THE BIOAVAILABILITY OF 90MX CRANBERRY POWDER AND QUERCETIN WHEN ADMINISTERED TO HORSES

by

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ABSTRACT OF THE THESIS

The Bioavailability of 90MX Cranberry Powder and Quercetin when Administered to Horses

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The subject of this thesis was to investigate the bioavailability of a commercially available cranberry powder (90MX) from Ocean Spray® and quercetin when administered to horses. The hypothesis states that flavonols from a commercially available 90MX cranberry powder and quercetin would both be bioavailable to the horse. Bioavailability was determined by the appearance and identification of flavonols in horse plasma, urine, and/or muscle after dosing via nasogastric tube. For the first study, three healthy unfit Standardbred mares were used in a random cross-over design. They received either 200 g of 90MX powder in 2 L of water (low dose = LD), 400 g of 90MX powder dissolved in 2 L of water (high dose = HD), or 2 L of water (control = CON). For the second study, the same three mares were given 6 g of quercetin in 2 L of water. Blood, urine, and muscle samples were collected pre-dosing and at 0.5, 1, 2, 4, 8, 16, and 24 h post-dosing. High performance liquid chromatography (HPLC) analysis of the samples showed trace amounts of flavonols in some plasma samples, but overall there

were no quantitatively significant increase flavonols found in horse urine or plasma. HPLC analysis of the muscle samples did show an increase in quercetin (Q), Q-3 arabinopyranoside (Q-3-AP), M-3 galactoside (M-3-G), Q-3 rhamnoside (Q-3-R), Q-3 arabinofuranoside (Q-3-AF), and Q-3 galactoside (Q-3-G). Peak quantities of each flavonol (μg/g) in horse muscle were Q (1.96; 5.33), Q-3-AP (0.24; 0.05), M-3-G (0.80; 0.37), Q-3-R (0.55; 0.24). Q-3-AF (0.12; 0.07), Q-3-G (2.45; 1.22) for the LD and HD, respectively. In study two, there was no quantitatively measureable quercetin in plasma, urine, or muscle samples. These data show for the first time the uptake of an ingested flavonol compound (cranberry juice) into horse muscle. This lays the foundation for further investigation into the effects of flavonols on horse muscle which has been physiologically stressed by exercise or other variables.

Keywords: flavonol, bioavailability, cranberry, quercetin, muscle samples, horse, equine

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Section 1

INFLAMMATION

The definition of inflammation has advanced through the decades. The original definition was attributed to Aulus Cornelius Celsus in 1478 who noted the characteristic swelling, redness, pain, and heat associated with an irritated area of the body (Silva, 1994). In the 19th century the "father of pathology", Rudolf Virchow, expanded the definition of inflammation to include the loss of function (Silva, 1994). A more recent definition describes inflammation as a series of local phenomena that develop as the result of primary lesions to the tissues that functions to restore tissue health (Silva, 1994). It is clear by the complexity of the definition that inflammation is a multifaceted biological response.

The inflammatory response is initiated as a protective attempt to remove irritants and initiate healing. Inflammation eliminates noxious compounds and damaged tissue (Moldoveanu *et al.* 2001). The response evolved both to protect the organism against infection and injury and to promote tissue repair (Moldovanu *et al.* 2001). When inflammation subsides it may leave some scar tissue resulting from the healing process, but it generally allows for a restoration of normal structure and function (Moldoveanu *et al.* 2001).

In addition to the original cardinal signs of inflammation other characteristics have been elucidated. These include activation of leukocytes and vascular endothelium, movement of fluid into tissue spaces, and the movement of leukocytes (particularly

neutrophils) to the affected site (Munford and Pugin, 2001). These combined physiological responses cause local hyperemia, pyrexia, edema, and pain (Moldoveanu *et al.* 2001; Munford and Pugin, 2001).

Loss of function is another one of the debilitating effects of inflammation. Despite the fact that inflammation can allow the body to heal properly, the pain, stiffness, and loss of range of motion associated with inflammation can limit athletic endeavors and impact daily activities. To further confuse the definition of inflammation all of the signs of inflammation do not occur each time there is an inflammatory response. Often inflammation occurs at the molecular level and does not result in any heat, redness, swelling, or pain (Gallin and Snyderman, 1999). A prime example is delayed onset muscle soreness (DOMS) which may result in pain, but not in any redness or swelling to that area (Gallin and Snyderman, 1999).

Stresses as diverse as psychological trauma, strenuous exercise, cold exposure, and major injury can all trigger a similar inflammatory response (Munford and Pugin, 2001). The coordination of local inflammation and systemic anti-inflammation enable the body to concentrate the response to a local area while preventing potentially damaging inflammation in uninvolved tissues (Munford and Pugin, 2001).

Section 2

CYTOKINES

Cytokines are hormone-like proteins that mediate the inflammatory responses and can be considered markers of inflammation (Cannon, 2000). They are produced by and mediate communication both between and within various immune and non-immune cells, as well as organs and organ systems throughout the body (Moldoveanu *et al.* 2001). Cytokines can be classified as either pro- or anti- inflammatory cytokines. These classifications typically serve as guidelines and the division, pro- or anti-inflammatory, is seldom apparent.

2.1 Pro-inflammatory Cytokines

Injection of tumor necrosis factor alpha (TNF-α), interleukin-1-beta (IL-1β), and interleukin-6 (IL-6) into lab rats will induce an acute phase response (Dinarello, 1992). These cytokines therefore have been referred to as inflammatory or pro-inflammatory cytokines (Ostrowski *et al.* 1999). TNF-α, IL-1β, and other pro-inflammatory molecules are extremely potent and can be effective at very low concentrations (Munford and Pugin, 2001). Locally, the pro-inflammatory cytokines stimulate leukocyte proliferation, cytotoxicity, release of proteolytic enzymes, synthesis of prostaglandins, and initiation of a cascade of secondary cytokines (Cannon, 2000). Systemically, these cytokines raise the thermoregulatory set-point (causing fever) and can mediate redistribution of iron from extracellular to intracellular sites (Cannon, 2000). Energy consumption is reduced via direct action of the cytokines on the central nervous system that reduce locomotor activity and increase slow wave sleep, allowing the body to protect itself against

pathogenic microbial invaders, in what has been termed a "wartime economy" (Cannon, 2000).

2.2 Anti-inflammatory Cytokines

An anti-inflammatory response is one that inhibits the production or action of pro-inflammatory mediators. Cytokines that act as anti-inflammatory mediators include interleukin-4 (IL-4), IL-6, interleukin -10 (IL-10), interleukin-11 (IL-11), interluekin-13 (IL-13), and transforming growth factor beta (TGF-β) (Munford and Pugin, 2001). These anti-inflammatory cytokines attenuate inflammation by restricting inflammatory cytokine production, up-regulating their soluble antagonist binding proteins, and by suppressing inflammatory cell activity (Elenkov *et al.* 2000; Moldoveanu *et al.* 2001).

2.3 The Pro-Inflammatory Cytokines

Tumor Necrosis Factor Alpha (TNF-α): TNF-*α* is an acute inflammatory cytokine that plays a role in muscle damage (Kimura *et al.* 2001), muscle proteolysis (Nawabi *et al*., 1990), impaired skeletal muscle glucose uptake (Steensberg *et al.* 2002), multiple sclerosis (Malamud *et al*. 2003), insulin resistance, obesity, and diabetes (Hotamisligil, 1999). The origin of TNF-α is less clear than other cytokines (Vassilakopoulos *et al.* 2003). It has been suggested that TNF-α is synthesized and released by mononuclear phagocytes and T-lymphocytes, Kupffer cells, neural cells, and endothelial cells (Moldoveanu *et al.* 2001). However, studies using flow cytometry have excluded monocytes as sources of exercise-induced TNF-α production (Starkie *et al.* 2000; 2001).

TNF-α is one of the major pro-inflammatory cytokines involved in the pathogenesis of chronic inflammatory diseases and is modulated by oxidative stress (Nair *et al.* 2006). It triggers the cellular release of other cytokines, chemokines, and

inflammatory mediators and displays antiviral and antimicrobial effects (Nair *et al.* 2006). TNF-α has additional effects on glucose metabolism, stimulating the down-regulation of GLUT4 (Coppack, 2001) and regulating leptin levels in humans (Hotamisligi, 1999). It can also increase muscle protein breakdown (Li, 2005).

Interferon-gamma (IFN-γ): IFN-γ is primarily an immune and inflammatory modulator with 100 to 1000 fold more activity than the other interferons (Moldoveanu *et al.* 2001). It is produced by natural killer (NK) cells, CD4+ helper cells, and CD8+ T cytotoxic cells (Moldoveanu *et al.* 2001). This cytokine is responsible for inducing nonspecific cell mediated mechanisms of host defense. It is considered a pro-inflammatory cytokine in part because it augments the synthesis of other inflammatory cytokines such as TNF-α, it up-regulates expression of the TNF receptors, and induces at least one form of nitric oxide synthase (iNOS) (Moldoveanu *et al.* 2001).

Interluekin-1(IL-1): There are two subtypes of IL-1 (IL-1α and IL-1β). IL-1α remains in the cytosol of cells in nearly all cases and IL-1β is only active upon cleavage by a protease found only in monocytes (Moldoveanu *et al.* 2001). IL-1 is produced by macrophages, monocytes, and dendritic cells.

The response to IL-1 can include hypotension, fever, lethargy, modulation of inflammation, and stimulation of cell proliferation (Moldoveanu *et al.* 2001). IL-1 up-regulates a wide variety of genes including those encoding cytokines (Moldoveanu *et al.* 2001). Thus, it augments its own expression as well as that of IL-2 and IL-6 (Moldoveanu *et al.* 2001). IL-1 can also induce enzymes which are required for the synthesis of leukotreines, prostaglandins, and nitric oxide (NO) (Moldoveanu *et al.* 2001). IL-1 has inflammatory and degenerative effects on joint surfaces and systemically

injected IL-1 stimulates bone resorption (Moldoveanu *et al.* 2001). It also promotes catabolism of lean tissue in synergy with TNF-α (Moldoveanu *et al.* 2001). IL-1β knockout mice lack the ability to generate a fever in response to local inflammation (Cannon, 2000).

2.4 Anti-inflammatory Cytokines

Interluekin-6 (IL-6): IL-6 has gone through a variety of identity changes overtime. It was originally classified as a pro-inflammatory cytokine because when injected in laboratory animals or humans it produced an acute phase response to inflammation (Dinarello, 1992). However, IL-6 does not directly induce inflammation (Ostrowski *et al.* 1999). Therefore, it has been reclassified as an inflammatory responsive cytokine (Ostrowski *et al.* 1999). IL-6 has also been called a myokine, since it is produced and released by skeletal muscle during prolonged exercise (Ostrowski *et al.* 1998; Steensberg *et al.* 2002; Pedersen *et al.* 2003). Skeletal muscle cells are most likely the source of this exercise-induced IL-6 production, with fast twitch fibers contributing more then slow twitch fibers (Hiscock, 2004; Pedersen *et al.* 2003). IL-6 is also produced by various immune cells such as T and B lymphocytes, NK cells, monocytes, smooth muscle cells, chondrocytes, glial cells, and adipose tissue (Moldoveanu *et al.* 2001; Langberg *et al.* 2002). Recent studies also indicate that IL-6 is produced by peritendinous tissue during exercise in humans (Langberg *et al.* 2002) and can be released from the brain during prolonged exercise (Nybo *et al.* 2002). The function of IL-6 can differ depending on the origin (Ndubuisi *et al*. 1998).

IL-6 may be one of the most significant blood borne activators, since production and release of IL-6 is sensitive to cellular reactions, to injury or infection, and several

authors have identified a correlation between the severity of tissue damage and blood concentrations of IL-6 (Munford and Pugin, 2001). The most important effects of IL-6 are the ability to suppress the synthesis of IL-1 and TNF-α by hepatocytes, B cells, and mononuclear phagocytes (Cohen and Cohen, 1996). IL-6 also attenuates the IL-1 signal by increasing synthesis of interleukin-1 receptor antagonist (IL-1ra) and TNF binding proteins (Moldoveanu *et al.* 2001). IL-6 has been implicated, along with TNF-α, in glucose homeostasis (Steinacker *et al.* 2004). It stimulates hepatic glucose release, can promote lipolysis in adipocytes, limit adipocyte size, and increase fat oxidation (Coppack, 2001; Petersen and Pedersen, 2005). IL-6 knock-out mice are incapable of generating a fever in response to any stimuli, further demonstrating the importance of this cytokine in the inflammatory process (Cannon, 2000).

Section 3

INFLAMMATION DURING EXERCISE

Exercise or physical activity is a potent activator of the immune system resulting in changes in circulating pro- and anti- inflammatory cytokine concentrations (Pedersen and Toft, 2000). Physical exercise induces a non-specific inflammatory response, which is manifested by elevated concentrations of circulating pro-inflammatory cytokines (Moldoveanu *et al.* 2000). This increase in pro-inflammatory cytokines is countered and balanced by an increase in post-exercise anti-inflammatory cytokines such as IL-1ra, IL-4, IL-10, interleukin-12 subunit p40 (IL-12p40), and monocyte chemotactic protein-1 (MCP-1) (Peake *et al.* 2005). This increase in anti-inflammatory cytokines has been observed (in humans) after various forms of exercise including brief maximal exercise, resistance exercise, intense eccentric cycling, endurance running, and endurance cycling (Peake *et al*. 2005). Further studies, examining the effects of exercise on cytokine systems, are needed to elucidate the significance of cytokine regulation by physical activity and to clarify the health implication of short and long term physical activity with respect to overall immune function and resistance to infection (Moldoveanu *et al.* 2001).

