Published in final edited form as:

Electrophoresis. 2013 June ; 34(11): 1693–1700. doi:10.1002/elps.201200567.

Heat-assisted extraction for the determination of methylarginines in serum by capillary electrophoresis

Thomas H. Linz^{1,3} and Susan M. Lunte^{1,2,3}

¹Department of Chemistry, University of Kansas, Lawrence, KS

²Pharmaceutical Chemistry, University of Kansas, Lawrence, KS

³Ralph N. Adams Institute for Bioanalytical Chemistry, University of Kansas, Lawrence, KS

Abstract

Methylarginines (MAs) are potent vasoconstrictors that have been reported to be present at elevated concentrations in the blood of patients suffering from cardiovascular disease (CVD). To determine the diagnostic potential of MAs for CVD, a method capable of rapidly quantifying their endogenous concentrations from serum samples is required. To that end, a heat-assisted extraction method was developed. Serum was first rapidly heated, causing it to congeal into a gel, and then subjected to solid-liquid extraction. The extraction solution was then derivatized with a fluorogenic dye and analyzed by CE-LIF to permit quantitation of the MAs. This heat-assisted extraction procedure allowed a no-net-dilution extraction of the analytes to be performed into a solvent compatible with the subsequent CE analysis. This enabled direct detection of low abundance analytes, such as MAs, without the need for a preconcentration step. This sample preparation method was compared with a commonly used solid-phase extraction method for MA analysis. Endogenous MA concentrations determined by both the heating and SPE methods were found to be in good agreement with each other and with values previously reported in the literature.

Keywords

Capillary electrophoresis; Fluorescence detection; Methylarginine; Sample preparation; Serum

1 Introduction

The enzyme nitric oxide synthase (NOS) utilizes arginine as a substrate to produce nitric oxide (NO) *in vivo* [1]. However, in the presence of methylarginines (MAs) (Figure 1), the generation of NO has been shown to be attenuated [2]. The compounds N^G-monomethylarginine (MMA) and asymmetric N^G,N^G-dimethylarginine (ADMA) are competitive inhibitors of NOS. A related compound, symmetric N^G,N^G-dimethylarginine (SDMA), does not directly inhibit NOS; however, it competes with arginine for cellular uptake via cationic amino acid transporters along with the other MAs [3, 4]. Since MAs inhibit NOS and compete with the enzyme substrate for uptake, elevated concentrations of these species diminish endogenous production of NO. A reduced bioavailability of NO has been associated with various pathological conditions, including the development of

Susan Lunte, Ralph N. Adams Professor of Chemistry and Pharmaceutical Chemistry, 2030 Becker Dr. MRB 220, Lawrence, KS 66047, slunte@ku.edu, Fax: 785-864-1916.

Conflict of Interest

The authors declare no conflict of interest.

cardiovascular diseases (CVDs) [5, 6]. Not surprisingly, elevated concentrations of MAs have been found in the blood of patients suffering from a number of CVDs including various forms of heart disease [7–13] and stroke [14, 15]. Because of the effect that MAs have on NO production and the onset of CVD, a rapid and inexpensive method to measure their concentrations in blood as a means of diagnosing CVD would be highly valuable.

Biological matrices, such as serum, often prove to be major obstacles when developing analytical methods because of their extreme complexity. Matrix effects can substantially diminish a measured signal for a variety of reasons, including incompatible pH or solvent composition [16]. This reduction in sensitivity could make the detection of low abundance endogenous compounds, such as MAs, difficult. To circumvent these issues, sample preparation steps must be integrated into the analytical method to isolate the small molecules of interest in a solution that is compatible with the analysis technique [17]. Often, a necessary first step is to remove macromolecules (*e.g.* proteins) from the sample so they do not interfere with the analysis. Conventional protein precipitation methods include the addition of an organic solvent to the sample or changing the pH of the sample. A subsequent centrifugation step then effectively isolates small molecules in the supernatant. Following this preparation, the analytes of interest are no longer in a macromolecule-rich environment; however, they are still in a potentially problematic matrix. The sample solution is now diluted and either contains an organic solvent or is at an extreme pH. These factors may present a challenge for subsequent quantitative analysis. Additional sample preparation steps are often undertaken to evaporate the organic solvent or remove interferents using solid-phase extraction (SPE), but all these methods further complicate the analysis, increasing the total analysis time and introducing additional potential sources of error.

