Determination of the Effect of Raspberry Ketone on Markers of Obesity in High-fat Fed C57BL/6 Male Mice

THESIS

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Abstract

Raspberry Ketone (RK) is a naturally occurring aroma compound in raspberries. Its chemical structure is similar to capsaicin and synephrine, which studies have shown to have weight loss effects. Preliminary studies indicate that RK may promote secretion of adiponectin, a fat regulating hormone, from adipocytes in cell culture and it has anti-obesity effects when fed to mice. Despite minimal published literature supporting RK’s utility as a fat and body-weight regulating compound, it is sold as a weight-loss supplement. These supplements are often marketed as adiponectin stimulating compounds, but there is no published study of RK’s effect on adiponectin *in-vivo*. Further, the mouse studies that have investigated RK’s biological action did not validate the presence of RK in the formulated diet. The goal of this study is two-fold: to develop an HPLC method for extraction and quantification of RK in high-fat mouse food, and to investigate the hypothesis that RK attenuates weight gain in mice fed a high fat diet (HFD).

A method for extracting RK from high-fat mouse diet was optimized by solubilizing with ethyl acetate, drying and reconstituting with equal parts methanol and hexanes. It was then analyzed using HPLC and an isocratic method equipped with a C18 column. It was determined that RK is stable to processing, but between 13 and 50% loss of compound occurred.
C56BL/6J mice were fed a HFD for 2 weeks then randomized by body weight to four groups: n = 10 HFD + 0% RK (control), n = 10 0.25% RK (LRK) or n = 9 1.74% RK (HRK) and a group n = 9 pair-fed control diet to the *ad-libitum* intake of the LRK group. Mice were fed experimental diets for 5 weeks. Food intake and body weight were measured daily. Adiponectin in blood plasma was measured by immunoassay at week 0 (baseline), week 2 and week 5. No effect of RK on plasma adiponectin levels was observed. Control group food intake was significantly higher than RK-fed mice (P = 0.0014), and there was a strong interaction between body weight and food intake (P < 0.0001) suggesting that body and tissue weight depression in RK-fed mice is due to lower energy intake, not the biological effect of RK. RK-fed mice showed attenuated weight gain compared to control mice in a dose-dependent manner. Pair-fed mice showed nearly identical weight gain to the LRK group. Epididymal fat depot weight was 22.5% smaller in HRK mice than control and inguinal fat depot weight was 41.9% (p = 0.013) lower in HRK mice. It is speculated that RK diet exhibited a weight-attenuating effect because of the reduced energy intake of mice fed RK-diet compared to control rather than a biological mechanism invoked by RK. RK does not appear to have an effect on plasma levels of adiponectin in the time-frame studied. This research is important for informing RK supplement consumers and manufacturers of its efficacy.
Dedicated to my parents
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1.1 Raspberry Ketone Overview

4-(4-hydroxyphenyl)butan-2-one, also called raspberry ketone (RK), rheosmin, rasketone, oxyphenylon, frambinone, p-hydroxybenzyl acetone and 4-(p-hydroxyphenyl)-2-butanone is a volatile aroma compound found predominantly in raspberries but also in other fruits including black raspberries and kiwifruit (Ulbricht et al. 2013). Its glucosidic derivatives are found in cranberries, rhubarb and pine (Borejsza-Wysocki et al. 1992). Its structure is pictured in Figure 1. It has gained significant popularity since its first discovery in 1957 ((Schinz and Seidel 1957), originally in both the flavor and aroma industry for use in food, beverages, cosmetics and fragrances. Raspberries’ aroma profile is composed of hundreds of compounds, 230 of which have been identified (Hrazdina 2006). These include esters, ketones, aldehydes, terpenoids and alcohols (Forney 2001). RK, however, is considered the primary compound associated with the characteristic raspberry flavor and aroma.
In a February 2012 episode titled “The No. 1 Miracle in a Bottle”, Dr. Mehmet Oz, a medical doctor and TV host, touted the “miraculous” weight-loss effects of RK supplements. This resulted in a tremendous increase in RK’s popularity. A similar result has occurred with other products Dr. Oz has endorsed, and thus it has colloquially come to be called “The Oz Effect”. Immediately after RK was highlighted on the show, Google Trends showed a substantial increase in the use of search terms, “raspberry ketones” from approximately 0 to 80 (based on Google Trends scale, relative to the total number of searches over time). This likely corresponded with a drastic increase in the number of RK sales from a natural product store (Bootsman et al. 2014).

Since the inception and rapid growth of the raspberry ketone supplement industry, the popular media has put forth claims about the health effects of this compound: raspberry ketone “increases metabolism and burns off excess fat”, “improves insulin sensitivity”, “helps control your appetite” (“BioActive Raspberry Ketones” 2014, “Lolo:
Do Raspberry Ketone supplements really work?” 2014). The Dr. Oz Show claims that “studies prove” that consuming raspberry ketone 30-minutes before a meal will “stop your body from absorbing the carbs and fats from you meal” (CellHealthMakeover.com). In nearly every case, there have been no peer-reviewed research publications supporting any of these claims, and the breadth of research does not exist to reach a scientific consensus.

1.1.1 Concentration in Raspberries

Although the compound alone does not make-up the whole of raspberry aroma, one study found, not surprisingly, that higher levels of RK are associated with more intense raspberry flavor and aroma in an organoleptic sensory study (Aprea et al. 2015). It logically follows that RK synthesis in raspberries increases as the fruit ripens, and it is associated with higher anthocyanin and sugar concentrations (Borejsza-Wysocki et al. 1992).

Concentrations of this compound in raspberries vary greatly but are typically found in very small quantities, a common characteristic of important plant volatile compounds (Goff and Klee 2006). Honkanen et al. (1980) found that RK in wild raspberries is approximately three times higher than the cultivar Preussen and the Ottawa cultivar contains only traces of RK (Honkanen et al. 1980). Quantitative analysis of RK by different groups yielded highly variable concentrations in red raspberries. One investigation of raspberries outside the United States found RK concentrations between 7 and 200µg, while Borejsza-Wysocki et al. (1992), in a study of American-grown
raspberries, determined concentrations between 0.9 and 17 µg/100g fruit. Variation could be due to differences in the ripeness of the berries sampled since riper raspberries contain more RK as RK develops during the ripening process. Further, different varieties contain differing amounts of RK. For this reason, extraction of RK from fruit is inconsistent and also very expensive. Anywhere from 0.5 to 100 kg of raspberries would be needed to obtain just 1 gram of RK. Not surprisingly, this makes natural RK much more expensive than synthetically made RK, with prices approximating $20 per gram for natural compound, extracted from fruit (Beekwilder et al. 2007).

1.1.2 Production of Raspberry Ketone

Because of the high cost of extracting RK from berries, it is most often made synthetically or with cell tissue cultures as they are more economical and consistent than isolating from raspberries. The highest yielding method for producing RK is through direct synthesis of the compound. Expected yields, as described by Smith (1996) are between 43 and 90%, based on starting material weight. However, many consumers do not consider RK produced using this chemical synthesis as “natural” for labeling-purposes (although governmental regulation on use of the term “natural” is lacking) (Beekwilder et al. 2007). Therefore significant research on alternative methods for RK production has been performed. Beekwilder et al. (2007) laid the groundwork for a method using microbial fermentation by examining the behavior of RK synthesis in-vivo. Using this novel concept, it may be possible to introduce the necessary genes and
enzymes for production to bacteria and yeast and encourage their expression for the microbial production of RK.

An alternate method to produce RK, also considered “natural”, is through cell suspension, cultivated from raspberry leaves. Cell suspensions do not undergo the same ripening processes as the fruit, which as discussed have a synergistic effect on production of RK. Although much greater than direct extraction of ketones from raspberries, this yield is not considered high enough for mass-production of RK (Borejsza-Wysocki and Hrazdina 1993).

