feeling sick, poor sleep, etc)																															

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Faculty of Medicine
Memorial University of Newfoundland

Consent to Take Part in Health Research
Consent Form

TITLE: Noninvasive Assessment of Menstrual Cycle Hormone Levels

INVESTIGATOR(S): Sarah J. Halfyard, Donald McKay Ph.D, Dr. Jerilynn Prior M.D.

You have volunteered to take part in a research study. It is important that procedures and tests are thoroughly explained to you and any questions and concerns addressed. This consent form explains the study.

As a volunteer, you are free to withdraw from the study at any time.

The researchers will:

- **discuss the study with you**
- **answer your questions**
- **keep confidential any information which could identify you personally**
- **be available during the study to deal with problems and answer questions**

1. Introduction/Background:

Often, ovulation is assumed to occur when menstrual cycles are regular. However, ovarian disturbances (short luteal phase [<10 days], anovulatory cycle [failure to ovulate], short/long cycle length) have been documented in healthy women who report regular cycles. Previous research has implicated ovarian disturbances with the development of infertility, osteoporosis, breast cancer, and heart disease. Current ovulation detection methods tend to involve any one or all of the following problems: are not reliable, are hard to do, are uncomfortable or invasive and are expensive.

This study proposes to document hormone levels obtained from sanitary products (tampons) obtained during the menstrual flow. The method is easy to remember and doesn't involve any invasive testing. Potentially this new method could be used to document how commonly ovarian disturbances happen in the general population of women. If proven accurate, this method could be used cycle by cycle over many years to advance our understanding of menstrual cycle variability.

2. Purpose of study:

The purpose of this study is to develop and validate a noninvasive method to determine hormonal, ovulatory and luteal function (i.e. function of the ovaries) of women by documenting hormone levels in finger-pricks and in menstrual flow.

3. **As a participant you understand that you are agreeing to do the following:**

a) Answer background questions regarding your health and history of menstruation and reproduction

b) Keep a daily Menstrual Cycle Diary for a three month period noting what you observe every day in the chart provided to you

c) Record morning temperature in your mouth first thing in the morning for a three-month period. You understand that the digital thermometer will be yours to keep if you finish the study

d) Have blood finger-prick samples taken daily for three days about 10-14 days after your period starts, about a week later and the first day of your period each month for two months

e) Monitor vaginal stretchiness of mucus and notify the principal investigator as soon as possible when maximal stretchiness of mucus is observed

f) Collect all used tampons during the first two full days of flow and storing them in your freezer in the containers that are provided to you. You will do this for three consecutive menstrual periods

4. **Length of time:**

You are participating in a three-month study. It will take about an hour at first to learn about the Menstrual Cycle Diary and to complete the questionnaires about your health, habits, past health and menstrual cycles. It will take no more than 2 minutes of your time each morning to take your temperature and usually no more than 3 minutes each day to fill out the daily Menstrual Cycle Diary. The investigator will come to your home or you may meet at the Health Sciences Centre for the finger-prick blood sampling, which should take no more than 5 minutes.

5. **Benefits:**

We cannot guarantee that participation in the study will benefit you personally.

6. **Risks, Discomforts and Inconveniences:**

You are asked to complete the daily Menstrual Cycle Diary during the entire duration of the study as well as collect basal temperature readings. You will collect used sanitary products during the first two days of flow for the entire three cycles. Finger prick blood samples will be taken daily for a total of seven days each cycle for two cycles. The finger-prick blood sampling is quick and easy and results in minimal discomfort.

7. **Liability statement:**

Signing this form gives us your consent to be in this study. It tells us that you understand the information about the research study. When you sign this form, you do not give up your legal rights. Researchers involved in this research study also still have their legal and professional responsibilities.

8. **Confidentiality:**

Unless required by law or hospital policy, only the principal investigator may have access to any confidential documents pertaining to your participation in this study that may identify you by name. Furthermore, your name will not appear in any report or article published as a result of this study.

Questions:

You have been given a copy of this consent form.

If you have any questions about taking part in this study, you can meet with the investigator who is in charge of the study. That person is:

Sarah J. Halfyard – (709) 738-2009 or (709) 777-8565

Or you can talk to someone who is not involved with the study at all, but can advise you on your rights as a participant in a research study. This person can be reached through:

Office of the Human Investigation Committee (HIC) at 709-777-6974
Email: jennifem@mun.ca

- **Exclusion of women who are pregnant or not regularly menstruating. Women who have significant health problems are also excluded. Women who are taking reproductive hormones including the birth control pill or shots are also not eligible.**

This study intends to document hormone levels of the menstrual cycle.

If you are pregnant you cannot take part in this study.

If intending to participate in this study you **must have stopped** taking any reproductive hormones (such as oral contraceptives (birth control pills), hormone patches, or other kinds of hormone therapy) at least 6 months before the start of this study. You **must not** take reproductive hormones at any time throughout the study.

If you do become pregnant or begin taking reproductive hormones during the study period you must **immediately** inform the study investigator.

Initials: _____

Signature Page

Study title: Noninvasive Assessment of Menstrual Cycle Hormone Levels

Name of principal investigator: Sarah J. Halfyard

To be filled out and signed by the participant:

	Please check as appropriate	
You have read the consent form [and information sheet].	Yes { }	No { }
You have had the opportunity to ask questions/to discuss this study.	Yes { }	No { }
You have received satisfactory answers to all of your questions.	Yes { }	No { }
You have received enough information about the study.	Yes { }	No { }
You understand that you are free to withdraw from the study	Yes { }	No { }

- at any time
- without having to give a reason

You understand that it is your choice to be in the study and that you may not benefit. Yes { } No { }

You agree to take part in this study. Yes { } No { }

_____ _____

Signature of participant Date

_____ _____

Signature of witness Date

To be signed by the investigator:

I have explained this study to the best of my ability. I invited questions and gave answers. I believe that the participant fully understands what is involved in being in the study, any potential risks of the study and that she has freely chosen to be in the study.

_____ _____

Signature of investigator Date

Telephone number: (709) 738-2009 or (709) 777-8565

Initials: _____

Faculty of Medicine
Memorial University of Newfoundland

Consent to Take Part in Health Research

TITLE: Non-invasive assessment of menstrual cycle hormone levels

INVESTIGATOR(S): Sarah J. Halfyard, Donald W. McKay, Ph.D., Jerilynn C. Prior, M.D.

You have been asked to take part in a research study. It is up to you to decide whether to be in the study or not. Before you decide, you need to understand what the study is for, what risks you might take and what benefits you might receive. This consent form explains the study.

As a volunteer, you are free to withdraw from the study at any time.

The researchers will:

- **discuss the study with you**
- **answer your questions**
- **keep confidential any information which could identify you personally**
- **be available during the study to deal with problems and answer questions**

1. Purpose of study:

The purpose of the study is to compare progesterone levels from finger-prick blood spots with progesterone levels from blood sampling. Similar readings will demonstrate the suitability of the finger-prick method as an accurate alternative to venous blood sampling.

2. As a participant you understand that you are agreeing to do the following:

a) To share the phase or cycle day of your menstrual cycle or term of pregnancy with the investigator

b) To provide, sequentially, up to 8 drops of blood by the finger-prick method and 6 teaspoons of blood by the venous sampling method. You will be asked to do this up to two times during different phases of your menstrual cycle (once during the late follicular phase and once during the mid-luteal phase). If you are pregnant, you will be asked to donate the blood and serum samples only once.

3. **Length of time:**

You are asked to provide finger-prick blood spot samples and a venous blood sample up to two times. It should not take more than 10 minutes to complete the spot test and blood collection each time.

4. **Possible risks and discomforts:**

The finger-prick blood sampling method is quick and easy and results in minimal discomfort. Blood collection by the venous sampling method results in minor discomfort and takes no more than 5 minutes to complete.

