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MOOD AND VIGILANCE FOLLOWING QUERCETIN SUPPLEMENTATION

BY

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THESIS

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ABSTRACT

There is a high degree of military interest in the effects of specific nutritional constituents on cognitive function. This double-blind, placebo-controlled study was undertaken to test whether quercetin aglycone affects mood and vigilance in humans. Block randomization was used to assign 57 Department of Defense personnel into three groups. Based on group assignment, each subject received a one-time dose of 200 mg caffeine plus 1,800 mg placebo, 2,000 mg quercetin or 2,000 mg placebo 1 hour prior to completing a 45-minute scanning visual vigilance task. Profile of Mood States (POMS) questionnaires were completed prior to treatment and immediately following the vigilance test to measure change in subjective mood ratings. Caffeine and quercetin concentrations were measured in plasma samples collected 2-hours after treatment. The caffeine group significantly outperformed the placebo group with regard to correct detection of stimuli as well as reaction time during the vigilance task; no other groups were significantly different from one another. There was a significant effect of group (caffeine, quercetin or placebo) on total mood disturbance (TMD). In addition to TMD, the following mood sub-scales were assessed: vigor-activity, fatigue-inertia, tension-anxiety, depression-dejection, anger-hostility and confusion-bewilderment. The caffeine group reported significantly lower TMD scores, higher vigor-activity scores and lower fatigue-inertia scores versus the placebo group. No other groups were significantly different from one another with regard to the other mood sub-scales. Mean plasma quercetin concentration in the quercetin-supplemented group (3.39μM; range=0.70 to 12.7μM) was significantly higher ($p<0.05$) than in the caffeine group and the placebo group. Mean plasma caffeine concentration (22.78μM; range=9.8 - 38.6μM) was

significantly higher ($p<0.05$) than in the quercetin group and the placebo group. Results of plasma quercetin and caffeine assays confirm quercetin aglycone and caffeine were readily absorbed from the oral supplements. The results also confirm that the psychometric tests employed in this study were sufficiently sensitive to detect differences in cognitive function among groups. There was a clear trend in the results of each psychometric test such that the placebo group consistently did not perform as well as the quercetin group; however, these effects were not statistically significant. In contrast, caffeine was associated with a significant positive change in mood and vigilance compared to the placebo group.

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CHAPTER 1. NUTRITION, COGNITIVE FUNCTION AND MILITARY RELEVANCE

Introduction

The food supply of an army can play a crucial and determining role in warfare. In addition to meeting the basic nutrient requirements of military personnel, a food supply can deliver nutritional factors for the purpose of enhancing a war fighter's physical and mental performance. Military leaders have long recognized the importance of food on the well-being and success of their troops. The Roman armies gained a decided advantage over their adversaries when they overcame many of the problems associated with troop feeding during the Roman Republican wars (264 – 30 BC) (Erdkamp 1998). In 1917, the Nutrition Division was established in the Office of the Surgeon General to safeguard the nutritional interests of the United States Army (Friedl and Hoyt 1997). Twenty years ago, in response to the increasing physical demands placed on American warfighters, the Committee on Military Nutrition Research (CMNR) was created to

Nutrition Research (CMNR) was created to advise the Department of Defense (DoD) on issues related to nutrition research. While the DoD was beginning to recognize the importance of ensuring optimal nutrition for military personnel, progress was being made elsewhere in characterizing the connection between nutrition and brain function (Poos MI 1999; Uauy et al. 2001). At the same time, weapons systems became more complex and the physical and mental demands placed on American forces grew exponentially. These simultaneous and complementary forces contributed to the current military interest in the effects of nutrition constituents on cognitive function and will no doubt continue to influence military nutrition research in the future.

Although the desire to improve mental performance through enhanced nutrition is not unique to the military, the concept is especially applicable to military scenarios given the demanding conditions to which American and allied forces are exposed. In a recent review on the use of caffeine in the military, the CMNR emphasized the rationale for enhancing performance by stating: "Today's military relies heavily on the use of computer-controlled systems that require highly trained and alert operators. In addition, there is a greater reliance on rapid mobility to enable deployment at any time to achieve the nation's military objectives. The urgency of maintaining and enhancing performance is also driven by longer periods of time with minimal sleep, shorter transition times and less recovery time between missions (Committee on Military Nutrition Research 2001)." Clearly, there is a potential benefit to be had by enhancing selected cognitive functions in military personnel.

The effect of lack of sleep and fatigue resulting from continuous and sustained operations is a long-standing concern, yet combat doctrine for North Atlantic Treaty

Organization (NATO) countries continues to emphasize the need for 24-hour war fighting capabilities. Several options are available for countering the effects of fatigue. Sleep is generally regarded as the preferred method; however, it is not always an option during military operations (Bonnet et al. 1995). Many pharmacologic agents have been tested in attempts to reduce performance-decrements associated with sustained operations, but most have been found to be impractical. For example, amphetamines have been shown to have beneficial effects on measures of performance such as reaction time and alertness, but with their high potential for abuse and other side effects, they are not good candidates for improving performance in military personnel.

Relevant to this issue is the possible use of nutritional supplements to reduce the breakdown of mental alertness and performance that usually accompanies fatigue. Various food components have been evaluated for their behavioral effects in humans, including carbohydrate, tyrosine, tryptophan and caffeine. Of these, caffeine, an adenosine receptor antagonist, is the most extensively studied, and has been shown repeatedly to have beneficial effects on performance parameters including mood and vigilance (De Valck and Cluydts 2001; French et al. 1994; Herz 1999; James 1998; Lieberman et al. 1987a; Lieberman et al. 1987b; Reyner and Horne 2000). Caffeine is considered safe as a food additive; however, development of tolerance may occur with repeated caffeine dosing, and it has been shown that withdrawal-like symptoms may be seen following even short-term cessation of caffeine-use by habitual caffeine consumers (Phillips-Bute and Lane 1997).

The ability of caffeine to inhibit adenosine receptors appears to be highly important in its effects on behavior and cognitive function. This ability results from the

competitive binding of caffeine and paraxanthine to adenosine receptors, and is of importance in contributing to central nervous system effects, especially those involving the neuromodulatory effects of adenosine.

Another adenosine receptor antagonist *in vitro* is the flavonoid quercetin (3,3',4',5,7-penthydroxyflavone) (Ji et al. 1996; Moro et al. 1998). Quercetin would be a desirable performance enhancer because it is already associated with other potential health benefits. These benefits are attributed to several mechanisms including anti-bacterial, anti-viral, anti-inflammatory, antiallergenic, vasodilatory action, antioxidant action, modulation of immune function, and reduction of platelet aggregation (Cook and Samman 1996). Because quercetin has a high affinity for the same adenosine receptor subtypes as caffeine, it is conceivable that it may bring about physiological effects similar to those seen following caffeine supplementation including enhancement of mood and vigilance.

The purpose of this study was to assess cognitive performance following oral supplementation of the flavonoid quercetin. The study schema is outlined in chapter 2, Figure 3. To date, no studies investigating the possible cognitive effects of quercetin have appeared in the literature. This thesis examines mood and vigilance following quercetin supplementation in a DoD population, and explores the hypothesis that oral consumption of the flavonoid quercetin improves mood and enhances vigilance in humans. The hypothesis that a flavonoid can influence specific cognitive functions is original; however, the proposed mechanism of action is similar to that of caffeine. Therefore, cognitive tests were selected for this quercetin study based on their successful use in prior caffeine studies (Lieberman et al. 1987a; Lieberman et al. 1987b).

Definitions

Cognition is a general term referring to the process of acquiring, storing, retrieving and revising knowledge, whereas cognitive function is a broad term encompassing a range of functions including intelligence, creativity, memory, executive function and cognitive resources (Bryan 1998). Of these, executive function is of particular interest because it integrates and controls other cognitive domains such as intelligence and memory, and is thought to be responsible for a variety of cognitive activities including vigilance. The present study relies on the use of validated psychometric tests for assessment of vigilance, a specific executive function incorporating fluid intelligence.

Horn and Cattell proposed the theory of fluid and crystallized intelligence in 1963 and refined it in 1966 (Horn and Cattell 1966). Fluid intelligence is thought to be independent of education, experience and cultural influence and generally refers to innate information processing ability (Santrock 1986). Fluid intelligence is demonstrated by information processing and the application of mental processes to situations requiring no previous knowledge (Bryan 1998; Santrock 1986). In contrast, crystallized intelligence refers to skills, abilities and understanding gained through education and observation (Santrock 1986). Enhancement of fluid intelligence and executive function has considerable potential for military application.

Mood is a complex interaction of sensory, physiological and emotional factors resulting in a persistent emotional state that influences our perceptions and behavior (Benton and Donohoe 1999). Tellegen described mood as being comprised of two perpendicular dimensions of affect: pleasure-displeasure, defined as the valence of one's

feelings, and arousal-sleepiness, defined as the physical energy level one feels (Tellegen 1985). Based on this model, overall subjective mood scores could be expected to improve in individuals following consumption of a moderate amount of a known stimulant. Perhaps this explains the mood-enhancement effect seen in the majority of caffeine supplementation studies.

Indicators of performance

Selection of the appropriate cognitive tests is essential to consistently measure the cognitive function effects of caffeine and other nutrition constituents being evaluated. Tests with substantial vigilance components have been successfully used by investigators to measure the effects of moderate doses of caffeine (Lieberman et al. 1987a; Lieberman et al. 1987b). Vigilance and mood were selected as the most appropriate measures of performance for this study due to the well-documented mood-enhancing effect of caffeine and the dose-dependent effect of caffeine on vigilance. In addition, vigilance is regarded as critical to the successful completion of many military tasks including, but not limited to, sentry duty and monitoring complex weapon systems. Because visual information is often critical for a number of military tasks such as sentry duty and instrument monitoring, a validated visual, as opposed to an auditory vigilance task, was selected for use in this study (Fine et al. 1994; Gilbert et al. 2000; Loke and Meliska 1984; Van Dongen et al. 2001).

Mood assessment is a validated method to assess effectiveness in military personnel. When mood is significantly impaired, physical and mental performance also deteriorates (Conway and Giannopoulos 1993; Opstad et al. 1978). The most common

way of measuring mood is to ask subjects to read a list of mood-descriptive adjectives and to rate their current feelings on each adjective. Of the commercially available and validated tools used to measure mood, the Profile of Mood States (POMS) appears most frequently in the literature. The POMS was selected for use in the present study because it is a rapid, reliable and economical method of detecting transient fluctuations in six mood states and it has been used successfully in other studies measuring the effect of caffeine on mood (Herz 1999; James 1998; Lieberman 2001; Lieberman et al. 1987a; Lieberman et al. 1987b; Loke 1988; Warburton et al. 2001).

Adenosine Receptors

Adenosine is a neuromodulator that is involved in a diverse array of functions in the central nervous system. It has an inhibitory effect on neuronal activity (Yarbrough and McGuffin-Clineschmidt 1981) and thus plays a role in promoting and maintaining sleep as well as regulating arousal state. Adenosine has been shown to be an inhibitor of locomotion and coordination in mice (Barraco et al. 1983) and a sleep-promoter in rats (Radulovacki et al. 1984). The physiological actions of adenosine in the periphery and central nervous system are mediated by four receptor subtypes that have been identified as: A_1, A_{2A}, A_{2B} and A_3 (Jacobson 1995; Ji et al. 1996). These receptors are present on basically every cell type and, therefore, are a desirable target for pharmacologic interventions with selective agonists and antagonists. Caffeine and other xanthines, are the classical adenosine antagonists and bind most efficiently with A_1 and A_{2A} receptors.

The hypothesis that quercetin can influence mood and vigilance is based on its high affinity for A_1 adenosine receptors *in vitro* (Ji et al. 1996). This may account, in

part, for the variety of biological activities associated with quercetin and other flavonoids. Ji et al., conducted a screening of various phytochemcials for adenosine receptor affinity utilizing radioligand binding assays in rat brain A_1, A_{2A}, A_3 and human brain A_3 receptors and found that several flavonoids exhibited a high affinity for the various receptor subtypes (Ji et al. 1996). According to Middleton: "It is estimated that the flavonoids have been persistent in nature for somewhat over 1 billion years and, therefore, it seems entirely likely that they have been interacting with evolving animal forms/species over the eons (Middleton 1988)." The effects of caffeine were recognized long before their antagonism of adenosine receptors was identified; therefore, it seems reasonable that activation of adenosine receptors may be somewhat dependent on other dietary factors as well, including phytochemicals such as quercetin. Based on this reasoning, it is entirely possible that the physiological effects attributed to quercetin may be due in part to an interaction with adenosine and its A_1 receptors.

The physiological effects of each adenosine receptor subtype have been characterized through studies utilizing receptor-specific ligands. Activation of the A_1 subtype is associated with the bradycardiac, cerebroprotective and antilipolytic effects of adenosine (Jacobson et al. 1992). Activation of the A_{2A} receptor results in hypotensive, antiplatelet aggregation (Hutchison et al. 1990) and memory retention and consolidation in mice (Kopf et al. 1999). The A_{2B} receptor has yet to be linked to specific physiological or behavioral responses because of the lack of specific agonists or antagonists. Klotz and Hannan et al. have suggested the A_{2B} receptor subtype may have important functions in the regulation of vascular tone and function of mast cells (Hannon et al. 1995; Klotz 2000). The A_3 subtype is linked to hypotension and release of

inflammatory mediators from mast cells (Hannon et al. 1995). The physiological actions and distribution of the adenosine receptor subtypes are reviewed in Table 1.

