Isotope dilution–mass spectrometric quantification of specific proteins: model application with apolipoprotein A-I


An enzymatic hydrolysis isotope dilution–mass spectrometric method was developed for reference quantification of specific proteins. The analytical procedure involved measuring a reproducibly hydrolyzed peptide (serving as the primary standard) unique to a specific protein. This new mass spectrometric method was evaluated by assessing the concentration of apolipoprotein (apo) A-I in the European Community Bureau of Reference (BCR) lyophilized Certified Reference Material (CRM 393). We used the method to make 96 measurements (4 replicate analyses of 4 enzymatic digests of 6 vials of BCR-CRM 393), which gave an average total protein mass of 1.048 mg (±1.0% at 99% confidence limits). The total overall analytical CV was 3.95%. The results of this evaluation of our model approach to determine the concentration of a specific protein in a purified preparation demonstrated that our new mass spectrometric method can be used to measure apolipoproteins and other specific proteins without the use of epitope immunoassay methods.

INDEXING TERMS: standardization • trypsin • definitive method

Studies on specific proteins indicate a need for a nonepitopic assay with reference or definitive method capabilities of determining absolute concentrations of the specific protein. Immunochemical methods based on epitope measurement are often subject to cross-reactivity, weak antibody affinity, or denatured epitopes, and methods based on purification procedures followed by total protein analysis produce variable results that may not be comparable. A need exists therefore for a highly accurate method such as mass spectrometry to be a point of reference. In this study, we wanted to determine whether isotope dilution–mass spectrometry (ID-MS) could be used to quantify a unique polypeptide formed by the enzymatic hydrolysis of a specific protein and thus measure the concentration of the specific protein itself.† The distinct polypeptide served as the primary standard for the enzymatic hydrolysis–chromatographic separation–ID-MS procedure.

MS has proved useful in many aspects of protein chemistry, e.g., determination of molecular mass [1], kinetic studies [2], sequence analysis [3], and posttranslational modification [4]. Indeed, ID-MS has been used as a definitive method for a variety of compounds, including cholesterol [5], cortisol [6], and dioxin [7]. Quantification of peptides has also been performed by ID-MS [8]. MS has not, however, been used to quantify specific proteins. Because controlled hydrolysis with trypsin can be used to produce unique peptides from specific proteins [9, 10] and because ID-MS can reliably quantify peptides [8], we decided to measure protein concentration by (a) conducting a restricted hydrolysis of the protein, (b) chromatographically isolating selected peptides characteristic of that protein, and (c) quantifying the peptides by ID-MS.

Apolipoproteins are clinically important proteins situated on the surface of lipoproteins that transport lipids in serum. Measurements of these clinical biomarkers with known function

† Nonstandard abbreviations: apo, apolipoprotein; BCR, European Community Bureau of Reference; BSA, bovine serum albumin; CF, continuous-flow; CRM, Certified Reference Material; FAB, fast atom bombardment; ID, isotope dilution; LC, liquid chromatography; MS, mass spectrometry; and TPCK, N-tosyl-l-phenylalalanine chloromethyl ketone.

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are used to complement cholesterol measurements in predicting risk and in diagnosing coronary heart and atherosclerotic peripheral vascular disease [11-16]. Considerable effort has been made to standardize or make harmonious the results of the various immunochemical methods for apolipoproteins [17-20]. For apolipoprotein (apo) A-I, the European Community Bureau of Reference (BCR) has certified a purified lyophilized apo A-I reference material (CRM 393); the concentration of this reconstituted solution has been verified by six laboratories using amino acid analysis [21]. Protein methods, including that of Lowry et al. [22], did not produce results that agreed well among themselves or with the results of the amino acid analysis [21]. The International Federation of Clinical Chemistry (IFCC) has now developed a lyophilized serum reference material for apo A-I, the World Health Organization–IFCC First International Reference Reagent for Apolipoprotein A-I [23]. The amino acid sequence for apo A-I has been known since 1983 [24]. An innovative ID-RIA method using radiolabeled purified apo A-I was reported for the measurement of apo A-I [25].