Thermal coagulation of serum is an attractive alternative method for removing macromolecules from solution due to the simplicity of the method. Rapidly heating serum provides excess energy into the system which can break the non-covalent forces crucial for maintaining protein tertiary structure. As a gel forms during the heating process, small molecules become entrapped in the pores of the cross-linked protein framework [18]. These molecules can later be removed from the gel by performing a solid-liquid extraction. Heating methods to congeal serum have been reported in the distant past, albeit sparingly, to measure small molecules including urate [19], glucose [20], and creatinine [21]. Limitations to the reported procedures, however, were that the samples still experienced appreciable dilution and underwent additional heating steps, which increased the time required for preparation.

Previous reports in the literature concerned with measuring MAs from blood samples employed either LC or CE coupled to spectroscopic or mass spectrometric detection [22, 23]. Regardless of analysis technique, SPE has still been the method of choice for sample preparation even though it is time-consuming and can therefore limit sample throughput [24]. The goal of this work was to create a rapid and inexpensive method to extract small molecules from serum into a simple matrix that could be analyzed directly to quantify endogenous MA concentrations by CE-LIF. A heat-assisted extraction method is described herein that provides a means of rapidly isolating small molecules in a solvent compatible with the analysis method without requiring further sample clean-up. This is especially beneficial for fluorescence detection schemes that require analyte derivatization prior to analysis because analytes can be extracted directly into a solution compatible with derivatization. Additionally, the lack of dilution afforded by this method obviates preconcentration and allows samples to be analyzed immediately.

2 Materials and methods

2.1 Reagents

Standards of MMA, ADMA, SDMA, and N^G-propylarginine (PA) were acquired from Enzo Life Sciences (Farmingdale, NY). Sodium tetraborate and sodium cyanide were purchased from Sigma Aldrich (St. Louis, MO). Naphthalene-2,3-dicarboxaldehyde (NDA) was purchased from Invitrogen (Carlsbad, CA). Sulfobutylether- β -cyclodextrin (SBE-CD) was acquired from Cydex Pharmaceuticals (Lenexa, KS). HPLC-grade dimethylsulfoxide, acetonitrile, methanol, and ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA). All solutions were made in 18.2 M Ω ·cm deionized water (Millipore, Billerica, MA) unless otherwise noted. Pooled serum samples from anonymous donors were obtained from Lawrence Memorial Hospital (Lawrence, KS).

2.2 Capillary electrophoresis

A Beckman P/ACE MDQ capillary electrophoresis instrument (Brea, CA) with a 50 μ m i.d. capillary segment (Polymicro Technologies, Phoenix, AZ) 65 cm in length (50 cm to window) was utilized in this study. The run buffer consisted of 15 mM sodium tetraborate, 10 mM SBE-CD, and 25% (v/v) DMSO. Samples were injected hydrodynamically at 1.0 psi for 5 s, and separations were carried out at an applied field strength of 430 V/cm. Fluorescent emission (>490 nm) was measured with an external fluorescence detector (Picometrics, Ramonville, France) following excitation with a 445 nm diode laser (CrystaLaser, Reno, NV). Both CE operation and LIF detection were controlled with 32 Karat software (Beckman).

Samples analyzed by CE were first derivatized with NDA/CN. NDA was dissolved in 1:1 acetonitrile:water; all other solutions were prepared in deionized water. The derivatization procedure entailed combining equal volumes of sample, 50 mM sodium tetraborate, NDA, and 5 mM NaCN and allowing the mixture to react for 10 min prior to injection. The initial NDA concentration was 1 mM when derivatizing standards and 5 mM when derivatizing serum samples. These NDA concentrations provided the maximal signal while preserving the best signal-to-noise (data not shown). Propylarginine was used as an internal standard for each analysis. Fluorescence signals from both standards and serum samples were normalized to the peak area of PA for quantitation. All standards/samples were measured in triplicate unless otherwise noted.

