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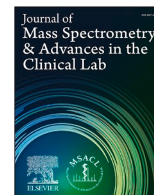

Effects of sample matrix in the measurement of antithrombin by LC-MS: A role for immunocapture

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ABSTRACT

Introduction: The sample matrix composition, which is greatly affected by the type of blood collection tube used during phlebotomy, is of major importance in laboratory testing as it can influence test results. We developed an LC-MRM-MS test to molecularly characterize antithrombin in citrate plasma. The test principle differs greatly from traditional laboratory tests and the influence of varying plasma sample matrices is largely unknown.
Objectives: To identify whether variations in sample matrix affect the LC-MRM-MS test for antithrombin and assess whether sample pre-processing by immunocapture reduces matrix-specific effects.
Methods: Samples (n = 45) originating from four different blood collection tubes (sodium citrate, lithium heparin, K_2-EDTA and K_2-EDTA with protease inhibitors) were processed directly or after immunocapture. Antithrombin was digested into proteotypic peptides, which were monitored by LC-MRM-MS. Results from lithium heparin and the K_2-EDTA matrices were compared to the standard sample matrix, sodium citrate, using Deming regression analysis and repeated measures one-way ANOVA.
Results: Deming regression analysis of directly processed samples revealed slopes deviating >5% from the line of identity for at least six out of 22 peptides in all matrices. Significant differences between all matrices were found upon analysis by ANOVA for at least 10 peptides. Pre-processing by immunocapture led to slopes within 5% of the line of identity for nearly all peptides of the matrices. Furthermore, significant differences between matrices after immunocapture were only observed for four peptides.
Conclusion: Variations in the sample matrix affect the measurement of antithrombin by LC-MRM-MS, but observed effects are greatly reduced upon pre-processing by immunocapture.

Introduction

Reliable results are of utmost importance to support medical decision-making and high-quality research. The pre-analytical phase is the largest contributor to errors in the laboratory testing process [1], and the process of phlebotomy including the correct choice of blood collection tubes is a critical aspect of the pre-analytical phase. A variety of CE-marked blood collection tubes for serum or plasma is commercially available and used routinely in clinical chemistry practice. These tubes may or may not contain anticoagulant, affecting the composition of the sample. Laboratory tests are validated in a specific sample matrix and the use of an alternative collection tube may influence the test results [2]. For haemostasis activity tests, it is essential that coagulation factors are not activated upon blood draw, and blood for these tests is therefore collected into tubes containing anticoagulant, typically sodium citrate. Alternative frequently used anticoagulant additives are lithium heparin and K_2-EDTA. Sodium citrate and K_2-EDTA both target calcium ions to prevent coagulation, as Ca^{++} is an important catalyst for many procoagulant proteins [3]. However, the mechanism by which the additives convert calcium differs, with sodium citrate forming a reversible complex with calcium, and K_2-EDTA chelating calcium. In contrast, lithium heparin inhibits coagulation by heparin activating the

Abbreviations: AT, antithrombin; BD, Becton, Dickinson and Company; CV, coefficient of variation; FA, formic acid; LC, liquid chromatography; MRM, multiple reaction monitoring; LUVDS, Leiden University Medical Center Voluntary Donor Service; PIC, protease inhibitor cocktail; QC, quality control; SST, system suitability test; VHH, single variable domain on a heavy chain.

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anticoagulant protein antithrombin (AT) [4].

We recently developed a next-generation protein diagnostic test for AT by liquid chromatography coupled to multiple reaction monitoring mass spectrometry (LC-MRM-MS) that measures AT at the molecular level through quantitation of proteotypic peptides, formed by trypsin digestion [5,6]. Traditional tests for AT are activity-based and only provide an overall indication of the functionality of AT. Diagnosing AT-deficient patients on the basis of activity tests may lead to underdiagnosis, as heparin-binding site mutations in AT markedly reduce the sensitivity of these tests [7,8]. In contrast, the LC-MRM-MS test provides information on the concentration (in μmol/L), possible mutations and glycosylation of antithrombin, enabling a molecularly based diagnosis [6]. Traditional activity-based haemostasis tests rely on clotting or substrate conversion, and variation in the anticoagulant mechanism or strength leads to disparities in test results [9,10]. As the LC-MRM-MS test does not rely on the activity of the AT protein, one could assume that the test is less affected by variation in the sample matrix. However, the influence of the sample matrix on trypsin digestion is still largely unknown, and variation in trypsin activity may affect the conversion of the AT protein into proteotypic peptides.

