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Limited Tryptic Digestion-Isotope Dilution Mass Spectrometry (LTD-IDMS): A Reagent-Free Analytical Assay To Quantify Hemagglutinin of A(H5N1) Vaccine Material

Hans C. Cooper[§],
National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia 30341, United States

Yuhong Xie[§],
Seqirus, a CSL Company, Cambridge, Massachusetts 02139, United States

Giuseppe Palladino,
Seqirus, a CSL Company, Cambridge, Massachusetts 02139, United States

John R. Barr,
National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia 30341, United States

Ethan C. Settembre,
Seqirus, a CSL Company, Cambridge, Massachusetts 02139, United States

Yingxia Wen,
Seqirus, a CSL Company, Cambridge, Massachusetts 02139, United States

Tracie L. Williams
National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia 30341, United States

Abstract

Avian influenza viruses, such as A(H5N1) and A(H7N9), are primary public health concerns due to their pandemic potential. Influenza vaccines represent the most effective response to this threat especially with timely provision. The current pandemic response timelines require a substantial period for strain-specific reference antigen and sera preparation for use with single-radial immunodiffusion (SRID), the accepted vaccine potency assay. To address this time lag, the isotope dilution mass spectrometry (IDMS) method was developed to quantify the absolute hemagglutinin (HA, the main influenza antigen) amount in the vaccine without the need for purified, inactivated, and calibrated virus reference antigens. However, an additional challenge in determining potency

Corresponding Author: Tracie L. Williams – *National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia 30341, United States*; Phone: 770-488-3542; tracie.williams@cdc.hhs.gov.

[§]Author Contributions

H.C.C. and Y.X.: These authors contributed equally.

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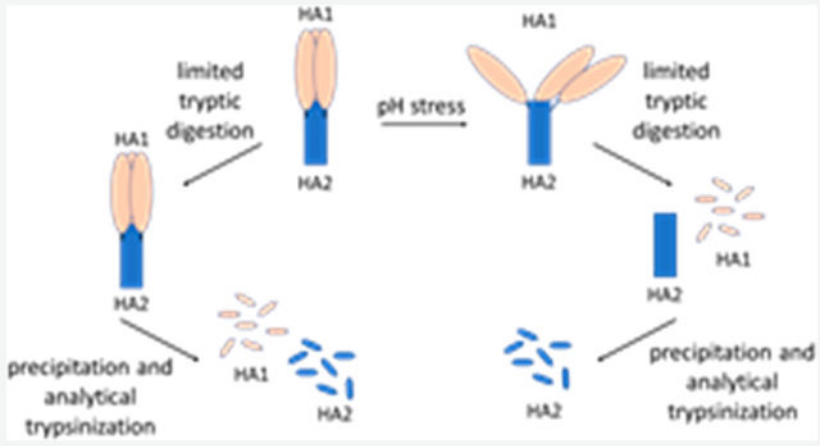
is to differentiate between vaccine antigens in their most potent form from other less potent, stressed antigen forms. The limited trypsin digestion (LTD) method has been developed and does not require strain-specific full-length reference antigens or antibodies; instead, stressed HA is selectively degraded, leaving the more potent form to be measured. LTD, followed by precipitation and IDMS, allows for efficient differentiation between potent and significantly less potent HA for vaccine release and potency testing across the vaccine's shelf life. In this study, we tested the LTD-IDMS assay on A(H5N1) vaccine material that had been stressed by low pH, heat, and multiple freeze-thaw cycles. The results showed that the LTD-IDMS method effectively quantified the potent HA in A(H5N1) vaccine material with results comparable to SRID. As such, it shows great promise to complement and potentially replace SRID in a pandemic when strain-specific reagents may not be readily available.

Graphical Abstract



Influenza poses one of the most immediate global threats to the human population. A(H5N1) and A(H7N9) avian influenza viruses are of concern to public health officials because of their pandemic potential.[1,2] The best defense against human transmission of influenza involves effective vaccination.[3,4] Influenza viruses can rapidly mutate; thus, it is not always feasible to accurately predict which strains and subtypes will pose a threat to the human population at any given time. It takes approximately 6 months to generate a vaccine and provide significant quantities to the public. Key steps for vaccine preparation include (1) generating candidate seed viruses, (2) developing strain-specific reference antigens and antisera to be used for single-radial immunodiffusion (SRID), the currently accepted means of potent hemagglutinin (HA) quantification,[5,6] and (3) formulation and delivering the final vaccine product. Of these timeline components, preparation of calibrated SRID reagents (primarily the antisera) typically requires 2–3 months for seasonal vaccines.[7,8] Reagents for pandemic influenza strains generally require more time to produce due to inexperience with a novel subtype in current vaccine production systems and issues related to safe transport of the virus material.[7,8] The ability to quickly develop, produce, and release an effective vaccine would be essential to limiting the spread of a pandemic strain.[9]

Due to these and other challenges with SRID, there has been a recent effort to develop an alternative potency assay that could provide a more rapid and accurate means of quantifying vaccine potency. Alternative methods need to address the challenge of developing suitable reagents in a timely manner. This can be accomplished in one of three ways: (1) using a whole virus reagent of a similar, but not exact, strain, (2) developing a method that can produce strain-specific reagents more quickly, potentially in smaller quantities, or (3) eliminating the need altogether for whole virus reagents.[10]

Due to the potential limited vaccine supply early in a pandemic, the use of an adjuvant for dose sparing may be important to increase the available doses. The A(H5N1) vaccines adjuvanted with MF59 have been stockpiled for years and have been shown to be well tolerated.[11] An alternate potency assay needs to be able to accurately quantify the amount of HA in an adjuvanted vaccine which may contain as little as 3.75 μg HA/0.5 mL.[11–13]

An alternative potency assay currently being considered is reversed-phase high-performance liquid chromatography (RP-HPLC) combined with either fluorescence or ultraviolet detection.[14,15] However, RP-HPLC has some significant limitations. First, it has the same need for reliable standardized reference antigens as SRID and thus the same time-consuming process of generating and calibrating those materials. Second, as a stand-alone method it cannot distinguish potent vaccine from vaccine that has been denatured by heat or pH stress.[16] Third, the RP-HPLC method uses hydrophobicity to separate the HA1 component from other proteins and is thus generally unable to fully distinguish HA1 different subtypes in multivalent vaccines.