We selected BCR CRM 393 for use in a model assessment of the proposed enzymatic hydrolysis–chromatographic–ID-MS method. Reproducible quantification required optimization of protein cleavage and choosing suitable peptide standards. Unlabeled and isotopically labeled standards were synthesized, purified, characterized, and quantified. Finally, we applied ID-MS to quantify apo A-I in the BCR CRM. We report here the results of our model application evaluation of whether enzymatic digestion, chromatographic separations, ID, and MS could be combined for highly accurate measurement of apo A-I.

**Materials and Methods**

**Production and Characterization of Specific Peptides**

**Enzymatic selective fragmentation of apo A-I.** Apo A-I was dissolved in 50 mmol/L NH₄HCO₃ buffer (pH 8.5) at 1 g/L. Trypsin [N-tosyl-l-phenylalanine chloromethyl ketone (TPCK)-treated; Worthington, Freehold, NJ] dissolved in the same buffer was added to the protein solution to yield an initial enzyme:substrate weight ratio of 1:100. After 3, 6, 9, and 12 h, additional aliquots of trypsin were added, bringing the final enzyme:substrate ratio to 1:20. The tryptic digest was incubated at 37 °C for 24 h (12 h after the last trypsin addition). The reaction was stopped by lowering the pH of the mixture to 5–6 by adding 5 μL of 50 mL/L acetic acid.

**Reversed-phase HPLC.** Peptide mixtures derived from digests of 1–10 nmol of apo A-I were analyzed and fractionated on a Hewlett-Packard (Palo Alto, CA) 1090 HPLC system equipped with a diode-array ultraviolet absorbance detector. The reversed-phase HPLC fractionation was performed on a fully end-capped Vydac wide-pore C₁₈ column [25 cm × 2.1 mm (i.d.), 5 μm particles, 30-nm pore size] with a Brownlee RP 100 guard column [30 mm × 2.1 mm (i.d.), 7 μm particles, 30-nm pore size]. Solvent A was 1 mL/L aqueous trifluoroacetic acid; solvent B was 90:10 (by vol) acetonitrile:solvent A. We used a gradient of 100% A at 0 min, 5% B at 1 min, 40% B at 60 min, and 100% B at 65 min.

**Fast atom bombardment (FAB) MS.** FAB mass spectra were obtained on a VG 70–4SE tandem magnetic-deflection MS (accelerating voltage 8 kV, mass range 3000 amu) equipped with a standard-flow FAB ion source and a high-voltage Cs ion gun. A VG 11–250J data system collected and processed all data. Typically, 2 μL of a fraction of the digested apo A-I containing ~0.1-1 nmol of peptide was placed on a stainless steel target in a "magic bullet" (5:1 dithiothreitol/dithioerythritol), and samples were analyzed at a resolution of 2000.

**Liquid chromatography (LC)-MS optimization.** LC-MS of tryptic digests of apo A-I with continuous-flow FAB (cf-FAB) was also performed to aid in peptide mapping the apo A-I CRM and to determine reproducible cleavage conditions. The HPLC utilized a 25 cm × 1 mm Brownlee microbore C₁₈ column (7 μm particles, 30-nm pore size) and a flow rate of 40 μL/min. Postcolumn splitting the effluent sent 5 μL/min to the cf-FAB probe; the remaining 35 μL/min was used to measure absorbance at 215 and 280 nm. Fractions (1-min) were collected with a Gilson (Middleton, WI) FC203 fraction collector. The solvent gradient in this analysis was the same as for the reversed-phase HPLC.

**Amino acid analysis.** We quantified the amount of the peptides with a Waters 720/730 system controller and data module with two Waters M6000A HPLC pumps equipped with a Waters 486 tunable absorbance detector and a Waters 710B WISP auto injector (all from Waters Chromatography Div., Millipore, Milford, MA). The Waters Pico-Tag method of amino acid analysis was used except with methylene chloride in the final extraction. The internal standard for quantification was norleucine, and the external standard was Pierce Amino Acid Standard H (Pierce Chemical Co., Rockford, IL).

