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Oral Nutritional Supplement Effects on Human Hydration Indices

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Oral Nutritional Supplement Effects on Human Hydration Indices

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ABSTRACT

Urine color (U_{col}) is a verified hydration status biomarker in laboratory and non-laboratory environments due to its practicality, ease of use, and the strong correlation that exists between U_{col} and both urine specific gravity (U_{sg}) and urine osmolality (U_{osm}). Although indicative of the sum total of daily fluid turnover, and solute and urochrome excretion, U_{col} may also reflect dietary composition. The purpose of this investigation was to determine the efficacy of U_{col} as a hydration status biomarker after nutritional supplementation with beetroot (880 mg), vitamin C (1000 mg), and riboflavin (200 mg). Twenty males (Mean ± SD; age, 21 ± 2 y; body mass, 82.12 ± 15.58 kg; height, 1.77 ± 0.06 m; BMI, 26.4 ± 4.3 $kg \cdot m^{-2}$) consumed a standardized breakfast and collected urine on one control day (CON) and one day after ingesting a standardized breakfast and a nutritional supplement (SUP), randomized over 3 weeks. Participants and researchers were blinded to the nutritional supplement provided, and participants recorded and replicated exercise and diet for one day before CON and throughout CON and SUP. U_{col}, U_{sg}, U_{osm}, and urine volume (U_{vol}) were measured on all 24-hour urine samples. U_{col} was found to be a significant predictor of 24-hour U_{sg} and U_{osm} in all CON and SUP days for beetroot and vitamin C (all $p<0.001$), though U_{col} was not a significant predictor in the riboflavin SUP day ($p>0.05$). Further, there was no significant difference between CON and SUP 24-hour U_{col} after beetroot ($p=0.431$) and vitamin C supplementation ($p=0.136$). Conversely, there was a significant difference after riboflavin supplementation ($p<0.001$). In conclusion, 24-hour U_{col} was validated as an effective hydration status biomarker after

beetroot and vitamin C supplementation, but not after riboflavin ingestion. When classifying hydration status based on urine color, users should consider recent use of nutritional supplements.

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Chapter 1

Review of Literature and Purpose

Introduction

Water is vital for life and many processes within the human body. The kidneys are the primary regulatory organs within the body responsible for the maintenance of body water and electrolytes. A variety of methods may be employed to measure one's hydration status at any given time. Since these methods assess a simple snapshot of one's hydration status, it is advantageous for investigators to utilize a variety of assessments during an investigation to obtain the most accurate assessment of hydration status.

In particular, urinary hydration biomarkers (i.e., urine volume, urine specific gravity, urine osmolality, and urine color) have been verified as useful methods to assess hydration status, in a variety of laboratory and non-laboratory environments, due to their practicality and ease of collection [1]. These methods, excluding osmolality, do not require the use of costly laboratory equipment or intricate technical skills. Thus, they are practical methods of hydration assessment when used appropriately and repeatedly throughout a research protocol [1]. Although total fluid intake is a major determinant of urinary volume and composition, dietary content is another important factor in urinary excretion [2-4]. It is possible that deviation from one's typical diet, or the ingestion of a nutritional supplement may alter urinary composition (i.e., urine color). The 8-color urine color chart (UCC) developed by Armstrong et al. [5] depicts typical urine colors ranging from pale yellow to brownish green. When used appropriately and

repeatedly throughout a protocol, urine color assessment proves to be a practical method to assess hydration status in laboratory and non-laboratory settings due to its high correlation with urine specific gravity and urine osmolality. Armstrong et al. have noted correlations between urine color and urine specific gravity, along with urine color and urine osmolality [5]. These particular indices were further validated as capable of assessing hydration status in highly trained males [6]. Expanding upon these findings, Perrier et al. reported that strong correlations exist between urine color and urine osmolality, urine specific gravity, 24-hour urine volume, and solute concentrations in healthy, sedentary males [6,7]. Based on these correlations and other studies, there exists an opportunity to identify daily drinking habits based on urine characteristics, such as urine color [8].

However, published findings have noted that particular medications, vitamins, nutritional supplements, and foods may alter urine color [2,6,9]. In particular, studies have identified changes in urine color after nutritional supplementation with beetroot, vitamin C, and riboflavin. Firstly, beetroot has been shown to change urine color to a pink or red hue [9-11]. Secondly, vitamin C supplementation has been shown to change urine color to an orange hue [12,13]. Lastly, riboflavin has been shown to alter urine color to a neon yellow hue [2,10,11,13].

Urine Formation and Composition

The kidney is the primary site of urine formation within the human body. The creation of urine begins when renal capillaries carry blood into one of the

nephrons within the kidney. First, blood enters Bowman's capsule, which is responsible for the first step of simple filtration. After blood plasma passes through Bowman's capsule, the filtrate reaches the proximal tubule, loop of Henle, and distal tubule where it undergoes further filtration, and sodium chloride and water may be reabsorbed. In so doing, urine is created through the removal of solutes, uric acid, and other waste materials by the kidneys [14].

Urine analysis provides a snapshot into endogenous metabolism, general health, and hydration status; thus, it is an effective biomarker when used frequently by health and science professionals. While the physical and chemical properties of urine created within the body are influenced by total fluid intake, they are also affected by diet, physical activity, and stress [2]. Although the types, amount, and concentration of compounds in urine may vary as a result of these factors, all urine includes water, urea, uric acid, and sodium chloride.

Urinary Hydration Biomarkers

It's crucial that professionals are trained in and utilize a variety of techniques to assess hydration status. This is because a sole, universal measure of urine composition does not exist for all situations [1]. The most commonly used urinary hydration biomarkers in health, laboratory, and field settings are: urine volume, urine specific gravity, urine osmolality, and urine color. While it's important to control for variability in analysis of these samples, it's also important to minimize variability in sample collection through the standardization of diet,

lifestyle, and collection times. These factors have been shown to play a significant role in previous clinical studies [3,15,16].

Urine volume is commonly used to estimate hydration status due to the minimal cost, instrumentation, and technical expertise necessary for evaluation. Urine volume for healthy males ranges between 800 and 2000 mL for a 24-hour period and is determined in part by nephron filtration rate, along with fluid and sodium balance. It is also determined by other factors such as posture, physical activity, and fluid volume and concentration of the extracellular space [2,5,17].

Urine specific gravity compares the density of urine to that of deionized water and is utilized by professionals due to its high correlation with urine color and urine osmolality, low cost, level of technical equipment, and training necessary to evaluate [5]. 24-hour urine specific gravity can range from less than 1.003 to greater than 1.030. However, typical urine specific gravity for a euhydrated individual ranges between 1.018 to 1.020 [18].

Urine osmolality is another accurate physiological measure of hydration status. This measure is typically used in controlled, laboratory studies and measures the concentration of solutes within urine. Previous investigations have noted a strong linear relationship between urine osmolality, urine specific gravity, and urine color [5]. 24-hour urine osmolality can range from less than 281 mOsm to greater than 1114 mOsm. However, typical urine osmolality for a euhydrated individual ranges between 587 and 766 mOsm [18].

Lastly, strong correlations exist between urine specific gravity, osmolality, and urine color [5]. 24-hour urine color can range from a pale yellow color

(indicating hyperhydration), to a brownish-green color (indicating hypohydration). Typical urine color for a euhydrated individual is light yellow, or golden, in color. Urine color is an effective hydration biomarker since it accurately reflects endogenous metabolism along with the total fluid intake and hydration status of adults [7,19,20].

Urine Color: Urochrome

Urochrome is the pigment responsible for the typical golden yellow color of urine. The UCC demonstrating the range of urochrome capable of being excreted in urine was developed by Armstrong et al. in 1994 [5]. This chart contains 8 shades of urine color (numbered from 1-8) and ranges from very pale yellow (indicating hyperhydration) to brownish green (indicating hypohydration) [5]. The normal range of urinary pigmentation is primarily a result of total fluid intake, solute excretion, and urochrome excretion. The UCC has not only been verified in males and females during daily activities and exercise, but also within an elderly population in the same scenarios [5,6,21-23] . These validations of the UCC make it a useful tool to monitor ones dynamic hydration status.