Table 1

Distribution and Function of Adenosine Receptors by Sub-Type

Receptor	Adenosine Affinity(Dun widdie and Masino 2001)	Physiological actions (Klotz 2000)	Distribution (Fredholm et al. 2000)
A_1	~ 70 nM	Involved in regulation of neuronal activity. Activation results in reduced neurotransmitter release and neuronal firing. Mediate neuroprotective effects of adenosine.	Brain (cortex, cerebellum, hippocampus); dorsal horn of spinal cord; eye, adrenal gland, atria.
A_{2A}	~ 150 nM	Responsible for adenosine-induced vasodilation and involved in regulation of neuronal activity.	Spleen, thymus, leucocytes (both lymphoctyes and granulocytes), blood platelets.
A_{2B}	~5100 nM	Thought to have important functions in the regulation of vascular tone and function of mast cells	Colon, bladder, lung, blood vessels, eye.
A_3	~ 6500 nM	Poorly characterized, but apparently modulates A_1 receptor function and possibly other receptors.	Low levels in thyroid, most of brain, adrenal gland, spleen, liver, kidney, heart, intestine.

Based on the variety of functions associated with adenosine receptors, it is clear that selective adenosine receptor antagonists could be of considerable physiological and therapeutic value. This thesis is concerned with the enhancement of cognition; however, it should be noted that adenosine receptor antagonists are also sought for renal protective (Suzuki et al. 1992), cerebroprotective (von Lubitz and Jacobson 1995), antiasthmatic (Beaven et al. 1994) and anti-inflammatory properties (Beaven et al. 1994).

The pharmacologic application of adenosine receptor antagonists is complicated by the observation that the effects of acute administration of a particular adenosine receptor ligand can be opposite to the chronic effects of the same ligand (Jacobson et al. 1996). For example, chronic ingestion of caffeine by humans can lead to tolerance and withdrawal symptoms (Jones et al. 2000; Phillips-Bute and Lane 1997) while acute, low-dose caffeine administration has a well-documented stimulating effect (Akerstedt and Ficca 1997; De Valck and Cluydts 2001; Fine et al. 1994; Kamimori et al. 2000; Kuznicki and Turner 1986; Lieberman et al. 1987b; Loke and Meliska 1984). In a review of effect differences between acute and chronic administration of adenosine agonists and antgaonists, Jacobson, et al, addressed the phenomenon of "effect inversion" (Jacobson et al. 1996). Effect inversion refers to the observation that the effects of long-term treatment with adenosine receptor antagonists can resemble the acute effects of adenosine receptor agonists, and vice versa (Jacobson et al. 1996). As previously mentioned, this is a complicating factor in the use of adenosine receptor antagonists for cognitive enhancement and other therapeutic applications. Jacobson has commented: "An increase in adenosine receptor function, after chronic administration of caffeine, might be expected to increase the threshold for the stimulatory action of caffeine to the point where the depressant action, which occurs at a different site (possibly inhibition of phosphodiesterases), overrides the stimulatory effects of adenosine receptor antagonism" (Jacobson et al. 1996). It is also possible that alteration in other receptors, in pathways modulated by adenosine receptors, accounts for the tolerance to caffeine.

Caffeine

Caffeine is a plant alkaloid with a physical structure of $C_8H_{10}N_4O_2$ (Figure 1) and a molecular weight of 194.19. It is a methylated xanthine (1,3,7-trimethylxanthine) and a mild stimulant that is often referred to in the literature as the most widely used psychoactive drug in the world (Committee on Military Nutrition Research 2001; Phillips-Bute and Lane 1997; Rees et al. 1999; Rogers and Dernoncourt 1998). It is found in more than 60 different plant species (Barone and Roberts 1996) and, therefore, occurs naturally in many foods including coffee, tea, cocoa, chocolate and soft drinks. In addition to being present in several prescription and over-the-counter drugs, caffeine is also a food additive.

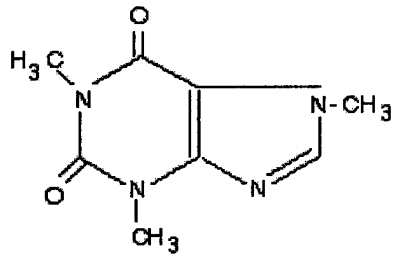


Figure 1. Chemical structure of caffeine.

Mechanism of Action

The Committee on Military Nutrition Research (CMNR) recently reviewed the use of caffeine in military operations. They concluded that in doses of 100 to 600 mg, caffeine could maintain cognitive performance, especially in situations of sleep deprivation (Committee on Military Nutrition Research 2001). This consensus was the

result of research conducted at a number of institutions, including several military laboratories, that demonstrated improved vigilance in rested subjects and more generalized effects on cognitive function in sleep-deprived individuals following caffeine consumption (Committee on Military Nutrition Research 2001; Lieberman et al. 1987a; Lieberman et al. 1987b; Penetar et al. 1994; Smith et al. 1994).

Early animal studies provided evidence that caffeine's effects on behavior and cognitive function are mediated by modulation of the inhibitory neurotransmitter adenosine (Hirsh 1984; Snyder et al. 1981). Snyder et al. observed that stimulant actions of 10 methylxanthines in mice correlate with affinities for adenosine receptors, thus supporting the idea that the stimulant effects of methylxanthines involve competitive binding at adenosine receptor sites (Snyder et al. 1981).

Caffeine is a relatively non-specific adenosine receptor antagonist, but it appears to have the greatest affinity for A_1 receptors versus the other sub-types. The A_1 adenosine receptor sub-type is localized to specific brain regions (see Table 1) and is involved in the regulation of arousal level. Caffeine, which readily crosses the blood-brain barrier, blocks the neuromodulatory effects of adenosine on brain neurons resulting in the well-documented effects on behavior and cognitive function (Committee on Military Nutrition Research 2001).

Caffeine in the diet

Reported levels of caffeine in commonly consumed beverages, foods and other products vary considerably. The variability is most often attributed to differences in the

reference volumes used, analytic techniques, product sources and preparation methods (Barone and Roberts 1996).

In 1995, Barone and Roberts estimated caffeine consumption in the United States (Barone and Roberts 1996). Due to the limited availability of caffeine consumption data, estimates were made indirectly through analysis of data from the United States Department of Agriculture (USDA) 1977-1978 Nationwide Food Consumption Survey, as well as food consumption data from surveys conducted from 1987 through 1989 by the Market Research Corporation of America. The mean daily caffeine intake for all US adults in the general population was reported to be approximately 3 mg/kg body weight and for adult consumers of caffeine containing products, approximately 4 mg/kg body weight. As expected, coffee accounted for the majority of caffeine consumption (approximately 2 mg/kg body weight). Among children younger than 18 years of age who were consumers of caffeine-containing foods, the mean daily caffeine intake was estimated to be 1 mg/kg body weight (Barone and Roberts 1996).

Absorption and metabolism of caffeine

Caffeine absorption is rapid and almost complete, reaching 99% in humans within 45 minutes of ingestion. Peak plasma concentration is typically reached between 15 and 120 minutes, but varies based on gastric emptying time and the presence of other dietary constituents (Committee on Military Nutrition Research 2001). Arnaud and Welsh found that plasma concentration is dose-dependent and equates to 8 to 10 mg/L (41 to 51 μM) for oral doses of 5 to 8 mg/kg (Arnaud 1987). Based on their findings, an 80 kg human

could be expected to have a plasma caffeine concentration of approximately 4 mg/L (21 μM) following a 200 mg caffeine dose.

Caffeine is readily reabsorbed by the renal tubules; therefore, only a small amount is excreted unchanged in the urine. Arnaud et al. surmised that caffeine's limited appearance in the urine indicates that metabolism is the rate-limiting factor in its plasma clearance (Arnaud 1987; Committee on Military Nutrition Research 2001). Caffeine half-life ranges from 2.5 to 4.5 h in humans and, with the exception of newborns, is not dependent on age (Arnaud 1987). There are, however, factors that influence caffeine metabolism. For example, it has been shown that caffeine half-life in adult males is reduced by up to 50% in smokers compared with non-smokers (Hart et al. 1976; Murphy et al. 1988). In contrast, caffeine half-life is increased by 50% in women taking oral contraceptives (Patwardhan et al. 1980).

Caffeine is metabolized by the liver to form dimethylxanthines, monomethyl- and dimethyl uric acids, dimethyl- and trimethylallantoin, and uracil derivatives (Arnaud 1987). Of these metabolites, formation of 1,7-dimethylxanthine (paraxanthine) is quantitatively the most important and represents the first step of caffeine metabolism in humans. Formation of paraxanthine accounts for approximately 75 to 80% of caffeine metabolism. This is significant, given the fact that paraxanthine is also pharmacologically active and may contribute to the behavioral effects of caffeine as well as contribute to the development of caffeine tolerance and withdrawal symptoms (Committee on Military Nutrition Research 2001). By contrast, an adenosine receptor antagonist that is not associated with the formation of pharmacologically active

metabolites would be desirable because of a reduced likelihood of tolerance and withdrawal phenomena.

Quercetin

Phenolic compounds comprise one of the largest and most ubiquitous groups of plant metabolites. They can be divided into at least ten different classes based on their general chemical characteristics. The largest class of phenolic compounds is the flavonoids, with over 5,000 described to date (Yang et al. 2001). The flavonoid class of phenolic compounds is characterized by a C_{15} (C_6-C_3-C_6) flavone nucleus: two benzene rings (A and B) linked through an oxygen-containing pyran or pyrone ring (C) (Figure 2). Flavonoids are further classified into flavonols, flavanols, flavones, isoflavones, flavanones and anthocyanins (Yang et al. 2001). The flavonol category includes quercetin, kaempferol and myricetin. Typically, quercetin (3,3′,4′,5,7-Penthydroxyflavone) is the main flavonol in the human diet and, as such, is one of the most thoroughly studied dietary flavonoids (Walle et al. 2001).

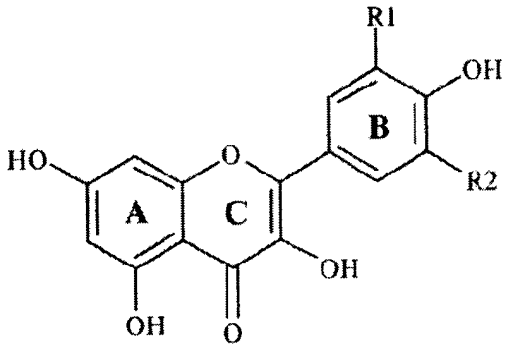


Figure 2. Chemical structure of flavonols: quercetin, R1 = OH, R2=H; kaempferol, R1=H, R2=H; myricetin, R1=OH, R2=OH.

Quercetin occurs naturally as *O*-glycosides, with D-glucose as the most common sugar residue (Hollman et al. 1999). Other sugar residues are D-galactose, L-rhamnose, L-arabinose, D-xylose and D-glucuronic acid. More than 170 different quercetin glycosides have been identified (Yang et al. 2001). Quercetin aglycone was used in this study because of its commercial availability as a dietary supplement. Use of the aglycone form also ensured standardization of quercetin doses administered to subjects and allowed for comparison of absorption estimates from this study with estimates from other studies utilizing quercetin aglycone (Erlund et al. 1999; Shoskes et al. 1999; Walle et al. 2001).

Mechanisms of Action

Quercetin may offer potential human health benefits through several mechanisms including anti-bacterial, anti-viral, anti-inflammatory, anti-allergy, vasodilatory action, antioxidant action, modulation of immune function, and reduction of platelet aggregation (Cook and Samman 1996). Quercetin has also been shown to inhibit lipid peroxidation (Terao and Piskula 1999; Yamamoto et al. 1999) and inhibit degranulation of mast cells, basophils and neutrophils *in vitro* and *in vivo* (Alexandrakis et al. 1999; Blackburn et al. 1987; Foreman 1984; Kimata et al. 2000). Such activity could, in part, account for quercetin's anti-inflammatory and anti-allergy properties. Another possible mechanism behind the observed health effects of quercetin is related to its affinity for the A_1 subtype of adenosine receptor. Quercetin and other flavonoids with adenosine receptor antagonist activity may influence the regulation of the physiological effects attributed to adenosine and its receptors. For example, caffeine and quercetin are both regarded as lipolytic

agents. It has been shown that caffeine increases lipolysis through inhibition of the enzyme cyclic 3',5'-nucleotide phosphodiesterase (PDE) and antagonism of the A_1 adenosine receptor subtype (Ryu et al. 2001). Kuppusamy and Das demonstrated that quercetin was associated with a dose- and time-dependent increase in lipolysis in rat adipocytes that was independent of PDE inhibition; however, the authors did not comment on the possibility that quercetin's antilipolytic properties were perhaps due in part to adenosine receptor antagonism (Kuppusamy and Das 1992).