Another accurate and precise quantitative method being considered for vaccine potency evaluation is isotope dilution mass spectrometry (IDMS). The IDMS method utilizes precisely characterized HA peptides for HA quantification. One key advantage of this approach is that each of these peptides is unique to each influenza subtype, so a single IDMS run can analyze each component in a multivalent vaccine.[17] This method also has a significant advantage: rapid and accurate strain-specific vaccine quantification without the need for standardized full-length reference antigens. Each HA subcomponent has two sections that are external to the viral envelope: a globular head structure that is exclusively composed of HA1 residues and an elongated stem which is comprised of residues from both the HA1 and the HA2 regions. By quantifying representative peptide sequences from both the HA1 and the HA2 regions of the inactivated Influenza vaccine and then comparing that with an isotopically labeled peptide version of the same peptide, IDMS can provide an accurate total HA content.[17,18] IDMS as a stand-alone method does have one key limitation, however: it has a limited ability to distinguish between stressed and unstressed HA.[19]

Limited tryptic digestion (LTD) is a means of treating a vaccine sample prior to IDMS analysis. This method assumes changes in the HA conformation (native versus denatured) will occur if the vaccine potency decreases by exposure to stress conditions or aging. An additional separation step, protein precipitation, is also included between LTD and IDMS. For HA that has not been exposed to stress conditions, the protein remains in its native conformation and LTD-IDMS results are identical to IDMS results because the HA is tightly folded and resistant to low trypsin levels. HA that has experienced a decrease in pH or other

stresses, however, undergoes unfolding in the HA1 region and is then susceptible to trypsin cleavage. Treating a vaccine sample that has experienced such a stress with limited tryptic digestion leads to digestion of the now exposed HA1 region, while the more protected HA2 region remains intact.[19–22] Protein precipitation separates the digested peptides from larger, intact, polypeptide pieces of the protein. Thus, the quantitative results obtained with LTD-IDMS using HA2 region peptides represent the total HA amount in the sample, while the results obtained using HA1 region peptides indicates the potent HA in the sample that generally correlates with the SRID results.

LTD-IDMS was previously used to measure A(H7N9) vaccine material that had been compromised by chemical or physical stressors.[19] Here, we expand the method and demonstrate its ability to quantify the A(H5Nl) subtype without the need for strain-specific reagents and in the presence of MF59. LTD-IDMS provides comparable potency measurements for an A/turkey/Turkey/1/2005 A(H5Nl) monovalent vaccine to those of SRID and to effectively distinguish between unstressed HA and chemically or physically stressed HA. This method shows promise as an alternative potency assay that can save time by eliminating the need to generate full-length strain-specific reagents and thus accelerate the vaccine delivery to the public.

MATERIALS AND METHODS

Influenza Reference Reagents.

SRID reference reagents were provided by the US Food and Drug Administration's Center for Biologics Evaluation and Research (FDA CBER, Silver Spring, MD, USA): sheep polyclonal reference antiserum to A/Indonesia/5/2005 A(H5N1), lot# H5-Ab-1115, and reference antigen A/turkey/Turkey/1/2005 A(H5N1), lot# H5-Ag-1510.

Influenza Vaccines.

A/turkey/Turkey/1/2005 A(H5N1) monobulk was produced from Madin-Darby Canine Kidney (MDCK) cells using a process like the Flucelvax subunit influenza vaccine process by Seqirus for research purposes only. Essentially, harvested viruses from MDCK cells were purified and inactivated. After cetyltrimethylammonium bromide (CTAB) splitting, the viral surface proteins were further purified and formulated in PBS buffer.

The nonadjuvanted and MF59-adjuvanted A(H5N1) vaccine were formulated by diluting the A(H5N1) monobulk material to appropriate concentrations using two different diluents: DPBS (Gibco, Catalog #14190–136, Thermo Fisher Scientific, Waltham, MA, USA) or adjuvant MF59 produced by Seqirus for research purpose only.

Samples Stressed by Exposure to Low pH Conditions.

For the first low-pH stress protocol utilizing DPBS as a diluent, two mixtures were prepared: a control group and a pH-stressed group. For the control group, 2.4 mL of DPBS was added to 2.4 mL of the A(H5N1) monobulk material to generate a solution with an HA concentration of approximately 175 μg/mL. To generate a pH-stressed group, 2.4 mL of the monobulk material was treated with 180 μL of 0.5 M citrate, pH 4.0 (Boston BioProducts,

Catalog #BB2032, Ashland, MA, USA), to reduce the sample to a pH of approximately 4.5. This stressed material was then incubated at room temperature with shaking at 2500 rpm on an Eppendorf MixMate for 30 min. The pH was checked using colorpHast pH indicator strips (EMD Chemicals Inc., Catalog #9578, Gibbstown, NJ, USA). Then 220 μL of Tris HCl buffer, 1 M, pH 8.5 (Boston BioProducts, Catalog #BBT-85, Ashland, MA, USA), was added to neutralize the pH to approximately 7.0. The pH was verified. An additional 2.0 mL of DPBS was added to the pH-stressed samples to bring the final volume to 4.8 mL. Both sample sets were then stored at 4 °C until analyzed.

For the second low-pH stress protocol utilizing MF59 as a diluent, 2.2 mL of the A(H5N1) monobulk material was diluted with an equal amount of MF59 to generate a solution with an HA concentration of approximately 175 μg/mL. To generate a pH-stressed sample, the diluted sample material was then treated with 180 μL of 0.5 M citrate, pH 3.0 (Boston BioProducts, Catalog #BB2028, Ashland, MA, USA), to reduce the sample pH to approximately 3.5. The pH was checked using colorpHast pH indicator strips. This MF59-diluted A(H5N1) bulk material was then incubated at room temperature with shaking at 2500 rpm on an Eppendorf MixMate for 30 min. Then 220 μL of Tris HCl buffer, 1 M, pH 8.5, was added to neutralize the pH to 7.0 and confirmed with pH indicator strips. In the control group, 400 μL of water was added to bring the final volume to 4.8 mL. Both sample sets were then stored at 4 °C until analyzed.

Sample Stress by Exposure to Elevated Temperature for Varied Amounts of Time.

For the heat stress protocol, the A(H5N1) monobulk material was diluted with an equal amount of DPBS to achieve a 50:50 mixture. This sample mixture was then divided into 4 sample pools and incubated in a water bath at 56 °C for 0 (control), 5, 24, and 63 h. The sample sets were promptly removed from heat at the appropriate times and returned to 4 °C, where they were stored until analyzed.

Sample Stress by Exposure to Multiple Freeze/Thaw Cycles.

For the freeze/thaw stress protocol, the A(H5N1) monobulk material was diluted with an equal amount of DPBS to achieve a 50:50 mixture. This sample mixture was then divided into 6 sample pools, exposed to an ethanol-dry ice bath for 20 min, and then allowed to thaw at room temperature for 30 min. This process was repeated for 0 (control), 1, 2, 3, 4, and 5 cycles. As each sample set completed its respective number of freeze/thaw cycles, it was promptly returned to 4 °C, where they were stored until analyzed.

