# DOES BLOOD SAMPLE HANDLING AND PROCESSING AFFECT LEVELS OF VITAMINS A, E, AND D?

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## Abstract

Proper handling of blood samples is critical for the most accurate results. Knowledge of which factors affect blood samples leads to a practical and efficient system of handling these samples. This study evaluated the effect of light, temperature, or type of blood collection tube on 25(OH) vitamin D, retinol, and alpha-tocopherol in lion blood samples. Blood samples were collected from 2 lions into serum tubes (RED) or tubes containing lithium heparin anticoagulant (GREEN). During processing (<1 h) each tube was either foil wrapped (FOIL), in a dark room without foil (DARK), or in a lighted room (LIGHT) and spent 30 min either at room temperature (20.6°C, ROOM) or in the refrigerator (7.6°C, COOL). Samples were analyzed for retinol and alpha-tocopherol by HPLC, and for 25(OH)D by radioimmunoassay. Results were rank transformed and compared by ANOVA. Light exposure did not decrease concentrations of any analyte ($p>0.05$). Temperature had no effect on any analyte ($p>0.05$). Alpha-tocopherol was higher ($p=0.011$) in RED tubes compared to GREEN. Retinol, alpha-tocopherol and vitamin D were higher ($p<0.002$) in the female lion compared to the male lion. These results indicate that wrapping blood samples in foil or processing them in the dark may be unnecessary. The difference between lions on similar diets indicates how other factors or management may influence circulating vitamin levels. Because this study was very limited in the number of samples, these results should be further validated to provide solid recommendations for handling blood samples for retinol, alpha-tocopherol, and vitamin D analysis.

## Introduction

Blood handling techniques need to be practical and at the same time ensure precise and repeatable results. Many analytes are fragile and may be affected by handling. Recommendations for handling blood samples for 25(OH)D (vitamin D), alpha-tocopherol (vitamin E), and retinol (vitamin A) analysis are to keep plasma blood samples at room temperature for less than 24 hours. In addition, serum should be stored in the cold and dark for less than 24 hours because detectable levels of fat soluble vitamins may decrease after a 24 hour time period. Fat soluble vitamins in both plasma and serum have also been shown to be less stable at room temperature than in cooler environments.[13,10] These recommendations may be difficult to adhere to in situations such as the field when temperature, light, and processing time cannot be controlled. To protect samples from light exposure, blood tubes may be wrapped in foil, however it is difficult to collect blood directly into foil wrapped tubes or to wrap tubes immediately after blood collection. This may also be impractical for large sample sets. Maintaining an ideal temperature during sample handling may also be difficult due to lack of refrigeration and/or the use of ice or ice packs.

Vitamins such as A, E, and D are needed for growth, reproduction, and overall health.[6] Analysis of circulating blood levels of vitamins A, E, and D can allow animal health managers to identify health risks and improve management or diet to ensure nutrient requirements are being met. Circulating vitamin D is directly affected by diet and exposure to sunlight in some species, but the effect of sunlight on vitamin D status may be limited in cats.[2] Vitamin D in lions and other exotic felids is important for calcium uptake. This may be especially important when captive diets contain significant quantities of muscle meat which is low in calcium.[16] Vitamin A is highly regulated and therefore circulating values may reflect recent intake or other physiological factors, but reflect vitamin A stores only at extremes. Vitamin A is primarily important in supporting vision, epithelial tissue, and glycoprotein including supporting normal brain function. Vitamin A deficiency in captive lions has been documented.[3] There is correlation among vitamin E levels in plasma, dietary intakes, and liver stores. However, differences among species and between individuals limit the use of single serum values for assessment.[6,4] Vitamin E levels in blood generally reflect dietary intake. Vitamin E in captive lions is important because it is an anti-oxidant which can reduce cell damage and age related disease processes.[1]

Inaccurate values for vitamin D, vitamin E, and vitamin A due to inappropriate sample handling are not useful and potentially misleading when trying to evaluate the nutrient status of the subject. The objective of this study was to test the effects of light, blood collection tube type, and temperature on the analysis of 25(OH)D (vitamin D), alpha-tocopherol (vitamin E), and retinol (vitamin A) in blood samples.