Urochome, composed of urobilin and a peptide, is the primary chemical responsible for the pigmentation of urine. Urochrome is created in part after heme, a cofactor responsible for the binding of iron in hemoglobin, is broken down in the liver, creating the bile pigment bilirubin. A portion of endogenous bilirubin undergoes further degradation, being released into the large intestine and feces. The remaining bilirubin in the liver re-enters the bloodstream. As the

nephrons of the kidneys filter this blood, the remaining bilirubin in the bloodstream is filtered and oxidized to create urobilin. The urobilin then combines with a peptide to create urochrome, the principal pigment responsible for the yellow color of urine.

Accordingly, when urochrome is excreted in a greater concentration over a standardized volume, urine appears darker, and more golden in color. Conversely, when urochrome is excreted in a lesser concentration, or in the same concentration but in a greater standard volume, urine appears lighter, and more straw-like in color. Overall, this means that one would expect the density of urochrome to decrease if there were an increase in the volume of urine excreted.

Factors Affecting Urine Color

Although urochrome is the primary determinant, urine color can also be influenced by other factors, including: endogenous metabolism, diet, exercise, and the intake of vitamins or nutritional supplements [4,5,9,20]. Whereas some of these factors may be controlled for in laboratory settings (i.e., diet, exercise, and nutritional supplement ingestion), investigators may be unable to control for others (i.e., endogenous metabolism) after data collection has begun. Thus, it is advantageous for investigators examining urine color to control for as many of these factors affecting urine composition as possible.

Metabolites expelled in urine vary widely among individuals; thus, it behooves investigators to control for as much intra- and inter-individual variation as possible [16]. Metabolities in renal filtrate can vary over time and are a direct

result of urine production. Thus, it is important to compare metabolites in a controlled setting, as well as an experimental setting to provide a basis for data collected. In order to control for differences of endogenous metabolism in experimental studies involving the analysis of urine, investigators need to first be judicious regarding investigation inclusionary criteria. That is, sex should be considered a particularly important factor when conducting this type of investigation since some metabolites (i.e; citrate, alanine, fumarate, carnitine, acetylcarnitine, taurine, and acetone) have been shown to be excreted in statistically different amounts in males and females [16,24,25].

Further, since the metabolites expelled in urine can vary widely between subjects, and even within subjects over an extended period of time [16], it is important to control for diet and fluid ingestion during investigations. This has been further verified by studies of urinary metabonomics in individuals who consume a variety of diets representative of different cultures [4,26,27]. Since dietary variation can greatly alter the short-term urinary metabolite profile of individuals, diet should be controlled for throughout an investigational protocol. However, it is important to remember that urine may be representative of biological processes that have been occurring for a longer time period than diet standardization. For example, the presence of creatine and creatinine in the urine may be a result of diet. However, these substances may present in a higher urinary concentration in males due to a higher average skeletal muscle mass [24,26]. Therefore, while diet standardization may control for a large fragment of urine composition, it is fairly impractical to control all urinary metabolite concentrations.

Since an alteration in exercise has been associated with a change in both the regulation of body water, along with psychological changes related to the drive to drink, it is important to standardize exercise during an experimental protocol involving the collection of urine. In studies of males during exercise in the heat, a mean increase in total water intake throughout a heat acclimation protocol has been shown [28]. Since psycho-physiological changes occur as a result of exercise, a change in an exercise regimen may stimulate not only a change in body water reserves, but also psychological stimuli driving (or not driving) one to drink. Thus, it is important for participants in studies involving the collection of urine to standardize their exercise regimen for the course of the investigation, to control these potential changes. After the development of consistent training schedules and fluid consumption regimens during exercise, significant changes in mean or baseline urine measurements should not necessarily be expected [5].

In particular, the metabolism and excretion of certain vitamins and nutritional supplements have been cited as potential reasons for deviation in typical urine color. Three specific nutritional supplements have been described in textbooks and scientific literature as capable of altering urine colors: vitamin C, riboflavin, and beetroot.

Nutritional Supplement Effects on Urine Color: Beetroot

Beetroot, beetroot juice, and beetroot supplements have gained increasing attention within the scientific community, and increasing popularity in

the general public, due to an increase in the number of studies evaluating it as a source of antioxidants, and also for its effects on blood pressure, as well as the potential increase in athletic performance. Beetroot (or simply, “beets” in the United States) is a magenta-colored vegetable that is grown in the ground, and is typically eaten cooked, pickled, raw, or juiced in a variety of dishes or as a sports supplement. A typical cup of beetroot contains approximately 58 kilocalories, 3.8 grams of dietary fiber, 9.19 grams of sugars, and 13 grams of carbohydrates. Further, approximately 200 mg of the beet pigment, betanin, can be found in a single beetroot. This pigment is responsible for most of the coloring in beetroot and is used in sauces, desserts, and other sweet, sugary foods as a red food colorant. In particular, beetroot juice blends and capsules have become most common on the nutritional supplement market. The typical dosage of beetroot juice (BRJ) in studies is 500 mL, which contains approximately 362.7 mg of betanin [29].

While the majority of studies performed on betanin utilize beetroot juice, the bioavailability, metabolism and absorption of this nutrient may differ among sources (i.e., capsule form, juice form, raw beetroot). These differences are likely a result of the variance in stability, during and after the manufacturing of beetroot products. Betanin instability has been shown to occur after heat treatment, light exposure, drastic pH changes, and when it is combined with other nutrients or foods.

Previous studies indicate an average urinary excretion of approximately 1.00% of betanin after beetroot juice, beetroot, or betanin ingestion [29-31]. This

indicates that renal clearance of beetroot accounts for a small portion of its' intake. The chemical properties of betanin have been likened to that of flavonoids, a large family of plant compounds that are metabolized in the gut. Thus, there is strong evidence suggesting the majority of betanin absorption occurs in the gut. In particular, the stomach (pH=2) has been cited as a possible site of betanin metabolism [32], because it is capable of degrading betanin, which is most stable at a pH of around 4-5. It is reasonable to surmise that those who show a slower change in gastric pH, coupled with a rapid gastric emptying rate, will show an increase in excretion of betanin. However, large interindividual differences in previous studies have made it difficult for studies to draw definite conclusions regarding the absorption, metabolism, and excretion of betanin.

A pink- or red-colored urine may result in some people who ingest beetroot, beetroot products, or products colored with betanin; this is referred to as beeturia. While it may be of concern to some, this is a commonly experienced side effect with no potential for short- or long-term negative health consequences. Due to the rise in popularity of beetroot products in recent years, and the potential impact of beetroot on urine color, the usefulness of the urine color chart after nutritional supplementation with beetroot should be evaluated.

Nutritional Supplement Effects on Urine Color: Vitamin C

Vitamin C is an essential, exogenous water-soluble antioxidant required by all humans, which is necessary for a variety of metabolic and enzymatic reactions. Common sources of vitamin C in the average American diet include

vegetables and fruits such as red peppers, broccoli, strawberries, oranges, and lemons. Although food sources alone are capable of fulfilling the daily requirement for this vitamin, many adults consume nutritional supplements along with, or in place, of dietary sources. In fact, vitamin C is the most widely consumed vitamin supplement throughout the world [33]. Although the ingestion of this vitamin within healthy adult males may come from a variety or combination of sources, studies indicate that the bioavailability and metabolism of vitamin C does not differ between nutritional sources [34-36].

Vitamin C includes two primary forms of the nutrient: ascorbic acid (reduced form) and dehydroascorbic acid (DHA) (oxidized form). The most active form of this vitamin in the body is ascorbic acid. When ingested, it undergoes immediate transport, metabolism, and excretion. Conversely, DHA (oxidized form) must undergo reduction to ascorbic acid within cells before transport, metabolism, and excretion. Thus, most vitamin C supplements on the market are already in the form of ascorbic acid.

Based upon the chemical properties, limited body pool size, and 10-20 day biological half-life of vitamin C, the National Institute of Health Recommended Dietary Allowance (RDA) for adult men is 90 mg/day. This recommendation is based on maintaining an effective concentration within the body, since the average adult body pool of vitamin C is approximately 1000 to 2000 mg. Further, this recommendation is based upon minimizing urinary excretion to ensure maximum absorption [36].