Sample Stress by Exposure to Freezing for Varied Amounts of Time.

For the freeze stress protocol, the A(H5N1) monobulk material was diluted with an equal amount of DPBS to achieve a 50:50 mixture. This sample mixture was then divided into 5 sample pools and exposed to −80 °C for 0 (control), 2, 5, 24, and 63 h. The sample sets were promptly removed from the −80 °C freezer at the appropriate times and returned to 4 °C, where they were stored until analyzed.

Limited Trypsin Digestion (LTD).

Following the above protocols, each of the sample sets were removed from 4 °Cand brought to room temperature. A bovine trypsin (Sigma, Catalog # T9201–100MG, St. Louis, MO, USA) solution was prepared by diluting the trypsin in 1 mM HCl (in water) (Sigma), to provide 40 U of trypsin activity per 100 μg of HA. An appropriate amount—dependent upon the initial starting concentration of HA in the vaccine material as measured by IDMS-- of the LTD solution was added to each of the samples. The samples were then incubated for 2 h in a 37 °C water bath. Following removal from the water bath, the trypsin activity was quenched by returning the sample to 4 °C, where they were stored until analyzed.

Precipitation.

Following LTD, a 200 μL aliquot from each sample condition was removed and readied for precipitation. To this, 800 μL of cold (−20 °C) acetone (Sigma-Aldrich, St. Louis, MO 63178) was added, and the samples were then incubated at −20 °C for 2 h. They were centrifuged for 20 min at 4 °C with a speed of 20 817 RCF. The supernatant was removed, allowing a pellet to remain at the bottom of the vial. Three pellet washes were performed with 800 μL of cold (−20 °C) ethanol and the same centrifugation parameters. Careful removal of the supernatant after each wash resulted in a relatively dry pellet that was stored at −20 °C until needed. The pellets were reconstituted in 200 μL of 0.1% RapiGest SF (Waters, Milford, MA, USA) in 50 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA).

SDS-PAGE.

Samples mixed with NuPAGE lithium dodecyl sulfate (LDS) sample buffer and reducing agent (Invitrogen, Grand Island, NY, USA) were heated at 90 °C for 5 min and then separated by NuPAGE 4–12% Bis-Tris gels with 2-(*N*-morpholino)ethanesulfonic acid (MES) running buffer (In-vitrogen). Protein bands were visualized using Coomassie blue staining and silver staining.

RP-HPLC.

Samples pretreated with 1% Zwittergent (volume/volume) at room temperature for 30 min followed by treatment with 25 mM dithiothreitol (DTT) at 90 °C for 10 min were injected onto a Pros R1/10 column (Applied Biosystems, Grand Island, NY, USA) and eluted with a gradient of 30–35% 0.1% trifluoroacetic acid (TFA) in 100% acetonitrile (Waters Alliance, Milford, MA, USA). Sample HA was quantified by comparing the area-under-curve (AUC) of the HA1 peaks to those from SRID standard reference materials.

SRID.

Agarose solution in phosphate-buffered saline (PBS), 1% weight/volume, was prepared by mixing 3.0 g of SeaKem Agarose (Lonza, Basel, CH) with 300 mL of PBS. The mixture was boiled to dissolve the agarose, cooled to 60 °C, and mixed with virus-specific sheep antiserum (SRID reference sheep antiserum lot # H5-Ab-1115) at the dilution recommended by the Center for Biologics Evaluation and Research (CBER) of the Food and Drug Administration (FDA). The agarose was poured into plastic casts to a uniform 2 mm

thickness and cooled at room temperature for 20 min. Then 4 mm diameter round wells were cut 10 mm apart in the solidified gel.

Sucrose-purified whole virus (SRID reference antigen lot # H5-Ag-1510) was diluted with PBS to 40 μg HA/mL. Reference and unknown samples were treated with Zwittergent 3–14 (Calbiochem-Novabiochem, Billerica, MA, USA) to a 1% final concentration and incubated for 30 min at room temperature. After treatment, the reference and samples were diluted 3:4, 2:4, and 1:4 in PBS and dispensed into the agarose wells at 20 μL of sample/well in duplicate.

SRID plates were incubated 16–24 h at 25 °C to allow complete diffusion of the antigen. Proteins that had not formed immune complexes were removed by blotting the gel with filter paper (VWR) for 6–16 h at 37 °C. The protein remaining in the gel was stained with Coomassie Brilliant Blue R250 (EMD Millipore, Billerica, MA, USA): 0.3% Coomassie solution in 45% methanol, 10% acetic acid for 5 min at room temperature on an orbital shaker, followed by 45% methanol, 10% acetic acid for 5 min. Gels were air dried, and the precipitin ring diameters were measured. HA concentration was calculated by the slope ratio method using CombiStat 5.0 software (European Directorate for the Quality of Medicines).

Analytical Digestion and Isotope Dilution Mass Spectrometry.

From the reconstituted samples above, 10 μL of each sample was combined with 10 μL of 0.2% RapiGest SF (Water Corp., Milford MA, USA) and then heated for 5 min at 100 °C. After cooling, 5 μL (86 pmol) of sequencing grade modified porcine trypsin (Promega, Madison, WI, USA) was added to each sample and incubated in a 37 °C for 2 h. After the 2 h incubation, 10 μL of 0.45 M HCl (Thermo Fisher Scientific, Waltham, MA, USA) was added to each sample to degrade the acid-labile RapiGest SF. These samples were incubated at room temperature for 30 min. A 10 μL aliquot of an A(H5N1) influenza heavy-isotope-labeled cocktail containing 4 peptides, each at a concentration of 0.5 pmol/mL in 0.1% formic acid (Thermo Fisher Scientific), was added to serve as an internal standard. A 55 μL amount of of 0.1% formic acid was added to yield a final volume of 100 μL. The contents were then transferred to an autosampler vial for LC/MS/MS analysis.

