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A fully validated liquid chromatography-mass spectrometry method for the quantification of the soluble receptor of advanced glycation end-products (sRAGE) in serum using immunopurification in a 96-well plate format

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ABSTRACT

The study of proteins is central to unraveling (patho)physiological processes and has contributed greatly to our understanding of biological systems. Corresponding studies often employ procedures to enrich proteins from their biological matrix using antibodies or other affinity binders coupled to beads with a large surface area and a correspondingly high binding capacity. Striving for maximal binding capacity may, however, not always be required or desirable, for example for proteins of low abundance. Here we describe a simplified immunoprecipitation in 96-well ELISA format (IPE) approach for fast and easy enrichment of proteins. The applicability of this approach for enriching low-abundant proteins was demonstrated by an IPE-based quantitative workflow using liquid chromatography-mass spectrometry (LC-MS) for the soluble Receptor of Advanced Glycation End-products (sRAGE), a promising biomarker in chronic obstructive pulmonary disease (COPD). The method was validated according to U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines and enabled accurate quantitation of sRAGE between 0.1 and 10 ng/mL in 50 µL serum. The assay showed substantial correlation with the two most commonly-used sRAGE immunoassays (ELISAs) (R²-values between 0.7 and 0.8). However, the LC-MS method reported 2–4 times higher sRAGE levels compared to the ELISAs, which is largely due to a suboptimal amount of capturing antibody and/or calibration strategy used by the immunoassays. In conclusion, our simplified IPE approach proved to be an efficient strategy for enriching the low-abundant protein sRAGE from serum and may provide an easy to use platform for enriching other (low-abundant) proteins from complex, biological matrices.

1. Introduction

Studying proteins in complex, biological matrices is a common feature of (bio)medical research and has contributed considerably to our current understanding of life processes. For studies on protein expression, structure, and interactions, a variety of analytical techniques is being deployed including gel electrophoresis, liquid chromatography and mass spectrometry, which may all require specific protein enrichment procedures depending on the aim of a study and/or the protein(s) of interest [1,2]. Immunopurification is an eminent example of such enrichment techniques and uses specific ‘bait’ proteins to selectively bind and purify the targets of interest (e.g. antigens) [3]. Antibodies are generally used to capture proteins or even protein complexes, though conversely, antigens may also serve as baits to capture antibodies which

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Abbreviations: BSA, bovine serum albumin; CML-BSA, Nε-(carboxymethyl)lysine-modified bovine serum albumin; COPD, chronic obstructive pulmonary disease; CV, coefficient of variation; EMA, European Medicines Agency; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; HMBG1, high mobility group box 1 protein; IPE, immunoprecipitation in 96-well ELISA format; LC-MS, liquid chromatography-mass spectrometry; LLOQ, lower limit of quantification; PBS, phosphate-buffered saline; PTM, post translational modification; QC, quality control; S100A12, protein S100-A12; SA1, serum amyloid A1; SIL, stable-isotope labeled; SNP, single nucleotide polymorphism; (s)RAGE, (soluble) receptor of advanced glycation end-products

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may be valuable for analyzing autoantibodies [4,5]. The former approach is a widely-used application of antibodies in basic and applied scientific research, and has contributed to the conception of antibodies being the ‘workhorses’ of (bio)medical experiments [6].