Quercetin in the diet

Hertog et al. determined the content of the flavonols quercetin, kaempferol and myrecetin in vegetables, fruits and beverages commonly consumed in the Netherlands (Hertog et al. 1993a; Hertog et al. 1992b). Quercetin levels in edible parts of most vegetables were generally below 10 mg/kg, except for onions (284-486 mg/kg), kale (110 mg/kg), broccoli (30 mg/kg), french beans (32-45 mg/kg) and slicing beans (28-30 mg/kg) (Hertog et al. 1992a). Justensen et al. reported similar levels of quercetin for these vegetables (Justensen et al. 1998). In most fruits, the quercetin content was 15 mg/kg, on average, except for different apple varieties in which the quercetin content was 21-72 mg/kg (Hertog et al. 1992a). Hertog et al. reported that red wines and grape juice had quercetin levels of 4 to 16 mg/L and of 7 to 9 mg/L, respectively (Hertog et al. 1993b). According to Justensen et al., the mean quercetin level in red wines was 8 mg/L (Justensen et al. 1998).

Vegetables and fruits, particularly onions and apples, account for the majority of quercetin consumed in the United States (Hertog et al. 1995). Hollman proposed that tea

was also a significant of quercetin in the typical American diet (Hollman et al. 1996). The estimated average daily intake of quercetin by an individual in the United States is 25 mg (Program 1992).

In addition to dietary sources, nutrition supplements containing quercetin are marketed worldwide and are likely to contribute to intakes of quercetin in excess of previous estimates. In 2000, over 130 preparations containing quercetin were registered as drugs worldwide. In 1998 sales of flavonoid supplements (including quercetin) were an estimated $430 million (Erlund et al. 2000).

Absorption of quercetin

As mentioned previously, quercetin occurs naturally in a glycosylated form. Hollman demonstrated that, depending on the nature and position of the sugar residue, quercetin absorption can occur in the small intestine (Hollman et al. 1999; Hollman and Katan 1997), or in the large intestine after bacterial deconjugation. Mechanisms of absorption are still under investigation; however, Hollman et al. suggested that the sodium/glucose transporter (SGLT1) is responsible for absorption of quercetin glucosides in the small intestine. Another study showed that lactase phloridzin hydrolase (LPH) present on the brush border has an affinity for flavonol glucosides *in vitro* (Day et al. 2000). The authors suggest that LPH may hydolyze quercetin glucosides *in vivo* allowing the quercetin aglycone to passively diffuse into the enterocyte. It should be noted that if quercetin aglycone is absorbed through passive diffusion, conjugation could occur within the enterocyte forming glucuronides and sulphates (Day and Williamson 2001). In addition, after absorption, quercetin would travel to the liver via the portal circulation

where it would undoubtedly undergo significant first pass metabolism resulting in conjugation, which raises the question of whether quercetin loses some of its physiological activity *in vivo*.

It is likely that absorption of quercetin glycosides and aglycone occurs at multiple sites and perhaps through a variety of mechanisms. A recent study investigated the absorption of quercetin, isoquercetin (3-*O*-glucose) and rutin (quercetin 3-*O*-glucose-rhamnose) in rat stomach following *in situ* gastric administration (Crespy et al. 2002). The authors found that the stomach rapidly absorbs quercetin aglycone. In contrast, the stomach did not absorb isoquercetin and rutin, thus supporting the idea of a requirement for an active transport mechanism or the need for hydrolysis of quercetin glycosides prior to absorption.

In other studies, absorption of quercetin aglycone was estimated at approximately 25% of the total amount consumed (Hollman et al. 1995; Hollman et al. 1997). Peak levels of plasma quercetin aglycone occur 2 to 5 hours following ingestion, and the elimination half-life is approximately 25 hours (Day et al. 2000). The variability in time to peak plasma concentration may be related to dose size. The rationale for using a high dose of quercetin in my study was based on the observation that the majority of quercetin in plasma is conjugated or tightly bound to albumin thus reducing the likelihood of quercetin crossing the blood-brain barrier. The goal was to reach a plasma quercetin concentration in excess of 2.4 ± 0.64 μM (quercetin's *in vitro* K_i at the A_1 adenosine receptor sub-type).

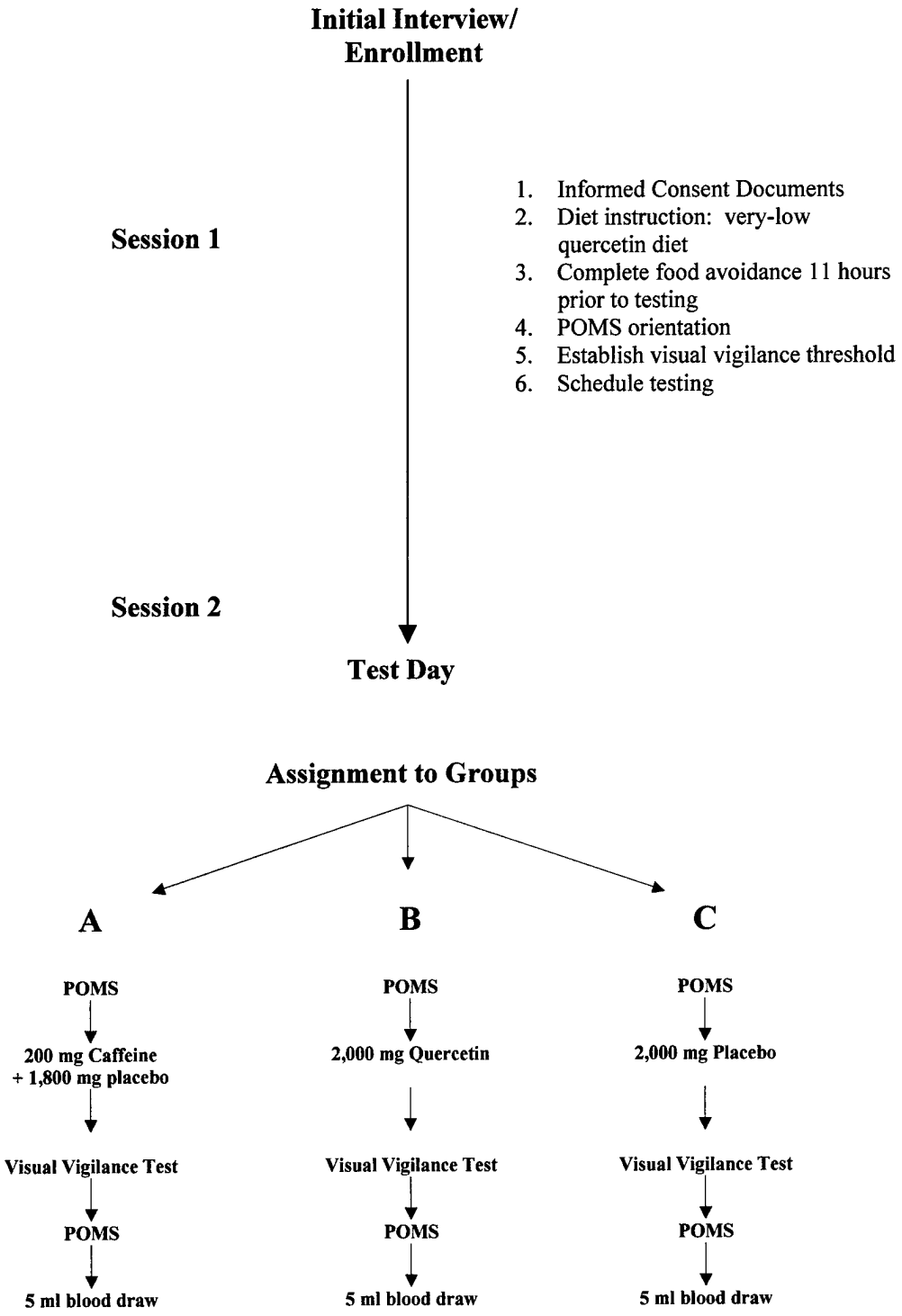
CHAPTER 2. METHODS

Experimental design

This study tested the hypothesis that quercetin supplementation improves visual vigilance and subjective mood ratings.

The hypothesis was evaluated using a computerized visual vigilance task and a computerized version of the Profile of Mood States Questionnaire to measure cognitive performance. In addition, plasma caffeine and quercetin concentrations were measured 2 hours after oral administration of 200 mg caffeine, 2,000 mg quercetin or placebo (methylcellulose) in fasting individuals who had followed a very-low quercetin diet for 49 hours prior to testing and fasted 11 hours prior to testing. The study schema is outlined in Figure 3.

Figure 3. Study schema



Materials and Methods

Subjects

Group size was calculated using 6 as the expected difference in the number of "hits" (correct responses) on the vigilance test and a pooled SD of 6. Using a one way ANOVA with three groups, alpha = 0.05, power = .80, 60 subjects were needed.

Fifty-seven subjects participated in this study. All were recruited from the pool of civilian and military employees of David Grant USAF Medical Center at Travis Air Force Base. Subjects were healthy, nonsmoking adults with no history of hypertension, heart disease, malabsorption disorders or vision impairment. Women were not pregnant or lactating. Subjects provided written informed consent for the study, which was approved by the University of California, Davis, Human Subjects Review Committee and the 60th Medical Group Institutional Review Board, Travis Air Force Base, California. Of the 57 subjects, 36 were female and 21 were male (Table 2). Ages ranged from 19 to 45 (mean =30.5, SD = 6.4), and body weight ranged from 50 to 109 kg (mean = 72.1 kg, SD = 13.2). The groups did not differ significantly with regard to weight (Table 3) or age (Table 4). Twenty-five were officers, 27 were enlisted and 5 were civilian employees of the medical group (Table 5). Subjects were assigned to one of three groups using block randomization.

Table 2

Composition of Groups by Gender

	Gender		
Group	**Male**	**Female**	**Total**
Caffeine	7	11	18
Quercetin	8	12	20
Placebo	6	13	19
Total	**21**	**36**	**57**

Table 3

Mean Weight of Groups

	Weight (kg)	
Group	**n**	**Mean**
Caffeine	18	75.0
Quercetin	20	71.8
Placebo	19	70.9
Total	**57**	**72.6**

Note. No significant differences in weight were found among the groups ($F_{2,54} = 4.25$; $p = .656$).

Table 4

Mean Age of Groups

	Age	
Group	**n**	**Mean**
Caffeine	18	29.6
Quercetin	20	31.0
Placebo	19	31.6
Total	**57**	**30.7**

Note. No significant differences in age were found among the groups ($F_{2,54} = 0.113$; $p = .893$).

Table 5

Group Composition by Rank

	Rank			
Group	**Officer**	**Enlisted**	**Civilian**	**Total**
Caffeine	7	7	4	18
Quercetin	9	10	1	20
Placebo	9	10	0	19
Total	**25**	**27**	**5**	**57**

Assessment of caffeine intake

Habitual daily caffeine intake was estimated using a self-report questionnaire (see Appendix A) of daily consumption of foods and beverages containing caffeine. This was collected at the time of enrollment in the study. Estimates were calculated using standard values for the caffeine content of foods and beverages based on those found in *Bowes and Church's Food Values of Portions Commonly Used, 17th edition.* Caffeine values used are listed in Table 6. To minimize tolerance and withdrawal, potential subjects were excluded from the study if their estimated average daily caffeine consumption exceeded 400 mg. Of the 57 subjects accepted into this study, 44% reported a daily caffeine intake over 200 mg. In general, younger subjects (ages 19-24) consumed less caffeine (n = 14, mean = 128 mg/day, SD = 116) than older subjects (ages 25-45) (n = 43, mean = 190 mg/day, SD = 126). Males reported caffeine intakes (n = 21, mean = 171 mg/day, SD = 134) similar to those reported by females (n = 36, mean = 179 mg/day, SD = 122). Groups (caffeine, quercetin and placebo) were not different with regard to amount of caffeine consumption ($F_{2,54} = .069$; $p = .933$). The results of this survey are similar to those reported by Lieberman et al., who found similar trends regarding age and caffeine use (Lieberman et al. 1987a).

Table 6

Caffeine Content of Selected Foods and Beverages

Food/Beverage	Caffeine (mg)
Coffee	
Brewed (6 oz)	103
Instant (1 rounded teaspoon)	57
Expresso (6 oz)	210
Cappuccino (6 oz)	30
Tea, 5 oz	
Brewed, black, 3 minutes	36
Instant (1 teaspoon)	31
Chocolate beverages	
Chocolate milk (8 oz)	8
Cocoa beverage (6 oz)	5
Carbonated beverages (regular and diet)	
Cola-type (12 oz)	46
Pepper-type (12 oz)	41
Mountain Dew (12 oz)	54
Mello Yello (12 oz)	52
Jolt (12 oz)	70
Chocolate	
Milk Chocolate (1 oz)	7
Dark Chocolate (1 oz)	21
Baker's Chocolate (1 oz)	25
Over the counter drugs (1 tablet)	
No-Doz	100
No-Doz, maximum strength	200
Vivarin	200
Anacin	32
Excedrin	64

Note. Caffeine data are from Bowes & Church's Food Values of Portions Commonly Used. (p. 381-383), A. D. Bowes and J. A. Pennington, 1998, Philadelphia: Lippincott.

Behavioral Tests

The United States Army Research Institute of Environmental Medicine (USARIEM), Natick MA, provided software to implement the Profile of Mood States questionnaire (Educational and Industrial Testing Service, San Diego, CA) on IBM PCs and compatibles. USARIEM also provided the Scanning Visual Vigilance Test (v2.0) written in Borland C++ (v3.1) for use on IBM PCs and compatibles. Computer-based cognitive tests were administered on one of two Dell™ (Round Rock, TX) OptiPlex™ GX110 computers. The computers were configured identically around the Intel® Pentium® III microprocessor. Dell 17" monitors (model number E771p) and Dell Quiet Key keyboards were used.