Separation of the targeted peptides was achieved by HPLC utilizing a NanoAcquity (Waters, Milford, MA, USA). The aqueous mobile phase (A) utilized was HPLC-grade water with 0.1% formic acid, and the organic phase (B) was acetonitrile with 0.1% formic acid (Thermo Fisher Scientific). A Waters reverse phase C-18, 3.5 mm particle size, 150 mm 1 mm i.d. Symmetry300 was used for peptide separation. The method gradient involved initial column conditions of 98% A for the first 5 min, followed by ramping up to 20% B within 20 min and finally to 25% B at 25 min. The column was then washed with 98% B for 10 min and then allowed to re-equilibrate to 98% A (initial conditions) for 20 min. The total run time for the method was 57 min. The column eluent was introduced into a Thermo Quantum TSQ Vantage triple-quadrupole tandem mass spectrometer with an electrospray interface (Thermo Fisher Scientific). The instrument, operating in positive ion mode, monitored for the precursor/product ion transition pairs for the native and labeled peptides as shown in Table 1. The data was processed with the Thermo Xcalibur Quan software.

RESULTS AND DISCUSSION

We have recently described a LTD-IDMS method applicable to A(H7N9) strains.[19] This study is an expansion of that work to another potential pandemic strain, A(H5N1), and a demonstration that the method also works in the presence of adjuvants that would be present due to the need for dose sparing during a pandemic. To accomplish this, the A/turkey/Turkey/1/2005 A(H5N1) monobulk was initially quantified by IDMS. Four sample sets were created: a nonstressed/no LTD control, a nonstressed with LTD group, a low-pH-stressed/no LTD group, and a low-pH-stressed with LTD group. Three mixtures were also made of samples that had not been stressed with samples that had been pH stressed. Limited tryptic digestion-IDMS was performed and results compared to SRID and LTD-RP-HPLC.

Initial Potency Quantification by IDMS for A/turkey/Turkey/1/2005 A(H5N1) Vaccine.

The A/turkey/Turkey/1/2005 A(H5N1) monobulk was quantified by an IDMS method previously described.[18] HA peptides were selected as the stoichiometric representative of the larger HA protein with the assumption that one mole of that specific peptide represented one mole of the protein. Thus, the peptides selected for this IDMS analysis did not contain sequences with likely modifications that would alter the peptide's mass and were in areas of the detergent denatured HA that are easily accessible for proteolytic digestion.[18] As such, peptides with the following characteristics were avoided: potential N-linked glycosylation sites, oxidizable residues (e.g., methionine, tryptophan), and peptides containing cysteines or located proximal to cysteines. Following these guidelines, four target peptides distantly located across the protein were selected for measurement: IQIIPK and LVLATGLR from the HA1 region and EFNNLER and EEISGVK from the HA2 region. The peptide locations within the A/turkey/Turkey/1/2005 A(H5N1) HA sequence are shown in Figure 1. ^{13}C- and ^{15}N-labeled peptides were also generated as internal standards that have the same chemical and chromatographic properties as the target HA peptide but a different molecular weight that is easily distinguished by mass spectrometry.

With the above target peptides established, the A/turkey/Turkey/1/2005 A(H5N1) monobulk was analyzed by the IDMS method. To confirm complete protein digestion, all four target peptides were quantified independently. These results were then used as a baseline for approximate vaccine concentration and to determine appropriate dilutions needed for the remaining experiments.

Potency Quantification by RP-HPLC, SRID, and LTD-IDMS of A/turkey/Turkey/1/2005 A(H5N1) Monobulk.

The LTD method is intended to distinguish conformational changes which may be indicative of immunologically active and inactive HA without the need for strain-specific antibodies.[19] In order to evaluate and compare LTD-IDMS with RP-HPLC and SRID using the A/turkey/Turkey/1/2005 A(H5N1) monobulk, four sample sets were created: (1) nonstressed material that was not treated with LTD (control), (2) nonstressed material that was treated with LTD, (3) material in which the pH was decreased between 3 and 4.5 briefly but with no LTD, and (4) pH-stressed material that was treated with LTD.

A more detailed analysis by LTD-IDMS for each of the four individual peptides is shown in Figure 2A. Separate analysis (n = 6, CV less than 7%) for each of the four target peptides showed agreement in both unstressed groups, confirming complete HA protein digestion in the region containing the target peptides. For the no pH-stressed/no LTD samples that included a precipitation step, peptide values obtained among the four peptides had a 15% RSD with identical results for the two HA1 peptides (both 138 μg/mL) and nearly identical for the two HA2 peptides (183 and 179 μg/mL). While we would expect the absolute values between all 4 peptides to have a < 15% RSD for fresh materials containing HA almost all in the native conformation, the material used in this study was an aged sample, so the lower results of the two HA1 peptides suggested the presence of preexisting, denatured HA. When the sample was stressed and LTD was not performed, one of the HA1 peptides (LVLATGLR) showed a decrease likely because it is located proximal to the cleavage site and the change in protein conformation made that peptide less accessible to enzymatic cleavage. The other HA1 peptide (IIQPDK) remained unchanged. When LTD was added as pretreatment for the pH-stressed samples followed by a precipitation step, the LTD-IDMS results showed that the two peptides, IQIIPK and LVLATGLR of HA1, substantially decreased in concentration, while the two peptides from the HA2 region, EFNNLER and EEISGVK, showed similar concentrations to nonstressed HA. As such, these results indicate that the LTD-IDMS specifically quantitated nondenatured HA represented by the HA1 peptides and total HA represented by the HA2 peptides.

Each of these sample groups was then analyzed by RP-HPLC and SRID. The results, shown in Figure 2B, demonstrate that RP-HPLC, SRID, and IDMS all measured HA similarly in both the non-pH-stressed (control) group and the non-pH-stressed/LTD group (Figure 2A). In the pH-stressed/no-LTD group, both RP-HPLC and IDMS demonstrated little change while SRID was diminished substantially. This is a consequence of SRID being an antibody-based assay, while RP-HPLC and IDMS are purely biophysical assays which, on their own, cannot detect sample stress. Finally, in the pH-stressed/LTD group all three methods showed similar results, demonstrating the LTD-IDMS quantified potency matched the results from SRID, the standard potency assay.

Potency Quantification by LTD-IDMS for A/turkey/Turkey/1/2005 A(H5N1) Vaccine in Mixtures.

To further evaluate the selectivity of potency quantification, LTD-IDMS was performed to quantify the nonstressed, native HA in a mixture of nonstressed and pH-stressed A/turkey/Turkey/1/2005 A(H5N1) monobulk (Figure 3A) material. A control group of 100% unstressed/0% pH-stressed served as the benchmark, and all other groups are shown relative to those results. The next three groups were blended mixtures using different percentages of unstressed and pH-stressed monobulk with the percent of each shown on the X axis. As shown in Figure 3A, the two HA1 peptides diminished as the percentage of pH-stressed material increased. One hundres percent pH-stressed material displayed results consistent with the stressed/LTD results shown in Figure 2B, as expected. The LTD-IDMS results for the HA2 peptides showed very little decrease in the preparations that contained stressed monobulk, demonstrating that the HA2 peptides can be used for total HA quantitation regardless of the sample quality and protein conformation.