5. **Benefits:**

This study will not benefit you personally.

6. **Liability statement:**

Signing this form gives us your consent to be in this study. It tells us that you understand the information about the research study. When you sign this form, you do not give up your legal rights. Researchers or agencies involved in this research study still have their legal and professional responsibilities.

7. **Confidentiality:**

Unless required by law or hospital policy, only the principle investigator, Sarah J. Halfyard, may have access to any confidential documents pertaining to your participation in this study that may identify you by name. Furthermore, your name will not appear in any report or article published as a result of this study.

8. **Questions:**

You have been given a copy of this consent form.

If you have any questions about taking part in this study, you can meet with the investigator who is in charge of the study at this institution. That person is:

Sarah J. Halfyard at 709-777-8565

Or you can talk to someone who is not involved with the study at all, but can advise you on your rights as a participant in a research study. This person can be reached through:

Office of the Human Investigation Committee (HIC) at 709-777-6974
Email: hic@mun.ca

Initials: _____

- **Exclusion of women who are not regularly menstruating. Women who have significant health problems are also excluded. Women who are taking reproductive hormones including the birth control pill or shots are also not eligible.**

This study intends to document the progesterone concentrations from blood spots and serum.

If intending to participate in this study you **must have stopped** taking any reproductive hormones (such as oral contraceptives (birth control pills), hormone patches, or other kinds of hormone therapy) at least 6 months before the start of this study. You **must not** take reproductive hormones at any time throughout the study.

If you begin taking reproductive hormones during the study period you must **immediately** inform the study investigator.

Initials: _____

Signature Page

Study title: Non-invasive assessment of menstrual cycle hormone levels

Name of principle investigator: Sarah J. Halfyard

To be filled out and signed by the participant:

Please check as appropriate

I have read the consent [and information sheet].	Yes { }	No { }
I have had the opportunity to ask questions/to discuss this study.	Yes { }	No { }
I have received satisfactory answers to all of my questions.	Yes { }	No { }
I have received enough information about the study.	Yes { }	No { }
I understand that I am free to withdraw from the study	Yes { }	No { }

- at any time
- without having to give a reason

I understand that it is my choice to be in the study and that I may not benefit. Yes { } No { }

I agree to take part in this study. Yes { } No { }

_____ _____

Signature of participant Date

_____ _____

Signature of witness Date

To be signed by the investigator:

I have explained this study to the best of my ability. I invited questions and gave answers. I believe that the participant fully understands what is involved in being in the study, any potential risks of the study and that he or she has freely chosen to be in the study.

_____ _____

Signature of investigator Date

Telephone number: (709) 777-8565

Initials: _____

CONFIDENTIAL

Noninvasive Assessment of Ovarian Cycle Hormone Levels

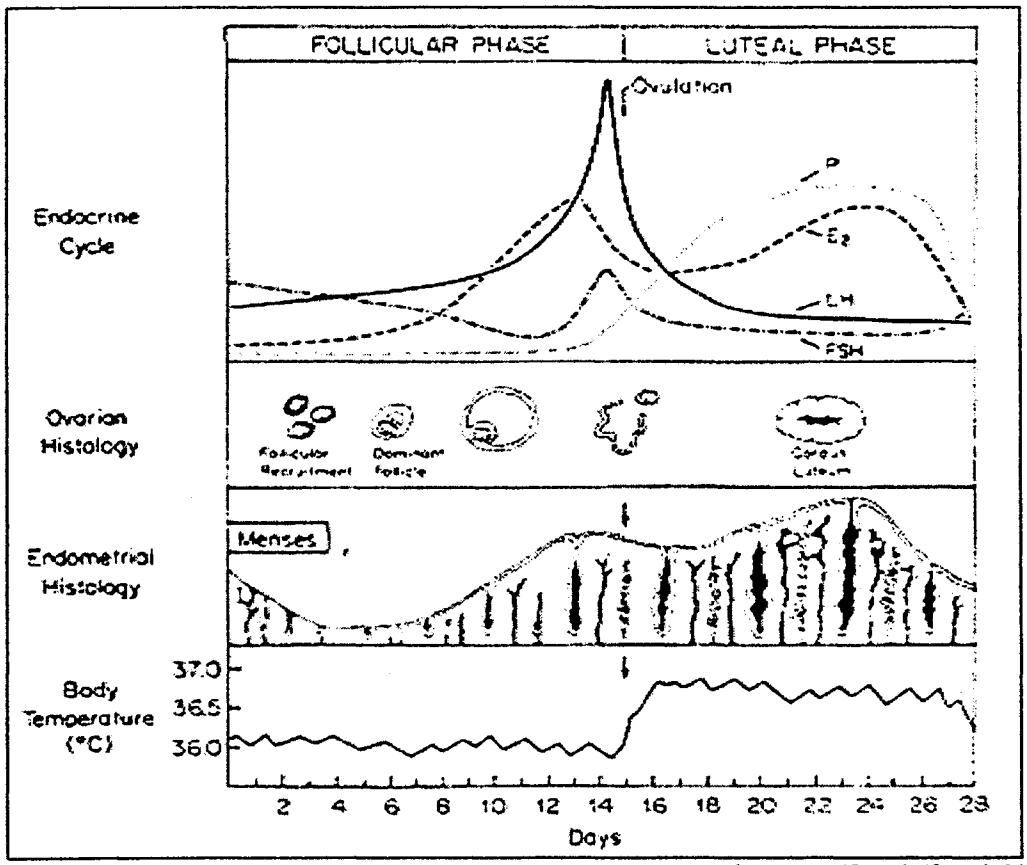
Explanation Sheet

Often, ovulation (release of an egg) is assumed to occur in women who have regular menstrual cycles. However, despite regular cycle length (21-36 days) ovulation may not occur. Furthermore, ovarian hormone production, especially progesterone, may be inadequate. Ovarian disturbances (i.e. short luteal phase [<10 days], anovulatory cycles, short/long cycle length) have been implicated in future health problems such as osteoporosis and breast cancer. Current ovulation detection methods can be variably reliable, tedious and inconvenient as well as invasive and expensive. Therefore, an easy-to-use, inexpensive, reliable, and noninvasive method to determine luteal function in women is needed.

The ovarian cycle has an average length of 28 days. The cycle is divided into two main phases: the follicular phase and the luteal phase. The follicular phase is characterized by a steady increase in blood levels of the ovarian hormone, estradiol, and a very low (almost absent) level of progesterone. During this phase the ovarian follicle that contains the oocyte begins to change and grow due to exposure to estradiol and the anterior pituitary hormone, follicle-stimulating hormone (FSH). Estradiol also causes the uterine endometrium to proliferate during this phase and vaginal mucous to become abundant and stretchy. At about mid-cycle (day 14), a surge in the release of the anterior pituitary hormone, lutenizing hormone (LH), precedes ovulation by a few hours. Ovulation marks the beginning of the luteal phase.

The luteal phase commonly lasts about 10-16 days and is characterized by a dominant progesterone presence and a lower but still significant blood level of estradiol. The remaining ruptured follicle transforms into the corpus luteum, which secretes progesterone. During this phase the endometrium takes on more secretory properties in preparation for possible implantation of an embryo. The corpus luteum functions for about two weeks and in the absence of pregnancy will degenerate at the end of the two-

week period. A decline in blood hormone levels ensues with the resultant sloughing off of the endometrium as menstrual flow. The first day of menstrual flow is considered the beginning of a new cycle.



www.endotext.org/female/female3/female3.htm

Progesterone has various effects on the body. One in particular is its effect on body temperature. Progesterone travels in the blood to reach the brain and acts on areas of the brain, such as the hypothalamus, that control temperature. During the follicular phase the temperature is relatively steady. Increased concentrations of progesterone in the luteal phase cause a small rise in basal (resting) body temperature (0.2-0.5 °C). The measurement of first morning temperature is used to determine whether a woman has ovulated during that cycle and if she has, the length of the luteal phase, which should be 10-16 days. The temperature data are analyzed with least squares statistics to determine the date of temperature rise and the length of the luteal phase.

