The use of LC-MS/MS for analysis of Nutritional Biomarkers (A, D, MMA) in Dry Matrix Spots (DMS)

MATTHEW K. FLESHMAN, FRANCISCO X. ARREDONDO, AND NEAL E. CRAFT.

Eurofins Craft Technologies, Inc. Wilson, NC 27893.

OBJECTIVE

To conduct time and temperature stability experiments on Nutritional Biomarkers (NBs) and inflammation markers extracted from VIVEBio Dry Matrix Spots (DMS). To establish LC-MS/MS methods to measure 25OH-Vitamin D3, Retinol and Methylmalonic acid (MMA) in Dried Matrix Spots (DMS).

METHODS

Blood samples were created at three hematocrits (20, 30 and 45%) by combining purchased red blood cells (RBC) and sera (4 QC serums). Blood samples of 35 µL were loaded onto VIVEBio PRISM™ blood separators. The underlying pads retaining the serum were stored at 45°, 23°, 4°. -20° and -70°C. Samples were pulled for testing on days 0, 1, 3, 7, 14, 28, 84 and 168 in duplicate for analysis of MMA and 25OH-Vitamin D.

The extraction of 25OH-Vitamin D3 and Retinol was achieved using a stable isotope dilution method by placing the pad in 500µL aqueous solution with isotopically labeled 25-OHD3 then sonicating for 30 min. Proteins were precipitated by vortexing with 500 µL MeOH. Hexanes (1 mL) were added with vortexing to extract lipids. Samples were centrifuged, the hexane layer transferred, and dried. Extracts were reconstituted in mobile phase and analyzed by LC-MS/MS. Baseline separation of 25-OHD3 and 25-OHD2 and their respective epimers was achieved using an Allure PFP Propyl 3µm, 2.1 x 50mm column with a methanol and water gradient. *(Chromatogram 1).*

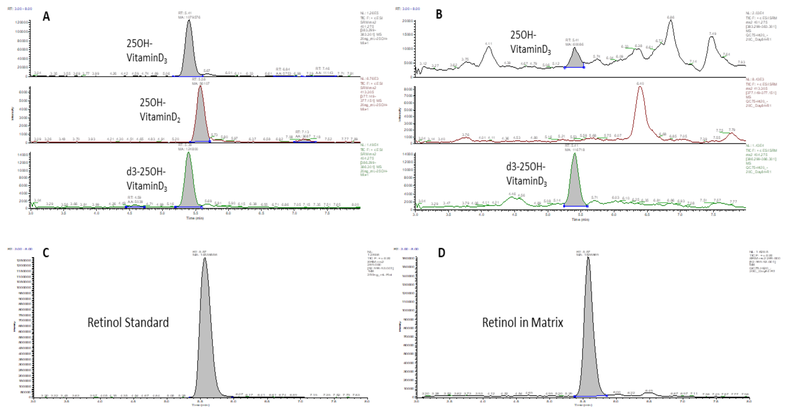
The extraction of MMA from DMS pads was performed using multiscreen 96-well plates. Pads were placed in the wells and 100 µL of d3-MMA working internal standard solution was added followed by 20 µL of DTT solution. Plates were shaken at room temperature for 1hr and then centrifuged to transfer eluent. Eluents were dried under nitrogen and 100 µL 3N HCl in butanol was added, then shaken and incubated at 65 ºC for 20 min. Plates were dried under nitrogen and stored at -70 ºC until analysis. Extracts were reconstituted in mobile phase and analyzed by LC-MS/MS. Separation of MMA was achieved using an Acquity UPLC BEH C18 1.7µm, 2.1 x 50mm column with an acetonitrile and water gradient. *(Chromatogram 2).*

RESULTS

Stability of 25OH-Vitamin D3 in the pads was influenced by time and temperature. Recovery decreased with both time and increasing temperature. Through day 14 recoveries dropped below 70% for the higher temperatures (23 and 45ºC). Stability then appears to level off for the remaining time points. The rate of the of decline was less at lower temperatures. Hematocrit levels had no affect on stability. Concentrations were normalized to each hematocrit and QC at time 0 then percent recovery of subsequent days is shown in *Figure 1* for each temperature.

Endogenous levels of MMA were minimal, thus serums used for the study were spiked with known amounts of MMA. Results show an initial drop in recovery on Day 3 to 60% and then recovery holds steady for the remainder of the time course (50-60%). There appears to be no affect of temperature or hematocrit on MMA stability. Concentrations were normalized to each hematocrit and QC values at time 0. Percent recovery on subsequent days is shown in *Figure 2* for each temperature.

RESULTS



Chromatogram 1. 25OH-Vitamin D standards (A) and DMS extract (B), and retinol standard (C) and retinol in DMS extract (D).

