A Quantitative LC-MS Method to Determine Lutein Levels in Whole Human Blood Using Dried Blood Spot Analysis (DBS)

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Introduction

Lutein is a natural compound included in one of two subgroups known as xanthophylls, owing to the yellow pigmentation. This class of compounds, known as carotenoids, can be found in green leafy vegetables. Sources of lutein include cale, spinach and collard greens, among others.

It is believed that lutein keeps eyes safe from oxidative stress and the high-energy photons of blue light. Further, studies have shown that an increase in macula pigmentation, such as lutein, may decrease the risk for disease such as macular degeneration (AMD). There's also evidence to suggest that consumption of lutein and zeaxanthin lowers the risk of cataract development. An additional benefit of consuming lutein and zeaxanthin is the improvement of visual performance and decrease in light sensitivity (glare).

Previous work in this group have included determination of lutein in tissues and human breast milk. In the work presented here, we have used dried blood spot (DBS) technology in conjunction with addition of an anti-oxidant that is pre-spotted on the DBS paper in order to stabilize the analyte and thereby preventing oxidation and degradation. Using this approach has led to reliable sensitivity and quantitation down to 10 ppb.

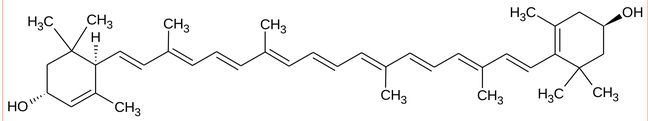

Fig. 1 Chemical structure of the xanthophyll, lutein (IUPAC: β,ε-carotene-3,3'-diol)

Experimental

Sample preparation

GE Whatman FTA™ DMPK-C Cards (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) were pre-treated with an anti-oxidant, one day prior to standard and QC addition, by spotting 20µL of a 0.5% t-butyl hydroquinone in ethanol solution. These where allowed to dry at ambient conditions.

Spiking solutions for an eight point calibration curve and QCs (at three concentration levels) were prepared by serial dilution from a stock solution, such that the final concentrations yielded calibration points at 5, 10, 25, 50, 100, 250, 400 and 500ppb and QCs at 10, 50, and 250 ppb. These spiking solutions (10µL), were each added to 90µL of K_2-EDTA whole human blood (BioreclamationIVT, Westbury, NY) and vortexed. Each whole blood sample was spotted (20µL) on the pre-treated DBS cards and allowed to dry overnight at ambient temperatures covered with aluminum foil. The subsequent day, the center of each spot was removed and transferred into a micro centrifuge tube using a 6 mm punch (Harris Uni-Core™). Ultrapure water (500µL) (Milli-Q™, Millipore Corporation, Billerica, MA) was added to each vial containing a punch. The vials were then vortexed on a Glas-Col Digital Pulse Mixer at 1000rpm for 10 minutes. The DBS punches were transferred into clean vials and 200µL of extraction solution was added (0.1% BTH in 80/20 MeOH/MTBE that was 100ppb in $^{13}C_{40}$-Lutein). These were again vortexed for 10 minutes at 1000rpm. The supernatant (150µL) was transferred into a Waters total recovery vial and analyzed by LC-MS/MS.

Results

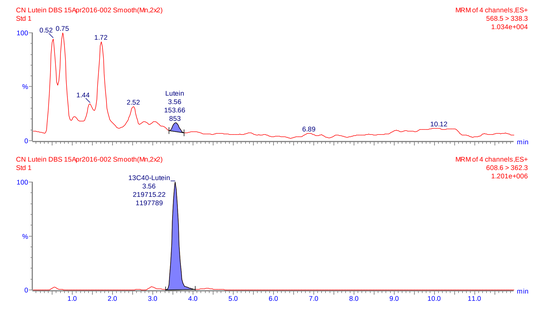

Fig. 2 Multiple reaction monitoring (MRM) chromatogram (5ppb) of lutein (analyte) and $^{13}C_{40}$-lutein (IS)

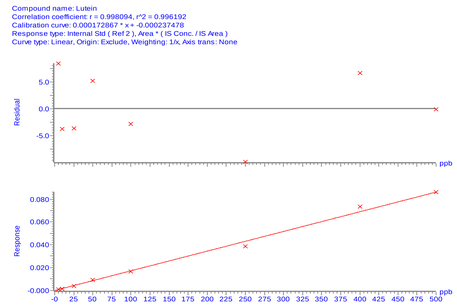

Fig. 3 Example DBS extracted calibration curve and residual plot for lutein

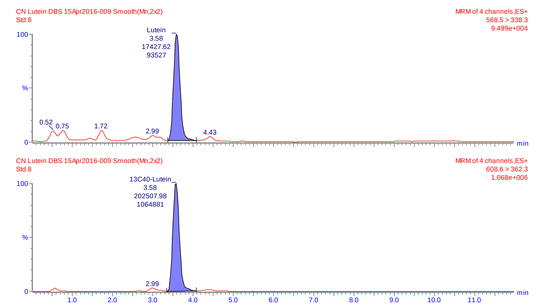

Fig. 4 Multiple reaction monitoring (MRM) chromatogram (500ppb) of lutein (analyte) and $^{13}C_{40}$-lutein (IS)