Collecting menstrual tampons:

Day 1 is considered the first day of your menstrual flow. On this day, you will begin to fill out the daily Menstrual Cycle Diary form. Menstrual tampons will be collected during your next three menstrual flows. Tampons will be provided for you. Please refrain from using tampons of a different brand during the study period. You will be provided with opaque, screw top containers and a marker. Each time you change your tampon place it in a container sealing it well. Label the container with your code #, the date and time. Then place the container in the freezer. When your menses is over, telephone the principal investigator, Sarah, at 738-2009 or 777-6585 so that arrangements can be made for the pick up of the samples and data sheets.

Blood sample collection:

Finger prick blood samples will be collected for 3 consecutive days at the onset of stretchy vaginal mucous (which, if you look at the above diagram, is just before the highest point for estrogen). We will collect again for the 3 consecutive days about a week later (when we are trying to catch the peak of progesterone) and once on the first day of your period (to compare with what we measure from your tampons). Finger prick blood sampling will be performed during your last two cycles of the three-month study period. Blood sampling can be performed at your home or at the Health Sciences Centre. Upon the observation of maximal mucous stretchiness, immediately contact the principal investigator, Sarah, at 738-2009 or 777-8565.

UNDERSTANDING YOUR MENSTRUAL CYCLE

Dr JC Prior© 1991, 2002

The menstrual cycle is created by over a dozen hormones changing in a complex and coordinated manner. Hormones of the ovary, pituitary, and uterus work together to create cyclic symptoms and signs during your cycle. Completing this form every day will help you to learn from and to notice important features about your own menstrual cycle.

At the top, write the month of the first day of flow (day 1). Please start filling in the form on the **evening** of the first day of your period.

Be sure to write something in every box. A blank tells you nothing about that day but a 0 is good evidence you didn't experience it! If you forget, skip that day. Don't try to remember later. Use a new chart for each cycle.

If no period occurs, continue recording on another sheet after day 30 and just re-number 1 to "31" for subsequent days until flow starts. If you have had no flow for several months, start using a new Diary sheet for each month.

The scale at the top of this Diary is from 0 to 4; zero represents something you did not experience and four represents the worst it has ever been for you. The scale at the bottom uses letters to indicate as factors go up or down from your usual '(U)' or normal state. Although the form is mostly self-explanatory, a few explanations and samples are provided.

Flow:

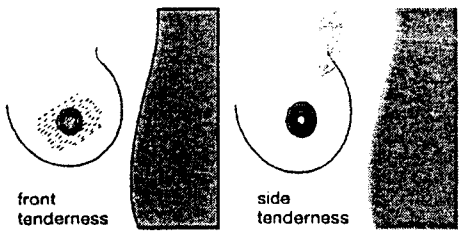
Because flow is important, you are requested to provide two indicators for it. The first is the number of regular sized, **soaked** pads and/or tampons you use each day of flow. If you use a combination of pads and tampons, enter the combined total for that day (4 tampons/3 pads = 7). The second is your assessment of flow. The score goes from 0 = none, 1 = spotting, to 4 which means clots or a change every hour.

Name: _____ Month: March Year: 1993

Cycle Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Date	12	13	14	15	16	Continued													
Tampons/pads/day	4	4	3	2	0														

Record 0 = none, 1 = minimal, 2 = moderate, 3 = moderately intense, 4 = very intense

Amount Flow	3	4	2	2	1														



Breast Tenderness:

Breasts can tell us a lot about estrogen and progesterone levels and how they are acting. You are being asked to note both front and/or side breast tenderness. In the diagram there is shading in the general areas of your breast that you should touch firmly with the palm of your hand to determine if you have any breast soreness or sensitivity there. There may be very little soreness (a "1") but that pressure will feel different from the way the same pressure feels on your leg, for example.

Fluid Retention:

This means feeling bloated or puffy or water weight gain and getting up to urinate at night.

Mucous Secretions:

The mouth of the uterus (cervix) makes a clear stretchy fluid when estrogen levels are high. Progesterone levels stop the production of stretchy mucous A "4" means you can stretch the mucus out for 6-8 cm (3-4 inches).

Record 0 = none, 1 = minimal, 2 = moderate, 3 = moderately intense, 4 = very intense

Amount Flow	3	4	2	2	1	0	0	0	0	0	0	0	0	0		0	0	0	0
Cramps	4	2	0	0	0	0	0	0	0	0	0	0	0	1		0	0	0	0
Breast Tenderness: Front	0	0	0	0	0	0	0	0	0	0	0	0	1	3		2	0	0	0
Breast Tenderness: Side	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	1
Fluid Retention	0	0	0	1	0	0	0	0	0	0	0	2	3	3		2	1	2	1
Mucous Secretion	0	0	0	0	0	0	0	0	0	0	0	1	1	3		0	0	0	0

Headache:
The score in this box is for any old ordinary kind of headache. However, if it was a **migraine**, put a small star or dot in the box as well as the score.

Feelings and Outside Stresses:
You are asked to record how you feel each day using a 0-4 scale on the top section of the form. In addition, we would like you to evaluate the amount of outside stress in your life (Stress - work, home, etc). These two things are sometimes different - your day may have been awful but you can still feel good about yourself and not depressed or anxious. Please write your comments at the bottom of the column. This may include any particular event that influenced how you felt that day (eg illness, a job promotion, winning a lottery, argument with partner).

Record M = much less, L = a little less, U = usual, Y = a little increased, Z = much increased

Appetite	L	U	U	U	U													
Breast Size	U	U	U	Y	Y													
Interest in sex	L	U	U	Y	U													
Stress - work, home, etc	L	L	U	U	Y													

Quantitative Basal Temperature (QBT) Monitoring: (called this because we use statistics to decide where the temperature increase occurs—Prior, Clinical Invest. Med., 1990). Progesterone makes the first morning temperature increase a small but reliable amount.

The following recommendations will assist you to accurately take and record your oral temperature.

1. Day 1 is the first day of your flow (and you should be starting on a new sheet).
2. Take your temperature in the morning, when you first wake.
3. Activity will raise your basal (resting) temperature. Although you may start your thermometer and head to the washroom, if you can, postpone this or getting out of bed until your temperature taking is finished.
4. Under 'Comments', please record any events that may affect your morning temperature (the time if you slept in or got up early, felt like you were getting the flu or had a very late night).

Using the digital thermometer:

1. Press the ON/OFF button and a beep will sound (88.88 will display when the thermometer is used for the first time).
2. After a few seconds the display will go blank.
3. Place the thermometer under your tongue.
4. When the peak temperature is reached (in about 1 minute), a beep will sound 3 times. The reading will not change while the power remains on.
5. Turn the thermometer off by pressing the green ON/OFF button. The reading will be stored in memory for 3 seconds the next time you switch the thermometer on. After 3 seconds, the display will go blank and the temperature will be permanently erased from memory.

Analyzing your temperature data:

If you would like to figure out whether you have ovulated and the length of your luteal phase (the time following ovulation) you can do that. First, compute the average of all the temperatures in your record, by adding them up and dividing by the number of days for which you have temperature readings. The average temperature you get can then be compared with the actual readings. If your temperature went above and stayed above that average until the day before the next flow you have ovulated. The higher temperatures should last 10-16 days. When there are between 3 and 9 days of higher temperatures, you have what is called a short luteal phase. This means that you have ovulated but the time of progesterone elevation is too short.

Enjoy the keeping of this daily Menstrual Cycle Diary and quantitative basal temperature record. You will learn new things about yourself!