3.1 Type of Exercise

The initial response to exercise is an increase in circulating pro-inflammatory cytokines (TNF-α, IL-β and IL-6) followed by the release of anti-inflammatory (regulating) cytokines like IL-4, IL-10, and IL-1ra (Pedersen *et al.* 1998). The nature of the response differs depending on the mode of exercise (Bruunsgaard, 1997; Brenner, 1998; 1999; Nieman, 1998). High-intensity eccentric (lengthening) exercise causes a more pronounced increase in the plasma levels of cytokines during and following

exercise than that produced by concentric (shortening) exercise (Bruunsgaard *et al.* 1997). High intensity running has a greater effect than lower intensities, or down-hill running, on the plasma concentrations of IL1-1ra and IL-10 (Peake *et al.* 2005). Cycling exercise for 30 min resulted in significant changes in several measured variables in blood and muscle tissue (Malm *et al.* 2000). Two hours of cycle ergometer exercise induced significant changes in IL-6 and TNF-α in humans, more-so then 5 min of cycle exercise, or a standard circuit-training routine (Brenner *et al.* 1999). The data that exists on the effects of different types of exercise on the cytokine response is numerous and varied. The only thing that is clear is that many types of exercise can induce a cytokine response and that this response is different depending on the mode of exercise.

3.2 TNF-α Response

TNF-α modulation by exercise appears to be influenced by the intensity and particularly the duration of the exercise stimulus (Moldoveanu *et al.* 2001). Prolonged endurance activity, resistance exercise, and a submax run all increase TNF-α release in humans (Louis *et al.* 2007; Molvoveanu *et al.*2001). There was a significant elevation is TNF-α mRNA in human muscle samples following 2 hr weight training (Neiman, 2004). There are conflicting findings and at least one study indicates that TNF-α does not significantly increase with exercise in humans (Steensberg *et al.* 2002; Petersen and Pedersen, 2005).

3.3 IL-1 Response

IL-1β is among the first cytokine to be released in response to exercise stress (Moldoveanu *et al. 2001).* IL-1β has been shown to increase in human plasma and blood mononuclear cells (BMNC) after running and cycling (Ostrowski *et al.* 1998; Ostrowski

et al. 1999; Moldoveanu *et al.* 2000). An increase in IL-1β mRNA was found in human muscle samples following a marathon (Ostrowski *et al.* 1998). Other studies have shown no increase with exercise, possibly because plasma concentrations hover around the level of detection (Petersen and Pedersen, 2005).

3.4 INF-γ Response

Most studies have failed to detect a difference in IFN-γ in response to exercise (Moldoveanu *et al.* 2001). Though, at least one study has shown an increase of IFN-γ in monocytes following 30 min of cycling (Zaldivar *et al.* 2006). A lower plasma concentration of this cytokine has been suggested as contributing to the "open window" effect for infections following exercise (Moldoveanu *et al.* 2001).

3.5 IL-6 Response

IL-6 is the first cytokine present in the circulation during exercise. The appearance of IL-6 in the circulation is by far the most marked and its appearance precedes that of the other cytokines (Petersen and Pedersen, 2005). Exercise generally causes a strong, but transient induction of IL-6 (Pedersen *et al.* 1998). The release of IL-6 is related to exercise intensity, duration, the mass of muscle recruited, and endurance capacity (Gokhale *et al.* 2007). A recent study investigating a 264 km foot race showed markedly elevated plasma IL-6, peripheral tissue damage, and significant changes in serum lipid levels in humans (Margeli *et al.* 2005). IL-6 was also elevated in humans after 24 h with both a resistance exercise protocol and a run at submax intensity (Louis *et al.*2007). IL-6 is released from muscle in response to both endurance and resistance type exercise (Hiscock *et al.* 2004). Plasma levels of IL-6 were significantly increased after eccentric exercise, but not after concentric exercise (Bruunsgaard *et al.* 1997). The

increase of IL-6 seen after eccentric exercise could be a result of inflammation in the muscle and the factor causing further muscle damage (Bruunsgaard *et al.* 1997). This is further supported by a significant increase in creatine kinase (CK), a marker of skeletal muscle damage, in the week following exercise (Bruunsgaard *et al.* 1997; Harris, 1998).

3.6 The Cytokine Response to Exercise in the Horse

*S*trenuous exercise induces a pro-inflammatory state in the horse (Donovan *et al.* 2007). Specifically, expression of mRNA for IL-6 and TNF-α in circulating leukocytes are significantly increased after exercise as are circulating concentrations of TNF-α and PGF2-α (Donovan *et al.* 2007). Short strenuous exercise induces a pro-inflammatory condition in the horse that persisted for approximately 2 h after exercise (Donovan *et al.* 2007). This data corresponds with some reports and contradicts others. The reason could be due to differences in type and intensity of exercise, sample collection times, or assay methods (Donovan *at al.* 2007).

TNF-α mRNA has been shown to increase in horse plasma following a graded exercise test (GXT) (Streltsova *et al.* 2006; Liburt *et al.* 2008). A GXT increased IFN-γ mRNA immediately following exercise, and caused no alteration in the mRNA of IL-6 (Streltsova *et al.* 2006).

Donovan's study recognized a three-fold increase in plasma endotoxin, which could have been responsible for the changes in cytokine gene expression. These three studies demonstrate that short strenuous exercise induces a pro-inflammatory condition in the horse (Streltsova *et al.* 2006; Donovan *et al.* 2007; Liburt *et al.* 2008).

Section 4

DELAYED ONSET MUSCLE SORENESS

Delayed onset muscle soreness (DOMS) is the feeling of pain and discomfort felt after a bout of eccentric exercise that usually manifests itself in the hours and days following (Armstrong, 1990). There is no pain immediately after exercise, but pain peaks 48-72 h post-exercise in humans and subsides 5-7 d later (Armstrong, 1990; Proske and Morgan, 2001; Close *et al.* 2005). A high incidence of DOMS can result in decreased exercise performance, increased muscle stiffness, and a loss of force development (Close *et al.* 2004). The minimal exercise parameters needed to induce DOMS have not been defined, though it is associated with unaccustomed, high-force muscular activity that involves lengthening muscle actions (Newham *et al.* 1983; Schwane and Armstrong, 1983; Bruunsgaard *et al.* 1997; Dannecker *et al.* 2005). This lengthening exercise (often termed negative or eccentric work) includes downhill running and lowering of weights (Close *et al.* 2005).

In addition to pain ratings, which are available only in human subjects, several other indicators of DOMS have been identified. The affected muscle is tender to local palpation, stretch, and contraction (Proske and Morgan, 2001). There is a change in ventilatory response, but not cardiovascular response, at the start of exercise in subjects suffering from DOMS and muscle damage (Hotta *et al.* 2006). The presence of disrupted sarcomeres in myofibrils, z-disc streaming, and damage to the excitation-contraction (E-C) coupling system in the muscle can be seen under electron microscopy in subjects experiencing DOMS (Proske and Morgan, 2001; Carlsson *et al*. 2006).

Despite more than a century of research there is still no consensus on the mechanism of DOMS (Close *et al.* 2005). Several potential mechanisms have been postulated, including lactate accumulation (Asmussen, 1953), involuntary muscle spasms (De Vries *et al.* 1998), connective tissue damage (Abraham 1977), muscle tissue damage (Hough *et al.* 1902), inflammation, (Smith, 1991) and increased muscle temperature (Davies and Barnes, 1972; Close *et al.* 2005).

DOMS is thought to begin with the over-stretching of the sarcomeres during repeated contractions, which leads to extensive damage, and could ultimately cause death of the muscle fibers (Proske and Morgan, 2001). This could cause the inflammatory response and edema that leads to exercise induced muscle damage (Proske and Morgan, 2001). Evidence exists that DOMS may not be a direct result of exercise induced muscle damage (EIMD), since there is a change in the stiffness of the muscle before there is any associated swelling (Proske and Morgan, 2001).

Delayed onset muscle soreness is thought to be a result of muscle damage during exercise (Proske and Morgan, 2001). It could result from lengthening activities and is unlikely to be caused by metabolic factors, but rather by mechanical factors (Close *et al.* 2005). One potential mechanism is that fewer motor units are recruited for a given work load during lengthening contractions, compared with shortening muscle actions, the force is therefore activated over a smaller cross sectional area of muscle (Close *et al.* 2005).

A common factor in many of the proposed mechanisms of DOMS is the production of free radicals, these free radicals have been proposed as another potential cause of DOMS (Close *et al*. 2005). Downhill running results in an increase in reactive oxygen species (ROS) production, as detected by electron spin resonance (ESR)

spectroscopy (Close *et al.* 2005). The role of free radicals in DOMS is still unclear with some research groups claiming they are pathological, while others suggest they are physiological (Close *et al.* 2005).

There are a number of potential explanations for the inconsistencies in the literature regarding the etiology of contraction-induced muscle damage and DOMS; these include human versus animal populations, gender, mode of inducing DOMS, and methods of assessment (Close *et al.* 2005). The most apparent inconsistencies can be found by the disparate exercise protocols used between studies (Close *et al.* 2005).

It has been implied that that there is a relationship between inflammation and DOMS. A secondary inflammatory process occurring post-exercise could be the source of ROS production and resulting DOMS (Smith, 1991; Close *et al.* 2003).

Section 5

FLAVONOLS

Several hundred different molecules having polyphenol structure (several hydroxyl groups on aromatic rings) have been identified in edible plants (Manach *et al.* 2004). Polyphenols are the active component in cranberries and they have the ability to modulate a wide range of enzymes and cell receptors (Middleton and Kandaswami, 2000). Four phenolic classes are identified in cranberries including phenolic acids, anthocyanins, flavonols and flavan-3-ols, which consist of monomers and polymer classes of proanthocyanidins (Duarte *et al.* 2006).

Flavonols can be absorbed in the intestine, possibly by the NA+/glucose co-transporter (SGLT1), but the bioavailability among them varies (Manach *et al.* 2004; Ruel and Couillard, 2007). The rate of absorption differs with respect to numerous factors, such as the type of flavonols, co-ingestion of certain macro or micronutrients, and the linkage of flavonols to sugars (Ruel and Couillard, 2007). Flavonols have a great affinity for proteins because they contain an aromatic nucleus and hydroxyl groups, and often circulate bound to albumin (Manach *et al.* 1995).

Flavonol absorption is accompanied by extensive conjugation and metabolism (Williamson and Manach, 2005). Thus, the forms appearing in the body are usually different than those originally ingested, and there is only limited information on the properties of conjugates. In addition, flavonols can become de-conjugated in the liver and at sites of inflammation, further influencing their activity (Williamson and Manach, 2005).The nature and position of the hydroxyl group will affect the subsequent biological activity of the flavonol, possibly reducing or abolishing the activity that was seen with

the aglycone form (Day *et al.* 2000). Studies have shown that glycosylated flavonols (those bound to a sugar) are more efficiently absorbed than their aglycone (pure) form (Hollman *et al.*, 1995; Ruel and Couillard, 2007). Therefore, it is important to consider the type of flavonol being fed, and the form it is in, to allow for more precise composition of an anti-inflammatory supplement.

Bioavailability assesses the absorption and metabolism of a substance by the appearance of it, or metabolites, in the body. Recent studies have indicated that dietary flavonoids have poor bioavailability and only reach low micromolar concentrations in human plasma (Lotito and Frei, 2006). In addition, the half-life in human plasma is relatively short, usually a few hours (Lotito and Frei, 2006). This quick disappearance from the plasma makes it difficult to track the distribution of flavonols in the body. Supplementation of 750 ml/d of cranberry juice for 2 wks was not shown to affect human plasma levels (Duthie *et al.,* 2006). The lack of a plasma response may indicate that flavonols are quickly excreted or partitioned to other areas of the body.

Recent studies show quercetin, isorhamnetin and kaempherol appear in urine samples from human subjects in 24-h samples and spot samples at days 2 and 3 (Mennen *et al.* 2007). A significant increase in quercetin-glucoronide was found in urine samples of human subjects following the consumption of 1200 mg/d of dried cranberry juice for a duration of 8 wks, but not with 400 mg/d (Valentova *et al.* 2007). Flavonols that are unabsorbed by the small intestine are believed to undergo microbial degradation in the large intestine (Ruel and Couillard, 2007). In addition to extensive metabolism in the liver and intestines, the microflora in the colon play a critical role in the metabolism of

polyphenols (Williams and Manach, 2005). These actions could significantly affect the bioavailablity of flavonols and the appearance of metabolites in urine samples.

Only a handful of studies have attempted to measure the uptake of flavonols by muscle tissue. A recent study showed an increase in flavonol distribution to murine tissue following intraperitoneal injection of a flavonol extract (2 mmole/kg of body weight) (Vorsa *et al.* 2007).

Section 6

QUERCETIN

Quercetin is a flavonol (aglycone form) that has shown numerous biological effects. Quercetin is found in very high concentration in onions, apples, tea, broccoli, and red wine (Willamson and Manach, 2005) and is also a flavonol found in the aglycone and glycoside forms in cranberries.

Consumption of quercetin has been shown to elicit a plasma response in humans. Quercetin from tea and onions can increase the plasma concentrations and urinary excretion 3-25 fold (De Vries *et al.* 1998). Quercetin is not commonly found in the plasma in its free form, or as the parent glucoside, but rather as a conjugate (Day and Williamson, 2001; Crespy *et al.* 2002; Williamson and Manach, 2005). Other studies have detected quercetin in plasma (0.75-1.5 umol/L) , after a 50 mg dose, and have identified it as having a relatively long (11-28 h) plasma half-life in humans (Scalbert and Williamson, 2000; Manach *et al.* 2005). Quercetin has also been detected in dog plasma following a 10 mg/kg body weight dose of quercetin (Anger *et al.* 2007).

Recent studies show quercetin, isohamnetin, and kaempherol appear in urine samples from human subjects in 24-h samples and spot samples at day 2 and day 3 (Mennen *et al.* 2007). Quercetin was also detected in low concentrations in the urine of humans given 0.3 g green tea extract (Ito *et al.* 2005).

Quercetin has been detected in muscle tissue from rats fed a quercetin diet for 11 wks (De Boer *et al.* 2005). Further information on the uptake of quercetin and conjugates into muscle tissue would offer invaluable insight into the absorption of flavonols.

Section 7

FLAVONOLS AND INFLAMMATION

The anti-inflammatory effects of quercetin are mediated by the inhibition of major pro-inflammatory cytokines, such as TNF-α, which is involved in the pathogenesis of chronic inflammatory diseases (Nair *et al.* 2006). Quercetin has shown the ability to inhibit TNF-α via modulation of the NF-kB pathway (NF-kB1 and iKB) (Comalada *et al.* 2006; Nair *et al*. 2006). TNF-α triggers the cellular release of other cytokines, chemokines, and inflammatory mediators, and displays antiviral and antimicrobiral effects (Nair *et al*. 2006). Quercetin significantly inhibits TNF-α production and gene expression in a dose-dependent manner (Nair *et al*. 2006). It significantly inhibits the expression of TNF-α mRNA in THP-1 cells (Huang *et al*. 2006). Quercetin has some of the highest inhibitory effects on TNF-α of any flavonol (Comalada *et al.* 2006).