A similar experiment was performed on the same A(H5N1) monobulk vaccine formulated with the MF59 adjuvant. The results for this experiment were very similar to the first group diluted with DPBS and are shown in Figure 3B. As such, taken together, these two separate analyses of different stressed sample mixtures confirmed that the degree to which the total HA from the A(H5N1) vaccine has been irreversibly shifted into its less immunogenically relevant conformational state can be quantified by LTD-IDMS.

Potency Quantification by LTD-IDMS for A/Turkey/Turkey/1/2005 A(H5N1) Vaccine Stressed by Heat, Freeze Stress, and Freeze/Thaw Cycles.

The LTD-IDMS method was originally developed by analyzing samples stressed with a pH decreased to between 3.5 and 4.5. The potential of LTD-IDMS to detect and quantify material that had been exposed to other sources of possible sample stress was then evaluated. These stresses included exposure to heat, freezing, and a series of freeze/thaw cycles. Three analytical methods were used to compare results: LTD-RP-HPLC, SRID, and LTD-IDMS.

For sample stress induced by heat, monovalent vaccine material was heated in a hot water bath at 56 °C for 5, 24, and 63 h. Heat stress was chosen to simulate the effect if cold chain storage procedures were not followed and vaccine was exposed to high temperatures in transit. Heat stress can cause a strain-dependent, variable level of potency reduction in different vaccines.[20–25] The objective was to identify both the effect of heat exposure and the ability of LTD-IDMS to detect HA structural changes. The results are shown in Figure 4A.

For sample stress induced by freezing, the samples were stored at −80 °C for 2, 5, 24, and 63 h. Freezing the sample simulates the scenario a vaccine could experience where shipping is not adequately controlled. Freezing a sample that has been stored in PBS decreases the pH of the sample.[26,27] Therefore, the LTD-IDMS results of samples that have been frozen would be expected to be like what was observed when the pH of the sample was intentionally decreased (Figure 2B). Results of this experiment are shown in Figure 4B.

Samples were also exposed to multiple freeze/thaw cycles as it is well known that proteins undergo degradation with such treatment.[26–30] Samples were placed in an ethanol/dry ice bath for 20 min and then thawed at room temperature for 30 min. Samples were removed after each cycle to test via LTD-IDMS, SRID, and LTD-RP-HPLC. The maximum number of freeze/thaw cycles was 5. The results are shown in Figure 4C.

Comparison of the methods meant that LTD-IDMS with 4 independent measurements had to be compared to two methods that only provided one reportable result. Since it had been established that it was the HA1 peptides that indicate HA stability/conformation, it was the average of the two HA1 peptides that was used to compare SRID and LTD-RP-HPLC results. The results indicated that relative HA potency quantified by LTD-IDMS correlates with relative SRID and LTD-RP-HPLC values, and absolute HA potency quantified by LTD-IDMS also correlates with SRID and LTD-RP-HPLC values (data not shown). These results further illustrated that LTD-IDMS has the capability to quantify native HA in stressed vaccines.

CONCLUSIONS

The greatest emerging threat from the influenza virus is currently thought to be from avian A(H5Nx) and A(H7N9) strains. We have recently described the LTD-IDMS method applicable to A(H7N9) strains. In this study we have expanded that work to A(H5N1) and demonstrated that the LTD-IDMS method could function effectively to rapidly determine the potency of the A(H5N1) strain in material- and time-limited scenarios. In order to accomplish this, we initially quantified the A/turkey/Turkey/1/2005 A(H5N1) monobulk by IDMS. This allowed rapid and accurate quantification of the absolute HA amount in the vaccine without the need for calibrated reference antigens. However, IDMS has a limited ability to distinguish between immunologically active versus inactive HA; LTD was developed to provide information about the conformation of the hemagglutinin protein.[19–22] To bridge LTD and IDMS and form the complete biophysical potency assay, precipitation was introduced post-LTD treatment to remove trypsin-digested HA1 peptides and retain trypsin-resistant native-conformation HA. As a result, precipitation enabled IDMS to quantify HA potency and mass in one single analytical run—HA1 peptides quantified HA in its conformationally correct state, and HA2 peptides quantified total HA in the starting material.

For A(H5N1) vaccine quantification, two HA1 peptides have been identified, IQIIPK and LVLATGLR, and two HA2 peptides, EFNNLER and EEISGVK. By comparing the relative proportion of each of these two peptide groups, it should be possible to identify how stress affects the vaccine. Thus, in an unstressed vaccine, all four peptides should produce quantitative results that are in close agreement. In contrast, vaccine material that has been stressed will contain misfolded hemagglutinin protein. The HA1 region of the protein (IQIIPK and LVLATGLR) is subject to greater unfolding and will be more easily digested by a limited trypsin exposure. The more protected HA2 peptides (EFNNLER and EEISGVK) will remain intact. This principal is fundamental to the LTD method and was applied in this study to demonstrate the ability to quantify both the native HA as well as the total HA in an influenza vaccine sample.

In this study, we were able to demonstrate that the limited tryptic digestion-isotope dilution mass spectrometry (LTDIDMS) can provide comparable potency measurements for a prepandemic A/turkey/Turkey/1/2005 A(H5Nl) monobulk to those of SRID and to effectively distinguish between immunologically active HA and chemically or physically stressed HA. As with the results from previous studies with an H7 vaccine, LTD-IDMS continues to show tremendous promise as a rapid and highly effective influenza vaccine alternative potency assay.

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ABBREVIATIONS

HA	hemagglutinin
IDMS	isotope dilution mass spectrometry
LTD	limited trypsin digestion
SRID	single-radial immunodiffusion
RP-HPLC	reversed-phase high-pressure liquid chromatography
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

REFERENCES

(1). Cumulative number of confirmed human cases of avian influenza A(H5N1) reported to WHO, 2003–2019; WHO, 2019.

(2). Webster RG; Govorkova EA N. Engl. J. Med. 2006, 355 (21), 2174–7. [PubMed: 17124014]

(3). Poland GA; Rottinghaus ST; Jacobson RM Vaccine 2001, 19 (17–19), 2216–20. [PubMed: 11257336]

(4). Zambon MC J. Antimicrob. Chemother. 1999, 44, 3–9.