**Materials and Methods**

Two young unrelated lions at the Fort Worth Zoo were anesthetized for a routine exam on 6/13/2013 (0.1 lion, 129 kg, 861 days of age, obese) and 7/11/2013 (1.0 lion, 163 kg, 707 days of age, overweight). The lions were managed separately and consumed similar diets containing a fortified meat mix (Nebraska Premium Feline, Nebraska Brand, North Platte, NE), horse muscle meat and shank bones, and whole rabbits. Diet meat and meat mixes were analyzed for retinol and alpha-tocopherol as part of standard quality control testing (Fort Worth Zoo, Nutritional Services Laboratory, Fort Worth, TX). The 0.1 lion had outside access from approximately 9 am to 4 pm and the 1.0 lion had outside access from approximately 4 pm to 9 am. The lions were fed between 4 and 5 pm daily, and both were fasted approximately 40 hours prior to anesthesia.

Blood was collected by jugular venipuncture into syringes and transferred into vacutainer tubes for serum (RED) or plasma using lithium heparin anticoagulant (GREEN). Blood tubes were assigned to temperature/light treatments according to Table 1 and Table 2. All tubes were transferred to the lab within 5 minutes, sat for 30 minutes and then were centrifuged for 10 minutes at 2016 x g. Serum or plasma was pipetted into 2 ml foil covered cryovials and frozen at -80°C for subsequent analysis.

In a room with no direct lighting, duplicate aliquots of 200ul of thawed serum or plasma were pipetted into 13 x 100 mm test tubes, extracted according to methods previously described[15] and analyzed for retinol and alpha tocopherol using a WatersTM2695® separations module HPLC, with a Grace/Vydac 201TP54® column and a WatersTM 2487® dual wavelength absorbance

detector. Plasma and serum samples were sent to the Diagnostic Center for Population and Animal Health at Michigan State University (Lansing, MI) for 25-hydroxyvitamin D analysis. Samples were analyzed using a DiaSorin™ Liaison® 25 OH Vitamin D TOTAL radioimunnoassay.

Data was rank transformed to reduce assumptions due to the small number of data points.[5] Ranks were compared by ANOVA with Turkey-Kramer multiple comparisons within lion (for tube and temperature effects) and across lions for light effects on GREEN and ROOM samples. Statistics were performed using Intercooled Stata 9.2 (STATACorp LP, College Station, TX) and graphs were plotted using Graphpad Prism 6.01 (Graphpad Software Inc., La Jolla, CA).

**Results**

Vitamin levels in lion diets and blood samples are shown in Table 3 and Table 4. Results of blood sample analysis for vitamin A, E, and D are shown in Figures 1, 2, and 3.

There was no effect of tube ($p=0.38$) on retinol and no effect of light ($p=0.25$) on retinol in the 0.1 lion (Figure 1). Retinol from the 1.0 lion was higher ($p=0.033$) in LIGHT samples than SEMILIGHT or DARK samples. There was no effect ($p=0.36$) of temperature on retinol. Retinol was also higher in the 0.1 lion compared to the 1.0 lion ($p<0.001$).

Alpha-tocopherol was higher ($p=0.011$) in RED tubes compared to GREEN tubes for 0.1 lion (Figure 2). There was no effect of temperature ($p=0.25$) in the 1.0 lion. Alpha-tocopherol was higher ($p=0.002$) in the 0.1 lion compared to the 1.0 lion. There was no effect of light exposure ($p=0.086$) on alpha-tocopherol for either lion.

Vitamin D (25(OH)D) was lower ($p=0.002$) in FOIL compared to LIGHT and DARK in the 0.1 lion but not affected by light exposure ($p=0.33$) or temperature ($p=0.93$) in the 1.0 lion (Figure 3). Vitamin D was lower ($p<0.001$) in the 1.0 lion compared to the 0.1 lion.

**Discussion**

The Fort Worth Zoo lion diet was comparable in vitamin A, E, and D to diets published for lions[6,7] although vitamin A and E levels were higher due to high levels in their commercial meat mix. Previously published values might also underestimate actual diet content as they represented manufacturers' guaranteed values rather than direct analysis. The diet vitamin A, E, and D levels met the expected nutrient requirements for cats and lions[1,2] and were well below potential toxic levels.[1,2]

The lack of higher circulating vitamin A in Fort Worth lions despite higher dietary vitamin A agrees with other studies which found that circulating vitamin A is not a marker of vitamin A status or dietary intake.[14] However, retinol was higher in 0.1 lion. The exact reasons for this are unclear but serum retinol may be affected by gender, age, or estrous cycle.[8]

Circulating alpha-tocopherol (vitamin E) levels in the Fort Worth lions were higher than previously published values.[6] This is likely due to the higher vitamin E content of the diet. The

0.1 lion was also slightly higher than the 1.0 lion, possibly due to consumption of extra diet from the other lion she was housed with. Circulating vitamin E may also be affected by gender, age, or stress level.[8,11] Although this study found higher vitamin E levels in RED serum tubes, this difference was small and a previous study using human blood did not find any difference between serum and plasma values.[12] Further evaluation is recommended before drawing conclusions from this finding.