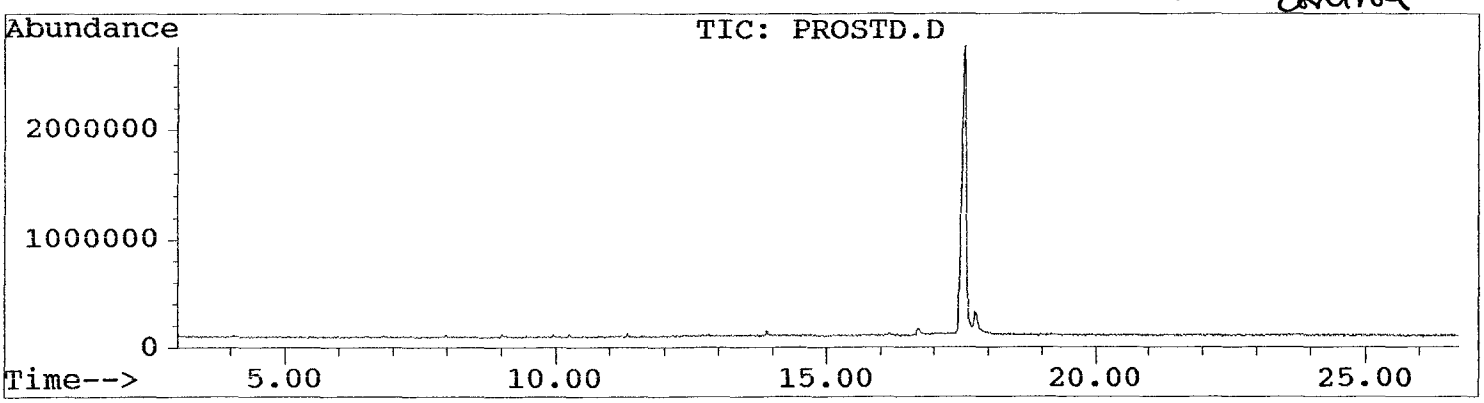
APPENDIX B

Gas Chromatography-Mass Spectrometry (GC-MS) Print-out Results. First print-out shows the results of GCMS of a progesterone standard (~0.3 mg/ml). The first chart shows the total ion counts over time. The greatest number of counts was at the 17 minute mark. Scan 1319 with the retention time of 17.569 min shows characteristic progesterone peaks at 124, 191, 272, and 314. Scan 1337 with the retention time of 17.768 min revealed the greatest abundance of progesterone at the 312 cluster. The second print-out shows the results for a concentrated sample (10X) of un-used (blank) tampon extracts. The first chart shows the total signal of the acetonitrile-extracted sample with peaks within the same time frame as the progesterone standard (17 min). Listed is the chromatogram of each ion present. Tested for the presence and abundance of progesterone-characteristic peaks within the specific time frame of the progesterone standard total ion peak revealed no signals characteristic of progesterone (see second graph). The last print-out shows the results of the concentrated (10X) hexane-extracted sample. The presence of progesterone was also not apparent in that sample.

Area Percent Report -- Sorted by Signal

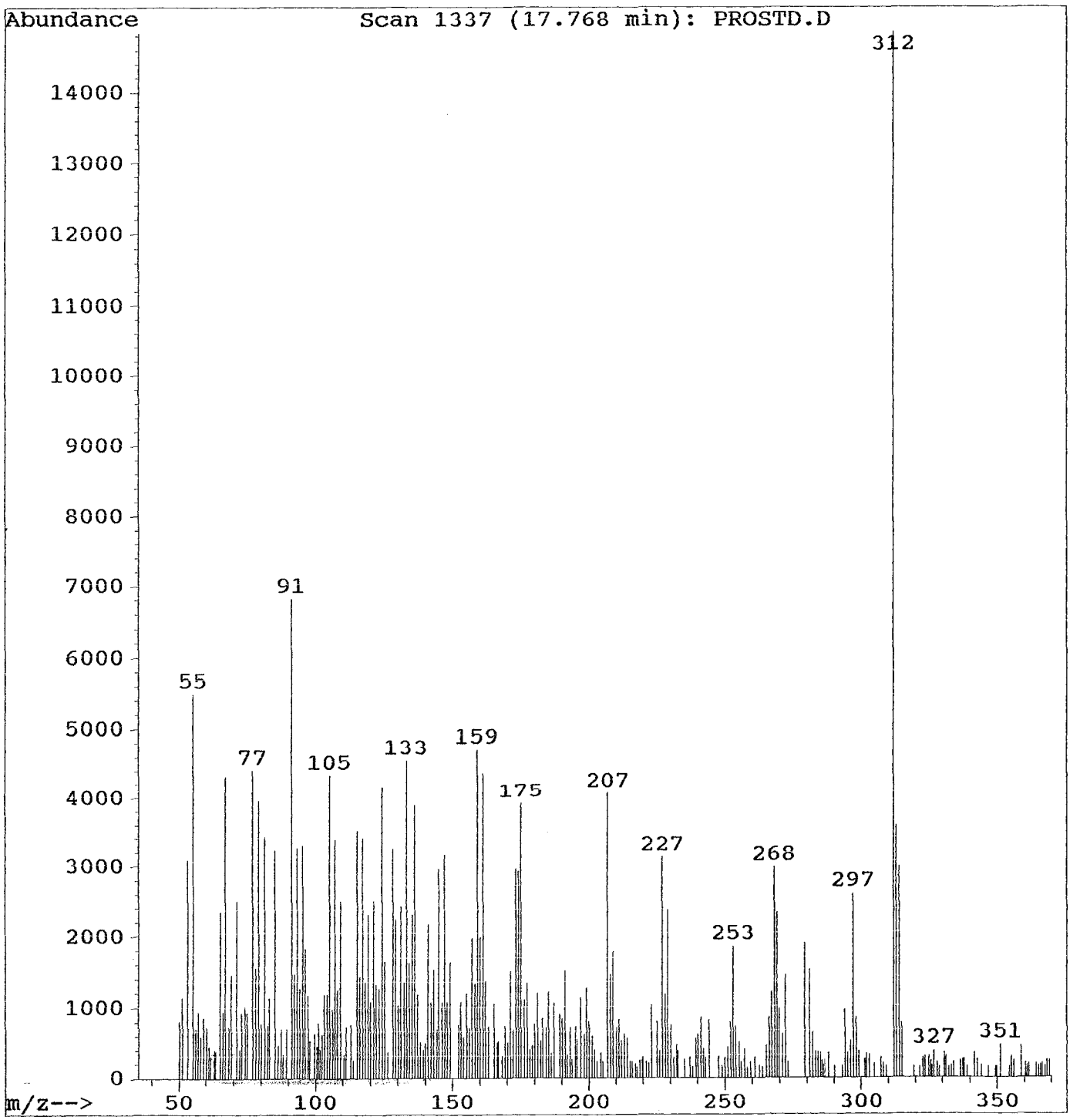
Information from Data File:
File : C:\HPCHEM\1\DATA\PROSTD.D
Operator : 1 winsor
Acquired : 2 Sep 103 9:26 am using AcqMethod LWINSOR
Sample Name: progesterone std ~0.3mg/ml
Misc Info : 0.5ul inj
Vial Number: 1
CurrentMeth: C:\HPCHEM\1\METHODS\LWINSOR.M

