

# Affimers as an Alternative to Antibodies in an Affinity LC–MS Assay for Quantification of the Soluble Receptor of Advanced Glycation End-Products (sRAGE) in Human Serum

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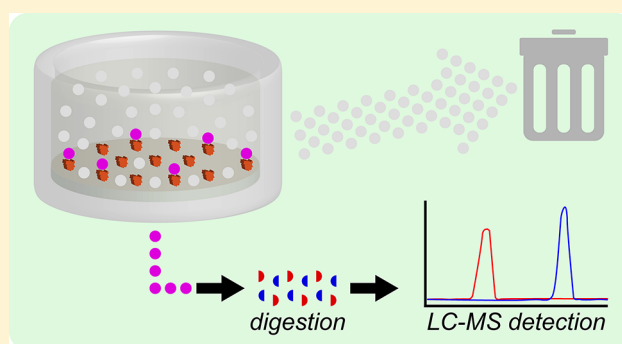
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## Supporting Information

**ABSTRACT:** Antibodies are indispensable tools in biomedical research, but their size, complexity, and sometimes lack of reproducibility created a need for the development of alternative binders to overcome these limitations. Affimers are a novel class of affinity binders based on a structurally robust protease inhibitor scaffold (i.e., Cystatin A), which are selected by phage display and produced in a rapid and simple *E. coli* protein expression system. These binders have a defined amino acid sequence with defined binding regions and are versatile, thereby allowing for easy engineering. Here we present an affimer-based liquid chromatography–mass spectrometry (LC–MS) method for quantification of the soluble Receptor of Advanced Glycation End-products (sRAGE), a promising biomarker for chronic obstructive pulmonary disease. The method was validated according to European Medicines Agency and U.S. Food and Drug Administration guidelines and enabled quantitation of serum sRAGE between 0.2 and 10 ng/mL. Comparison between the affimer-based method and a previously developed, validated antibody-based method showed good correlation ( $R^2 = 0.88$ ) and indicated that 25% lower sRAGE levels are reported by the affimer-based assay. In conclusion, we show the first-time application of affimers in a quantitative LC–MS method, which supports the potential of affimers as robust alternatives to antibodies.

**KEYWORDS:** affimers, biomarker, COPD, immunoprecipitation, LC–MS, quantification



## INTRODUCTION

Antibodies have found numerous applications in present-day biomedical research owing to their capability of binding antigens with high affinity and specificity. These affinity reagents are, for example, widely used for the enrichment of target molecules, the detection of target molecules, and the analysis of cells.<sup>1</sup> Antibodies have furthermore become well-rooted in clinical practice, as exemplified by the more than 60 therapeutic antibodies reaching the market since the first therapeutic antibody, Muromonab-CD3, received regulatory approval from the United States (U.S.) Food and Drug Administration (FDA) in 1986.<sup>2</sup>

The possibilities and importance of antibodies are beyond dispute, although these proteins are not without their limitations.<sup>2</sup> For example, antibodies are large and complex proteins with a molecular weight around 150 kDa featuring several disulfide bonds and N-glycosylated asparagine residues.<sup>3</sup> Antibody production accordingly is a challenging and costly process. Their production is furthermore difficult to

control, as reflected in an increasing number of reports describing reproducibility issues.<sup>2–5</sup> Consequently, considerable efforts have been expended in recent years to develop nonantibody affinity ligands, which resulted in an impressive number of putative antibody alternatives, including adnectins, affibodies, anticalins, avimers, DARPin, fynomers, knottins, and kunitz domains.<sup>2,6</sup> Affimers that are derived from the cysteine protease inhibitor Cystatin A represent another example of antibody alternatives. These innovative affinity ligands can be produced quickly, relatively easily, and without the use of animals. Affimers are small, versatile, and stable proteins that can be engineered to bind target proteins with high affinity and selectivity.<sup>6</sup> Their efficiency of binding linear targets (e.g., peptides) is, however, limited. Moreover, examples of their application are still limited in number because affimer technology has only been established recently,

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although the few available examples do indicate that affimers represent attractive alternatives to antibodies, at least for some applications.<sup>7–15</sup>