Quercetin acts as an inhibitor of mast cell secretion which causes a decrease in the release of tryptase and IL-6 and the down-regulation of histidine decarboxylase (HDC) mRNA from human mast cell (HMC)-1 cells (Kempuraj *et al*. 2006). Thus, quercetin could play a therapeutical role against neurological diseases mediated by mast cell degranulation and pathological conditions associated with chronic inflammation (Kempuraj *et al.* 2006).

Quercetin also has the ability to decrease the expression of IL-1β mRNA levels in THP-1 cells (Huang *et al.* 2006). These results indicate that quercetin could regulate inflammation by decreasing TNF-α and IL-1β mRNA as well as protein expression induced by S100B in THP-1 cells (Huang *et al*. 2006).

There have been several *in vitro* studies investigating the inhibitory activity of flavonoids on cytokine production in different macrophage cell lines such as RAW 264.7 (Blonska *et al.* 2003; Kim *et al.* 2004; Comalada *et al*. 2006). It has been established that the inflammatory response may be resolved through the clearance of the inflammatory cells or through the release of endogenous anti-inflammatory mediators such as IL-10 and TGF-β, thus the effects of flavonols on IL-10 secretion is important (Comalada *et al.* 2006). Quercetin is able to stimulate the LPS-induced secretion of the anti-inflammatory cytokine IL-10 at low concentrations (Comalada *et al.* 2006).

This wide variety of effects makes flavonols promising anti-inflammatory compounds that warrant further evaluation of their clinical and therapeutical potential as a class of anti-inflammatory agents (Comalada *et al.* 2006).

Section 8

RATIONALE

There is an abundance of data suggesting that flavonols have anti-inflammatory properties. The following studies are designed to test the bioavailability of flavonols in an equine model. Once bioavailability as been determined, their efficacy as anti-inflammatory substances can be evaluated, specifically in relation to exercise-induced inflammation.

Section 9
REFERENCES

Abraham. W. 1977. Factors in delayed onset muscle soreness. *Medicine and Science in Sports and Exercise* 9, 11-20.

Anger, M., G. Abraham, R. Ungermach, R. Cermak. 2007. Bioavailability of the flavonol quercetin from different quercetin glycosides in dogs. *Proceedings of the 11th ESVCN Congress,* 119.

Armstrong, R. 1990. Initial events in exercise-induced muscular injury. *Medicine and Science in Sports and Exercise* 22, 429-435.

Asmussen, E. 1953. Positive and negative muscular work. *Acta Physiol Scand* 28, 364-382.

Brenner. 1998. Autonomic regulation of the circulation during exercise and heat exposure. *Sports Medicine* 26, 85-99.

Brenner, I., V. Natale, P. Vasiliou, A. Moldoveanu, P. Shek, R. Shephard. 1999. Impact of three different types of exercise on components of the inflammatory response. *European Journal of Applied Physiology* 80, 452-460.

Bruunsgaard, H., H. Galbo, J. Halkjaer-Kristensen, T. Johansen, D. MacLean, B. Pedersen. 1997. Exercise-induced increase in serum interleukin-6 in humans is related to muscle damage. *Journal of Physiology* 499, 833-841.

Cannon. J. 2000. Inflammatory cytokines in nonpathological states. *News in Physiological Science* 15, 298-303.

Close, G., T. Ashton, T. Cable, D. Doran, D. MacLaren. 2004. Eccentric exercise, isokinetic muscle torque and delayed onset muscle soreness: the role of reactive oxygen species. *European Journal of Applied Physiology* 91, 615-621.

Close, G., T. Ashton, A. McArdle, D. MacLaren. 2005. The emerging role of free radicals in delayed onset muscle soreness and contraction-induced muscle injury. *Comparitive Biochemistry and Physiology* 142, 257-266.

Cohen M., S. Cohen. 1996. Cytokine function: a study in biologic diversity. *American Journal of Clinical Pathology* 105, 589-598.

Comalada, M., I. Ballester, E. Bailon, S. Sierra, J. Xaus, J. Galvez, F. Sanchez de Medina, A. Zarzuelo. 2006. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: Analysis of the ctructure-activity relationship. *Biochemical Pharmacology* 72, 1010-1021.

Coppack, S. 2001. Pro-inflammatory cytokines and adipose tissue. *Processings of the Nutrition Society* 60, 349-356.

Crespy, V., C. Morand, C. Besson, C. Manach, C. Demigne, C. Remesy. 2002. Quercetin, but not its glycosides, is absorbed from the rat stomach. *Agricultural and Food Chemistry* 50, 618-621.

Dannecker, E., H. Hausenblas. T. Kaminski, M. Robinson. 2005. sex differences in delayed onset muscle pain. *Clinical Journal of Pain* 21, 120-126.

Davies, C., C. Barnes. 1972. Negative (eccentric) work. I. Effects of repeated exercise. *Ergonomics* 15, 3-14.

Day, A., Y. Bao, M. Morgan, G. Williamson. 2000. Conjugation position of quercetin glucuronides and effect on biological activity. *Free Radical Biology and Medicine* 29, 1234-1243.

Day, A., G. Williamson. 2001. Biomarkers for exposure to dietary flavonoids: a review of the current evidence for identification of quercetin glycosides in plasma. *British Journal of Nutrition* 86, S105-S110.

De Vries, J., P. Hollman, S. Meyboom, M. Buysman, P. Zock, W. van Staveren, M. Katan. 1998. Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake. *American Journal of Clinical Nutrition* 68, 60-65.

Dinarello, C. 1992. Role of interleukin-1 in infectious diseases. *Immunological Reviews* 127, 119-146.

Dinarello, C. 2000. Pro-inflammatory cytokines. *Cardiopulmonary and Critical Care Journal* 118, 503-508.

Donovan, D., C. Jackson, P. Colahan, N. Norton, D. Hurley. 2007. Exercise-induced alterations in pro-inflammatory cytokines and prostaglandin F2α in horses. *Veterinary Immunology and immunopathology* 118, 263-269.

Duarte, S., S. Gregoire, A. Singh, N. Vorsa, K. Schaich, W. Bowen, H. Koo. 2006. Inhibitory effects of cranberry polyphenols on formation and acidogenicity of *Streptococcus mutans* biofilms. *2006 Federation of European Microbiological Societies* 257, 50-56.

Duthie, S., A. Jenkinson, A. Crozier, W. Mullen, L. Pirie, J. Kyle, L. Yap, P. Christen, G. Duthie. 2006. The effects of cranberry juice consumption on antioxidant status and biomarkers relating to heart diesae and cancer in healthy human volunteers. *European Journal of Nutrition* 45, 113-122.

Elenkov, I., G. Chrousos, R. Wilder. 2000. Neuroedocrine regulation of IL-12 and TNF-α/IL-10 balance. *Annals of the New York Academy of Sciences* 917, 94-105.

Gallin, J., R. Snyderman. 1999. Inflammation: Basic Principles and Clinical Correlates. 3rd Edition. Lippincott, Williams & Wilkins, Philadelphia.

Gokhale, R., S. Chandrashekara, K.C. Vasanthakumar. 2007. Cytokine response to strenuous exercise in athletes and non-athletes – an adaptive response. *Cytokine* 40, 123-127.

Gregoire, S., A. Singh, N. Vorsa, H. Koo. 2007. Influence of cranberry phenolics on glucan synthesis by glucosyltransferase and *Streptococcus mutans* acidogenicity. *Journal of Applied Microbiology* 103, 1960-1968.

Harris, P., D. Marlin, J. Gray. 1998. Plasma aspartate aminotransferase and creatine kinase activities in Thoroughbred racehorses in relation to age, sex, exercise and training. *The Veterinary Journal* 155, 295-304.

Hiscock, N., M. Chan, T. Bisucci, I. Darby, M. Febbraio. 2004. Skeletal myocytes are a source of interleukin-6 mRNA expression and protein release during contraction: evidence of fiber type specificity. *FASEB* express article published online April 1, 2004.

Hollman, P., J. de Vries, S. van Leeuwen, M. Mengelers, M. Katan. 1995. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *American Journal of Clinical Nutrition* 62, 1276-1282.

Hotamisligil, G. 1999. Mechanisms of TNF-α-induced insulin resistance. *Experimental and Clinical Endocrinology and Diabetes* 107, 119-125.

Hotta, N., K. Sato, Z. Sun, K. Katayama, H. Akima, T. Kondo, K. Ishida. 2006. Ventilatory and circulatory responses at the onset of exercise after eccentric exercise. *European Journal of Applied Physiology* 97, 598-606.

Hough, T. 1902. Ergographic studies in muscular soreness. *American Journal of Physiology* 7, 76-92.

Huang, S., C. Wu, G. Yen. 2006. Effects of flavonoids on the expression of the pro-inflammatory responses in human monocytes induced by ligation of the receptor for AGEs. *Molecular Nutrition and Food Research* 50, 1129-1139.

Kimura, H., M. Suzui, F. Nagao, K. Matsumoto. 2001. Highly sensitive determination of plasma cytokines by time-resolved fluoroimmunoassay; Effect of bicycle exercise on plasma level of interleukin-1α (IL-1α), tumor necrosis factor α (TNF-α), and interferon γ (IFNγ). *Analytical Sciences* 17, 593-597.

Kempuraj, D., M.L. Castellani, C. Petrarca, S. Frydas, P. Conti, T.C. Theoharides, J. Vecchiet. 2006. Inhibitory effect of quercetin on tryptase and interleukin-6 release, and histidine decarboxylase mRNA transcription by human mast cell-1 cell line. *Clinical and Experimental Medicine* 6, 150-156.

Langberg, H., J. Olesen, C. Gemmer, M. Kjaer. 2002. Substantial elevation of interleukin-6 concentration in peritendinous tissue, in contrast to muscle, following prolonged exercise in humans. *Journal of Physiology* 542, 985-990.
Li, Y., Y. Chen, J. John, J. Moylan, B. Jin, D. Mann, M. Reid. 2005. TNF-α acts vis p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. *FASEB* 19, 362-370.

Liburt, N., K. McKeever, J. Streltsova, M. Gordon, H. Manso Filho, D. Horohov, R. Rosen, W. Franke, A. Singh, N. Vorsa. 2008. Effects of ginger and cranberry extracts on markers of inflammation and performance following intense exercise in horses.

Lotito, S. and B. Frei. 2006. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: Cause, consequences, or epiphenomenon? *Fee radical biology and Medicine* 41, 1727-1746.

Louis, E., U. Raue, Y. Yang, B. Jemiolo, S. Trappe. 2007. Time course of proteolytic, cytokine, and myostatin gene expression after acute exercise in human skeletal muscle. *Journal of Applied Physiology* 103, 1744-1751.

Malamud, V., A. Vaaknin, O. Abramsky, M. Mor, L. Burgess, A. Ben-Yehudah, H. Lorberboum-Galski. 2003. Tryptase activates peripheral blood mononuclear cells causing the synthesis and release of TNF-α, IL-6 and IL-1β: possible relevance to multiple sclerosis. *Journal of Neuroimmunology* 138, 115-122.

Malm, C., P. Nyberg, M. Engstrom, B. Sjodin, R. Lenkei, B. Ekblom, I. Lundberg. 2000. Immunological changes in human skeletal muscle and blood after eccentric exercise and multiple biopsies. *Journal of Physiology* 529, 243-262.

Manach, C., C. Morand, O. Texier, M. Favier, G. Agullo, C. Demigne, F. Regerat, C. Remesy. 1995. Quercetin metabolites in plasma of rats fed diets containing rutin and quercetin. *The Journal of Nutrition* 125, 1911-1922.

Manach, C., A. Scalbert, C. Morand, C. Remesy, L. Jimenez. 2004. Polyphenols: food sources and bioavailability. *American Journal of Clinical Nutrition* 79, 727-747.

Manach, C., G. Williamson, C. Morand, A. Scalbert, C. Remesy. 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *American Journal of Clinical Nutrition* 81, 230S-242S.

Margeli, A., K. Skenderi, M. Tsironi, E. Hantzi, A. Matalas, C. Vrettou, E. Kanavakis, G. Chrousos, I. Papassotiriou. 2005. Dramatic elevations of interleukin-6 and acute-phase

reactance in athletes participating in the ultradistance foot race Spartatholon: Severe systemic inflammation and lipid and lipoprotein changes in protracted exercise. *Journal of Clinical Endocrinology and Metabolism* 90, 3914-3918.

Middleton, E., C. Kandaswami, T. Theoharides. 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Reviews* 52, 673-751.

Mennen, L., D. Sapinho, H. Ito, P. Galan, S. Hercberg, A. Scalbert. 2007. Urinary excretion of 13 dietary flavonoids and phenolic acids in free-living healthy subjects-variability and possible use as biomarkers of polyphenols intake. *European Journal of Clinical Nutrition* 62, 519-525.

Moldoveanu, A., R. Shephard, P. Shek. 2000. Exercise elevates plasma levels but not gene expression of IL-1β, IL-6, and TNF-α in blood mononuclear cells. *Journal of Applied Physiology* 89, 1499-1504.

Moldoveanu, A., R. Shephard, P. Shek. 2001. The cytokine response to physical activity and training. *Sports Medicine* 31, 115-144.

Morand, C., C. Manach, V. Crespy, C. Ramesy. 2000. Quercetin 3-O-beta-glucoside is better absorbed than other quercetin forms and is not present in rat plasma. *Free Radical Research* 33, 667-676.

Munford, R., J. Pugin. 2001. Normal responses to injury prevent systemic inflammation and can be immunosuppressive. *American Journal of Respiratory and Critical Care Medicine* 163, 316-321.

Nair, M., S. Mahajan, J. Reynolds, R. Aalinkeel, H. Nair, S. Schwartz, C. Kandaswami. 2006. The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via moducation of the NF-κβ system. *Clinical and Vaccine Immunology* 13, 319-328.