**Nitrogen analysis.** An Antek (Houston, TX) Chemiluminescence Nitrogen Analyzer at the University of Wisconsin was used to quantify labeled and unlabeled standard peptides. After pyrolysis of the peptides, the resulting nitric oxides reacted with ozone to form excited nitrogen dioxide molecules. When these excited molecules, which contained metastable electrons, relaxed to the ground state, they released energy (as photons) that could be detected by a photomultiplier tube equipped with optical filters for wavelength specificity [26].

**Preparation of reference solutions.** After purifying the synthetic unlabeled and labeled peptides by C₁₈ reversed-phase HPLC, we weighed the purified peptides and used them to prepare stock solutions. The molar amount of each peptide in the solutions was carefully quantified by a combination of repetitive nitrogen analysis and repetitive quantitative amino acid analysis. These stock solutions were then used to prepare high- and low-concentration standards that tightly bracketed the target value of the BCR CRM for apo A-I.

**Analytical Procedure**

One vial of BCR CRM 393 (containing ~1 mg of apo A-I) was dissolved in a freshly prepared ammonium bicarbonate buffer
(pH 8.5). To this solution we added two labeled peptides: 29.6 µg of Leu[CD3]-Ala[13C5]-Glu-Tyr-His-Ala[13C5]-Lys (the quantification peptide) and 43.7 µg of Ala[13C5]-Thr-Glu-His-Leu[CD3]-Ser-Thr-Leu[CD3]-Ser-Glu-Lys (the confirmation peptide). This mixture was then divided into four aliquots and trypsin, in the same buffer (1:100, trypsin:apo A-I), was added to each. Additional portions of trypsin were added 3, 6, 9, and 12 h later, making the final enzyme:substrate ratio 1:20. The selective hydrolysis proceeded for 24 h at 37 °C, after which the reaction was quenched with 5 µL of 50 mL/L acetic acid. The resulting mixture of peptides from each reaction was then purified by C18 reversed-phase HPLC. The fractions containing isolate-labeled and unlabeled Leu-Ala-Glu-Tyr-His-Ala-Lys and Ala-Thr-Glu-His-Leu-Ser-Thr-Leu-Ser-Glu-Lys were then analyzed by FAB-MS. Spectra were collected by taking 15 scans in multichannel-analysis mode at a resolution of 3000. Low- and high-concentration standards were always run before and after each unknown and were used in the quantification. Recoveries were determined by adding an isotope-labeled peptide (Leu[CD3]-Ser-Pro-Leu[CD3]-Gly-Glu-Glu-Met-Arg) to the HPLC fractions containing the unlabeled peptide and analyzing by FAB-MS. This procedure was repeated for each of six vials of the BCR CRM for apo A-I.

**PEPTIDE ANALYSIS**

**Mapping.** Determinations of peptide molecular mass by FAB-MS coupled with LC-MS techniques, such as cf-FAB-MS and sequence analysis by tandem MS, were used for the structural characterization of apo A-I. The scheme used to characterize the BCR apo A-I CRM included enzymatic hydrolysis by trypsin, a proteolytic enzyme that specifically cleaves at the C-terminus of lysine and arginine residues except when these residues are followed by a proline. The mixture of peptides that resulted from the trypsin digest was then separated by HPLC on a wide-pore C18 reversed-phase column (Fig. 1). Fractions were collected at 1-min intervals, and each fraction was concentrated and analyzed by FAB-MS. The FAB-MS spectra indicate the molecular masses of the peptides produced during the cleavage reaction. These molecular mass values were then fitted (based on established rules for the specific enzymatic cleavage reactions) to the known or deduced sequence of the protein, and the sequence of the protein and the chemical or enzymatic cleavage conditions were entered into a computer program (Mac Bio Ion; PE Sciex, Thornhill, ON, Canada) to generate lists of expected peptide products. FAB-MS peptide mapping data for apo A-I are summarized in Fig. 2. Approximately 97% of the sequence of the BCR apo A-I was confirmed by a combination of FAB-MS and cf-FAB-MS analyses of the tryptic digests. All significant signals in the FAB-MS spectra could be assigned to the deduced sequence of apo A-I. The tryptic digest of the BCR apo A-I CRM yielded a NH2-terminal peptide with an MH+ ion at m/z 1227. Extended or NH2-terminally modified forms of this peptide were not observed. From this we conclude that the NH2-terminal amino acid was aspartic acid and that the protein has a homogeneous terminus. The tryptic digest yielded a COOH-terminal tetrapeptide, which, again, did not show any extended or modified forms. FAB-MS data did not indicate the presence of the oxidized forms of the two methionines (Met86 and Met149), suggesting that the oxidized products (sulfoxides) are not present in the purified protein.