All behavioral testing was conducted in one of two designated rooms located in David Grant United States Air Force Medical Center's Clinical Investigation Facility. The rooms were configured to be as similar as possible with regard to lighting, temperature and external noise level. Each room was illuminated with a 40-watt incandescent light bulb. Ambient air temperature was measured with mercury thermometers previously checked for accuracy and concurrence. Computer monitors were positioned approximately 53 cm from the subject.

Profile of Mood States Questionnaire

The Profile of Mood States (POMS) questionnaire was used to measure subjective mood states (McNair et al. 1971). Volunteers rated a series of 65 mood-related adjectives on a five-point scale ranging from "not at all" to "extremely," in response to the question, "How are you feeling right now?" Previous research has demonstrated that the adjectives

factor into six mood sub-scales (tension-anxiety, depression-dejection, vigor-activity, fatigue-inertia, anger-hostility and confusion-bewilderment). In addition to the six mood sub-scales, a total mood disturbance (TMD) score can be calculated by summing the scores across all six factors (weighting vigor negatively). Four of the sub-scales (tension-anxiety, depression-dejection, vigor-activity and fatigue-inertia) and TMD have been shown to be sensitive to caffeine administration (Fine et al. 1994; Lieberman et al. 1987a; Lieberman et al. 1987b). This study was conducted using a computerized version of the POMS. Responses and scores were written to an ASCII report file upon test completion. Data from the POMS were viewed and printed using Windows™ Notepad.

Subjects completed the POMS a total of three times during the study. The first session was completed during the initial enrollment session and was intended only to familiarize them with the format of the computerized questionnaire. The results of the first POMS questionnaire were not included in the data analysis. Each subject completed the POMS two additional times (upon arrival and following completion of the 45-minute vigilance task) on their scheduled test day. Treatment (caffeine, quercetin or placebo) was administered immediately following completion of the first POMS. The second POMS was completed approximately 1 hour and 50 minutes after the first one. The scores collected on the subject's test day were included in the data analysis.

Although POMS scores are not typically influenced by learning effects (McNair et al. 1971), the risk of this confounding factor was minimized by limiting subjects to one practice session. In an attempt to measure a true baseline mood score, each subject completed the computerized questionnaire before performing any other task related to the study. Subjects were asked to complete the POMS following a review of the instructions

(see instructions, Appendix B). The baseline scores were used to measure change in mood following treatment with caffeine, quercetin or placebo and completion of the 45-minute scanning visual vigilance test. In this regard, the vigilance test served a dual purpose in that it provided a source of stress and was expected to have a negative impact on mood scores. The TMD score was used as a single global assessment of affective state while the six individual POMS factors were analyzed to assess changes in tension-anxiety, depression-dejection, vigor-activity, fatigue-inertia, anger-hostility and confusion-bewilderment. It should be noted that although caffeine is not typically shown to influence anger-hostility and confusion bewilderment scores on the POMS, the data analysis for these factors was still performed.

USARIEM Scanning Visual Vigilance Test

Description. The USARIEM Scanning Visual Vigilance Test is a variable-length detection task designed to assess the ability of individuals to maintain visual alertness for sustained periods of time and was designed to be sensitive to changes in vigilance produced by subtle variations in performance, such as those produced by low doses of centrally acting food constituents, drugs or environmental stress. The test runs on a standard IBM compatible personal computer, presents a simple, near-threshold stimuli at pseudo-random locations on the monitor and can run up to 2 hours. It minimizes the cognitive load and thereby emphasizes the vigilance aspects of the task. The test requires the subject to detect a faint dot that appears randomly on a computer screen for 2 seconds. Average presentation of the dot for this study was once a minute for 45 minutes. Upon detection of the dot the subject pressed the space bar on the keyboard.

Threshold test. Due to differences in visual ability, it was first necessary for each subject to complete a threshold test to determine their ability to detect blocks of various shades against a gray background. The threshold test lasted for 7 to 10 minutes and was completed by each subject during session one (Figure 3). A percent correct goal of greater than 80% was used to establish the brightness of the stimuli. The test presented a stimulus once every 12 seconds on average. The brightness of the stimuli changed according to subject's response to previous stimuli. A threshold setting was established when the subject continuously attained the percent correct goal (80%) on a certain stimuli brightness setting. Figure 4 is an illustration of the vigilance test.

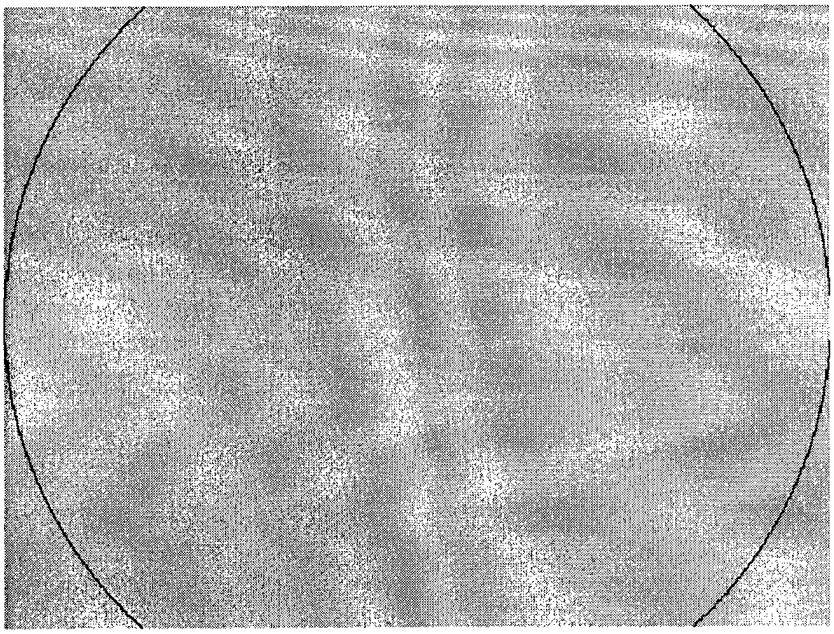


Figure 4. Illustration of USARIEM Scanning Visual Vigilance Test. The test requires the subject to detect a faint dot that appears randomly on a computer monitor for 2 seconds. Average presentation of the dot for this study was set at one per minute. Upon detection of the dot the subject responded by pressing the space bar on the keyboard.

Actual test. Individual threshold settings were used during the test session when the task was run for 45 minutes beginning exactly 1-hour after oral administration of 200 mg caffeine + 1,800 mg placebo, 2,000 mg quercetin or 2,000 mg placebo. The task is not copyrighted and was obtained from USARIEM personnel.

Quercetin, caffeine and placebo encapsulation

Premium Ingredients, Ltd. (Franklin Park, IL) provided the quercetin dihydrate and caffeine anhydrous for this study. Certificates of analysis for quercetin and caffeine indicated they were 100% and 99.36% pure respectively. Methylcellulose, a pharmacologically inert substance, was used as the placebo and was provided by Longwood Pharmacology Research, Inc.

A registered pharmacist supervised the encapsulation of quercetin, caffeine and placebo in identical, opaque, gelatin capsules at Longwood Pharmacology Research, Inc. (Boston, MA). Treatments were administered in eight identical, gelatin capsules (250 mg each).

The goal was to reach a plasma quercetin concentration in excess of 2.4 ± 0.64 μM (quercetin's *in vitro* K_i at the A_1 adenosine receptor sub-type). The rationale for using a high dose was based on the fact that the majority of quercetin in plasma is conjugated and/or tightly bound to albumin thus reducing the likelihood of quercetin crossing the blood-brain barrier.

Safety

Subjects assigned to the quercetin group ingested a 2,000 mg one-time dose of quercetin aglycone. As a precaution, a medical monitor was available during all test sessions. No adverse events occurred during this study and no subjects reported any adverse reactions following quercetin, caffeine or placebo ingestion.

Plasma Analysis

Enzyme Multiplied Immunoassay Technique (EMIT®) Caffeine Assay

To provide consistency and expertise, Pennington Biomedical Research Center was commissioned to perform the analysis of plasma caffeine concentration. After coordination, 53, 1 ml samples were packaged on dry ice and shipped overnight for analysis of plasma caffeine concentration. Determination of caffeine concentration was completed using the Syva EMIT® Caffeine Assay from Behring Diagnostics (Cupertino, CA).

The EMIT® Caffeine Assay is a homogenous enzyme immunoassay technique used for determining caffeine concentration in biological fluids. The assay is based on competition between caffeine in the sample and caffeine labeled with the enzyme glucose-6-phosphate dehydrogenase (G6P-DH) for antibody binding sites. Enzyme activity decreases upon binding to the antibody, so the caffeine concentration in the sample can be measured in terms of enzyme activity. Active enzyme converts oxidized nicotinamide adenine dinucleotide (NAD) to NADH, resulting in an absorbance change that can be measured spectrophotometrically. Endogenous serum G6P-DH does not interfere because the coenzyme functions only with the bacterial enzyme employed in the

assay. Results from the EMIT® Caffeine Assay are regarded as accurate, reliable and reproducible. Early studies compared the EMIT® Caffeine Assay with high-performance liquid chromatography and found the EMIT® assay provided better reproducibility and precision (Miceli et al. 1984; Zysset et al. 1984). Table 7 summarizes the content of the EMIT® Caffeine Assay kit.

Table 7

Contents of the EMIT® Caffeine Assay Kit

Component	Content
Antibody/substrate reagent A	Sheep antibodies reactive to caffeine, glucose-6-phosphate, NAD, Tris buffer, bulking agents, stabilizers and 0.05% sodium azide.
Enzyme reagent B	Caffeine labeled with glucose-6-phosphate dehydrogenase, Tris buffer, bulking agents, stabilizers and 0.05% sodium azide.
EMIT® drug assay buffer concentrate	Tris buffer, surfactant and 0.75% sodium azide.
Caffeine calibrators	Caffeine, human serum, and 0.1% sodium azide.

When reconstituted, the caffeine calibrators contain caffeine concentrations as shown in Table 8.

Table 8

Caffeine Calibrators Included in the EMIT® Caffeine Assay Kit

Calibrators	0	1	3	7	15	30
Caffeine (µg/mL)	0	1.0	3.0	7.0	15.0	30.0
Caffeine (µmol/mL)	0	5.2	15.0	36.0	77.0	155.0

The immunoassay was accomplished on a Synchron® CX5 chemistry analyzer (Beckman Coultier Inc. Brea, CA). Blood samples were collected into 5-milliliter BD

Vacutainers ™ (Franklin Lakes, NJ) containing EDTA. Samples were centrifuged at 1000 × g for 15 min at room temperature using a GFMD (Dallas, TX) Silencer 2200 centrifuge system. The plasma samples were immediately frozen and stored at -80° C.

Reagents, standards and buffer concentrates were reconstituted and/or diluted according to manufacturer directions. After reconstitution, reagents and calibrators were allowed to equilibrate at room temperature (20° - 25° C) for 1 hour before use.

The Synchron® CX5 was calibrated prior to analysis. The math model in the Synchron® CX5 was checked to verify agreement with the model number found on the reagent package. Change in absorbance at 340 nm was measured over a fixed time interval. According to the EMIT® caffeine assay protocol, the change in absorbance is directly proportional to the concentration of caffeine in the sample and is used to calculate caffeine concentration. The results generated by the Synchron® CX5 chemical analyzer for this study needed no further calculations.

Plasma quercetin

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were high performance liquid chromatography (HPLC) grade unless otherwise stated. Samples were prepared for HPLC with Varian (Harbor City, CA) Bond Elut™ C18 solid phase extraction (SPE) cartridges. Chromatography was carried out using an Agilent 1100 HPLC system, equipped with a quaternary pump, temperature controlled auto sampler, thermostatic column compartment and diode array detector (Hewlett Packard, Wilmington, DE) in series with an ESA (Chelmsford, MA) Coulochem II coulometric detector with an ESA CoulArray 5600 multielectrode array detector. Data were collected

using CoulArray for Windows data acquisition software (ESA). A reversed phase C18 Inertsil® ODS-3 analytical column (5μm, 250 x 4.0 mm) (Chrom Tech, Inc., Apple Valley, MN) with a C18 5μm guard column (Western Analytical Products, Inc, Murietta, CA) was used to effect the separation.

The HPLC analysis of plasma quercetin concentration followed procedures described by Erlund et al. (Erlund et al. 1999). The objective was to demonstrate absorption of quercetin following oral supplementation; therefore, the method of analysis was not required to differentiate between free and conjugated forms of quercetin in plasma. Quercetin conjugates were hydrolyzed by incubating 1 ml of EDTA plasma with 110 μl of 0.78 M sodium acetate buffer (pH 4.8), 100 μl of 0.1 M ascorbic acid and 40 μl of a crude preparation from *Helix pomatia*, containing 4000 U β-glucuronidase and 200 U sulfatase activity (type HP-2, Sigma), for an average of 10 hours at 37° C. Incubation times were allowed to range from 9 to 11 hours based on the observation of Erlund et al. (Erlund et al. 1999) that maximal recovery of quercetin was possible after a minimum incubation time of 8 hours with no degradation until the 17 hour time point. For this study, incubation times ranging from 9 to 11 hours were selected to ensure maximal recovery of quercetin and based on availability of required laboratory equipment.