To evaluate the exchangeability of plasma matrices on the quantitation of AT peptides by LC-MRM-MS, we examined the effect of four different sample matrices (sodium citrate, lithium heparin, K_2-EDTA and K_2-EDTA with protease inhibitor cocktail). Immunocapture facilitates the isolation of AT from the varying sample matrices and allows for trypsin digestion to take place in a harmonized matrix. By comparing the results of the method without and with immunocapture prior to trypsin digestion, the effects of the immunocapture on matrix-specific variances in next-generation protein diagnostics by MS could be assessed.

Materials and methods

Plasma samples

Volunteers were recruited from the Leiden University Medical Center Voluntary Donor Service (LUVDS), which is coordinated by the biobank facility of the Leiden University Medical Center. Institutional Review Board approval was obtained, and donors gave broad consent. Forty-five apparently healthy volunteers were included (ages 28–69; 66.6 % female), and blood was collected at the in-house blood collection facility. Four different Becton, Dickinson and Company® (BD) plasma tubes were used: 3.2 % (g/v) Sodium Citrate (BD363048, 2.7 mL), 17 U/mL Lithium Heparin (BD367374, 3 mL), 1.8 mg/mL K_2-EDTA without (BD368861, 4 mL) and with Protease Inhibitor Cocktail (BD366422, 2 mL). Samples were processed according to validated standard operating procedures at the Department of Clinical Chemistry and Laboratory Medicine. After blood draw, samples were inverted six times and centrifuged for 10 min at 2350 g, and plasma was transferred to a clean tube, mixed, and aliquoted immediately to prevent variation in the freeze/thaw cycles between analyses. Samples were drawn, processed, and frozen at −80 °C within three hours and analysis was performed within 13 months of storage.

LC-MRM-MS analysis

The 45 samples, processed in eight experimental batches, were measured in duplicate for the direct method, and singlicate for the immunocapture method. Direct processing of samples for AT LC-MRM-MS analysis without immunocapture was performed as described by Ruhaak et al. (2018) [5], with the additional inclusion of stable-isotope labelled peptides for all peptides (not glycopeptides). Processing of samples with immunocapture of AT, as well as LC-MRM-MS analysis, was performed as described by Kruijt et al. (2022) [6]. In short, immunocapture is performed from 50 μL of 200x diluted plasma using a single variable domain on a heavy chain (VHH) anti-antithrombin antibody coupled to streptavidin-coated plates. For the direct method, 8 μL of 20x diluted plasma is used. Both for immunocaptured samples as well as directly processed samples, reduction mix is added to reduce disulfide bonds, which contains sodium deoxycholate, TCEP bond-breaker and stable-isotope labelled peptides (which serve as an internal standard), in ammonium bicarbonate. Subsequent alkylation and trypsin digestion result in the formation of proteotypic peptides after which the digestion is quenched by acidification. Samples are injected into an Agilent 1290 UPLC system and peptides are separated on a C18 reversed phase column using a 19-minute gradient with 1 % Methanol and 0.05 % Formic Acid (FA) in water as eluent A and 95 % Methanol and 0.05 % FA in water as eluent B. A total of 19 peptides and 4 glycopeptides (3 glycopeptides for the direct method) are measured in multiple reaction monitoring mode on a triple-quadrupole mass spectrometry system. For each peptide two or three transitions are monitored, of which one is used for quantitation and the remaining one or two for qualification.

Calibration and quality control

Three native citrate plasma samples with known AT concentrations of 0.66, 1.25, and 1.39 μmol/L were run in duplicate in each experiment to generate a calibration curve that is forced through the origin. Calibrators were targeted using an antithrombin standard that was value-assigned based on a multicentric consensus approach anchored to a World Health Organization standard. System performance was monitored using a system suitability test (SST) sample, which was run in fivefold before and after each experimental run. The SST was assessed on area, relative response, ion ratios, retention time, and carryover. To ensure data validity, two quality control (QC) samples were run in triplicate in each experiment (one at the start, middle, and end of the experiment) and monitored through Levey-Jennings plots (Fig. S1). Quality control results complied with target values and showed an average coefficient of variation (CV) of 4.1 % and 3.4 % for QC1 and QC2, respectively.

