

# Spot ferritin assay for serum samples dried on filter paper<sup>1,2</sup>

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**ABSTRACT** A spot method was developed for analyzing ferritin from 20- $\mu$ L serum samples ( $n = 71$ ) that were spotted and dried on filter paper and stored frozen (2 d). Spot samples were thawed, incubated in a buffer containing cellulase, and centrifuged and the supernate assayed for ferritin by a commercial radioimmunoassay. The geometric means ( $\pm 1$  SD) for ferritin analyzed with the spot and traditional methods were 49.4 (range: 14.9–164.0) and 47.5 (range: 14.4–156.0)  $\mu$ g/L, respectively. The two methods correlated strongly ( $r = 0.98$ ,  $P = 0.0001$ ). Storage of spot samples ( $n = 31$ ) under various conditions (at room temperature, refrigerated, or frozen for 2 wk, or at room temperature for 4 wk) in airtight bags before analysis yielded ferritin values that were not significantly different from those obtained by the traditional method. Ferritin values from spotted samples stored at room temperature for 4 wk before being analyzed were only 2.2  $\mu$ g/L higher than those from samples analyzed by the traditional method. With iron depletion defined as a serum ferritin concentration  $< 15$   $\mu$ g/L, this method corresponded absolutely with the traditional method in classifying individuals as iron sufficient or deficient. Thus, the spot ferritin method (with samples stored at room temperature for 4 wk) offers a reliable, accurate, and practical tool for iron status assessment in field studies. Capillary blood can be sampled to avoid the costs and concerns associated with venipuncture and spotted serum samples can be stored at room temperature for  $\geq 4$  wk, eliminating the need for freezing during storage and transportation. *Am J Clin Nutr* 1998;67:88–92.

**KEY WORDS** Iron status assessment, serum ferritin, filter paper method, population studies, spot ferritin method, assay

## INTRODUCTION

Iron deficiency anemia continues to be a major nutritional and public health problem, particularly in developing countries (1–3). Assessment of iron deficiency is often based on a battery of laboratory tests spanning various stages of iron deficiency (4). Serum ferritin is a sensitive indicator of decreased body iron stores and detects the first stage of iron deficiency (4–6). A serum ferritin concentration  $< 12$ – $15$   $\mu$ g/L usually reflects iron deficiency (5, 6). However, the feasibility of assessing serum ferritin in field studies, especially in developing countries, is often limited by a lack of on-site refrigeration or freezing facilities and costs and concerns associated with blood sampling by venipuncture.

Several spot or dot methods using blood or serum samples dried on filter paper have been developed and validated to screen for antibody titers in surveys in various countries (7–10). A similar approach has been used for assessing nutrient status; for instance, a dried blood spot method was developed for measuring retinol-binding protein as an indicator of vitamin A status (11). Filter paper methods offer the advantages of ease in collection, storage, transport, and handling of samples in field studies. Furthermore, these methods may be suitable for storage of spotted samples at room temperature, eliminating the need for refrigerating or freezing samples before assay and making such tests available for use in remote areas in field surveys.

The purpose of the present study was to develop a spot method for measuring serum ferritin concentrations. The availability of such a method would facilitate the storage, transport, and handling of samples. Furthermore, samples of capillary blood could be taken by finger prick, reducing costs and avoiding concerns associated with venipuncture in some individuals or population groups. Specific aims of this study were 1) to develop a method for measuring ferritin in serum samples blotted and air-dried on filter paper and 2) to determine the stability of the spotted serum samples stored under various conditions. Ferritin values obtained by the spot method from serum samples spotted on filter paper were compared with values obtained in the traditional manner from serum samples stored frozen in microfuge tubes until analysis by radioimmunoassay.

## MATERIALS AND METHODS

Serum samples obtained from 71 subjects (ranging from infants to adults) who had participated in previous studies or were participating in ongoing studies of iron status assessment were spotted in duplicate (20  $\mu$ L each) on filter paper (Whatman no. 1; Whatman Inc, Clifton, NJ) and stored frozen ( $-20^{\circ}\text{C}$ ) for 2 d. Informed consent was obtained from subjects by following protocols approved by the Institutional Review Board of the University of California, Davis, and the Pennsylvania State University. Serum from these 71

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Received January 29, 1997.

Accepted for publication August 25, 1997.

subjects was also portioned into microfuge tubes and stored frozen ( $-20^{\circ}\text{C}$ ) until assayed with a commercially available radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles).