**Optimization of enzymatic cleavage.** The LC-MS data also aided in the final choice of cleavage reaction conditions. The tryptic cleavage reactions were allowed to proceed from 3 to 24 h with substrate:enzyme ratios from 100:1 to 20:1. The enzymatic digestion reactions were then monitored by both absorbance detection and cf-FAB-MS. Both the ultraviolet chromatogram and the mass spectra were then carefully examined for partial cleavages and chymotryptic activity. The shorter reaction times and lower enzyme:substrate ratios tended to yield less-complete cleavages at several sites. At longer reaction times and higher enzyme:substrate ratios, chymotryptic activity sometimes occurred. When we used a 24-h reaction time with a substrate:enzyme ratio of 20:1 and TPCK-treated trypsin, no chymotryptic activity was observed. Thus, these conditions were chosen because they yielded reproducible quantitative cleavage at most sites.

**Identification of peptides.** Several peptides were identified and sequenced by tandem MS. Three peptides were chosen for quantification: L109-A-E-Y-H-A-K115, A106-T-E-H-L-S-T-L-S-E-K206, and L144-S-P-L-G-E-E-M-R147. These peptides, sequenced by tandem MS with use of high-energy collisions on the VG 70-4SE four-sector mass spectrometer, were chosen because they responded well in FAB-MS, were well separated by reversed-phase HPLC, and were reproducible and quantitatively formed.

**Synthesis and purification of selected peptides.** We also synthesized the three peptides that were selected for quantification (L-A-E-Y-H-A-K, L-S-P-L-G-E-E-M-R, and A-T-E-H-L-S-T-L-S-E-K), as well as isotopically labeled peptides of 6–9 mass units greater mass for use as comparison reagents in the quantification by ID-MS. The synthetic labeled peptides were purified by C18 reversed-phase HPLC.

**Results**

Fig. 1. Absorbance (at 215 nm) chromatogram of a tryptic digest of apo A-I, indicating peptides used for quantification (shaded peaks).

At least six labels in stable positions were placed within each labeled peptide so that the mass of the labeled peptides was not subject to interference from the isotope cluster of the endogenous unlabeled peptides. Because all of the peptides contained Leu, Ala, or both, we used [CD$_3$]leucine and [L$^{13}$C$_6$]alanine to synthesize the labeled peptides. Thus, the sequence of the labeled peptides corresponded to the sequence of the native peptides to which the labeled leucine and labeled alanine had been added. This yielded the sequences L(CD$_3$)-A($^{13}$C$_6$)-E-Y-H-A($^{13}$C$_6$)-K, L(CD$_3$)-S-P-L(CD$_3$)-G-E-E-M-R, and A($^{13}$C$_6$)-T-E-H-L(CD$_3$)-S-T-L(CD$_3$)-S-E-K, which were confirmed by high-energy collision-induced dissociation MS of unlabeled and labeled peptides.