Besides bait proteins, immunopurification strategies require a (solid) support to which a bait protein is or can be coupled thereby allowing to separate the bait/target-complex from the original matrix [7]. Examples of such supports comprise gel-based (e.g. agarose) and magnetic beads each having specific advantages and disadvantages in terms of binding capacity, protocol flexibility and throughput as well as the degree and extent of non-specific binding [8]. As alternative to bead-based supports, (adsorptive) microtiter plates commonly used for immunoassays, have also been employed for immunoaffinity enrichment purposes [9–12]. Some hybrid assays based on immunoaffinity enrichment and digestion in microtiter plates followed by LC-MS detection demonstrated efficient enrichment of low abundant proteins [9,10]. A similar approach (referred to as immunoprecipitation in 96-well ELISA format, or IPE) showed matching capabilities, though IPE features a decoupled enrichment and digestion strategy and thereby also allows to study intact proteins, for example by top-down proteomics or Western Blot analysis [11]. This approach utilizes microtiter plates coated with Protein (A)/G to which antibodies are bound and covalently coupled with disuccinimydyl suberate (DSS). Although this methodology has many potential applications, examples of such applications are absent in literature, which may be because IPE’s advantages compared to (magnetic) beads-based alternatives are less pronounced due to its dependence on Protein (A)/G-coated plates.

The soluble Receptor of Advanced Glycation End-products (sRAGE) is a potential biomarker for chronic obstructive pulmonary disease (COPD) and an example of a clinically relevant protein of low abundance [13]. sRAGE is formed after proteolytic cleavage of membrane-bound RAGE, a pattern recognition receptor involved in pro-inflammatory signaling pathways [14]. In addition, sRAGE can be formed upon alternative splicing of the AGER gene thereby leading to a RAGE splice variant known as endogenous secretory RAGE (esRAGE) [15]. Circulating RAGE has anti-inflammatory properties by acting as a decoy receptor for pro-inflammatory RAGE ligands and also by inhibiting homo-dimerization of membrane-bound RAGE which is presumed to be essential for RAGE activation [16–18]. In several (large-scale) biomarker studies, sRAGE was put forward as useful biomarker in COPD, particularly with respect to the presence and progression of emphysema, and sRAGE was consequently considered for biomarker qualification by the U.S. Food and Drug Administration (FDA) [19,20]. However, current knowledge about sRAGE is strongly depending on measurements with “research-grade” enzyme-linked immunosorbent assays (ELISA) from a single vendor, and appropriately validated assays are lacking [19]. Furthermore, it is known that sRAGE is regulated by alternative splicing and post translational modifications, including proteolytic cleavage and N-linked glycosylation [21]. Circulating RAGE thus likely comprises a series of related proteins (also referred to as ‘protein species’ or ‘proteoforms’) with potentially different functions, activities or ligand specificities [21–23]. It is therefore not only essential that sRAGE assays for clinical use are adequately validated, but these assays also need to be adequately characterized with respect to the exact forms of circulating RAGE that are being quantified.

In this study, we present an efficient, fast, and easy to use enrichment strategy for proteins in complex matrices on the basis of anti-bodies directly immobilized on high affinity microtiter plates. This methodology was combined with liquid chromatography-mass spectrometry (LC-MS) for quantifying sRAGE in human serum based on specific peptides in its N-terminal region which is essential for the binding of most RAGE ligands [14,24]. The method was validated according to FDA and European Medicines Agency (EMA) guidelines with a lower limit of quantification (LLOQ) of 0.1 ng/mL [25,26]. The validated LC-MS assay for sRAGE is expected to contribute to the development of sRAGE as biomarker in COPD.

2. Experimental

2.1. Chemicals and materials

Recombinant human RAGE encompassing the extracellular domain of this protein (Cat. No. C423; UniProtKB ID ‘Q15109’; Ala23-Ala344 with C-terminal hexa-histidine tag) was purchased from Novoprotein (Summit, NJ, U.S.A.), monoclonal anti-RAGE antibody (Cat. No. MAB11451; clone 176902) was obtained from R&D Systems (Abingdon, U.K.), and stable-isotope-labeled RAGE peptides (i.e. IGEPLVLRK*& VLSQPGGPGPWSVAR*) were synthesized by Pepscan Presto (Lelystad, The Netherlands). Acetonitrile (ACN; LC-MS grade) was obtained from Biosolve ( Valkenswaard, The Netherlands) and sequencing grade (bio)medical experiments [6].