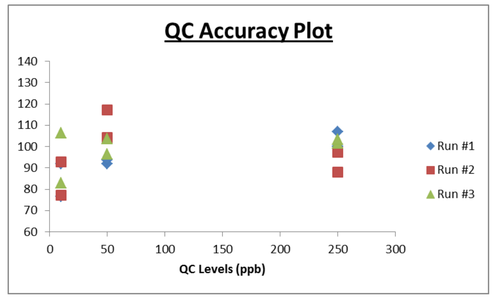

Fig. 5 Percent accuracy of lutein spiked in whole blood samples

Fig. 6 A pre-treated, spotted DBS Card

	QC-10 (ppb)	QC-50 (ppb)	QC-250 (ppb)
Run#1	8	46	250
	9	47	267
Run#2	8	59	220
	9	52	243
Run#3	8	52	254
	11	48	259
Inter Run Mean:	9	51	249
Inter Run SD:	1.1	4.7	16.2
Inter Run %RSD:	**12.9**	**9.3**	**6.5**

Table 1. Summary of inter run accuracy and precision for lutein in whole human blood

Instrument Conditions

Chromatography

Chromatographic separation was carried out on a YMC_{30}Carotenoid HPLC column (100 x 2.0mmID, 3µm, YMC America, Inc., Allentown, PA) with a flow rate of 0.5mL/min at a temperature of 40˚C. The mobile phases for this method consist of A: 10mM NH_4OAc in 95/5 MeOH/Milli-Q water, and, B: 10 mM NH_4OAc in 80/15/5 MTBE/MeOH/Milli-Q™ water.

The following gradient profile was used:

Time(min):	Flow Rate:	%A:	%B:	Curve:
Initial	0.5	100	0	6
0.50	0.5	100	0	6
7.50	0.5	55	45	6
9.00	0.5	0	100	6
10.00	0.5	0	100	6
10.01	0.5	100	0	6
12.00	0.5	100	0	6

Table 2. Summary of LC gradient profile

Mass spectrometry

One multiple reaction monitoring (MRM) transition was recorded for each compound (Table 2) on a Waters Xevo® TQ-S instrument.

Table 3. Multiple reaction monitoring (MRM) transitions used for considered analytes and internal standard

Compound	Polarity	MRM[a]	CE[b] (eV)	Dwell Time (s)
Lutein	ESI+	568.5 > 338.3	20	0.35
$^{13}C_{40}$-Lutein	ESI+	608.6 > 362.3	20	0.35
[a]Multiple reaction monitoring				
[b]Collision energy				

Data analysis

Samples were quantified by the internal standard method against spotted calibration curves in matrix (i.e., K_2-EDTA whole human blood).

Discussion

- Quadratic regression with $1/x^2$ weighting, gave best fit with $R^2 \geq 0.99$ for this compound with standard's and QC's accuracy ≤15% (≤20% at the LLOQ).
- Acceptable accuracy and precision across three independently extracted sample runs established ruggedness of the method.
- Mean recoveries (extraction efficiency) for QC's were 88% for QC-10's, 101% for QC-50's and 100% for QC-250's.
- Pre-treatment of DBS cards greatly increased limit of detection (LOD) for three runs from approximately 250ppb down to 5ppb with LLOQ at 10ppb.
- Elevated background seen across runs most likely contributed to low biased values for lower portion of curve. Suspected instrument maintenance issue.
- Slightly higher backpressure seen on column used through out these three runs may have affected chromatography slightly.
- The use of t-butyl hydroquinone (TBHQ) pre-treatment increased sensitivity by approximately 50 times.

Conclusion

DBS spotted lutein blood samples showed potential oxidation of terminal ring structures. Oxidative losses can be minimized by incorporating an anti-oxidant treatment. Pre-treatment of the DBS cards with TBHQ proved to give the stability to the analyte necessary to improve sensitivity in the assay by approximately 50 times. Future work to lower the high background, thereby lowering the baseline, may improve the sensitivity in this assay even more.

The present DBS based analysis of lutein demonstrated the feasibility of sensitive detection in blood and using 20µL volume of whole human blood samples. With further optimization, the DBS technique can be used to monitor the levels of lutein present in blood on a routine basis.

The use of minimally invasive DBS based analysis offers the potential convenience of "at home" sampling by the consumer and ship the sample to a designated central facility for analysis. The same technique also offers the possibility of obtaining blood samples from neonates and infants and potentially broaden clinical trials.

Selected references

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