(5). Wood JM; Schild GC; Newman RW; Seagroatt V J. Biol. Stand. 1977, 5 (3), 237–242. [PubMed: 408355]

(6). Engelhardt OG; Edge C; Dunleavy U; Guilfoyle K; Harvey R; Major D; Newman R; Penn R; Skeldon S; Storey C; Wheeler J; Wood J; Minor P Vaccine 2018, 36 (29), 4339–4345. [PubMed: 29895503]

(7). Weir JP; Gruber MF Influenza Other Respir. Viruses 2016, 10 (5), 354–360. [PubMed: 27426005]

(8). Gerdil C Vaccine 2003, 21, 1776–1779. [PubMed: 12686093]

(9). Hardy S; Eichelberger M; Griffiths E; Weir JP; Wood D; Alfonso C Influenza Other Respir. Viruses 2011, 5 (6), 438–42. [PubMed: 21668676]

(10). Minor PD Vaccines (Basel, Switz.) 2015, 3 (1), 90–104.

(11). Oshansky CM; Zhou J; Gao Y; Schweinle JE; Biscardi K; DeBeauchamp J; Pavetto C; Wollish A; Webby RJ; Cioce V; Donis RO; Bright RA Vaccine 2019, 37 (3), 435–443. [PubMed: 30553570]

(12). Sun X; Belser JA; Pulit-Penaloza JA; Creager HM; Guo Z; Jefferson SN; Liu F; York IA; Stevens J; Maines TR; Jernigan DB; Katz JM; Levine MZ; Tumpey TM Virology 2017, 508, 164–169. [PubMed: 28554058]

(13). Velkov T; Ong C; Baker MA; Kim H; Li J; Nation RL; Huang JX; Cooper MA; Rockman S Mol. Immunol. 2013, 56 (4), 705–19. [PubMed: 23933511]

(14). Garcia-Canas V; Lorbetskie B; Girard M J. Chromatogr A 2006, 1123 (2), 225–32. [PubMed: 16677659]

(15). KAPTEYN J; SAIDI M; DIJKSTRA R; KARS C; TJON J; WEVERLING G; DEVOCHT M; KOMPIER R; VANMONTFORT B; GUICHOUX J Vaccine 2006, 24 (16), 3137–3144. [PubMed: 16490287]

(16). Wood JM; Weir JP Influenza Other Respir. Viruses 2018, 12 (2), 195–201. [PubMed: 29356318]

(17). Wen Y; Han L; Palladino G; Ferrari A; Xie Y; Carfi A; Dormitzer PR; Settembre EC Vaccine 2015, 33 (41), 5342–5349. [PubMed: 26348403]

(18). Williams TL; Pirkle JL; Barr JR Vaccine 2012, 30 (14), 2475–82. [PubMed: 22197963]

(19). Morgenstern K; Xie Y; Palladino G; Barr JR; Settembre EC; Williams TL; Wen Y Vaccine 2018, 36 (41), 6144–6151. [PubMed: 30194004]

(20). Wiley DC; Skehel JL J. Mol. Biol. 1977, 112 (2), 343–7. [PubMed: 875021]

(21). Skehel JJ; Bayley PM; Brown EB; Martin SR; Waterfield MD; White JM; Wilson IA; Wiley DC Proc. Natl. Acad. Sci. U. S. A. 1982, 79 (4), 968–972. [PubMed: 6951181]

(22). Wen Y; Palladino G; Xie Y; Ferrari A; Ma X; Han L; Dormitzer PR; Settembre EC Vaccine 2016, 34 (29), 3388–95. [PubMed: 27154389]

(23). Norrgran J; Williams TL; Woolfitt AR; Solano MI; Pirkle JL; Barr JR Anal. Biochem. 2009, 393 (1), 48–55. [PubMed: 19501563]

(24). Santana WI; Williams TL; Winne EK; Pirkle JL; Barr JR Anal. Chem. 2014, 86 (9), 4088–95. [PubMed: 24689548]

(25). Williams TL; Luna L; Guo Z; Cox NJ; Pirkle JL; Donis RO; Barr JR Vaccine 2008, 26 (20), 2510–20. [PubMed: 18440105]

(26). Gomez G; Pikal MJ; Rodriguez-Hornedo N Pharm. Res. 2001, 18 (1), 90–7. [PubMed: 11336359]

(27). Kolhe P; Amend E; Singh SK Biotechnol. Prog. 2010, 26 (3), 727–733. [PubMed: 20039442]

(28). Pikal-Cleland KA; Cleland JL; Anchordoquy TJ; Carpenter JF J. Pharm. Sci. 2002, 91 (9), 1969–79. [PubMed: 12210044]

(29). Pikal-Cleland KA; Rodriguez-Hornedo N; Amidon GL; Carpenter JF Arch. Biochem. Biophys. 2000, 384 (2), 398–406. [PubMed: 11368330]

(30). Coenen F; Tolboom JT; Frijlink HW Vaccine 2006, 24 (4), 525–31. [PubMed: 16150515]

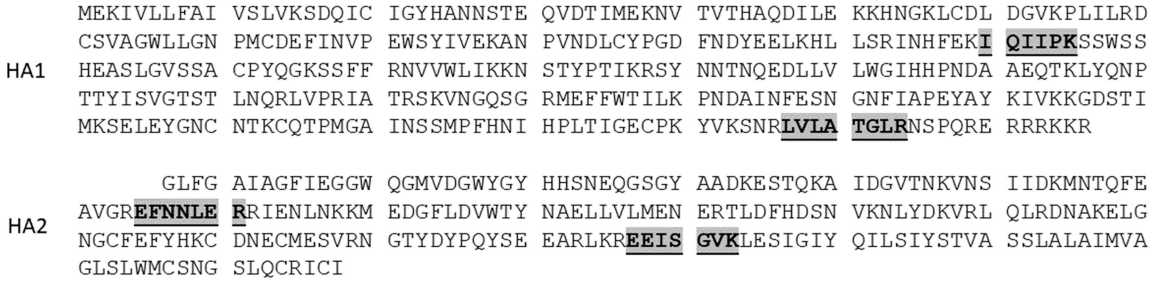

Figure 1.

A/turkey/Turkey/1/2005 A(H5N1) HA amino acid sequence. Four target peptides utilized for quantitation are shown in grayscale: Two HA1 peptides are IQIIPK and LVLATGLR, and two HA2 peptides are EFNNLER and EEISGVK.