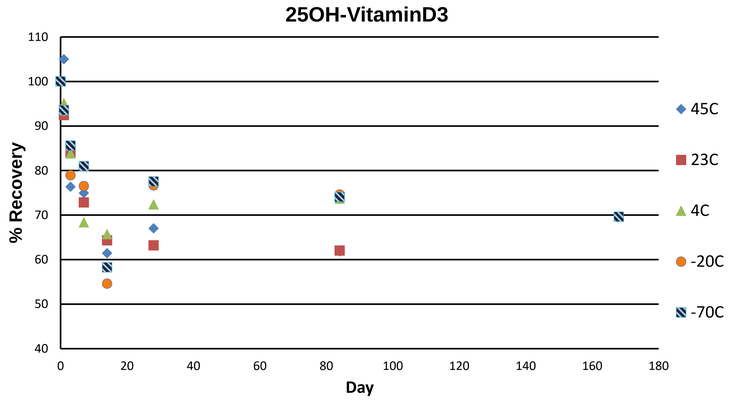
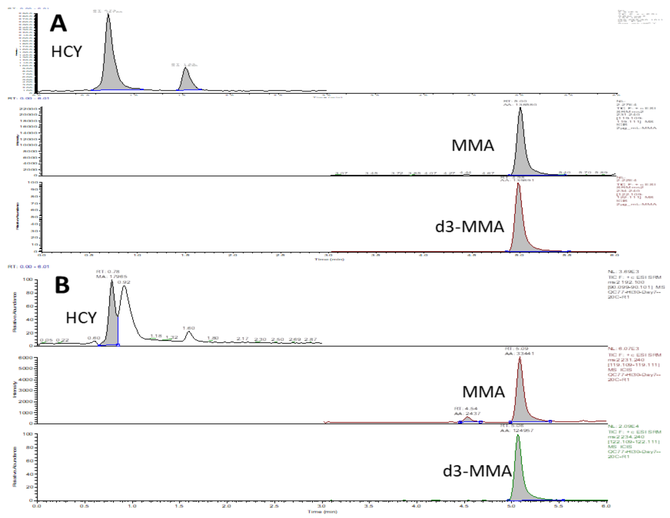


Figure 1. Time and Temperature Dependence of 25 OH-D3 in DMS



*Chromatogram 2. Chromatograms of MMA and HCY standards (**A**) and in DMS extract (**B**).*

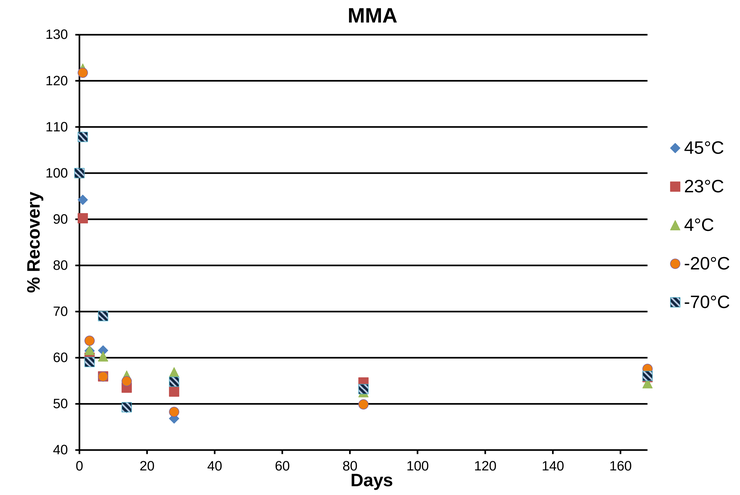


Figure 2. Time and Temperature Dependence of MMA in DMS

CONCLUSIONS

With the current platform of dried matrix spots it is possible to quantify 25OH-Vitamin D3 and retinol in a single extraction. The preparation involves a single tube extraction that could be automated if high through-put analysis is needed. The stability of 25OH-Vitamin D3 and retinol are both time and temperature dependent in the DMS.

The MMA method was adapted from CDC's MMA method in dried blood spots.(1) Our method is designed for high through-put extraction and analysis and is also able to measure total homocysteine. It is sensitive enough to detect normal levels of MMA using ≤10µL serum. Further research is needed to explore why recovery declines during the first day. One confounder in this study is the estimate of serum volume that is transferred to the puck. We retained an aliquot of each eluted puck in order to measure sodium content to estimate the serum volume. This correction will be applied during final analysis. Some samples were excluded from the study as it was apparent that there was poor transfer of serum to the collection disk. In preliminary studies, the 25OHD and MMA dried extracts were found to be stable at -70°C for >30 days

REFERENCES

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ACKNOWLEDGEMENTS

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