Progesterone control

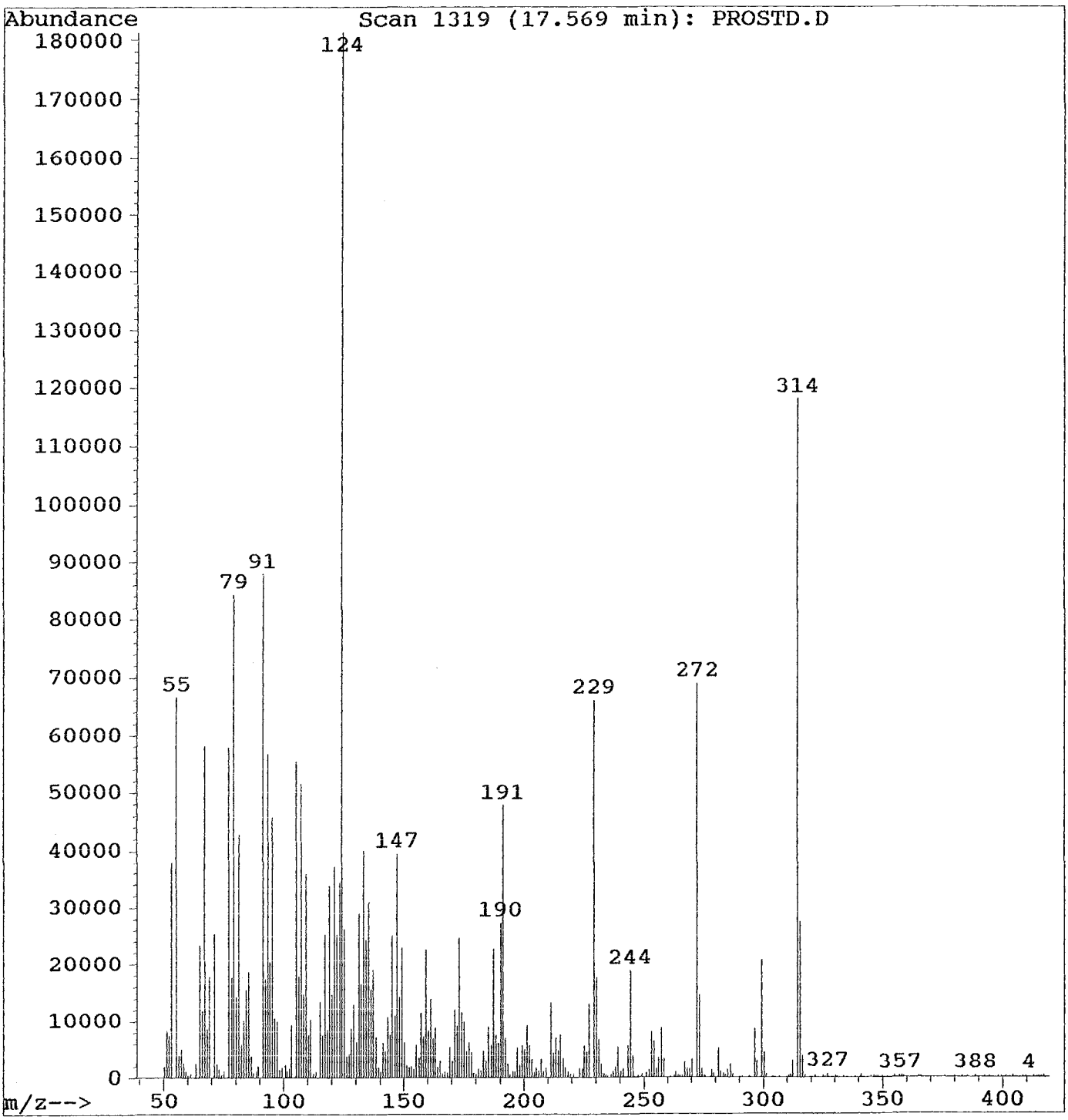


Retention Time	Area	Area %	Ratio %
Total Ion Chromatogram			
16.707	2065844	1.233	1.338
17.572	154346421	92.119	100.000
17.776	11138089	6.648	7.216

File : C:\HPCHEM\1\DATA\PROSTD.D
Operator : 1 winsor
Acquired : 2 Sep 103 9:26 am using AcqMethod LWINSOR
Instrument : 5970 - In
Sample Name: progesterone std ~0.3mg/ml
Misc Info : 0.5ul inj
Vial Number: 1



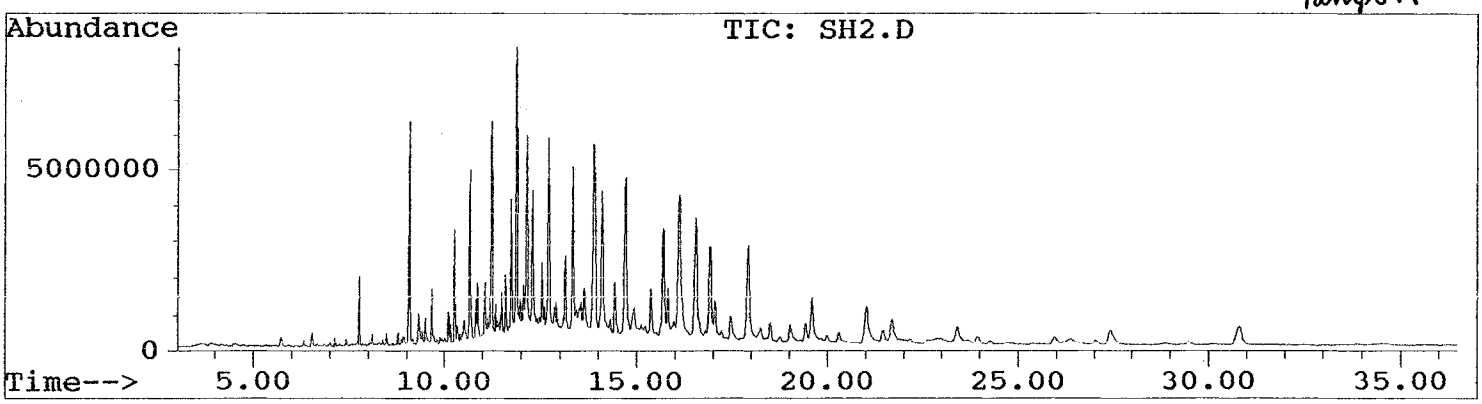
File : C:\HPCHEM\1\DATA\PROSTD.D
Operator : 1 winsor
Acquired : 2 Sep 103 9:26 am using AcqMethod LWINSOR
Instrument : 5970 - In
Sample Name: progesterone std ~0.3mg/ml
Misc Info : 0.5ul inj
Vial Number: 1



Area Percent Report -- Sorted by Signal

Information from Data File:
File : C:\HPCHEM\1\DATA\SH2.D
Operator : lwinsor
Acquired : 16 Sep 103 9:46 am using AcqMethod LWINSOR
Sample Name: Sarah H ~~4~~ 2 (Aceto)
Misc Info :
Vial Number: 1
CurrentMeth: C:\HPCHEM\1\METHODS\LWINSOR.M

(Aceto tube)
10X Concentrated blank (un-used) tampon



Retention Time	Area	Area %	Ratio %
Total Ion Chromatogram			
5.722	8380812	0.163	2.944
6.539	8604037	0.168	3.022
7.150	3500720	0.068	1.230
7.431	2981529	0.058	1.047
7.772	20525306	0.400	7.209
8.127	5493844	0.107	1.930
8.496	6315960	0.123	2.218
8.786	5521833	0.108	1.940
8.931	8072530	0.157	2.835
9.083	90782087	1.771	31.887
9.327	24883777	0.485	8.740
9.414	9245975	0.180	3.248
9.504	22466841	0.438	7.891
9.672	32686696	0.638	11.481
10.095	18761580	0.366	6.590
10.144	10117006	0.197	3.554
10.256	52874403	1.031	18.572
10.337	11374189	0.222	3.995

Area Percent Report -- Sorted by Signal

Information from Data File:
File : C:\HPCHEM\1\DATA\SH2.D
Operator : lwinsor
Acquired : 16 Sep 103 9:46 am using AcqMethod LWINSOR
Sample Name: Sarah H 1 (Aceto)
Misc Info :
Vial Number: 1
CurrentMeth: C:\HPCHEM\1\METHODS\LWINSOR.M

