

James M. Hempe^{1,3}
Randall D. Craver^{1,2,3}

¹Department of Pediatrics

²Department of Pathology
Louisiana State University
School of Medicine, New
Orleans, LA, USA

³Children's Hospital, New
Orleans, LA, USA

Separation of hemoglobin variants with similar charge by capillary isoelectric focusing: Value of isoelectric point for identification of common and uncommon hemoglobin variants

Clinical assays for the primary evaluation of congenital hemoglobin (Hb) disorders must detect and identify a variety of Hb variants. We analyzed hemolysates containing Hb variants with similar charge to evaluate the diagnostic sensitivity and specificity of automated capillary isoelectric focusing (CIEF). Peak separation was observed for each variant in samples containing Hb S, D, and G. The calculated isoelectric points (*pI*) of these variants were significantly different such that each could be identified in a single run with *pI* as the sole criterion of identification. The *pI* of Hb C was significantly different from that of Hb E, C-Harlem, and O-Arab. Hb E, C-Harlem, and O-Arab had similar *pI* and were not readily differentiated. Hb Köln, M-Saskatoon, Aida, and S/Aida hybrid were readily separated from common Hb variants and detected by CIEF. We conclude that CIEF exhibits both diagnostic sensitivity and specificity, and that *pI* is an objective and specific criterion of Hb variant identification.

Keywords: Hemoglobin variant / Hemoglobinopathy / Congenital hemoglobin disorder / Capillary electrophoresis / Capillary isoelectric focusing / Isoelectric focusing / Clinical diagnosis EL 3820

1 Introduction

Laboratory diagnosis of congenital hemoglobinopathies and thalassemias requires both identification of abnormal hemoglobin (Hb) variants and quantification of major and minor variants over a wide range of concentrations [1–3]. Most clinical laboratories use a progression of analytical methods to evaluate these disorders, beginning with alkaline electrophoresis and followed for more precise identification as needed by acid electrophoresis, gel isoelectric focusing, and/or HPLC. These assays are commonly supplemented by minicolumn ion exchange chromatography and/or alkali denaturation if quantification of the minor Hb variants A₂ and F is needed. Quantification of Hb A₂ and F is often used to help diagnose thalassemia syndromes, or to longitudinally follow changes in Hb F concentration in patients with sickle cell disease that are treated with hydroxyurea.

The combination of alkaline and acid electrophoresis is used in most laboratories as primary and secondary analytical methods for structural Hb variant identification

because the assays are well characterized, easy to perform, and relatively inexpensive. Both methods must be used, however, because the separation of Hb variants with similar charge is relatively poor with either method alone. For example, Hb S cannot be differentiated from Hb D or G, and Hb C cannot be differentiated from Hb E, C-Harlem, or O-Arab by alkaline electrophoresis alone [2]. This lack of diagnostic specificity means that laboratories using alkaline electrophoresis as their primary assay must perform a secondary assay, usually by acid electrophoresis, to positively identify Hb S, C, and E, the three most common abnormal Hb variants in the world.

The need for multiple assays increases turn-around-time for results and also adds to test costs for labor, supplies, and administrative management of multiple procedures. Also, because both alkaline and acid electrophoresis are labor-intensive methods, they are not amenable to automation of either sample handling or postanalytical data processing. Automation of clinical assays not only decreases labor costs, but also makes possible the use of computerized diagnostic algorithms for objective interpretation of analytical results. The use of peak elution time as a criterion of Hb variant identification, for example, is one reason for the commercialization and increasing use of HPLC for primary hemoglobinopathy assessment [4–8]. Some HPLC methods, however, require separate programs to identify and quantify both major (e.g., Hb A, S, E, and C) and minor (e.g., Hb A₂ and F) variants. Also, capital equipment and reagent costs are relatively high for HPLC compared to conventional electrophoresis meth-

Correspondence: Dr. J. M. Hempe, Department of Pediatrics, LSU School of Medicine, 1542 Tulane Avenue, New Orleans, LA 70112, USA
E-mail: jhempe@lsu.mc.edu
Fax: +504-733-4036

Abbreviations: CAP, College of American Pathologists; Hb, hemoglobin; RBC, red blood cells

ods, although the cost increase is somewhat offset by the lower labor costs afforded by automation.