Data analysis

Results from LC-MRM-MS measurements were analyzed using Agilent Masshunter Workstation Quantitative Analysis for QQQ (version 10.0). Data was analyzed using R (version 4.1.0) in R studio (version 1.4.1717). Sodium citrate-based calibrators were used, which contain 1:9 sodium citrate to blood and are thus diluted when compared to the other matrices. Due to this dilution effect, concentrations of samples from lithium heparin, K_2-EDTA and K_2-EDTA with protease inhibitor were corrected (original concentration/$1\frac{1}{9}$ = corrected concentration) to agree with citrate sample concentrations. Deming regression analysis was performed with regression lines forced through the origin. Deviation from linearity >5 % was considered significant based on expert opinion, conforming to the third tier of the Milan Hierarchy on performance specifications, which sets the performance specifications based on state-of-the-art [11,12]. Comparison of groups was performed using repeated measures one-way ANOVA followed by multiple comparison pairwise T-tests using Bonferroni multiple testing correction. Graphs were created using Graphpad Prism (version 9.0.1).

Results

Samples were collected from 45 apparently healthy volunteers in four types of blood collection tubes containing different anticoagulants: sodium citrate, lithium heparin, K_2-EDTA and K_2-EDTA with protease inhibitor cocktail (PIC), referred to here as Citrate, LiHe, K_2-EDTA and K_2-EDTA + PIC. As sodium citrate is a liquid anticoagulant, plasma is diluted for this matrix. However, calibrators are native sodium citrate plasma samples, as the test is based on sodium citrate, leading to an overestimation of the concentration of the alternative matrices. Results

were adjusted to correct for this overestimation effect. A five-letter code, indicating the first five amino acids of the sequence, is used to name the monitored peptides, with the full sequences reported elsewhere [6]. Samples originating from the four different blood collection tubes were processed either directly or after immunocapture. The AT derived glycopeptide GP-KANK is not measured in the direct method due to lack of sensitivity, resulting in a total of 22 or 23 peptides being measured with the direct or immunocapture method, respectively. Of note, the role of the various monitored peptides differs. Peptide LVSAN is used for quantitation (with peptides EVPLN and HGSPV acting as confirmation and backup peptides), the glycopeptides are used for assessing the glycosylation status of the protein, and the remaining peptides are used to identify mutations. To this end, no clinically significant variation between matrices for the quantitative peptides is allowed, whereas mutations may still be identified if minor variation between matrices is observed, as mutations lead to expected concentration differences of approximately 50 or 100 % in peptides used for mutation identification.

Results of LC-MRM-MS analysis without immunocapture were assessed to reveal potential matrix interference. Deming regression analysis of the various sample matrices compared to the standard matrix, Citrate, revealed aberrances; LiHe, K_2-EDTA and K_2-EDTA + PIC had 10, 6 and 8 peptides, respectively, with slopes deviating >5 % from the line of identity (Table 1). To illustrate that the differences do not only arise compared to Citrate, but also between the three alternative matrices, the maximum variation between slopes of the three comparisons was calculated. The difference between slopes was >0.05 for 11 out of 22 peptides, with the largest Δ of 0.19 found for peptide IPEAT, originating from a slope of 1.076 for LiHe versus Citrate compared to 0.891 for K_2-EDTA + PIC versus Citrate. Overall, slopes either deviated >5 % from the line of identity or differed >5 % in between slopes for 16 out of 22 peptides. Specifically, quantitative peptide LVSAN was not clearly affected by matrix effects based on linear regression analysis, illustrated by slopes for LiHe, K_2-EDTA and K_2-EDTA + PIC matrices of 0.979, 1.029 and 1.019, respectively (Table 1 and Fig. 1A). Peptide IPEAT, however, which is used for mutation identification, showed slopes of 1.076, 0.974 and 0.891 for LiHe, K_2-EDTA and K_2-EDTA + PIC matrices, respectively (Table 1 and Fig. 1C), indicating clear matrix effects. For LVSAN, a large fraction of LiHe samples showed lower concentrations compared to the other matrices while IPEAT showed clustering of all sample matrices, visible in the bias plot (Fig. 1E and G). The clustering of samples by matrix indicates that the observed variation in slopes is not due to technical variation but matrix-specific. To statistically substantiate the observed matrix effects, one-way repeated measures ANOVAs were performed with subsequent multiple comparison pairwise T-tests with Bonferroni correction. Pairwise t-tests revealed significant differences between matrices for at least 10 peptides for every comparison, resulting in a total of 85 significant differences in peptides between matrices out of 132 comparisons (Table S1). In contrast to linear regression analysis, significantly higher concentrations were observed for peptide LVSAN upon Student's T-test for K_2-EDTA samples both without ($p = 5.36e^{-6}$, $p = 1.14e^{-4}$) and with PIC ($p = 0.009$, $p = 0.003$) compared to Citrate and LiHe samples (Fig. 1I), indicating minor matrix effects. Conversely, peptide IPEAT showed higher concentrations for LiHe compared to Citrate ($p = 2.81e^{-6}$), while the K_2-EDTA matrices had significantly lower concentrations than Citrate ($p = 0.05$ and $p = 2.66e^{-13}$) (Fig. 1K).