The amount of vitamin C excreted depends primarily upon the amount of the supplement ingested. In doses up to approximately 100 mg·day^{-1}, the majority of vitamin C is metabolized and absorbed by the kidneys, with the renal excretion of this vitamin increasing proportionately with higher intakes up to approximately 80 mg·day^{-1} [37,38].

Conversely, in doses beyond approximately 200 mg·day^{-1}, absorption decreases, more vitamin C is degraded in the intestine, and more is eventually excreted. This may account for some of the deleterious side effects of large doses of vitamin C. The Tolerable Upper Intake Level (UL) for vitamin C in adult men is established at 2000 mg·day^{-1}, based upon the increased potential for adverse effects. Some particular effects noted beyond this dosage include gastrointestinal distress and diarrhea. Decreasing the amount of vitamin C can effectively mitigate these adverse effects, which are not indicated as serious or life threatening.

Vitamin C supplementation reportedly changes urine color to an orange hue in humans [12,13]. Since this supplement is the most widely used nutritional supplement in the world, it is advantageous to evaluate the effects of vitamin C consumption on the reliability of the urine color chart.

Nutritional Supplement Effects on Urine Color: Riboflavin

Riboflavin is a highly regulated, water-soluble vitamin involved in a variety of human metabolic pathways. Common sources of riboflavin within the average American diet include dairy products such as milk, fortified breads, and breakfast

cereals. Many adults also ingest riboflavin in the form of nutritional supplements, and it is especially prevalent in multivitamins. Riboflavin is converted to the coenzyme flavin mononucleotide (FMN) within the small intestine and other organs [39]. FMN is then converted to flavin adenine dinucleotide (FAD), which is the active flavocoenzyme in body tissues. The National Institute of Health RDA for riboflavin in adult men is 1.3 mg·day^{-1}. This recommendation is based on maximal intestinal absorption, and maintaining an effective body pool reserve, which is approximately 27 mg. Further, this recommendation is based on avoiding a clinical deficiency, which occurs at less than 0.5 to 0.6 mg·day^{-1} [39,40].

When ingested with food, riboflavin ingestion is proportional to absorption. When this vitamin is ingested in excess of body stores, the excess is excreted in the urine. While the amount of riboflavin excreted in urine is primarily affected by the amount ingested, it is also affected by other macronutrients and physical activity level. Diets with a low fat-to-carbohydrate ratio coincide with a decreased riboflavin requirement, though the mechanism for this is not currently known. Further, adults who participate in a high level of physical activity excrete more of this vitamin than their sedentary counterparts [39].

Since riboflavin is a highly regulated nutrient within the body, the processes utilized to maintain a safe body pool reservoir are also effective in excreting an excess of this vitamin. That said, a UL for riboflavin does not currently exist, because no short- or long-term adverse effects have been associated with ingestion of up to 60 mg [39].

Identifiable by their neon yellow color, riboflavin nutritional supplements reportedly alter urine to a brighter than expected neon yellow color after nutritional supplementation [2,10]. Thus, it is possible that a brighter urine color as a result of riboflavin ingestion could falsely indicate that an individual is more hypohydrated than is actually true. The body reservoir for riboflavin is saturated at a dose of approximately 27 mg. Thus, it does not require a large dose of this vitamin to reach the RDA. Further, ~98% of riboflavin is excreted within 24 hours, and ~51% of this supplement passes via renal clearance [41]. Thus, it is reasonable to assume that urinalysis for 24 hours after nutritional supplementation may reveal changes in urine composition or color. Since renal excretion of this nutritional supplement is crucial in its removal from the human body, it's beneficial to evaluate the urine color before and after nutritional supplementation.

Statement of Problem and Research Hypothesis

It is known that certain vitamins, nutritional supplements, and foods may alter urine color [2,6,9]. As described above, changes in urine color may occur following nutritional supplementation with beetroot, vitamin C, and riboflavin. However, the quantitative measures of discoloration have not been studied, and the doses that may affect measurements are currently unknown.

Urine specific gravity (U_{sg}) and urine osmolality (U_{osm}) were used as the accepted standards of hydration status assessment in this investigation, due to the strong correlation with urine color (U_{col}). The purpose of this study is to

determine if 24-hour U_{col}, U_{sg}, and U_{osm} remain valid indices of hydration status after nutritional supplementation with commercially available beetroot, vitamin C, and riboflavin. If a change in 24-hour U_{col} is observed, it may be necessary to modify the use of the current UCC, or add prerequisites to its use. Further, if supplementation with leads to a change in other urinary hydration indices (i.e., 24-hour U_{sg} and U_{osm}), it may be necessary to modify the interpretation and use of particular urinary hydration indices in laboratory settings.

Since beetroot is not recognized by the National Institute of Health as a nutritional supplement, it does not have an established UL. However, past studies do not note any toxicity values with beetroot, the standard dosage of beetroot juice given in studies approximating 500 mL. For the purposes of this study, and to demonstrate ecological validity, participants will be given the recommended dosage of beetroot capsules (880 mg/day). The doses of vitamin C and riboflavin given in this investigation are based upon the National Institute of Health Tolerable Upper Limits (UL), along with the recommended doses on the label of each supplement [36,39]. The established upper limit of Vitamin C is 2000 mg/day [36], and average dose recommended on Vitamin C labels is between 500-1000 mg/day. For the purposes of this investigation, participants will be given 1000 mg/day in tablet form. Lastly, while an UL for riboflavin does not exist, previous studies have noted that there's the potential for adverse side effects beyond 400 mg/day [42]. For this investigation, participants will be ingesting 200 mg/day in tablet form, which should not induce any negative side effects.

There are two major hypotheses for this investigation (I: ordinary; II: null hypothesis):

1) (A) (I) 24-hour urine color will be altered by beetroot, vitamin C, and riboflavin supplementation.

(II) 24-hour urine color will not be altered by beetroot, vitamin C, and riboflavin supplementation.

(B) (I) 24-hour urine color will change by at least one unit on the urine color chart after beetroot, vitamin C, and riboflavin supplementation.

(II) There will be no change in 24-hour urine color after beetroot, vitamin C, and riboflavin supplementation.

2) (I) 24-hour urine specific gravity and osmolality will not be altered by beetroot, vitamin C, and riboflavin supplementation.

(II) 24-hour urine specific gravity and osmolality will be altered by beetroot, vitamin C, and riboflavin supplementation.

The information gained from this investigation could provide further guidance for hydration assessment in laboratory settings.

Chapter 2

Manuscript

Abstract

Urine color (U_{col}) is a verified hydration status biomarker in laboratory and non-laboratory environments due to its practicality, ease of use, and the strong correlation that exists between U_{col} and both urine specific gravity (U_{sg}) and urine osmolality (U_{osm}). Although indicative of the sum total of daily fluid turnover, and solute and urochrome excretion, U_{col} may also reflect dietary composition. The purpose of this investigation was to determine the efficacy of U_{col} as a hydration status biomarker after nutritional supplementation with beetroot (880 mg), vitamin C (1000 mg), and riboflavin (200 mg). Twenty males (Mean ± SD; age, 21 ± 2 y; body mass, 82.12 ± 15.58 kg; height, 1.77 ± 0.06 m; BMI, 26.4 ± 4.3 $kg \cdot m^{-2}$) consumed a standardized breakfast and collected urine on one control day (CON) and one day after ingesting a standardized breakfast and a nutritional supplement (SUP), randomized over 3 weeks. Participants and researchers were blinded to the nutritional supplement provided, and participants recorded and replicated exercise and diet for one day before CON and throughout CON and SUP. U_{col}, U_{sg}, U_{osm}, and urine volume (U_{vol}) were measured on all 24-hour urine samples. U_{col} was found to be a significant predictor of 24-hour U_{sg} and U_{osm} in all CON and SUP days for beetroot and vitamin C (all $p<0.001$), though U_{col} was not a significant predictor in the riboflavin SUP day ($p>0.05$). Further, there was no significant difference between CON and SUP 24-hour U_{col} after beetroot ($p=0.431$) and vitamin C supplementation ($p=0.136$). Conversely, there was a

significant difference after riboflavin supplementation ($p<0.001$). In conclusion, 24-hour U_{col} was validated as an effective hydration status biomarker after beetroot and vitamin C supplementation, but not after riboflavin ingestion. When classifying hydration status based on urine color, users should consider recent use of nutritional supplements.

