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SHORT COMMUNICATION

An innovative, time- and cost-saving method for the quantification of asymmetric dimethylarginine in serum by high-performance liquid chromatography without evaporation

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A new time- and energy-saving method for the determination of asymmetric dimethylarginine in human serum is presented. Here, a newly developed eluent was used in the sample cleanup of the solid-phase extraction whose composition makes an evaporation step redundant. After derivatization, asymmetric dimethylarginine was quantified by high-performance liquid chromatography with fluorescent detection. The conditions of the solid-phase extraction lead to a relative recovery of asymmetric dimethylarginine of 101%. A concentration of 25 ng/mL was found as the limit of quantification and the batch was highly linear from 25 to 800 ng/mL with the correlation coefficient $R^2 = 0{,}9999$. Intra-assay coefficients of variation <2.1% and inter-assay coefficients of variation <3.1% indicate a high precision. Since no evaporation is necessary compared to previously published methods, this newly presented method does not only save time, but also is a cost- and energy-saving alternative for the routine quantification of asymmetric dimethylarginine in serum.

KEYWORDS
asymmetric dimethylarginine, evaporation, high-performance liquid chromatography, solid-phase extraction

1 | INTRODUCTION

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase (NOS) [1] and therefore, plays an important role inter alia in endothelial function. In addition to ADMA, there are the methylated arginine derivatives symmetric dimethylarginine (SDMA) and monomethylarginine (L-NMMA). However, ADMA is a much more potent inhibitor of NOS than SDMA [2] and the concentration of ADMA in the circulation is significantly higher than that of L-NMMA [2,3]. So, ADMA has the greatest physiological and pathophysiological importance of these derivatives.

Various methods for measuring the serum concentration of ADMA are described in the literature. The most commonly used method for this purpose is the

Article Related Abbreviations: ADMA, asymmetric dimethylarginine; CV, coefficient of variation; L-NMMA, monomethylarginine; MEA, monoethylarginine; NOS, nitric oxide synthase; SDMA, symmetric dimethylarginine; SPE, solid-phase extraction

determination by HPLC with fluorimetric detection after solid-phase extraction (SPE) [4,5]. Furthermore, it is also possible to quantify ADMA by LC–MS [6], GC–MS [7], or ELISA [8]. However, an HPLC method with fluorimetric detection is more cost-saving than a method using mass spectrometry [5] and more precise than an ELISA assay [9]. In previously described HPLC methods, the sample preparation was carried out by SPE and subsequent evaporation of the SPE eluent [1,10–14]. The eluents used for the SPE process usually contain ammonia and methanol [4]. A disadvantage of these methods is that the evaporation step takes a lot of time and thus extends the total analysis time. Therefore, these methods are only of limited use for routine determinations of ADMA concentrations and for large-scale studies.

For this reason, a time-saving and reliable assay to determine concentrations of ADMA by using a newly developed SPE eluent, which makes the evaporation step redundant, is presented.

2 | MATERIALS AND METHODS

2.1 | Materials and equipment

NG,NG-Dimethylarginine dihydrochloride (ADMA), phthaldialdehyde, and 3-mercaptopropionic acid were obtained from Sigma–Aldrich (Seelze, Germany). NG-Monoethyl-L-arginine TFA (MEA, internal standard) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Methanol, acetonitrile, potassium chloride, potassium borate, and potassium hydroxide were obtained from Merck (Darmstadt, Germany). Hydrochloric acid 0.1 M was purchased from VWR Chemicals (Darmstadt, Germany) and potassium dihydrogen phosphate from Carl Roth (Karlsruhe, Germany).

Water was deionized and filtered using the water purification system Arium® Basic from Sartorius (Goettingen, Germany). HPLC was carried out by a Shimadzu (Duisburg, Germany) Prominence® system with an RF-10AXL® fluorescence detector. The software LabSolutions® from Shimadzu was used for data acquisition and evaluation.