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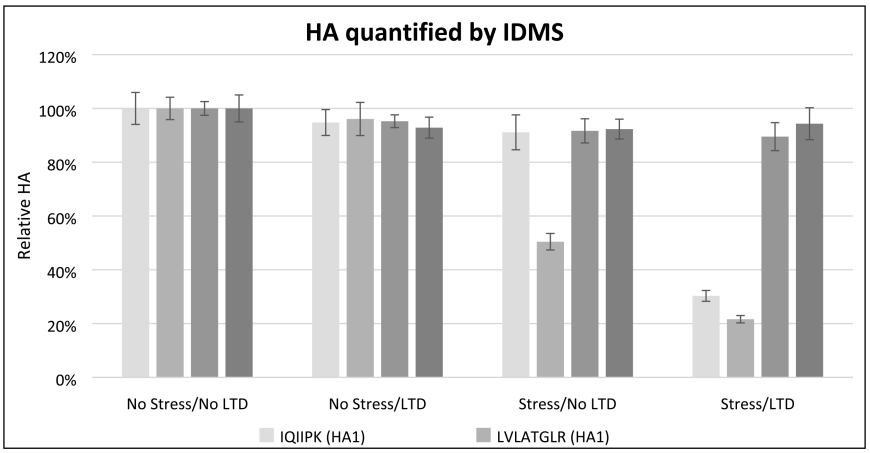

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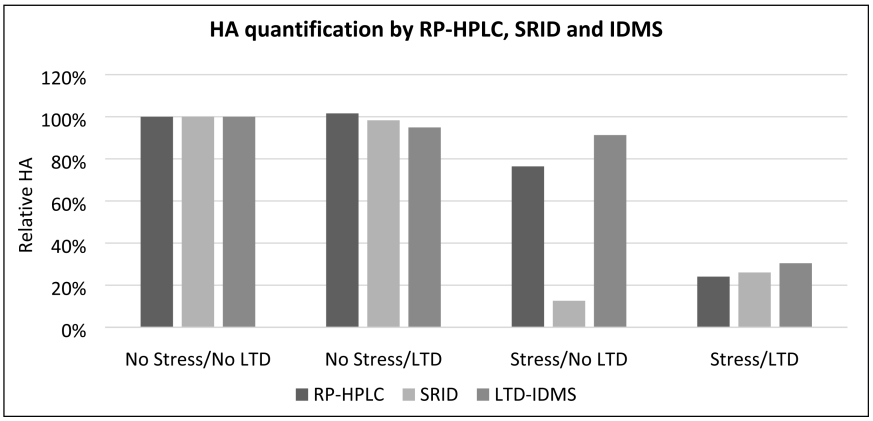
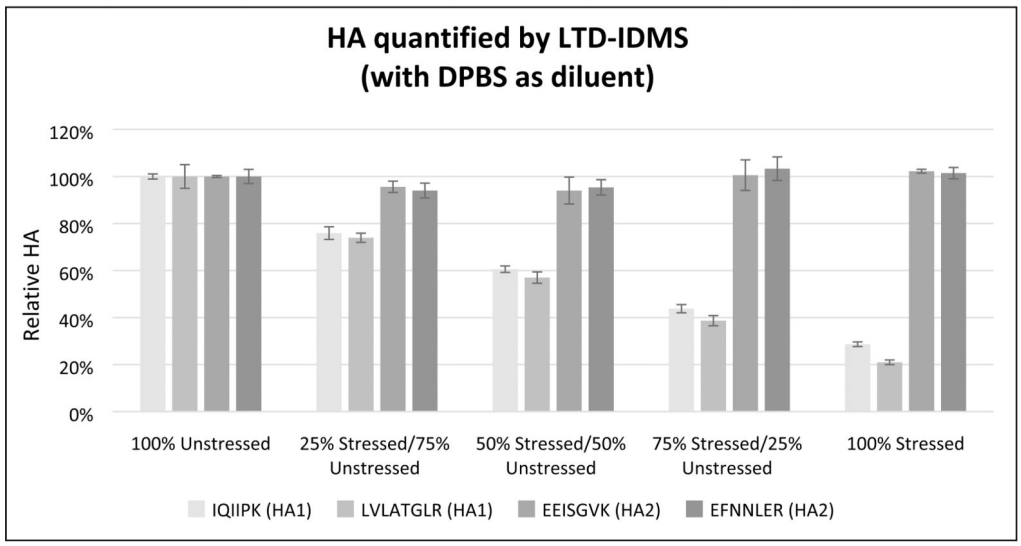


Figure 2.

Relative HA quantification for A/turkey/Turkey/1/2005 A(H5N1) monobulk. Nonstressed and low-pH-stressed monobulk vaccine materials were pretreated with and without LTD as indicated. (A) Relative HA quantification by IDMS following precipitation, reconstitution, and analytical digestion using four reference peptides: IQIIPK and LVLATGLR from the HA1 sequence and EFNNLER and EEISGVK from the HA2 sequence for measurement. Six biological replicates are represented in this data. (B) HA quantification by RP-HPLC, SRID, and LTD-IDMS. Duplicate analyses were performed by SRID and RP-HPLC. LTD-IDMS was repeated 6 times.

A



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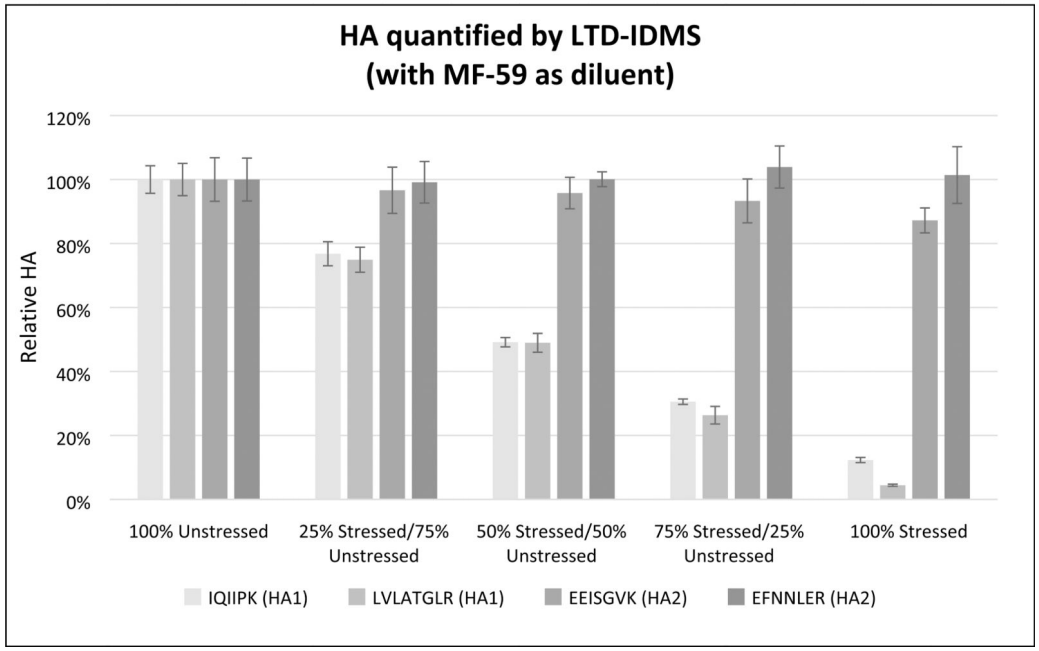

Figure 3.