Introduction

Water is vital for human metabolism, movement of substances into and out of cells, cardiovascular function, temperature regulation, and many other homeostatic processes. Because the kidneys are primarily responsible for the maintenance of body water and electrolytes, urinary hydration biomarkers (i.e., urine volume, U_{vol}; urine specific gravity, U_{sg}; urine osmolality, U_{osm}; and urine color, U_{col}) have been identified as valid indices of hydration status in a variety of laboratory and non-laboratory environments. When used appropriately, a moderate to strong correlation has been shown ($r=0.46$-0.82) between U_{col} and both U_{sg} and U_{osm} in a variety of populations including healthy, sedentary males [5,7]. In particular, U_{col} is a practical method of hydration assessment for athletes and laypeople because it is easy to measure and does not require laboratory equipment or technical expertise [1]. One validated urine color chart (UCC) depicts typical urine colors ranging from pale yellow to brown, and represents the sum total of daily fluid turnover, solute excretion, fluid intake volume, and urochrome excretion [5].

Although U_{col} represents endogenous metabolism, dietary content may also influence urinary composition [2-4]. In particular, previous studies have identified changes in urine color after nutritional supplementation with beetroot, vitamin C, and riboflavin. Although many professional organizations recommend seeking out balanced nutrition in the form of whole foods, many members of the general population utilize a variety of nutritional supplements regularly for many reasons [43]. In previous nutritional studies, beetroot has reportedly changed urine color to a pink or red hue [9-11], vitamin C supplementation has changed urine color to an orange hue [12,13], and riboflavin colored urine neon yellow [2,10,11,13]. The purpose of this study was to assess if U_{col}, U_{sg}, and U_{osm} remained valid indices of hydration status after nutritional supplementation with beetroot (880 mg), vitamin C (1000 mg), and riboflavin (200 mg).

Methods

Subject Characteristics

Twenty males (demographics in Table 1) participated in this investigation. Participants were instructed to limit exercise to 7 hours per week, refrain from ingesting nutritional and/or vitamin supplements, as well as beetroot or beetroot derived products at the beginning of this investigation, and refrain from drinking alcoholic beverages for 48 hours prior to all visits. Exclusionary criteria included the use of tobacco products, history of renal, heart or bladder disease, and the use of prescription or over-the-counter medications. The University of Connecticut Institutional Review Board for Human Studies approved this

investigation. All participants attended an informational briefing, and then gave written informed consent prior to participation.

Table 1: Participant Demographic Information

	Mean ± SD	Range (Minimum, Maximum)
Age (y)	21 ± 2	6 (19, 25)
Body Mass (kg)	82.12 ± 15.58	69.22 (59.60, 128.82)
Height (m)	1.77 ± 0.06	0.22 (1.65, 1.87)
BMI ($kg \cdot m^{-2}$)	26.4 ± 4.3	18.5 (20.2, 38.7)

Abbreviations: BMI= Body Mass Index

Overview of Investigational Design

This investigation consisted of two phases. The first phase, lasting one week, consisted of 1 visit during which participants were instructed on how to record exercise and diet. During the second phase, lasting three weeks, participants collected urine during one control day (CON) and one day on which they consumed a provided nutritional supplement (SUP). CON and SUP days were repeated for each nutritional supplement; supplements were given in a randomized order and a minimum of 72 hours separated nutritional supplement trials. Researchers and participants were blinded to the nutritional supplement

provided. Participants recorded and replicated exercise and diet for one day before CON as well as during CON and SUP.

On CON and SUP, participants reported to the laboratory fasted at least four hours and consumed a standardized breakfast consisting of one plain bagel, 2 tablespoons of peanut butter, 1 banana, and .5 liters of water (total macronutrients: 475 kcal, 69 g carbohydrates, 16 g protein, 18 g total fat). On SUP, participants consumed one of the nutritional supplements (880 mg Eclectic Institute Fresh Freeze-Dried Beet Juice, Nature's Bounty 1000 mg vitamin C, or Nature's Bounty 200 mg riboflavin) with breakfast. These supplement doses were based upon the National Institute of Health Tolerable Upper Limits (UL), and the recommended doses on the label of each supplement bottle, which provides ecological validity [36,39].

Following their visit to the laboratory, participants collected urine for 24 hours and recorded the time of each void on a urine survey form.

Measurements and Outcome Variables

Urine variables were measured after each CON and SUP day. 24-hour U_{vol} was measured gravimetrically (Ranger 3000, Ohaus, USA), 24-hour U_{sg} was assessed via hand-held refractometer (Reichert TS 400, Reichert Technologies, USA), and 24-hour U_{col} was evaluated by the same researcher using an 8-category urine color chart (Urine Color Chart, Human Hydration, LLC., USA). 24-hour U_{osm} was measured in 24-hour samples in triplicate, using freezing-point depression (Model 3250, Advanced Instruments, Inc., USA).

Alongside urine measurements, 3-day diet records were recorded and analyzed with Nutritionist Pro™ Software (V.6.1, Axxya Systems, USA) for macronutrients (carbohydrates, g; protein, g; fat, g), total kilocalories (kcal), and total water intake (TWI, L). TWI was defined as the amount of fluids (water and all other beverages) consumed, along with the moisture content present in all foods. Diet recording and analysis allowed researchers to confirm diet replication during each week of participation.

Moreover, participants were asked to record and replicate their exercise alongside diet replication.

Height was also measured with a stadiometer (Adult Mechanical Model, Detecto Scales, USA) at the Preliminary Procedures visit, and body mass (Model 349KLX, Healthometer Professional, USA) was measured at each visit.

Statistical Analyses

All statistical analyses were completed with SPSS® Statistical Software (V.22.0.0, IBM Corp., USA). Given an *a priori* α level set at $p<0.05$, effect size of 1 unit on the UCC, and power set at 0.9, the necessary sample size required to detect significance was calculated to be 13 participants (G*Power, V.3.1.9, Germany).

Dependent sample t-tests were used to assess potential changes in demographics, dietary composition, and U_{vol} from CON to SUP days within (and not between) weeks. Three dependent t-tests, per group comparison, were also employed in order to evaluate differences between 24-hour U_{col}, U_{sg}, and U_{osm} on

CON days versus SUP days for each nutritional supplement. Further, linear regression analyses were utilized to evaluate if 24-hour U_{sg} was a significant predictor of participants' 24-hour U_{osm} for all supplements on both CON and SUP days. These analyses were also utilized to evaluate 24-hour U_{col} as a predictor of 24-hour U_{sg} and U_{osm}.

Results

A total of twenty males (nutritional supplement, [n=x]; beetroot, [n=16]; vitamin C, [n=14]; riboflavin, [n=15]) participated in this investigation; their starting age, body mass, height, BMI, and diet composition are reported in Table 1. Demographics did not significantly differ between CON and SUP days.

The mean kilocalories, protein, carbohydrates, fat, and TWI ingested by participants did not significantly differ between CON and SUP days within any week. 24-hour U_{vol} was also similar on CON versus SUP days for all nutritional supplements (all $p>0.05$).