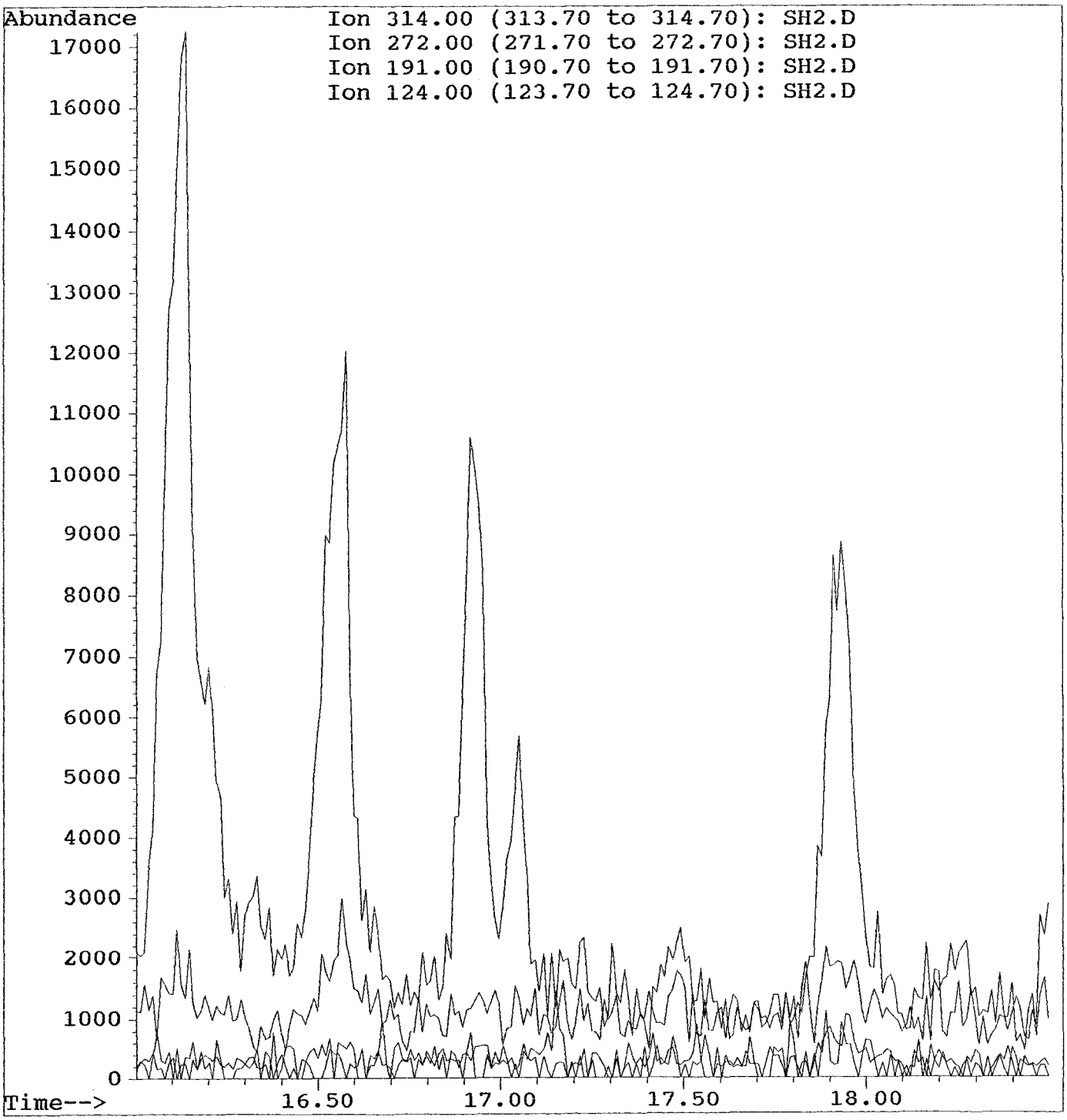
Retention Time	Area	Area %	Ratio %
10.399	5811125	0.113	2.041
10.516	36749292	0.717	12.908
10.674	109004478	2.126	38.287
10.823	29441642	0.574	10.341
10.868	38691944	0.755	13.590
11.054	43764159	0.854	15.372
11.122	19390452	0.378	6.811
11.225	166118883	3.241	58.348
11.328	31868647	0.622	11.194
11.409	14111442	0.275	4.957
11.479	42713699	0.833	15.003
11.581	51668557	1.008	18.148
11.734	93034314	1.815	32.678
11.874	243321161	4.747	85.465
11.969	32794638	0.640	11.519
12.055	48735919	0.951	17.118
12.133	184218947	3.594	64.706
12.287	149523933	2.917	52.519
12.468	21730077	0.424	7.633
12.527	53982209	1.053	18.961
12.585	27304163	0.533	9.590
12.713	205001518	3.999	72.005
12.851	34261397	0.668	12.034
12.894	27524116	0.537	9.668
13.146	107581063	2.099	37.787
13.355	145991131	2.848	51.278
13.438	33906868	0.661	11.910
13.553	65931731	1.286	23.158
13.644	98180104	1.915	34.485
13.903	284703628	5.554	100.000
14.118	204457097	3.989	71.814
14.319	31248157	0.610	10.976
14.443	72753207	1.419	25.554
14.731	228078816	4.449	80.111
14.938	82999169	1.619	29.153

Area Percent Report -- Sorted by Signal

Information from Data File:
File : C:\HPCHEM\1\DATA\SH2.D
Operator : lwinsor
Acquired : 16 Sep 103 9:46 am using AcqMethod LWINSOR
Sample Name: Sarah H 1 (Aceto)
Misc Info :
Vial Number: 1
CurrentMeth: C:\HPCHEM\1\METHODS\LWINSOR.M

Retention Time	Area	Area %	Ratio %
15.125	25642084	0.500	9.007
15.383	87883630	1.714	30.868
15.712	148537156	2.898	52.173
15.823	57225034	1.116	20.100
15.978	37919253	0.740	13.319
16.130	283954173	5.539	99.737
16.567	214223888	4.179	75.245
16.938	123461003	2.408	43.365
17.047	54896786	1.071	19.282
17.462	48489334	0.946	17.032
17.937	166245818	3.243	58.393
18.252	31034607	0.605	10.901
18.503	31840069	0.621	11.184
19.030	31972055	0.624	11.230
19.439	23585446	0.460	8.284
19.606	72963068	1.423	25.628
20.312	12324524	0.240	4.329
21.025	76773995	1.498	26.966
21.456	15300028	0.298	5.374
21.697	37487805	0.731	13.167
23.422	28976083	0.565	10.178
25.964	13675740	0.267	4.804
27.435	36384624	0.710	12.780
30.823	56175471	1.096	19.731

File : C:\HPCHEM\1\DATA\SH2.D
Operator : lwinsor
Acquired : 16 Sep 103 9:46 am using AcqMethod LWINSOR
Instrument : 5970 - In
Sample Name: Sarah H 1 (Aceto)
Misc Info :
Vial Number: 1