Nawabi, D., K. Block, M. Chakrabarti, M. Buse. 1990. Administrati0n of endotoxin, tumor necrosis factore, or interleukin 1 to rats activates skeletal muscle branched-chain α-keto acid dehydrogenase. *Journal of Clinical Investigations* 85, 256-263.

Newham, D., D. MPhil, R. Edwards. 1983. Large delayed plasma creatine kinase changes after stepping exercise. *Muscle and Nerve* 6, 380-385.

Nieman, D., S. Nehlsen-Cannarella, O. Fagoaga, D. Henson, A. Utter, J. Davis, F. Williams, D. Butterworth. 1998. Influence of mode and carbohydrate on the cytokine response to heavy exertion. *Medicine and Science in Sports and Exercise* 30, 671-678.

Nieman, D., J. Davis, V. Brown, D. Henson, C. Dumke, A. Utter, D. Vinci, M. Downs, J. Smith, J. Carson, A. Brown, S. McAnulty, L. McAnulty. 2004. Influence of carbohydrate

ingestion on immune changes after 2 h intensive resistance training. *Journal of Applied Physiology* 96, 1292-1298.

Nybo, L., B. Nielsen, B. Pedersen, K. Moller, N. Secher. 2002. Interleukin-6 release from the human brain during prolonged exercise. *Journal of Physiology* 542, 991-995.

Ostrowski, K., T. Rohde, M. Zacho. S. Asp, B. Pedersen. 1998. Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running. *Journal of Physiology* 508, 949-953.

Ostrowski, K., T. Rohde, S. Asp, P. Schjerling, B. Pedersen. 1999. Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. *Journal of Physiology* 515, 287-291.

Peake, J., K. Suzuki, M. Hordern, G. Wilson, K. Nosaka, J. Coombes. 2005. Plasma cytokine changes in relation to exercise intensity and muscle damage. *European Journal of Applied Physiology* 95, 514-521.

Pedersen, B., T. Rohde, K. Ostrowski. 1998. Recovery of the immune system after exercise. *Acta Physiolo Scand* 162, 325-332.

Pedersen. B., A. Steensberg, R. Keller, C. Keller, C. Fischer, N. Hiscock, G. van Hall, P. Plomgaard, M. Febbraio. 2003. Muscle-derived interleukin-6: lipolytic, anti-inflammatory and immune regulatory effects. *European Journal of Physiology* 446, 9-16.

Pedersen, B. and A. Toft. 2000. Effects of exercise on lymphocytes and cytokines. *British Journal of Sports Medicine* 34, 246-251.

Petersen, A., B. Pedersen. 2005. The anti-inflammatory effect of exercise. *Journal of Applied Physiology* 98, 1154-1162.

Proske, U., D. Morgan. 2001. Muscle damage from eccentric exercise: Mechanism, mechanical signs, adaptation and clinical applications. *Journal of Physiology* 537, 333-345.

Ruel, G., C. Couillard. 2007. Evidences of the cardioprotective potential of fruits: The case of cranberries. *Molecular Nutrition and Food Research* 51, 692-701.

Scalbert, A., G. Williamson. 2000. Chocolate: Modern science investigates an ancient medicine. *Journal of Nutrition* 130, 2073S-2085S.

Schwane, J., R. Armstrong. 1983. Effect of training on skeletal muscle injury from downhill running in rats. *Journal of Applied Physiology* 55, 960-975.

Silva, R. 1994. A brief survey of the history of inflammation. *Agents Actions* 43, 86-90.

Smith, L. 1991. Acute inflammation: the underlying mechanism in delayed onset muscle soreness? *Medicine and Science in Sports and Exercise* 23, 542-551.

Starkie, R., D. Angus, J. Rolland, M. Hargreaves, M. Febbraio. 2000. Effect of prolonged, submaximal exercise and carbohydrate ingestion on monocyte intracellular cytokine production in humans. *Journal of Physiology* 528, 647-655.

Starkie, R., J. Rolland, D. Angus, M. Anderson, M. Febbraio. 2001. Circulating monocytes are not the source of elevations in plasma IL-6 and TNF-α levels after prolonged running. *American Journal of Physiology – Cell Physiology* 280, C769-C774.

Steensberg, A., G. van Hall, T. Osada, M. Sacchetti, B. Saltin, B. Pedersen. 2000. Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. *Journal of Physiology* 529, 237-242.

Steensberg, A., C. Keller, R. Starkie, T. Osada, M. Febbraio, B. Pedersen. 2002. IL-6 and TNF-α expression in , and release from, contracting human skeletal muscle. *American Journal of Physiology, Endocrinology, and Metabolism* 283, E1272-E1278.

Steinacker, J., W. Lormes, S. Reissnecker, Y. Liu. 2004. New aspects of the hormone and cytokine response to training. *European Journal of Applied Physiology* 91, 382-391.

Streltsova, J., K. McKeever, N. Liburt, M. Gordon, H. Manso Filho, D. Horohov, R. Rosen, W. Franke. 2006. *Equine and Comparative Exercise Physiology* 3, 121-130.

Valentova, K., D. Stejskal, P. Bednar, J. Vostalova, C. Cihalik, R. Vecerova, D. Koukalova, M. Kolar, R. Reichenbach, L. Sknouril, J. Ulrichova, V. Simanek. 2007. Biosafety, antioxidant status, and metabolites in urine after consumption of dried cranberry juice in healthy women: a pilot double-blind placebo-controlled trial. *Agricultural and Food Chemistry* 55, 3217-3224.

Vassilakopoulos, T., M. Karatza, P. Katsaounou, A. Kollintza, S. Zakynthinos, C. Roussos. 2003. Antioxidants attenuate the plasma cytokine response to exercise in humans. *Journal of Applied Physiology* 94, 1025-1032.

Vorsa, N., A. Singh, E. Shabrova, K. Schaich, H. Jin, L. Quadro. 2007. Bioavailability and tissue distribution of cranberry flavonols glycosides in mice. *FASEB Abstract.*

Williamson, G., C. Manach. 2005. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *American Journal of Clinical Nutrition* 81 (S), 243S-55S.

Zaldivar, F., J. Wang-Rodriguez, D. Nemet, C. Schwindt, P. Galassetti, P. Mills, L. Wilson, D. Cooper. 2006. Constitutive pro- and anti- inflammatory cytokine and growth factor response to exercise in leukocytes. *Journal of Applied Physiology* 100, 1124-1133.

CHAPTER 2: THE BIOAVAILABILITY OF 90 MX CRANBERRY POWDER AND QUERCETIN WHEN ADMINISTERED TO HORSES

Section 1

ABSTRACT

These two studies were designed to investigate the bioavailability of a commercially available cranberry juice powder (90MX) from Ocean Spray® and pure quercetin when administered to horses. The hypothesis stated that compounds from a commercially available 90MX cranberry powder and quercetin would both be bioavailale to the horse. Bioavailability was determined by the appearance and identification of flavonols in horse plasma, urine, and/or muscle after dosing via nasogastric tube. For the first study, three healthy unfit Standardbred mares were used in a random cross-over design. They received either 200 g of 90MX powder in 2 L of water (low dose = LD), 400 g of 90MX powder dissolved in 2 L of water (high dose = HD), or 2 L of water (control = CON). For the second study, the same three mares were given 6 g of quercetin in 2 L of water. Blood, urine, and muscle samples were collected pre-dosing and at 0.5, 1, 2, 4, 8, 16 and 24 h post-dosing. Study One: High performance liquid chromatography (HPLC) analysis of the samples showed trace amounts of flavonols in some plasma samples, but overall there were no quantitatively significant increase of flavonols in horse urine or plasma samples. HPLC analysis of the muscle samples did show an increase in quercetin (Q), Q-3 arabinopyranoside (Q-3-AP), M-3 galactoside (M-3-G), Q-3 rhamnoside (Q-3-R), Q-3 arabinofuranoside (Q-3-AF), and Q-3 galatoside (Q-3-G). Peak quantities of each of the flavonols were Q (1.96; 5.33), Q-3-AP (0.24; 0.05), M-3-G

(0.80;0 .37), Q-3-R (0.55; 0.24), Q-3-AF (0.12; 0.07), Q-3-G (2.45; 1.22) µg/g for the LD and HD, respectively. Study Two: There were no quantitatively significant increases in quercetin levels in the horses' plasma, urine, or muscle samples during the quercetin study. These data show for the first time the uptake of an ingested flavonol compound (cranberry juice) into horse muscle. This lays the foundation for further investigation into the effects of flavonols on horse muscle which has been physiologically stressed by exercise or other variables.

Keywords: flavonol, bioavailability, cranberry, quercetin, muscle samples, horse, equine

Section 2

INTRODUCTION

Inflammation is a physiological response to injury or irritation that is dependent on the duration and intensity of the event (Shephard, 1996). The cytokine response to inflammation is generally a protective response that is designed to destroy and eliminate foreign substances or organisms in the body (Goodman, 2003). Exercise-induced increases in inflammatory cytokines can result in muscle and body soreness that can affect an athlete's ability to perform at peak potential (Pedersen, 2000). For this reason, the ability to reduce exercise-induced inflammation and the pain associated with it is a top priority for doctors, sports therapists, and athletes.

Non-steroidal anti-inflammatory drugs (NSAIDs) have long been the pharmacological choice to help control this type of inflammation, utilizing the ability to reduce the production of prostaglandins (PGs). However, many NSAIDs block PGs at the cyclooxygenase (COX) 1 and/or the COX-2 level (Vane and Botting, 2003). Blocking COX-1 can also block the beneficial effects of PGs which include protecting the stomach mucosa from hydrochloric acid, maintaining kidney function, and aggregating platelets (Bjarnason *et al.* 1993; Vane and Botting, 2003). This can result in heart burn, ulcers, and kidney damage (Bjarnason *et al.* 1993; McLaughlin *et al.* 1998; Vane and Botting, 2003). The use of NSAIDs has also been implicated in a loss of muscle function. NSAIDs can allow a short-term gain in function and range of motion, by blocking pain; however, masking pain can result in a subsequent loss of muscle function (Mishra *et al.* 1995). Therefore, researchers and pharmaceutical companies have actively sought out alternatives to NSAID use, such as dietary supplements with anti-inflammatory actions.

These non-drug anti-inflammatory substances, such as food extracts, could allow for a reduction in pain and maintenance of exercise ability without severe side-effects.

A common property among these food extracts is a relatively high content of flavonols. Flavonols are found in many plants and are especially rich in seeds, citrus fruits, olive oil, tea, and red wine (Middleton *et al.* 2000). Cranberries are one of the most important sources of flavonols, especially of the flavonol quercetin (Duarte *et al.* 2006; Ruel and Couillard, 2007). Flavonols have various biological and pharmacological effects including those associated with their anti-inflammatory, antioxidant, antitumor, antiangiogenic, antiallergic, and antiviral properties (Middleton *et al.* 2000).

Several *in vitro* studies have documented the anti-inflammatory activity of flavonols, including the ability to inhibit TNF-α, inducible nitric oxide synthase (iNOS) expression, and nitric oxide (NO) production in LPS-activated macrophages (Blonska *et al.* 2003; Kim *et al.* 2004; Comalada *et al.* 2006). Quercetin has been found to promote expression of the anti-inflammatory cytokine IL-10 (Comalada *et al.* 2006). It also has the ability to induce a reduction in the release of IL-6 (Kempuraj *et al.* 2006) and inhibit TNF-α production (Nair *et al.* 2006). However, these *in vitro* effects on markers of inflammation often contradict those results found *in vivo* studies. Discrepancies between *in vivo* and *in vitro* findings may be largely accounted for by absorption and metabolism in a biological system. The possible value of a food component is largely determined by its bioavailability. The bioavailability, to humans, of different flavonols has been a topic of many research papers (Manach *et al.* 2005; Williamson and Manach, 2005; Lotito and Frei, 2006; Ito *et al.* 2005; Valentova *et al.* 2007).

Flavonols are absorbed in the intestine, possibly by the NA+/glucose co-transporter (SGLT1), but the bioavailability among them varies (Manach *et al.* 2004; Ruel and Couillard, 2007). The rate of absorption differs with respect to numerous factors, such as the type of flavonols, co-ingestion of certain macro or micronutrients, and the linkage of flavonols to sugars (Ruel and Couillard, 2007). Flavonols have a great affinity for proteins because they contain an aromatic nucleus and hydroxyl groups, and often circulate bound to albumin (Manach *et al.* 1995).

Flavonol absorption is accompanied by extensive conjugation and metabolism (Williamson and Manach, 2005). Thus, the forms appearing in the body are usually different than those originally ingested, and there is only limited information on the properties of conjugates. In addition, flavonols can become de-conjugated in the liver and at sites of inflammation, further influencing their activity (Williamson and Manach, 2005).The nature and position of the hydroxyl group will affect the subsequent biological activity of the flavonol, possibly reducing or abolishing the activity that was seen with the aglycone form (Day *et al.* 2000). Studies have shown that glycosylated flavonols (those bound to a sugar) are more efficiently absorbed than their aglycone (pure) form (Hollman *et al.*, 1995; Ruel and Couillard, 2007). Therefore, it is important to consider the type of flavonol being fed, and the form it is in, to allow for more precise composition of an anti-inflammatory supplement.

Bioavailability assesses the absorption and metabolism of a substance by the appearance of it, or metabolites, in the body. Recent studies have indicated that dietary flavonoids have poor bioavailability and only reach low micromolar concentrations in human plasma (Lotito and Frei, 2006). In addition, the half-life in human plasma is

relatively short, usually a few hours (Lotito and Frei, 2006). This quick disappearance from the plasma makes it difficult to track the distribution of flavonols in the body. Supplementation of 750 ml/day of cranberry juice for 2 wks was not shown to affect human plasma levels (Duthie *et al.,* 2006). The lack of a plasma response may indicate that flavonols are quickly excreted or partitioned to other areas of the body. However, consumption of pure quercetin has been shown to elevate plasma levels in humans. Quercetin from tea and onions can increase the plasma concentrations and urinary excretion 3-25 fold (De Vries *et al.* 1998). More data on the appearance of metabolites in plasma is needed for a complete understanding of the bioavailability of flavonols and the comparative value of using conjugates versus pure quercetin as a potential anti-inflammatory agent.