### Spot ferritin assay

On the day of the assay, spot samples were brought to room temperature, cut around the spot circumference, placed in glass vials with 750  $\mu\text{L}$  ammonium acetate buffer (pH 5.5) containing 25 mg cellulase (obtained from *Penicillium funiculosum*; Sigma Chemical Co, St Louis), incubated in a water bath at  $\geq 37^{\circ}\text{C}$  for 4 h, transferred to microfuge tubes (pH 8), and refrigerated until the next day. The samples were then centrifuged at  $481 \times g$  (1500 rpm) for 5 min ( $25^{\circ}\text{C}$ ) and ferritin was assayed in 200  $\mu\text{L}$  of the supernate by radioimmunoassay.

### Optimal storage conditions for spot ferritin samples

Serum samples from 31 subjects, ranging in ferritin concentration from 5 to 600  $\mu\text{g/L}$ , were spotted on filter paper (Whatman no. 1) and stored under five different conditions in airtight plastic bags (Ziploc; DowBrands, Indianapolis). Two 20- $\mu\text{L}$  spotted serum samples from each subject were stored under each of the following conditions: frozen at  $-20^{\circ}\text{C}$  for 2 d, frozen at  $-20^{\circ}\text{C}$  for 2 wk, refrigerated ( $4^{\circ}\text{C}$ ) for 2 wk, stored at room temperature ( $25^{\circ}\text{C}$ ) for 2 wk, and stored at room temperature ( $25^{\circ}\text{C}$ ) for 4 wk. After storage, ferritin concentrations were analyzed after the samples were brought to room temperature by using the spot ferritin assay described above.

### Reliability of the spot ferritin assay

The reliability of the spot ferritin assay was determined by computing the CV between duplicate samples. The batch-to-batch variation for the traditional method and the spot ferritin method (on samples spotted and frozen for 2 d or spotted and stored at room temperature for 4 wk) was determined by analyzing 10 samples in duplicate by these methods in five different runs.

### Statistical analyses

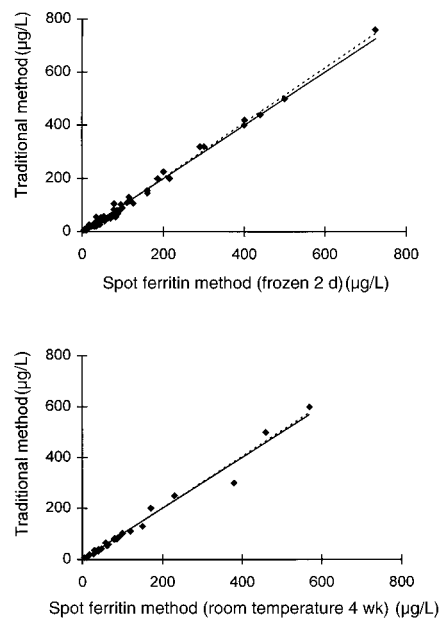
All analyses were done with SAS on the university mainframe computer (12). Data were logarithmically transformed because serum ferritin concentrations are consistent with a log-normal distribution. A paired  $t$  test was used to test the difference between ferritin measured by the traditional method and that measured by the spot ferritin method. Pearson correlation analysis was carried out to examine the relation between the traditional method and the spot ferritin method under various storage conditions. The relation between the two methods was also evaluated with linear regression analysis. The line of regression was tested against the line of unity (slope = 1, intercept = 0) by simultaneously testing the hypotheses of slope = 1 and intercept = 0. The mean differences in ferritin values obtained with the traditional and spot ferritin methods on serum samples spotted and stored under various conditions were examined by analysis of variance (ANOVA) with storage condition and sample identification as main effects and use of Tukey's method for post hoc analysis (12). The accuracy of the spot ferritin method in classifying subjects as iron sufficient or iron deficient (based on a definition of iron depletion as serum ferritin  $< 15 \mu\text{g/L}$ ) was compared with that of the traditional method. Furthermore, the approach of Bland and Altman (13) was followed to compare the spot ferritin method with spotted serum samples stored at room

temperature for 4 wk before analysis with the traditional method by plotting the difference in ferritin values against the mean ferritin value by the two methods for each sample ( $n = 31$ ).