Vitamin D concentration in blood from the 1.0 lion was within previously published ranges of vitamin D for captive lions, but the 0.1 lion had notably higher circulating levels.[6] The 0.1 lion had access to more direct sunlight than the 1.0 lion due to their times on exhibit, however it has been found that cats have limited ability to synthesize vitamin D from sunlight.[16] The 0.1 lion had access to the diet of another lion and may have consumed extra vitamin D compared to the 1.0 lion. It is not clear why blood samples covered in foil would have lower concentrations of vitamin D than those exposed to light for as little as 5 min (DARK) especially as it has been shown in previous studies that exposure to light would be expected to decrease vitamin D values.[17] The difference may have resulted from cross-reactivity with other light sensitive analytes during the radioimmunoassay analysis for vitamin D.[9]

Our results showed that processing blood samples in the light did not result in loss of vitamin A, E, or D. Processing samples in the light would be simpler than current practices of foil-wrapping samples and processing in the dark.[6] Also using serum or plasma tubes only affected alpha-tocopherol levels. This is important because it allows the sampler to use the most convenient tube type when collecting and processing blood for vitamins A or D. Due to the limited number of samples in this study the results should be considered preliminary and further tests should be conducted to validate these results and establish more concrete recommendations for handling blood samples for vitamin A, E, and D. However, if these results are further validated, the process for handling blood samples can become much easier and the integrity of samples would not be lost when collected in less than ideal conditions where following specific light and temperature protocols is not possible.

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**Table 1**. Light exposure of tubes. All tubes were handled and processed at room temperature (21°C, ROOM) or refrigerated (7.6°C, COOL).

| Treatment | | Transferred to lab (5 min) | Sitting (30 min) | Centrifuged (10 min) | Pipetted (5 min) |
|---|---|---|---|---|---|
| ROOM | FOIL | Dark | Dark | Dark | Dark |
| | LIGHT | Light | Light | Dark | Light |
| | DARK | Light | Dark | Dark | Dark |
| COOL | SEMILIGHT | Light | Dark (7.6°C) | Dark | Light |
| | DARK | Light | Dark (7.6°C) | Dark | Dark |

**Table 2**. Number of blood tubes collected and analyzed for each subject and treatment combination.

| | 0.1 Lion | | 1.0 Lion | |
|---|---|---|---|---|
| Temperature | ROOM | | ROOM | COOL |
| Tube | RED | GREEN | GREEN | |
| FOIL | 3 | 3 | | |
| DARK | 3 | 3 | 6 | 6 |
| LIGHT | 3 | 3 | 6 | 6 |

**Table 3**. Comparison of lion diets from current study (FWZ diet) with published values and nutrient recommendations for cats.

| | | % of diet DM | Retinol IU/kg DM | Alpha-tocopherol IU/kg DM | Cholecalciferol (vitamin D3) IU/kg DM |
|---|---|---|---|---|---|
| FWZ Diet | Nebraska premium feline diet[1] | 68% | 53,511 | 1018 | 1120 |
| | Horse muscle meat[1] | 16% | 6614 | 70 | NA |
| | Rabbit[2] | 16% | NA | NA | NA |
| | Minimum for FWZ diet | | 37446 | 703 | 762 |
| | Published Lion diets[3] | | 8000 | 55 | 1000 |
| | NRC requirements for cats[4] | | 3550 | 57 | 250 |
| | Suggested toxic levels | | 100,000[6] | 2200[4,5] | 33,000[4] |

[1] Nebraska Brand, North Platte, NE; vitamin D3 based on analysis by the manufacturer
[2]Various sizes, analysis not available
[3]Crissey et. al., 2002 Slifka personal communication, 2013
[4]NRC 2006
[5]Based on other species
[6]Vitamin tolerance of animals, 1987

**Table 4**. Comparison of circulating vitamin levels in captive lions in the current study (FWZ) and published data (mean±sem).