Recent studies show quercetin, isorhamnetin, and kaempherol appear in urine samples from human subjects in 24-hr samples and spot samples at days 2 and 3 (Mennen *et al.* 2007). A significant increase in quercetin-glucoronide was found in urine samples of human subjects following the consumption of 1200 mg/day of dried cranberry juice for a duration of 8 wks, but not with 400 mg/d (Valentova *et al.* 2007). Quercetin was also detected in low concentrations in the urine of humans given 0.3 g green tea extract (Ito *et al.* 2005). Flavonols that are unabsorbed by the small intestine are believed to undergo microbial degradation in the large intestine (Ruel and Couillard, 2007). In addition to extensive metabolism in the liver and intestines, the microflora in the colon play a critical role in the metabolism of polyphenols (Williams and Manach, 2005). These actions could significantly affect the bioavailablity of flavonols and the appearance of metabolites in urine samples.

Only a handful of studies have attempted to measure the uptake of flavonols by muscle tissue. A recent study showed an increase in flavonol distribution to murine tissue following intraperitoneal injection of a flavonol extract (2 mmole/kg of body weight) (Vorsa *et al.* 2007). The uptake of flavonols into muscle tissue is further supported in rats fed a quercetin diet for 11 wks (De Boer *et al.* 2005). We are unaware of any study that has measured flavonol distribution in horse muscle. Prior work has examined the effects of cranberry on the reduction in markers of exercise-induced inflammation in blood (Liburt *et al.* 2008). However, those studies did not provide any information on the uptake of flavonols into muscle, which may be the more appropriate target for an anti-inflammatory supplement.

We are aware of only one study that has attempted to measure flavonols in horse urine and plasma after dosing with cranberry (Liburt *et al.* 2008). This work focused on the same 90MX powder used in our study, but the extract was prepared differently, which may have affected bioavailability. In that study the flavonols from the 90MX powder were bioavailable to the horse, with a total of 12 quercetin and one myricetin based flavonol compounds detected in urine and plasma samples (Liburt *et al.* 2008).

Data on absorption and metabolism of flavonols is limited and varied for a variety of reasons, including the method used to detect the flavonols in biological substances, the type and amount of flavonol fed, the method of ingestion, and numerous other discrepancies in methodology between research groups. Most *in vitro* biological activity has been associated with the aglycone form; however, in nature flavonols are mainly found glycosylated (Day *et al.* 2000). For these reasons, our first study will use the same 90MX powder that Liburt *et al.* 2008 used, dissolved in tap water. This mixture is similar

to the commercial application for the drink. The second study will analyze pure quercetin dissolved in tap water. The combination of the two studies will allow for a more concise look at the bioavailability of pure quercetin compared to the glycoside forms.

This study addressed two hypotheses; 1) flavonols from a mixture of 90MX cranberry powder will appear in the plasma, urine, and muscle of horses after dosing via oral lavage and 2) quercetin will appear in the plasma, urine, and muscle samples of horses dosed with pure quercetin.

Section 3

MATERIALS AND METHODS

The same three unfit Standardbred mares (~500 kg, 16-19 years old) were used for both studies. All practices were approved by the Rutgers University Institutional Review Board for the Care and Use of Animals.

Study One:

The three mares were assigned to one of three treatments in a random cross-over design. The mares were kept on dry lots and fed a commercial pellet (2 kg/d) and free choice hay and water. The mares were brought in 12 h before the study began and housed overnight in 3 x 3 m box stalls within the lab. Pellets were withheld the night before the study until completion; however, hay and water were available free choice. On the morning of the study the horses were dosed via nasogastric tube with one of three treatments; control (CON = 2 L of tap water), low dose (LD = 200 g of 90MX cranberry powder in 2 L of tap water) or high dose (HD = 400 g of 90MX cranberry powder in 2 L of tap water).

Study Two:

To test the bioavailability of pure quercetin, the same three mares were used in the second study. The mares were kept on dry lots and fed a commercial pellet (2 kg/d) and free choice hay and water. The mares were brought in 12 h before the study began and housed overnight in 3 x 3 m box stalls within the lab. Pellets were withheld the night before the study until completion; however, hay and water were available free choice. The morning of the study the horses were dosed via nasogastric tube with 6 g of quercetin in 2 L of water.

3.1 Collection of Samples

During each study blood, urine, and muscle samples were collected pre-dosing and at 0.5, 1, 2, 4, 8, 16 and 24 h post-dosing. Blood samples (50mL) were obtained via venipuncture, collected into 10mL BD Vacutainer tubes (BD Diagnostics, Plymouth, UK) containing K_2 EDTA, and immediately centrifuged at 15,000 g. Plasma was separated into six separate aliquots, frozen on dry ice, and then stored at -80º C for later analysis.

Urine samples were obtained using a 24-french Foley bladder catheter (Jorgensen Laboratories, Loveland, CO) that was inserted before dosing. After placement of the catheter a pre-dose sample and all additional urine was emptied from the bladder to allow for the approximation of total urine output during the 24 h period. A 150 mL aliquot of urine was collected into conical tubes at each time point, immediately frozen on dry ice, and stored at -80º C for later analysis.

Muscle samples were collected from the gluteal muscle at each of the time points mentioned above. All biopsies were obtained under aseptic conditions, using sterile techniques, and a local subcutaneous lidocaine (Abbott Laboratories, North Chicago, IL) injection. A small stab incision was made in the skin and fascia to allow for insertion of the biopsy needle. The samples were taken using a Bergstrom biopsy needle (6mm, KRUUSE Worldwide Veterinary Supplier, Denmark) at a depth of 5 cm. Each sample was at least 5 cm from surrounding samples. Approximately 200 mg of muscle (38.0 – 439.0 mg) was collected at each time point and placed into a CryoTube (Nalge Nunc International, Rochester, NY). The muscle was immediately frozen on dry ice and then stored at -80º C.

3.2 Preparation of plasma samples for flavonol detection.

Plasma samples were thawed and 300 µL aliquoted into 2.0 mL snap cap tubes. Nine hundred µL of acetonitrile and 100 µL of acetic acid were added to each sample. The samples were then vortexed (Vortex Genie2, Fischer Scientific, Pittsburgh, PA) for ~1 min and placed in a ultra sonic cleaner (sonicator) (Heat Systems Ultrasonics, Farmingdale, NY) for 20 min. After sonication the samples were vortexed again to ensure thorough mixing and centrifuged (AccuSpinMicro 17R, Fischer Scientific Inc. Pittsburgh, PA) for 10 min at 12,000 g. The supernatant of each sample was transferred to a separate tube and 900 µL of acetonitrile and 100 µL of acetic acid were added to the pellet. This extraction process was repeated a total of three times. All the supernatant for each sample was transferred to the same snap-cap tube. The tubes were then dried (Savant SPD 2010 Speed Vac Concentrator, Thermoelectron Corp., Waltham, MA) until only a pellet remained. The pellet was reconstituted with 100-200 µL of methanol. Samples were vortexed, sonicated for 20 min, centrifuged for 10 min, and the supernatant was harvested and placed in HPLC tubes. All reagents used for all of the extractions were HPLC grade (Sigma-Aldrich, St. Louis, MO).

3.3 Preparation of urine samples for extraction of conjugates.

Urine samples were thawed and 16 mL of each sample was combined with 60 mL of ethyl acetate, 400 µL of 20% phosphoric acid and 400 µL of 20% ascorbic acid. Samples were then placed on a magnetic stirrer (Corning Inc., Lowell, MA) for 20 min at room temperature. The supernatant was poured off into a separate collection tube. The extraction was repeated using the remaining liquid and 40 mL of ethyl acetate two more times, for a total of three extractions. The collected supernatant for each sample was

concentrated using a rotoevaporator (Julabo F25, Yamoto, Orangeburg, NY) until dry. Following, the supernatant was reconstituted using 1 mL of methanol. The resulting rinse was transferred to a snap cap tube and dried until only a pellet remained. The pellet was mixed with 200 µL of methanol. Tubes were then vortexed for 10 min, sonicated for 20 min, and centrifuged for 10 min. The supernatant was extracted and placed in HPLC tubes for analysis.

3.4 Preparation of urine samples for the extraction of flavonols.

For the extraction of flavonols 500 µL of hydrocholoric acid was added to each of the 4 mL urine samples. The samples were then placed in a water bath at 80º C for 30 min. After cooling the tubes were rinsed using 20 mL of ethyl acetate and the rinse was collected into a flask. The resulting rinse was placed on a magnetic stirrer for 10 min, following which the top layer was decanted off into another flask. The remaining layer was combined with 20 mL of ethyl acetate and mixed for 10 min before the top layer was poured off again. The combined top layer was concentrated using a rotoevaporator until dry. The tube was rinsed with 1 mL of methanol and the rinse was dried to a pellet. Two hundred µL of methanol was added to the pellet. The samples were vortexed for 2 min, sonicated for 20 min, vortexed again for 2 min, and centrifuged for 10 min. They were then placed in HPLC tubes for analysis of flavonol content.

3.5 Preparation of muscle samples for detection of flavonols.

Muscle samples were weighed, transferred to a conical bottom tube, and mixed with 15 mL of buffer (50% methanol in 0.1 M phosphate buffer (pH 7.00) containing 20 mM sodiumdithiocarbamate). The biopsy samples were then homogenized (Cyclone I.Q.^{2}, VirTis, Gardiner, NY) for a minimum of 10 min or until the muscle pieces were

completely broken down. The samples were vortexed and gently shaken to ensure complete mixing. Following this the samples were centrifuged (Marathon 21 K/R, Fischer Scientific Inc. Pittsburg, PA) for 20 min at 12,000 g. The resulting supernatant was collected into a round bottom flask. The pellet was mixed with another 15 mL of buffer, vortexed for 5 min, mixed again for 30 min, and centrifuged for 20 min. The supernatant was added to that previously collected and the pellet was discarded. The collected liquid was rotoevaporated down to 10 mL. Then the pH was adjusted to 3.0 using acetic acid. Ethyl acetate was added up to the 45 mL mark and samples were vortexed for 5 min. The top aqueous layer was decanted into another tube. This ethyl acetate extraction was repeated two more times for a total of three extractions. After the third extraction the collective top layer was rotoevaporated until dry. The tubes were rinsed with 1 mL of methanol and the rinse was collected in a 2.0 mL snap cap tube and dried. The resulting pellet was reconstituted with 100 µL of methanol. Samples were vortexed, sonicated for 20 min, centrifuged for 10 min, and the supernatant was harvested and placed in HPLC tubes for flavonols analysis.

3.6 High Performance Liquid Chromatography (HPLC) Analysis of Samples

Blood, urine, and muscle samples were analyzed in the same machine under the same conditions. Analytical detection of flavonol glycosides was completed with HPLC (chromatograph from Waters, Milford, MA) using a C18 Luna column (4.6 x 150 mm; particle size 5 µm; Phenomenex, Torrance, CA). Each sample (~200 µL) was injected into the column, and a gradient elution was used for fractional separation with two solvents at a flow rate of 1 mL/min. Solvent A consisted of 10% methanol in deionized water adjusted to a pH of 3.5 with formic acid. Solvent B consisted of 20% deionized

water (pH 3.5), 20% methanol, and 60% acetonitrile. The gradient consisted of 0 min at 100% A, followed by 5 min at 85% A, 10 min at 80% A, 20 min at 75% A, 25 min at 73% A, 27 min at 60% A, 30 min at 60% A, 35 min at 50% A, 40 min at 10% A, 45 min at 0% A, and 50 min at 100% A and holding at 100% A for a final 55 min. Five min of equilibration at 100% A was performed before and after each injection. Scanning by photo diode array (PDA) detector utilized 366 nm for flavonol glycosides.

3.7 Identification of Flavonol Glycosides

The methods used to extract the flavonols were previously validated (Gregoir*e et al.* 2007). A methanol-water-acetonitrile-formic acid solvent system and gradient procedure provided for the successful separation of flavonols from each other, and yielded over 98% purity of the flavonols (Gregoire *et al.* 2007). Flavonol glycosides were identified using in-house standards and published methods (Gregoire *et al.* 2007).

3.8 Data Analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical significance was evaluated by comparison to the pre-dose control using a paired t-test. Significance was set at $P < 0.1$.

Section 4

RESULTS

The 90MX cranberry powder was bioavailable to the horse when given via nasogastric tube. Bioavailability was determined by the appearance of six flavonol compounds; quercetin-3 galactoside (Q-3-G), quercetin-3 arabinofuranoside (Q-3-AF), quercetin-3 rhamnoside (Q-3-R), myricetin-3-galactoside (M-3-G), quercetin-3 arabinopyranoside (Q-3-AP), quercetin (Q), in horse muscle (Fig. 1, Table 1). The peaks identified by HPLC correspond with published in house standards (Gregoire *et al.* 2007) (Table 1).

There were no flavonol peaks with the CON treatment (Fig. 2), indicating that nothing in the horses' diet contained the flavonols identified in the muscle samples. This further supports the bioavailablity of the 90MX cranberry powder. The HPLC profile of the 90MX cranberry powder was identified with corresponding peaks (Fig. 3, Table 2).

Peak concentrations (Table 3) of flavonols in horse muscle were calculated as the mean of the highest concentration reached for each horse. Peak concentrations for the LD (μg/mg) were 2.31 ± 2.14 for Q-3-G, 0.12 ± 0.07 for Q-3-AF, 0.54 ± 0.51 for Q-3-R, 0.78 ± 0.76 for M-3-G, 0.19 ± 0.17 for Q-3-AP, and 1.93 ± 1.83 for Q. Peak concentrations for the HD (μg/mg) were 1.22 ± 0.83 for Q-3-G, 0.07 ± 0.04 for Q-3-AF, 0.23 ± 0.20 for Q-3-R, 0.37 ± 0.21 for M-3-G, 0.05 ± 0.05 for Q-3-AP, and 5.25 ± 5.19 for Q (Table 3). There was no significant difference ($P > 0.1$) between the LD and HD for any of the flavonols (Fig. 4-9, Table 4).

There was no dose response in either the urine or plasma samples from study 1 (data not shown). Total urine output was calculated for the 24 hr period of the study CON

(1883 mL ± 1099 mL), 200 g (2698 mL ± 944 mL), and 400 g (2329 mL ± 750 mL). There was no significant difference in urine output between doses.

In the second study, quercetin did not appear to be bioavailable to horse in the aglycone form. There were no flavonols peaks in urine, muscle, or plasma samples that corresponded with the identified flavonols (data not shown).