## RESULTS

The geometric means and ranges for serum ferritin ( $n = 71$ ) in traditionally handled frozen samples and in spotted samples stored frozen for 2 d before analysis were 47.5 (14.4–156.0) and 49.4 (14.9–164.0)  $\mu\text{g/L}$ , respectively. These mean ferritin values obtained by the two methods were not significantly different ( $P > 0.10$ ) and as shown in **Figure 1**, the two methods were strongly correlated ( $n = 71$ ,  $r = 0.98$ ,  $P = 0.0001$ ). When tested against the line of unity (slope = 1, intercept = 0), the slope and intercept of this regression line were not different from 1 and 0, respectively ( $P > 0.10$ ).

The geometric means  $\pm 1$  SD for serum ferritin ( $n = 31$ ) measured by the traditional and spot ferritin methods after storage under various conditions are shown in **Table 1**. Serum ferritin values obtained with use of the spot ferritin assay irrespective of the condition under which the samples were stored for up to 4 wk were not significantly different from those obtained with use of the traditional method. The traditional method for serum ferritin correlated strongly with the spot ferritin method for all storage conditions ( $r > 0.95$ ,  $P = 0.0001$ ; Table 1). Even when the spotted serum samples were kept at room temperature for 4 wk in airtight bags before being analyzed with the spot ferritin assay, the serum ferritin values obtained corre-



**FIGURE 1.** Relation between serum ferritin values from samples and measured by the traditional method and the spot ferritin method. Top panel: spotted samples analyzed with use of the spot ferritin method after being stored frozen for 2 d. The regression line (dashed line) is compared with the line of unity (solid line) with a slope of 1 and intercept of 0.  $\text{Log } y = 0.05 + 0.98 \times (\text{log } x)$  describes the regression equation between the two methods ( $R^2 = 0.97$ ). Bottom panel: spotted samples analyzed with use of the spot ferritin method after storage at room temperature for 4 wk. The regression line (dashed line) is compared with the line of unity (solid line) with a slope of 1 and intercept of 0.  $\text{Log } y = -0.03 + 1 \times (\text{log } x)$  describes the regression equation between the two methods ( $R^2 = 0.99$ ).

**TABLE 1**  
Serum ferritin concentrations measured by the traditional method and the spot ferritin method under various storage conditions

| Method                            | Value<br>$\mu\text{g/L}$       | Correlation with<br>traditional method: |
|-----------------------------------|--------------------------------|---|
|                                   |                                | $r$ ( $P$ )                             |
| Traditional method ( $n = 31$ )   | 55.0 (15.5–200.0) <sup>1</sup> | 1.0 (0.0000)                            |
| Spot ferritin method ( $n = 31$ ) |                                |   |
| Frozen 2 d                        | 57.5 (16.2–204.2)              | 0.99 (0.0001)                           |
| Frozen 2 wk                       | 57.5 (15.8–208.9)              | 0.99 (0.0001)                           |
| Refrigerated 2 wk                 | 51.3 (12.3–213.8)              | 0.96 (0.0001)                           |
| Room temperature 2 wk             | 51.3 (13.5–195.0)              | 0.98 (0.0001)                           |
| Room temperature 4 wk             | 58.9 (16.6–208.9)              | 0.99 (0.0001)                           |

<sup>1</sup> Geometric  $\bar{x}$ ; range in parentheses. Means are not significantly different from one another.

lated highly with those obtained with use of the traditional method ( $n = 31$ ,  $r = 0.99$ ,  $P = 0.0001$ ; Figure 1). Furthermore, the line of regression describing the relation between this spot ferritin (with storage of spotted samples at room temperature for 4 wk) method and the traditional method was not significantly different from the line of unity (slope = 1 and intercept = 0,  $P > 0.10$ ).

Because the usefulness of serum ferritin measurements in defining iron depletion lies on the lower end of the distribution, we examined the relation between the spot ferritin method and the traditional method in the range of 0–30  $\mu\text{g/L}$ . The spot ferritin and traditional methods remained highly correlated even in this low range:  $r = 0.91$  and  $P = 0.0001$  for spotted samples stored frozen for 2 d before analysis ( $n = 24$ ) and  $r = 0.93$  and  $P = 0.0001$  for spotted samples kept at room temperature for 4 wk before the spot ferritin assay ( $n = 10$ ).