After incubation, each plasma sample was diluted with 2 ml of phosphate buffer (70 mM, pH 2.4) and added to a Bond Elut C18 SPE column that had been preconditioned with 6 ml of HPLC-grade methanol and 6 ml phosphate buffer. Up to 12 samples were processed simultaneously utilizing a vacuum manifold system designed for use with SPE columns. Samples were drawn through the SPE columns at approximately 1.5 ml per minute. Columns were washed with 9 ml of phosphate buffer and 0.5 ml of

water and then allowed to dry before elution of quercetin. Quercetin was eluted into a 12 ml plastic tube with 2 ml of methanol. A 12 ml plastic tube was used for compatibility with the vacuum manifold system and for ease of handling during the drying process. The methanol eluate was dried under a stream of nitrogen at room temperature using an N-Evap evaporator (Organomation, Berlin, MA). When the tube was completely dry, 1 ml toluene-dichloromethane (80:20, v/v) and 200 µl of 5.3 M acetic acid - 32 mM oxalic acid (80:20, v/v) (pH 2.4) were added. The samples were vortexed for 1 minute and transferred to 2 ml microcentrifuge tubes. Samples were centrifuged at 1000 × g for 15 minutes at 4° C. Of the lower phase, 150 µl was transferred into Ultrafree®-MC centrifugal filter devices (Fisher Scientific) before centrifuging at 10,100 × g for 5 min at 4° C. Filtered samples were transferred into 200 µl vial inserts for HPLC analysis.

The mobile phase was comprised of two solvent solutions: solvent A, 59% methanol in phosphate buffer (70 mM, pH 2.4); and solvent B, phosphate buffer (70 mM, pH 2.4). For isocratic elution (1 ml/min), the mobile phase was 95% A and 5% B. When solvent A was used alone, quercetin eluted at approximately 7 minutes; however, interference from other compounds in the plasma extract made a later elution time more desirable. The mixture of solvent A and B resulted in an elution time of 11.7 minutes for quercetin with no interference from other compounds. For electrochemical detection, using the multielectrode array detector, the following potentials were used: +100, + 175, +200, +250, +300, +350, +700, +800 mV.

The plasma quercetin peaks were quantified using the standard additions method. Four plasma standards were prepared in duplicate by addition of quercetin standard in methanol to 1 ml aliquots of very low-quercetin plasma. The very-low quercetin plasma

pool was obtained from a volunteer who had been following a strict, very-low quercetin diet for 72 hours. Final concentrations included 0.0 μM (control), 1.0 μM, 2.0 μM and 5.0 μM. The standards were subjected to the same hydrolysis, extraction and analysis procedures as the other samples in this study. The standard curve was obtained by plotting peak height versus quercetin concentration.

CHAPTER 3. RESULTS

Behavioral data

Three criteria were used to measure performance on the USARIEM Visual Vigilance Test: number of correct detections (hits), response time and number of false responses. Performance criteria were compared in each group (caffeine, quercetin and placebo) using analysis of variance (ANOVA) or Kruskal-Wallis one-way ANOVA. Mood scores (total mood disturbance, tension-anxiety, depression-dejection, vigor-activity, anger-hostility, confusion-bewilderment and fatigue-inertia) were compared among groups using Kruskal-Wallis one-way ANOVA. Alpha was set at .05 and all analyses were performed using routines available in SPSS 10.0.5 for Windows™. Data were tested for normality and homogeneity of variance. An ANOVA was used in the analysis of data that passed these tests while the nonparametric equivalent was used to analyze data that failed.

Correct detections

A one-way ANOVA was performed to determine if group assignment (caffeine, quercetin or placebo) had an effect on the dependent variable (number of correct detections or "hits"). There was a statistically significant effect of group on the number of hits ($F_{(2,54)}$=4.37; p=.017). Post hoc testing using the Bonferroni procedure showed there was a significantly higher (p=.014) number of hits with caffeine (mean=37.8, SD=3.3) compared to the placebo group (mean=31.7, SD=7.5). The other groups were not statistically different from one another. See Figure 5.

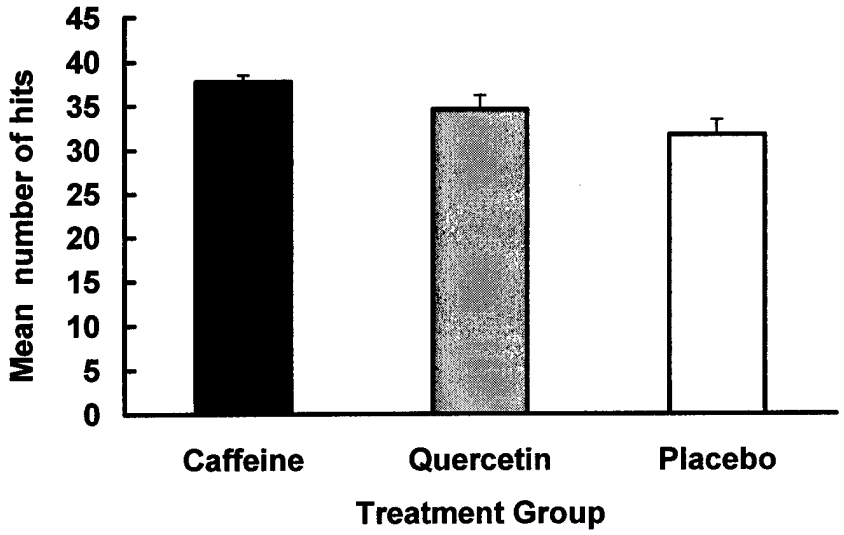


Figure 5. Mean hits (+SE) for caffeine (n=18), quercetin (n=20) and placebo (n=19) groups. The caffeine group significantly outperformed the placebo group (p=0.014); no other groups were significantly different from one another.

Response times

A 3 × 3 repeated-measures ANOVA was performed using group (caffeine, quercetin and placebo) as the between subjects factor, and 15 minute blocks of time (block 1, block 2 and block 3) as the repeated measures within subjects factor. There was no statistically significant interaction between group and block of time ($F_{(2,54)}$=.246; p=.783). There was a main effect of group ($F_{(2,54)}$=7.69; p=.001) and a main effect of time ($F_{(1,54)}$=15.68; p<.001). Post hoc adjustment for multiple comparisons using the Bonferroni procedure showed a significantly lower response time with caffeine compared to placebo (p=.001) and a significant difference between time block 1 compared with block 2, and time block 1 compared with time block 3 (p<.01). See Figure 6.

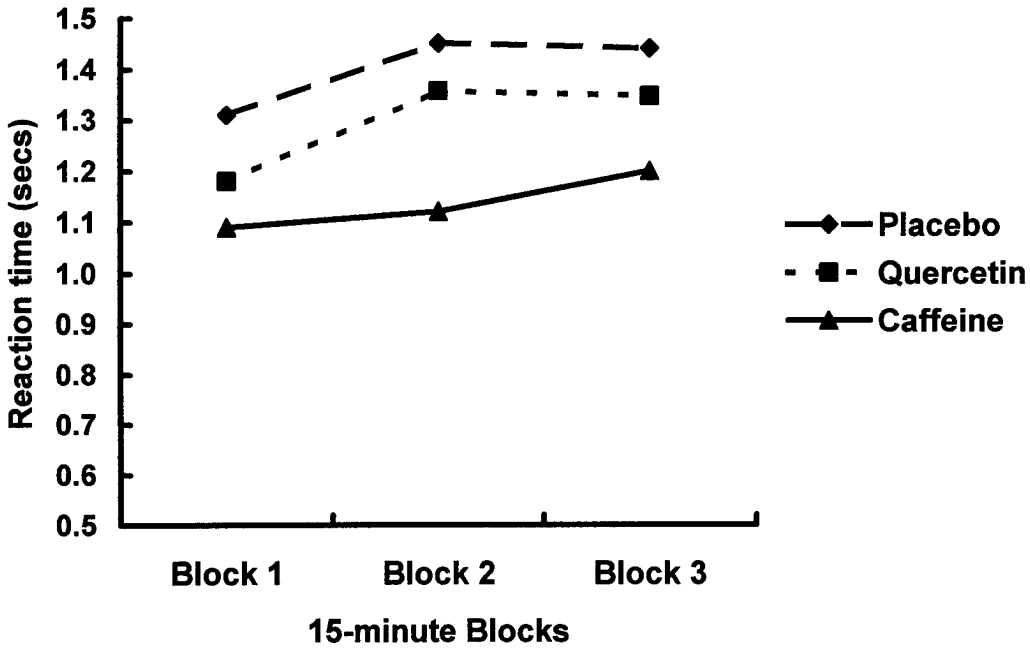


Figure 6. Response time in seconds for caffeine (n=18), quercetin (n=20) and placebo (n=19) groups. The caffeine group significantly outperformed the placebo group (p=0.001); however, no other groups were different from one another.

False responses

A false response was recorded when the subject pressed the space bar when there was no stimuli. A Kruskal-Wallis one-way ANOVA on ranks was performed to determine if group assignment had an effect on the dependent variable (number of false responses). There was no statistically significant effect of group on the number of false positives ($F_{(2,54)}$=2.08; p=.09). See Figure 7.

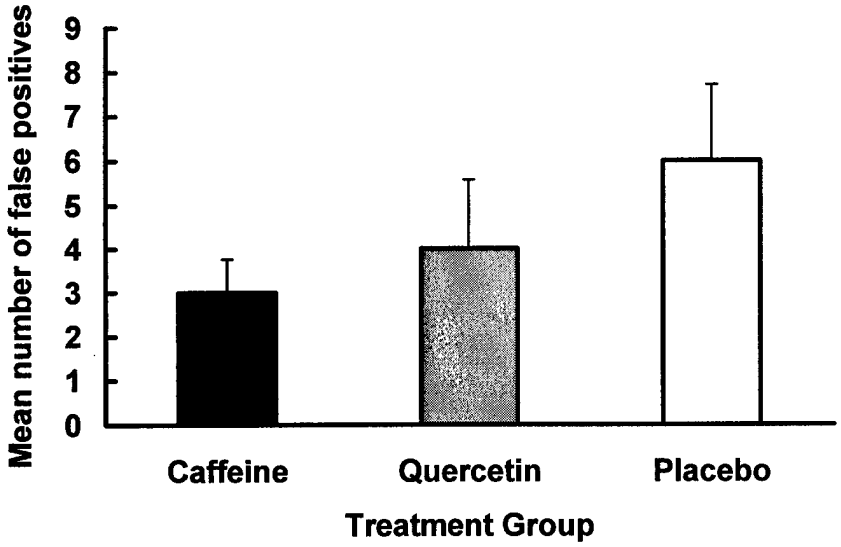


Figure 7. Mean false positives (+SE) for caffeine (n=18), quercetin (n=20) and placebo (n=19) groups. There was not a significant effect of group on the number of false positives (p=0.09).

Changes in POMS total mood disturbance (TMD) scores

Changes in POMS TMD scores were analyzed using a Kruskall-Wallis one-way ANOVA on ranks to determine whether there was any effect of group on mood change from before to after the task. There was a significant effect of group on TMD change score ($p=.021$). Post hoc testing using Dunn's Method of multiple comparisons showed a significant decrease in the caffeine group's TMD scores relative to the placebo group TMD scores ($p<0.05$). No other groups were significantly different from one another.

As stated earlier, the TMD score is based on six individual POMS factors (tension-anxiety, depression-dejection, vigor-activity, fatigue-inertia, anger-hostility and confusion-bewilderment). The TMD was used as a single global assessment of affective state. A negative TMD change score indicated improved mood whereas a positive TMD score represented a worsened mood. See Figure 8.

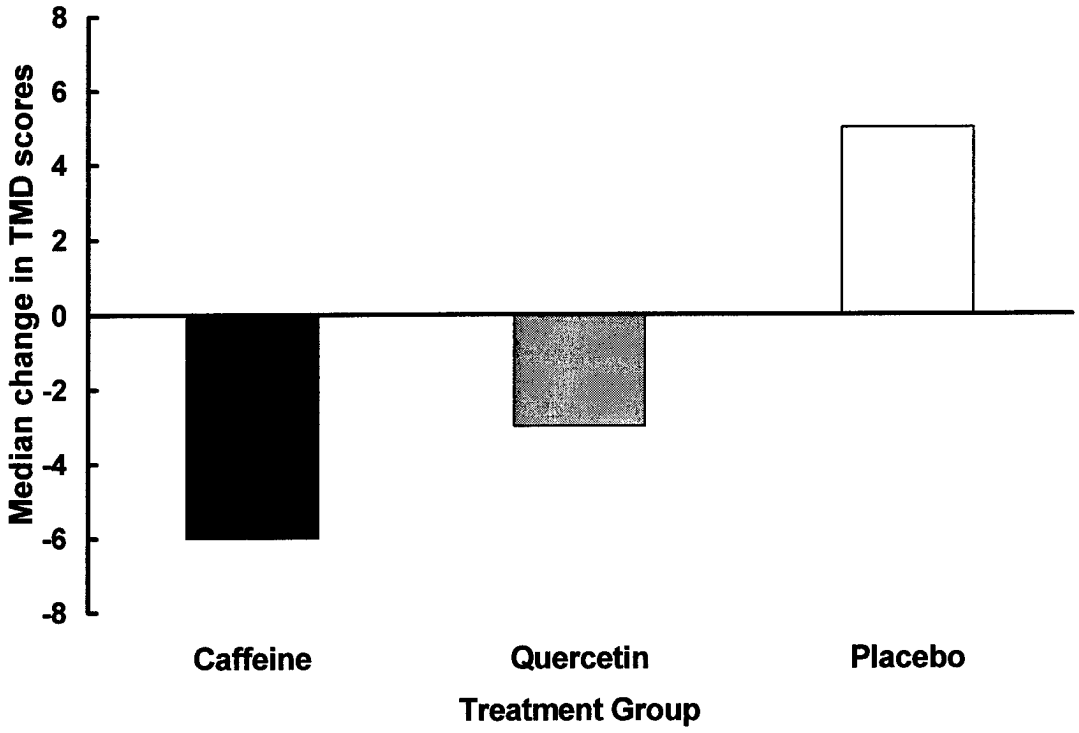


Figure 8. Median change in total mood disturbance (TMD) scores for caffeine (n=18), quercetin (n=20) and placebo (n=19) groups. There was a significant effect of group ($p=0.021$) such that the caffeine group scores showed significant improvement from before to after the vigilance task relative to the placebo group, which showed a worsening mood from before to after the vigilance task ($p<0.05$).