Table 2: Nutritional Information and Total Water Intake during CON and SUP Days

Nutritional Supplement	Day	Kilocalories (kcal) 24-hour Mean ± SD	Carbohydrates (g) 24-hour Mean ± SD	Protein (g) 24-hour Mean ± SD	Fat (g) 24-hour Mean ± SD	TWI (L) 24-hour Mean ± SD
Beetroot (n=16)	CON	2802 ± 562	322 ± 118	137 ± 35	112 ± 33	3.308 ± 1.012
	SUP	2802 ± 562	322 ± 118	137 ± 35	112 ± 33	3.308 ± 1.012
Vitamin C (n=14)	CON	2664 ± 514	306 ± 106	131 ± 35	107 ± 35	3.323 ± 1.027
	SUP	2670 ± 513	306 ± 106	131 ± 35	107 ± 34	3.304 ± 1.047
Riboflavin (n=15)	CON	2726 ± 568	314 ± 103	136 ± 36	108 ± 38	3.435 ± 1.033
	SUP	2756 ± 610	314 ± 103	137 ± 36	109 ± 40	3.454 ± 1.074

Abbreviations: CON, Control Day; SUP, Supplementation Day, TWI, Total Water Intake.

There was no significant difference between CON and SUP 24-hour U_{col} after beetroot (mean U_{col} difference; 0 ± 1, t=-0.808, p=0.431) and vitamin C supplementation (mean U_{col} difference; 0 ± 1, t=-1.581, p=0.136). Conversely, there was a significant difference between CON and SUP 24-hour U_{col} after nutritional supplementation with riboflavin (mean U_{col} difference; -1 ± 1, t=-5.514, p<0.001) (Table 4).

Table 4: Analysis of 24-hour Urine Variables during CON and SUP Days

Nutritional Supplement	24-hour Urinalysis	CON Mean ± SD	SUP Mean ± SD
Beetroot (n=16)	U_{col}	3 ± 1	3 ± 1
	U_{sg}	1.014 ± 0.005	1.015 ± 0.005
	U_{osm} (mOsm·kg^{-1})	560 ± 205	593 ± 221
	U_{vol} (L)	2.036 ± 0.898	1.891 ± 0.675
Vitamin C (n=14)	U_{col}	3 ± 1	3 ± 1
	U_{sg}	1.013 ± 0.006	1.016 ± 0.008
	U_{osm} (mOsm·kg^{-1})	524 ± 241	585 ± 282
	U_{vol} (L)	2.255 ± 0.901	1.985 ± 0.941
Riboflavin (n=15)	U_{col}	3 ± 1 *	4 ± 0 *
	U_{sg}	1.014 ± 0.005	1.014 ± 0.006
	U_{osm} (mOsm·kg^{-1})	547 ± 223	578 ± 255
	U_{vol} (L)	2.176 ± 0.825	2.203 ± 0.768

*Significance at p<0.05
Abbreviations: CON, Control Day; SUP, Supplemented Day; U_{col}, Urine Color; U_{sg}, Urine Specific Gravity; U_{osm}, Urine Osmolality; U_{vol}, Urine Volume

24-hour Urine Variable Relationships

24-hour U_{sg} explained 98% (Beetroot), 99% (Vitamin C), and 97% (Riboflavin) of the variance in 24-hour U_{osm} during all CON days. 24-hour U_{sg} also explained 99% (Beetroot), 86% (Vitamin C), and 98% (Riboflavin) of the variance in 24-hour U_{osm} during all SUP days.

Further, 24-hour U_{col} was found to be a significant predictor of both 24-hour U_{sg} and U_{osm} in all CON and SUP days for beetroot and vitamin C (all $p \leq 0.015$). However, 24-hour U_{col} was not a significant predictor of 24-hour U_{sg} or U_{osm} in the riboflavin SUP day (all $p \geq 0.097$) (Table 3).

Table 3: Analysis of 24-hour U_{col} as a predictor of 24-hour U_{sg} and 24-hour U_{osm}

Nutritional Supplement	24-hour Urinalysis	CON			SUP		
		F	df	R^2	F	df	R^2
Beetroot (n=16)	U_{sg}	13.797	15	.48*	9.122	15	.38*
	U_{osm}	12.835	15	.46*	7.007	15	.32*
Vitamin C (n=14)	U_{sg}	6.831	13	.34*	26.076	13	.68**
	U_{osm}	5.943	13	.31*	13.486	13	.51*
Riboflavin (n=15)	U_{sg}	33.255	14	.70**	1.854	14	.12
	U_{osm}	26.075	14	.65**	1.693	14	.11

*Significance at $p<0.05$
**Significance at $p<0.001$
Abbreviations: U_{col}, Urine Color; U_{sg}, Urine Specific Gravity; U_{osm}, Urine Osmolality CON, Control Day; SUP, Supplementation Day
]

Discussion

Previous publications have noted that the ingestion of certain nutritional supplements may alter U_{col} [5,9]. Thus, diet standardization in nutritional or hydration studies is crucial, as it controls for metabolite excretion, as well as intra-individual variation in urinary hydration biomarkers [4,15,16]. The purpose of this investigation was to determine the efficacy of 24-hour U_{col}, U_{sg}, and U_{osm} as indices of hydration status after nutritional supplementation with beetroot, vitamin C, and riboflavin. Major findings indicate that nutritional supplementation with either beetroot or vitamin C ingestion may not be capable of altering 24-hour U_{col}. On the contrary, riboflavin ingestion may be capable of altering 24-hour U_{col} by making the urine appear more concentrated, thus altering the efficacy of the UCC. Lastly, 24-hour U_{sg} and U_{osm} remained strong biomarkers for hydration status assessment before and after nutritional supplementation with beetroot, vitamin C, and riboflavin.

U_{col} as a Hydration Status Biomarker

U_{col} has been validated as an effective hydration status biomarker due to the strong correlation with both U_{sg} and U_{osm} in healthy adults [5,7]. Not only reflective of the sum total of daily fluid turnover, U_{col} is also indicative of endogenous metabolism, along with solute and urochrome excretion [7,44]. This investigation further substantiates U_{col} as an effective biomarker of hydration status in healthy male adults due to the strong correlation found between Ucol and U_{sg} and U_{osm} on all CON days (r=0.56-0.84).

Effects of Nutritional Supplementation on Urine Color: Beetroot

Whereas previous investigations have shown that renal metabolism plays a fairly insignificant role in betanin excretion, it is purported that the majority of betanin metabolism occurs within the gut. This is likely due to gastric pH at the time of ingestion, along with gastric emptying rate [29,31,32]. Since a change in U_{col} was not observed in this investigation, it is possible that the acidity of the stomach (pH ~ 2) degraded the betanin (which is most stable at a pH of about 4-5), so that beeturia could not result.

Interestingly, previous studies have utilized either raw beetroot or beetroot juice; whereas beetroot capsules were utilized in order to maintain the double-blinded nature of this investigational design. Since extreme temperatures, light treatment, and pH fluctuations are capable of altering betanin stability, it is possible that this pigment was damaged or unstable within capsules prior to ingestion. This may also explain the non-significant change of urinary hydration biomarkers after nutritional supplementation.

Effects of Nutritional Supplementation on Urine Color: Vitamin C

The most widely used nutritional supplement in the world, vitamin C, is frequently consumed by athletes and non-athletes alike [33]. Previous investigations indicate that renal clearance plays a larger role in excretion in dosages beyond 200 mg/day [37,38]. Despite previous accounts of a change in U_{col} after supplementation with vitamin C, this investigation did not identify a

significant change before and after supplementation. This may be the result of intestinal excretion of this vitamin.

It has been shown that the absorption of vitamin C may be related to both body pool size, as well as carbohydrate intake. This is because vitamin C competes with glucose for uptake via GLUT1 transporters. It may be reasonable to assume that participants had small body pool sizes of vitamin C prior to this investigation, which lead to the increased absorption of vitamin C, and lack of (visible) excretion.

Effects of Nutritional Supplementation on Urine Color: Riboflavin

When taken with food, riboflavin ingestion has been noted to be proportional to absorption in doses up until about 27 mg/day, with excess excreted in the urine within 24 hours [41]. This investigation revealed a significant change in U_{col} after nutritional supplementation with riboflavin, as the mean U_{col} changed from a unit of 3 to 4, falsely indicating a more hypohydrated state. Overall, the role of the renal system in riboflavin excretion may be a primary explanation for the significant change observed in U_{col} before and after nutritional supplementation.

Limitations and Future Research Directions

Although the participants in this investigation did replicate their diet throughout the investigation, they did not ingest the same amount of

macronutrients and micronutrients. There were not intra-individual changes noted in diet, though there were inter-individual differences.