2.2. Serum samples

Serum was obtained from healthy volunteers and was pooled for preparation of the quality control (QC-)samples. Pooled serum was used directly as QC-medium sample, diluted eight times with 1% BSA in 1 × PBS, pH 7.4 (Surrogate Matrix) for preparation of the QC-low sample, or fortified with 5 ng/mL recombinant RAGE to obtain the QC-high sample. Recovery and spike recovery experiments were carried out using six different sources of human serum from (seemingly) healthy subjects (all from Seralab). Spike recovery experiments were furthermore performed on a lipemic serum sample (triglyceride content > 150 mg/dL; obtained from Seralab) and a hemolytic sample which was prepared by adding freeze-thawed whole blood (2%) to human serum.

2.3. Calibrants and internal standard

Lyophilized RAGE was dissolved in Milli-Q water to obtain a 200 µg/mL solution (based on the quantity as declared by the supplier) which was diluted to 100 µg/mL with 1 × PBS, pH 7.4 (PBS Buffer) after checking protein purity by SDS-PAGE and MALDI-TOF MS, which did not reveal the presence of proteins other than sRAGE. The resulting stock solution was sequentially diluted to 100 ng/mL with Surrogate Matrix (see Serum samples section above), 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. For the internal standard (IS), SIL-peptides (supplied as 5 pmol/mL solutions in 5% ACN) were mixed 1:1 and diluted to 5 fmol/µL with 1% dimethyl sulfoxide (DMSO) in water.

2.4. Simplified IPE protocol

(1: plate coating) The plate was coated using 100 µL aliquots of PBS Buffer containing 0.5 µg of antibody (from a 200 µg/mL stock solution; antibody was reconstituted in PBS Buffer) which were added to microplate wells, followed by overnight incubation at room temperature.

(2: plate blocking) After removal of unbound antibody by three washing steps with 300 µL Wash Buffer (0.05% Tween-20 in PBS Buffer), uncoated surface was blocked with 300 µL Surrogate Matrix for 60 min while shaking on a plate shaker (600 RPM; room temperature).

(3: immunocapture) Wells were washed three times with 300 µL Wash Buffer, and 100 µL of Sample Solution (for which 60 µL of serum was pre-mixed with 60 µL Surrogate Matrix to allow quantitative transfer of Sample Solution) was added to the wells for the immunocapture of sRAGE (120 min; 600 RPM; room temperature).

(4: analyte collection) The wells were washed three times with 300 µL Wash Buffer, and sRAGE was eluted from the antibodies with 100 µL 0.1% aqueous trifluoroacetic acid (TFA) solution (10 min; 600 RPM; room temperature).
The IPE eluate was collected in low binding tubes (Eppendorf; Cat. No. 022431081) using low binding tips (WVR; Cat. No. 613-0891), and the samples were dried in a vacuum centrifuge at 60 °C.

2.5. In-solution digestion

Proteins were reconstituted in 50 µL Digestion Buffer (100 fmol/mL). SIL-peptides in 50 mM ammonium bicarbonate (ABC). Subsequently, disulfide bonds were reduced with 10 mM dithiothreitol (DTT) (5 µL 110 mM DTT in 50 mM ABC) for 30 min (60 °C; 600 RPM), and thiols were alkylated with 20 mM iodoacetamide (IAM) (5 µL 240 mM IAM in 50 mM ABC) for 30 min in the dark (room temperature). After quenching the excess of IAM with a 0.5 M excess of DTT (6 µL 110 mM DTT in 50 mM ABC), 100 ng trypsin was added to each sample, and proteins were digested by overnight incubation (37 °C; 600 RPM). The IPE digests were acidified by adding 2 µL of 50% formic acid (FA), and 15 µL of sample was analyzed by LC-MS.