2.3 Heat-assisted extraction procedure

To prepare the serum samples, 100 μ L aliquots of pooled serum were transferred into 2 mL polypropylene microcentrifuge tubes (Fisher Scientific) to which 5 μ L of 10 μ M PA was added. The tubes were immersed in a beaker of boiling water (100 °C) for 1.5 min. During the heating process, the liquid serum quickly congealed to form a solid gel. Once the serum gel was formed, 100 μ L of water was added to each vial and vortexed for ~20 s to dislodge the clot from the bottom of the vial and break it into small pieces; however, complete homogenization was not achieved. Samples were then centrifuged to sediment the aggregated proteins, and the supernatants were decanted into separate tubes for subsequent analysis. The volume recovered following the extraction was slightly greater than the initial volume of water added to the vial. An illustration depicting the overall sample preparation scheme is shown in Figure 2a.

2.4 Solid-phase extraction procedure

For the SPE procedure, 100 μ L aliquots of pooled serum and 5 μ L of 10 μ M PA were first transferred into microcentrifuge tubes. Proteins were then precipitated by adding 200 μ L methanol to each vial. Samples were centrifuged to pellet the precipitated proteins, and the

supernatant was decanted. This sample was then subjected to SPE without further pretreatment.

HyperSep Retain CX strong cation exchange (SCX) SPE cartridges (Thermo Scientific, Waltham, MA) were utilized to isolate MAs present in the serum samples. SPE was performed based on a modified procedure from the manufacturer. Our procedure included an initial wash step to desorb an interfering compound that was found to leach out of the stationary phase. This was performed by first hydrating the SPE resin with 5% NH_4OH in 1:1 methanol:water followed by equilibration with 1:1 methanol:water. After binding and rinsing the serum supernatant, analytes were eluted in 1 mL of 10% NH_4OH in 1:1 methanol:water. Samples were evaporated to dryness using a Savant SpeedVac SC110 centrifugal evaporator (Thermo Scientific) and resuspended in 100 μL of water. A schematic illustrating the steps required for SPE is shown in Figure 2b.

3 Results and discussion

3.1 Separation of MAs by CE

The CE-LIF separation method for MAs was adapted from work previously reported by our group [25]. The modified run buffer composition described here contained a high percentage of DMSO, which separation optimization studies showed dramatically increased the peak capacity compared to the previous method. This improved separation efficiency was necessary for the analysis of complex serum-derived samples. A sample electropherogram and the validation parameters are shown in Figure 3 and Table S1, respectively. The linearity of the method was determined by constructing a calibration curve over a clinically relevant concentration range (50–1200 nM) (Figure S1). This method provided good linearity ($R^2 = 0.999$) and precision. The average peak area deviation for each analyte was <5% over the concentration range studied. The number of theoretical plates for each analyte was ~150,000 plates/m. The limits of detection ($S/N = 3$) were determined experimentally and found to be 5–8 nM, all of which are well below the expected endogenous concentrations.

3.2 SPE optimization

The SPE recoveries for the compounds of interest were determined prior to the analysis of serum samples. Similar recoveries of ~90% were observed for standards of SDMA, ADMA, and MMA; however, PA exhibited a much lower recovery (52%) than did the MAs (Table S2). To account for these recovery discrepancies, calibration standards were subjected to the SPE procedure to generate a second calibration curve. This curve helped to ensure accurate quantitation for serum samples that underwent SPE. Table S2 shows the SPE recoveries of the analytes as well as the calibration parameters. While the recoveries of the standards were reasonably high for the MAs, the linearity ($R^2 = 0.988$ – 0.991) and precision (%RSD = 27–30%) of the method were poor. The predominant reason for this originated from high inter-cartridge variability, which increased the imprecision of the method. The full calibration curve is shown in Figure S2.

3.3 Sample preparation considerations

Solid-phase extraction is a sample preparation technique that has been widely used for decades. Despite its prevalence, however, SPE suffers from many drawbacks. In the case of common SCX SPE procedures used for MA determination, analytes are isolated in an eluent containing a high percentage of ammonia and methanol [26, 27]. This is problematic when utilizing amine-based derivatization chemistry because ammonia reacts with NDA/CN and methanol interferes with the derivatization reaction [28]. Both of these factors decreased the sensitivity of the analysis. Additionally, the procedure required desorbing the analytes in an

elution volume an order of magnitude higher than the volume of the initial sample to ensure that maximum recovery was obtained. This presents a challenge when analyzing MAs present endogenously at nanomolar concentrations because any appreciable dilution may reduce the concentrations below the detection limits of the method. To circumvent dilution and to remove the ammonia and methanol from the sample, an evaporation step needed to be performed prior to derivatization. This added significantly to the total time of the preparation method.