Recently, we reported on the development and validation of an antibody-based immunoaffinity liquid chromatography–mass spectrometry (LC–MS) method for quantification of the soluble Receptor of Advanced Glycation End-products (sRAGE) in human serum.<sup>16</sup> sRAGE has anti-inflammatory properties by acting as a decoy receptor for pro-inflammatory ligands in the lungs and is considered to be a promising biomarker candidate for chronic obstructive pulmonary disease (COPD) on the basis of findings in several large-scale clinical studies.<sup>17–21</sup> In addition, sRAGE is considered to be an interesting biomarker as well for other diseases including diabetes mellitus, autoimmune diseases, and neurological diseases.<sup>22</sup> The corresponding findings are, however, all based on data obtained with a single sRAGE immunoassay.<sup>20</sup> In our initial report on the antibody-based LC–MS method, we presented data that triggered concerns about the accuracy of the immunoassay.<sup>16</sup> Because our antibody-based LC–MS method relies on antibodies from the vendor of the sRAGE immunoassay, we felt the need for complementary, antibody-free strategies for enriching sRAGE to support the further development of sRAGE as a biomarker.

In this study, we describe the application of affimers in an LC–MS method for the quantification of sRAGE in human serum. The affimer-based method met the requirements as stipulated in the European Medicines Agency (EMA) and FDA guidelines on bioanalytical method validation,<sup>23,24</sup> and its measurements correlated well with those carried out using a previously developed antibody-based LC–MS method. The successful application of affimers in a quantitative sRAGE method is expected to contribute to further elucidating the role of sRAGE in COPD pathophysiology and in facilitating further development for this highly promising COPD biomarker candidate.

## METHODS

### Chemicals and Materials

Recombinant human sRAGE (rh-sRAGE; cat. no. C423; UniProtKB ID Q15109; Ala23-Ala344 with C-terminal hexahistidine tag) was purchased from Novoprotein (Summit, NJ), anti-sRAGE affimers (raised against recombinant human sRAGE from Novoprotein, cat. no. C423) were produced and supplied by Avacta Life Sciences (Wetherby, U.K.), and stable-isotope-labeled RAGE peptides (i.e., IGEPLVLK\* and VLSPQGGPWDSVAR\*) were synthesized by Pepscan Presto (Lelystad, The Netherlands). Acetonitrile (ACN; LC–MS grade) was obtained from Biosolve (Valkenswaard, The Netherlands), and sequencing grade modified trypsin was purchased from Promega (Madison, WI). Nunc-Immuno MicroWell 96-well plates with MaxiSorp coating (cat. no. M9410), bovine serum albumin (BSA; cat. no. A7638), Trizma base (tris; cat. no. T6791), and phosphate-buffered saline (PBS; 10×; cat. no. D1408) as well as all other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

### Serum Samples

Pooled human serum from Seralab (West Sussex, U.K.) was diluted with 10 mM tris-buffered saline, pH 7.5 (TBS buffer) containing 1% BSA for the preparation of the low-level quality control (QC-low) sample or was fortified with recombinant

RAGE at two levels to obtain the midrange (QC-medium) and high-level (QC-high) quality-control samples. Recovery and spike recovery experiments were carried out using six different sources of human serum from healthy subjects (all from Seralab). Recovery and spike recovery experiments were furthermore performed on a lipemic serum sample (triglyceride content >150 mg/dL; obtained from Seralab) and a hemolytic sample that was prepared by adding freeze–thawed whole blood (2%) to human serum.

### Calibrants and Internal Standard

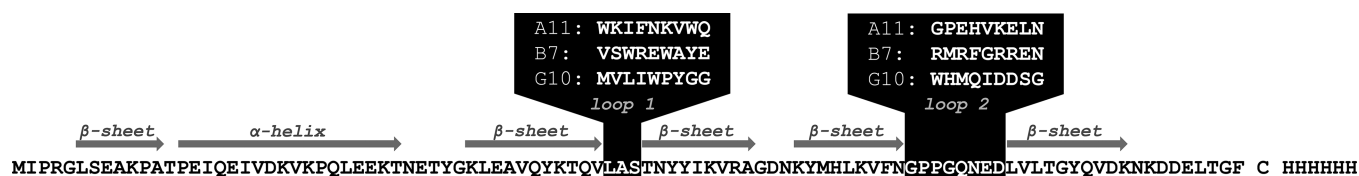
Lyophilized sRAGE was dissolved in Milli-Q water to obtain a 200 µg/mL solution (based on the quantity as declared by the supplier) that was diluted to 100 µg/mL with 1× PBS, pH 7.4 (PBS buffer) after verifying the absence of proteins other than sRAGE using SDS-PAGE and MALDI-TOF MS. The resulting solution was sequentially diluted to 100 ng/mL with 1% BSA in TBS buffer (Surrogate Matrix), and calibration samples were prepared at 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 8.0, and 10.0 ng/mL in Surrogate Matrix. The internal standard (IS) stock solution was prepared by mixing equimolar amounts of the two SIL-peptides (supplied as 5 pmol/µL solutions in 5% ACN) and subsequently diluting these peptides to 5 fmol/µL with 1% dimethyl sulfoxide (DMSO) in water.