We have previously reported that computer-assisted capillary isoelectric focusing (CIEF) is an excellent method for the clinical assessment of structural hemoglobinopathies and thalassemias because it is an automated, high resolution, and microanalytical (therefore low use of consumables) assay with good precision for simultaneous quantification of both major and minor Hb variants [9–12]. The experiments described in this report highlight the use of CIEF as a primary analytical method for the specific identification of common and uncommon structural Hb variants. We emphasize the value of isoelectric point (*pI*) as a reproducible, objective, and specific criterion of Hb variant identification for automated post-analytical data processing and results interpretation.

2 Materials and methods

2.1 Samples

Blood was obtained from Children's Hospital Laboratory in accordance with the ethical guidelines of that institution from patients with normal Hb phenotype, sickle cell trait, Hb E trait, Hb O-Arab trait, Hb S trait/Hb Aida trait, Hb S/C-Harlem disease, or Hb S/C disease transfused with normal blood. The sample from the patient with Hb S trait/Hb Aida trait was sent to a commercial reference laboratory (Mayo Medical Laboratories, Rochester, MN) to verify the identify of the Hb variants. Proficiency testing samples, from the College of American Pathologists (CAP; Northfield, IL), containing unusual Hb variants (Hb Köln trait and Hb M-Saskatoon trait) were also analyzed, as were lyophilized commercial controls (Isolab, Akron, OH) containing Hb A and either Hb D-Punjab or Hb G-Philadelphia. Hemolysates were prepared from blood by mixing 10 μ L of packed red blood cells (RBC) with 200 μ L of hemolyzing reagent (10 mmol/L KCN, 5 mmol/L EDTA) as previously described [13]. Lyophilized controls were reconstituted with hemolyzing reagent. Where necessary, hemolysates were stored at -70°C prior to analysis (storage at -20°C is not suitable). In one study, twenty-four samples were prepared by mixing variable amounts of hemolysate containing Hb S, D, or G ($n = 11$ each). Different mixture ratios were used to produce samples containing two abnormal variants (*i.e.*, SD, SG, or DG) at different concentrations to preclude identification based on proportions or electropherogram similarity. The samples in this study were randomly analyzed over a 5-day period. In a second study, hemolysate from a patient with Hb S/C disease transfused with normal blood (containing Hb A, S, and C) was mixed with hemolysate containing either Hb E, O-Arab, or C-Harlem ($n = 10$ each) to assess the

separation of these more positively charged Hb variants by CIEF. All of the samples in this study were randomly assayed in a single analytical run.

2.2 Capillary electrophoresis

CIEF (pH 6–8/3–10, 10:1) was conducted using a P/ACE 2200 capillary electrophoresis system and System Gold software (Beckman Instruments, Fullerton, CA) as described elsewhere [12, 13]. The proportions of different Hb variants present in normal and abnormal controls were compared to expected values to verify assay performance with each analytical run. Quantification was based on the integration of peak area absorbance at 415 nm and expressed as a percent of total Hb.

2.3 Estimation of *pI* with external standards

In each study, *pI* was estimated using a control hemolysate stored at -70°C as an external standard. For each analytical run, the migration times and *pI* of Hb variants present in the control (Hb A₂, S, F, A, and A_{1c} using *pI* of 7.412, 7.210, 7.060, 6.973, and 6.935, respectively) were entered into the System Gold software. Linear regression analysis was used to calculate the *pI* of Hb variants in subsequent unknown samples based on corrected migration times using Hb A as a reference peak as previously described [13]. In each study, differences between estimated *pI* for different Hb variants were determined by one-way ANOVA (SAS Institute, Cary, NC). *Post hoc* comparison of treatment means was by least-squares analysis with significant differences between treatments determined at $p < 0.05$.