Heat-induced coagulation of serum is a simple alternative sample preparation method for serum samples providing that the small molecules of interest are stable at high temperatures and can survive the initial heating process. Following heating, the intact molecules can be readily extracted from the gel into an external solvent. By putting forethought into the experimental setup, no dilution is necessary during the extraction step. Additionally, choosing an appropriate extraction solvent will allow the resulting sample matrix to be compatible with the subsequent derivatization procedure. This will allow samples to be analyzed directly without requiring the solvent to first be evaporated.

3.4 Heat-assisted extraction optimization

The application of extreme heat to a serum sample can cause protein denaturation and subsequent aggregation. When interested in measuring small molecules, maintaining the native conformations of the proteins in the sample is of little concern. However, the stability of the small molecules of interest is crucial for the implementation of this procedure. Therefore, compounds that are liable to heat-induced degradation or oxidation would be poor candidates for this sample preparation procedure. To ensure that MAs are thermally stable, 500 nM MA standards were placed in boiling water for 2 min, and then derivatized and analyzed via CE-LIF. It was determined that the heating procedure did not affect the integrity of the molecules since no loss was observed between the heated and non-heated samples (Figure S3).

The heating time required to generate protein aggregation was also evaluated. It was determined that 30 s was insufficient to induce complete formation of a gel as evidenced by the serum still appearing slightly “runny” and a large broad peak spanning through the electropherogram. Extending the heating time to 1 min alleviated this problem. There was no difference between samples heated between 1 and 4 min (data not shown), so 1.5 min was chosen as a middle point for further experiments.

After the serum was heated and congealed, water was added over the gel and briefly vortexed to extricate the coagulum from the bottom of the container. The time the water was incubated with the serum gel (at room temperature) was optimized to maximize the amount of MAs extracted into solution. Results from this experiment showed that there were no significant increases in MA recoveries for extractions over a 1 h period and that all MAs were extracted at similar rates (Figure 4). Since a steady-state recovery (within error) was reached within 5 min, this duration was used in further experiments to expedite the analysis. It should be noted that a 15 min centrifugation step was performed to sediment the proteins from solution after the incubation period. This step increased the time that the extraction solvent remained in contact with the solid, and was not accounted for in Figure 4.

The recovery of MAs from the serum gel ranged between 52 and 58% using the method described above. Because of this, the incorporation of an internal standard was crucial to obtaining accurate results. Since a suitable internal standard was identified (*i.e.* PA), no further attempts were made to improve the recovery of MAs. To increase the extraction efficiency, however, a heated extraction could have been performed to help resolubilize the analytes of interest [20]. Previous methods have reported that complete recovery could be

achieved by adding water to the coagulated serum and extracting the mixture at 37 °C for 30–60 min or at room temperature overnight [29–31]. In the interest of minimizing the complexity of the analysis and the total analysis time, this was not performed in our method. In the future, though, smaller volumes of serum could be used to increase the solvent accessible surface area, which would help increase recovery and would require even less blood to be drawn initially from patients.

3.5 Sample preparation method comparison

Extracted serum samples derived from both the SPE and heating procedures were compared to determine the differences in their pH values and conductivities. The pH values (colorpHast strips, EM Reagents, Cherry Hill, NJ) showed that both samples were slightly basic regardless of the extraction procedure (Table 1). The pH from the heated sample matched well with a previously reported value acquired using a thermal coagulation method that attributed the basic pH to a release of CO₂ during the heating process [30]. The conductivity of each sample was also measured. For comparison purposes, the conductivity values from the extracted serum samples were normalized to that of the 50 mM borate used in the NDA derivatization reaction. Table 1 shows that serum that underwent the heating method had a conductivity similar to that of borate while the SPE sample was over an order of magnitude less conductive. This result is not surprising because samples bound to the SPE stationary phase should have had all of the residual ions from the serum rinsed to waste before the analytes of interest were desorbed from the resin. In the heating procedure, however, the salts in the serum gel were able to readily redissolve into the extraction solvent. While the conductivities of the heat-prepared samples were substantially higher, they were on par with that of the borate used for derivatization and were not found to hinder the CE analysis.