Change in POMS vigor-activity scores

Changes in POMS vigor-activity scores from before to after the vigilance task were analyzed by using Kruskal-Wallis one-way ANOVA to determine whether there was any effect of treatment group. There was a statistically significant effect of group on vigor-activity change scores ($p=.037$). Post hoc testing using Dunn's Method of multiple comparisons showed vigor-activity scores were significantly improved in the caffeine group relative to the placebo group ($p<0.05$). No other groups were significantly different from one another. See Figure 9.

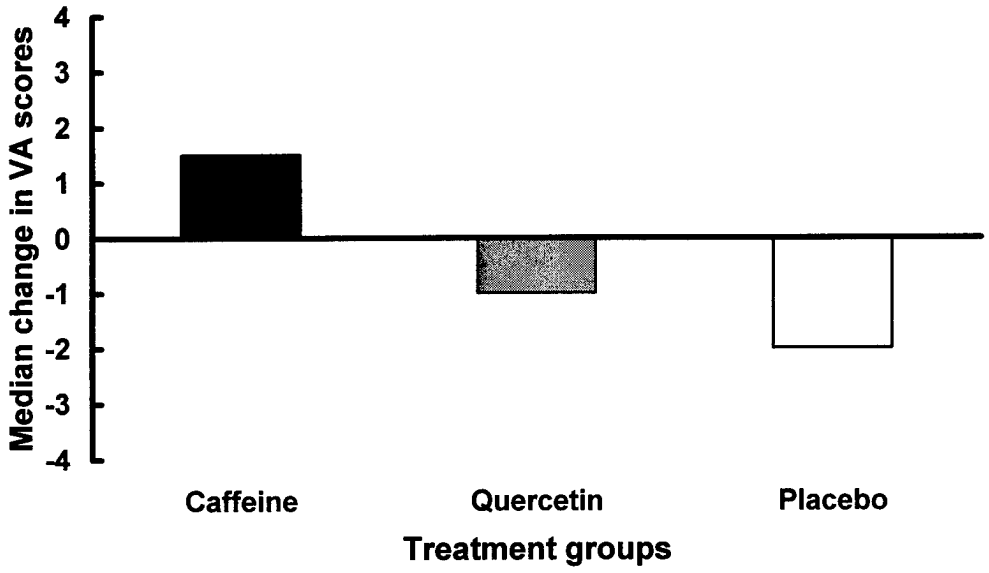


Figure 9. Median change in vigor-activity scores for caffeine (n=18), quercetin (n=20) and placebo (n=19) groups. The caffeine group had significantly higher vigor-activity change scores relative to the placebo group ($p<0.05$); no other groups were significantly different from one another.

Change in POMS fatigue-inertia scores

Changes in POMS fatigue-inertia scores from before, to after, the vigilance task were analyzed by using Kruskal-Wallis one-way ANOVA to determine whether there was any effect of treatment group on the change in fatigue-inertia mood factor. There was a statistically significant effect of group on fatigue-inertia change scores ($p=.003$). Post hoc testing using Dunn's Method of multiple comparisons showed fatigue-inertia scores were significantly higher in the caffeine group relative to the placebo group and relative to the quercetin group ($p<0.05$). The quercetin and placebo groups were not significantly different from one another ($p>0.05$). See Figure 10.

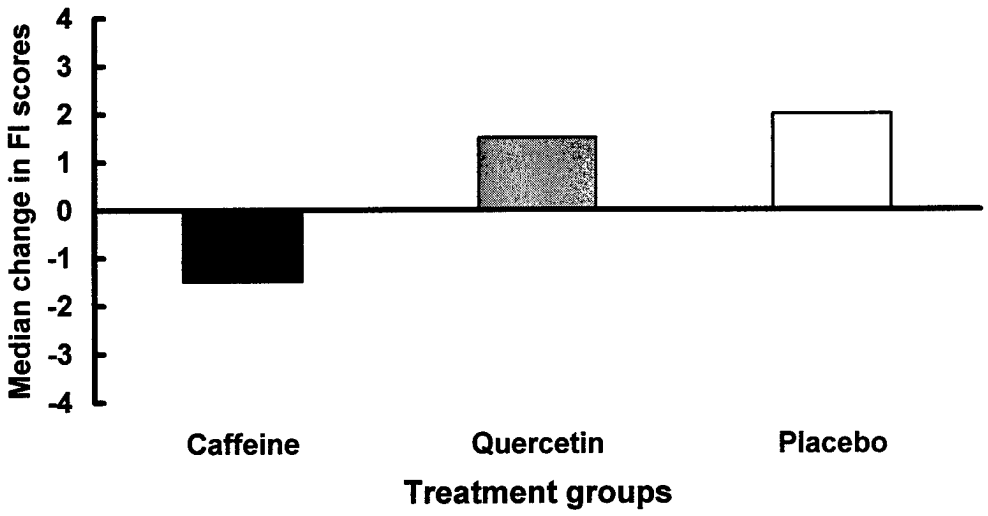


Figure 10. Median changes in fatigue-inertia scores for caffeine (n=18), quercetin (n=20) and placebo (n=19) groups. The median change in fatigue-inertia scores for the caffeine group was significantly different relative to the placebo group and relative to the quercetin groups ($p<0.05$); the placebo and quercetin groups were not different from one another ($p>0.05$).

Summary of POMS results by sub-scale

Table 9 summarizes the results of the POMS for total mood disturbance (TMD) as well as the six sub-scales.

Table 9

Summary of POMS Results.

Score Type	Median	25%	75%	Chi-Square	df	p
Total Mood Disturbance				7.702	2	.021*
Caffeine	-6.0	-16.0	4.0			
Quercetin	-3.0	-13.0	10.0			
Placebo	5.0	0.0	12.8			
Vigor-Activity				6.594	2	.037*
Caffeine	1.5	-1.0	6.0			
Quercetin	-1.0	-4.5	3.0			
Placebo	-2.0	-5.0	0.5			
Fatigue-Inertia				11.407	2	.003*
Caffeine	-1.5	-4.0	-1.0			
Quercetin	1.5	-2.0	4.5			
Placebo	2.0	0.0	4.0			
Tension-Anxiety				4.244	2	.120
Caffeine	2.5	-1.0	4.0			
Quercetin	0.0	-2.0	1.5			
Placebo	0.0	-1.0	2.8			
Depression-Dejection				2.774	2	.250
Caffeine	-1.0	-3.0	0.0			
Quercetin	0.0	-2.0	-.5			
Placebo	0.0	-2.0	0.0			
Anger-Hostility				6.582	2	.061
Caffeine	-0.5	-3.0	0.0			
Quercetin	-1.0	-3.5	0.0			
Placebo	0.0	-1.0	0.0			
Confusion-Bewilderment				3.960	2	.138
Caffeine	0.0	-1.0	0.0			
Quercetin	-1.0	-2.0	2.0			
Placebo	1.0	0.0	2.0			

Note. The "*" indicates the mood factors where a significant effect of group was detected. The other factors were assessed using Kruskal-Wallis one-way ANOVA; however, none of the groups were significantly different from one another.

Plasma Analysis

EMIT® Caffeine Assay

Plasma caffeine concentrations are reported in μM. A one-way ANOVA was performed to determine if group assignment (caffeine, quercetin or placebo) had an effect on the dependent variable (plasma caffeine concentration). There was a statistically significant effect of group ($F_{(2,54)}$=4.37; p=.001). Post hoc testing using Dunn's Method of multiple comparisons showed mean plasma caffeine concentration was significantly higher in the caffeine group relative to the placebo group ($p<0.05$) as well as the quercetin group ($p<0.05$). There was no difference in plasma caffeine concentration between quercetin and placebo groups ($p>0.05$). See Figure 11.

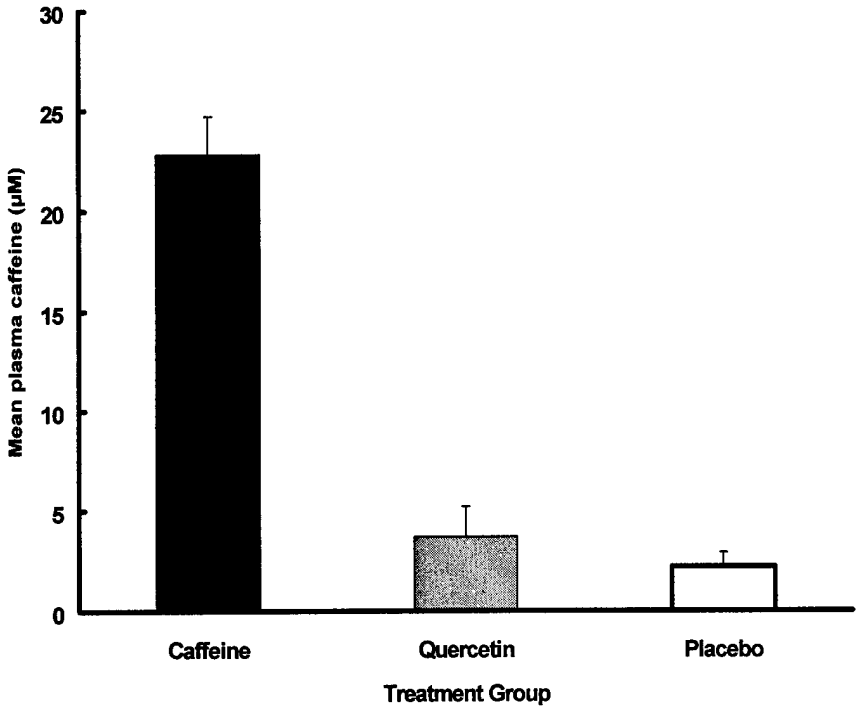


Figure 11. Mean plasma caffeine concentrations (+SE) in for caffeine (n=18), quercetin (n=17) and placebo (n=17) groups. Caffeine concentration was significantly higher in the caffeine group relative to the quercetin group ($p<0.05$) and placebo group ($p<0.05$).

Plasma quercetin assay

A one-way ANOVA was performed to determine if group assignment (caffeine, quercetin or placebo) had an effect on the dependent variable (plasma quercetin concentration). There was a statistically significant effect of group ($F_{(2,54)}$=4.37; p=.001) on plasma quercetin concentration. Post hoc testing using Dunn's Method of Multiple Comparisons showed plasma quercetin concentration was significantly higher in the quercetin group relative to the caffeine group ($p<0.05$) as well as the placebo group ($p<0.05$). There was no difference in plasma quercetin concentration between the caffeine and placebo groups ($p>0.05$). See Figure 12.

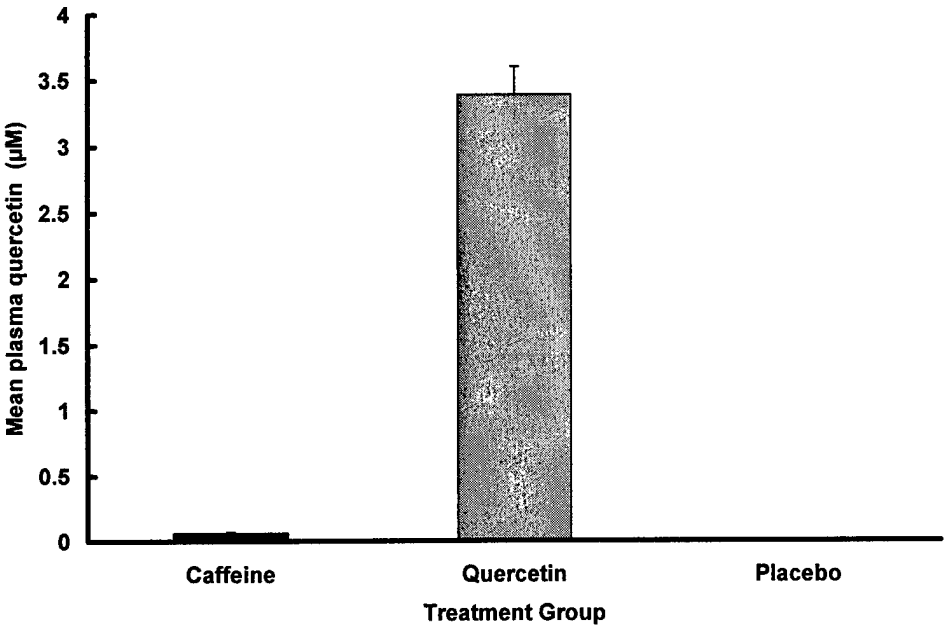


Figure 12. Mean plasma quercetin concentration (+SE) for caffeine (n=18), quercetin (n=17) and placebo (n=17) groups was significantly higher in the quercetin group relative to the caffeine and placebo groups ($p<0.05$).