2.6. 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, U.S.A.). Chromatographic separation was achieved on a C18-bonded Waters iKey HSS T3 Separation Device (1.8 µm particles, 100 Å pore size, 150 µm × 100 mm; Cat. No. 186007261) which was kept at 40 °C, using 0.1% FA in H₂O as mobile phase A and 0.1% FA in ACN as mobile phase B. Samples were loaded onto a Dionex Acclaim PepMap100 C18 trap column (5 µm particles, 100 Å pore size, 300 µm × 5 mm; Cat. No. 160454) for 2.5 min with 3% B at 20 µL/min. Subsequently, peptides were separated on the analytical column at 3 µL/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, capillary voltage 3.5 kV, cone voltage 30 V, source offset 50 V, source temperature 120 °C, cone gas (nitrogen) flow 150 L/h, sheath (nanoflow) gas (nitrogen) flow 0.2 bar, and collision gas (argon) flow 0.15 L/min. MRM transitions and settings for IPE-lyk (selected for quantification) and VLCspQGGPQDSVAR (selected for confirmation) are presented in Table S1 (Supplementary Information). 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.

2.7. Method validation

The method was validated according to FDA and EMA guidelines, and the following criteria were addressed: selectivity (e.g. spike recovery and ligand challenge tests), accuracy & precision, recovery, calibration curve, and stability (e.g. 13 days benchtop, 10 × freeze-thaw, 4 months – 20 °C & – 80 °C storage, 28 days autosampler (10 °C), and 443 days stock stability) [25,26]. For the recovery experiment, 10 µL 50 ng/mL sRAGE was added to 240 µL serum (addition of calibration curve, and stability (e.g. 120 °C, cone gas (nitrogen). For recovery and selectivity experiments, samples were incubated for at least 30 min following addition of sRAGE or the ligands prior to initiating sRAGE immunocapture.

2.8. Method comparison

For method comparison, 40 serum samples were analyzed from a cross-sectional study (NCT00807469) within the University Medical Center Groningen (UMCG) [27]. For this study, ethical approval 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 in plastic coagulation tubes (Becton Dickinson), which were incubated for at least 30 min at room temperature prior to centrifugation at 1000 × g and 4 °C for 10 min. After collecting the serum fraction, samples were aliquoted into 1.4 mL polypropylene storage tubes (Thermo Fisher, Cat. No. 3712), and stored at −80 °C until further analysis. In all 40 samples, sRAGE was quantified by LC-MS as well as with the R&D Systems Human RAGE DuoSet (Cat. No. DY1145) and Quantikine (Cat. No. DRG00) ELISA kits, both performed according to manufacturer’s instructions.

3. Results and discussion

3.1. Development of the simplified immunoprecipitation in 96-well ELISA format (IPE) approach for sRAGE in serum

As serum sRAGE levels are reported to be in the low to sub ng/mL range, enrichment of sRAGE prior to LC-MS analysis is required [16]. For this purpose, we adopted a strategy which builds upon the widely-used microtiter plate-based format that forms the basis of most immunoassays. Our strategy furthermore represents a simplified version of the previously described immunoprecipitation in 96-well ELISA format (IPE) [11] for which we particularly aimed to circumvent the need to use Protein (A/G) coated plates (comparable with previously published approaches [9,10]) as non-occupied Protein (A/G) residues may favor non-specific binding and thus may lead to increased background signals [28]. Accordingly, the resulting approach initially follows the typical steps of an ELISA, but instead of adding a secondary antibody for signal amplification and detection, enriched proteins are eluted from the capturing antibody. Proteins can subsequently be studied in their intact form by mass spectrometry and Western Blot analysis, or can be digested with a protease (e.g. trypsin) and analyzed by LC-MS, which was the selected strategy for our sRAGE assay. Optimization of the amount of antibody per sample (see Fig. S1) revealed that less than 0.5 µg of antibody was needed to detect sRAGE at clinically relevant levels with sensitivity down to 0.1 ng/mL (see Fig. 1). The required amount of antibody obviously depends greatly on the dissociation constant (Kₐ) and quality of an antibody [28], yet 0.25–0.5 µg of antibody per sample corresponds well with the amounts that are generally used for immunoassays and is furthermore eight to twenty times lower than the amounts that were previously used in IPE protocols [11].