A substantial savings in both sample preparation time and the cost of analysis was also realized with the heating method compared to SPE. The heating procedure could be performed in ~30 min with the majority of the time allotted for the centrifugation cycle and the time spent waiting for the water bath to come to a boil. This was in stark contrast to the SPE procedure where the time required to prepare the necessary solutions, perform SPE, and then evaporate the samples to dryness was ~4 h. The time difference was even further accentuated when one considers that in the SPE protocol, standards also had to undergo SPE to permit accurate quantitation of unknowns. Additionally, a significant financial savings was obtained by forgoing SPE entirely since the need for cartridges and solvents was obviated. The production of chemical solvent waste generated during SPE was also eliminated by implementing the heating procedure in its place.

3.6 Determination of serum MAs

Following the optimization of the heating procedure, the method was applied to the determination of MAs in serum. A single lot of pooled serum was divided into two fractions to undergo either SPE or heat-assisted extraction. Sample electropherograms from each method are shown in Figure 5. Concentrations of SDMA, ADMA, and MMA from samples that underwent the heating procedure were quantified using the calibration curve from the non-extracted standards (Figure S1). Similarly, the endogenous MA concentrations from samples that were prepared with SPE were determined from the calibration curve constructed from standards subjected to SPE themselves (Figure S2). The calculated values from the two methods are reported in Table 2. A comparison shows that the mean values are similar in the two sample preparation methods even though the absolute peak areas were higher in SPE-prepped samples than in heat-prepped samples. This discrepancy arises from the differences in recoveries between the two methods. The SPE recoveries for MAs were ~90% (Table S2) while the recoveries from heat-prepped samples were only ~55% (Figure

4). The calibration curves compensated for this, and following normalization of the peak areas, no significant differences in MA concentrations were determined between the two sample preparation methods. It should also be noted that the precision of the heat-assisted extraction method was better than that from the more conventionally accepted SPE method as evidenced by lower relative standard deviations for each analyte of interest.

The MA concentrations determined in this experiment are within the range of those reported in the literature. Average endogenous concentrations compiled from a number of reports found that SDMA, ADMA, and MMA were present at 480 nM [7, 8, 10, 15, 27, 32–37], 605 nM [7–10, 12, 14, 15, 27, 32–40], and 142 nM [7, 12, 35], respectively, while the concentration ranges were 370–750 nM, 340–1030 nM, and 70–195 nM for each analyte, respectively. The large concentration ranges were most likely due to a combination of the different clinical populations used in each study and inherent patient-to-patient variability. The MA concentrations found in this study were all below the average inter-study values, but still fell mostly within the reported ranges.

4 Concluding remarks

Matrix effects can have a substantial impact on the quality of an analytical measurement. To diminish their influence, sample preparation steps must be undertaken, which can be major obstacles to sample throughput. As a result, this study was conducted to develop a rapid and inexpensive method to extract small molecules from serum samples by means of a heat-assisted extraction procedure. This procedure was found to effectively isolate small molecules in an aqueous solution compatible with the derivatization reaction with no-net-dilution. This allowed low abundance biomarkers of CVD to be extracted much more quickly and inexpensively than with a commonly employed SPE method. Serum concentrations of MAs determined by the two methods were in good agreement with each other and with previously reported values in the literature after analysis by CE-LIF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Dr. Roger Dreiling, Connie Broers, and Faith Nilhas at Lawrence Memorial Hospital for providing blood samples for this project, as well as Nancy Harmony for her assistance in the preparation of this manuscript. Funding for this work was provided by NIH NS61202, NIH NS42929, and the Ralph N. Adams Institute for Bioanalytical Chemistry. THL would also like to thank the American Heart Association for providing a predoctoral fellowship to sponsor this work.