3 Results

3.1 Identification of common Hb variants by CIEF

Figure 1 shows Hb variant separation by CIEF in samples containing Hb A and Hb variants S and D, S and G, or D and G. Despite the small differences in net surface charge between Hb S, D, and G, each Hb variant was separated from each of the other abnormal variants. Near-baseline resolution was observed between Hb S and G while Hb D and G were separated only at the peak. Some peak separation was apparent in all three samples analyzed containing Hb D and G, even when the D:G ratio was disproportionate (2:1 or 1:2). Identification of α -variants like Hb G-Philadelphia is facilitated by the presence of the α -variant of Hb A₂ (also called Hb $\alpha\text{A}_2'$ or G₂).

Figure 2 shows Hb variant separation by CIEF in samples containing hemolysate from the patient with Hb S/C dis-

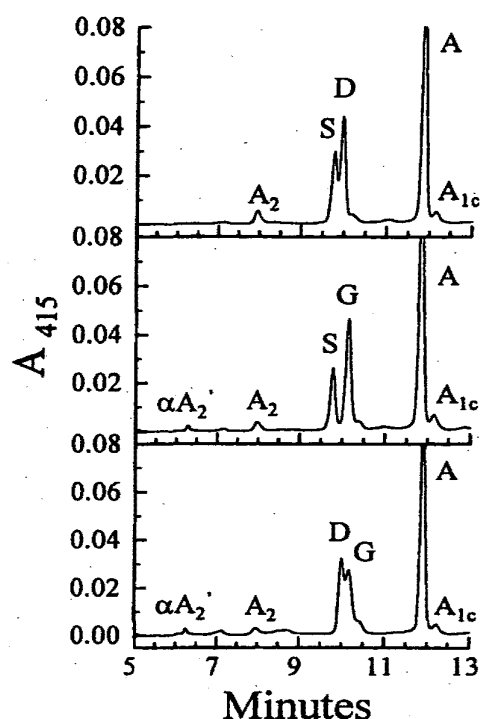


Figure 1. CIEF of Hb S, D, and G. Hemolysates containing Hb A and either Hb S, D, or G were mixed and analyzed. Peaks representing each of these common Hb variants were identifiable regardless of the combination of Hb variants present in the sample.

ease transfused with normal blood (containing Hb variants A, S, and C) mixed with hemolysate containing either Hb E, O-Arab, or C-Harlem. In each case, Hb C was separated from the other abnormal variant in the sample with baseline or near-baseline resolution. The Hb variants E, O-Arab, and C-Harlem, in contrast, all migrated in approximately the same positions. Neither peak separation nor abnormal peak shape was observed in samples containing mixtures of these hemolysates, *i.e.*, containing Hb E and C-Harlem, C-Harlem and O-Arab, or E and O-Arab (data not shown).

3.2 Precision of estimated pI for Hb variant identification

Table 1 shows the precision of pI estimates using Hb variants in the abnormal control as external standards for each analytical run. The pI estimates for Hb S, D, and G were all significantly different from each other and there was no overlap in the observed ranges. The calculated pI of Hb S differed from that of the Hb D and G by 0.025 and 0.042 pH units, respectively. The calculated pI of Hb D and G differed by 0.017 pH units. In conducting this study

with randomized and blinded samples, the medical technologist operating the instrument correctly identified all 33 abnormal Hb variants present in 24 samples based on pI.