Section 5

DISCUSSION

The purpose of these studies was to determine the bioavailability flavonols in cranberry juice and quercetin when administered to a horse. Flavonols, including those found in cranberry juice, have been shown to have anti-inflammatory properties in numerous studies (Middleton *et al.* 2000; Blonska *et al.* 2003; Kim *et al.* 2004; Comalada *et al.* 2006; Kempuraj *et al.* 2006; Nair *et al.* 2006). Just because flavonols are available in the diet does not mean that they will have any intrinsic value to those ingesting them, due to a low biological activity, poor absorption from the intestine, higher metabolism, or rapid elimination (Manach *et al.* 2004). Therefore, the bioavailability of flavonols is an important consideration when examining their relevance as an anti-inflammatory agent.

The most significant finding in the first study was that the flavonols in 90MX cranberry powder from Ocean Spray® were bioavailable to the horse. Bioavailability was determined by the appearance of five quercetin compounds and quercetin in horse muscle at numerous time points post-dosing. This data supports a study which found 6.5 nmole/g of flavonols in murine tissue following intraperitoneal injection of 2 mmole/kg of body weight of a flavonol extract consisting of 8 flavonols (Vorsa *et al.* 2007). It also corresponds with a study indicating the uptake of quercetin (0.12 nmol/g and 1.21 nmol/g) in mice fed a 0.1% or 1% quercetin diet, respectively, for 11 wks (De Boer *et al.* 2005). To our knowledge no other studies has demonstrated the uptake of flavonols into equine muscle tissue.

While trace amounts of flavonols could be detected in some plasma samples from the first study, the study failed to find a dose response in horse plasma. This could be due to

the rapid clearance of flavonols from plasma to be redistributed in other tissue, such as the muscle, liver, or kidneys. This data agrees with other studies which have shown low, or no, plasma response after ingestion of flavonols (Duthie *et al.* 2006; Lotito and Frei, 2006). Intact glycosides of quercetin were not recovered in plasma or urine after ingestion as pure compounds or from the complex food (Manach *et al.* 2004). It is possible that this study did not collect blood samples soon enough (30 min) to observe the plasma response. Studies in rats have indicated that isoflavone metabolites appear in plasma only 3 min after administration, indicating that absorption in the stomach of the rat is quick and effective (Crespy *et al.* 2002). The metabolic process which serves to detoxify many xenobiotics increases biliary and urinary elimination (Manach *et al.* 2004). This process is very efficient and could cause the compounds to appear absent in blood or present in very low concentrations (Manach *et al.* 2004).

As with plasma, the first study did not show a significant increase in any of the detectable metabolites in horse urine. This is in contrast to studies showing that flavonols are eliminated mainly in urine and bile (Manach *et al.* 2004; Vorsa *et al.* 2007). Both of the current studies attempted to collect total urine from t = 0 until the completion of the study, so it is unlikely that the collection of samples did not include the time points that were most relevant to the elimination of flavonols, unless elimination happened after the 24 h period.

The variation between horses was large. Other studies have reported very high inter-individual variability in humans and it is possible that some individuals, due to particular polymorphisms for intestinal enzymes or transporters, are better absorbers of flavonols (Manach *et al.* 1995; Manach *et al.,* 2005).

Horses on the CON treatment showed no evidence of any of the flavonols present in the 90MX cranberry powder. Further supporting the uptake of the powder through the gastrointestinal tract and dispelling the idea of the intake of naturally occurring flavonols through hay or grain. Both the hay and grain used in these studies were analyzed for naturally occurring flavonols similar to those tested in this study, and none were found (data not shown).

The second study reports that pure quercetin did not appear to be bioavailable to the horse, as there was no occurrence of any of the identified metabolites in urine, plasma, or muscle samples. Recent studies have shown quercetin plasma levels (0.14 to 0.42 μmol/L) in human subjects being fed (8 – 4000 mg; 0.14 mg/kg) pure quercetin (Gugler *et al.* 1975; Erlund *et al.* 2000; Goldberg *et al.* 2003; Manach *et al.* 2005). The occurrence of quercetin in the plasma is usually quick and short-lived, and it is possible that the first time point chosen for this study was not early enough to identify quercetin in horse plasma (Manach *et al.* 2005). The elimination of quercetin is quite slow, with reported half-lives in urine samples ranging from 11 to 28 h (Manach *et al.* 2005). This could mean that in the horse, elimination is even slower, leading to the absence of flavonols in horse urine samples, since these studies were only carried out to 24 h. The relatively slow elimination of quercetin in the body could account for the loading effect seen in some studies and the reason that repeated intakes of quercetin produced increases in plasma levels (Manach *et al.* 2005).

These studies lay the foundation for further investigation into the bioavailability of different flavonols, and the continuance to examine the effects of flavonols on horse muscle which has been physiologically stressed by exercise or other variables.

Section 6

REFERENCES

Bjarnason, I., J. Hayllar, A. MacPherson, A. Russell. 1993. Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. *Gastroenterology* 104 (6), 1832-1847.

Blonska, M., Z. Czuba, W. Krol. 2003. Effect of flavone derivatives on interleukin-1β protein synthesis in stimulated RAW 264.7 macrophages. *Scandanavian Journal of Immunology* 57, 162-166.

Comalada, M., I. Ballester, E. Bailon, S. Sierra, J. Xaus, J. Galvez, F. Sanchez de Medina, A. Zarzuelo. 2006. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: Analysis of the structure-activity relationship. *Biochemical Pharmacology* 72, 1010-1021.

Crespy, V., C. Morand, C. Besson, C. Manach, C. Demigne, C. Remesy. 2002. Quercetin, but not its glycosides, is absorbed from the rat stomach. *Agricultural and Food Chemistry* 50, 618-621.

Day, A., Y. Bao, M. Morgan, G. Williamson. 2000. Conjugation position of quercetin glucuronides and effect on biological activity. *Free Radical Biology and Medicine* 29, 1234-1243.

De Boer, V., A. Dihal, H. Van der Woude, I. Arts, S. Wolffram, G. Alink, I. Rietjens, J. Keijer, P. Hollman. 2005. Tissue distribution of quercetin in rats and pigs. *Nutrition Metabolism* 135, 1718-1725.

De Vries, J., P. Hollman, S. Meyboom, M. Buysman, P. Zock, W. van Staveren, M. Katan. 1998. Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake. *American Journal of Clinical Nutrition* 68, 60-65.

Duarte, S., S. Gregoire, A. Singh, N. Vorsa, K. Schaich, W. Bowen, H. Koo. 2006. Inhibitory effects of cranberry polyphenols on formation and acidogenicity of *Streptococcus mutans* biofilms. *2006 Federation of European Microbiological Societies* 257, 50-56.

Duthie, S., A. Jenkinson, A. Crozier, W. Mullen, L. Pirie, J. Kyle, L. Yap, P. Christen, G. Duthie. 2006. The effects of cranberry juice consumption on antioxidant status and biomarkers relating to heart diesae and cancer in healthy human volunteers. *European Journal of Nutrition* 45, 113-122.

Erlund, I., T. Kosonen, G. Alfthan, J. Maenpaa, K. Perttunen, J. Kenraali, J. Parantainen, Aro. 2000. Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy vounteers. *European Journal of Pharmacology* 56, 545-553.

Goldberg, D., J. Yan, G. Soleas. 2003. Absorption of three wine-related polyphenols in three different matrices by healthy subjects. *Clinical Biochemistry* 36, 79-87.

Goodman, R., J. Pugin, J. Lee, M. Matthay. 2003. Cytokine-mediated inflammation in acute lung injury. *Cytokine and Growth Factor Reviews* 14, 523-535.

Gregoire, S., A. Singh, N. Vorsa, H. Koo. 2007. Influence of cranberry phenolics on glucan synthesis by glucosyltransferases and *Streptococcus mutans* acidogenicity. *Journal of Applied Microbiology* 103, 1960-1968.

Gugler, R., M. Leschik, H. Dengler. 1975. Disposition of quercetin in man after single oral and intravenous doses. *European Journal of Pharmacology* 9, 229-234.

Hollman, P., J. de Vries, S. van Leeuwen, M. Mengelers, M. Katan. 1995. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *American Journal of Clinical Nutrition* 62: 1276-1282.

Ito, H., M. Gonthier, C. Manach, C. Morand, L. Mennen, C. Remesy, A. Scalbert. 2005. Polyphenol levels in human urine after intake of six different polyphenols-rich beverages. *British Journal of Nutrition* 94, 500-509.

Kempuraj, D., M.L. Castellani, C. Petrarca, S. Frydas, P. Conti, T.C. Theoharides, J. Vecchiet. 2006. Inhibitory effect of quercetin on tryptase and interleukin-6 release, and histidine decarboxylase mRNA transcription by human mast cell-1 cell line. *Clinical and Experimental Medicine* 6, 150-156.

Kim, S., H. Park, H. Kim. 2004. Inhibition of nitric oxide production from lipopolysaccharide-treated RAW 264.7 cells by synthetic flavones: structure-activity relationship and action mechanism. *Archives of Pharmacal Reseach* 27 (9), 937-943.

Liburt, N., K. McKeever, J. Streltsova, M. Gordon, H. Manso Filho, D. Horohov, R. Rosen, W. Franke, A. Singh, N. Vorsa. 2008. Effects of ginger and cranberry extracts on markers of inflammation and performance following intense exercise in horses.

Lotito, S. and B. Frei. 2006. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: Cause, consequences, or epiphenomenon? *Fee Radical Biology and Medicine* 41, 1727-1746.

Manach, C., C. Morand, O. Texier, M. Favier, G. Agullo, C. Demigne, F. Regerat, C. Remesy. 1995. Quercetin metabolites in plasma of rats fed diets containing rutin and quercetin. *The Journal of Nutrition* 125 (7), 1911-1922.

Manach, C., A. Scalbert, C. Morand, C. Remesy, L. Jimenez. 2004. Polyphenols: food sources and bioavailability. *American Journal of Clinical Nutrition* 79, 727-747.

Manach, C., G. Williamson, C. Morand, A. Scalbert, C. Remesy. 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *American Journal of Clinical Nutrition* 81 (S), 230S-242S.

McLaughlin, J., L. Lipworth, W. Chow, W. Blot. 1998. Analgesic use and chronic renal failure: A critical review of the epidemiologic literature. *Kidney International* 54, 697-686.

Mennen, L., D. Sapinho, H. Ito, P. Galan, S. Hercberg, A. Scalbert. 2007. Urinary excretion of 13 dietary flavonoids and phenolic acids in free-living healthy subjects-variability and possible use as biomarkers of polyphenols intake. *European Journal of Clinical Nutrition* 62 (4), 519-525.

Middleton, E., C. Kandaswami, T. Theoharides. 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Reviews* 52, 673-751.

Mishra, D., J. Friden, M. Schmitz, R. Lieber. 1995. Anti-inflammatory medication after muscle injury. A treatment resulting in short-term improvement but subsequent loss of muscle function. *The Journal of Bone and Joint Surgery* 77, 1510-1519.

Nair, M., S. Mahajan, J. Reynolds, R. Aalinkeel, H. Nair, S. Schwartz, C. Kandaswami. 2006. The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via moducation of the NF-κβ system. *Clinical and Vaccine Immunology* 13 (3), 319-328.

Pedersen, B., L. Hoffman-Goetz. 2000. Exercise and the immune system: regulation, integration, and adaptation. *Physiological Reviews* 80 (3) 1055-1081.

Ruel, G., C. Couillard. 2007. Evidences of the cardioprotective potential of fruits: The case of cranberries. *Molecular Nutrition and Food Research* 51, 692-701.

Shephard, R., S. Rhind, P. Shek. 1995. The impact of exercise on the immune system: NK cells, interleukin 1 and 2, and related responses. *Exercise and Sports Science* 23, 215-241.

Vane, J. and R. Botting. 2003. The mechanism of action of aspirin. *Thrombosis Research* 110, 255-258.

Valentova, K., D. Stejskal, P. Bednar, J. Vostalova, C. Cihalik, R. Vecerova, D. Koukalova, M. Kolar, R. Reichenbach, L. Sknouril, J. Ulrichova, V. Simanek. 2007. Biosafety, antioxidant status, and metabolites in urine after consumption of dried

cranberry juice in healthy women: a pilot double-blind placebo-controlled trial. *Agricultural and Food Chemistry* 55, 3217-3224.

Williamson, G., C. Manach. 2005. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *American Journal of Clinical Nutrition* 81 (S), 243S-55S.

Vorsa, N., A. Singh, E. Shabrova, K. Schaich, H. Jin, L. Quadro. 2007. Bioavailability and tissue distribution of cranberry flavonols glycosides in mice. *FASEB Abstract.*

Section 7

FIGURES

7.1 Tables

Name of Compound	Peak Number
M-3 Galactoside	1
Quercetin-3 Galactoside	2
Quercetin-3 Arabinopyranoside	3
Quercetin-3 Arabinofuranoside	4
Quercetin-3 Rhamnoside	5
Quercetin	6

Table 1: The flavonols detected in horse muscle and the peak number corresponding to Fig. 1.

Peak no.	Name of Compound	Retention Time (min)	λmax(nm)	mg/gram
1	Myricetin-3-β-galactoside	23.772	209.1,265.4,357.3	0.115
2	Myricetin-3- α –arabinofuranoside	25.277	252.8,351.3	0.014
3	Quercetin-3-β-galactoside	28.056	206.2,256.1,355.2	0.357
4	Quercetin-3- α –xylopyranoside	30.592	257.6,356.0	0.04
5	Quercetin-3- α –arabinopyranoside	31.499	206.2,255.3,357.7	0.0097
6	Quercetin-3- α –arabinofuranoside	32.506	206.2,255.3,357.7	0.099
7	Quercetin-3-rhamnopyranoside	33.469	206.9,256.0,351.1	0.100
8	3-methoxyquercetin-3-β-galactoside	33.967	207.3,262.1,354.2	0.0010
9	Dimethoxymyricetin-hexoside	34.742	254.9,363.3	0.0043
10	Methoxymyricetin-pentoside	35.173	208.3,253.5,358.1	0.0023
11	3-methoxyquercetin-3-α-xylopyranoside	36.209	211.7,255.4,359.1	0.021
12	Q-3-O-(6”-p-coumaroyl)-β-Galactoside	37.516	254.6,375.7	0.17
13	Quercetin	40.778	255.8,367.2	0.53
14	Q-3-O-(6”-p-benzoyl)-β-Galactoside	42.136	255.4,357.8	0.089
15	Methoxy Kaempferol derivative	42.799	247,272.1,368.2	0.045
16	Methoxy Kaempferol derivative	43.975	242,272.1,367.2	0.048

Table 2: The flavonol peaks in the 90MX cranberry powder, as identified in Fig. 3.