### PROTEIN MOLECULAR MASS

The molecular mass of the BCR apo A-I CRM was recorded on a Sciex API III tandem mass spectrometer. Ionization of apo A-I, accomplished by electrospray ionization, was analyzed by scanning the first quadrupole. Ions for [M + 12]$^+$ to [M + 32]$^+$ were observed. This spectrum indicated that the measured average molecular mass of apo A-I in CRM 393 was 28 078.63 Da, in good agreement with the calculated average molecular mass of 28 078.69 [24]. In this case, the difference between the average calculated molecular mass and the measured molecular mass was only 0.0002%. Careful examination of each peak in the spectrum showed no evidence of posttranslational modification or extended forms of apo A-I.

### QUANTIFICATION OF APO A-I

We analyzed a total of six BCR apo A-I CRM 393 vials according to the strategy outlined in Materials and Methods. Each vial was split into four aliquots, each of which underwent separate tryptic digestion. We performed four MS analyses for both the quantification peptide (A-T-E-H-L-S-T-L-S-E-K) in each tryptic digest, which allowed us to perform complex statistical analysis of the entire method. The results for each ID-MS analysis of each digest from every vial are given in Table 1. The average total protein mass value for the total of 96 measurements (4 analyses of 4 digests of 6 vials) of the BCR apo A-I CRM was 1.048 mg/vial ± 0.010 mg (or 1.048 mg ± 1%) at the 99% confidence limits. The average amount of total protein in each vial as calculated from 16 measurements on 4 digests of the primary quantification peptide was 1.048 mg for vial 1, 1.068 mg for vial 2, 1.077 mg for vial 3, 1.000 mg for vial 4, 1.076 mg for vial 5, and 1.020 mg for vial 6. The CV of the mean of these means for each vial is 3.1%. The average amount of total protein in each vial as calculated from the confirmation peptide (A-T-E-H-L-S-T-L-S-K) was 0.957 mg for vial 1, 1.004 mg for vial 2, 1.080 mg for vial 3, 1.038 mg for vial 4, 1.103 mg for vial 5, and 1.084 mg for vial 6. The average total protein mass of apo A-I calculated from the confirmation peptide was 1.044 mg/vial, which is within the confidence limits for the protein mass value calculated from the primary quantification peptide (A-T-E-H-L-S-T-L-S-K). The results of quantifying the synthetic peptide L-S-P-L-G-E-E-M-R indicated that the recovery through the purification of the peptides was generally 70–80%. All data from each digest were used because there were no unusually low recoveries and no MS runs that were found to be statistically out of acceptable limits. However, results of the analyses of digest 1/vial 6, digest 3/vial 4, and digest 4/vial 4 tended to be lower than the results of other digest/vial analyses (Table 1).

Variances for vial to vial, digest to digest, and MS run to MS run were calculated. As we expected, the largest variance was digest to digest, and smallest was MS run to run. The variance components as percentages of total variance were 13.3% run to run, 42.6% vial to vial, and 44.1% digest to digest. The total analytical CV was 3.95% for all 96 measurements on 6 vials and
3.1% for the mean of means for each vial. The results from the total statistical analysis of this system are summarized in Table 2. The distribution of values is mainly concentrated around the mean (Fig. 3); a slight skew toward the low end, however, could possibly be from slightly incomplete cleavages.

**Discussion**

Our major goal in this study was to develop an MS-based Reference Method for quantification of specific proteins, for which we used apo A-I in the development and model evaluation of the method. Currently, specific proteins are routinely measured by immunoassays linked to total protein measurements of the purified specific protein [20, 23]. The accuracy with which a specific protein can be measured by a total protein method is limited by the accuracy of the analytical method used for total protein and by the purity of the isolated specific protein being analyzed; the accuracy of such a measurement by epitopic immunoassay can suffer from cross-reactivities, nonequivalent binding, and unknown denaturation of epitopes. We, therefore, devised a new analytical approach to determine whether a reproducible hydrolyzed peptide unique to the specific protein could be measured by MS. This approach would provide a procedure not dependent on epitopes, could use a unique hydrolyzed peptide as the primary standard, and could be based on the peptide structure of the specific protein.