Further, this investigation recorded the efficacy of U_{col} as a hydration status biomarker after nutritional supplementation in healthy, college-aged males eating a standardized breakfast and replicated diet. Future research should evaluate the efficacy of this hydration status biomarker in sub-populations known to ingest nutritional or vitamin supplements at a higher propensity than the remainder of the population. In particular, women (pregnant and non-pregnant), and athletes (before, during, and after exercise) may be interesting groups to investigate.

Conclusion

This investigation holds particular relevance for clinicians, recreational enthusiasts, athletes, and members of the general population who utilize nutritional supplements and consciously monitor urine as a biomarker of hydration status. Whereas 24-hour U_{col} was verified as an effective hydration status biomarker after supplementation with beetroot and vitamin C, accurate U_{col} analysis after riboflavin ingestion proves trivial since it may be directly altered by supplementation, thus falsely representing hydration status. Therefore, it may be necessary to add a prerequisite to the use of the UCC, which asks users to consider recent use of nutritional supplements when classifying hydration status. If riboflavin has been ingested recently, it may behoove users to classify hydration status based on other hydration status biomarkers, such as U_{sg}, or by a combination of hydration status biomarkers, such as U_{sg} and U_{col}.

Chapter 3

Conclusion

This investigation effectively assessed the efficacy of 24-hour U_{col} as a hydration status biomarker after nutritional supplementation with beetroot, vitamin C, and riboflavin. It was hypothesized that: (1) 24-hour U_{col} would be altered (by at least one unit) after nutritional supplementation, and (2) 24-hour U_{sg} and U_{osm} would not be altered by nutritional supplementation. The major findings of this investigation indicate that 24-hour U_{col} remains an effective biomarker before and after nutritional supplementation with beetroot and vitamin C, but not after riboflavin supplementation. Consequently, those evaluating 24-hour U_{col} as a reflection of hydration status after riboflavin supplementation should exercise prudence and consider assessing hydration status with another biomarker. This is because 24-hour U_{col} after riboflavin supplementation may falsely indicate that someone is more hypohydrated than is actually true.

Further research should investigate the effectiveness of U_{col} as a hydration status biomarker after supplementation with different forms (i.e., tablets, capsules, whole foods, powders) of each nutritional supplement studied in this protocol. Furthermore, research should evaluate the efficacy of this hydration status biomarker in sub-populations known to ingest nutritional or vitamin supplements at a higher propensity than the remainder of the population. In particular, women (pregnant and non-pregnant), and athletes (before, during, and after exercise) may be interesting groups to investigate.

Additionally, research should evaluate the efficacy of this hydration status biomarker in those eating and drinking *ad-libitum*.

Until further research is conducted, those assessing U_{col} should take the findings of this thesis into serious consideration. The evidence presented herein suggests that nutritional supplementation with riboflavin has the potential to influence U_{col}, thus indicating that one is more hypohydrated than is actually true.

Chapter 4

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Appendix A: Medical History Questionnaire

HUMAN PERFORMANCE LABORATORY MEDICAL HISTORY QUESTIONNAIRE

Study: Oral Nutritional Supplement Effects on Human Hydration Indices Subject #: _____

Name: _____ Sex: ___ Age: _____ DOB: _____

Street: _____

City: _____ State: ___ Zip: _____ Phone: _____

Email: _____

PLEASE ANSWER ALL OF THE FOLLOWING QUESTIONS AND PROVIDE DETAILS FOR ALL "YES" ANSWERS IN THE SPACES AT THE BOTTOM OF THE FORM.

YES	NO		
☐	☐	1.	Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?
☐	☐	2.	Has your doctor ever denied or restricted your participation in sports or exercise for any reason?
☐	☐	3.	Do you ever feel discomfort, pressure, or pain in your chest when you do physical activity?
☐	☐	4.	In the past month, have you had chest pain when you were not doing physical activity?
☐	☐	5.	Do you lose your balance because of dizziness or do you ever lose consciousness?
☐	☐	6.	Does your heart race or skip beats during exercise?
☐	☐	7.	Has a doctor ever ordered a test for you heart? (i.e. EKG, echocardiogram)
☐	☐	8.	Has anyone in your family died for no apparent reason or died from heart problems or sudden death before the age of 50?
☐	☐	9.	Have you ever had to spend the night in a hospital?
☐	☐	10.	Have you ever had surgery?

11. Please check the box next to any of the following with which you have ever been diagnosed or treated.

☐ High blood pressure	☐ Elevated cholesterol	☐ Diabetes
☐ Asthma	☐ Epilepsy (seizures)	☐ Kidney problems
☐ Bladder Problems	☐ Anemia	☐ Heart problems
☐ Coronary artery disease	☐ Lung problems	☐ Chronic headaches

YES	NO		
☐	☐	12.	Have you ever gotten sick because of exercising in the heat? (i.e. cramps, heat exhaustion, heat stroke)
☐	☐	13.	Have you had any other significant illnesses not listed above?
☐	☐	14.	Do you currently have any illness?
☐	☐	15.	Do you know of any other reason why you should not do physical activity?

16. Please list all medications you are currently taking. Make sure to include over-the-counter medications and birth control pills.

Drugs/Supplements/Vitamins	Dose	Frequency (i.e. daily, 2x/day, etc.)

ADDITIONAL DETAILS:

17. Please list all allergies you have.

Substance	Reaction

YES	NO	18.	Have you smoked?	If yes, #/day	Age Started	If you've quit, what age?
☐	☐		Cigarettes			
☐	☐		Cigars			
☐	☐		Pipes			

19 Do you have a family history of any of the following problems? If yes, note who in the space provided.

☐	High blood pressure		☐	Heart disease	
☐	High cholesterol		☐	Kidney disease	
☐	Diabetes		☐	Thyroid disease	

20. Please check the box next to any of the following body parts you have injured in the past and provide details.

☐	Head		☐	Hip		☐	Calf/shin	
☐	Neck		☐	Thigh		☐	Shoulder	
☐	Upper back		☐	Knee		☐	Upper arm	
☐	Lower back		☐	Ankle		☐	Elbow	
☐	Chest		☐	Foot		☐	Hand/fingers	

YES	NO		
☐	☐	21.	Have you ever had a stress fracture?
☐	☐	22.	Have you ever had a disc injury in your back?
☐	☐	23.	Has a doctor ever restricted your exercise because of an injury?
☐	☐	24.	Do you currently have any injuries that are bothering you?

25	Do you consider your occupation as?	☐	Sedentary (no exercise)
		☐	Inactive-occasional light activity (walking)
		☐	Active-regular light activity and/or occasional vigorous activity (heavy lifting, running, etc.)
		☐	Heavy Work-regular vigorous activity

26. List your regular physical activities

Activity	How often do you do it?	How long do you do it?	How long ago did you start?

ADDITIONAL DETAILS:

Appendix B: 24-hour Diet Record

24-HOUR DIET RECORD: INSTRUCTIONS

- **Please record dietary intake (include all foods and beverages)**

1 All foods and beverages consumed should be recorded. Be sure to include all water and all beverages consumed.

2 **Be very specific**. Make sure you include:

- The **type** of food/beverage
- The **amount** of each food/beverage
- The **preparation method** (i.e., fried, baked)
- The **brand name** of the food (if applicable)
- The **time** it was eaten
- The restaurant you ate it at (i.e., Subway, Applebees, Red Robin)

3 Record food/beverage consumption **during or after each meal/snack** instead of waiting until the end of the day.

4 Use nutrient descriptors (e.g., low-fat, low-carb, fat-free, light, reduced calorie, etc.).

5 **Include miscellaneous items** such as condiments (ketchup, salad dressing, mayonnaise, jams, creams, sugar), and chewing gum.

YOU MUST REFRAIN FROM THE USE OF any vitamins, supplements, any prescription or over the counter medications, beetroot, beetroot derivative products, or an excess of red-colored foods.