Relative HA potency quantification for A/turkey/Turkey/1/2005 A(H5N1) mixed preparations by LTD-IDMS. Nonstressed monobulk was mixed with low-pH-stressed monobulk as indicated. (A) Relative HA quantification by LTD-IDMS with DPBS as diluent. LTD-IDMS results with HA1 peptides decrease with the reduction of unstressed HA, while HA2 peptides remain relatively consistent. HA2 peptides provide a measure of total HA, while HA1 peptides measure stability. Six biological replicates are represented in

this data. (B) HA quantification by LTD-IDMS with MF59 as diluent to formulate MF59 adjuvated vaccine. Six biological replicates are represented in this data.

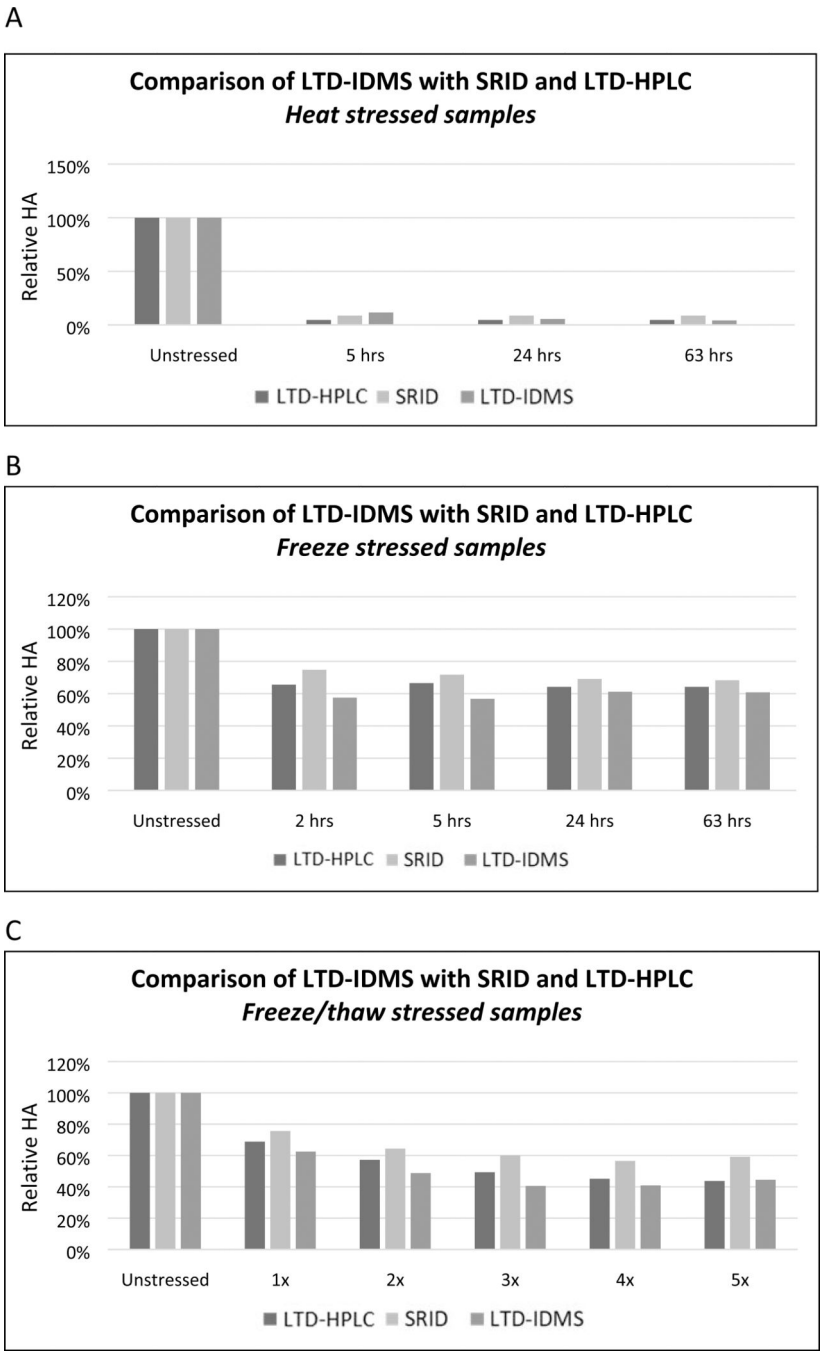

Figure 4.

Relative HA quantification by LTD-HPLC, SRID, and LTD-IDMS of A/turkey/Turkey/1/2005 A(H5N1) monobulk subjected to heat and freeze stressing. (A) Heat stressing: samples were heated in a hot water bath at 56 °C for the specified amount of time. Objective was to identify both the effect of heat exposure and the ability of LTD-IDMS to detect structural changes in the vaccine relative to other modalities. (B) Freeze stressing: samples were frozen at −80 °C for the specified time. Objective was to identify both the effect of exposure to a single freezing event and the ability of LTD-IDMS to detect structural

changes in the vaccine relative to other modalities. (C) Freeze/thaw stressing: samples were frozen in an ethanol/dry ice bath for 20 min and then thawed at room temperature for 30 min for the specified number of cycles. Objective was to identify both the effect of exposure to multiple freeze/thaw events and the ability of LTD-IDMS to detect structural changes. Duplicate analyses were performed by SRID and RP-HPLC. Triplicate analyses were performed by LTD-IDMS.

Table 1.

Target peptides used for the LTD-IDMS quantification of A/turkey/Turkey/1/2005 (H5N1) HA

target peptide	precursorion *m/z*	fragmention *m/z*	fragmention *m/z*	fragmention *m/z*	Collision energy (eV)
IQIIPK	356.242	357.25	470.334	598.392	14
IQII<u>P</u>K	360.242	365.264	478.348	606.407	14
EEISGVK	381.206	390.234	503.318	632.361	16
EEISG<u>V</u>K	384.213	396.248	509.332	638.375	16
LVLATGLR	421.777	446.272	517.309	630.393	17
LVLATG<u>L</u>R	425.285	453.289	524.326	637.41	17
EFNNLER	461.225	531.288	645.331	792.399	19
EFNN<u>L</u>ER	464.733	538.305	652.348	799.417	19