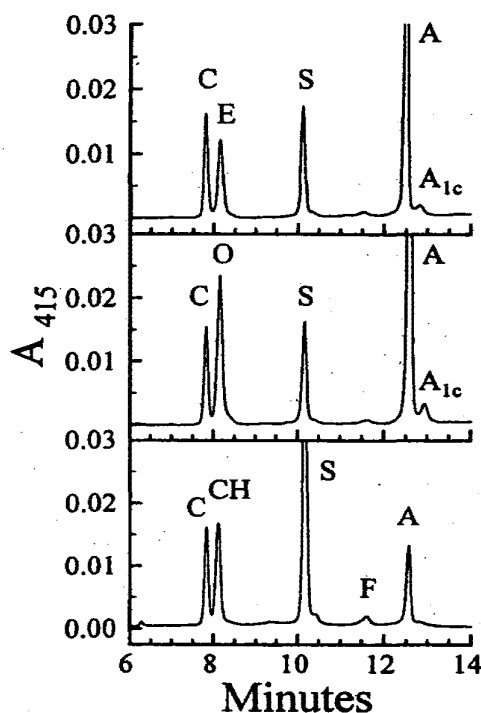


Figure 2. CIEF of Hb C, E, O-Arab, and C-Harlem. Hemolysate prepared from blood collected from a transfused patient with Hb S/C disease (containing Hb A, S, and C) was mixed with hemolysate containing Hb A and either Hb E, O-Arab, or C-Harlem prior to analysis by CIEF. Hb C was readily separated from the other abnormal Hb variants. Peak migration for Hb E, O-Arab, and C-Harlem was similar.

Table 1. Precision of pI estimates for common Hb variants with similar charge

Hb variant	pI mean (SD)	95% Tolerance interval mean $\pm$ 2 SE	Observed range
Experiment 1 (n = 11 each)			
S	7.209 (0.004) ^a	7.206–7.211	7.202–7.215
D	7.183 (0.003) ^b	7.181–7.185	7.178–7.190
G	7.166 (0.004) ^c	7.164–7.169	7.162–7.174
Experiment 2 (n = 10 each)			
C	7.440 (0.003) ^a	7.439–7.442	7.434–7.447
C-Harlem	7.410 (0.002) ^b	7.408–7.411	7.406–7.413
O-Arab	7.408 (0.002) ^{b,c}	7.407–7.410	7.406–7.412
E	7.406 (0.002) ^c	7.405–7.407	7.404–7.410

a) b) c) Values within an experiment having different superscripts are significantly different ($p < 0.05$).

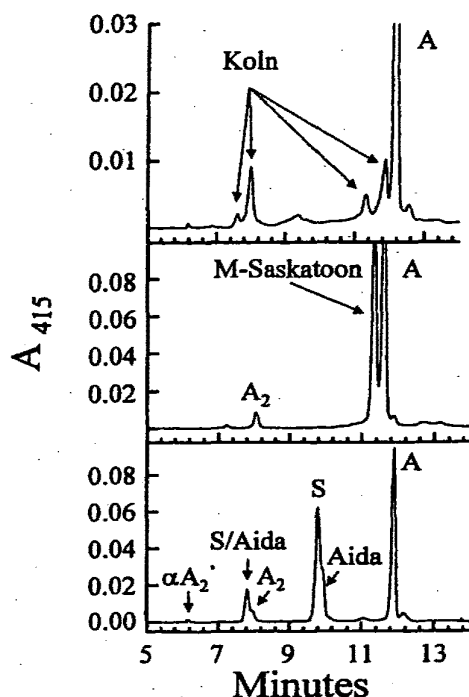


Figure 3. CIEF of Hb Köln, M-Saskatoon, Aida, and S/Aida hybrid. Analysis of hemolysates containing these rare Hb variants demonstrates the excellent separation and diagnostic sensitivity of CIEF for unusual Hb variants with *pI* similar to that of more common Hb variants.

In contrast, the estimated *pI* for Hb C was significantly higher than that of Hb C-Harlem, O-Arab, or E. The estimated *pI*s for Hb C-Harlem and E were also significantly different, but overlap in the observed *pI* ranges indicates lack of specificity for this identification based on *pI* alone. The *pI* for Hb C-Harlem and E were not significantly different from that of Hb O-Arab.