3.2. Selection of internal standard and calibration matrix

Stable-isotope-labeled (SIL) versions of authentic analytes are the internal standards of choice to enable adequate correction for variability arising from sample preparation or LC-MS, particularly when an immunopurification step is included for analyte enrichment [29,30].
Unfortunately, such standards are often not available (in sufficient quality) for intact proteins, as is the case for sRAGE. Consequently, SIL versions of selected proteotypic peptides were employed as internal standards which were added to the samples after the IPE procedure. To check whether this strategy introduces a bias, recovery of sRAGE during IPE was evaluated during method validation revealing a consistently high and precise recovery of sRAGE using the IPE protocol (see the Assay characteristics section below).

For selection of proteotypic peptides, Skyline software (version 3.5.0.9319; UniProtKB reference protome: UP000005640 (canonical + isoforms)) and the online MRM Peptide Picking Tool (version 1.0; UniProtKB entry: Q15109) were employed [31,32]. Trypsin was selected as protease, and peptides were evaluated on the basis of their uniqueness, presence in the mature protein, presence in relevant isoforms (e.g. sRAGE), peptide length, absence of post translational modification (PTM) sites (e.g. N-linked glycosylation), and location close to or, preferably, within the N-terminal domain of sRAGE which is most relevant for ligand binding [14,24]. In addition, genetic variation was considered by consulting the ExAC Browser (version 0.3; Ensembl gene: ENSG00000204305) and peptides encompassing highly prevalent SNP-sites (e.g. rs2070600 SNP leading to the Gly82Ser substitution) were excluded [33]. Upon in silico evaluation of proteotypic peptides as well as empirical assessment of their ESI-ionization properties, the following two peptides were selected for further method development: r. IGEPLVLK.c (30–37) and k. VLSPQGGGWDSVAR.v (63–77). Since the IGEPLVLK peptide performed best in terms of accuracy and precision during method validation, this peptide was selected for sRAGE quantitation (quantifier peptide), whereas the VLSPQGGGWDSVAR peptide was used to confirm the presence of sRAGE (qualifier peptide).

With respect to selection of the calibration matrix, employing an authentic matrix for preparation of the calibration samples would be preferable, yet an analyte-free, authentic matrix could not be obtained [29]. Accordingly, several surrogate matrices were evaluated including complex matrices (e.g. fetal calf serum) as well as a simple, artificial matrix consisting of 1% BSA in PBS. The recovery of spiked (recombinant human) sRAGE from these matrices was comparable to that of spiked sRAGE from human serum (see Fig. S2). Therefore, 1% BSA in PBS was selected as surrogate matrix and its suitability was demonstrated during method validation (see the Assay characteristics section below).

### 3.3. Assay characteristics

A concise summary of the validation results is presented in Table 1 while a full overview is given in Tables S2 to S19 (Supplementary Information). Accurate quantification of sRAGE was demonstrated for a 1/x weighted linear calibration model using 8 non-zero standards between 0.1 ng/mL (LLOQ: CV & bias ± 10%) and 10 ng/mL (see Tables S2 and S3). Evaluation of accuracy and precision revealed comparable biases and CVs for all three QC-levels (approximately ± 5% and 10%, respectively) suggesting that methodological variation due to the IPE procedure (for which an internal standard to compensate for methodological variability is absent) is constant for low, midrange, and high sRAGE levels and within the approved limits for regulated bioanalysis (see Tables S4 to S6). The extent of the IPE recovery was consistently high ranging from 81% to 84% and precise (CVs of 6–7%) regardless of whether the average of duplicate measurements or individual replicates were considered (see Table S16). Accordingly, these data justify quantifying sRAGE by single measurements which entails that LC-MS analysis of samples from one 96-well microtiter plate (i.e. 81 clinical samples, 9 calibrants, and 6 QC-samples) can be performed within two days.