Flavonol	Time to Peak (hrs)		Peak Concentration		24 hr Concentration	
	LD	HD	LD	HD	LD	HD
Q-3 Galactoside	4	1	2.31 ± 2.14	1.22 ± 0.83*	0	0.03 ± 0.03
Q-3 Arabinofuranoside	4	1	0.12 ± 0.07*	0.07 ± 0.04*	0	0
Q-3 Rhamnoside	4	1	0.54 ± 0.51	0.23 ± 0.20	0	0.02 ± 0.02
M-3 Galactoside	4	1	0.78 ± 0.76	0.37 ± 0.21*	0	0
Q-3 Arabinopyranoside	4	16	0.19 ± 0.17*	0.05 ± 0.05	0.02 ± 0.02	0
Quercetin	4	4	1.93 ± 1.83*	5.25 ± 5.19*	0.10 ± 0.04	0.16 ± 0.13

Table 3: The peak time and peak concentration (μg/g) in horse muscle for each of the flavonols in the LD and HD from study 1. The data is expressed as the means ± SE mean. n = 3. An asterisk (*) indicates the mean peak concentration was significantly different from the pre-dosing control.

Time (hrs)	0	0.5	1	2	4	8	16	24
Q-3 Galactoside (2)								
Low Dose	0	0.390±0.29	0.041±0.05	0.215±0.18	2.314±3.83	0.0004±.0007	1.113±1.80	0±0
High Dose	0	0.081±0.05	1.012±1.37	0.170±0.19	0.985±1.61	0.110±0.14	0.240±0.39	0.027±0.05
Q-3 Arabinofuranoside (4)								
Low Dose	0	0.018±0.03	0±0	0.029±0.03	0.095±0.15	0±0	0.051±0.04	0±0
High Dose	0	0±0	0.048±0.08	0.0002±0.0004	0.039±0.07	0±0	0.024±0.04	0±0
Q-3 Rhamnoside (5)								
Low Dose	0	0.106±0.13	0.049±0.06	0±0	0.536±0.9	0.003±0.004	0.351±0.6	0.013±0.02
High Dose	0	0.020±0.03	0.234±0.36	0.050±0.06	0.212±0.37	0±0	0.008±0.01	0.018±0.03
M-3 Galactoside (1)								
Low Dose	0	0.028±0.05	0±0	0±0	0.775±1.34	0±0	0±0	0±0
High Dose	0	0.019±0.03	0.369±0.37	0±0	0.110±0.19	0±0	0±0	0±0
Q-3 Arabinopyranoside (3)								
Low Dose	0	0±0	0.050±0.04	0.026±0.04	0.194±0.34	0.015±0.03	0.047±0.08	0.016±0.03
High Dose	0	0.008±0.01	0.017±0.03	0.024±0.04	0.021±0.04	0.025±0.04	0.045±0.08	0±0
Quercetin (6)								
Low Dose	0	0.682±0.97	0.028±0.03	0.023±0.02	1.933±3.2	0.046±0.05	0.083±0.04	0.097±0.07
High Dose	0	0.034±0.05	0.826±1.21	0.082±0.08	5.255±9.06	0.023±0.04	0.047±0.08	0.162±0.22

Table 4: The mean concentration (μg/g) for each flavonols found in horse muscle following study 1. Data is expressed at each of the eight selected time points as the mean ± standard deviation of the mean. n = 3.

7.2 Figures

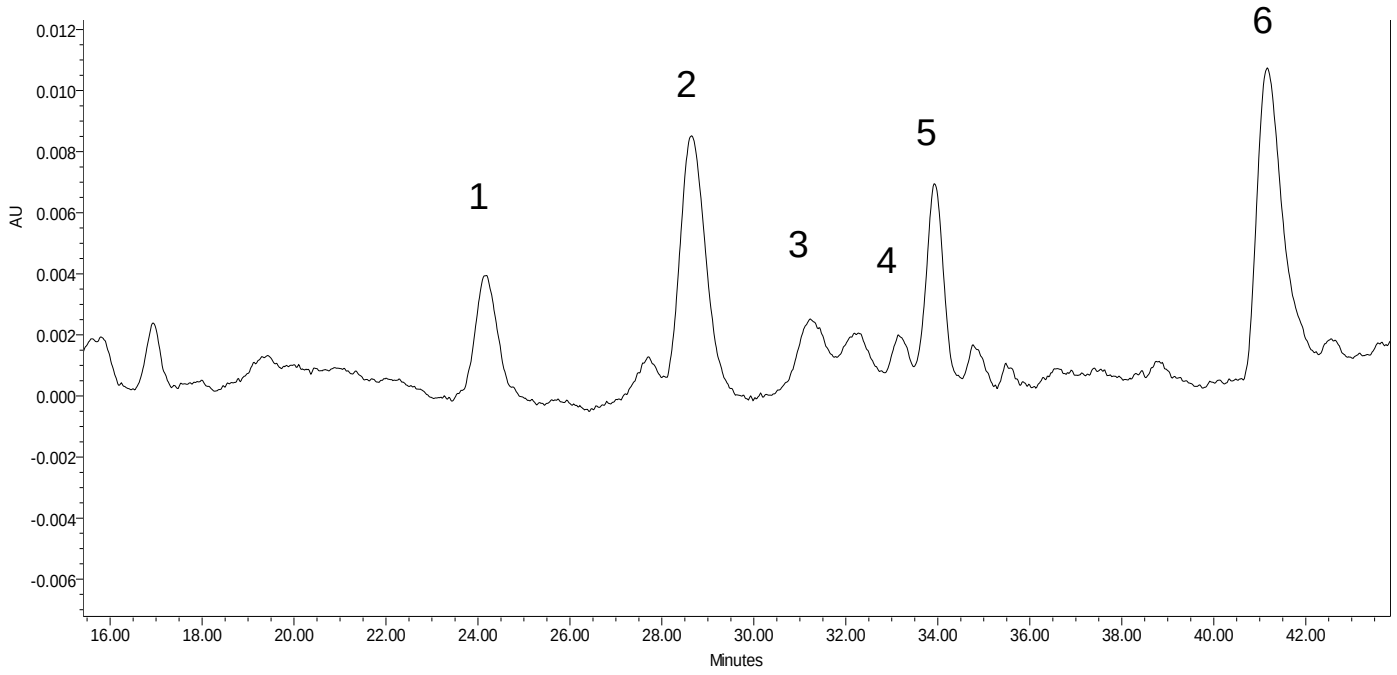

Fig. 1: An example HPLC chromatogram of flavonols present in horse muscle at 4 hrs post dosing. The peaks are identified in Table 1.

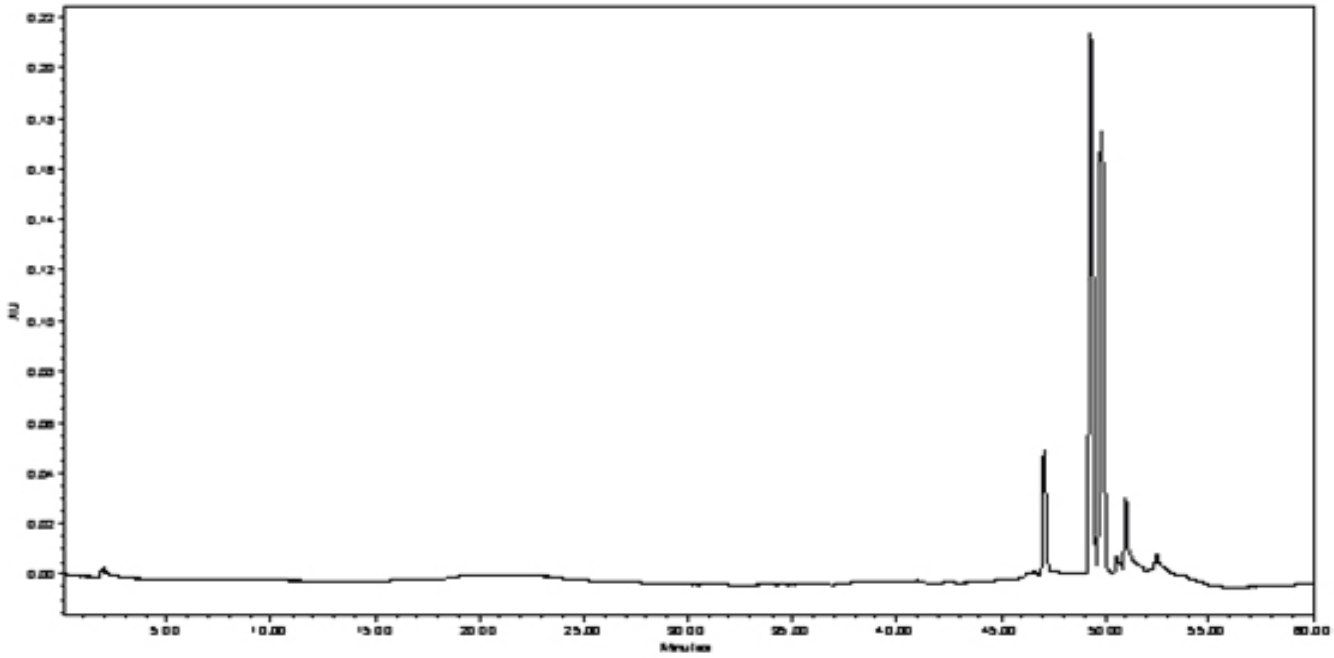

Fig. 2: An example chromatogram of a muscle sample from a horse given the CON treatment, showing no peaks corresponding with any identified flavonols.

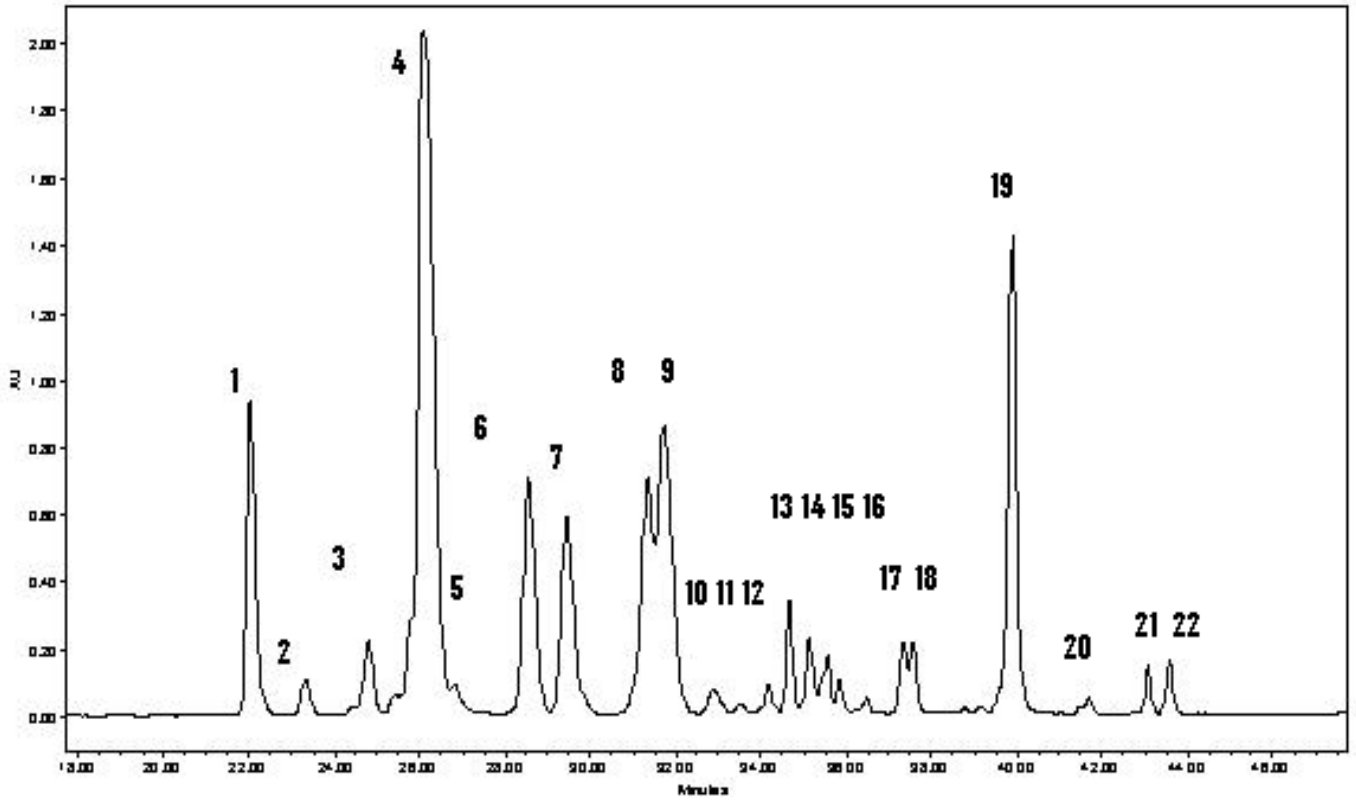

Fig. 3. The HPLC fingerprint of the 90mx cranberry powder. Structure based on liquid choromotography – mass spectrometry data. Peaks are identified corresponding to flavonols in Table 2.