3.3 Identification of uncommon Hb variants by CIEF

Electropherograms from three unusual Hb variants analyzed in our laboratory are shown in Fig. 3 to further demonstrate the value of CIEF for primary Hb variant analysis. Hb Köln is an unstable Hb variant that arises from a mutation in the β -globin gene (98 β -Val $\rightarrow$ Met) and is typically found in individuals of European ancestry [1–3]. In this example from a patient with Hb Köln trait, Hb A was evident, as were four peaks (*pI* 7.428, 7.407, 6.993, 7.056) attributable to Hb Köln, presumably related to the loss of β globin heme. Hb A₂ was not measurable because of comigration with the *pI* 7.407 Hb Köln peak. Hb M-Saskatoon results from a mutation in the β -globin gene (63 β -His $\rightarrow$ Tyr) that produces an abnormal variant (*pI* 7.003)

with high oxygen affinity and a surface charge between that of Hb F and A. Only 12% of participating laboratories correctly identified Hb M-Saskatoon as an Hb M variant in the CAP proficiency survey from which this sample was obtained (HG-B, 1997). The presence of Hb M-Saskatoon was detected by only 8% of laboratories using either alkaline electrophoresis alone or the combination of alkaline and acid electrophoresis, 21% of those using HPLC, and 55% of laboratories using some form of IEF.

The Hb S trait/Aida trait is a rare double heterozygous condition with a clinical presentation similar to that of Hb S trait. In the case presented here, the affected patient had one normal β -globin allele, one abnormal β -globin allele (Hb S, 6 β -Glu $\rightarrow$ Val), three normal α -globin alleles, and one abnormal α -globin allele (Hb Aida, 64 α -Asp $\rightarrow$ Asn). This combination of normal and abnormal alleles produced four major $\alpha\beta$ Hb variants, including Hb A, S, Aida ($\alpha^{\text{Aida}}\beta^{\text{A}}$, *pI* 7.188, apparent as a large shoulder on Hb S), and S/Aida hybrid ($\alpha^{\text{Aida}}\beta^{\text{S}}$, *pI* 7.430, apparent as a small peak cathodic to Hb A₂). Two minor $\alpha\delta$ Hb variants are also apparent, including normal Hb A₂ and an α -variant of Hb A₂ (Hb $\alpha\text{A}_2'$, $\alpha^{\text{Aida}}\gamma$, *pI* 7.617).

4 Discussion

Hb S, E, and C, respectively, are the three most common structural Hb variants in the world and are frequently encountered in most clinical hematology laboratories [1, 2]. Although the gene frequencies for Hb D, G, C-Harlem, and O-Arab are far lower, these variants are nevertheless occasionally encountered. The results show that Hb C, S, D, and G can be confidently identified by CIEF in a single analytical run based on automated postanalytical *pI* calculation. Analysis of several thousand patient samples by CIEF in our laboratory confirms that *pI* is a reproducible, objective, and specific criterion for identification of these four common abnormal Hb variants, and that confirmation by a second assay is unnecessary. In contrast, Hb E, C-Harlem, and O-Arab have similar *pI* and are not readily differentiated by CIEF alone. Further testing may be necessary when an abnormal Hb variant with a *pI* $\approx$ 7.410 is encountered, but the extent and manner of the testing should be decided on a case-by-case basis. For example, Hb C-Harlem can be differentiated from Hb E and O-Arab in the absence of Hb S by a positive sickle solubility test. Patient history may also help logically discriminate between these variants since Hb E is more common in individuals of Asian heritage while Hb C-Harlem is usually encountered in individuals of African heritage. However, conventional acid electrophoresis or another assay may be needed to firmly differentiate between Hb E, C-Harlem, and O-Arab.