Sample stability was assessed under conditions going beyond what is relevant for typical clinical assays after 13 days of storage on the benchtop (room temperature), 4 months of storage in the freezer (−20 °C and −80 °C), and upon 10 freeze-thaw cycles (See Tables S9 to S14). These stability assessments indicated that sRAGE is a rather stable biomarker with respect to IPE enrichment and the proteotypic peptide that is used for sRAGE quantification by LC-MS.

Selectivity of the sRAGE method was studied by spike-recovery and ligand challenge testing. Spike-recovery experiments were carried out using six different sources of serum which were processed in duplicate. The observed biases were within ± 15% when either the average sRAGE level of both replicates or the levels of the individual replicates were assessed. With respect to these data, it should be noted that one of the samples (subject c3, see Table S17) contributed mostly to the observed (negative) bias. The reason for this difference is currently unclear. Even though the overall bias is within acceptable limits, this observation indicates that employing a whole-protein SIL internal standard should be considered as relevant future enhancement for our assay. Moreover, spike-recovery assessment was extended to a hemolytic and a lympic sample which yielded biases within ± 15% as well (see Table S18). For evaluation of potential interfering ligands, we selected high mobility group box 1 protein (HMGB1) which is the most studied and characterized RAGE ligand [21,34], S100 calcium-binding protein A12 (S100A12) and serum amyloid A1 (SAA1) as examples of damage-associated molecular pattern (DAMP) proteins known to bind RAGE [35,36], and N-((carboxymethyl)lysine-modified bovine serum albumin (CML-BSA) was included as a surrogate for advanced glycation end-product-modified proteins [171]. All ligands were added at 4 µg/mL (> 10,000-times molar excess). Furthermore, we aimed to mimic potential interferences arising from cigarette smoking by challenging samples with cigarette smoke extract (CSE) in view of future studies on COPD patients. Since many RAGE ligands are DAMPs which are released upon cigarette smoke-induced cell death, we also studied potential interferences by challenging samples with lysed human alveolar epithelial A549 cells [37,38]. Ultimately, none of the tested ligands affected the measured sRAGE levels (see Table S19), as reported previously for the Quantikine sRAGE ELISA as well [39].

### 3.4. Method comparison

Agreement between the LC-MS sRAGE assay and two widely-used sRAGE immunoassays (i.e. R&D Systems DuoSet and Quantikine ELISAs for RAGE) was assessed on the basis of 40 clinical samples using linear regression and Bland-Altman plots (see Fig. 2). These comparisons revealed substantial correlations between the LC-MS assay and the DuoSet and Quantikine ELISAs with coefficients of determination of 0.72 and 0.79, respectively, as well as substantial correlation between the immunoassays (R² = 0.80). However, sRAGE levels obtained with the ELISAs were considerably lower than those obtained by the LC-MS assay with average relative differences of − 84% and − 131% (2.0 and 3.9 times lower sRAGE levels) for the Quantikine and DuoSet ELISAs, respectively.

Possible explanations for the observed differences in sRAGE levels

![Fig. 1. Selected Ion Chromatograms of the sRAGE-derived proteotypic peptides (A) IGEPLVLK and (B) VLSPQGGGWDSVAR at 0.1 ng/mL in Surrogate Matrix (1% BSA in 1 × PBS, pH 7.4).](image-url)
### Table 1
Summary of validation data *a*.