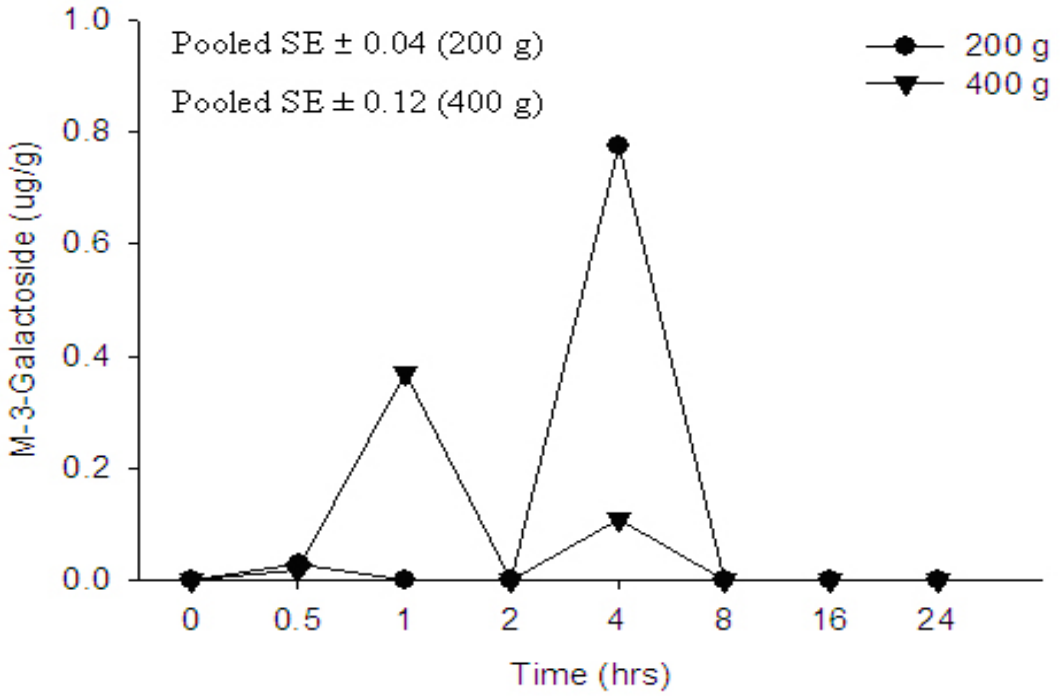

Fig 4: The dose response for M-3-Galactoside in horse muscle. n = 3.

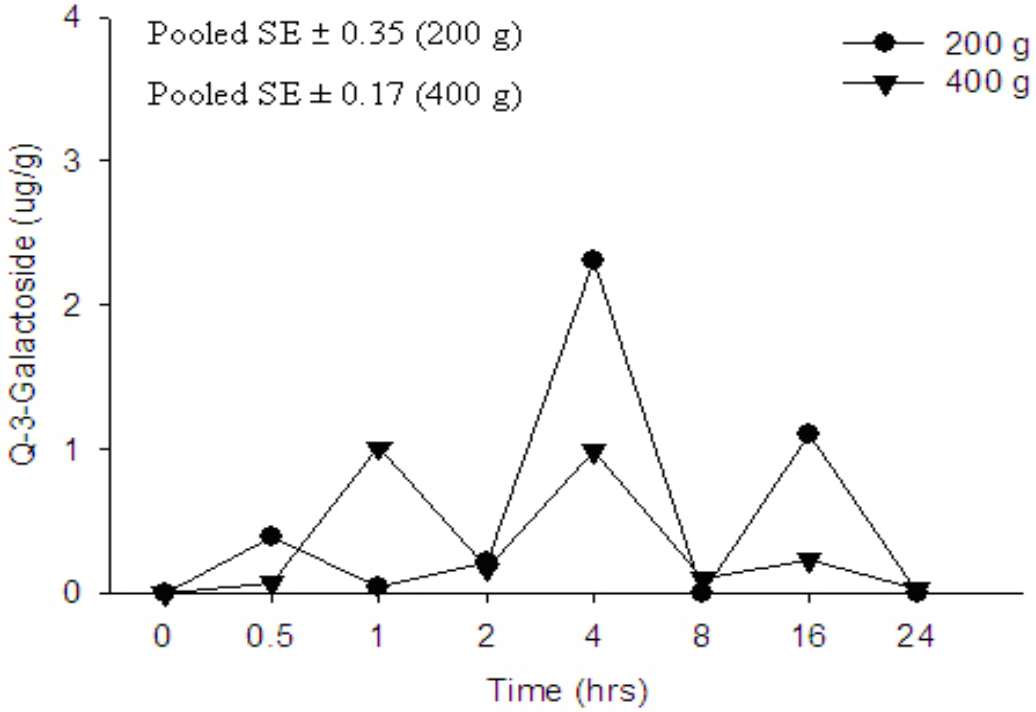

Fig. 5: The dose response in horse muscle of Q-3-Galactoside. n = 3.

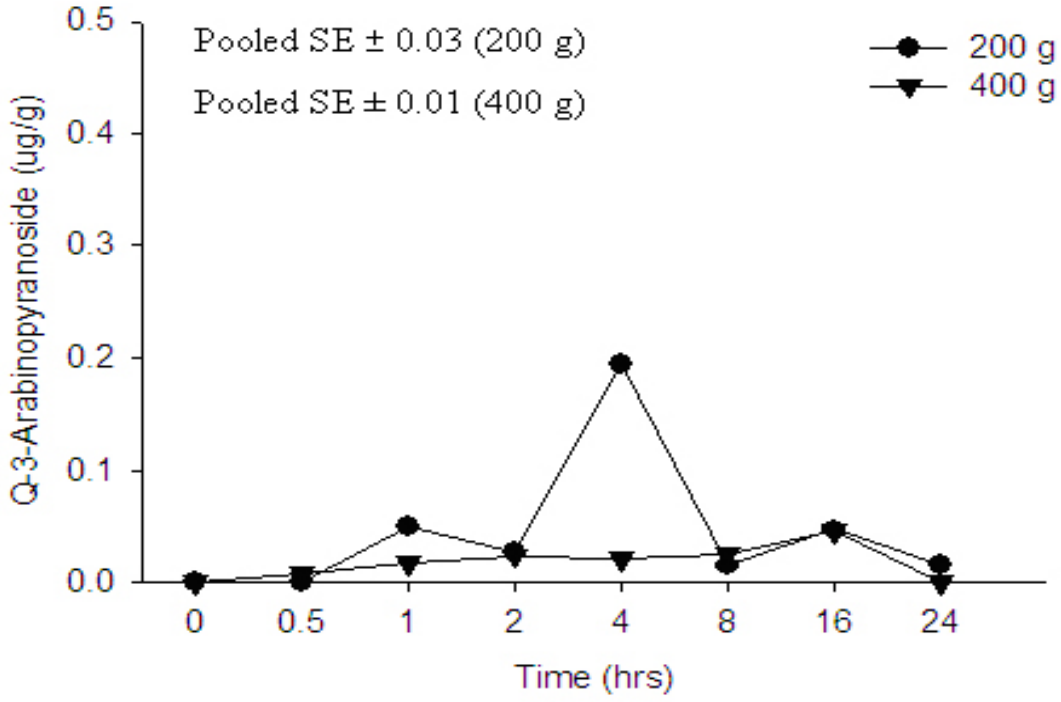

Fig. 6: The dose response in horse muscle of Q-3-Arabinopyranoside. n = 3.

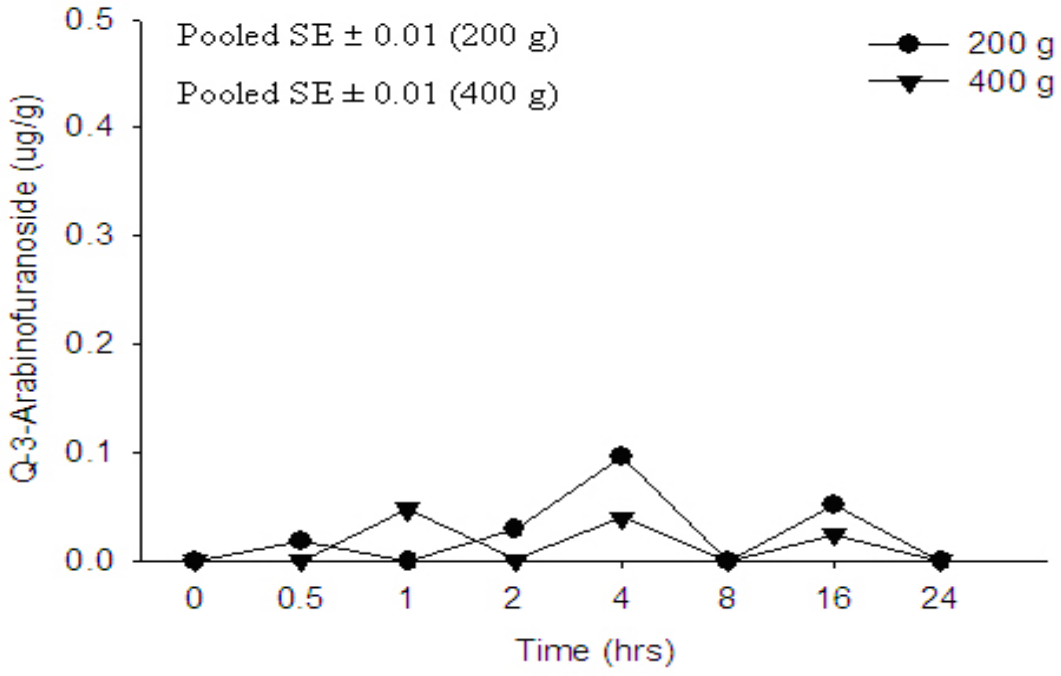

Fig. 7: The dose response in horse muscle of Q-3-Arabinofuranoside. n = 3.

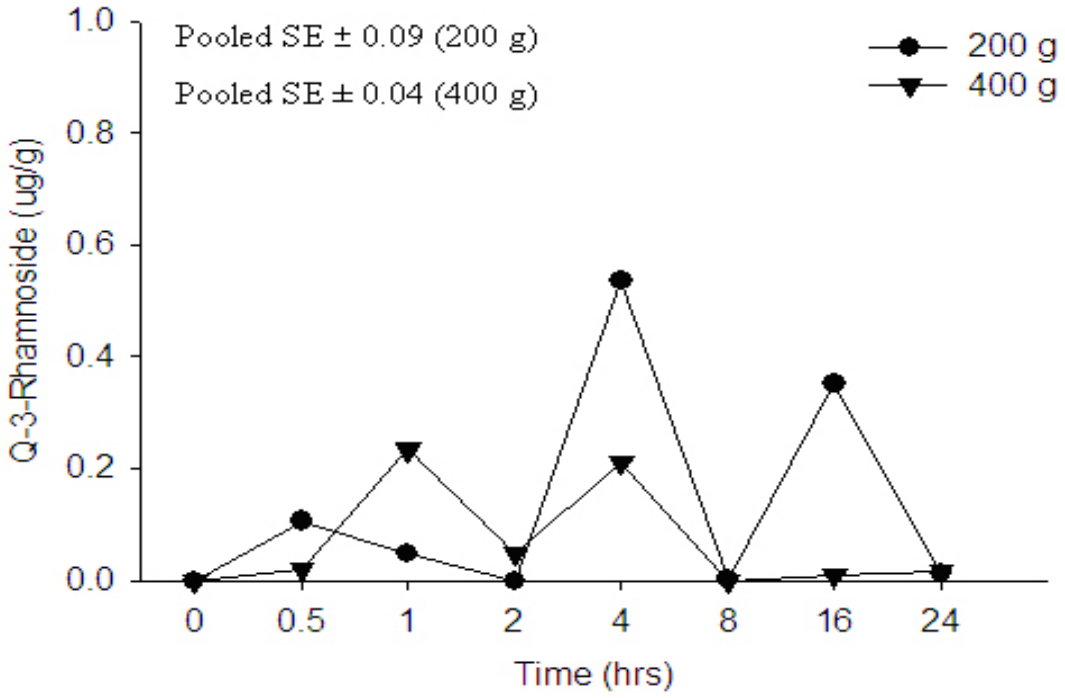

Fig. 8: The dose response of Q-3-Rhamnoside in horse muscle. n= 3.

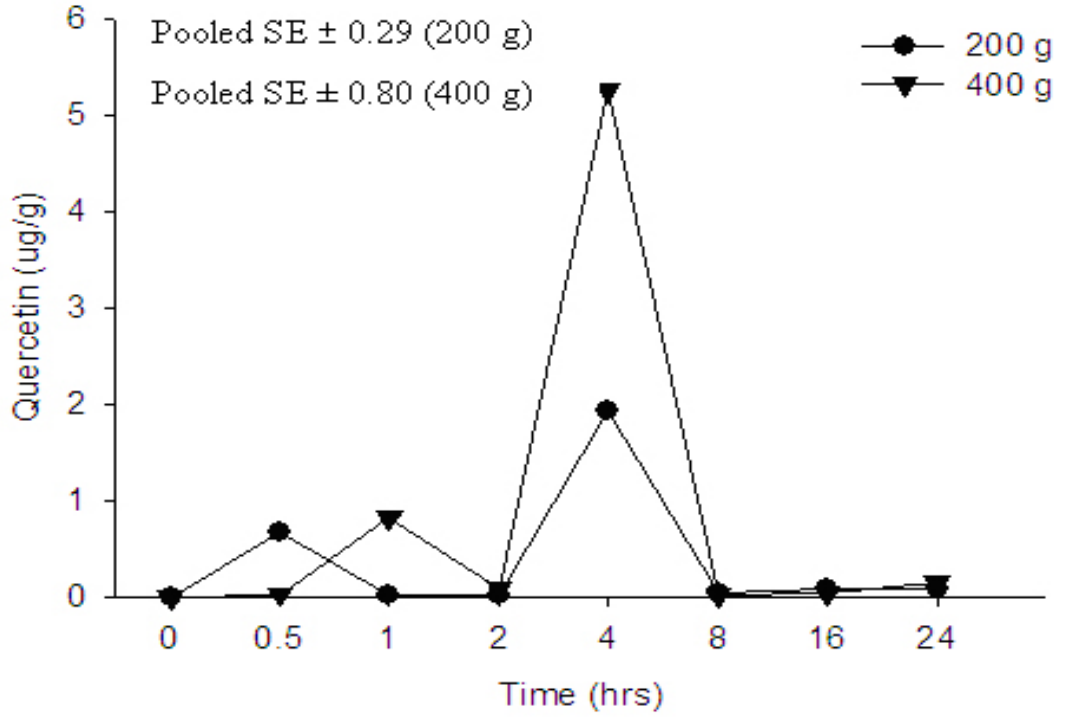

Fig. 9: The dose response of Quercetin in horse muscle. n=3.