The mean difference in ferritin value (traditional minus the spot ferritin method, with spotted samples kept at room temperature for 4 wk before analysis) was  $-2.2 \mu\text{g/L}$  for samples with ferritin concentrations ranging from 0 to 100  $\mu\text{g/L}$  (Figure 2). The partition of subjects as iron sufficient or iron deficient by the traditional and spot ferritin methods with use of various storage conditions was similar (Table 2). Furthermore, under all storage conditions the spot ferritin method accurately classified individuals as iron deficient or iron sufficient (Table 2). The sensitivity and specificity of the spot ferritin method under various storage conditions ranged from 80% to 100% and from 96% to 100%, respectively.

The CVs between duplicate measures with the traditional method, the spot ferritin method with samples frozen for 2 d, and the spot ferritin method with samples kept at room temperature for 4 wk were 2.5%, 3.0%, and 3.1%, respectively. The CVs for batch-to-batch variation with the traditional procedure and with the spot ferritin method with samples frozen for 2 d or kept at room temperature for 4 wk were 3.0%, 3.6%, and 5.0%, respectively.

## DISCUSSION

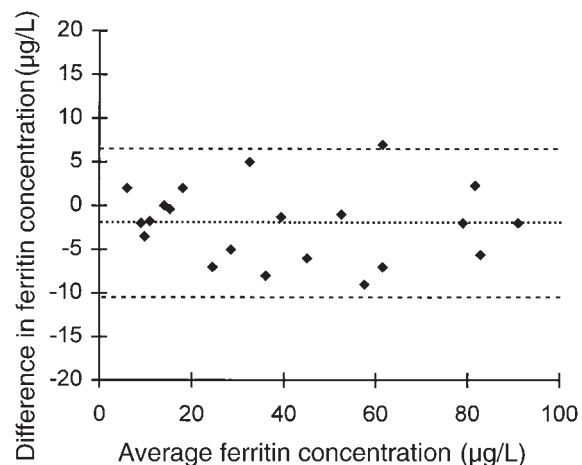
A method was developed for measuring ferritin from serum samples spotted on filter paper, air-dried, and stored under various conditions in airtight polyethylene bags. Ferritin was measured in the supernate after samples were incubated with a buffer containing cellulase to digest the filter paper. This use of the enzyme cellulase to break down the cellulose in filter paper before the radioimmunoassay assay is a unique approach. Pre-

liminary findings on a spot method for serum ferritin in which an elution approach was used have also been reported (14).

In practice, a whole-blood spot ferritin assay would be more convenient, eliminating the need for separation of serum before spotting the sample on filter paper. Preliminary results in our laboratory (N Ahluwalia, unpublished observations, 1996) and those reported by Flowers and Cook (14), however, indicate that whole-blood spot ferritin assays give inconsistently higher results than the traditional method. The whole-blood spot ferritin method was unsatisfactory because of the confounding effect of variable release of intracellular ferritin from red blood cells among subjects. Consequently, the spot ferritin assay is valid for use only with serum samples.

Ferritin values for spotted serum samples stored under various conditions (kept frozen for 2 d, kept refrigerated or frozen for 2 wk, or kept at room temperature for 2 or 4 wk) and analyzed by the spot ferritin method were not significantly different from those obtained for serum samples stored frozen and analyzed in the traditional manner. Values obtained with use of the spot ferritin method under various storage conditions correlated strongly with those obtained with use of the traditional method ( $r > 0.95$ ,  $P < 0.0002$ ) in the wide range of serum ferritin (5–800  $\mu\text{g/L}$ ) examined. Because the usefulness of serum ferritin in defining iron depletion lies at the lower end of this concentration range, the relation between the traditional and the spot ferritin methods was explored for ferritin concentrations in the range of 0–30  $\mu\text{g/L}$ . In this range, the spot ferritin method with spotted samples stored frozen for 2 d or kept at room temperature for 4 wk remained strongly correlated ( $r = 0.91$  and  $0.93$ , respectively) with the traditional method. The mean difference and the 95% prediction interval for the difference in ferritin values by the traditional and spot ferritin (with samples stored at room temperature for 4 wk) methods in the low ferritin range (0–30  $\mu\text{g/L}$ ) were  $-2.1 \mu\text{g/L}$  and  $-8.2$  to  $4.0 \mu\text{g/L}$ , respectively, with use of the approach of Bland and Altman (13).