High Performance Liquid Chromatography (HPLC)

Quercetin peaks were identified in the plasma extracts by comparison with a quercetin standard. The retention time of quercetin was 11.7 minutes. Representative chromatograms of high-quercetin and low-quercetin plasma extracts are shown in Figures 13 through 16

As stated previously, the following potentials were used for electrochemical detection, using the multielectrode array detector: +100, + 175, +200, +250, +300, +350, +700, +800 mV. Figures 13 through 15 depict only the results produced by the +200 mV detector (channel 3). Figure 13 is a representative chromatogram of the plasma quercetin concentration in a subject from the quercetin-supplemented group. The peak in Figure 13 equates to a plasma quercetin concentration of 7.7 μM. In contrast, Figures 14 and 15 are representative of the caffeine-supplemented and placebo groups respectively. Figures 14 and 15 do not have the characteristic quercetin peak at 11.7 minutes.

Figure 16 is a representative chromatogram depicting the results from all eight channels on the multielectrode array detector. The quercetin peak is clearly visible on all channels at 11.7 minutes.

The peaks in Figure 13 and Figure 16 correspond with a quercetin standard and were quantified using the standard additions method described in chapter 2.

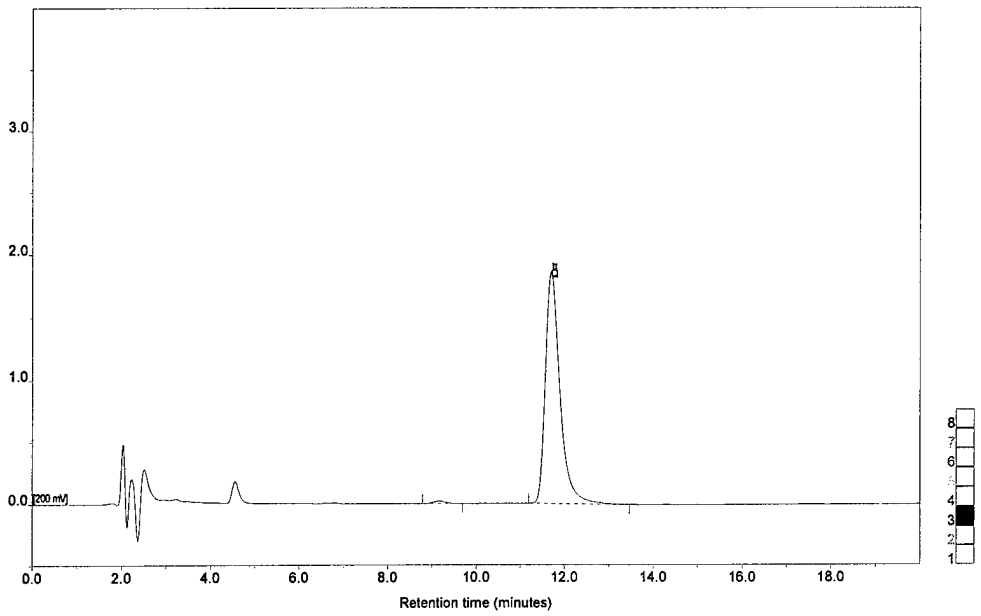


Figure 13. Representative chromatogram of plasma quercetin concentration in a subject from the quercetin treatment group. Quercetin had a retention time of 11.7 min and this peak corresponded to a plasma quercetin concentration of 7.7 μM. The x axis represents response. The Y axis represents retention time in minutes.

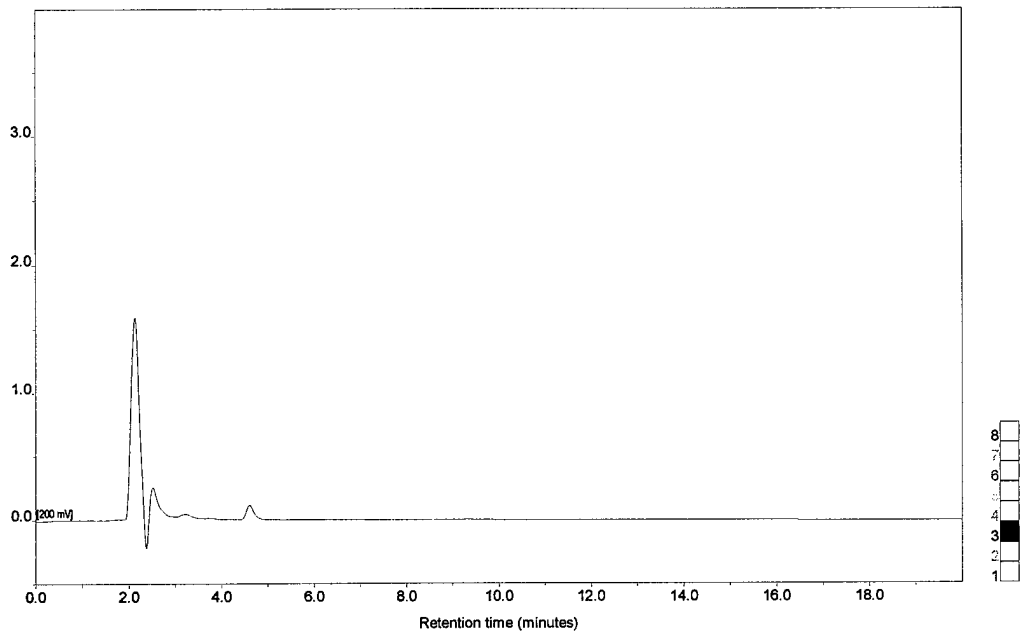


Figure 14. Representative chromatogram of plasma quercetin concentration in a subject from the caffeine-supplemented treatment group. Quercetin has a retention time of 11.7 minutes, but was not detected in this sample. The x axis represents response. The Y axis represents retention time in minutes.

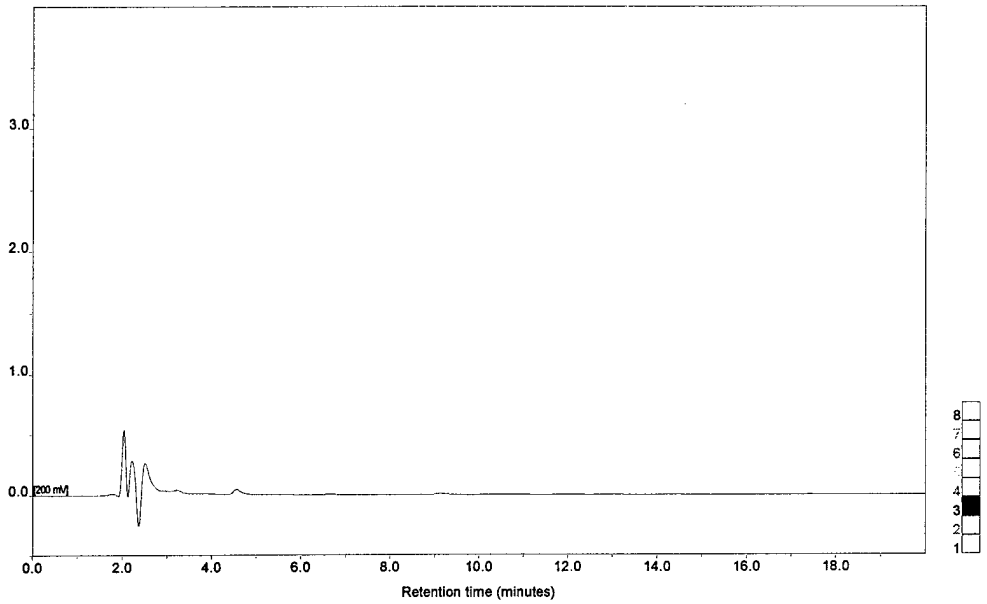


Figure 15. Representative chromatogram of plasma quercetin concentration in a subject from the placebo group. Quercetin has a retention time of 11.7 minutes, but was not detected in this sample. The x axis represents response. The Y axis represents retention time in minutes.

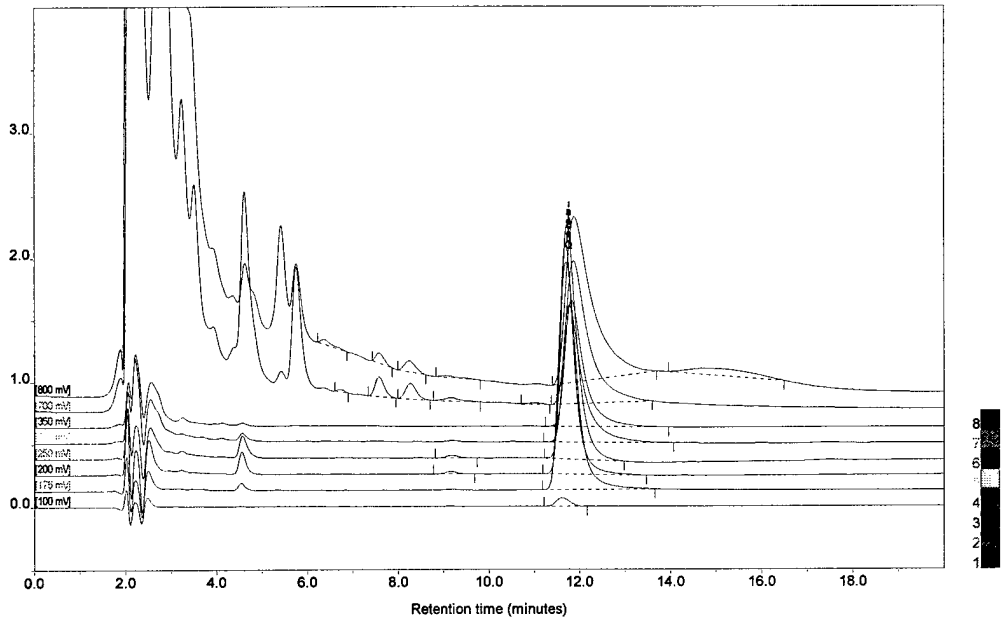


Figure 16. Representative chromatogram of plasma quercetin concentration in quercetin-supplemented subjects. Quercetin had a retention time of 11.7 min. This chromatogram displays all channels. The x axis represents response. The Y axis represents retention time in minutes.

CHAPTER 4. DISCUSSION

Quercetin administration did not result in significant improvement of performance on the vigilance task or measures of mood state relative to the placebo group. However, caffeine did significantly improve performance on a variety of behavioral measures related to vigilance and mood state. This finding is supported by similar previous research on the use of caffeine. Lieberman and others showed that performance on visual vigilance tasks was improved by the administration of as little as 64 mg of caffeine (Fine et al. 1994; Lieberman et al. 1987a; Lieberman et al. 1987b). The 200 mg caffeine dose used in this study proved sufficient to improve performance relative to the placebo group. By comparison, the findings of Baker and Theologus. and Childs also showed improved performance on vigilance tasks following caffeine administration in average doses of 200 mg (Baker and Theologus 1972; Childs 1978).

Vigilance

Sensitivity of the USARIEM Scanning Visual Vigilance Test was adequate to detect significant differences between the caffeine and placebo groups with regard to number of hits (correct detections) and response time. These findings are consistent with several studies showing caffeine's influence on tasks requiring sutained vigilance (Committee on Military Nutrition Research 2001; Lieberman et al. 1987a; Lieberman et al. 1987b; Penetar et al. 1994; Smith et al. 1994).

Although performance of the quercetin group was intermediate to the performance of the other two groups, the difference between the quercetin group and the placebo group failed to reach significance on any of the measured vigilance parameters. Despite this observation, it should not be concluded that quercetin aglycone supplementation does not enhance vigilance or response time. It is possible the plasma concentrations of quercetin aglycone achieved in this study were not adequate to elicit an effect, or perhaps the vigilance test was conducted prior to reaching maximum plasma quercetin concentrations.

Mood state

Results showed caffeine, but not quercetin, was associated with significant improvement in various aspects of mood. Specifically, subjective measures of total mood disturbance (TMD), vigor-activity and fatigue-inertia improved over baseline following caffeine administration. This supports the findings of Herz, who also showed a significant change in only the vigor-activity and fatigue inertia sub-scales of the POMS following caffeine administration (TMD was not discussed) (Herz 1999). Other

Other caffeine research has shown that in addition to TMD, vigor-activity and fatigue inertia, the tension-anxiety and depression-dejection sub-scales of the POMS are also typically improved by moderate doses of caffeine (Fine et al. 1994; Lieberman et al. 1987a; Lieberman et al. 1987b).