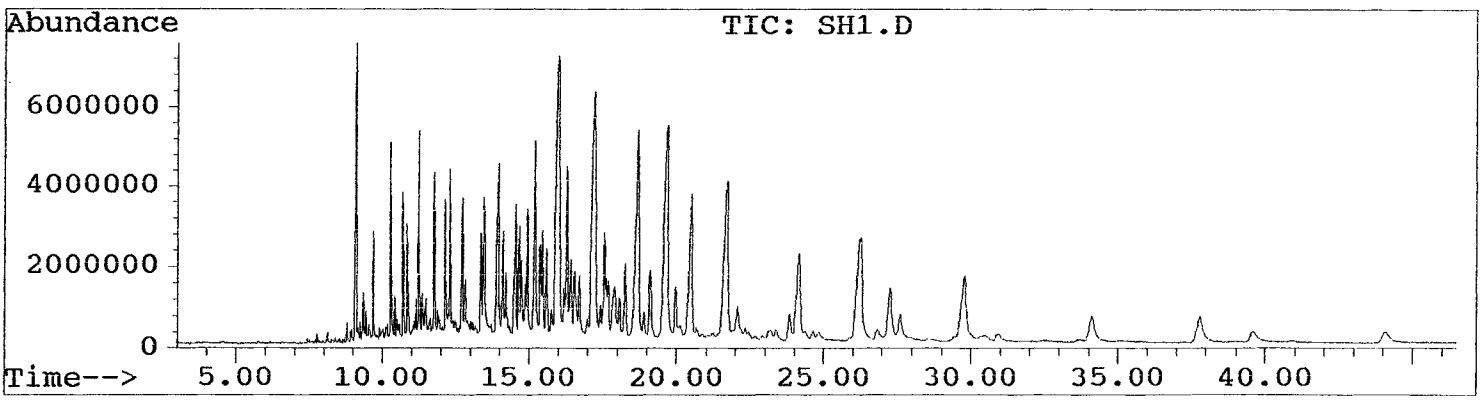


SP = sanitary product

Area Percent Report -- Sorted by Signal

Information from Data File:
File : C:\HPCHEM\1\DATA\SH1.D
Operator : lwinsor
Acquired : 16 Sep 103 8:40 am using AcqMethod LWINSOR
Sample Name: Sarah H 1 (hex)
Misc Info :
Vial Number: 1
CurrentMeth: C:\HPCHEM\1\METHODS\LWINSOR.M

(hex-tube)
10X concentrated un-used SP



Retention Time	Area	Area %	Ratio %
Total Ion Chromatogram			
7.786	2644222	0.029	0.406
8.148	5936108	0.066	0.911
8.804	10365142	0.115	1.590
8.955	12312462	0.136	1.889
9.108	182949992	2.025	28.073
9.186	8260904	0.091	1.268
9.254	7780817	0.086	1.194
9.353	35239048	0.390	5.407
9.438	19443252	0.215	2.983
9.539	20841736	0.231	3.198
9.694	53166736	0.589	8.158
9.908	9405683	0.104	1.443
10.033	18834793	0.209	2.890
10.125	14466761	0.160	2.220
10.168	10296535	0.114	1.580
10.278	91994821	1.018	14.116
10.354	9017185	0.100	1.384
10.412	19558935	0.217	3.001

Area Percent Report -- Sorted by Signal

Information from Data File:
File : C:\HPCHEM\1\DATA\SH1.D
Operator : lwinsor
Acquired : 16 Sep 103 8:40 am using AcqMethod LWINSOR
Sample Name: Sarah H 1 (hex)
Misc Info :
Vial Number: 1
CurrentMeth: C:\HPCHEM\1\METHODS\LWINSOR.M

Retention Time	Area	Area %	Ratio %
10.485	16340727	0.181	2.507
10.537	25523226	0.283	3.916
10.691	82471897	0.913	12.655
10.844	78698420	0.871	12.076
11.146	62045996	0.687	9.521
11.239	122745497	1.359	18.835
11.348	21903957	0.242	3.361
11.382	14273808	0.158	2.190
11.467	47779092	0.529	7.331
11.613	25456942	0.282	3.906
11.757	109848987	1.216	16.856
11.871	49864650	0.552	7.652
12.138	103720007	1.148	15.915
12.253	16620799	0.184	2.550
12.307	112788845	1.249	17.307
12.492	26891726	0.298	4.126
12.725	109116752	1.208	16.743
12.827	57071707	0.632	8.757
12.915	29619016	0.328	4.545
13.042	16150884	0.179	2.478
13.102	13811448	0.153	2.119
13.375	86154886	0.954	13.220
13.468	171397751	1.898	26.300
13.957	209922952	2.324	32.212
14.129	90209783	0.999	13.842
14.231	64119459	0.710	9.839
14.559	138475627	1.533	21.248
14.673	83765499	0.927	12.853
14.743	66243918	0.733	10.165
14.868	49491126	0.548	7.594
14.939	122082378	1.352	18.733
15.202	225139370	2.493	34.547
15.375	85255052	0.944	13.082
15.452	96074389	1.064	14.742
15.591	96260598	1.066	14.771

Area Percent Report -- Sorted by Signal

Information from Data File:
File : C:\HPCHEM\1\DATA\SH1.D
Operator : lwinsor
Acquired : 16 Sep 103 8:40 am using AcqMethod LWINSOR
Sample Name: Sarah H 1 (hex)
Misc Info :
Vial Number: 1
CurrentMeth: C:\HPCHEM\1\METHODS\LWINSOR.M

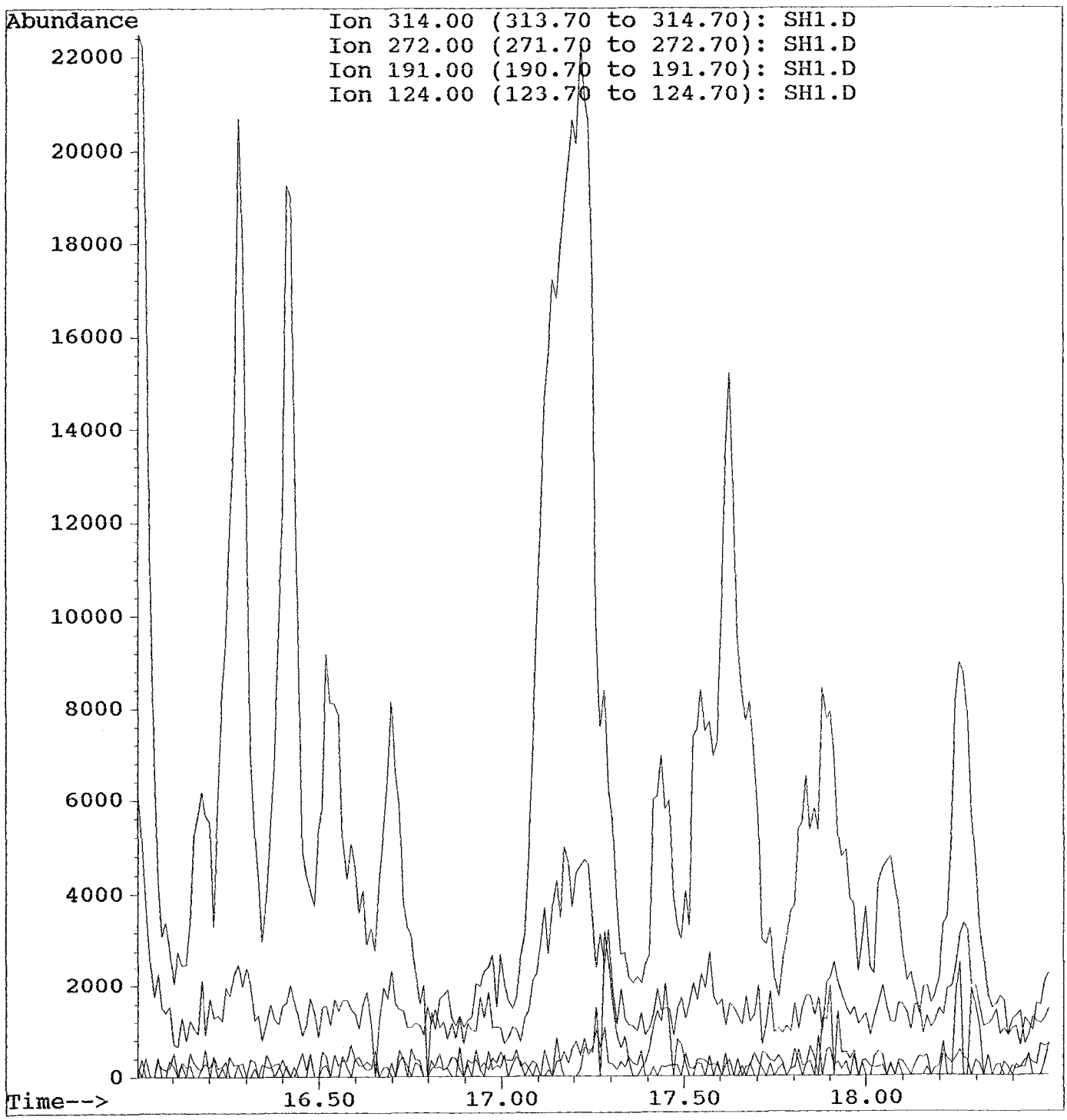
Retention Time	Area	Area %	Ratio %
15.762	41350017	0.458	6.345
16.002	651696452	7.215	100.000
16.180	44043857	0.488	6.758
16.285	180778018	2.001	27.740
16.418	102240708	1.132	15.688
16.541	105578999	1.169	16.201
16.705	80378499	0.890	12.334
16.972	31650487	0.350	4.857
17.233	597755859	6.618	91.723
17.441	37805497	0.419	5.801
17.556	109755523	1.215	16.842
17.622	46090056	0.510	7.072
17.682	57779796	0.640	8.866
17.900	122650227	1.358	18.820
18.064	61206213	0.678	9.392
18.264	96665860	1.070	14.833
18.704	427347237	4.731	65.575
18.902	39341511	0.436	6.037
19.112	111702282	1.237	17.140
19.700	547946938	6.066	84.080
20.000	76333021	0.845	11.713
20.148	27539624	0.305	4.226
20.520	276177515	3.058	42.378
20.696	23457789	0.260	3.599
21.716	404821353	4.482	62.118
22.075	93606870	1.036	14.364
22.329	19382879	0.215	2.974
23.172	29388268	0.325	4.510
23.382	20203287	0.224	3.100
23.845	46089445	0.510	7.072
24.167	214883255	2.379	32.973
24.378	19089249	0.211	2.929
24.658	17403218	0.193	2.670
24.850	18206705	0.202	2.794
26.239	192493041	2.131	29.537