Thus, on average, the spot ferritin method (with samples stored at room temperature for 4 wk) gave ferritin values that



**FIGURE 2.** Relation between the difference in serum ferritin values determined by the traditional and spot ferritin methods (with spotted samples kept at room temperature for 4 wk) against the mean serum ferritin concentration by the two methods for samples with concentrations in the 0–100  $\mu\text{g/L}$  range. The dotted and dashed lines represent the mean and mean  $\pm 2$  SD, respectively, of the difference in ferritin values by the two methods.

TABLE 2

Number of subjects classified as iron sufficient or iron deficient by the traditional compared with the spot ferritin method

| Method                                     | Iron deficient<br>(serum ferritin < 15 µg/L) | Iron sufficient<br>(serum ferritin ≥ 15 µg/L) | Sensitivity <sup>1</sup> | Specificity <sup>2</sup> |
|--|--|---|--------------------------|--------------------------|
| Traditional method (n = 71)                | 13   | 58  | 13/13 (100)              | 58/58 (100)              |
| Spot ferritin method (frozen 2 d) (n = 71) | 12   | 59  | 12/13 (92)               | 58/58 (100)              |
| Traditional method (n = 31)                | 5  | 26  | 5/5 (100)                | 26/26 (100)              |
| Spot-ferritin method (n = 31)              |  |   |                          |                          |
| Frozen 2 d                                 | 4  | 27  | 4/5 (80)                 | 26/26 (100)              |
| Frozen 2 wk                                | 5  | 26  | 5/5 (100)                | 26/26 (100)              |
| Refrigerated 2 wk                          | 6  | 25  | 5/5 (100)                | 25/26 (96)               |
| Room temperature 2 wk                      | 6  | 25  | 5/5 (100)                | 25/26 (96)               |
| Room temperature 4 wk                      | 5  | 26  | 5/5 (100)                | 26/26 (100)              |

<sup>1</sup> Proportion of subjects classified as iron deficient by the traditional method who were classified correctly by the method under study. Percentage in parentheses.


<sup>2</sup> Proportion of subjects classified as iron sufficient by the traditional method who were classified correctly by the method under study. Percentage in parentheses.

were 2.2 µg/L higher than those obtained with use of the traditional method. The effect of this difference on the classification of subjects as iron deficient or iron sufficient could be of greater importance. However, with use of serum ferritin < 15 µg/L as the definition of iron depletion, the classification of subjects as iron deficient or iron sufficient by the spot ferritin method under various storage conditions was similar to that by the traditional method.

Comparison of the performance of a test in classifying individuals with that of a standard test may be a better reflection of its accuracy. Under various storage conditions the spot ferritin method was highly sensitive and specific in relation to the traditional assay for classifying subjects as iron sufficient or iron deficient. Specifically, all subjects with low serum ferritin concentrations (< 15 µg/L) by the traditional method (n = 5) also had low ferritin concentrations (< 15 µg/L) by the spot ferritin method with spotted serum samples kept at room temperature for 4 wk (100% sensitivity). Moreover, all subjects classified as being iron sufficient by the traditional ferritin assay also had serum ferritin ≥ 15 µg/L with the spot ferritin method with samples kept at room temperature for 4 wk (100% specificity). Thus, the spot ferritin method with spotted samples kept at room temperature for 4 wk was highly accurate and corresponded absolutely with the traditional method in classifying individuals as iron sufficient or iron deficient (sensitivity and specificity of 100%).

Furthermore, the spot ferritin method is reliable: the CVs for duplicate samples and for batches were similar to those obtained after traditional storage and analysis and were < 5%. The fact that samples can be stored on filter paper at room temperature for ≤ 4 wk before being assayed may make this assay useful in remote areas of developing countries where access to refrigerators or freezers is limited. In such circumstances, the spot ferritin method may be of particular interest because serum samples could be obtained, spotted on filter paper, air-dried, and stored in airtight polyethylene bags for ≤ 4 wk at room temperature before being assayed at a central or reference laboratory.

In summary, we developed a spot ferritin method that is both accurate and reliable. The small sample volume needed for the assay means that blood can be collected by capillary sampling (15, 16). If a microcentrifuge is not available, serum can be spotted from capillary tubes allowed to stand vertically at room tem-

perature. Thus, the availability of this spot method may facilitate the use of serum ferritin measurement in field assessment of iron status. 

We thank Janet Peerson, Department of Nutrition, Program in International Nutrition, University of California, Davis, for her guidance in statistical analyses.

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