| | Values summarized in Crissey et al., 2002 | FWZ 0.1 | FWZ 1.0 |
|---|---|---|---|
| Retinol, µg/mL | 0.13 ± 0.03<br>0.14<br>0.23 ± 0.02 | 0.19 ± 0.01 | 0.14 ± 0.01 |
| Alpha-tocopherol, µg/mL | 8.4 ± 2.1<br>5.2<br>14.8 ± 1.8 | 18.1 ± 0.5 | 16.8 ± 0.2 |
| 25(OH)D, ng/mL | 36.9 ± 5.1 | 70.5 ± 5.2 | 32.7 ± 0.3 |

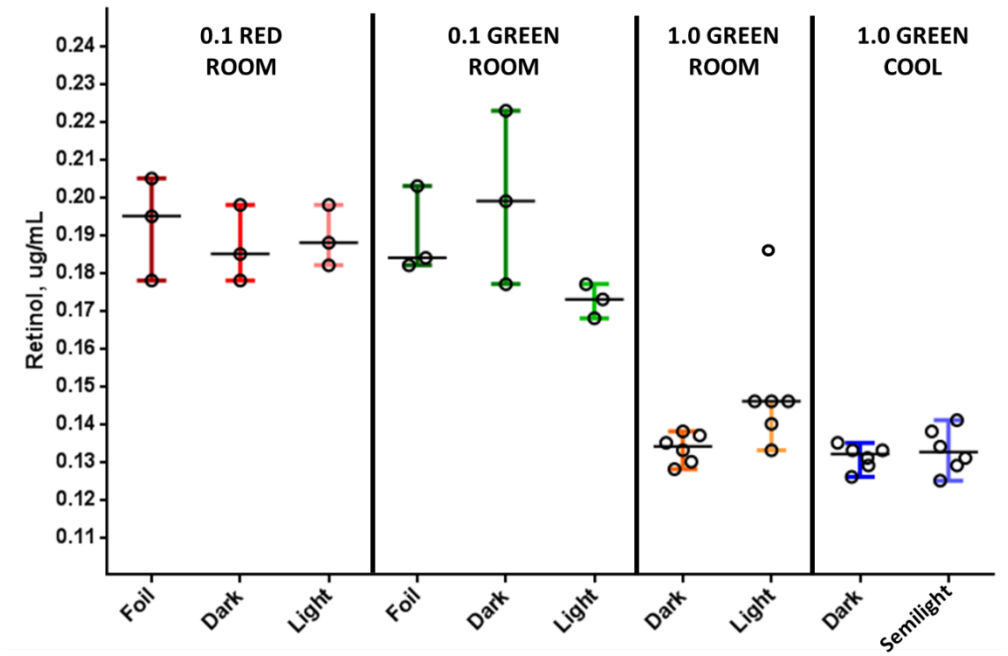


**Figure 1**. Retinol concentrations in blood samples from 1.1 lions collected in serum (RED) or lithium heparin (GREEN) tubes, covered in foil (FOIL) or no foil and processed at 21°C (ROOM) or 7.6°C (COOL) in a dark (DARK) or lighted (LIGHT) room.