The fundamental role of a primary assay for Hb variant analysis is to rapidly and inexpensively identify individuals with a structural hemoglobinopathy or thalassemia syndrome. The assay must exhibit a high probability of detecting an abnormal condition (diagnostic sensitivity) even if the specific underlying mutation (diagnostic specificity) cannot always be immediately determined [13]. A low-cost primary assay that can rapidly and specifically identify all of the more than 600 known structural Hb variants [1-3] is technically unfeasible at present. Such a highly specific assay is also clinically unnecessary since many abnormal Hb variants are extremely rare and unlikely to be encountered by most laboratories in years of operation except as part of a proficiency testing program. With CIEF, most abnormal Hb variants are readily separated from normal adult Hb A and thus structural hemoglobinopathies are readily detected. The diagnostic sensitivity of CIEF for unusual Hb variants is clearly evidenced by the analyses of Hb Köln trait, Hb M-Saskatoon trait, and the double heterozygous condition Hb S trait/Aida trait. The diagnostic specificity of CIEF for rare Hb variants is presently limited only because many variants have not been analyzed by this method and information about their *pI* is not yet available. The *pI* of 46 Hb variants analyzed in our laboratory were previously reported [12], and the diagnostic specificity of the assay can be expected to improve as information about more unusual Hb variants becomes available.

A major advantage of CIEF over other analytical methods is that it economically combines excellent diagnostic sensitivity with a level of diagnostic specificity suitable for primary assessment of most common congenital Hb disorders in a single automated assay. It is important to note that this includes simultaneous detection of both structural hemoglobinopathies and thalassemia syndromes. The data presented in this report verify the use of *pI* for specific diagnosis of common structural hemoglobinopathies. We have previously demonstrated that CIEF is characterized by precise and simultaneous quantification ($CV < 5\%$) of both major and minor Hb variants [10, 12]. The assay is therefore also useful for the diagnosis of thalassemia syndromes, especially β -thalassemias where elevated amounts of normal minor variants (Hb A₂ and F) are typically diagnostic. Low levels of Hb A₂ are also precisely measured by CIEF, but the use of these data for the detection of mild α -thalassemia syndromes has not been studied. CIEF can, however, identify Hb H and thus detect the more severe forms of α -thalassemia that cause Hb H disease [12, 14].

A number of other investigators have also reported the use of various capillary electrophoresis methods for the analysis of Hb variants [14-21]. In our opinion, however, none of these assays offer the sensitivity, specificity, pre-

cision, or throughput of the assay presented here. The CIEF method used in these experiments was optimized to meet the needs of a small to average-size hospital laboratory with a sample throughput of less than 40 samples per day. This is the approximate number of samples that can be analyzed in one day, by one technologist (~ 3 h actual labor time required), using one instrument operating at the rate of three samples per hour with some unattended overnight instrument operation. This throughput routinely permits same day or next day results and is more than sufficient for most laboratories since more rapid reporting of results does not normally influence patient outcome or cost efficiency. Rapid laboratory results are sometimes desirable, however, to minimize the hospital stay of some patients, specifically those with sickle cell disease undergoing multiple transfusions to optimize the ratios of Hb A and S prior to surgical intervention. In such cases, we have often found it advantageous that the assay is simple to set up even for analysis of a single sample. Also, unlike conventional gel electrophoresis or IEF, additional samples can be easily added to a run in progress.

Finally, we would like to note that the experiments described in this report were initiated to address questions posed by the hematology checklist of the CAP Laboratory Accreditation Program. Specifically, question 02:3812 requires that all samples identified to contain Hb S should be "further examined to ascertain whether the 'Hb S' band or peak contains solely Hb S or both Hb S and Hb D or Hb G." Our results show that CIEF can readily detect the simultaneous presence of Hb S and either Hb D or G, and that further examination of a sample showing a single peak identified as Hb S is unnecessary. Furthermore, question 02:3814 asks "are all samples that appear to have Hb S in the primary screening (by whatever method) further examined to confirm the presence of Hb S by solubility testing or other acceptable methods?" Again, because Hb S can be readily differentiated from Hbs D and G by CIEF based on *pI*, misidentification of Hb S is highly unlikely and has not been encountered in over five years of using CIEF in our laboratory. Failure to comply with either of these checklist requirements, however, represents a phase II deficiency that could lead to loss of laboratory accreditation. We therefore routinely perform a sickle solubility each time a patient with Hb S is initially identified in our laboratory. Based on the results presented here, however, we would submit that compliance with this rule is unnecessary and represents an added expense and a competitive disadvantage for laboratories using CIEF or other high resolution separation methods like HPLC [4-6]. We therefore recommend that the CAP guidelines should be amended to provide dispensation from these rules for laboratories using more advanced analytical technologies.