Results from the various total protein methods currently being used to quantify purified specific proteins such as apolipoproteins are often not comparable. A CDC-IFCC international survey of seven protein methods used to measure proteins in purified delipidated HDL, isolated LDL, and purified bovine serum albumin (BSA) demonstrated that the CV for results from all methods was ~18% for HDL and BSA and ~50% for LDL [27]. The total CV of the Lowry et al. method [22] was ~11% for HDL and BSA solutions and ~23% for LDL samples [27]. The accuracy of the apo A-I values assigned to reference materials by proposed reference methods will be influenced by the accuracy of the Lowry et al. method [22], used to measure the amount of total protein in the purified apo A-I [28, 29]. This uncertainty about the accuracy of the total protein measurement of apolipoproteins by different methods can be decreased by developing a highly accurate method that can be used to evaluate the accuracy of the various protein methods.

We chose to use the well-characterized, purified, lyophilized specific protein apo A-I in BCR CRM 393 for developing and evaluating the proposed enzymatic hydrolysis–HPLC–ID-MS method to measure the concentration of a specific protein.

### Table 1. Analytical results by isotope dilution–mass spectrometry.*

<table>
<thead>
<tr>
<th>Source</th>
<th>Digest 1</th>
<th>Digest 2</th>
<th>Digest 3</th>
<th>Digest 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among-vial</td>
<td>1.039</td>
<td>1.028</td>
<td>1.051</td>
<td>1.065</td>
</tr>
<tr>
<td>Among-digest (within-vial)</td>
<td>1.045</td>
<td>1.061</td>
<td>1.051</td>
<td>1.051</td>
</tr>
<tr>
<td>Run-to-run</td>
<td>1.042</td>
<td>1.022</td>
<td>1.073</td>
<td>1.069</td>
</tr>
<tr>
<td>Total</td>
<td>1.042</td>
<td>1.023</td>
<td>1.050</td>
<td>1.048</td>
</tr>
</tbody>
</table>

* The quantification results included are for each mass spectral run on each digest for every vial, corrected to mg/vial.

### Table 2. Statistical data for the quantification of BCR apo A-I standard.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square</th>
<th>Component of variance</th>
<th>% of overall variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among-vial</td>
<td>5</td>
<td>0.01622</td>
<td>0.000793</td>
<td>42</td>
</tr>
<tr>
<td>Among-digest (within-vial)</td>
<td>18</td>
<td>0.00353</td>
<td>0.000820</td>
<td>44</td>
</tr>
<tr>
<td>Run-to-run</td>
<td>72</td>
<td>0.000249</td>
<td>0.000249</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>95</td>
<td>0.001711</td>
<td>0.001862</td>
<td>100</td>
</tr>
</tbody>
</table>

Mean = 1.048 mg/vial  
SDoverall = 0.04136 mg/vial  
CVoverall = 3.946%  
Confidence limits (99%)overall = ± 0.010 mg
During initial studies to determine and validate the quality of the BCR CRM (e.g., protein sequence, molecular mass, and purity), the high purity of this apo A-I CRM was confirmed by our molecular mass characterization and peptide mapping and sequencing. The agreement of results for apo A-I as measured by electrospray ionization MS and as calculated from the predicted sequence [24] indicated that the BCR apo A-I is a very pure protein that did not contain any extended forms. Moreover, the peptides used for quantifying apo A-I were unique to apo A-I; comparing these peptide sequences with the Swiss protein database showed that they are found only in apo A-I.

Optimization of enzymatic cleavage conditions permitted reproducible formation of specific peptides. A 24-h reaction time with repeated additions of trypsin at 37 °C gave consistent results with a 20:1 substrate/enzyme ratio. Three peptides were consistently produced and well separated from other peptides. The enzymatic digest, though, potentially the most variable part of this method, represented ~44% of the small overall total variance (CV 3.95%) (Table 2). Excellent reproducibility was achieved by carefully choosing the quantification peptides and optimizing the reaction conditions. The other peptide-containing HPLC fractions were also carefully examined to specifically ensure that no partial cleavages had occurred around the quantification peptides.