Table 1
Deming regression analysis of different matrices compared to Citrate.

	No Immunocapture				With Immunocapture			
	Slope vs Citrate				Slope vs Citrate			
Peptide	LiHe	K_2-EDTA	K_2-EDTA + PIC	Δ slope	LiHe	K_2-EDTA	K_2-EDTA + PIC	Δ slope
ADGES	0.973	1.045	1.026	0.07	1.002	0.997	0.986	0.02
ANRPF	0.971	1.016	1.008	0.05	1.011	1.014	0.992	0.02
DDLYV	0.967	0.983	0.946	0.04	0.954	0.951	0.951	0.00
DIPMN	1.000	1.042	1.026	0.04	1.024	1.019	1.003	0.02
ELFYK	0.977	1.027	1.012	0.05	1.010	1.008	0.993	0.02
EVPLN	0.971	1.031	1.030	0.06	1.003	1.004	0.985	0.02
FATTF	0.976	1.025	0.982	0.05	0.979	0.978	0.963	0.02
FDTIS	0.971	1.033	1.006	0.06	0.990	0.978	0.991	0.01
GDDIT	0.936	0.976	0.936	0.04	0.971	0.973	0.973	0.00
HGSPV	1.065	1.060	1.045	0.02	1.036	1.004	0.992	0.04
IEDGF	0.927	0.964	0.948	0.04	0.976	0.973	0.964	0.01
IPEAT	1.076	0.974	0.891	0.19	1.031	1.017	1.004	0.03
LPGIV	0.965	0.945	0.862	0.10	0.963	0.968	0.957	0.01
LQPLD	0.936	0.972	0.976	0.04	0.973	0.977	0.970	0.01
LVSAN	0.979	1.029	1.019	0.05	1.001	0.999	0.981	0.02
SLNPN	0.970	1.043	1.021	0.07	0.976	0.981	0.969	0.01
TSDQI	1.050	0.959	0.957	0.09	1.002	0.994	0.982	0.02
VAEGT	0.932	0.945	0.900	0.05	0.972	0.961	0.956	0.02
VANPC	0.977	1.053	1.027	0.08	0.996	0.998	0.996	0.00
GP-KANK	NA	NA	NA	NA	1.005	1.002	0.985	0.02
GP-LGACN	0.850	0.891	0.878	0.04	0.977	0.982	0.974	0.01
GP-SLTFN	0.855	0.902	0.906	0.05	0.950	0.962	0.957	0.01
GP-WVSNK	0.949	1.013	1.014	0.06	0.977	0.978	0.981	0.00

Slopes are depicted per peptide and Δ slope is calculated as the maximal difference between slopes of LiHe and **K_2-EDTA** matrices. Red color and underscore indicate a difference >5 % from 1 for slope or >0.05 for Δ slope.