2.2 | Preparation of standards

Stock solutions of ADMA (100 µg/mL = 0.494 mM) and monoethylarginine (MEA, 1 mM) were prepared in 10 mM hydrochloric acid and stored at -80°C. Calibration standards of ADMA with 50, 100, and 200 ng/mL were prepared by diluting the stock solution with water. A working solution of the internal standard MEA (5 µM) was prepared by diluting the stock with water. The use of the non-endogenous internal standard MEA was first described by Blackwell et al. [10].

2.3 | Sample preparation

The SPE procedure and derivatization were executed according to Teerlink et al. [1] with modifications. For extraction the cartridges Oasis® MCX 1cc 10 mg 30 µm from Waters (Eschborn, Germany) were used. The novel SPE eluent was prepared with 0.4 g potassium chloride, 5.96 mL water, 0.04 mL 5 M potassium hydroxide solution, and 4 mL methanol.

First, the cartridges were washed with 0.5 mL SPE eluent and equilibrated with 0.5 mL water. Serum (0.1 mL) or calibration standard was mixed with 0.05 mL MEA working solution and 0.35 mL water until loading on the cartridge. After two washing steps with 0.5 mL 0.1 M hydrochloric acid and 0.5 mL methanol, the analytes were eluted in HPLC vials with 0.3 mL SPE eluent [1].

A stock solution of the derivatization reagent was prepared with 10 mg *O*-phthaldialdehyde (OPA), 0.2 mL methanol, 1.8 mL potassium borate buffer 0.2 M pH 9.5, and 10 µL 3-mercaptopropionic acid. A working solution was prepared by diluting the stock solution 1:5 with the borate buffer.

Working solution (0.3 mL) was added to the HPLC vials containing the analyte and incubated for 3 min at room temperature [1]. Then, the vials were transferred to the autosampler.

2.4 | Chromatography

HPLC was carried out by a Shimadzu (Duisburg, Germany) Prominence® system consisting of the degasser DGU-20A 3R®, the two pumps LC-20AT®, the autosampler SIL-20AC HT®, the column oven CTO-10AS VP®, the system controller CBM-20A® and the fluorescence detector RF-10AXL®. As analytical column, a Kinetex® EVO C18 2.6 µm 150 × 4.6 100A protected by a SecurityGuard Ultra® cartridge for EVO-C18 (Phenomenex, Aschaffenburg, Germany) was used.

Mobile phase was a potassium phosphate buffer 25 mM pH 6.5 containing 9.7% acetonitrile. Five microliter was injected into the system and separated at a flow rate of 1 mL/min and a column temperature of 40°C. Runtime was 13 min and fluorescence was measured at excitation of 340 nm and emission of 455 nm. ADMA was quantified on the basis of the ratio of peak areas. An isocratic washing step was performed by increasing the proportion of acetonitrile to 35% for 10 min.

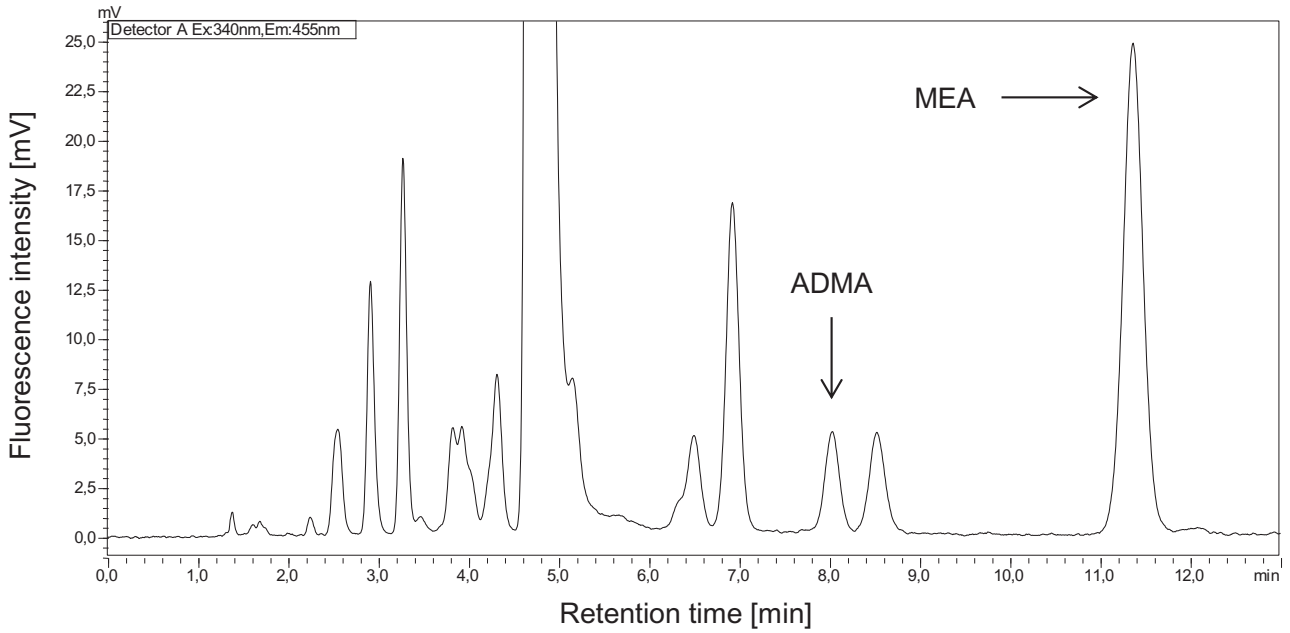

FIGURE 1 Representative chromatogram of a serum sample analyzed with the described method for ADMA determination. The concentration of ADMA in this serum sample is 92.9 ng/mL. Analytical column: Kinetex® EVO C18 2.6 µm 150 × 4.6 100 A; mobile phase: potassium phosphate buffer 25 mM pH6.5 containing 9.7% acetonitrile; flow rate: 1 mL/min; runtime: 13 min; column temperature: 40°C; fluorimetric detection at excitation of 340 nm and emission of 455 nm

Afterward, the column was equilibrated for 6 min with 9.7% acetonitrile.

2.5 | Method validation

To analyze the LOQ, the baseline noise was determined and compared to the height of the ADMA peak. The LOQ was defined as the concentration point where the signal-to-noise ratio was 10.

The LOD has not been determined for this method as all human serum contains ADMA. Only the concentration of ADMA can be altered by pathological conditions. To date, no diseases are known to cause ADMA to be undetectable in serum.

For analysis of assay linearity, a calibration curve was constructed using six calibration points (25, 50, 100, 200, 400, 800 ng/mL), thereby receiving equation and correlation coefficient of the calibration curve.

For ADMA, no certified reference standards were available to check accuracy. So, for each calibration point, the ADMA concentration was calculated from the signal using the equation of the linear calibration curve. Accuracy was analyzed by comparison of the calculated ADMA concentration with the real ADMA concentration and deviation was expressed as bias.

Precision of the method was tested using two different serum samples that contained a high or a low ADMA concentration. Both serum samples were measured three times a day on 6 days and the intra-assay and the inter-assay coefficients of variation (CVs) were calculated.

The effect of the matrix on linearity was analyzed according to Teerlink et al. [1]. To various volumes (25, 50, 100, 150, 200, 250 µL) of a serum pool, 50 µL MEA working solution was added and adjusted to a total volume of 500 µL with water. It was investigated if plots of the calculated amount of ADMA versus the volume of serum pool were linear [1].

3 | RESULTS

3.1 | Sample preparation

The chosen conditions resulted in 93.4% ($n = 6$) SPE recovery of ADMA and 92.8% ($n = 6$) SPE recovery of MEA. Thus, the relative recovery of ADMA was 101%. Evaporation of the eluent after SPE was not necessary, since it contained no ammonia that could react with the derivatization reagent.

3.2 | Chromatography

In Figure 1, a typical chromatogram of a serum sample is shown. The chromatographic conditions after SPE

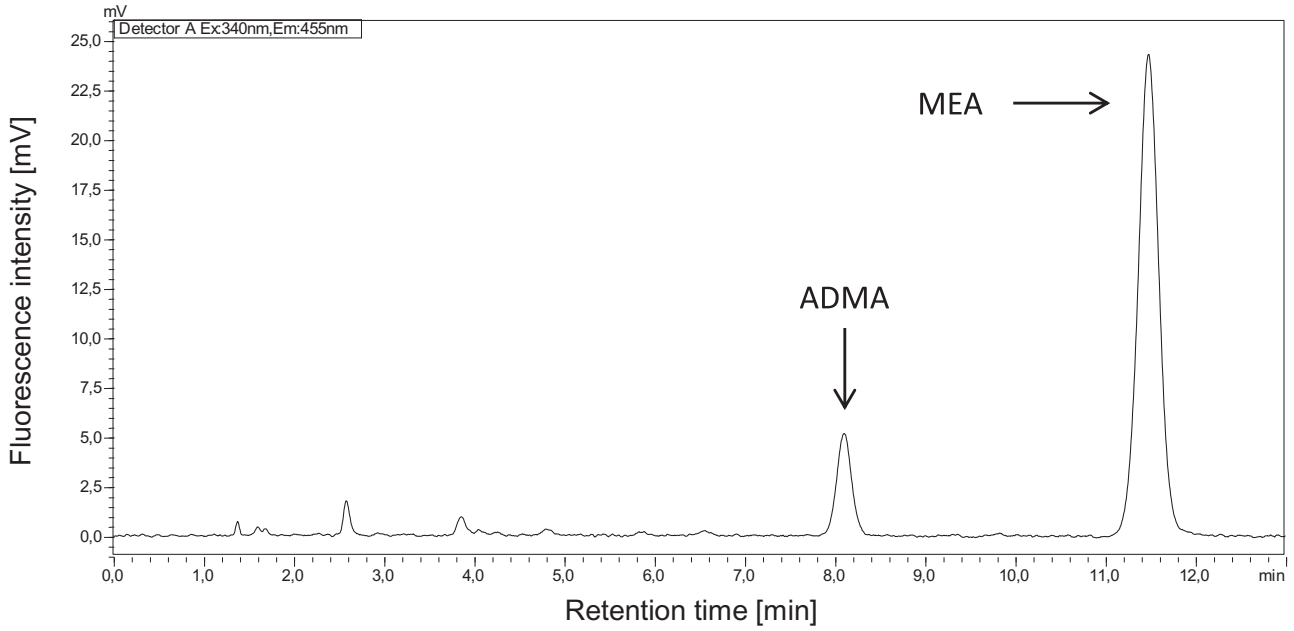

FIGURE 2 Representative chromatogram of an aqueous calibration solution that has been prepared in the same way as a serum sample with the described method. This solution contains ADMA at a concentration of 100 ng/mL. Analytical column: Kinetex® EVO C18 2.6 µm 150 × 4.6 100A; mobile phase: potassium phosphate buffer 25 mM pH6.5 containing 9.7% acetonitrile; flow rate: 1 mL/min; runtime: 13 min; column temperature: 40°C; fluorimetric detection at excitation of 340 nm and emission of 455 nm

procedure and derivatization led to baseline separation of the ADMA peak and the MEA peak. The separation performance, expressed as calculated number of theoretical plates, was 10 425 and thus correspond to the state of the art. In the serum, the chromatogram of which is shown in Figure 1, an ADMA concentration of 92.9 ng/mL was measured. For comparison, a chromatogram of an aqueous calibration solution containing 100 ng/mL ADMA is shown in Figure 2.

3.3 | Method validation

An ADMA concentration of 25 ng/mL was found as the LOQ. The calibration curve of the ratios of peak area of ADMA and MEA against ADMA concentration shown in Figure 3 was highly linear with $R^2 = 0.9999$ in the range from 25 to 800 ng/mL. Outliers were not obtained. The coefficients of variation (CVs) of the calibration concentrations were low with a maximum of 2.8%. This high linearity was to be expected, since fluorescence detectors usually show linear detector response over several orders of magnitude [4].

Accuracy and precision data of the method are shown in Table 1. The deviation between calculated ADMA concentration and real ADMA concentration was 12% at the LOQ and 5.6% at a concentration of 50 ng/mL. For all other concentrations of the calibration curve (100–800 ng/mL), the bias was less than 3.0% indicating the accuracy of the method.

Intra-assay CVs of the concentration were <2.1% and inter-assay CVs were <3.1%. Thus, the developed method allows a precise measurement of the ADMA concentration.

Plots of calculated ADMA concentration versus volume of serum pool were linear with $R^2 = 0.9978$. So, there are no significant matrix effects.

4 | DISCUSSION

The applied cartridges were based on Oasis® MCX that is a reversed-phase, strong cation-exchange polymer containing sulfonic acid groups. Due to their high acidity, the sulfonic acid groups deprotonate and are negatively charged during the SPE process. Since deprotonated sulfonic acid groups can interact well with positively charged protonated bases such as guanidine groups, this polymer is suitable to separate the basic arginine derivatives from other amino acids.

In the standard protocol of Oasis® MCX an eluent containing ammonia is recommended. However, the use of ammonia requires a time-consuming evaporation step after the SPE as it can react with the derivatization reagent. In previously published methods, the eluent often contains about 40% water in addition to methanol

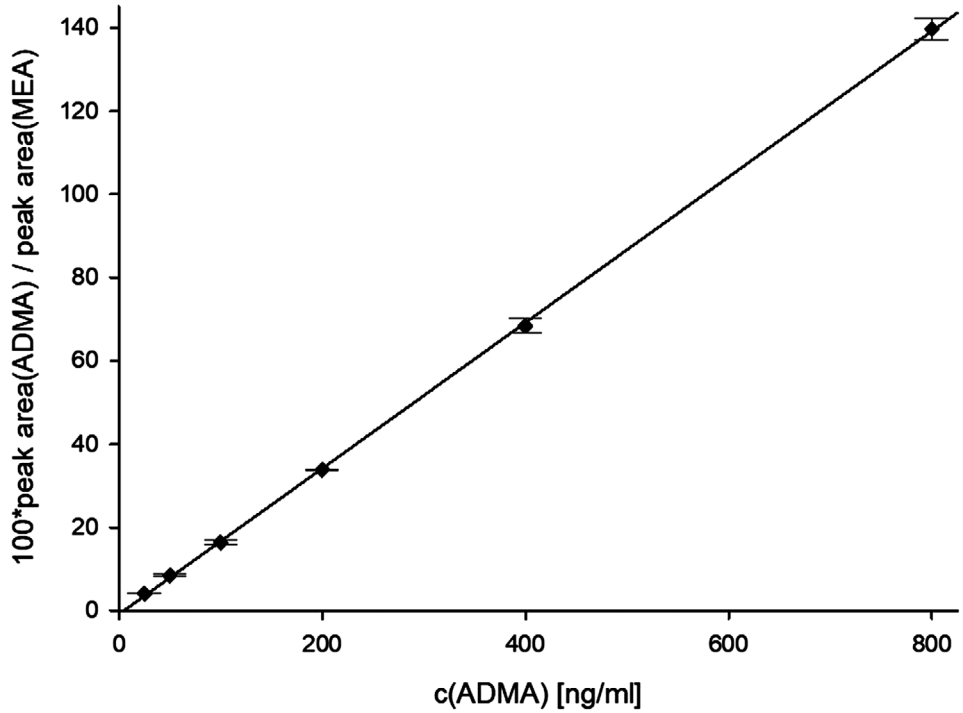

FIGURE 3 Linearity of the method: calibration curve (ratio of peak area of ADMA and MEA versus ADMA concentration) with ADMA concentrations ranging from 25 to 800 ng/mL. The standard deviations are shown as bars at the respective concentrations. The equation of the linear regression line was $y = 0.1750x - 0.8012$ whereby x = c(ADMA) and $y = 100 \times$ peak area(ADMA)/peak area(MEA). c(ADMA), concentration of ADMA

TABLE 1 Accuracy and Precision data of the described method. These data show the reliability of the method

Accuracy	**c(ADMA) [ng/ml]**	**Bias [%]**
(n = 3)	25	12.0
	50	5.6
	100	3.0
	200	1.1
	400	2.3
	800	1.2
Precision	**serum sample with**	**CV [%]**
CV intra-assay (n = 3 × 6 [a])	Low ADMA	2.1
	High ADMA	1.9
CV inter-assay (n = 6 × 3 [a])	Low ADMA	3.1
	High ADMA	2.8

[a]For the calculation of CVs, serum samples were measured 3 times a day on 6 days.Abbreviations: c(ADMA), concentration of ADMA; CV, coefficient of variation.

and ammonia [1,10,13]. Due to this high proportion of water, evaporation of the eluent is a time-consuming step, leading to longer sample preparation times. Thus, a newly developed eluent without ammonia was used for the presented SPE procedure. To remove the analyte from the strong cation exchange polymer, the eluent should be basic and have a high cation concentration. These conditions were fulfilled by adding potassium hydroxide and potassium chloride to methanol and water. By applying the newly developed eluent, the evaporation after SPE was not necessary resulting in a significantly shorter sample preparation time. Consequently, energy can be saved, and costs are reduced, since time-consuming evaporation would also be associated with power consumption. The recoveries of ADMA (93.4%) and MEA (92.8%) after SPE under these conditions are adequate.

In comparison to the method of Teerlink et al. [1], further modifications were made: instead of L-NMMA, the non-endogenous substance MEA was used as the internal standard. As a result, the developed method is less susceptible to errors than methods using endogenous substances such as L-NMMA or homoarginine. These arginine derivatives may be elevated in pathological conditions, such as renal failure, where ADMA may also be elevated [10]. The relative SPE recovery of ADMA of 101% demonstrates the utility of MEA as the internal standard.

Teerlink et al. used Oasis MCX 30 mg for the SPE method [1] while Oasis MCX 10 mg is used for the present method. Therefore, only 0.1 mL serum instead of 0.2 mL is needed for the determination of ADMA.

Another advantage of this method is the use of a semiporous column with a small particle size of 2.6 µm (Kinetex® EVO C18 2.6 µm) instead of a 5 µm column [1] for chromatography. The high resolution of that column allows application of a comparatively high proportion of acetonitrile in the eluent, leading to a shorter run time.

In Teerlink's method, the chromatographic separation was performed at a column temperature of 30°C [1]. The increase of column oven temperature during the development of the present method up to 40°C has shortened the run time without loss of resolution.

Furthermore, the number of calibration points has been increased from one [1] to three, resulting in a more accurate evaluation.

The determined LOQ of 25 ng/mL is sufficient for the measurement of ADMA in plasma [4]. Since the concentration of ADMA in plasma does not differ significantly from that in serum [1], this value is also adequate for the quantification of ADMA in serum. The concentration of ADMA in serum is expected to be within the established linear range of 25–800 ng/mL [4,15].

The calculated inter-assay and intra-assay CVs of this method are very low with values <3.1 and <2.1%, respectively. The resulting high precision enables a reliable determination of the serum concentration of ADMA [10].

5 | CONCLUDING REMARKS

In summary, a reliable method for quantification of ADMA in serum was developed by introducing a new eluent for the SPE procedure. The main advantages of this method are savings in time, cost, and energy, as evaporation of the new eluent is redundant. Thus, this method is suitable for routine analysis of serum concentrations of ADMA and for large scale studies. Nevertheless, further improvements of the method are possible, such as automatic derivatization or automation of the SPE process. Since no evaporation is required, the method should be well suited for automatization.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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