For peptide mapping, we used FAB-MS to determine the molecular masses of the peptides produced by enzymatic cleavage reaction. By using established rules for calculating the molecular masses of peptides formed by specific enzymatic cleavage, we matched the molecular masses determined by FAB-MS to the known or deduced sequence of the protein. The sequence of the protein and chemical or enzymatic cleavage conditions were then entered into a computer program designed to give lists of expected peptide products. These lists of peptides and molecular masses aid in interpreting the FAB-MS data and mapping the peptide sequence of the protein.

About 97% of the BCR apo A-I CRM sequence was confirmed by a combination of FAB-MS and cf-FAB-MS of the tryptic digests. All significant signals in the FAB-MS spectra could be assigned to the deduced sequence of apo A-I. The 3% of the BCR CRM that was not mapped consisted of only three small hydrophilic peptides (two dipeptides and one tetrapeptide). Small hydrophilic peptides are generally very difficult to detect by FAB-MS. No posttranslational modifications such as glycosylation or phosphorylation were observed.

We used several criteria to select peptides for use in quantifying the BCR apo A-I, including FAB-MS response, retention time, sequence, and (most importantly) reproducible quantitative enzymatic cleavage. For the peptide V97-K107, the HPLC chromatogram suggested that this peptide was formed reproducibly (retention time 39 min) and was well separated from other peptides; the FAB-MS data, however, indicated that this LC peak often contained two peptides: V97-K107 and V97-K108. A signal at m/z 1353 corresponded to V97-K107, and that at m/z 1381 referred to V97-K108. We rejected peptides such as V97-K107 because they often yielded partial cleavages. We also paid careful attention to the formation of chymotryptic fragments. Tryptic digests often show a small amount of chymotryptic activity caused by a trace contamination by chymotrypsin. However, the trypsin utilized in this study contained a chymotrypsin inhibitor (TPCK), and no problems with superimposed chymotryptic activity were observed.

The quantification results obtained by ID-MS in this study were in good agreement with the amino acid analysis results reported for the BCR apo A-I CRM [21]. The average of the results of the BCR amino acid analysis indicated that the quantity of apo A-I per vial was 1.06 ± 0.05 mg (37.7 ± 1.8 μmol) at the 95% confidence limits, whereas in this study, using ID-FAB-MS, we found an average of 1.048 ± 0.010 mg (37.4 ± 0.4 μmol) per vial at the 99% confidence limits. In the BCR study [21], the mean of means of 4 measurements per vial for 8 vials of 1.06 g/L ± 0.05 indicated a CV near 5%. Our vial-to-vial CV (for mean of means for 6 vials) of 3.1% was below, but in general agreement with, this published value for 8 vials of BCR CRM 393. Thus, ID-FAB-MS yielded average mass values in excellent agreement with the BCR amino acid analysis results but with tighter confidence limits.

Future investigations will examine the utility of the ID-MS assay for measuring apo A-I and other apolipoproteins in complex biological matrices. Initially, lipoprotein fractions of serum partially purified by ultracentrifugation could be examined to determine potential obstacles to their use in serum analysis. However, whether or not the ID-MS assay will prove useful with complex matrices, we have shown here that the developed ID-MS for apo A-I demonstrates the applicability of the model to the evaluation and certification of purified specific proteins used for primary standards. Potentially, this MS technique could be applicable to the measurement of other functional proteins such as enzymes that possess unique peptides.

Accurate determination of the concentration of a specific protein present in reference materials is critical for scientifically sound accuracy bases and for their use in the standardization of routine clinical measurements. Because the concentration of apo A-I has been shown to be an important indicator of risk for coronary heart disease, much effort has been spent on standardizing different analytical methods and in developing a highly purified preparation apo A-I. We therefore selected apo A-I for use in developing and evaluating the proposed enzymatic hydrolysis–ID-MS procedure. Not only did our findings with the
proposed MS method agree with the published results on apo A-I in the lyophilized BCR CRM 191 (with amino acid analysis used for total protein determination), but most importantly, our findings suggest that ID-MS has the potential to become an important tool in standardizing measurements of other specific proteins quantified in the clinical laboratory.

References