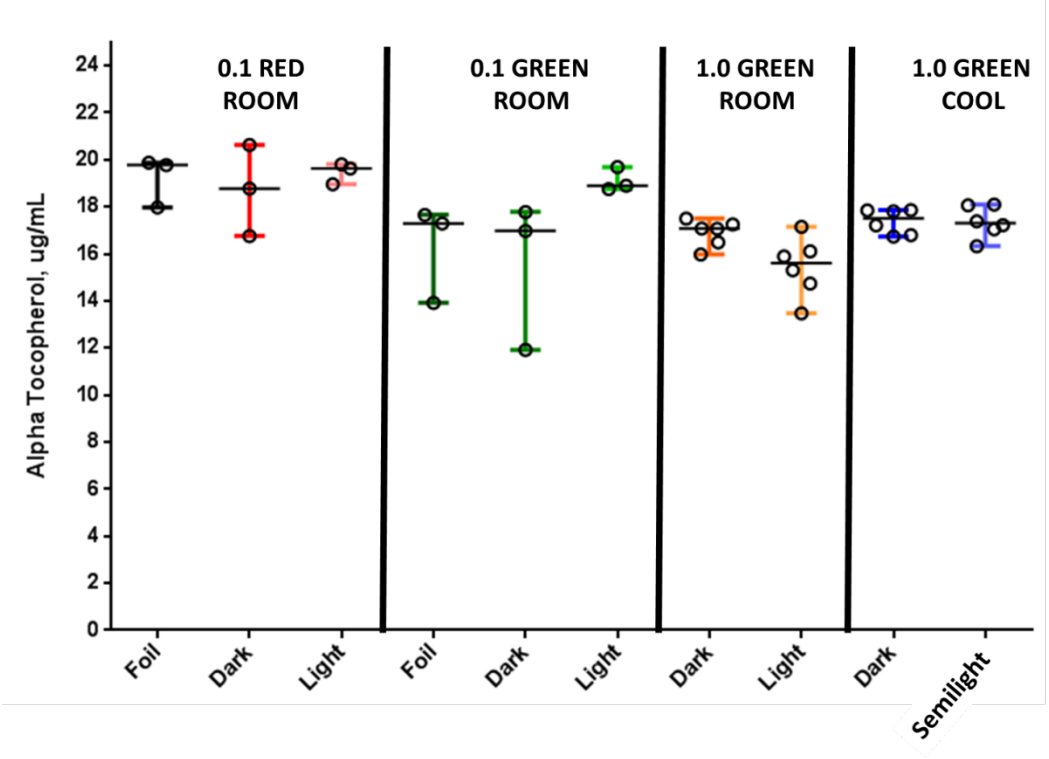


**Figure 2**. Alpha-tocopherol concentrations in blood samples from 1.1 lions collected in serum (RED) or lithium heparin (GREEN) tubes, covered in foil (FOIL) or no foil and processed at 21°C (ROOM) or 7.6°C (COOL) in a dark (DARK) or lighted (LIGHT) room.

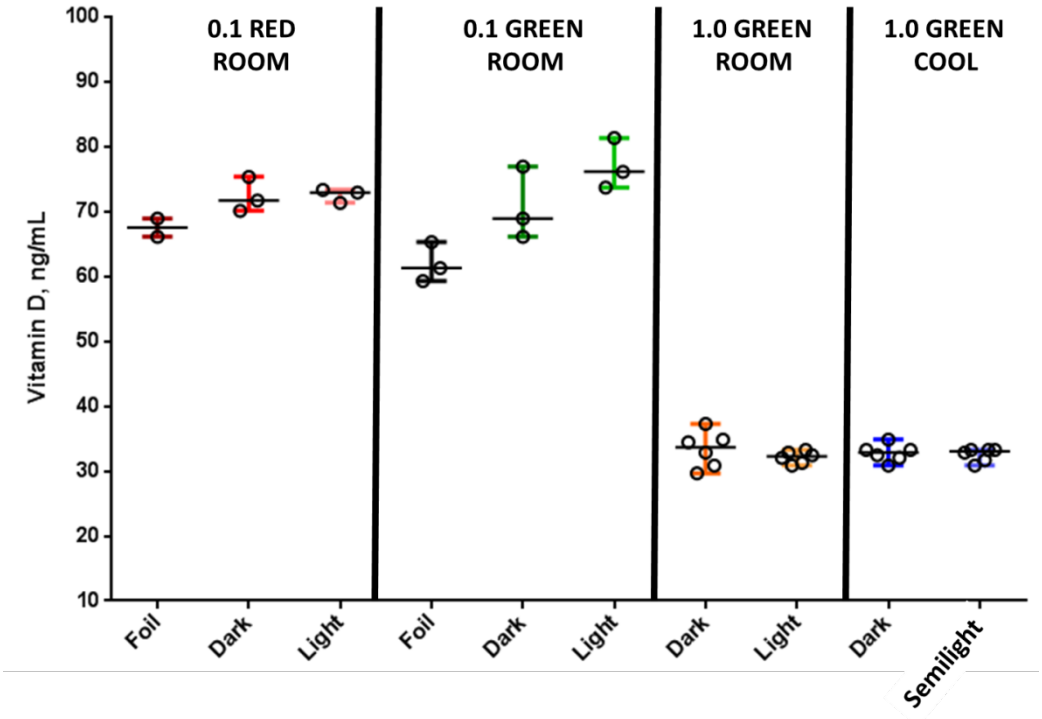


**Figure 3**. Vitamin D (25-OH-D) concentrations in blood samples from 1.1 lions collected in serum (RED) or lithium heparin (GREEN) tubes, covered in foil (FOIL) or no foil and processed at 21°C (ROOM) or 7.6°C (COOL) in a dark (DARK) or lighted (LIGHT) room.