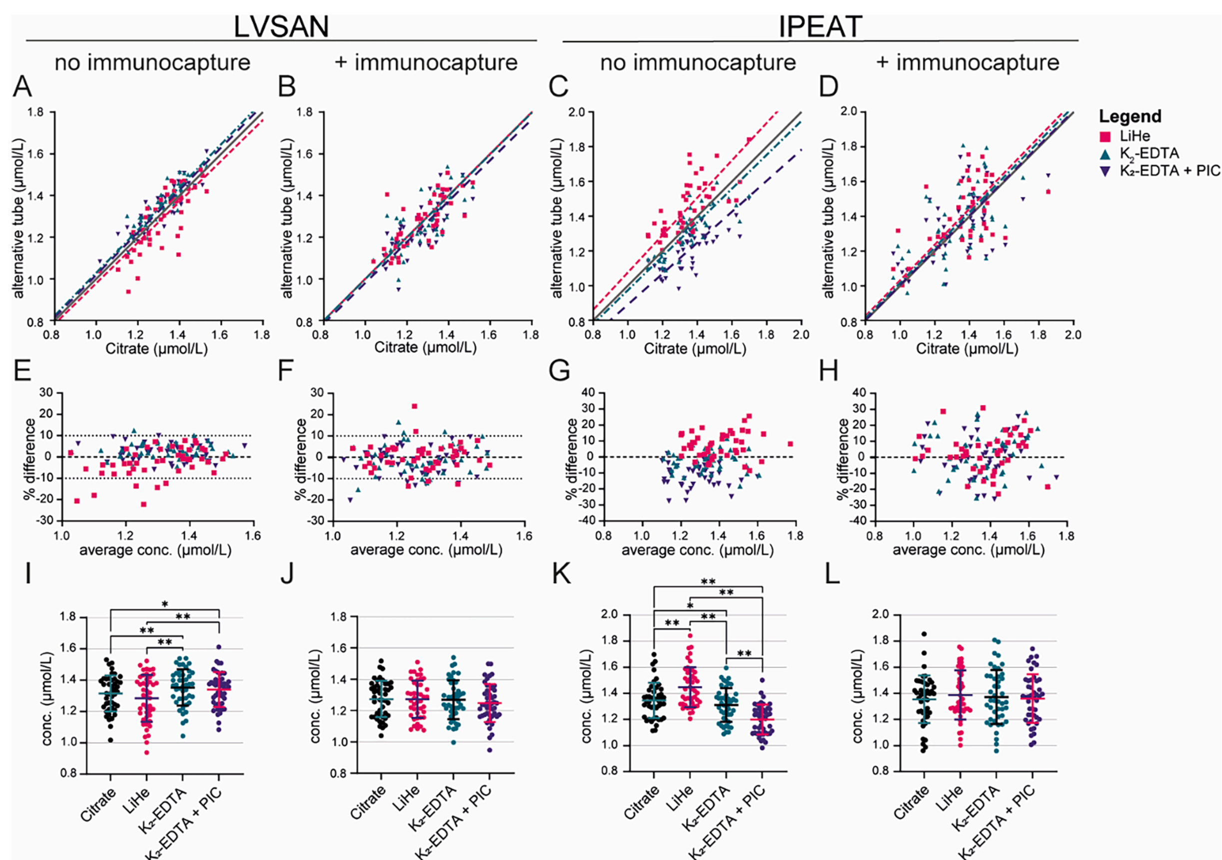

Fig. 1. Matrix-dependent differences in concentration of AT peptides LVSAN and IPEAT as determined by LC-MRM-MS. A-D) Deming regression analysis of quantifying peptide LVSAN (A-B) and qualifying peptide IPEAT (C-D) for the method without and with immunocapture. Grey line indicates line of identity. E-H) Corresponding % difference plots. Outer dotted lines in E and F indicate total allowable error (10 %). I-L) Corresponding results of the pairwise comparisons of the four matrices. * Indicates $p \leq 0.05$. ** Indicates $p \leq 0.005$.