Abbreviations

MA	methylarginine
CVD	cardiovascular disease
NOS	nitric oxide synthase
ADMA	asymmetric dimethylarginine
MMA	monomethylarginine
SDMA	symmetric dimethylarginine
PA	propylarginine
NDA	naphthalene-2,3-dicarboxaldehyde

SBE-CD	sulfobutylether- β -cyclodextrin
SCX	strong cation exchange

References

1. Dusting GJ. J Vasc Res. 1995; 32:143–161. [PubMed: 7772676]
2. Tran CTL, Leiper JM, Vallance P. Atheroscler Suppl. 2003; 4:33–40. [PubMed: 14664901]
3. Bogle RG, MacAllister RJ, Whitley GSJ, Vallance P. Am J Physiol. 1995; 269:C750–C756. [PubMed: 7573406]
4. Zakrzewicz D, Eickelberg O. BMC Pulm Med. 2009; 9:5. [PubMed: 19178698]
5. Vallance P, Leiper J. Arterioscler, Thromb, Vasc Biol. 2004; 24:1023–1030. [PubMed: 15105281]
6. Culotta E, Koshland DE Jr. Science. 1992; 258:1862–1865. [PubMed: 1361684]
7. Wang Z, Tang WHW, Cho L, Brennan DM, Hazen SL. Arterioscler, Thromb, Vasc Biol. 2009; 29:1383–1391. [PubMed: 19542023]
8. Ajtay Z, Scalera F, Cziraki A, Horvath I, Papp L, Sulyok E, Szabo C, Martens-Lobenhoffer J, Awiszus F, Bode-Boeger SM. Int J Mol Med. 2009; 23:651–657. [PubMed: 19360324]
9. Krempf TK, Maas R, Sydow K, Meinertz T, Boeger RH, Kaehler J. Eur Heart J. 2005; 26:1846–1851. [PubMed: 15860520]
10. Zeller M, Korandji C, Guillard JC, Sicard P, Vergely C, Lorgis L, Beer JC, Duviard L, Lagrost AC, Moreau D, Gamber P, Cottin Y, Rochette L. Arterioscler, Thromb, Vasc Biol. 2008; 28:954–960. [PubMed: 18276906]
11. Miyazaki H, Matsuoka H, Cooke JP, Usui M, Ueda S, Okuda S, Imaizumi T. Circulation. 1999; 99:1141–1146. [PubMed: 10069780]
12. Chirinos JA, David R, Bralley JA, Zea-Diaz H, Munoz-Atahualpa E, Corrales-Medina F, Cuba-Bustanza C, Chirinos-Pacheco J, Medina-Lezama J. Hypertension. 2008; 52:1051–1059. [PubMed: 18852383]
13. Dueckelmann C, Mittermayer F, Haider DG, Altenberger J, Eichinger J, Wolzt M. Arterioscler, Thromb, Vasc Biol. 2007; 27:2037–2042. [PubMed: 17569878]
14. Yoo JH, Lee SC. Atherosclerosis. 2001; 158:425–430. [PubMed: 11583722]
15. Leong T, Zylberstein D, Graham I, Lissner L, Ward D, Fogarty J, Bengtsson C, Bjorkelund C, Thelle D. Arterioscler, Thromb, Vasc Biol. 2008; 28:961–967. [PubMed: 18292394]
16. Shihabi, ZK. Handbook of Capillary Electrophoresis. 2. CRC; 1997.
17. Chiu ML, Lawi W, Snyder ST, Wong PK, Liao JC, Gau V. JALA. 2010; 15:233–242.
18. Huggins C, Cleveland AS, Jensen EV. JAMA, J Am Med Assoc. 1950; 143:11–15.
19. London M, Marymont JH Jr. Clin Chem. 1964; 10:298–305. [PubMed: 14165559]
20. Marymont JH Jr, London M. Clin Chem. 1964; 10:942–949. [PubMed: 14228273]
21. London M, Freiburger IA, Marymont JH Jr. Clin Chem. 1967; 13:970–975. [PubMed: 6054702]
22. Schwedhelm E. Vasc Med. 2005; 10(Suppl 1):S89–95. [PubMed: 16444874]
23. Martens-Lobenhoffer J, Bode-Boeger SM. J Chromatogr, B: Anal Technol Biomed Life Sci. 2007; 851:30–41.
24. Teerlink T. J Chromatogr, B: Anal Technol Biomed Life Sci. 2007; 851:21–29.
25. Linz TH, Snyder CM, Lunte SM. J Lab Autom. 2012; 17:24–31. [PubMed: 22357605]
26. Vallance P, Leone A, Calver A, Collier J, Moncada S. Lancet. 1992; 339:572–575. [PubMed: 1347093]
27. Teerlink T, Nijveldt RJ, de JS, van LPAM. Anal Biochem. 2002; 303:131–137. [PubMed: 11950212]
28. Matuszewski BK, Givens RS, Srinivasachar K, Carlson RG, Higuchi T. Anal Chem. 1987; 59:1102–1105. [PubMed: 3592222]
29. Marymont JH, London M. Am J Clin Pathol. 1963; 39:342–348. [PubMed: 13933236]
30. Marymont JH Jr, London M. Clin Chem. 1964; 10:937–941. [PubMed: 14228272]