This research was supported by Children's Hospital of New Orleans.

Received October 15, 1999

5 References

- [1] Bunn, H. F., Forget, B. G., *Hemoglobin: Molecular, Genetic and Clinical Aspects*, W. B. Saunders Company, Philadelphia, PA 1986.
- [2] Fairbanks, V. F., *Hemoglobinopathies and Thalassemias*, Brian C. Decker, New York 1980.
- [3] Schmidt, R. M., in: Bick, R. L. (Ed.), *Hematology: Clinical and Laboratory Practice*, CV Mosby, St. Louis, MO 1993, pp. 327-390.
- [4] Huisman, T. H., *Acta Haematol.* 1987, 78, 123-126.
- [5] Ou, C. N., Rognerud, C. L., *Clin. Chem.* 1993, 39, 820-824.
- [6] Riou, J., Godart, C., Hurtrel, D., Mathis, M., Bimet, C., Bardakdjian-Michau, J., Prehu, C., Wajcman, H., Galacteros, F., *Clin. Chem.* 1997, 43, 34-39.
- [7] Fucharoen, S., Winichagoon, P., Wisedpanichkij, R., Sae-Ngow, B., Sriphanich, R., Oncoung, W., Muangsapaya, W., Chowthaworn, J., Kanokpongsakdi, S., Bunyaratvej, A., Piankijagum, A., Dewaele, C., *Clin. Chem.* 1998, 44, 740-748.
- [8] Eastman, J. W., Wong, R., Liao, C. L., Morales, D. R., *Clin. Chem.* 1996, 42, 704-710.
- [9] Craver, R. D., Abermanis, J. G., Warrier, R. P., Ode, D. L., Hempe, J. M., *Am. J. Clin. Pathol.* 1997, 107, 88-91.
- [10] Hempe, J. M., Craver, R. D., *Clin. Chem.* 1994, 40, 2288-2295.
- [11] Hempe, J. M., Granger, J. G., Warrier, R. P., Craver, R. D., *J. Capil. Electrophor.* 1997, 4, 131-135.
- [12] Hempe, J. M., Granger, J. G., Craver, R. D., *Electrophoresis* 1997, 18, 1785-1795.
- [13] Hempe, J. M., Craver, R. D., in: Palfrey, S. M. (Ed.), *Clinical Applications of Capillary Electrophoresis*, Humana Press, Totowa, NJ 1999, pp. 81-98.
- [14] Zhu, M., Wehr, T., Levi, V., Rodriguez, R., Shiffer, K., Cao, Z. A., *J. Chromatogr.* 1993, 652, 119-129.
- [15] Molteni, S., Frischknecht, H., Thormann, W., *Electrophoresis* 1994, 15, 22-30.
- [16] Zhu, M., Rodriguez, R., Wehr, T., Siebert, C., *J. Chromatogr.* 1992, 608, 225-237.
- [17] Wu, J., Pawliszyn, J., *Electrophoresis* 1995, 16, 670-673.
- [18] Mohammad, A. A., Okorodudu, A. O., Bissell, M. G., Dow, P., Reger, G., Meier, A., Guodagno, P., Petersen, J. R., *Clin. Chem.* 1997, 43, 1798-1799.
- [19] Castagnola, M., Messana, I., Cassiano, L., Rabino, R., Rossetti, D. V., Giardina, B., *Electrophoresis* 1995, 16, 1492-1498.
- [20] Mario, N., Baudin, B., Aussel, C., Giboudeau, J., *Clin. Chem.* 1997, 43, 2137-2142.
- [21] Jenkins, M. A., Hendy, J., Smith, I. L., *J. Capil. Electrophor.* 1997, 4, 137-143.

BEST AVAILABLE COPY