To evaluate whether removal of AT from the matrix prior to further sample processing eliminates the observed matrix effects, the samples were also analyzed after pre-processing by immunocapture. The results of LC-MRM-MS analysis after immunocapture revealed high similarity between matrices. For only one peptide, GP-SLTFN, the slope deviated >5 % from the line of identity (Table 1). The overall difference between the three matrices was minor, with the largest difference between slopes being 0.04 for peptide HGSPV. Specifically, peptide LVSAN showed slopes of 1.001, 0.999 and 0.981 for LiHe, K_2-EDTA and K_2-EDTA + PIC matrices respectively (Table 1 and Fig. 1B and 1F), while peptide IPEAT showed slopes of 1.031, 1.017 and 1.004 for LiHe, K_2-EDTA and K_2-EDTA + PIC matrices (Table 1 and Fig. 1D and 1H). One-way repeated measures ANOVAs with subsequent multiple comparison pairwise T tests with Bonferroni correction revealed significant differences for only 10 out of 138 comparisons (Table S1). No significant differences between the matrices were found for LVSAN and IPEAT (Fig. 1J and 1L). Of note, the additional sample processing step did confer a slight increase in imprecision; as illustrated by the variation in QCs being 3.7 % and 2.5 % for the direct method versus 4.7 % and 5.7 % for the immunocapture method.

Discussion

Accurate medical test results are essential for clinicians to establish a correct diagnosis and offer the right treatment to patients. The pre-analysis, including correct sample collection and blood collection tube choice, is a major contributor to the quality of test results, which are validated for a specific matrix. However, alternative sample matrices may be used occasionally, for example for economic or practical considerations leading to a planned change of sample matrix, but may also be forced, for instance due to shortages (as has been a major hurdle during the COVID-19 pandemic) [13]. To make optimal use of patient material from clinical trials, samples may be shared and re-analyzed for new studies. However, the appropriate sample matrix may not be available for follow-up analysis; especially in the case of rare diseases for which only limited cohorts exist, such as AT deficiency. In these situations, knowledge on the interchangeability of sample matrices is required to assess how strict we must be when diverting to a different matrix, and a strategy to eliminate matrix-induced discrepancies would be highly valuable.

In this study, four different plasma matrices were compared for the determination of AT by an in-house-developed LC-MRM-MS test. The results showed that statistically significant differences arose between samples of the four matrices when samples were processed directly and measured by LC-MRM-MS. This matrix effect is well known for many haemostasis tests due to the varying strength of the different anticoagulants affecting the activity of the analyte, but had not yet been known for an LC-MRM-MS test with trypsin digestion in the preanalytical phase. It is known that components of the matrix may influence the digestion, which is a critical sample processing step [14]. The effect of anticoagulants on the tryptic peptide formation was recently investigated and revealed alterations in digestion kinetics and a possible (indirect) effect of K_2-EDTA on trypsin activity [15]. Immunocapture of AT before further processing nearly completely eliminated the observed differences between matrices. Therefore, we hypothesize that, unlike traditional haemostasis tests, interference is not caused by an effect of anticoagulants on the activity of AT, but rather on trypsin activity.

Although an additional immunocapture step introduces a slight

increase in imprecision and requires additional time and resources, it also increases the sensitivity of the LC-MRM-MS analysis and omits the need for additional sample clean-up steps, such as solid-phase extraction. Furthermore, the elimination of the sample matrix by immunocapture enables harmonization of results between varying sample matrices. This allows measurement in an alternative sample matrix, although it should be noted that verification is still required before measuring clinical samples in a different matrix than what has been validated. The addition of an immunocapture step reduces the chance of pre-analytical errors leading to erroneous patient results, as it may reduce the effects of interfering compounds present in the sample matrix.

Conclusion

LC-MRM-MS based tests, similar to traditional haemostasis activity tests, are affected by a change in sample matrix. The increased interest in precision diagnostics is reflected in the enhanced use of LC-MRM-MS as an enabling technology for molecular characterization of the measurands intended to be detected and/or quantified. Fortunately, owing to the versatility of the LC-MRM-MS test, we can circumvent matrix effects from different anticoagulants by introducing an immunocapture step. Specifically, LC-MRM-MS combined with immunocapture eliminates interference from matrix components and anticoagulants when measuring AT, allowing for high quality precision diagnostic results for patient care and clinical studies.

CRediT authorship contribution statement

M. Kruijt: Investigation, Formal analysis, Writing – original draft. **N.P.M. Smit:** Conceptualization, Writing – review & editing. **J.J. van Ham:** Resources. **C.M. Cobbaert:** Writing – review & editing. **L.R. Ruhaak:** Funding acquisition, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2023.01.002.

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