Quercetin administration was not associated with a significant change in TMD or the mood sub-scales measured by the POMS questionnaire; however, the results were intermediate to those of caffeine and placebo. This trend was also present in the results of the visual vigilance test. There is no clear explanation for this trend; however, it is possible that an effect threshold for plasma quercetin aglycone concentration exists and that it was approached, but not exceeded in all subjects prior to the start of testing. This idea is supported by previous caffeine research showing a plasma caffeine concentration threshold of 5-10μM that is required before central nervous stimulation is observed (Committee on Military Nutrition Research 2001). The intermediate nature of the quercetin group's performance also calls into question the sufficiency of the group size. As stated previously, 60 subjects were needed for this study (20 per group) based on a one way ANOVA (alpha=0.05, power=0.80); however, only 57 subjects ultimately completed the study. Perhaps the effect size from quercetin is smaller than predicted and a larger population is required to detect changes in subjective mood scores. Another very likely possibility is that conjugated quercetin is not able to cross the blood-brain barrier in order to compete for A_1 adenosine receptor binding sites. In contrast, it is reasonable to propose that quercetin aglycone can cross the blood-brain barrier. The earlier works of Lieberman et al. and Pardridge supports this concept. Their findings suggest that the selectively permeable nature of the blood-

brain barrier allows for a wide variety of biologically relevant endogenous and exogenous substances to enter the brain (Lieberman et al. 1984; Pardridge 1986). Lipid soluble compounds such as melatonin are capable of crossing the blood-brain barrier (Lieberman et al. 1984), so it is conceivable that quercetin aglycone does as well. This is a question worthy of further investigation because of the possibility that many of the physiological actions of the flavonoids are related to their affinity for adenosine receptors.

Plasma quercetin concentrations

Mean plasma quercetin concentration obtained *in vivo* (3.7 μM) was higher than that which is required to reach K_i at adenosine receptors *in vitro* (2.4 ± 0.64 μM) (Ji et al. 1996). However, because quercetin is typically conjugated and strongly bound to albumin in the blood, it is not known whether any *in vivo* properties would be similar to those seen *in vitro*. The results of this study suggest that quercetin's affinity for adenosine receptors *in vitro* may not be of physiological significance *in vivo* with regard to specific cognitive functions.

Predictability of quercetin plasma concentration following supplementation was restricted by a lack of information regarding absorption of a large, oral dose of quercetin aglycone. However, Erlund et al. observed a mean plasma quercetin concentration of approximately 70 μg/L or 0.23 μM in human subjects 2 hours after ingesting a 100 mg dose of quercetin aglycone (Erlund et al. 1999). Based on the assumption that quercetin aglycone absorption is somewhat linear and plasma quercetin concentrations are dose-dependent, the mean plasma quercetin concentration achieved in this study was expected

to be approximately 20-fold those reported by Erlund et al. (Erlund et al. 1999). The findings of Erlund et al. are strongly supported by the observation that mean plasma quercetin concentration in the quercetin-supplemented group was 3.7 μM compared to the predicted value of 4.6 μM.

Plasma samples were not analyzed for the presence of quercetin aglycone prior to hydrolysis with β-glucuronidase and sulfatase, however, based on the findings of Erlund et al., quercetin aglycone accounts for approximately 8% of the total plasma quercetin value (Erlund et al. 2000) 2 hours after ingestion of a quercetin aglycone supplement. Therefore, mean quercetin aglycone concentration was estimated at 0.30 μM in the quercetin-supplemented group. This equates to approximately 12% of the concentration required to reach the K_i at adenosine receptors *in vitro* (2.4 ± 0.64 μM). By comparison, caffeine has a K_i of 29 μM, however, physiological effects have been observed *in vivo* at 5-10 μM (Committee on Military Nutrition Research 2001) or 17-34% of the K_i. Based on this simple comparison, plasma concentration of quercetin aglycone may not have been sufficient at test time to influence the cognitive functions measured in this study. Indeed, maximum plasma quercetin concentration may not have occurred until several hours after ingestion.

A significant mean plasma quercetin concentration was clearly demonstrated in this study; however, one limitation was that total plasma quercetin was measured only one time after ingestion of the supplement. This poses the obvious risk of missing the true peak plasma quercetin concentration. Erlund et al. demonstrated a considerable increase in plasma quercetin concentration 30 minutes after ingestion of 8 mg, 20 mg and 50 mg quercetin aglycone with a peak plasma concentration at approximately 1.9 hours

following the 8 mg dose. However, Erlund et al. also found that the absorption curve for quercetin from quercetin aglycone was biphasic and that peak plasma concentration may not occur until 6 to 8 hours following ingestion of a large dose of quercetin aglycone (Erlund et al. 2000). Future studies should consider testing 5 to 6 hours post-ingestion of quercetin aglycone supplements.

Plasma caffeine concentrations

Plasma caffeine concentrations were highly predictable and the results of this study compared favorably with the findings of Arnaud who showed plasma caffeine concentration is dose-dependent and equates to a range of 41 to 51 μM for oral doses of 5 to 8 mg/kg (Arnaud 1987). In chapter 2, plasma caffeine concentration was predicted based on an 80 kg human receiving a 200 mg caffeine supplement. The estimated plasma caffeine concentration is 4 mg/L or 21 μM. Actual plasma caffeine concentrations measured in the present study were very close to predicted values. The mean weight of subjects in the caffeine-supplemented group was 72.6 kg and the mean plasma caffeine concentration 2 hours after ingestion of a 200 mg supplement was 4.4 mg/l or 22.8 μM.

CHAPTER 5. SUMMARY AND CONCLUSIONS

This study was undertaken to test the hypothesis that quercetin aglycone enhances mood and vigilance in humans. Although other studies have assessed the effects of various phytochemicals on cognitive functions (e.g., the effect of soy on memory), this is the first report evaluating specific cognitive functions in humans following supplementation with a flavonoid. In addition, this is one of very few human studies to measure plasma quercetin concentrations and apparently the first to measure plasma quercetin concentrations following a large oral dose of quercetin aglycone. This study is also unique in that it incorporated both caffeine as a positive control and a placebo as a control.

Caffeine, but not quercetin, did significantly improve performance on a variety of behavioral measures related to vigilance and mood state. The intermediate nature of the quercetin-supplemented group's performance raises concerns over sample size

adequacy as well as quercetin aglycone dose sufficiency. Conjugation of quercetin *in vivo* may also be a contributing factor in quercetin's failure to produce effects similar to those seen following caffeine supplementation. Considering the pharmacologic dose used in this study, it is not likely that dietary quercetin or quercetin aglycone supplementation will be sought for enhancement of mood or vigilance; however, the idea of quercetin and its conjugates acting on adenosine receptors *in vivo* should be investigated further.

Future studies should address the permeability of the blood-brain barrier to quercetin and other flavonoids. Behavioral studies similar to this one should consider measuring quercetin aglycone in plasma samples prior to measuring total quercetin following enzymatic hydrolysis.

The military will more than likely continue to maintain an interest in the effect of dietary constituents on certain cognitive functions. Ever-increasing complexity of weapon systems combined with an increasing operational tempo make the concept of performance enhancement through nutrition a very relevant concept. For now, it would appear that caffeine is still an effective choice for enhancing performance with only a minimal risk of side effects.

This study addressed only a few of the many cognitive functions that may be influenced by an acute pharmacologic dose of a dietary constituent. Therefore, despite caffeine's proven effectiveness in mood and vigilance enhancement, there is more work to be done in the area of nutrition and cognitive function. Future studies should also compare chronic and acute effects of dietary components on specific parameters of physical and cognitive performance.

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Appendix A

Instructions:

Answer each of the following questions as accurately as you are able. If you are unsure about the question or if you desire assistance, please ask the investigator.

Name: _____

Telephone Number: _____

1. What is your age in years? _____

2. What is your gender?

   ☐ Male

   ☐ Female

3. What is your height? _____ feet _____ inches

4. What is your weight in pounds? _____

5. What is your rank? _____

6. Approximate numbers of hours you sleep per night if you are on your own schedule:

   _____ hours

7. Do you currently take any medications or dietary supplements?

   ☐ Yes

   ☐ No **(go to question 9)**

8. Please list the medications and/or dietary supplements and the reasons for taking them.

   Medication/Dietary Supplement Reason

   _____

   _____

   _____

   _____

   _____

9. Have you ever experienced an allergic reaction to a medication?

   ☐ Yes

   ☐ No (please go to the next section)

10. Please list the medication and describe the reaction:

    _____

    _____

    _____

    _____

11. Place an "**X**" next to any of the following conditions that apply to you:

    _____ High blood pressure (hypertension)

    _____ Heart disease or history of irregular heartbeat (dysrythmia)

    _____ Malabsorption disorder such as irritable bowel disease or diverticulitis

    _____ Vision impairment such that you have difficulty viewing a computer monitor

The following question is for females only; males please go to the next section (page 4)

12. Are you pregnant, thinking of becoming pregnant in the next 2 months, or currently breastfeeding? (Females only)

☐ Yes

☐ No

IF YES, you SHOULD NOT participate in this study. STOP NOW and see the investigator (Maj Craig Olson) or the medical monitor (Lt Col Mary Nelson).

If no, please continue on to page 4.

Caffeine Use

This section will help us estimate your usual caffeine consumption. Indicate how often you usually eat or drink the foods or medication listed.
Instructions:
First, indicate your serving size by checking the box that most closely describes what you normally consume. **Second**, indicate the number of times you usually have the item in that amount. **Third**, indicate the time period for the number of times you usually have that item in that amount.

COFFEE / TEA	**Times**	
Brewed Coffee, Regular		
☐ I never use this product		
☐ Cup (5 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Small Mug (8 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Medium Mug (12 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Large Mug (16 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Jumbo Mug (22 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
Instant, Regular		
☐ I never use this product		
☐ Cup (5 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Small Mug (8 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Medium Mug (12 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Large Mug (16 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Jumbo Mug (22 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**

COFFEE / TEA	Times	
Espresso		
☐ I never use this product		
☐ Cup (5 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Small Mug (8 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Medium Mug (12 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Large Mug (16 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Jumbo Mug (22 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
Cappuccino		
☐ I never use this product		
☐ Cup (5 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Small Mug (8 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Medium Mug (12 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Large Mug (16 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Jumbo Mug (22 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
Brewed Tea		
☐ I never use this product		
☐ Cup (5 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Small Mug (8 fl oz)	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**

☐ Medium Mug (12 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Large Mug (16 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Jumbo Mug (22 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week

COFFEE / TEA	**Times**	
Instant Tea		
☐ I never use this product		
☐ Cup (5 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Small Mug (8 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Medium Mug (12 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Large Mug (16 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Jumbo Mug (22 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
Iced Tea		
☐ I never use this product		
☐ Sm. Glass (5 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Reg. Glass (8 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Med. Glass (12 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Lg. Glass (16 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Jumbo Glass (22 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week

COCOA	Times	
Chocolate Milk		
☐ I never use this product		
☐ Sm. Glass (5 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Reg. Glass (8 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Med. Glass (12 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Lg. Glass (16 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Jumbo Glass (22 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week

COCOA	Times	
Cocoa / Hot Chocolate		
☐ I never use this product		
☐ Cup (5 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Small Mug (8 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Medium Mug (12 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Large Mug (16 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week
☐ Jumbo Mug (22 fl oz)	1 2 3 4 5 6 7 8 9 10 11 12 13 14	Day Week

SOFT DRINK	Times	
Cola-Type (reg or diet)		
☐ I never use this product		
☐ 12 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 16 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 20 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 1 liter	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 2 liter	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
Dr. Pepper-Type		
☐ I never use this product		
☐ 12 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 16 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 20 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 1 liter	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 2 liter	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**

SOFT DRINK	Times	
Mountain Dew		
☐ I never use this product		
☐ 12 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 16 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 20 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 1 liter	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 2 liter	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
Mello Yello		
☐ I never use this product		
☐ 12 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 16 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 20 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 1 liter	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 2 liter	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
Jolt		
☐ I never use this product		
☐ 12 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 16 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 20 fluid ounces	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ 1 liter	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**

CANDY	Ounces	
Milk Chocolate – solid ☐ I never use this product **Typical Bar = 1.5 ounce**	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
Dark Chocolate – solid ☐ I never use this product **Typical Bar = 1.5 ounce**	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
Other Chocolate Candy ☐ I never use this product **Typical Bar = 1.5 ounce**	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
Chocolate Desserts ☐ I never use these **Serving examples:** **1/2 cup choc pudding** **1/12 of a chocolate cake** **2 Tbsp chocolate frosting** **2 Tbsp chocolate syrup**	**0 1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**

MEDICATIONS	Number of Pills/Tablets/Capsules	
☐ NoDoz (Regular) ☐ I never use this product	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Vivarin / NoDoz (Max) ☐ I never use this product	**1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**

☐ Anacin ☐ I never use this product	**0 1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Dristan ☐ I never use this product	**0 1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Triaminicin ☐ I never use this product	**0 1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Excedrine ☐ I never use this product	**0 1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**
☐ Weight Control Aids ☐ I never use this product	**0 1 2 3 4 5 6 7 8 9 10 11 12 13 14**	**Day Week**

Thank you very much for taking the time to provide this information. The answers you have given will be useful for completing this study, and interpreting the results.

Please return to:

Maj Craig A. Olson
Clinical Investigation Facility
Tube Station 10
423-7262

Appendix B

POMS Instructions for Study Participants

You are about to begin the Profile of Mood States Questionnaire. The questionnaire is designed to evaluate different aspects of your mood, so it's important you respond to each question as honestly as you can. Your responses should reflect how you feel RIGHT NOW.

You will be presented with a series of 65 adjectives. Each adjective will be preceded by the phrase "Do you Feel:" with a list of possible responses ranging from "Not at all" to "Extremely." Move the highlighted bar by pressing the "up" or "down" arrow on the keyboard until you have highlighted the response that most closely reflects the way you feel. Press "enter" to record your selection and advance to the next question.

The computer will inform you when you have completed the questionnaire. If you have any questions or problems during this test, please notify me immediately.

You may start when you're ready.