### Affimer-Based sRAGE Capture and In-Well Digestion

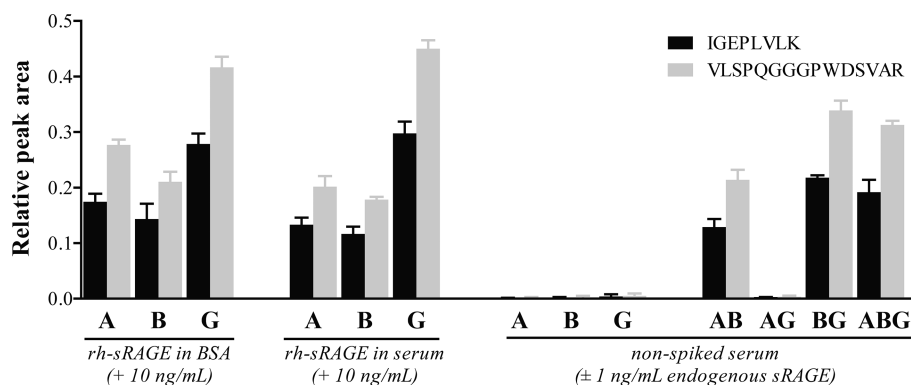
(1) Plate Coating: Microplate wells were coated overnight (room temperature) with 100 µL of PBS buffer containing 0.5 µg of the affimers B7 and G10. (Affimers were supplied as 1 mg/mL solutions in citrate-buffered saline, pH 6.5 containing 5 mM TCEP and 0.02% sodium azide.) (2) Plate Blocking: After removal of unbound affimers by three washing steps with 300 µL of wash buffer (0.05% Tween-20 in TBS buffer), uncoated surface was blocked with 300 µL of blocking buffer (0.01% BSA in TBS buffer) for 30 min while shaking on a plate shaker (600 rpm; room temperature). (3) sRAGE Capture: Wells were washed three times with 300 µL of wash buffer, and 100 µL of sample solution (for which 60 µL of serum was premixed with 60 µL of Surrogate Matrix to allow quantitative transfer of Sample Solution) was added to the wells for the capture of sRAGE (120 min; 600 rpm; room temperature). (4) Disulfide Bond Reduction: After three washing steps with 300 µL of wash buffer, 100 µL of digestion buffer (50 fmol/mL SIL-peptides in 50 mM ammonium bicarbonate (ABC) containing 10 mM TCEP) was added to the wells, and disulfide bonds were reduced following 30 min of incubation (600 rpm; room temperature). (5) Cysteine Alkylation: Thiols were alkylated in 20 mM iodoacetamide (IAM) (5 µL of 420 mM IAM in ABC) for 30 min in the dark (600 rpm; room temperature), after which nonreacted IAM was quenched with a 0.5 molar excess of DTT (5 µL of 210 mM DTT in ABC). (6) Trypsin Digestion: 100 ng of trypsin was added to each sample, plates were sealed with a 96-well cap mat (Screening Devices B.V., cat. no. SD964075), and proteins were digested following overnight incubation in an oven kept at 37 °C. Next, plates were briefly centrifuged in a plate centrifuge to spin down droplets and condensation, and the digests were acidified through the addition of 5 µL of 25% formic acid (FA).

### LC–MS

Analyses were performed with a Waters Ionkey/MS system using an ACQUITY M-Class UPLC and a XEVO TQ-S mass spectrometer (Milford, MA). Chromatographic separation was achieved on a C18-bonded Waters iKey HSS T3 separation



**Figure 1.** Amino acid sequence of the affimer scaffold in which the added C-terminal cysteine residue and a hexahistidine tag as well as the sequences of the inserted loops relevant for sRAGE binding are indicated.



**Figure 2.** Performance evaluation of the individual affimers A11, B7, and G10 (indicated as A, B, and G, respectively) as well as all affimer combinations based on the enrichment of endogenous sRAGE or recombinant human sRAGE (rh-sRAGE) from 1% BSA in TBS (BSA) and human serum, which contained endogenous sRAGE at a level of  $\sim 1$  ng/mL. The Figure shows the mean relative peak areas (plus standard deviations;  $N = 3$ ) of the proteotypic sRAGE peptides IGEPLVLK (in black; selected as quantifier peptide) and VLSPQGGPWDSVAR (in gray; selected as qualifier peptide due to its deamidation-sensitive “QG” sequence motive and due to a single nucleotide polymorphism leading to the substitution of the C-terminal arginine by a cysteine (e.g., rs116828224) occurring in 0.6% of the population).

device (1.8  $\mu\text{m}$  particles, 100  $\text{\AA}$  pore size, 150  $\mu\text{m} \times 100$  mm; cat. no. 186007261) that was kept at 40  $^{\circ}\text{C}$  using 0.1% FA in  $\text{H}_2\text{O}$  as mobile phase A and 0.1% FA in ACN as mobile phase B. Twenty  $\mu\text{L}$  of sample was loaded onto a Dionex Acclaim PepMap100 C18 trap column (5  $\mu\text{m}$  particles, 100  $\text{\AA}$  pore size, 300  $\mu\text{m} \times 5$  mm; cat. no. 160454) for 2.5 min with 3% B at 20  $\mu\text{L}/\text{min}$ . Subsequently, peptides were separated on the analytical column at 3  $\mu\text{L}/\text{min}$  with a 10 min linear gradient from 3 to 33% B, after which the column was cleaned (0.6 min at 60% B and 2.1 min at 95% B) and equilibrated (4.3 min at 3% B). Mass spectrometric detection was performed using the following conditions: ESI positive, precursor ion and fragment ion windows at unit mass resolution (0.7 amu), span 0.2, capillary voltage 3.5 kV, cone voltage 30 V, source offset 50 V, source temperature 120  $^{\circ}\text{C}$ , cone gas (nitrogen) flow 150 L/h, sheath (nanoflow) gas (nitrogen) flow 0.2 bar, and collision gas (argon) flow 0.15 mL/min. MRM transitions and settings for IGEPLVLK (selected for quantification) and VLSPQGGPWDSVAR (selected for confirmation) are presented in Table S-1. The Ionkey/MS system was operated under the Waters MassLynx software suite (version 4.1), and the TargetLynx module of this package was used for data processing.

### Method Validation

The method was validated according to EMA and FDA guidelines, and the following criteria were addressed: selectivity (e.g., spike recovery and ligand challenge tests), accuracy and precision, recovery, calibration curve, and stability (e.g., 28 days benchtop, 10 $\times$  freeze–thaw, and 13 days autosampler (10  $^{\circ}\text{C}$ )).<sup>23,24</sup> For the recovery experiment, samples were fortified with 5 ng/mL sRAGE either before or after sRAGE capture to obtain the precapture and postcapture spiked samples, respectively. The sRAGE dilutions for the recovery experiment

were prepared in 50 mM ABC because adding Surrogate Matrix to the postcapture spiked samples would introduce excessive BSA to the samples, thereby interfering with digestion and LC–MS analysis. sRAGE in Surrogate Matrix was used for the spike-recovery experiments, and the corresponding spiking procedure was similar to that of the precapture spiked samples of the recovery experiment. Ligand challenge tests were performed by adding 200 ng of fully reduced HMGB1 (HMGBiotech, Milano, Italy; cat. no. HM-116), S100A12 (Novoprotein; cat. no. C743), serum amyloid A1 (SAA1; Novoprotein; cat. no. C633), and N<sup>ε</sup>-(carboxymethyl)lysine-modified bovine serum albumin (CML-BSA; Academy Bio-Medical, Houston, TX; cat. no. 30P-CML-BS102) to the samples. For recovery and selectivity experiments, samples were incubated for at least 30 min following the addition of sRAGE or the ligands prior to initiating sRAGE capture.

### Method Comparison

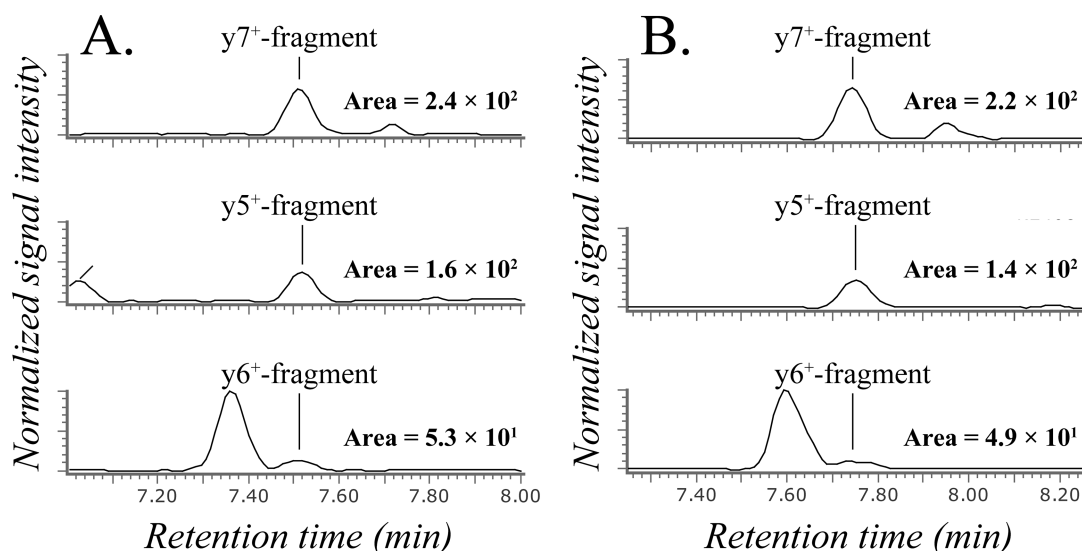
For method comparison, 40 serum samples were analyzed from a cross-sectional study (NCT00807469) within the University Medical Center Groningen (UMCG).<sup>25</sup> Ethical approval for this study has been granted by the UMCG’s review board (METc 2008/136), and the study adheres to the Declaration of Helsinki. Blood samples were collected as previously described.<sup>16</sup> In all 40 samples, sRAGE was quantified using the affimer-based sRAGE assay as well as with the previously described antibody-based sRAGE assay.<sup>16</sup>

## RESULTS AND DISCUSSION

### Affimer-Based sRAGE Enrichment

The affimers were supplied with an added C-terminal cysteine residue (see Figure 1) which allows coupling of the affimers to





**Figure 3.** Selected ion chromatograms of the y7<sup>+</sup> (quantifier), y5<sup>+</sup> (qualifier 1), and y6<sup>+</sup> (qualifier 2) fragments of the sRAGE-derived proteotypic peptide IGEPLVLK at 0.1 ng/mL in Surrogate Matrix obtained by (A) the affimer-based method and (B) the antibody-based method.<sup>16</sup> The y axes of the three MRM traces were linked and scaled to the highest observed signal observed in these traces. The presented peak areas represent the average values for all samples that were measured for the lower limit of quantification (LLOQ) determination. No statistically significant differences ( $p < 0.05$ , two-tailed Student's *t* test) between both methods were observed for all three fragments.

elucidating the mechanism behind the multiaffimer requirement would be desirable to exploit the affimers to their full potential. We furthermore acknowledge that calibration materials for complex analytes such as proteins are potential sources of bias because it is implausible that one recombinant protein can adequately represent all different forms of the analyte (so-called “proteoforms” or “protein species”)<sup>27,28</sup> that are present in biological samples.

#### Quantitative Assay Development

We aimed to develop an alternative for our previously developed antibody-based sRAGE LC–MS assay and accordingly adopted the same internal standard quantification approach using stable-isotope-labeled (SIL) peptides. The two peptides we selected for sRAGE quantification (i.e., IGEPLVLK and VLSPQGGPWDSVAR) are located in the N-terminal part of sRAGE and thereby reflect the protein domain to which most sRAGE ligands bind.<sup>29,30</sup> Further details of the selection of the SIL peptides can be found in the publication of the antibody-based method.<sup>16</sup>