YOU MUST ALSO REFRAIN FROM DRINKING ALCOHOL FOR 48 HOURS PRIOR TO EACH LABORATORY VISIT

- **If you have any questions about completing your 24-hour Diet Record, please e-mail onsuconn@gmail.com**

BAD

Time	Detailed food/beverage description: brand name, restaurant, method of preparation, flavor, condiments	Amount
7:30 am	Oatmeal	1 bowl
	Low fat milk	8 oz
	Omelette with ham and cheddar cheese	3 eggs
	Orange juice	12 oz
11:30am	Turkey sandwich	1
	Regular chips	1 bag
	Tomato soup	1 ½ cups
	Dunkin Donuts Coffee	Medium
2:00 pm	Yogurt	1 container
	Granola	Handful
6:20 pm	Salad	1 large
	Chicken breast chunks	Large handful
	Frozen mixed vegetables	1 cup
	Red wine	1 glass
9:05pm	Popcorn	2 cups
	Ice cream	¾ cup
	water	1 bottle

GOOD

Time	Detailed food/beverage description: brand name, restaurant, method of preparation, flavor, condiments	Amount
7:30 am	Regular oatmeal- Quick 1 minute Quaker oats made with 2 cups water	1 cup oats dry
	Hood LightBlock 1% lowfat milk, vitamins A, C, & D (fortified)	8 oz
	Omelette with 2 Tbsp Hillshire Farm diced ham and 2 oz Cabot 50% reduced fat sharp cheddar cheese	3 whole eggs (large)
	Tropicana Pure Premium orange juice with calcium & vitamin D	12 oz
11:30am	Turkey sandwich: Arnold 100% whole wheat bread Butterball extra thin sliced honey roasted smoked turkey breast (deli meat) Kraft mayonnaise Iceberg lettuce	2 slices 7 slices 1 Tbsp 1 leaf
	Lays classic potato chips	1 oz bag
	Campbell's Select soup-Tomato Garden flavor	1 ½ cups
	Dunkin Donuts Medium Coffee made with cream, 1 splenda packet	14 oz
2:00 pm	Dannon fruit on the bottom yogurt- strawberry	1- 6 oz container
	Bear Naked Banana Nut flavored Granola	¼ cup
6:20 pm	Salad: 2 cups romaine lettuce medium red tomato cucumber Pepperidge Farm Zesty Italian croutons, Newman's Own Ranch dressing	1 large 3 slices 3 slices 8 2 Tbsp
	Tyson premium chunk chicken breast (canned chunks)	4 oz.
	Mixed frozen vegetables (Bird's Eye broccoli, cauliflower, and carrots) boiled in salt (1t) water then sautéed in 1 Tbsp Stonehouse California olive oil	1 cup
	Red wine, Yellow Tail Australian Merlot	9-10 oz.
9:05pm	Act II light butter popcorn	2 cups, popped
	Hood regular ice cream- cookies n' cream	3/4 cup
	Dasani water	500 mL bottle

24-HOUR DIET RECORD

Time	Detailed food/beverage description: brand name, restaurant, method of preparation, flavor, condiments	Amount

24-HOUR DIET RECORD

Time	Detailed food/beverage description: brand name, restaurant, method of preparation, flavor, condiments	Amount

Appendix C: 24-hour Urine Survey Form

24-HOUR URINE SURVEY FORM

Record the date and time, each time you urinate during the next 24 hours. Please remember to note any pertinent information (illness, exercise, travel, stress) in the “Notes” column.

DATE	TIME	CUP #	NOTES

REMINDER: Please do not ingest any vitamin supplements, nutritional supplements, prescription or over the counter medications, beetroot, beetroot derivative products, or an excess of red-colored foods.

Appendix D: 7-Day Exercise Log

7-DAY EXERCISE LOG: INSTRUCTIONS

1. Include all aspects of your exercise (don't forget warm-ups and cool-downs).
2. **Be specific.**
    a. If you lifted weights, include what lifts you performed or machines you used, and then specify the reps/sets and duration of your workout.
    b. If you ran, biked, or swam, include the distance, estimated average speed (if known), heart rate (if known), type of workout, total time, and intensity.
    c. Provide detailed descriptions of the types of activities you did during each workout.

BAD EXAMPLE

ACTIVITY	DURATION, SETS/REPS, INTENSITY
DATE: 7/1/11	
Run	30 minutes
Ab workout	20 minutes
Weight lifting	20 minutes

GOOD EXAMPLE

ACTIVITY	DURATION, SETS/REPS, INTENSITY
DATE: 7/1/11	
Endurance paced run with hills	30 minutes, 4 miles, average heart rate: 130 beats/min, easy
Ab workout (planks x 6, 6inches x 3, Russian twists x 3)	18 minutes total (45 seconds on, 45 seconds off), hard
Weight lift, cable machines, (flys, lat pull downs, bicep curls, tricep dips, knee extensions, hamstring curls, hip abductors, hip adductors)	20 minutes total, 2x12 on each machine, light weights, easy

7-DAY EXERCISE LOG

ACTIVITY	DURATION, SETS/REPS, INTENSITY
DATE:	
DATE:	
DATE:	
DATE:	
DATE:	
DATE:	
DATE:	

Appendix E: Consent Form

Consent for Participation in a Research Study

UCONN
UNIVERSITY OF CONNECTICUT

Principal Investigator: Lawrence E. Armstrong, Ph.D.
Student Researchers: Lindsay A. Ellis, BS, Brandon A. Yates, BA
Study Title: Oral Nutritional Supplement Effects on Human Hydration Indices

Introduction

You are invited to participate in a research study to assess the changes in human urine volume, urine concentration, and urine color after nutritional supplementation with Vitamin C, Vitamin B-2, and Beetroot. You are being asked to participate because you are a male, 19-30 years of age.

This consent form will give you the information you will need to understand why this study is being done and why you are being invited to participate. It will also describe what you will need to do to participate and any known risks, inconveniences or discomforts that you may have while participating. We encourage you to take some time to think this over and to discuss it with your family, friends and doctor. We also encourage you to ask questions now and at any time. If you decide to participate, you will be asked to sign this form and it will be a record of your agreement to participate. You will be given a copy of this form.

Why is this study being done?

The purpose of this study is to explain changes in urine after supplementation with Vitamin C, Vitamin B-2, and Beetroot. If these supplements change urine (i.e., volume, concentration, and color), it may be necessary to modify the use of urine color and other hydration measures.

What are the study procedures? What will I be asked to do?

You may be included in this investigation if you are a male between the ages of 19-30 years. The Medical Monitor will screen your medical history questionnaire to ensure that you meet the following criteria: 1) no tobacco use; 2) no antibiotic use; 3) no physical illness at the time of testing; 3) no allergies to beetroot, bananas, wheat, or gluten; 4) no diagnosis of renal, heart, or bladder disease; 5) no daily use of over the counter, or prescription medications that affect total body water, fluid-electrolyte balance, or renal function. These include anti-hypertensive

medications, non-steroidal anti-inflammatory drugs, diuretics (which are primarily marketed towards weight loss), and electrolyte supplements such as potassium, magnesium, or salt tabs 6) no greater than 7 hours of exercise per week. Also, you must be willing to abstain from all alcohol consumption for 48 hours before each visit.

If you agree to take part in this investigation, you will be asked to refrain from the use of nutritional supplements, vitamins, over the counter and prescription medications that alter fluid-electrolyte balance or renal function, beetroot, beetroot derivatives and an excess of red-colored foods for the duration of the investigation. 10 visits to the Human Performance Laboratory will be involved: 1 preliminary procedure visit during Week A (30 minutes), 3 visits during Week B (30 minutes each on average), 3 visits during Week C (30 minutes each on average), and 3 visits during Week D (30 minutes each on average), totaling about 8 hours over the entire investigation. The week of Preliminary Procedures and the 3 visits during Weeks B, C, and D are described below.