<table>
<thead>
<tr>
<th>QC-low</th>
<th>QC-medium</th>
<th>QC-high</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV</td>
<td>Bias b</td>
<td>CV</td>
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<tr>
<td>12%</td>
<td>−5%</td>
<td>11%</td>
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</tr>
<tr>
<td>7%</td>
<td>0%</td>
<td>12%</td>
</tr>
<tr>
<td>7%</td>
<td>−3%</td>
<td>6%</td>
</tr>
</tbody>
</table>

**Accuracy & precision** (3 runs, in 6-fold)

- **Autosampler stability 10 °C (28 days, in 3-fold)**
- **Bench-top stability room temperature (13 days, in 3-fold)**
- **Freeze-thaw stability −20 °C (10 cycles, in 3-fold)**
- **Storage stability −20 °C (4 months, in 3-fold)**
- **Storage stability −80 °C (4 months, in 3-fold)**

<table>
<thead>
<tr>
<th>stock stability −20 °C (443 days, in 5-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV</td>
</tr>
<tr>
<td>10 ng/mL calibrant</td>
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</tbody>
</table>

**Recovery (6 different plasma samples, 1 or 2 technical replicates)**

<table>
<thead>
<tr>
<th>Recovery</th>
<th>CV</th>
<th>Replicate 1</th>
<th>CV</th>
<th>Replicate 2</th>
<th>CV</th>
<th>Average of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bias</td>
<td>81%</td>
<td>6%</td>
<td>78%</td>
<td>14%</td>
<td>83%</td>
<td>6%</td>
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<td>−13%</td>
<td>−12%</td>
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<tr>
<td>−13%</td>
<td>−8%</td>
<td></td>
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</table>

**Spike recovery (6 different plasma samples, 1 or 2 technical replicates)**

| Bias | −10% | −13% | −12% |

**Lipemic sample spike recovery (in 2-fold)**

| Bias | −10% | −13% | −8%  |

**Hemolytic sample spike recovery (in 2-fold)**

| Bias | −10% | −13% | −8%  |

**0.2 ng/mL calibrant**

| HMGB1 challenge test (4 µg/mL, in 5-fold) | 6% | 10% | CML-BSA challenge test (4 µg/mL, in 5-fold) | 12% | 4% |
| S100A12 challenge test (4 µg/mL, in 5-fold) | 4% | 12% | CSE challenge test (5%, in 5-fold) | 6% | −8% |
| SAA1 challenge test (4 µg/mL, in 5-fold) | 9% | 7%  | Cell lysate challenge test (5%, in 5-fold) | 6% | −1% |

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*a* An extensive summary of the validation results is presented in Tables S2 to S19 (Supplementary Information).

*b* The average value of measured concentrations during the precision and accuracy experiments was used as nominal concentration.

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**Fig. 2.** Comparisons between the quantitative IPE LC-MS sRAGE method (average levels), the R&D Systems Human RAGE DuoSet ELISA, and the R&D Systems Human RAGE Quantikine ELISA using (A) linear regression and (B) Bland-Altman plots. Comparisons between the results of the individual IPE replicates and data from both ELISAs are shown in Fig. S3 (Supplementary Information).
obtained by the assays include different specificities for the antibodies used in the assays (information on the epitope is not available), incomplete capture of sRAGE from serum due to an insufficient amount of primary antibody in the immunoassays as well as differences between the declared and the actual protein quantities of the sRAGE stocks used for preparation of the calibration curves. The latter two explanations were investigated by increasing the amount of antibody for the DuoSet ELISA whose design is rather flexible thereby allowing to deviate from recommended protocol, from 0.1 µg (manufacturer’s recommendation; this amount was actually insufficient for the IPE method, see Figure S1) to 0.5 µg per sample (i.e. the amount used for IPE). Furthermore, the DuoSet sRAGE standard was quantified using the LC-MS assay, and ELISA data were corrected for the observed difference between the expected and the actual sRAGE concentrations (4.0 and 2.9 ng/mL, respectively) as measured by LC-MS. Upon increasing the amount of antibody, signals for the ELISA increased significantly (see ‘0.1 µg’ and ‘0.5 µg’ in Fig. 3) yielding sRAGE levels comparable with those obtained by the LC-MS assay for the QC-low sample, albeit to a lesser extent for the QC-medium and QC-high samples. Referencing the ELISA protein stock against that of the LC-MS assay, however, led to a downward adjustment of the sRAGE levels determined by ELISA (see ‘0.1 µg corr.’ and ‘0.5 µg corr.’ in Fig. 3). Increasing the amount of capturing antibody in the ELISA was thus the most important factor to bring the results more in line with the IPE assay. However, it is conceivable that optimizing the calibration strategy of the ELISA, for example by employing a surrogate matrix which better resembles the complexity and composition of serum than the surrogate matrix used for the ELISA (i.e. 1% BSA in PBS), furthermore represents a strategy to improve the accuracy of this particular assay.

The remaining bias may be the result of the different detection principles of both assay platforms. Whereas readouts of an ELISA are indirect and rely on the ability of a detection antibody to bind the target molecule in order to generate a signal, LC-MS-based approaches analyze the target molecule based on protein-specific peptides. This readout is thus based on confined chemical information which in case of our method relates to whether a circulatory RAGE molecule (enriched by IPE) contains the IGEPVLVLK sequence. Considering this principle, a form of RAGE lacking the N-terminal domain which is essential for binding of RAGE to most of its ligands, will not be picked up by the MS-based assay, as both the quantifier and qualifier peptides are located in the N-terminal domain [14, 24]. Furthermore, the MS-based method does not discriminate between circulating RAGE formed upon shedding of membrane-bound RAGE by metallopeptases and esRAGE which is produced by alternative splicing of the AGER gene, since the N-terminal domain is preserved in both proteoforms [15]. Thus, results obtained with the different assay platforms are prone to be different due to the distinct detection principles of both platforms and the expected heterogeneity of circulating RAGE molecules.

3.5. Perspectives for the IPE assay

The IPE approach is easy to set up and use, antibody efficient, and a robust strategy for enriching low-abundant proteins like sRAGE from highly complex biological samples such as serum. It is conceivable that this assay format can be applied to other low-abundant proteins in other biological matrices and possibly also for studying protein-protein interactions or with the purpose of finding missing proteins (of low abundance) [40, 41]. In fact, the IPE principle is currently being investigated for pull-down assays of low-abundant, intracellular proteins with encouraging results (data not shown). With respect to future applications, it should however be noted that the IPE Wash Buffer contains Tween-20 which proved to be necessary for recovery of sRAGE, but which may interfere with LC-MS analyses. In our case, Tween-20 did not present difficulties, and remaining detergent in the LC system was removed by regularly flushing the LC flow paths with high percentages of eluent B. This may not be possible for some other applications, and washing conditions may need to be optimized accordingly. Moreover, the use of surfactants that can be removed more easily or that are more compatible with ESI-MS comprises another area that needs to be investigated.

4. Conclusions

The simplified immunoprecipitation in 96-well ELISA format (IPE) methodology is an easy and efficient platform for immunoaffinity enrichment. Its potential for enriching proteins from complex matrices was demonstrated in a mass spectrometry-based workflow to quantify the low-abundant biomarker sRAGE in human serum. The method was validated according to U.S. Food and Drug Administration and European Medicines Agency guidelines and enabled quantification of sRAGE in human serum at clinically relevant levels (between 0.1 and 10 ng/mL). Comparison of this assay with the two most widely-used sRAGE ELISAs revealed substantial correlation between all three assays; however, IPE LC-MS reported 2–4 times higher sRAGE levels compared to the ELISAs. This discrepancy can largely be explained by an insufficient amount of capturing antibody per well used by the ELISAs to capture all sRAGE in serum samples, though an improper calibration strategy of the ELISA and the different detection principles of both assay platforms may provide partial explanations as well. In conclusion, the IPE format allows to efficiently establish immunoaffinity enrichment strategies which may simplify and foster the study of proteins in complex matrices.