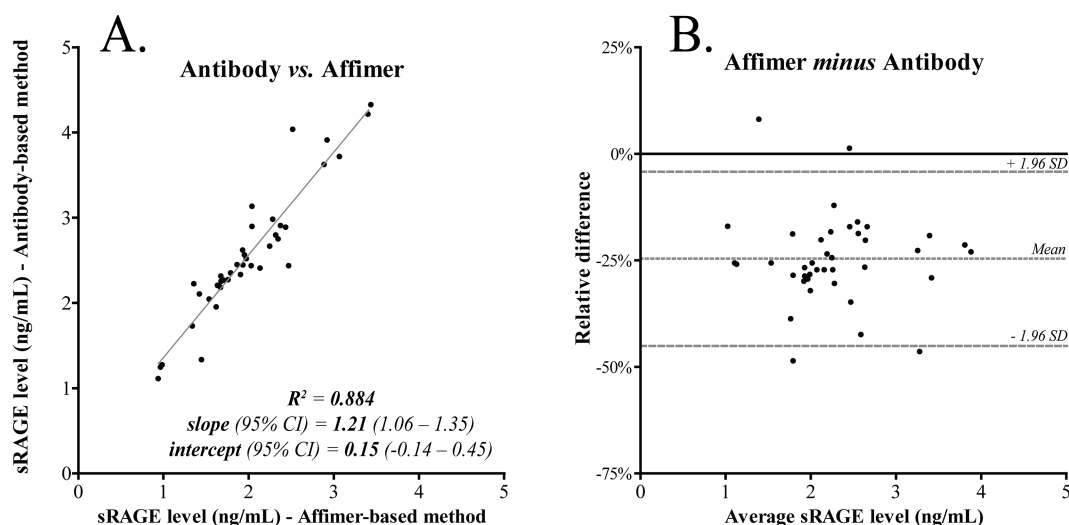
Affimer titration experiments indicated that 0.5 μg of the affimers is sufficient for the reliable and reproducible enrichment of endogenous sRAGE across the entire concentration range that is relevant for sRAGE quantification in human serum. (See Figure S-2.) For the preparation of the calibration curves, we tested 1% BSA in TBS and fetal calf serum (FCS) as surrogate matrices given that analyte-free, authentic matrix could not be obtained. The slopes of calibration curves prepared by spiking recombinant sRAGE in the BSA-based matrix and in human serum were similar, as judged from the overlapping 95% confidence intervals. (See Figure S-3.) The enrichment of sRAGE from FCS was, however, slightly, yet significantly less efficient compared with serum. (See Figure S-3.) Consequently, the suitability of 1% BSA in TBS as surrogate matrix was tested further during method validation. Corresponding spike-recovery experiments yielded an acceptable bias of ±15% (see the Method Validation section), and we therefore employed 1% BSA in

TBS as surrogate matrix for the preparation of calibrants by analogy to our antibody-based sRAGE LC–MS assay.

#### Method Validation

Table 1 features a concise summary of the validation results, whereas a full overview is given in Tables S-3–S-15. The affimer-based LC–MS method for sRAGE quantification was validated according to EMA and FDA guidelines on bioanalytical method validation.<sup>23,24</sup> Accurate quantification of sRAGE was demonstrated for a 1/*x* weighted linear calibration model using seven nonzero standards between 0.2 (LLOQ: CV and bias <20%) and 10 ng/mL. (See Tables S-3 and S-4.) We did not reach the LLOQ of our previously developed antibody-based sRAGE LC–MS method<sup>16</sup> (i.e., 0.1 ng/mL) because the 0.1 ng/mL calibration standard in the third accuracy and precision run deviated too much from the predicted sRAGE level. (See Table S-16.) This result was the sole obstacle to demonstrating an LLOQ of 0.1 ng/mL (see Table S-17), and we therefore did include a summary of the validation results based on an LLOQ of 0.1 ng/mL in the Supporting Information. (See Table S-18.) Levels of background noise were furthermore comparable for both methods, and peak areas were similar, too, as is exemplified by the LLOQ selected ion chromatograms presented in Figure 3. With respect to the required LLOQ, we can state that we did not observe sRAGE levels below 0.4 ng/mL in ~1000 samples from various clinical studies, and we therefore consider that an LLOQ of 0.2 ng/mL will not affect the applicability of the affimer-based method.

The evaluation of accuracy and precision revealed acceptable biases and CVs (within ±15%), which were slightly higher for the QC-low compared with the QC-medium and QC-high samples. (See Tables S-5–S-7.) The recovery of the affimer-based enrichment procedure was high (>90%) and precise (CVs < 10%) when considering the average of duplicate measurements as well as when based on the individual replicates. (See Table S-11.) Furthermore, the assessment of sample stability after 28 days of storage on the benchtop



**Figure 4.** Comparison between the affimer-based sRAGE LC–MS assay and a previously developed antibody-based LC–MS method for sRAGE quantification<sup>16</sup> using (A) linear regression and (B) the Bland–Altman plot.

(room temperature) and upon 10 freeze–thaw cycles indicated that sRAGE is a rather stable biomarker based on the sites that are recognized by the affimers and the proteotypic peptide that is used for sRAGE quantification by LC–MS. (See Tables S-9 and S-10.)

To assess the selectivity of the sRAGE method, spike-recovery and ligand challenge testing experiments were carried out. The spike-recovery experiments that included six different sources of serum, a lipemic sample, and a hemolytic sample, revealed acceptable biases within  $\pm 15\%$ . (See Tables S-12 and S-14.) Ligand challenge testing was performed by fortifying 0.2 ng/mL calibrants with >10 000-times molar excesses of high mobility group box 1 protein (HMGB1), which is the most studied and characterized RAGE ligand,<sup>31,32</sup> S100 calcium-binding protein A12 (S100A12), and serum amyloid A1 (SAA1) as examples of damage-associated molecular pattern (DAMP) proteins known to bind RAGE<sup>33,34</sup> or N $\epsilon$ -(carboxymethyl)lysine-modified bovine serum albumin (CML-BSA), which we included as a surrogate for advanced glycation end-product-modified proteins.<sup>18</sup> Ultimately, none of the tested ligands affected the measured sRAGE levels. (See Table S-15.)

#### Method Comparison

The agreement between the affimer-based LC–MS sRAGE assay and the previously developed antibody-based LC–MS sRAGE assay<sup>16</sup> was assessed on the basis of 40 clinical samples using linear regression and Bland–Altman plots. (See Figure 4.) The comparison between both methods revealed good correlation ( $R^2 = 0.88$ ) but also showed that  $\sim 25\%$  lower sRAGE levels were reported by the affimer-based assay. Given that the same sRAGE stock was used for the preparation of the calibrants for both methods, the observed difference likely represents a biology-based bias rather than a calibration-related artifact. Whereas the reason for this slight bias remains unclear at present, it is conceivable that the different affinity ligands enrich a different subset of sRAGE proteoforms or that sRAGE molecules bound to specific sRAGE ligands are enriched with different efficiencies by the two methods. Thereby, these data emphasize the need to differentiate between sRAGE proteoforms and to study the “sRAGE ligandome” to gain a better

insight into the biological role and clinical biomarker potential of this protein.

#### CONCLUSIONS

Affimers are attractive alternatives to antibodies for binding target proteins with high affinity and specificity. Their exceptional thermal and chemical stability and the fact that affimers can be produced easily in bacterial cultures (*E. coli*) in a reproducible and scalable manner are clear advantages toward their use as reagents in clinical and bioanalytical assays. In our study we showed the application of affimers in an LC–MS-based method for the quantification of the low-abundant biomarker sRAGE in human serum. The novel method was validated according to EMA and FDA guidelines and enabled the quantification of serum sRAGE at clinically relevant levels between 0.2 and 10 ng/mL. Moreover, the method showed good correlation with a previously developed, fully validated, antibody-based LC–MS method for serum sRAGE quantification, although 25% lower sRAGE levels were reported by the affimer-based method. In conclusion, affimers are small and versatile affinity ligands with significant potential for biomedical applications as alternatives to antibodies.

#### ASSOCIATED CONTENT

##### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.8b00414.

Results of affimer batch equivalence testing (Figure S-1), affimer titration experiments (Figure S-2), and surrogate matrix evaluation experiments (Figure S-3), MRM transitions and settings (Table S-1), summary of method optimization experiments and outcomes (Table S-2), complete overview of validation data supporting the data presented in Table 1 (Tables S-3–S-15), and calibration curve results (Table S-16), accuracy and precision results for the lower limit of quantification (LLOQ) determination (Table S-17), and a summary of validation data (Table S-18) based on 0.1 ng/mL as LLOQ. (PDF)

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### Notes

The authors declare no competing financial interest.

Targeted proteomics data have been deposited in the PeptideAtlas SRM Experiment Library (PASSEL) repository under accession code "PASS01202".

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