1.1.3 Biosynthesis of Raspberry Ketone

Understanding the biosynthesis of 4-(4-hydroxyphenyl)butan-2-one led Borejsza-Wysocki and Hrazdina (1993) to investigate ways of increasing yield. RK structure is thought to be derived from phenylpropanoid metabolism, the mechanism that converts phenylalanine to coumaroyl-CoA. Phenylpropanoid metabolism results in thousands of plant compounds, particularly key phenolics (Weisshaar and Jenkins 1998). 4-(4-hydroxyphenyl)butan-2-one is likely a result of a branch mechanism of the well-known chalcone synthase (CHS) reaction, the same mechanism responsible for the synthesis of the flavonoid naringenin (Borejsza-Wysocki and Hrazdina 1993). The basic steps for this synthesis are summarized in Figure 2: condensation of malonyl-CoA with p-coumaryl-CoA to form the intermediate p-hydroxyphenylbut-3-ene-2-one. This compound is then converted to p-hydroxyphenylbutan-2-one, raspberry ketone. Experiments showed that malonyl-CoA and p-coumaryl-CoA, when applied to raspberry cells or raspberry fruit
extracts, formed the known RK precursor, \( p \)-hydroxyphenylbut-3-ene-2-one \((p\)-hydroxybenzylacetone\). This elucidated the mechanism that leads to the formation of RK, allowing for a better understanding of how to produce synthetic RK at higher quantities. Theoretically, increasing gene expression in raspberry cultures for benzalacetone synthase (BAS), the rate-limiting enzyme that converts malonyl-CoA and \( p \)-coumaryl-CoA to \( p \)-hydroxybenzylacetone would result in higher RK production from tissues. However, identification of that gene is still underway, and even with its discovery, production in cultures or tissues is still drastically less than its direct chemical synthesis. These developments are of specific interest to the supplement industry, the current largest user of raspberry ketone, as it represents a strong market for “natural” products.
Figure 2: Mechanism of chemical synthesis of raspberry ketone in fruit. PAL = phenylalanine ammonia lyase; CHS = chalcone synthase; BAS = benzylacetone synthase; BAR = benzylacetone reductase.
1.2 Biological Function of Raspberry Ketone

Somewhat extensive research has been done investigating the odor thresholds of RK and other volatiles in raspberries (Larsen and Poll 1990) as RK is most commonly used for aroma and fragrance at very low levels. Even its utility as a skin whitener and an agent for increasing skin elasticity and promoting hair growth has been investigated (Harada et al. 2008; Lin et al. 2011). Another study explored its action on osteoblast differentiation in stem cells, promoting bone formation (Takata and Morimoto 2014). However, its biological activity has not been studied until more recently. As research is still being pioneered in this area, the mechanisms of action and fate of RK in-vivo has not been elucidated.

1.2.1 Anti-obesity Effect of Capsaicin and Synephrine

The rationale for RK’s purported anti-obesity effect is its slight chemical similarity to two compounds, synephrine and capsaicin (Figure 3), both of which have been associated with weight-loss effects. All three structures contain a hydroxyphenyl group and bonded oxygen: raspberry ketone and capsaicin contain carbonyl groups, and synephrine contains an alcohol. Capsaicin, the compound that is responsible for the pungency in chili peppers, has reported effects on lipid metabolism by increasing lipid mobilization and catecholamine secretion in rats (Kawada et a. 1986; 1988). In humans, it increased diet-induced thermogenesis and lipid oxidation (Yoshioka et al. 1995; 1998). A meta-analysis on the breadth of literature on capsaicin suggests that it contributes to about a 10 kcal negative energy balance when used at an acceptable dose (due to its
pungency), but this only results in approximately a loss of 0.5 kg per 6.5 years, and despite its minor effectiveness researchers question its utility long-term given free-will and food choices available (Ludy et al. 2012). Synephrine is an alkaloid derived from citrus and is found, along with other stimulants, in the supplement Bitter Orange. This weight-loss supplement gained popularity after the Ephedra ban (due to its link with cardiovascular sequelae) because it has been considered an Ephedra-substitute (Gange et al. 2006). Syneprine affects energy metabolism by acting as an $\alpha$-adrenergic agonist, but its weight-loss effects have not been firmly established (Haaz et al. 2006). In fact, synephrine and Bitter Orange have been found to be deleterious to health and associated with cases of angina (Gange et al. 2006).
Figure 3: Chemical structures of synephrine, capsaicin, and raspberry ketone
1.2.2 Adiposity and Obesity

There are numerous mechanisms that affect body weight, specifically adiposity, and lipid metabolism. For this reason, finding the correct mechanism that a compound utilizes is likened to finding a needle in a haystack. The early stages of research that seek to elucidate these mechanisms focus on determining what are not affected. RK research, as it is in its elemental stages, is no different.

Some obesity research focuses on dietary fat intake as a main cause. At approximately 9 kcal/gram it is calorically dense and has been shown in many animal and human studies to contribute largely to obesity (Ghibaudi et al. 2002). Some studies have supported the notion that even at an equal caloric intake to a low-fat diet, diets high in fat contribute to greater adiposity without significant change in body weight (Boozer et al. 1995). Overconsumption of fat is also very common as it contributes a desirable mouthfeel to foods and is a good vehicle for delivering flavors (Drewnowski 1992) which could possibly contribute to the common hyperphagia of high-fat foods. However, the chemical nature of specific fats (i.e. saturated, unsaturated, monounsaturated, cis, trans, long-chain, short-chain, etc.) has important implications for development of adiposity and markers of metabolic disease (Bourgeois et al. 1983).

Lard in particular, the rendered adipose tissue from swine, is highly associated with development of diet-induced obesity. While the composition of lard is dependent on the food intake of the pigs resulting in some variability, it characteristically contains a high degree of saturated and monounsaturated fatty acids (25-28% palmitic acid, 12-14% stearic acid, 44-47% oleic acid) ("Fat Content and Composition of Animal Products: \[\text{\ldots}\] \ldots\)
Proceedings of a Symposium” 1976). This particular fatty acid composition has been shown to successfully induce obesity in mice (Gajda 2008).

Study of adiposity has revealed that certain fat depots may be more pathogenic than others. Visceral adipose tissue (VAT), fat mainly surrounding internal organs and located in the abdominal region, is most commonly associated with metabolic disease as it acts as an endocrine organ, secreting adipokines that regulate fat metabolism. Additionally, these hormones contribute to metabolic disease as discussed in the next section (Fox et al. 2007). This type of fat is in contrast to subcutaneous adipose tissue (SCAT), fat located just below the skin (mainly in the thighs and buttocks in humans). Studies show that VAT is a stronger predictor of mortality (Ibrahim 2009). Strong evidence suggests that VAT plays a key role in the development of dyslipidemia, insulin resistance and inflammation (Cancello et al. 2006). Patterns of weight gain and loss are also different for VAT and SCAT. Some studies suggest that SCAT accumulation is the first site of increased fat mass from a positive energy balance and once this site begins to reach storage capacity, fat mass accumulates in VAT. Visceral tissue also typically shows higher concentrations of hormone receptors that participate in adipokine signaling (Ibrahim 2010). Saturated fatty acid (from lard) intake is not the only contributing factor to of adiposity and body weight gain. Metabolism of all macronutrients is highly complex and integrated. In addition to fat intake, a meta-analyses of studies on carbohydrate intake supports that sugar (simple carbohydrates) intake is a large determinant of body weight (Te Morenga et al. 2013).
1.2.3 Adiponectin

In addition to being energy reservoirs, fat cells act as autocrine signaling cells. Lipolysis is orchestrated by these signaling molecules: prostaglandins, adenosine and cytokines (including tumor necrosis factor alpha (TNFα) and adiponectin). Obesity induced by high-fat diet causes suppression of adiponectin gene expression. It is well-known that insulin stimulates adiponectin gene expression, though this mechanism is not well elucidated. Increase in insulin-resistance, a marker of obesity and metabolic disease, depresses adiponectin levels. PPARγ plays an additional role in the expression of adiponectin as it is considered a positive regulator of adiponectin expression. It is suppressed in obese individuals (Shehzad et al. 2012). Adiponectin is then an important regulator of lipid metabolism, and it is secreted exclusively by adipocytes. By activating AMPK, it can both increase fatty acid oxidation in mitochondria and decrease fatty acid synthesis. Figure 4 provides a schematic of how some of these signaling pathways are regulated. With close examination of these pathways, it is clear that increased levels of TNFα is associated with obesity (Arner 2005). Adiponectin is inversely related to obesity and decreased levels are therefore associated with obesity-related conditions including diabetes, insulin resistance, non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease. Through its activation of AMPK and PPARα, studies have shown adiponectin to increase free fatty acid (FFA) oxidation and decreased FFA transport into the liver (Shehzad et al. 2012). Animal studies have shown that administering adiponectin to obese mice has reversed insulin resistance, decreased gluconeogenesis, and reduced circulating FFAs by promoting fatty acid oxidation (Park 2010). This has led researchers
to dub adiponectin “a potent insulin enhancer” (Wolf 2003). It has further anti-diabetic and anti-obesity action by decreasing hepatic glucose output and stimulating production of anti-inflammatory factors such as IL-10 (Ibrahim 2009).

Morimoto et al. (2005) noted that RK attenuated weight gain in mice fed high levels of fructose. This led to the assumption that RK’s biological mechanism is through increasing lipolysis rather than prevention of lipid anabolism. Morimoto’s study also examined the effect of RK on fat cells and found that it increased lipolysis with a dose-response effect (Morimoto et al. 2005). Park (2010), in the only published study that has examined the utility of RK as an adiponectin stimulant, looked at the direct application of RK to 3T3-L1 adipocytes to determine if it increases adiponectin secretion and therefore increased lipolysis. RK was found to stimulate lipolysis and fatty acid oxidation and increase cellular secretion of adiponectin when applied at 10 µM (Park 2010). Although the results in-vitro are promising, RK in-vivo would need to be absorbed during digestion and make its way into adipocytes at a level high enough to exhibit activity. This study looked concentrations on cell medium at 1 and 10 µM. Only one published study has examined the metabolism of raspberry ketones and their destiny within the body. Researchers examined RK’s metabolic fat in rats, guinea pigs, and rabbits. The results showed that approximately 90% of the administered dose was excreted in urine within 24 hours of consumption. Excretion was mainly the intact compound, followed by its carbinol metabolite (Sporstøl and Scheline 1982) indicating a high possibility of limited absorption of the compound.
Figure 4: Regulation of fatty acid oxidation and fatty acid synthesis by the hormone, adiponectin. AMPK = AMP-activated protein kinase; IL-6 = pro-inflammatory cytokine interleukin-6, TNFα = adipokine tumor necrosis factor alpha; IL-8 = chemokine interleukin-8; AdipoR1 and AdipoR2 = adiponectin receptor proteins; SREBP1c = sterol regulatory element-binding protein; PPARα = peroxisome proliferator-activated receptor alpha
1.2.4 Impact of High Fat Diets on Mouse Models of Obesity

High-fat (HF) diets, containing 45-60% kcal from fat, have been used for over 70 years to induce obesity in mice for their use in scientific studies. By comparison, control diets are typically a cereal-based “chow” diet (nutritional composition variable) or a purified ingredient diet (little variability) that is low in fat (10% kcal from fat) (Ulman 2011). HF diets in mice have a myriad of effects from development of insulin resistance to metabolic syndrome. Studies show that prolonged feeding of HF diet (30% of calories coming from fat) to rodents beginning at a young age can result in 10-20% body weight gain. Most published studies that examine adipokine levels have determined that adiponectin levels are down-regulated in obese mice, as expected. Similar studies have found that the same metabolic effects that occur with adiposity occur in rodents fed HF diet. C57BL/6 mice have historically made the best model to resemble human obesity in its mimic of insulin resistance, hyperglycemia, etc. and their body weight is sensitive to diet (Surwit et al. 1998; Buettner et al. 2007). Certain studies have also noted that this strain shows preferential accumulation of abdominal fat (in the mesenteric fat pad mainly, to a lesser extent in inguinal tissue), more-so than other strains (Surwit et al. 1998). Adipocyte amount and size increases, contributing largely to the increase in body weight exhibited by rodents fed a HF diet. Adiposity begins with a positive energy balance in which the body stores the extra energy as lipid. When mice and other animals are fed high-calorie diets, they typically reduce consumption. The high caloric-density of HF food and the metabolic propensity to more efficiently utilize energy allows them to
maintain a positive energy balance (Corbett et al. 1986). HF diets also have a noted effect on rodent liver. Additionally, hepatic steatosis, or fatty liver, is common in HF-fed mice.

1.2.5 Effect of Raspberry Ketone on Obesity and Fatty Liver

Although reasoning and evidence for RK’s effect on energy metabolism and obesity is scant, a few studies delve into the biological mechanism behind its supposed effect. The first and perhaps most-cited article on the topic of RK and obesity is on research performed by Morimoto et al. (2005). In this study, researchers looked at the effect of RK on obesity and lipid metabolism in male ICR mice. In the first portion of the study, mice were fed a high-fat diet with or without RK at various concentrations between 0 and 2% for 10 weeks. In the second study, mice were fed high-fat diet for six weeks ad libitum, and then divided into two groups fed the same high-fat diet with or without RK for five more weeks. At the conclusion of these studies, mice fed 2% RK had a lower body weight elevation than mice fed the same diet without RK. Further, visceral adipose and liver weight in RK-fed mice was significantly lower than control diet (high-fat) fed mice. Hepatic histology showed that at 1% RK in the diet, lipid droplets in the liver were normal-sized compared to enlarged hepatocytes in livers of mice fed control high-fat diet and it also reduced hepatic triacylglycerol content. One concern with the findings in this study was that there was no dose-dependent response for body weight. Mice fed 0.5%, 1% and 2% did not show increased weight-attenuation, respectively. However, diets containing 1% and 2% RK appeared to prevent fatty liver. Based on these results, Morimoto concluded that 1% RK is a sufficient dose to see biological weight-
attenuation and prevention of fatty liver. Another study looked specifically at the effect of RK on nonalcoholic steatohepatitis (fatty liver) in high-fat fed Sprague-Dawley rats. In this study, rats were fed high fat diet for 4 weeks and then intragastrically administered 0.5%, 1% or 2% RK each day for 4 weeks. Each parameter measured in this study, including total cholesterol, triglycerides, free fatty acids, liver function parameters, TNFα, blood glucose, insulin, and adiponectin, was significantly improved at all three levels of RK. Histology of liver tissue showed reduced fatty deposition of liver cells. This research group concluded that RK has strong protective effects on the liver and can also diminish already active damage (Wang et al. 2012).

In a separate study by the Morimoto group, rats were fed corn oil tablets with the addition of 5% and 1% RK to study how it affects plasma triacylglycerol content. Reduced triacylglycerol (TAG) content would suggest RK has an inhibitory effect on lipid absorption. At 5% RK concentration, TAG was lower in plasma, however this was not observed at 1% (Morimoto et al. 2005). Further investigation revealed that dietary fat absorption is not the primary mechanism by which RK exhibits anti-obesity effects since 5% is much higher than the 1% dose necessary in the previous mouse study to see effects. The problem with these findings is that 5%, and even 1%, RK is an unreasonable dose, 4-20 times higher than an actual physiological dose.

Neither this study nor others looked at the absorption of RK or its metabolites into adipocytes or liver tissue. Thus, the studies examining RK’s effect on fat and NAFLD may not be physiologically relevant as the concentration of compound that is actually feasibly able to make its way into tissues is not likely enough to have an effect.
A single human clinical trial on the *in-vivo* effects of RK supplemented subjects for 4 weeks and then determined the impact on body weight and body fat. Results showed that RK significantly reduced subjects’ body weight and body fat. It also reduced triglycerides and arteriosclerosis. However, this study was published in Japanese only, is poorly cited and very little information on the study method is available. A review paper of RK alludes to this study, and gives a very rough description (Ulbricht et al. 2013). Because of these gross holes in this study, it was not considered credible enough to validate the efficacy of RK.

1.2.6 Toxicology of Raspberry Ketone

Raspberry Ketone (under the name 4-(p-hydroxyphenyl)-2-butanone received GRAS (Generally Recognized As Safe) in 1965 from FEMA (Flavor and Extract Manufacturers Association). Most supplements are taken at a higher level, about 1000 mg/day, about 40 times the maximal level when used as a flavoring agent. Based on toxicological studies on rats, it is unlikely that RK would cause permanent adverse effects, especially at the equivalent recommended daily dose for humans. Acute toxicity is estimated at 0.35 g/kg for females and 0.7 g/kg for males, requiring a human to consume approximately 20-43 grams of RK (or take 40 individual RK supplement tablets, each 500 mg). Subacute toxicity studies exhibited no-effect at 100 mg/kg/day in rats (about 0.2% of the diet) (Gaunt et al. 1970). For reference, this would be equivalent to prolonged consumption of about 6200 mg of RK per day, or 6 times the recommended dose. There has been a recent concern over the safety of raspberry ketone supplements,
particularly as they are often paired with other weight-loss compounds such as caffeine, garcinia, acai and African mango. A 24-year-old woman died from consuming a fatal dose of a combination of raspberry ketone, caffeine, and resveratrol. It is unclear the role that RK played in her lethality (Birch 2014).

1.3 Chemical Analysis of Raspberry Ketone

Fruit aroma volatiles are present in minute quantities in fruit, making up only about 0.001% to 0.01% of the fruit’s weight (Buttery 1981). Volatile compounds possess high vapor pressures at room temperature, causing molecules to vaporize where they can be detected by humans – thus, smell (Forney 2001). Due to the volatile nature of these compounds, the extraction and analysis needs to be tailored so as to preserve as much of the compound as possible. Propensity to degrade is also an important consideration when choosing an extraction and analytical method. Studies identifying the primary odor components in raspberries, and particularly detection of RK, are more prevalent than studies looking at the biological effect of RK. The use of differing analytical methods has yielded some conflicting results. Some studies have found that α- and β-ionone are the most important components of raspberry aroma and not RK (Pyysalo 1976). Others have found that raspberry ketone is by and far the most important compounds and other aroma compounds are not present at detectable levels (Maquin et al. 1981; Braun and Hieke 1977). These differences are due in part to raspberry variety as the aroma profile differs greatly across cultivars. Method of chemical analysis can also play a large role. Some of the first analyses identifying RK utilized thin layer chromatography (TLC) (Schinz and
Seidel 1957). Contemporarily, analysis using gas chromatography (GC) is probably the most common method for analyzing RK, but recently high performance liquid chromatography (HPLC) methods have been used as well. Most of these methods isolate RK from berries themselves to be analyzed. Extraction methods of RK from high-fat mouse diet has not been investigated.

Direct solvent extraction of RK is typically done using diethyl ether, methanol or ethyl acetate, all organic solvents, as it is not soluble in water (Perez 1983; Larsen et al. 1991; Klesk et al. 2004; Beekwilder et al. 2007; Malowicki et al. 2008).

1.3.1 Gas Chromatography

A number of studies have used gas chromatography (GC) to detect and quantify RK in many different matrices – food and otherwise, and is the most popular method for RK analysis. GC is well-suited for analysis of volatile compounds because the compound must become immediately volatilized in the inlet to be able to move through the column to the detector. Use of GC for analysis of chemical compounds is advantageous because of its high sensitivity and low injection volume. This makes detecting substances at very low concentrations possible and requires typically only a small amount of sample. Additionally, analysis with GC is very fast which is advantageous for volatile or thermally unstable compounds.

One limitation to using GC is that it is a separation technique, not an identification technique. When coupled with more powerful analytical instrumentation such as mass spectrometry (m/s), infrared (IR) or nuclear magnetic resonance (NMR)
compound identification becomes possible. However, without this additional capacity, unambiguous identification is not possible. Retention times, the time at which the compound elutes or reaches the detector, can be useful in identifying compounds. However, many compounds have the same retention time. Relating retention times to a standard may elucidate compound identification. Raspberry Ketone typically has ionic masses at $m/z$ 107, 121 and 164 if m/s is used in selected-ion-monitoring mode (Beekwilder et al. 2007).

Injection solvent is an important consideration for GC. As very small quantities are injected, solvents that evaporate quickly will create problems with quantitation. Ethyl acetate is commonly used as solvent for building a standard curve and solubilizing RK for injection as RK is soluble in ethyl acetate and it does not evaporate too quickly. As described, volatilization of compound is necessary for detection. Schmidlin-Meszaros (1971) found that RK was not readily volatilized and therefore had difficulties detecting the compound by GC. However, many other authors did not find volatilization to be a problem (Bruchmann and Klob 1973; Braun and Hieke 1977; Perez 1982). Polar or highly polar columns are best for analyzing ketones in general, but previously published studies have achieved separation on non-polar columns as well as polar (Sigma Aldrich Column Selection Guide). Previous studies also report inlet temperature values at about 45°C, a ramp of about 3-6°C/min, at a final temperature between 190°C and 250°C (Larsen and Poll 1990; Klesk et al. 2004; Malowicki et al. 2008). Quantitation is performed by measuring peak area.
1.3.2 *High-performance Liquid Chromatography (HPLC)*

HPLC analysis can be applied to a much wider range of compounds than GC as it can be used for any substances that are not volatile (or have not been derivatized). In general, LC is best used on more polar compounds and GC is best used for less polar compounds. There are, however, certain compounds that can be analyzed by either GC or LC, like RK ("Considerations for Selecting GC/MS or LC/MS for Metabolomics" 2007). Considerations to be made for HPLC are to account for RK’s volatility. Preparation should be done so as to not volatilize the compound and therefore lose quantitation power. Care should be applied in preparation for any analytical method used. Isocratic elution, in which the mobile phase and stationary phase composition remain the same throughout the method, is best utilized for separating very few compounds. On the other hand gradient mode is useful for separating many compounds with varying polarities. Solvent composition has a strong effect on chromatographic elution as well. Lili et al. (2011) did some work comparing the behavior of RK on mass spectrometric signal when different solvents were used. They found that methanol-water as mobile phase achieved the highest m/s signal and best peak shape (in comparison to acetonitrile-water). They also determined that spiking 0.1% formic acid in water resulted in the strongest signal. Formic acid improves peak shape by preventing the analyte of interest from interacting with non-capped silanols in the C18 column (Fujii et al. 1996).
1.4 Problem Statement

Recent mouse and cell studies suggest that raspberry ketone (RK) may have anti-obesity effects. The structure of RK, a flavor compound found in raspberries, loosely resembles the structures of capsaicin and synephrine. Both of these compounds, present in chili peppers and citrus, respectively, have shown anti-obesity effects in previous studies. Very few animal studies have examined the ability of RK to attenuate weight gain despite its widespread use as a weight loss supplement. Further, the studies that do exist do not verify the presence of RK in the formulated diet.

Reaching a scientific consensus on the efficacy of nutraceuticals requires a breadth of research, including cell, animal and human studies. Research on the biological effects of this compound is greatly lacking. Moreover, beyond verifying the ability of RK to attenuate weight gain, it is important to make steps toward elucidating its biological mechanism. A single cell study suggests it is through increasing secretion of the fat-regulating hormone adiponectin from adipocytes. However, this observation has not been studied \textit{in-vivo}.

Based on these gaps in scientific understanding of RK’s behavior, the objective of this study is to investigate the anti-obesity effects of RK using a mouse-model. It is hypothesized that in a dose-dependent manner RK will attenuate weight gain in C57BL/6 mice fed a high-fat diet for 7 weeks.
Aim 1: Develop a method using HPLC for detecting and quantifying Raspberry Ketone in high-fat mouse food.

1. Optimize the extraction of RK from high-fat mouse diet
2. Use HPLC to quantify RK in mouse diet pre and post-processing

Aim 2: Determine the biological effect of RK on adiposity and obesity in high-fat fed C57BL/6 male mice in a 7-week study.

1. Collect data on food intake, body weight, and fat depot and liver weights in mouse study.
2. Measure mouse adiponectin levels in blood plasma.
3. Compare fat content in livers of mice fed RK versus control diets.
Chapter 2: Materials and Methods

2.1 Analysis of Raspberry Ketone

2.1.1 Chemicals

HPLC grade ethyl acetate (Sigma Adrich; St. Louis, MO), methanol, 99% formic acid, water, and phosphate buffered saline (Fisher Scientific; Pittsburgh, PA) were used for their respective experiments. Food Grade Raspberry Ketone and analytical standard were obtained from Sigma Aldrich (St. Louis, MO).

2.1.2 Extraction of Raspberry Ketone from Mouse Diet

Mouse diet pellets were ground in an electric coffee grinder into a fine powder. 1 g of powder was weighed in a plastic centrifuge tube. 4mL of ethyl acetate was added to each tube, sonicated for 10 minutes, shaken on a vortex machine for 10 minutes, and centrifuged at for 10 minutes or until solid material formed a pellet. The supernatant was collected with a Pasteur pipet and transferred to a clean glass vial. This extraction process was repeated three times for each sample, yielding approximately 12 mL of pooled organic solvent mixture.

Pooled solvent mixtures were dried under a stream of nitrogen gas until completely dry. They were then reconstituted with 10 mL of hexanes and 10 mL of
methanol. A portion of the alcohol layers were collected and passed through 0.22 μm, 4 mm nylon filters before injection onto the HPLC system.

2.1.3 GC-MS Analysis

Analysis of food grade and pure analytical RK standard was performed using an Agilent 6890 Series gas chromatogram equipped with an Agilent 5973 Mass Selective Detector (MSD). Peak separation was achieved using an HP-5 column (Agilent Technologies; Santa Clara, CA). 1 μL was injected into the inlet, at a temperature of 250°C and column temperature was 45°C. After injection, column temperature was held for 2 minutes, and then ramped 10°C/min to 220°C and held for 5 minutes. Electron impact m/s data from m/z 35-300 were collected at an ionization voltage of 70 eV.

2.1.4 HPLC Analysis

Raspberry Ketone was detected and quantified in formulated mouse diet by HPLC with a photodiode array detector (PDA) set to detect data from wavelengths of 276 to 294 nm (Agilent Technologies 1100 Series; Santa Clara, CA) using a Symmetry® C18 column (3.5μm, 4.6 x 75mm; Waters Corp, Milford, MA) maintained at 40°C. Optimization of the mobile phase was achieved by testing several eluents. The mobile phase consisted of a 55:45 ratio of 0.1% formic acid in water (A) to methanol (B). Elution of RK was achieved using an isocratic method. The injection volume was 10μL and a flow rate of 1 ml/min. A standard curve was constructed based on peak area using serial dilutions of analytical standard dissolved in methanol.

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2.2 Mouse Study

2.2.1 Animals and Experimental Design

Animals used in this experiment were treated according to standards established by the American Association for Laboratory Animal Science and procedures were approved by the Institutional Animal Care Utilization Committee of The Ohio State University (2013A00000036). 38 five-week-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were housed in individual cages in a vivarium at Ohio State University (Columbus, OH) at 22 ± 0.5°C on a 12 hour light/dark cycle. The mouse study is outlined in Figure 5. Mice were fed standard chow diet for one week to allow them to acclimate to the new environment and given free access to water. The first two weeks of the study mice were fed high-fat control diet (45% kcal from fat, 24% wt) ad libitum to allow for accumulation of adiposity and markers of metabolic disease. The fat content was composed of 12% soybean oil (unsaturated fat) and 88% lard (saturated fat). After 2 weeks, mice were randomized by weight (average weight = 21.7 - 22.8 g) to four different experimental groups: high-dose raspberry ketone (HRK; n = 10), low-dose raspberry ketone (LRK; n = 9), control (n = 10), and pair-fed (PF; n = 9) (individual mice were matched by weight and pair-fed to the LRK group with control diet). Diet compositions are summarized in Table 2.

HRK dose was based on previous literature suggesting the minimum amount of RK needed to elicit a response (Morimoto et al. 2005). The LRK dose approximated what a physiologically-relevant dose would be for humans, based on normal daily RK intake. Information on converting human to mouse dose is from the U.S. Department of Health...
and Human Services (2005) based on body surface area of a mouse versus a human. The following assumptions were made: human dose is 1000 mg/day, human weight is approximately 50 kg, Animal and Human $K_m$ taken from Table 1, daily mouse food intake is about 2 g/day (based on food intake results), mice weigh approximately 20 g.

The following equation was used to calculate an equivalent mouse dose:

$$\text{Human Equivalent Dose (HED)} \left( \frac{\text{mg}}{\text{kg}} \right) = \frac{\text{Animal Dose} \left( \frac{\text{mg}}{\text{kg}} \right) \times \frac{\text{Animal } K_m}{\text{Human } K_m}}{\text{Animal } K_m = 3}$$

$$\text{Human } K_m = 37$$

$$\text{HED} = \frac{1000 \text{ mg}}{50 \text{ kg}} = 20 \text{ mg/kg}$$

$$\text{Animal Dose} = 246.7 \text{ mg/kg} \times 0.02 \text{ kg} = 4.934 \text{ mg/day } \div 2000 \text{ mg food} = 0.002467$$

Therefore, the physiologically-relevant dose used for RK (LRK) was 0.25%. Diet intervention occurred the remaining 5 weeks of the study as shown in Figure 5. Body weight and food intake were measured daily throughout the study.
Table 1: Conversion factors for calculating human and animal equivalent doses based on Body Surface Area (U.S. Department of Health and Human 2005)

<table>
<thead>
<tr>
<th>Species</th>
<th>To Convert Animal Dose in mg/kg to Dose in mg/m², Multiply by ( k_m )</th>
<th>To Convert Animal Dose in mg/kg to HED(^a) in mg/kg, Either:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Divide Animal Dose By</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Multiply Animal Dose By</td>
</tr>
<tr>
<td>Human</td>
<td>37</td>
<td>---</td>
</tr>
<tr>
<td>Child (20 kg)(^b)</td>
<td>25</td>
<td>---</td>
</tr>
<tr>
<td>Mouse</td>
<td>3</td>
<td>12.3</td>
</tr>
<tr>
<td>Hamster</td>
<td>5</td>
<td>7.4</td>
</tr>
<tr>
<td>Rat</td>
<td>6</td>
<td>6.2</td>
</tr>
<tr>
<td>Ferret</td>
<td>7</td>
<td>5.3</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>8</td>
<td>4.6</td>
</tr>
<tr>
<td>Rabbit</td>
<td>12</td>
<td>3.1</td>
</tr>
<tr>
<td>Dog</td>
<td>20</td>
<td>1.8</td>
</tr>
<tr>
<td>Primates:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkeys(^c)</td>
<td>12</td>
<td>3.1</td>
</tr>
<tr>
<td>Marmoset</td>
<td>6</td>
<td>6.2</td>
</tr>
<tr>
<td>Squirrel monkey</td>
<td>7</td>
<td>5.3</td>
</tr>
<tr>
<td>Baboon</td>
<td>20</td>
<td>1.8</td>
</tr>
<tr>
<td>Micro-pig</td>
<td>27</td>
<td>1.4</td>
</tr>
<tr>
<td>Mini-pig</td>
<td>35</td>
<td>1.1</td>
</tr>
</tbody>
</table>

\(^a\) Assumes 60 kg human. For species not listed or for weights outside the standard ranges, HED can be calculated from the following formula:

\[
\text{HED} = \text{animal dose in mg/kg} \times (\text{animal weight in kg/human weight in kg})^{0.33}
\]

\(^b\) This \( k_m \) value is provided for reference only since healthy children will rarely be volunteers for phase 1 trials.

\(^c\) For example, cynomolgus, rhesus, and stump tail.
C5BL/6 mice divided into 4 experimental diet groups, randomized by weight:

- **Control**: n = 10
- **LRK (0.25% RK)**: n = 10
- **Pair-fed**: n = 9
- **HRK (1.74% RK)**: n = 9

1 week
- All mice: Chow diet

2 weeks
- All mice: Purified high-fat diet; n = 38

5 weeks
- Experimental diet

Week 0: Blood plasma collected to measure blood glucose and adiponectin

Week 2: Blood collected to measure blood glucose and adiponectin

Week 5: Necropsy – Blood collected to measure blood glucose and adiponectin. Liver, quadriceps, epididymal and inguinal fat collected, weighed and frozen

Body weight and food intake measured daily

Figure 5: Mouse study outline
2.2.2 Experimental Diets

Mouse diet (purchased from Research Diets Inc., New Brunswick, NJ) was processed by mixing materials with 10% water, pelletizing and drying for 2 days at 70-80°F at low humidity. Food-grade synthetic raspberry ketone (≥98% pure) was purchased from Sigma Aldrich (St. Louis, MO). Each group’s diet is summarized in Table 2. All diets had the same caloric content, 4.7 kcal/g.
Table 2: Diet Composition of mice experimental diets

<table>
<thead>
<tr>
<th></th>
<th>Control/ PF</th>
<th>HRK</th>
<th>LRK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% wt</td>
<td>% kcal</td>
<td>% wt</td>
</tr>
<tr>
<td><strong>Protein</strong></td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Casein</td>
<td>23.3</td>
<td>22.8</td>
<td>23.2</td>
</tr>
<tr>
<td>L-cystine</td>
<td>0.34</td>
<td>0.34</td>
<td>0.34</td>
</tr>
<tr>
<td><strong>Carbohydrate</strong></td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>8.5</td>
<td>8.3</td>
<td>8.4</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>11.6</td>
<td>11.4</td>
<td>11.6</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20.1</td>
<td>19.7</td>
<td>20</td>
</tr>
<tr>
<td><strong>Fat</strong></td>
<td>45</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>2.9</td>
<td>2.85</td>
<td>2.9</td>
</tr>
<tr>
<td>Lard</td>
<td>20.6</td>
<td>20.3</td>
<td>20.6</td>
</tr>
<tr>
<td>Raspberry Ketone</td>
<td>0</td>
<td>2.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5.8</td>
<td>5.7</td>
<td>5.8</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>1.16</td>
<td>1.14</td>
<td>1.16</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>1.16</td>
<td>1.14</td>
<td>1.16</td>
</tr>
<tr>
<td>DiCalcium Phosphate</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Calcium Carbonate</td>
<td>0.64</td>
<td>0.63</td>
<td>0.64</td>
</tr>
<tr>
<td>Potassium Citrate</td>
<td>1.9</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>0.23</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>FD&amp;C Yellow Dye #5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FD&amp;C Red Dye #40</td>
<td>&lt;0.01</td>
<td>0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FD&amp;C Blue Dye #1</td>
<td>0</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

100 100 100 100 100 100 100

33
2.2.3 Measuring Fasting Blood Glucose

Blood glucose was measured at 2, 4 and 7 weeks from the start of the study. Mice were fasted by moving to a clean cage without food for 5-6 hours. Blood was drawn via tail-bleed and blood glucose levels were analyzed using a One Touch® Blood glucose meter and strips (Lifescan, Milpitas, CA).

2.2.4 Measuring Adiponectin

Fasting adiponectin was measured at 0, 2 and 5 weeks. Mice were fasted by moving to a clean cage without food for 5-6 hours. Blood was drawn via tail bleed and transferred to EDTA tubes (collected from retro-orbital bleeding at necropsy). They were stored at approximately 4°C for 2 hours and then centrifuged to isolate blood plasma. Samples were stored at -80°C until analyzed for adiponectin using a colorimetric ELISA kit (Millipore Mouse Adiponectin ELISA, Darmstadt, Germany).

2.2.5 Necropsy

After 7 weeks, mice were fasted for 5-7 hours before necropsy was performed. Mice were anesthetized by isoflurane for retro-orbital eye bleed. They were then euthanized by CO₂ and cervical dislocation. Liver, epididymal fat depot, inguinal fat depot, and quad muscles were removed, rinsed with PBS, weighed, and snap frozen in liquid nitrogen. Samples were stored at -80°C for future analysis. For statistical analysis, tissue weights were normalized to body weight (\( \frac{\text{tissue weight}}{\text{body weight}} \)). Portions of epididymal adipose and liver were removed for future histological analysis.
2.2.6 Measuring Total Hepatic Lipids

Frozen livers were ground to a fine powder in liquid nitrogen using a Cell Crusher™ and transferred to pre-weighed glass vials. Samples were then homogenized with 5 mL of phosphate buffered saline (PBS) using a probe sonicator for 30 seconds and extracted with 10.5 mL of 2:1 mixture of chloroform-methanol. The entire mixture was probe sonicated for 30 seconds and centrifuged for 10 minutes at 2000 x g to achieve separation of three phases: methanol – top, solid matter – middle, chloroform – bottom. The top layer was removed and reserved and the bottom chloroform layer was transferred to a clean, pre-weighed glass vial. The methanol layer was then added back to the original glass vial and the extraction process with chloroform-methanol was repeated. The reserved chloroform layer that contained dissolved hepatic lipids was dried under a flow of nitrogen and weighed. % lipid was determined by weight of lipids divided by the weight of liver tissue extracted.

2.2.7 Statistical Analysis

Descriptive analysis was performed using IBM SPSS Statistics software. Statistically significant differences (p<0.05) were calculated using one-way ANOVA and Tukey’s post-hoc test. Blood plasma samples with adiponectin levels that were outliers, fell outside of the standard curve or had a % coefficient of variation (CV) greater than 20 were removed. For graphs, error bars were constructed using standard deviation or standard error of the mean (\( SE = \frac{SD}{\sqrt{n}} \)), where SD is standard deviation and n is the size.
of the group. Food intake and body weight were analyzed using two-way ANOVA to determine interaction between two variables.
Chapter 3: Results and Discussion

3.1 Analysis of Raspberry Ketone

One of the main goals of this study was to analyze raspberry ketone in formulated high-fat mouse diet. This is an important step to verify the levels at which RK is present in experimental diets. Two methods were investigated for their use in mouse food analysis: gas chromatography and high-performance liquid chromatography.

3.1.1 Gas Chromatography (GC)

A method for analyzing RK using GC was successfully developed. As discussed in Chapter 1, previously published methods had temperature ramps between 3 and 6°C/min. However, the present study found that temperature ramps in this range resulted in a very wide and tailing peak. A narrower peak with less tailing was achieved by increasing the temperature ramp 10°C/min (Figure 6). This also greatly reduced analysis time. However, when separating compounds, having a fast temperature ramp can lead to decreased resolutions in complex mixtures such as foods. This study also found that analysis on a non-polar column was not a problem. Although polar columns are recommended for the analysis of ketones, separation was achieved on a non-polar hp-5 column.
Figure 6: GC chromatogram of pure analytical raspberry ketone for selected ion $m/z$ 107
3.1.2 Extraction Method Development

Optimization of extraction of raspberry ketone from high-fat mouse diet for detection and quantification was successfully achieved. HPLC analysis of ketone was the preferred method over gas chromatography (GC) for several reasons. First, resource constraints (no automatic sampler) required the use of an internal standard to quantify RK with GC which complicated quantitation and created more sources of error. Second, better peak shape was achieved on the HPLC than GC. As can be seen in Figure 6, tailing peaks was a consistent problem when using GC. On HPLC however, Gaussian shaped peaks were achieved.

Several methods of extraction and sample preparation were explored to determine which method resulted in the highest recovery of RK, reproducible results, and best peak shape. Several previous studies have extracted RK from fresh or frozen raspberries for subsequent HPLC or GC analysis. The techniques used in these extractions were much more complex than the extraction performed in this study, requiring techniques such as solvent assisted flavor evaporation (SAFE), solid phase micro extraction (SPME) or stir bar sorptive extraction (SBSE) to release RK from a complicated food matrix (Engel et al. 1999; Malowicki et al. 2008). Extracting RK from high-fat formulated mouse diet has different method requirements. Considerations should be made for the high triglyceride content that has a tendency to “trap” the RK, volatility of RK, miscibility of solvents and their performance on-column. The extraction method that best fit this application was using ethyl acetate, a solvent often used in RK extractions (Beekwilder et al. 2007).
While other studies have used diethyl ether as an extraction solvent, this study found ethyl acetate to adequately solubilize RK (Larsen et al. 1991; Klesk et al. 2004). Ethyl acetate is more advantageous for multiple reasons. Diethyl ether is flammable and explosive, thus creating a safety hazard. Additionally, ethyl acetate is less volatile than diethyl ether. Highly volatile solvents make quantitation less accurate as evaporation of solvent will alter concentration. Both solvents have similar polarities, so they have very similar abilities to solubilize RK (“Solvent Miscibility Table” 2014).

Reconstitution with only methanol after extraction of RK from mouse diet resulted in a large triglyceride fat globule, insoluble in alcohol. When a sample from the methanol portion was quantified using HPLC, recovery was highly variable, between 26 and 90% (SD = 20%). It was found that by reconstituting with equal parts methanol and hexanes (10 mL each), which solubilized the triglycerides, recovery and precision improved. These results were also reproducible, as shown in Table 3. The recovery, 68%, was used as a divider to determine RK concentration in formulated mouse diet. To explicate the consistent 68% recovery for this method, an experiment was conducted to determine recovery of RK that has been solubilized directly into 10 mL each of methanol and hexane. This revealed 70% recovery (SD = 0.75%) suggesting that the RK partitions between the two immiscible phases, hexanes and methanol, with the majority of compound in the alcohol phase. With this information, it was assumed that approximately 3% of compound is lost during transfers for each extraction or while being dried under nitrogen.
Based on extraction efficiency and known loss, the following calculation was used to determine the concentration of RK in mouse diet:

\[ RK \text{ Concentration} = \frac{Calculated \text{ Concentration}^*}{0.68} \]

*Calculated concentration is determined by the area under the curve on the HPLC chromatogram and the equation of the standard curve.

Table 3: Extraction recovery of raspberry ketone spiked into high-fat mouse diet as determined by analysis using HPLC

<table>
<thead>
<tr>
<th>Approximate Spiked % RK</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>68.9 ± 1.2</td>
</tr>
<tr>
<td>1.0</td>
<td>67.8 ± 4.7</td>
</tr>
<tr>
<td>1.5</td>
<td>68.8 ± 3.5</td>
</tr>
</tbody>
</table>
3.1.3 HPLC Analysis

A main goal of this study was to quantify RK in formulated mouse diet using HPLC. A method was determined that allowed for comparison of RK before and after processing. Figure 7 shows example chromatograms of RK before processing and after processing. The pre-processing sample was composed of ground high-fat control diet, spiked with RK at concentrations between 0.25 and 2%. The post-processing sample was formulated diet produced by Research Diets. The spectra remained stable after processing indicating RK’s stability to diet processing. By comparing retention time with a known pure standard, it was determined that RK was in the mouse food. Retention time was between 1.6 and 1.8 minutes for both experiments. Quantification was performed using peak area.
Figure 7: Top – HPLC chromatogram of RK peak in spiked food sample, not processed; Bottom – HPLC chromatogram of RK in processed mouse food.
3.1.4 Formulated Mouse Diet Quantification

Loss of RK to processing necessitated production of several batches of mouse diet. The high dose diet (LRK) was formulated to contain 2.0% RK. The actual RK concentration in HRK food, determined by analysis post-processing was 1.74% (SD = .03%), a loss of 0.26%. The low dose diet (LRK) was formulated to contain 0.25% RK, however after analysis post-processing, only 0.15% (SD = 0.005%) was detected. Two more batches were made, one with 0.33% RK added and the final with 0.5% RK added to achieve the final desired concentration of 0.25%. These results are summarized in Table 4.

Table 4: RK extraction recovery in processed high-fat mouse diet

<table>
<thead>
<tr>
<th>% RK in Formula</th>
<th>% Detected</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 ± .03</td>
<td>1.74</td>
<td>87</td>
</tr>
<tr>
<td>0.25 ± .005</td>
<td>0.15</td>
<td>60</td>
</tr>
<tr>
<td>0.33 ± .005</td>
<td>0.16</td>
<td>48</td>
</tr>
<tr>
<td>0.50 ± .04</td>
<td>0.25</td>
<td>50</td>
</tr>
</tbody>
</table>
3.2 Mouse Study

3.2.1 Food Intake

Cumulative food intake and feed efficiency (ratio of cumulative caloric intake to body weight (BW) gain) was calculated for all groups. Figure 8 displays cumulative food intake over the course of the experimental diet period of the study (weeks 0 – 5). In accordance with experimental design, the LRK and pair-fed groups show nearly identical cumulative food intake (no statistical difference). ANOVA statistical test revealed that LRK/pair-fed mice had significantly lower food intake than control mice (p = 0.00014). Data is not available for the food intake of the HRK group as mice exhibited eating behavior that made it extremely difficult to measure daily consumption. Observational data of the HRK group food intake was that food intake was likely less than the LRK and control groups. It is possible that the mice found the food unpalatable with a high level of RK due to irritation or distaste the mice have for its aroma. No previous mouse RK studies have noted this as a problem.

Feed efficiency for control, LRK and pair-fed groups was calculated and compared (Figure 9, Table 5). The calculation can be found below Figure 9. Looking at this ratio helps determine if the treatment, RK, has an effect on body weight independent of energy intake (Ellacot et al. 2010). Feed efficiency for the control group was lower than the LRK group, though not statistically significant. Because there is a known interaction between body weight and food intake, it is expected that there is feed efficiency is not affected by RK itself, but rather energy intake. In the future, more
definitely measures may be taken to determine the precise effect of RK, if any, on body weight gain. This can be achieved by measuring RK metabolites in tissues that may suggest higher or lower intake by mice.
Figure 8: Cumulative food intake over 5 weeks of experimental diet for control, LRK and pair-fed mice. Different letters represent statistically significant differences as determined by Tukey’s post-hoc test (p = .00014).
Figure 9: Feed efficiency of control, LRK and pair-fed mice, error bars represent standard error of the mean. No statistically significant differences between groups were found using Tukey’s post-hoc test.

Feed Efficiency = \frac{\text{Cumulative caloric intake}}{\text{Body Weight Gain}}

Table 5: Feed Efficiency of Control and RK Diets

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LRK</th>
<th>Pair-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>59.24 ± 9.93</td>
<td>73.22 ± 17.52</td>
<td>67.78 ± 16.4</td>
</tr>
</tbody>
</table>
3.2.2 Body Weight

All groups experienced body weight gain induced by high-fat diet over the course of the study, as expected (Figure 10). Table 6 summarizes percent gain and weight gain velocity for all groups. Body weight differences were considered significant for control, LRK/pair-fed and HRK groups ($p < 0.0001$). The LRK group weight gain was very similar to its pair-fed group. This was expected as the two groups had nearly identical caloric intake. If RK had been exhibiting an effect at this physiological level, the LRK group would have shallower weight gain than its pair-fed group. A two-way ANOVA was done to determine interaction between food intake for different treatment group and its effect on body weight. This revealed that there was a significant interaction between body weight and the statistically significant differences in food intake. This suggests that the depressed body weight of the LRK group compared to control was arguably due to lower energy intake rather than the effect of RK. Explaining the lower body weight in the HRK group is not as clear because only observational food intake data exists. This observational data supports that the HRK group ate significantly less than the control group, which would result in lower body weight.

The pair-fed group experienced some patterns of depressed body weight compared to the LRK group. This could be due to their pattern of eating that is different from *ad-libitum*. Researchers have determined that pair-fed mice exhibit “gorging” behavior marked by consuming all of their food shortly after it is presented to them and then experiencing a prolonged period of fasting during the day. In contrast, mice fed *ad-libitum* exhibit nibbling behavior throughout dark periods and also intermittently during
the day. This results in a longer fed-state for these mice. Therefore the metabolic states of pair-fed mice could be in an extreme metabolic state directly before being fed, the time at which they were weighed (Kliewer et al. 2015). Body weight data indicated that mice from all groups experience weight loss between 2.15 and 4.14% of their body weight during fasting. Body weight depression can help explain the consistently lower body weight seen in pair-fed mice, particularly between days 6 and 17 most prominently. All mice were fasted on day 21 for blood draw, thus explaining the large dip in body weight on this day. This dip in body weight can be seen in Figure 10 at day 21 for all mice.

Velocity of weight gain was determined by taking the slope of weight gain for each mouse (change in weight/time) and multiplying by 365.25. Values represent weight gain velocity per year:

\[
\frac{\Delta \text{Body Weight (g)}}{\Delta \text{Time (days)}} \times 365.25
\]
Table 6: Percent weight gain and velocity of weight gain for all groups. Different letter superscripts represent statistically significant differences.

<table>
<thead>
<tr>
<th>% weight gain</th>
<th>Velocity of Weight Gain (g/year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.1 ± 5.0</td>
</tr>
<tr>
<td>LRK</td>
<td>22.5 ± 5.6</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>23.6 ± 5.6</td>
</tr>
<tr>
<td>HRK</td>
<td>12.8 ± 2.8</td>
</tr>
</tbody>
</table>
Figure 10: Body weight gain of mice week 0 through week 5. Each point represents group average for that day. Different letters represent statistical differences in body weight gain between treatment groups (P < 0.0001)
3.2.3 Tissue Weight

Differences in quadriceps, liver, inguinal fat pad and epididymal fat pad weight, normalized to body weight, are displayed in Figure 11 and tissue weights that have not been normalized to body weight are in Figure 12. No significant differences were observed in quadriceps weight among groups indicating that lean muscle mass was not affected by RK diet. Body weight differences and increases are the result of differences in fat depot weight, as determined by the fat tissue weight differences in RK-fed mice in this study. In human studies, weight gain and loss induced by increases or decreases in energy intake resulted in total changes in body fat and no significant change in lean body mass (Singh et al. 2012). It was determined that different groups had different energy intake, therefore differences in fat pad depot weight is expected between groups.

HRK mice had significantly higher liver weights than control mice. One previous study that examined the effect of RK on mouse livers in mice found that liver weight decreased with RK treatment. It was speculated that the initiation of nonalcoholic steatohepatitis (fatty liver) from increased triglycerides and free fatty acids in the blood (sourced from the high-fat diet) causes triacylglycerol to be deposited in the liver more thickly than normal diet. Treatment with RK reduced fatty deposition on the liver surface and thereby decreased liver weight (Wang et al. 2012). It would therefore be expected that the HRK group had the lowest liver weights and the control group the highest since energy and dietary fat intake was lowest for the HRK group and the highest in control mice. However the present study found opposite results from these. When liver weight is normalized to body weight, HRK liver weights are significantly lower than control liver
weights. Examining liver tissue weights that have not been normalized to body weight reveals minimal differences in weight ($p = 0.743$). Considering the fact that the HRK group’s body weights were significantly lower than the control group, it would logically follow that liver weight, normalized to body weight would be larger.

One reason for increased liver weight in HRK mice may be that the high level of ketone induced toxicity, causing the livers to enlarge. Liver enlargement is a common occurrence in toxicological studies and has often been observed in mice (Gray et al. 1977; Aphale et al. 1998). However, several other studies fed mice equivalent and higher amounts of RK for the same length of time and did not report any toxicity and increased liver weight does not necessarily mean mice ingested toxic doses of RK (Sporstøl and Scheline 1982; Morimoto et al. 2005; Lili et al. 2011).

It is also speculated that since there was a decrease in inguinal fat in HRK mice, the fat could have re-deposited in the liver. To investigate whether lipid content differed among groups, indicating the ability of RK to protect mice from developing NAFLD as reported, or to determine if the liver fat content is higher, total lipid analysis was performed on livers from three groups – HRK, LRK and control. This analysis provided a rough comparison of fat content across groups. The preliminary results are summarized in Table 7. However, only three samples from each group have been analyzed and although there seems to be a trend that HRK mice have lower fat, data is not powered to draw firm conclusions. If these trends continue, it would validate that the mice in the HRK were protected from NAFLD since it is defined by greater than 5% fat in the liver. Future experiments to determine lipid content in more livers would be necessary to
determine if any differences exist among groups. Liver tissue was also preserved in paraffin blocks that could be stained with hematoxylin and eosin to determine if RK prevented enlargement of adipocytes or prevented the genesis of new fat cells.

Table 7: Fat concentration in mouse liver tissue

<table>
<thead>
<tr>
<th></th>
<th>%</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.731</td>
<td>4.567 ± 1.391</td>
</tr>
<tr>
<td>(n = 3)</td>
<td>7.244</td>
<td></td>
</tr>
<tr>
<td>LRK</td>
<td>8.041</td>
<td>2.344 ± 2.392</td>
</tr>
<tr>
<td>(n = 3)</td>
<td>6.380</td>
<td></td>
</tr>
<tr>
<td>HRK</td>
<td>3.376</td>
<td>1.572 ± 1.993</td>
</tr>
<tr>
<td>(n = 3)</td>
<td></td>
<td>6.403</td>
</tr>
</tbody>
</table>
Figure 11: Mouse tissue weights normalized to body weight; different letters represent significant differences; error bars represent standard error of the mean (Liver – $p = 0.026$; Inguinal – $p = 0.013$)
Figure 12: Mouse tissue weights; different letters indicate significant differences (Inguinal – p = 0.017); error bars represent standard error of the mean.
In humans, the location of preferential fat depot weight gain or loss when there is an energy surplus or deficit can be indicative of risk for metabolic diseases (dyslipidemia, hypertension, cardiovascular disease, insulin resistance, etc.). The pattern of weight gain seen in the mice in this study mimics common patterns of human weight gain. Human clinical trials have shown that when humans gain weight over comparable time period (8 weeks) from positive energy balance, lower body fat mass increases significantly more than visceral fat mass (Singh et al. 2012). Lower body fat is considered subcutaneous as it lies underneath the skin and not surrounding organs. In the present study, inguinal fat pad weight, normalized to body weight and not normalized (Figures 11 and 12), was significantly larger in control mice than mice fed HRK diet (p = .013). Epididymal fat pad weight, considered visceral fat, in HRK mice smaller but not statistically significant (normalized to body weight: p = 0.259; not normalized: p = 0.169) compared to control possibly because of the large SD within the group. Tukey’s post-hoc test was used to determine significance and this test is typically very sensitive to variability. Standard error of the means for epididymal weight was much larger for this particular tissue than all other tissue weights, as seen by the large error bars.

Differences in inguinal fat depot weight could be an indicator of where fat mass is preferentially stored during weight gain, independent of the interaction with RK, and HRK mice simply did not gain as much body weight as control mice. Studies have shown that subcutaneous fat stores fervently absorb circulating free fatty acids and triglycerides – two prominent components in high-fat mouse diet (Chaston and Dixon 2008). Further,
epididymal fat depots in all groups were 54 – 66% larger than the inguinal fat depot. Subcutaneous fat stores aside from the inguinal fat pad were not weighed.

Fat mass can increase by two mechanisms: increase in the size of the individual fat cells (hypertrophy) or increase in the number of fat cells (hyperplasia). Some research supports that in humans, adipocyte number is set during childhood and adolescence and during adulthood the number of fat cells remains stable. Therefore, increases or decreases in fat depot weight are the results of lipolysis or lipogenesis within fat cells (Spalding et al. 2008). Other research however challenges this notion as studies revealed that overfeeding resulted in hyperplasia of lower body fat (subcutaneous fat), even in adults (Tchoukalova et al. 2014). These conflicting theories cause ambiguity in determining the mechanism by which a factor attenuates weight gain. The proposed mechanism of RK action is by increasing secretion of adiponectin. Adipokines are active in both visceral and subcutaneous adipose tissue (Ibrahim 2010).

3.2.4 Adiponectin

If adiponectin was the mechanism by which RK can attenuate weight gain, it was expected that control mice would have lower circulating levels of plasma adiponectin at weeks 2 and 5, and RK-fed mice would have elevated plasma levels. Additionally, it was expected that adiponectin levels in RK-fed mice would increase over the course of the study (week 5 > week 2 > week 0). In a dose-response, HRK mice would have higher adiponectin levels than LRK mice, which would be higher than control mice. Circulating plasma adiponectin levels are shown in Figure 13. Most plasma adiponectin levels were
within a normal range for mice (5,000 – 15,000 ng/ml) (Berg et al. 2001). Although control mice showed a significant increase in adiposity and body weight throughout the 5 weeks of intervention, adiponectin levels at week 5 were very similar to levels at week 0 (Fig 14). Similarly, HRK mice showed no statistically significant change in adiponectin levels at the end of the study as they did at baseline, before starting a RK diet. The LRK group shows an increase in levels over the course of the diet intervention, however the levels are not statistically significant (p = 0.259). The pair-fed group follows the same trend as the LRK group, possibly from experiencing a fasting period each day, but these difference were also not considered statistically significant. Adiponectin levels were compared using one-way ANOVA for treatment and time and using a two-way ANOVA to compare treatment x time across groups. No statistically significant differences were found among or within groups. Although it was expected that HRK would have higher adiponectin levels, there was also an observed decrease in adipose tissue in these mice that could be a confounder that makes differences between groups unclear.

There are several possible reasons this study showed no trends in adiponectin levels across groups. First, the length of study and time points between measurements could be too short to see a lasting change in adiponectin levels. Future work could look at mRNA expression for adiponectin in visceral versus subcutaneous fat tissue to determine if its expression is upregulated in RK groups compared to control. Second, variation within groups may have been too large to see a significant difference.

The previous study that suggested the mechanism by which RK attenuates weight gain is through regulation of adiponectin was performed on 3T3-L1 adipocytes. In this
study, RK was applied directly to adipocytes (Park 2010). *In-vivo*, to see a similar effect, RK must be absorbed by the fat tissue at levels high enough to elicit a response. A study on the metabolism of RK found that up to 90% of the compound was excreted in rodents within 24 hours of consumption. Thus, it is probable that the compound is only minimally absorbed by tissues and the levels of absorption are not high enough to evoke a quantifiable effect on adiponectin secretion (Sporstøl and Scheline 1982). One way to verify absorption into tissues is to analyze the tissues for RK and its metabolites to determine the RK concentration and compare this level to the cell study previously performed.
Figure 13: Plasma adiponectin in control, LRK, pair-fed and HRK mice; error bars represent standard error of the mean. No statistically significant differences were found between groups (treatment), between weeks (time) or interaction between weeks and group (treatment x time) were found using Tukey’s post-hoc test.
3.2.5 Blood Glucose

Metabolic syndrome is characterized, among other symptoms, by high fasting glucose. Therefore, it was expected that RK-fed mice would have lower fasting blood glucose than control and pair-fed mice. The group averages are shown in Figure 14. All groups showed an increase in blood glucose, which was expected as they showed stronger signs of obesity and metabolic syndrome as the study went on, as induced by the high-fat diets. It is difficult to discern whether RK was affecting blood glucose however, especially since not all groups had the same baseline. Further, results could be confounded by stress experienced by mice which can cause sudden and significant hyperglycemia (Surwit et al. 1988). Before blood was collected by tail bleeding, mice were restrained and anesthetized. This process can be very stressful for mice and therefore cause variation in blood glucose.
Figure 14: Blood glucose measurement at week 0 (baseline), week 2 and week 5
Chapter 4: Conclusion

The main goal of this research was two-fold: to determine a method for measuring raspberry ketone in high-fat diet and to use that formulated diet to determine if RK attenuates weight gain in high-fat fed mice. In the first aim of this work, existing methodology for the analysis of RK using GC-MS and HPLC was adjusted for the specific needs of this project and its resources. The extraction method development built off of elements used to extract RK from fresh fruit and optimized it for extraction from a high saturated fat food matrix. Using this extraction and HPLC methodology, as it is described in this work, RK was analyzed in high-fat mouse diet and its behavior due to processing of the diet was monitored. It was determined that significant loss can occur, but the compound is stable to mild processing and drying conditions.

The biological action of raspberry ketone, if any, remains unclear. Results of this study indicate that mice fed high-fat diet with RK exhibited attenuated weight-gain in a dose-response manner; however, it is highly presumed that the body and tissue weight differences are due to a decrease in energy intake for RK-fed mice compared to control. Statistical analysis revealed that LRK-fed mice had significantly lower daily food intake than control mice and there was a strong interaction between food intake and body weight. Body weight gain was lowest for a high RK dose, followed by a low RK dose,
and the control group had the steepest body weight gain. RK appeared to have no effect on plasma levels of adiponectin. At a physiologically relevant dose, RK showed no effect on adipose tissue weight or adiponectin levels.
References