Area Percent Report -- Sorted by Signal

Information from Data File:
File : C:\HPCHEM\1\DATA\SH1.D
Operator : lwinsor
Acquired : 16 Sep 103 8:40 am using AcqMethod LWINSOR
Sample Name: Sarah H 1 (hex)
Misc Info :
Vial Number: 1
CurrentMeth: C:\HPCHEM\1\METHODS\LWINSOR.M

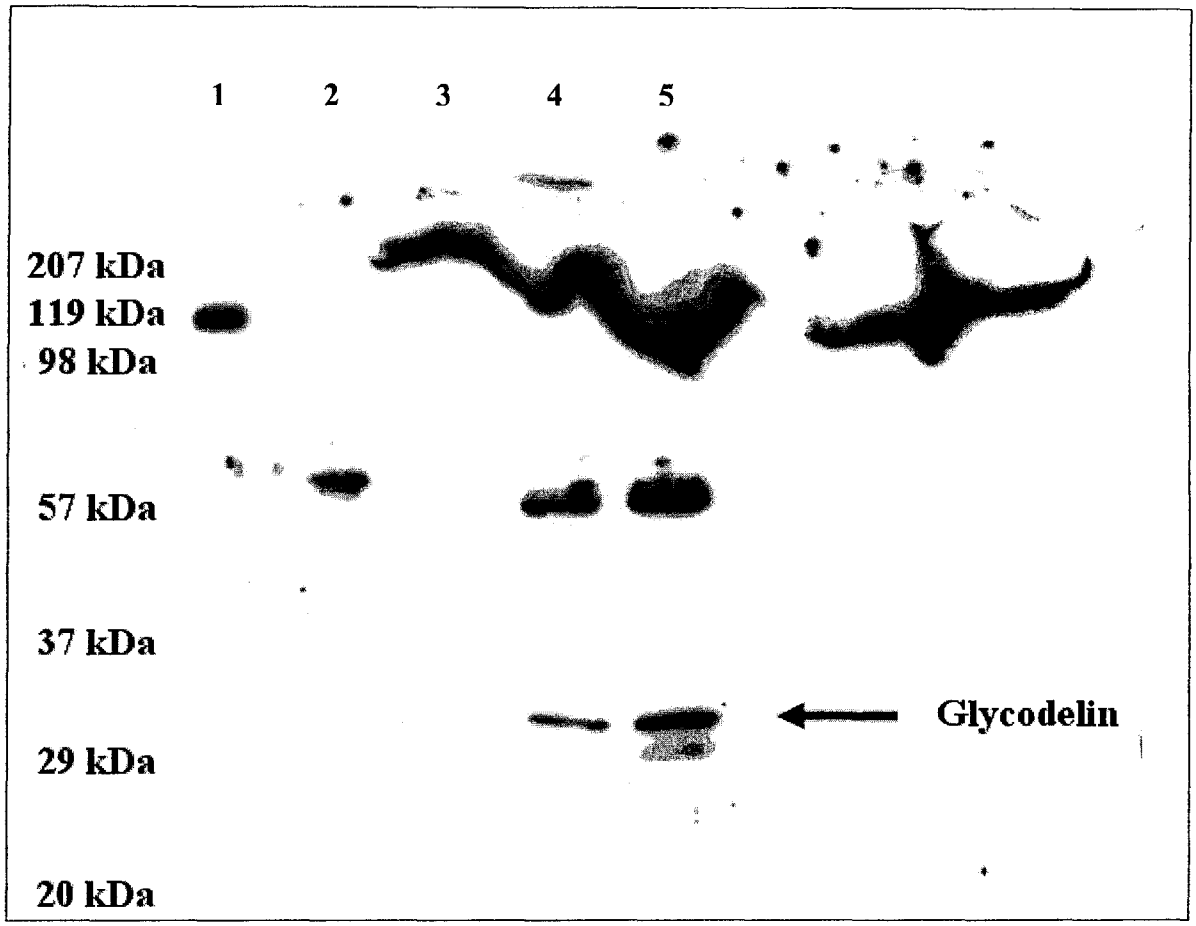
Retention Time	Area	Area %	Ratio %
26.276	144909787	1.604	22.236
26.846	25125547	0.278	3.855
27.279	116205583	1.287	17.831
27.619	50415251	0.558	7.736
29.790	211024059	2.336	32.381
30.909	17844303	0.198	2.738
34.116	71466437	0.791	10.966
37.804	92192175	1.021	14.146
39.594	30246983	0.335	4.641
44.072	40531454	0.449	6.219

File : C:\HPCHEM\1\DATA\SH1.D
Operator : lwinsor
Acquired : 16 Sep 103 8:40 am using AcqMethod LWINSOR
Instrument : 5970 - In
Sample Name: Sarah H 1 (hex)
Misc Info :
Vial Number: 1



APPENDIX C

Western Blot of Glycodelin from Two Extracted Tampon Samples. Two tampons collected during menses were extracted and analyzed for glycodelin. Protein separation and transfer was carried out using SDS-PAGE and western blot. Lane 1 contains the reference protein ladder with the approximate molecular weights shown to the left; lanes 2 and 3 contain unfiltered residue from two tampon extracts, A and B, respectively; lanes 4 and 5 are concentrated samples A and B, respectively, collected from the Centricon-Plus 20 protein filter. Lanes 4 and 5 show dark bands near 28kDa, the molecular weight of glycodelin.


1
2
3
4
5
207 kDa
119 kDa
98 kDa
57 kDa
37 kDa
29 kDa
20 kDa
Glycodelin