31. Benotti J, Pino S, Gardyna H. Clin Chem. 1966; 12:37–39. [PubMed: 5900598]
32. Boger RH, Bode-Boger SM, Szuba A, Tsao PS, Chan JR, Tangphao O, Blaschke TF, Cooke JP. Circulation. 1998; 98:1842–1847. [PubMed: 9799202]
33. Valtonen P, Karppi J, Nyyssoenen K, Valkonen VP, Halonen T, Punnonen K. J Chromatogr, B: Anal Technol Biomed Life Sci. 2005; 828:97–102.
34. Martens-Lobenhoffer J, Bode-Boeger SM. Clin Chem. 2006; 52:488–493. [PubMed: 16384887]
35. Weaving G, Rocks BF, Bailey MP, Titheradge MA. J Chromatogr, B: Anal Technol Biomed Life Sci. 2008; 874:27–32.
36. Marra M, Bonfigli AR, Testa R, Testa I, Gambini A, Coppa G. Anal Biochem. 2003; 318:13–17. [PubMed: 12782025]
37. Schwedhelm E, Maas R, Tan-Andresen J, Schulze F, Riederer U, Boeger RH. J Chromatogr, B: Anal Technol Biomed Life Sci. 2007; 851:211–219.
38. Schnabel R, Blankenberg S, Lubos E, Lackner KJ, Rupprecht HJ, Espinola-Klein C, Jachmann N, Post F, Peetz D, Bickel C, Cambien F, Tiret L, Muenzel T. Circ Res. 2005; 97:e53–e59. [PubMed: 16100045]
39. Trapp G, Sydow K, Dulay MT, Chou T, Cooke JP, Zare RN. J Sep Sci. 2004; 27:1483–1490. [PubMed: 15638156]
40. Causse E, Siri N, Arnal JF, Bayle C, Malatray P, Valdiguie P, Salvayre R, Couderc F. J Chromatogr, B: Biomed Sci Appl. 2000; 741:77–83. [PubMed: 10839134]

Figure 1.
Structures of the methylarginines of interest and their average concentrations in blood.

Figure 2. Schematic of the (a) heat-assisted extraction and (b) solid-phase extraction sample preparation methods used to extract MAs from serum.

Figure 3.
A representative electropherogram of the separation of 500 nM SDMA, ADMA, MMA, PA, and arginine derivatized with NDA/CN.

Figure 4. Evaluation of the effect of extraction times on MA recovery. Congealed serum was incubated with water for the indicated time ($n = 3$ for each point) and then analyzed via CE-LIF.

Figure 5.
Representative electropherograms of serum samples prepared by the (a) SPE or (b) heating procedures.

Table 1

Comparison of the conductivity and pH of extracted serum samples as compared to the borate used for derivatization.

Sample	Normalized conductivity	pH
50 mM borate	1.0	9.2
Heated serum	1.1	8.4
SPE serum	0.07	7.6

Table 2

Endogenous serum concentrations of MAs. MAs from a single lot of pooled serum were isolated by either the heating or SPE sample preparation methods (n = 3 samples for each method). Each sample was analyzed by CE-LIF in duplicate.

Compound	Heat (nM)	SPE (nM)
SDMA	436 ± 46	385 ± 64
ADMA	374 ± 49	307 ± 42
MMA	53 ± 16	71 ± 43