		Test Participant Activities					
Project Phase	**Visit and Week**	**Exercise Log**	**24-hour Diet Record**	**24-hour Urine Time Sheet**	**24-hour Urine Collection & Log**	**Consume Breakfast**	**Oral Intake: Vitamin C, Vitamin B-2, Beetroot**
Preliminary Procedures	1-A	7 days	3 days	3 days			
Treatment	1-B	X	X			X	
	2-B		X		X	X	X
	3-B		X		X		
Treatment	1-C	X	X			X	
	2-C		X		X	X	X
	3-C		X		X		
Treatment	1-D	X	X			X	
	2-D		X		X	X	X
	3-D		X		X		
(Make-Up,	*(1-E)*	*(X)*	*(X)*			*(X)*	
if necessary)	*(2-E)*		*(X)*		*(X)*	*(X)*	*(X)*
	(3-E)		*(X)*		*(X)*		

Visit 1-A (Week A): Preliminary Procedures:
You will meet with investigators and then complete a 7-day exercise log for Week A. This log will document your exercise each day. You also will complete 3 consecutive 24-hour diet records, which will document your food and beverage intake. You will also complete 3 consecutive 24-hour urine time sheets, which will record each time you void. You will be asked to return these records at Visit 1-B. You will also be instructed to start your exercise and diet log on the day before Visit 1.

Visit 1-B, 1-C, 1-D, (1-E) Procedures:

During each of these visits, you will submit your previous exercise log, diet records, and urine time sheets or urine logs. The primary student investigator will copy your diet log and you will be asked to replicate for the remainder of the next day. If you deviate from the first diet, you will be asked to record any changes on the diet log. You will then have your height and weight measured. You will eat a standard breakfast consisting of a plain bagel, 2 tbsp peanut butter or cream cheese, a banana, and 500mL of water. You will then be asked to collect your urine in a jug and in sample cups for the following 24 hours, starting after eating breakfast and ending after your first void the next morning. You will be asked to write down the times you urinate on a 24-hour urine log. You will also be asked to record your diet for the next day, and continue recording your exercise on the 7-day exercise log.

Visit 2-B, 2-C, 2-D, (E) Procedures:
During each of these visits, you will submit the previous day's 24-hour urine collection and 24-hour urine log. You will also submit the second day of your diet log for the appropriate week. You will be asked to replicate this diet, and the times you ate, for the next day. If you deviate from the first diet, you will be asked to record any changes on the diet log and adhere to this diet on the third diet day of diet records. You will have your weight measured. You will eat the standard breakfast, along with ingesting one of three commercially available nutritional supplements (i.e., 1000 mg Vitamin C tablet, 200 mg Vitamin B-2 tablet, 880 mg Beetroot capsule) in the presence of investigators. You and the student investigator will not be aware of the particular supplement that you are ingesting. However, another student investigator will randomize the order that you take the supplements so that you ingest 1000 mg Vitamin C one week, 200 mg Vitamin B-2 another week, and 880 mg Beetroot the last week. After ingesting breakfast and the assigned supplement, you will be asked to collect your urine in a jug and sample cups for the following 24 hours, starting after eating breakfast and ending after your first void the next morning. You will be asked to write down the times you urinate on a 24-hour urine log. You will also be asked to record your diet for the next day, and continue recording your exercise on the 7-day exercise log.

Visit 3-B, 3-C, 3-D, (3-E) Procedures:
During each of these visits, you will submit the previous day's 24-hour urine collection and 24-hour urine log. You will also submit the third day of your diet log for the appropriate week. You will have your weight measured. You will be given paperwork for the following week, as well.

Make-up week: (Week E):
If you do not complete Week B, C, or D, you will be asked to participate in the make-up week. This time period allows you to complete the part of data collection that you did not successfully finish.

What are the risks or inconveniences of the study?

You will be asked to refrain from taking any nutritional supplements, vitamins, and over the counter medications during the time of this investigation. This prevents a low risk to you since you will be able to obtain all necessary nutrients from your daily diet.

You may feel inconvenienced by having to carry 24-hour urine collection containers during daily activities. In order to reduce this inconvenience, you will be provided with an opaque bag for the transport of these containers.

You may feel inconvenience by the study since we ask that you visit the laboratory on 10 separate occasions, for a total time commitment of about 8 hours.

One expected effect of beetroot ingestion may be slightly pink or red urine or stool. This isn't a threat to your health, and is consistent with other studies done in the past.

The fluid restriction and exercise-induced dehydration will be inconvenient, but pose no risk to your health.

If you have any questions, or would like to report possible side effects of nutritional supplementation, you can e-mail the student investigator, Lindsay Ellis at Lindsay.ellis@uconn.edu.

What are the benefits of the study?

You will receive the results of your own tests after you complete the investigation. You will be provided with values that help describe how well hydrated you may be. This may lead you to modify your present method of daily hydration. This investigation will also provide information to investigators about the effectiveness of urine hydration indices when nutritional supplementation is used. This study will advance knowledge and provide insight into the use of hydration markers in athletic, industrial, and field settings.

Will I receive payment for participation? Are there costs to participate?

You will receive monetary compensation for your participation in this investigation. You will be compensated $25.00 for completing the Preliminary Procedure Phase.

You will be compensated $30.00 each week (Week B, C, D) of the Treatment Phase that you successfully complete. You will not receive compensation if you do not complete Week B, C, or D of the Treatment Phase. If you have to participate in a Make-up week, you will be compensated $30.00 upon completion.

You will receive a completion bonus of $25.00 if all study requirements are met; this means that your total participation stipend will be $140.00 maximum. At the end of the study, you also will receive $2.00 per visit, to reimburse parking fees for the South Parking Garage on the Storrs Campus.

If you remove yourself from this study, or the Principal Investigator removes you, your stipend will be *pro rated* to reflect the number of visits you successfully completed. All of your fees will be paid during your final visit to the Human Performance Laboratory.

How will my personal information be protected?

Your personal data and results will be protected like medical records, will be confidential, coded and password protected, and will be stored securely. Knowledge of your results will not be shared with anyone except assigned UConn investigators. The only record linking your information to the research will be the consent document, a list of your names and identifying code numbers, communications that are sent via e-mail, and a receipt for payment if you choose to participate. All of these records will be stored in the Department of Kinesiology in a locked office, and on computers that are password-protected. Only specific members of the research staff will have access to the passwords. These records will be kept for one calendar year after all associated publications have been published. Publications or presentations will not identify you by name.

You should also know that the UConn Institutional Review Board (IRB) and Research Compliance Services may inspect study records as part of its auditing program, but these reviews will only focus on the researchers and not on your responses or involvement. The IRB is a group of people who review research studies to protect the rights and welfare of research participants.

What happens if I am injured or sick because I took part in the study?

In the event you become sick or injured during the course of the research study, immediately notify the principal investigator or a member of the research team. If you require medical care for such sickness or injury, your care will be billed to you or to your insurance company in the same manner as your other medical needs are addressed.

If, however, you believe that your illness or injury directly resulted from the research procedures of this study, you may be eligible to file a claim with the State of Connecticut Office of Claims Commissioner. For a description of this process, contact Research Compliance Services at the University of Connecticut at 860-486-8802.

Can I stop being in the study and what are my rights?

You do not have to be in this study if you do not want to. If you agree to be in the study, but later change your mind, you may drop out at any time. There are no penalties or consequences of any kind if you decide that you do not want to

participate. If necessary, Principal Investigator Armstrong may remove you from this study at any time. Examples of withdrawal considerations are safety/medical concerns, lack of adherence to study protocol, missed visits, disruptive behavior during study procedures, and/or unanticipated illness.

Whom do I contact if I have questions about the study?

Take as long as you like before you make a decision. We will be happy to answer any question you have about this study. If you have further questions about this project or if you have a research-related problem, you may contact the principal investigator, Lawrence E. Armstrong, 860-486-2647 or the student researcher Lindsay A. Ellis, 860-486-2543. If you have any questions concerning your rights as a research subject, you may contact the University of Connecticut Institutional Review Board (IRB) at 860-486-8802.

Documentation of Consent:

I have read this form and decided that I will participate in the project described above. Its general purposes, the particulars of involvement and possible hazards and inconveniences have been explained to my satisfaction. I understand that I can withdraw at any time. My signature also indicates that I have received a copy of this consent form.

_____	_____	_____
Participant Signature:	Print Name:	Date:
_____	_____	_____
Signature of Person Obtaining Consent:	Print Name:	Date: